ADVANCES IN SECOND MESSENGER AND PHOSPHOPROTEIN RESEARCH

Formerly
ADVANCES IN CYCLIC NUCLEOTIDE AND PROTEIN PHOSPHORYLATION RESEARCH

SIXTH INTERNATIONAL CONFERENCE ON CYCLIC NUCLEOTIDES, CALCIUM AND PROTEIN PHOSPHORYLATION

Volume Editors

Robert Adelstein
Claude Klee
Mártin Rodbell

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

ADA204189

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) AFOSR-TR- 88-1232-Vol-21A
6a. NAME OF PERFORMING ORGANIZATION: Fdn. for Adv. Educ. in the Sciences; 6b. OFFICE SYMBOL (If applicable):	7a. NAME OF MONITORING ORGANIZATION: AFOSR/NL
6c. ADDRESS (City, State, and ZIP Code): Natl. Inst. of Health, One Cloister Court, Bethesda, MD 20814-1460	7b. ADDRESS (City, State, and ZIP Code): Bldg. 410, Bolling AFB, DC 20332-6448
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: AFOSR; 8b. OFFICE SYMBOL (If applicable): NL	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-86-0343
8c. ADDRESS (City, State, and ZIP Code): Bldg. 410, Bolling AFB, DC 20332-6448	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO. A1; WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification) Vol 21A

Sixth International Confernece on Cyclicnucleotides, Calcium & Protein Phosphorylation

12. PERSONAL AUTHOR(S): Dr. Rodbell — Abstracts Volume.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 1 Sep 86 TO 31 Aug 87	Nov 16 1988	35

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The Sixth International Conference on Cyclic Nucleotides, Calcium and Protein Phosphorylation Advances in Second Messenger and Phosphorprotein Research was held in Bethesda Maryland, 2-7 September 1986. This conference and the proceedings ~~from same~~ focused on the role of cyclic nucleotides, calcium and protein kinases as second messengers in signal transduction systems. A book by the same title, containing 29 chapters resulting from presentations at the meeting, was published by Raven press and constitutes the main substances of the final technica' report of this conference.

Cont'd Pg. iv

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL: Dr. William O. Berry	22b. TELEPHONE (Include Area Code): (202) 767-5021 — 22c. OFFICE SYMBOL: NL

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

UNCLASSIFIED

Advances in Second Messenger and Phosphoprotein Research

formerly Advances in Cyclic Nucleotide and Protein Phosphorylation Research

Series Editors: Paul Greengard and G. Alan Robison

Paul Greengard and G. Alan Robison, Editors
ADVANCES IN CYCLIC NUCLEOTIDE AND PROTEIN PHOSPHORYLATION RESEARCH (Vol. 20)
382 pp. (1986) ISBN 0-88167-242-4 (Order Code 1698)

"This volume continues the tradition of high quality which this series has established....All the chapters can be recommended to either the expert in the field looking for an up-to-the-minute overview of the subject or the neophyte looking for guidance to a complex literature....The depth of coverage of the topics makes the purchase of this volume appear to be a wise decision."
—Journal of Medicinal Chemistry

Volume 20 of this series features in-depth analyses of the biological and chemical properties of forskolin and the mechanisms involved in calcium mobilizing agonist responses, as well as important findings on early events in the action of insulin. Reviews of current and ongoing research on cyclic AMP examine the desensitization of hormonal stimuli coupled to regulation of cAMP levels and the relationship of cyclic AMP to learning in *Drosophila*. Another highlight of the book is a study of choline plasmalogens, glycerophospholipid methylation, and receptor-mediated activation of adenylate cyclase.

Dermot M. F. Cooper and Kenneth B. Seamon, Editors
DUAL REGULATION OF ADENYLATE CYCLASE (Advances in Cyclic Nucleotide and Protein Phosphorylation Research, Vol. 19)
352 pp., 116 illustrations (1985) ISBN 0-88167-079-0 (Order Code 1526)

"Recommended to anyone with an interest in adenylate cyclase."
—Quarterly Review of Biology

This volume provides an in-depth review of contemporary research on adenylate cyclase and its dual regulation. Coverage encompasses all relevant aspects of dually regulated adenylate cyclase systems, from their molecular characterization to their physiological significance, and represents the full range of conceptual and technical approaches to the study of these complex systems.

The book systematically progresses from receptor biochemistry and pharmacology to purification and characterization of adenylate cyclase components, whole cell studies, and neurophysiological approaches. Regulation of cyclic AMP production in intact model cells by adenylate cyclase and phosphodiesterase is examined, as well as correlations between cyclic AMP fluctuations and changes in metabolic processes in whole cells and brain slices. Detailed attention is devoted to the relationship of neuronal firing to changes in cyclic AMP production. The contributors also suggest applications of approaches used in the study of adenylate cyclase systems to the study of analogous regulatory systems, including those involved in calcium mobilization.

RAVEN PRESS 1185 Avenue of the Americas, New York, New York 10036

ISBN 0-88167-430-3 (Order Code 1741)

price $39.00

ADVANCES IN SECOND MESSENGER AND PHOSPHOPROTEIN RESEARCH

Volume 21A

Abstracts Volume.

formerly *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*

Advances in Second Messenger and Phosphoprotein Research

formerly *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*

Series Editors

International Advisory Board

Sold by:
Raven Press
1185 Avenue of the Americas
New York, NY 10036
Price: [redacted]
Volume 21A: $39.00

Advances in Second Messenger and Phosphoprotein Research

Volume 21A

formerly *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*

Abstracts Volume
Sixth International Conference on
Cyclic Nucleotides, Calcium and Protein Phosphorylation
Bethesda, Maryland, September 2–7, 1986

Volume Editors

Robert S. Adelstein, M.D.
Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, Maryland

Claude B. Klee, M.D.
Laboratory of Biochemistry
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Martin Rodbell, Ph.D.
National Institute of Environmental Health Sciences
National Institutes of Health
Research Triangle Park, North Carolina

Raven Press New York

Raven Press, 1185 Avenue of the Americas, New York, New York 10036

Made in the United States of America

International Standard Book Number 0-88167-430-3

This volume contains abstracts of papers presented at the ~~Sixth International~~ Conference on Cyclic Nucleotides, Calcium and Protein Phosphorylation, held September 2-7, 1986 in Bethesda, Maryland.

The material contained in this volume was submitted as previously unpublished material, except in the instances in which credit has been given to the source from which some of the illustrative material was derived.

Great care has been taken to maintain the accuracy of the information contained in this volume. However, Raven Press cannot be held responsible for errors or for any consequences arising from the use of the information contained herein.

Materials appearing in this book prepared by individuals as part of their official duties as U.S. Government employees are not covered by the above-mentioned copyright.

10 9 8 7 6 5 4 3 2 1

.1 **The EGF-receptor kinase: Structure, evolution and properties of various receptor mutants,** J. Schlessinger, Division of Molecular Biology, Research, Meloy Laboratories, 4 Research Court, Rockville, MD 20850

The EGF-receptor is a 170 KD membrane glycoprotein which has 3 major functional domains, a xtracellular, glycosylated EGF-binding domain, a single hydrophobic trans-membrane region and a cytoplasmic kinase domain. The extracellular domain of EGF-receptor contains 2 cysteine rich clusters which reveal internal homology and repetition of the cysteine residues. Questions concerning the mechanism of action and regulation of EGF-receptor were addressed by exploring properties and cellular effects of various EGF-receptor mutants introduced into cultured cells lines. Transient expression of intact EGF-receptor and various EGF-receptor mutants in COS-1 cells was achieved by using shuttle vector containing the SV-40 origin of replication. The same vector together with the DHFR gene were used to obtain stable cell lines expressing different amounts of EGF-receptor and its various mutants in CHO cells, which are normally devoid of EGF-receptor. A retroviral shuttle vector was used to express intact EGF-receptor and EGF-receptors mutants in NIH-3T3 cells and for obtaining retroviruses containing sequences coding for the intact receptor and its various mutants. Initially we have generated constructs with deletions in the cytoplasmic domain of the EGF-receptor including a receptor mutant which has only 8 amino acids in the cytoplasmic domain (devoid of Thr 654). We have also introduced specific linkers into different restriction sites along the full size cDNA of EGF-receptor. Using this approach we have explored the role of various receptor domains in the regulation of receptor internalization, endocytosis and transformation.

.2 RECONSTITUTION OF THE DELIPIDATED ß-ADRENERGIC RECEPTOR WITH THE DELIPIDATED G_s: REQUIREMENTS FOR SPECIFIC LIPIDS. Michael Schramm and Jorge Kirilovsky, Department of Biological Chemistry, Institute of Life Sciences, Hebrew University, 91904 Jerusalem, Israel.

Detergent solubilized preparations of the ß-adrenergic receptor (R) and of the regulatory protein (G_s) were extensively delipidated of phospholipids and cholesterol. Reconstitution of an R-G_s system was subsequently performed in presence of a mixture of natural phosphatidyl-ethanolamine, -choline and -serine or the synthetic dioleoyl derivatives of the same phospholipids. In both cases, an additional lipid was required for the agonist-dependent activation of G_s. The requirement could be fulfilled by α-tocopherol or by oleic acid. Cholesterol and cholesteryl esters failed to support activity. Inclusion of the non-phospholipid lipid in the reconstituted system enhanced the isoproterenol-dependent activation of G_s 16-35 fold. Upon relipidation with the defined phospholipid mixture plus α-tocopherol or oleic acid, and addition of isoproterenol, the kinetics of activation of G_s by GTPγS were similar to those found in native membranes. The rate was largely dependent on the addition of the agonist. Efficient functional reconstitution of R-G_s was thus achieved in a totally defined lipid system. Experiments designed to study the role of the non-phospholipid lipid in the reconstitution of R-G_s interaction, suggest that the effect of this lipid is not explained by: 1) enhancement of ligand binding to R; 2) stabilization of the system; 3) irreversible structural changes; 4) modification of vesicle permeability to GTPγS; 5) modulation of average fluidity. It is tentatively concluded that the non-phospholipid lipid is essential for function of the hormone-R complex. Further experiments suggest that such a lipid might fulfill the same role in native membranes.

.3 ANALYSIS OF cDNAs FOR THE α SUBUNIT OF G_s. Alfred G. Gilman, Janet D. Robishaw, and Michael P. Graziano. Department of Pharmacology, University of Texas Health Science Center at Dallas, Dallas, TX 75235

Most cells contain two forms of the α subunit of the G protein (G_s) that stimulates adenylate cyclase; their apparent molecular weights are 45,000 and 52,000. Two cDNAs that correspond to distinct mRNAs for the α subunit of G_s have been cloned from a bovine adrenal library and sequenced. The sequences of the two cDNAs, designated pG_s-1 and pG_s-s, are identical except for a single stretch of 46 nucleotides in the coding region, where 4 are altered and 42 are deleted in pG_s-s. Expression of pG_s-s and pG_s-1 in COS-m6 cells yields protein products with apparent molecular weights of 45,000 and 52,000, respectively, based on their mobility in SDS-polyacrylamide gels. The two cDNAs have also been expressed in E. coli, using prokaryotic expression vectors containing either tac or T7 promoters. Again, products with apparent molecular weights of 45,000 and 52,000 are detected. We conclude that pG_s-s and pG_s-1 encode the 45 kDa and 52 kDa forms of $G_{s\alpha}$ respectively, that the two forms differ by the alteration of two and the deletion of 14 amino acid residues (73-86 in pG_s-1), and that the apparent difference in molecular weight of 7000 between the two forms of $G_{s\alpha}$ is in large part due to anomalous electrophoretic behavior in SDS. There appear to be at least two mRNAs for $G_{s\alpha}$ that arise from a single gene by alternative internal RNA splicing. Analysis of the activities of the two forms of $G_{s\alpha}$, expressed separately in E. coli, is in progress.

.4 ROLES OF GTP REGULATORY PROTEINS IN RECEPTOR-MEDIATED ADENYLATE CYCLASE INHIBITION, PHOSPHOLIPASE C ACTIVATION AND CELL PROLIFERATION

Michio Ui, Fumikazu Okajima, Toshiaki Katada & Toshihiko Murayama. Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Receptor-mediated inhibition of membrane adenylate cyclase via an inhibitory GTP regulatory protein (Gi or Ni) is reversed by prior exposure of the membranes or the membrane-donor cells to islet-activating protein (IAP), pertussis toxin, since Gi is unable to couple to receptors any longer when its GTP-binding α-subunit has been ADP-ribosylated by the A-component of the toxin. Several kinds of Gi ($\alpha\beta\gamma$) have been purified from rat and porcine brains as the specific IAP substrate, and the cDNAs of their α-subunits have been cloned. Some of them competed with the α-subunit of stimulatory Gs for the same sites on the adenylate cyclase catalyst. βγ-subunits of these IAP substrates also interacted with the catalyst directly leading to inhibition of the cyclase activity. Thus, Gi inhibits adenylate cyclase by multiple mechanisms when it is dissociated into α- and βγ-subunits upon coupled receptor stimulation. IAP also abolished the chemotactic peptide (fMLP) receptor-coupled activation of phospholipase C that is involved in phosphatidylinositol 4,5-bisphosphate breakdown and Ca mobilization in neutrophils. The IAP substrates purified from rat brain were coupled to fMLP receptors when they were reconstituted into IAP-treated neutrophil membranes. Moreover, DNA synthesis in 3T3 fibroblasts as initiated by competence factors in the presence of a progression factor such as insulin or epidermal growth factor was inhibited by IAP treatment of the cells. IAP was effective in this regard even when it was added to culture medium 3 h after the addition of growth factors, suggesting that an IAP substrate G-protein is also involved in slow proliferative responses of cells.

.5 THE STRUCTURE AND REGULATION OF PROTEIN PHOSPHATASE-1

Protein phosphatase-1 (PP-1) is one of four phosphatase catalytic C-subunits which dephosphorylate a variety of regulatory proteins. Several forms of PP-1 have been identified in which the C-subunit interacts with proteins which either have a regulatory function or target the phosphatase to different subcellular locations. In skeletal muscle a major proportion of the PP-1 is bound to glycogen because the C-subunit is complexed with a glycogen-binding G-subunit. The G-subunit is phosphorylated very efficiently by cyclic AMP-dependent protein kinase and the effects of phosphorylation on PP-1 activity will be described. A second form of PP-1 is found in the muscle cytosol. This form is inactive, because the C-subunit is complexed to inhibitor-2 (I-2). The primary structure of I-2 and an analysis of its in vivo phosphorylation state will be presented. A third form of PP-1 has recently been identified in skeletal muscle, and its structure and localisation will be given. In mammalian liver a significant proportion of the PP-1 is found associated with microsomes as well as glycogen. The dephosphorylation of glycogen synthase by hepatic glycogen and microsomal PP-1 is exquisitely sensitive to inhibition by phosphorylase a, 50% inhibition occurring at <10nM, over 1000-fold lower than the Km for phosphorylase a as a substrate. This, and other evidence, demonstrates that phosphorylase a is an allosteric inhibitor of hepatic PP-1. The role of this allosteric inhibition in the hormonal regulation of liver function will be discussed.

Supported by the Medical Research Council, London, British Diabetic Association and Royal Society.

.6 INOSITOL PHOSPHOLIPIDS, PROTEIN PHOSPHORYLATION, AND STIMULUS-RESPONSE COUPLING. Ushio Kikkawa and Yasutomi Nishizuka, Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan.

Receptor-mediated hydrolysis of inositol phospholipids was recently realized to be a common mechanism for transducing various extracellular signals into the cell. At an early phase of cellular responses, inositol-1,4,5-trisphosphate mobilizes Ca^{2+}, whereas diacylglycerol (DG) activates protein kinase C. These two intracellular mediators are generated from the hydrolysis of phosphatidylinositol-4,5-bisphosphate, PIP_2. Using Ca^{2+}-ionophore and permeable DG or tumor-promoting phorbol ester (as a substitute for active DG), it has been repeatedly shown that both Ca^{2+} mobilization and protein kinase C activation are essential and often act synergistically to induce many of cellular responses such as exocytosis and release reactions. Similarly, these two singal pathways appear to be essential for long-term responses as well. However, additional receptor occupation by growth factor is necessary to induce full activation of cell proliferation. Immunocytochemical studies with monochonal antibodies suggest that this enzyme seems to be absent or in very low concentration in the nucleus; probably another step in signal translation is needed for the ultimate activation of nuclear events. The phosphorylation by protein kinase C of some proteins such as membrane receptors is apparently related to down-regulation or negative feedback control of cellular functions. In fact, protein kinase C appears to show dual actions in the positive as well as the negative phase of regulation. Results will be summarized of further studies on the structure, mode of activation, intracellular localization, and possible role of this protein kinase in stimulus-response coupling.

.7 INDUCTION OF C-FOS: A POSSIBLE NUCLEAR EVENT IN SIGNAL TRANSDUCTION. Tom Curran, James I. Morgan, Lidia Sambucetti and Robert Franza[1]. Roche Institute of Molecular Biology, Nutley, NJ 07110; [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

A transient elevation of c-fos mRNA and protein levels is elicited by diverse extracellular stimuli. These increases may be greater than 100-fold depending on the basal levels of expression in unstimulated cells. The response is triggered by a selection of polypeptide growth factors, some of which promote mitogenesis, while others promote differentiation. Agents such as phorbol esters, cAMP and calcium ionophores can also stimulate c-fos expression, although the magnitude of the effect varies with the cell line studied. In PC12 cells, induction is mediated by diverse pathways, one of which involves a calcium-dependent stimulation of calmodulin. The fos protein in stimulated cells exists as a protein complex which binds to DNA. The interaction of the fos protein with DNA supports the hypothesis that it may be involved in linking cell surface events to long-term alterations in gene expression.

.8 STRUCTURE-FUNCTION OF Ha ras: Irving Sigal[1], Jackson Gibbs[1], Mark Marshall[1], Graham Smith[1], Frances Jurnak[2] and Edward Scolnick; [1]Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania; [2] University of California, Riverside, California.

The ras protooncogene proteins have biochemical properties that are similar to those of the guanine nucleotide-binding regulatory proteins of the G protein family. Like other G proteins, ras proteins specifically bind GDP and GTP and have a low GTP-hydrolytic activity. Oncogenic forms of the ras proteins have decreased GTPase activities (Trends Biochem. Sci. 1985, 10: 350-353). To identify residues involved in nucleotide binding, GTP hydrolysis and protein-protein interactions, we have systemically varied the conserved amino acid residues of Harvey (Ha) ras. The mutant proteins have been expressed in E. coli for biochemical analysis and microinjected into NIH3T3 cells and expressed in yeast cells for assaying biological potency. This analysis has identified residues Lys16 and Asp119 within the nucleotide-binding site and indicated that Lys16 interacts with the phosphate group of GDP, and Asp119 interacts with the purine-2-amino group (PNAS 1986, 83: 952-956). This mode of interaction is analogous to that observed for EF-Tu*GDP by x-ray crystallography. Substitutions at either position 63, 65, 66, 67, 70 or 73 dramatically reduce the affinity of Ha for the neutralizing monoclonal antibody Y13-259 but do not alter biological activity. In contrast, substitutions within the region 32-41 while not affecting the known intrinsic biochemical activities of Ha do reduce the ability of the protein to function in mammalian and yeast cells. This conserved region of the ras proteins is a possible site for protein-protein interaction and for conferring ras biological function.

1 THE EFFECTS OF THE IRREVERSIBLE MUSCARINIC LIGAND ACETYLETHYLCHOLINE MUSTARD ON PRE- AND POSTSYNAPTIC RESPONSES. Stephen P. Baker, Philip Posner and Edwin M. Meyer. Depts. of Pharmacology and Physiology, University of Florida, College of Medicine, Gainesville, FL 32610.

The pharmacological effects of agonists that bind irreversibly are unclear at the receptor-transduction level. We have investigated the presynaptic and postsynaptic effects of a muscarinic agonist, acetylethylcholine mustard (Aech-M), which binds irreversibly to muscarinic receptors. As measured by several radiolabeled ligands, Aech-M induced a dose-dependent (0.01-10 μM) loss of cardiac and brain muscarinic receptors that was time-dependent, blocked by atropine and attenuated by guanine nucleotides. Aech-M (10-100 μM) produced a transient decrease in spontaneous atrial beating rate whereas carbachol and ACh produced sustained decreases. After pretreatment of atria with Aech-M (10-100 μM), there was a 10- to 20-fold increase in the carbachol EC_{50} value for the inhibition of the atrial beating rate. In addition, there was a 40-50% loss of muscarinic receptors. Presynaptically, Aech-M induced a protracted (up to 60 min) attenuation (40-50%) of ACh release from rat cortical synaptosomes with a potency similar to that of oxotremorine. Oxotremorine inhibited Ach release for a shorter duration. The ACh release-inhibition induced by 50 μM Aech-M was only acutely blocked by 1 μM atropine, was observed after washout of free drug, and was not due to inhibition of choline-uptake or acetylation. These presynaptic effects of Aech-M on ACh release were compatible with the observed time-dependent (IC_{50}, 9 μM after 5 min at 37°) and irreversible binding characteristics of the drug in synaptosomal membranes. The data suggest that Aech-M initially acts postsynaptically as an agonist followed by a long-term antagonist effect and presynaptically as an irreversible agonist.

2 **EVIDENCE FOR INCREASED LEVELS OF DIGITALIS LIKE FACTORS IN PLASMA OF PREGNANT RATS**

A. Binet, V. Querol-Ferrer, J. Baranès and P. Braquet
IHB Research Labs, 17 avenue Descartes, F-92350 Le Plessis Robinson (France)

For several years, numerous studies have demonstrated the presence of "digitalis-like" factors (D.L.F.) in mammalian blood and urine that is released by volume expansion. Recent evidence suggests that this factor is an inhibitor of Na, K-ATPase, enzyme which represents the biochemical basis of transmembrane sodium and potassium transport. This compound interacts with the ouabain binding site of the enzyme and shows a natriuretic effect by limiting tubular sodium reabsorption.

Pregnancy is known to be a volume expanded state, without increase in arterial blood pressure.

In this study the presence of D.L.F. has been investigated in plasma of rat during the course of pregnancy.

Blood samples were obtained from reorbital sinus, under ether anesthesia and collected in heparinized tubes. The deproteinized plasma (treatment by two volumes of ethanol for 4 min) were on a sephadex G25 column. Inhibitory effect of D.L.F., eluted in the post salt fraction was measured on purified Na, K-ATPase from dog kidney, using ATP-γ32 P.During the last week of pregnancy a significant increase of D.L.F. concentration was observed (8.4 $\pm$ 1.4 vs 4.61 $\pm$ 0.39 nM of ouabain equivalent, $p < 0.01$) a simultaneous increase in K^+ plasma levels appeared (+ 48 %) without any change in Na^+ level. Evidence for increase of endogenous Na, K-ATPase inhibitor is provided in plasma of pregnant rats.

3 **THE MECHANISM OF ACTIVATION OF THE INSULIN RECEPTOR KINASE: ROLE OF DISULFIDE BONDS.**

Marianne Boni-Schnetzler and Paul F. Pilch, Boston University Schol of Medicine.

The insulin receptor possesses a large number of cysteine residues in the α subunit. None of these are reactive with N-ethylmaleimide in the native receptor suggesting that normally most, if not all cysteines, exist as disulfide-linkages. These bonds are known to link the receptor in its $\alpha_2\beta_2$ tetrameric form, and are also likely to play a role in the structure of the ligand binding domain. We have performed a detailed kinetic analysis on the ability of the reducing agent, dithiothreitol (DTT) to reduce disulfides and to effect the functional properties of the receptor, namely ligand binding and kinase activity. We have correlated these functional data with the structure of the receptor as analyzed by velocity sedimentation analysis and SDS-PAGE. We find that $\alpha\beta$ receptor dimers isolated from sucrose gradients retain insulin binding of slightly lower ligand affinity as compared to intact tetramers. The $\alpha\beta$ dimers are as active as tetrameric receptor in basal kinase function. If autophosphorylated in the presence of insulin prior to reduction, the dimer remains as an activated kinase towards exogenous substrates. However, dimers isolated from gradients do not exhibit an insulin-dependent kinase activation. We interpret these data to indicate that the interaction of two $\alpha\beta$ halves is necessary for the ligand-dependent kinase activation that follows insulin binding. In addition, because $\alpha\beta$ dimers retain insulin binding, and because insulin binding continues to decrease with time of DTT exposure after the receptor is entirely in the $\alpha\beta$ dimer form, we conclude that the class 1 disulfides that link two $\alpha\beta$ halves are not requisite for ligand binding. Rather, maintenance of the multiple disulfides in the "cross-linking" domain of the α subunit appear critical for insulin binding.

4 PURIFICATION AND CHARACTERIZATION OF THE INSULIN-SENSITIVE "LOW K_m" cAMP PHOSPHODIESTERASE FROM RAT ADIPOSE TISSUE.

E. Degerman+, P. Belfrage+, and V. C. Manganiello*. + Department of Physiological Chemistry, Lund, Sweden and *Laboratory of Cellular Metabolism, NHLBI, NIH, Bethesda, MD. 20892

Particulate, but not soluble, "low K_m" cAMP phosphodiesterase (PDE) activity of rat adipocytes was increased 50-100% during incubation (10 min) of intact cells with 1-3 nM insulin; activation was less with higher or lower concentrations of insulin. Activation was maintained during solubilization with an alkyl polyoxyethylene non-ionic detergent C_1E_{12} and NaBr and chromatography on DEAE. Solubilized enzyme was purified to apparent homogeneity by DEAE, gel permeation, and affinity chromatography (K_m for cAMP $\sim$ 0.4 µM; sp. act. (with 0.5 µM [^{3}H]cAMP) $\sim$ 5.5 µmol/min/mg). M_r of the holoenzyme (from chromatography on Sephadex G-200) was $\sim$ 120-130,000; from polyacrylamide slab gel electrophoresis in SDS was estimated a subunit M_r of $\sim$ 63-65,000. Activity (with 0.5 µM [^{3}H]cAMP) was rather sensitive to inhibition by p-chloromercuribenzoate (IC_{50}, $\sim$ 1 µM) and less so by 2,2'-Dithiobis-(5-Nitropyridine)(160 µM), N-ethylmaleimide (525 µM) and iodoacetamide (750 µM). PDE activity was also rather sensitive to inhibition by cilostamide (IC_{50}, $\sim$ 40 nM) and the "cardiotonic" drugs CI 930 (450 nM) and milrinone (630 nM) but rather insensitive to RO 20-1724 (190 µM). Based on effects of these inhibitors, the hormone-sensitive "low K_m" particulate cAMP PDE from rat adipocytes seems to be analogous to the insulin-activated particulate PDE from 3T3-L1 adipocytes and the cilostamide-sensitive soluble "low K_m" cAMP PDE from bovine liver (designated as III-C), platelets, heart, and other tissues. Purification of this PDE should facilitate studies designed to probe the mechanism(s) of activation of the enzyme by insulin and other effectors.

5 MOLECULAR GENETICS OF THE β2 ADRENERGIC RECEPTOR. R.A.F. Dixon[1], I.S. Sigal[1], E. Slater[2], C. Strader[2]. Merck Sharp & Dohme Research Laboratories, [1]Virus and Cell Biology; West Point, Pennsylvania and [2]Biochemistry Endocrinology; Rahway, New Jersey.

Cloning of the gene for the β-adrenergic receptor (βAR) (Nature [1986] 321: 75) allowed the determination of the primary sequence of the receptor protein. Hydropathicity analysis of the βAR polypeptide suggests the presence of seven transmembrane helices and supports a structure for the receptor that is similar to the structure of rhodopsin. Using rhodopsin as a model, regions of the βAR protein have been selected which might be involved in ligand binding, in receptor-G-protein interaction or in receptor regulation. To examine the structure-function relationships of the βAR, mutations have been introduced into the βAR gene, and the mutated proteins expressed in mammalian cells. The mutant proteins were characterized by their ability to bind ligands and to stimulate adenylate cyclase. To further probe the structure of the βAR, antisera have been raised against E.-coli-expressed peptides corresponding to the βAR sequence. These sera are being used to determine the folding of the βAR protein within the membrane.

6 ATRIAL NATRIURETIC FACTOR (ANF) STIMULATES CYCLIC GMP ACCUMULATION AND EFFLUX IN C6-2B RAT GLIOMA CELL CULTURES AND THESE RESPONSES ARE ANTAGONIZED BY CHLORPROMAZINE. R.R. Fiscus, B.T. Robles, R.L. Del Vecchio, S.A. Waldman and F. Murad. Dept. Physiol., Loyola Univ. Med. Ctr., Maywood, IL 60153; Dept. Med. & Pharmacol., Stanford Univ., VA Med. Ctr., Palo Alto, CA 94304.

Previous studies from our laboratory have shown that ANF activates particulate (P), but not soluble (S), guanylate cyclase (GC) in rat aorta, kidney and several other tissues and that one class of ANF receptor co-purifies with PGC. Also, we have shown that the ANF analog atriopeptin II (AP-II) causes relaxation of precontracted rat aorta that is correlated with elevation of cGMP and activation of cGMP-dependent protein kinase, suggesting that these molecular events may mediate at least some of ANF's actions. Since ANF is located in rat brain and may serve as a neurotransmitter or neuromodulator, we studied its molecular actions in two neurally-derived cell lines, C6-2B rat glioma and PC-12 rat pheochromocytoma, which contain high levels of PGC. In PC-12 cell cultures AP-II (100 nM, 20 min) elevated cGMP in both cells and media by 12 fold. In C6-2B cell cultures AP-II (100 nM, 20 min) elevated cGMP 4 fold in cells and 47 fold in media. All responses were enhanced by 10 min preincubation with isobutylmethylxanthine (250 uM). Both cellular and extracellular cGMP responses to AP-II in C6-2B cell cultures were antagonized by 10 min preincubation with chlorpromazine, but not methylene blue (an inhibitor of SGC), at 1 and 10 uM (IC50 = 1 uM). Cellular accumulation of cGMP to sodium nitroprusside (1 uM, 2 min, an activator of SGC) was antagonized by 10 min preincubation with methylene blue, but not chlorpromazine, at 1 and 10 uM. These data indicate that intracellular and/or extracellular cGMP may mediate actions of ANF in neural tissues and that chlorpromazine selectively antagonizes ANF-induced cGMP accumulation and efflux. Supported by NIH (HL28474, AM30787), Veterans Administration and Council for Tobacco Research, U.S.A.

7 THE INVASIVE ADENYLATE CYCLASE OF B. PERTUSSIS: PROPERTIES OF CELLULAR PENETRATION. E. Friedman*, Z. Farfel** and E. Hanski*, *Department of Hormone Research, Weizmann Institute of Science, Rehovot, and **Clinical Pharmacology Unit, Sheba Medical Center, Tel-Aviv University, Israel.

Bordetella pertussis produces a calmodulin-sensitive adenylate cyclase (AC), which is thought to enter eukaryotic cells and produce high levels of cAMP. AC activity in lysates from lymphocytes exposed to a partially purified bacterial AC is insensitive to NEM, readily inactivated by acetic anhydride and relatively stable to SDS, similarly to the bacterial AC and in contrast to the intrinsic membrane-bound enzyme, thus providing a direct evidence for enzyme penetration. In human lymphocytes, a constant intracellular level of AC activity is reached within 20 min of cell incubation with the bacterial AC and maintained for at least 2 h, provided the enzyme is present in the cell medium. Upon its removal, a rapid decline ($t_{1/2}$≈15 min) in AC activity is observed. This decline reflects intracellular inactivation process rather than a release of the enzyme to the medium. In turkey erythrocytes, no intracellular inactivation is observed and AC activity increases linearly even after 6 h of incubation. Subcellular fractionation of human lymphocytes and erythrocytes exposed to the bacterial AC shows that the majority (83-89%) of intracellular AC activity is associated with postnuclear (160,000 x g) pellet. AC activity in plasma membrane-enriched fraction of lymphocytes reaches a constant level after 20 min of cell exposure to the enzyme, similarly to the accumulation of the enzyme in whole cells. We suggest that cAMP production by the invasive enzyme, as well as its intracellular inactivation occur while it is associated with plasma membrane.

8 REGULATION OF E. COLI ADENYLATE CYCLASE (AC) ACTIVITY BY FACTORS WITH SHARED FUNCTIONS
Prasad Reddy and Alan Peterkofsky, NIH, Bethesda, MD

In Escherichia coli, cAMP plays an important role in expression of genes for carbon metabolism. The major control for adjusting cellular levels of this nucleotide is via AC, which is regulated by high molecular weight (proteins involved in sugar transport and protein synthesis) and low molecular weight (sugars, inorganic orthophosphate and GTP) factors.

Regulation by low molecular weight factors can be inhibitory or stimulatory and can be demonstrated in permeable cells but not in extracts. Sugars that are transported by the phosphoenolpyruvate:glycose phosphotransferase system (PTS) inhibit AC. Inorganic orthophosphate (Pi) substantially stimulates AC activity. The effects of Pi and PTS sugars are related since inhibition by PTS sugars is not observed unless the AC activity is stimulated by Pi. GTP, which is a key regulator of eucaryotic AC, stimulates AC activity.

The regulation by the low molecular weight effectors is mediated by some protein factors. Inhibition by PTS sugars or stimulation by Pi of AC activity depends on the presence of functional PTS proteins. Furthermore, E. coli strains deleted for such proteins have low AC activity. The suggestion from these observations that the regulatory form of AC consists of a complex with PTS proteins has been supported by in vitro reconstitution experiments.

The stimulation of AC activity in permeable cells by GTP is probably mediated by a GTP-binding protein. Our search for such a mediator has led to the finding that the protein synthesis elongation factor EF-Tu specifically stimulates the AC activity of a purified preparation of the enzyme. The functional form of AC therefore appears to be a complex with a variety of high and low molecular weight regulators all of which serve other functions in the cell.

9 **PROPERTIES AND REGULATION BY CALMODULIN OF A PARTIALLY PURIFIED ADENYLATE CYCLASE SOLUBILIZED FROM BULL SPERM MEMBRANES.** Nira B. Garty and Yoram Salomon, Department of Hormone Research, The Weizmann Institute of Science, 76100 Rehovot, Israel.

Membrane-associated adenylate cyclase (AC) was solubilized and subsequently purified from membranes prepared from frozen bull-sperm ejaculate. The aim of this purification was to obtain a calmodulin (CaM)-free AC preparation in which regulation of AC by CaM could be studied. Nearly 50-60% of the total membrane-associated AC could be solubilized by salt extraction of the sperm membranes in the absence of detergent. The soluble enzyme was rapidly inactivated at high dilutions. Subsequent purification steps were, therefore, performed in 0.5 mg/ml BSA. Purification, expressed in terms of AC/CaM ratios, increased 2000-4000-fold with a 3-10% yield, using DEAE, lectin and phenyl-Sepharose chromatography.

Enzyme activity is stimulated 2- to 5-fold by purified testicular CaM, which shifts the Km (Mg-ATP) from 8.1 to 1.8 mM. The Km for Mn-ATP is 0.53 mM. Mellitin, a bee venom peptide, strongly inhibited AC activity by 66% in the absence of added CaM and totally abolished CaM-dependent enzyme activity. Mellitin inhibition could be reversed by the addition of equimolar concentrations of CaM. AC activity was resistant to high non-physiological concentrations (10 mM) of $CaCl_2$. The molecular weight of this AC is estimated to be 45 Kd by gel filtration.

To the best of our knowledge, this is the first demonstration of the involvement of CaM in regulation of mammalian sperm AC, thus suggesting a physiological mechanism for the control of this enzyme.

10 EFFECTS OF ATRIAL NATRIURETIC FACTOR (ANF) ON EXOCRINE PANCREAS. S. Heisler, H. Kopelman* J.G. Chabot and G. Morel . Unité de Biorégulation cellulaire, CHUL, Sainte-Foy, Québec, *Department of Pediatrics, Montreal Children's Hospital, Montréal, Québec, C.N.R.S., Laboratoire d'Histologie-Embryologie, Université Lyon-Sud, Lyon, France.

We have recently identified immunoreactive ANF in pancreatic acinar cells. The current study consequently was designed to determine if the atrial peptide had any effect(s) on the function of exocrine pancreas. Two study models were used: a) isolated rat pancreatic acinar cells, and b) cannulated rabbit pancreatic duct in vivo. When acinar cells were exposed to rat$_{-9}$ANF (8-33), a concentration-dependent increase in cGMP formation was observed (EC_{50} about 5×10^{-9} M). The peak cGMP response occurred within 2.5 min of exposure of cells to ANF. When rabbits received rat ANF (8-33) i.v. (0.01-0.25 µg/min), cGMP was secreted into pancreatic juice. ANF, alone, did not affect protein or fluid secretion from rabbit pancreas; when co-infused with secretin (0.1 CU/min), which is not an acinar cell secretagogue in rabbits, ANF did not alter the secretin-enhanced secretion of pancreatic juice. In isolated acinar cells, ANF, and by extension cGMP, did not alter a) basal or agonist (carbachol ± DbcAMP, CCK-OP or forskolin)-stimulated amylase secretion, b) [^{3}H]-leucine incorporation into trichloroacetic acid-precipitable protein, or c) [^{3}H]-thymidine incorporation into DNA. These data suggest that cGMP a) is not co-secreted with digestive enzymes into pancreatic juice, b) does not have an intraluminal effect on fluid secretion, and c) is not involved in the secretory function of acinar cells. The mechanism by which acinar cells secrete cGMP into pancreatic juice, as well as the biological significance of both intracellular (acinar), and intraluminar cGMP remain to be elucidated.

11 GENETIC ANALYSIS OF MUTANTS IN CALCITONIN AND VASOPRESSIN RECEPTOR FUNCTION
D.A.Jans, T.J.Resink* and B.A.Hemmings; Friedrich Miescher-Institut, Basel, Switzerland; *ZLF, Kantonsspital, Basel, Switzerland.

In the porcine kidney cell line, LLC-PK_1, the hormones calcitonin and vasopressin activate the cAMP-dependent protein kinase (cAMP-PK) which in turn leads to a 200-fold increase in production of urokinase-type plasminogen activator (uPA). LLC-PK_1 mutant cell lines affected in hormonal responsiveness were analyzed for uPA production, cAMP-PK activation, adenylate cyclase (AC) activation and hormone binding activity. The M18 mutant was found to lack both vasopressin and calcitonin binding activity but showed normal non-receptor mediated stimulation of AC and cAMP-PK activation. The FIB6 and FIB5-N4 mutants lacked calcitonin binding activity, but showed increased vasopressin-binding activity as well as vasopressin-mediated AC and cAMP-PK activation. Somatic cell hybrids between LLC-PK_1 cells and each of the three mutants were derived. The hybrid cell lines from each fusion were found to have normal vasopressin and calcitonin binding activity and hormonal responsiveness. Somatic cell hybrids between M18 and FIB6 also showed normal activities, indicating complementation of the recessive mutations in the two mutants, whereas hybrids between FIB6 and FIB5-N4 showed no complementation of receptor activity. Therefore, two different mutations (those in M18, and FIB6/FIB5-N4) were defined which concomitantly affect metabolism of both vasopressin and calcitonin receptors. This implies the existence of common processing steps for the two receptors prior to the mature membrane form.

12 HOMOLOGY BETWEEN THE HUMAN AND HAMSTER β_2-ADRENERGIC RECEPTORS AND RHODOPSIN REVEALED BY MOLECULAR CLONING.
Brian K. Kobilka, Henrik G. Dohlman, Thomas Frielle, Mark A. Bolanowski, Richard A.F. Dixon, Irving Sigal, Paul Keller, Marc G. Caron, Robert J. Lefkowitz. Duke Univ. Med. Ctr., Durham, NC and Merck Sharp & Dohme Research Laboratories, West Point, PA.

Agonist stimulation of the β_2-adrenergic receptor (β_2-AR) and light activation of rhodopsin lead to coupling of these receptors with GTP binding proteins which mediate transmembrane signaling. Both of these receptors are inactivated by stimulus promoted phosphorylation leading to homologous desensitization of the β_2-AR and light adaptation in the retina. To study the structural features responsible for observed similarities in function of these two receptors we cloned the gene and cDNA for the human β_2-AR. Hydrophobicity analysis of the deduced amino acid sequence for the human β_2-AR reveals 7 hydrophobic domains similar to those observed in the hamster β_2-AR and the visual pigments. These may represent membrane-spanning regions. Amino acid homology between the human and hamster β_2-ARs is 94% in the hydrophobic domains and the putative cytoplasmic loops. There is 75% homology in the extracytoplasmic region and the carboxy terminus. The overall amino acid homology between the β_2-AR and rhodopsin is much less, however 3 of the membrane-spanning regions share ~30% homology. Both mammalian β_2-AR and rhodopsin have a threonine and serine rich carboxy terminus which may be a substrate for specific kinases. These common structural features allow us to make tentative assignments of functional domains.

13 PURIFICATION AND CHARACTERIZATION OF THE RAT OVARIAN RECEPTOR FOR LUTEINIZING HORMONE: STRUCTURAL STUDIES OF SUBUNIT INTERACTION
Satoshi Kusuda and Maria L. Dufau, Mol. Endo. Section, Endo. & Reprod. Res. Branch, NICHD, NIH, Bethesda, MD 20892

We have purified the luteinizing hormone (LH)/human choriogonadotrophin (hCG) receptor by sequential affinity column on wheat germ lectin-Sepharose and hCG-Sepharose. The method was designed to allow also the purification of lactogen receptor from the initial starting material. The purified LH/hCG receptor retained full binding affinity and was identified as a single protein of Mr=70,000 ± 2,400 on SDS gel electrophoresis. The technique is simple and allows rapid purification of microgram amounts of biologically active receptor suitable for further molecular characterization, microsequencing and functional reconstitution studies. Cross-linking studies performed after binding of hCG (radiolabeled in the individual subunit α or β) crosslinked or uncrosslinked to the corresponding unlabeled subunit prior to binding to the purified LH/hCG receptor indicated that the hCG α-subunit undergoes predominant interaction with the receptor molecule. The influence on the β-subunit in this interaction seems to occur mainly through its association with the α-subunit, presumably by conferring specificity to the α-subunit for its hormonal interaction with the receptor. The α-subunit, which is identical within species, has an important role in the receptor binding interaction and biological activity of glycoprotein hormones.

14 CALCIUM AND pH-DEPENDENT AGGREGATION OF CALMODULIN, G. William Lauderdale and Richard M. Hyslop, Department of Chemistry, University of Northern Colorado, Greeley, CO 80639.

Calmodulin has been isolated from bovine testes by a modification of published procedures. The protein was homogeneous based on isoelectric focusing and two-dimensional electrophoresis. The purified protein has an apparent molecular weight of approximately 16,700 daltons as determined by molecular exclusion chromatography. The protein undergoes reversible self-association as a function of calcium concentration and pH of the medium. The largest polymeric form detected by molecular exclusion high pressure liquid chromatography has an apparent molecular weight of approximately 240,000. The biological activities of the polymeric forms as well as possible physiological functions will be presented.

15 EFFECT OF α-HALOACETYL ANALOGS OF FORSKOLIN ON ^{3}H-FORSKOLIN BINDING SITES. A.L. Laurenza and K.B. Seamon Center for Drugs and Biologics, FDA, Bethesda, MD 20892

The 7-bromoacetyl-7-desacetyl (BrAcFsk) and 7-chloroacetyl-7-desacetyl (ClAcFsk) analogs of forskolin were synthesized as alkylating agents to study forskolin binding sites. The inactive 1,9-dideoxy-7-bromoacetyl analog of forskolin (1,9-dideoxy-BrAcFsk) was synthesized as a control for the bromoacetyl group. Forskolin inhibited the binding of ^{3}H-forskolin to human platelet membranes with a Ki of about 40μM while the BrAcFsk and ClAcFsk had Ki's of about 0.5 μM. The 1,9-dideoxy-BrAcFsk was ineffective at inhibiting ^{3}H-forskolin binding. BrAcFsk and ClAcFsk activated adenylate cyclase in platelet membranes with an EC_{50} of about 10 μM. The binding of $_3$H-forskolin was determined in membranes from intact human platelets that had been preincubated with 10 μM BrAcFsk or ClAcFsk. The binding of ^{3}H-forskolin to membranes from pretreated platelets was reduced 95% after treatment with BrAcFsk and 60% after pretreatment with ClAcFsk while pretreatment with 1,9-dideoxy-BrAcFsk did not reduce ^{3}H-forskolin binding. The loss of binding occured within 5 minutes for BrAcFsk and within 15 minutes for ClAcFsk and was due to a decrease in the Bmax with no change in Kd. The irreversible loss of ^{3}H-forskolin binding sites produced by BrAcFsk could be prevented by the inclusion of 200 μM forskolin in the pretreatment buffer. Tritiated BrAcFsk (^{3}H-BrAcFsk) was synthesized in order to identify proteins alkylated. Proteins labelled with ^{3}H-BrAcFsk were separated on a molecular size column and identified by SDS-PAGE and fluorography. The major proteins identified had molecular weights of 135,000 daltons and 50,000 daltons and comigrated with adenylate cyclase activity. These results suggest that the 135,000 dalton protein may be the catalytic subunit of adenylate cyclase.

16 EFFECTS OF ATRIOPEPTIN II AND CYCLIC GMP ON CA^{2+} LEVELS AND CA^{2+} ATPASE IN RAT AORTA AND CULTURED SMOOTH MUSCLE CELLS. Thomas M. Lincoln, Subhash Rashatwar, and Trudy L. Cornwell. Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, AL 36688.

Atriopeptin II (ANP) produced time- and concentration-dependent increases in rat aortic cGMP content. Associated with the increase in cGMP was a decrease in tone and phosphorylase a content which appeared to be secondary to the decrease in cytosolic Ca^{2+}. Other effectors of cGMP in aortic smooth muscle produced similar changes in Ca^{2+} and Ca^{2+}-dependent processes. The mechanisms by which cGMP lowered cell Ca^{2+} in response to ANP appeared to be related to the removal of Ca^{2+} from the cytoplasm as opposed to the inhibition of uptake or release of Ca^{2+}. To further test this idea, we have examined the effects of cGMP-dependent protein kinase (cGPK) on Ca^{2+}ATPase from cultured smooth muscle cells. Ca^{2+}ATPase was stimulated up to 4-fold by 0.02 μM cGPK and 2-fold by calmodulin. The inclusion of both calmodulin and cGPK in the assay resulted in an additive stimulation of Ca^{2+}ATPase activity suggesting that this enzyme may be regulated by a dual mechanism. Stimulation of Ca^{2+}ATPase by cGPK was observed at all Ca^{2+} concentrations (10^{-8} to 10^{-6} M), and kinetic analysis revealed that cGPK increased the V_{max} for ATP hydrolysis from 8 to 18.1 nmol P_i formed/min/mg while the K_m for ATP remained unchanged by cGPK. Cyclic AMP-dependent protein kinase and protein kinase C were either ineffective or less efective than the cGPK in stimulating Ca^{2+}ATPase. These results suggest a possible mechanism of action for cGMP in mediating ANP relaxation through activation of a Ca^{2+}ATPase and subsequent Ca^{2+} eflux from the cell. (Supported by Grants from NSF and NIH.)

17 **CHARACTERIZATION OF STRUCTURE AND ACTIVATION OF A_1 ADENOSINE RECEPTORS BY AGONIST PHOTOAFFINITY LABELLING.** Martin J. Lohse, Karl-Norbert Klotz and Ulrich Schwabe. Pharmakologisches Institut der Universität Heidelberg, FRG.

A_1 adenosine receptors which mediate an inhibition of adenylate cyclase by adenosine have been photoaffinity labelled with R-2-azido-N^6-p-hydroxyphenylisopropyladenosine (R-AHPIA). The label identifies a Mr=35,000 protein as the binding subunit of the A_1 receptor. The electrophoretic mobility of this band is altered by treatment with neuraminidase but not with other glucosidases, suggesting the presence of complex-type carbohydrate chains. Total chemical deglycosylation shows a core protein of Mr=32,000. The covalent occupation of the receptor with R-AHPIA leads to a persistent activation, seen as a persistent reduction of cAMP levels of isolated adipocytes. The analysis of this activation with a model of pharmacological agonism suggests the presence of spare receptors. Spare receptors are also suggested from a comparison of direct binding and response data, indicating that the occupation of 5% of the receptors leads to a 50% reduction of cAMP levels. These data suggest that the A_1 receptor is activated according to the occupation theory. A large amount of spare receptors exists for the reduction of cAMP-levels in isolated adipocytes.

18 SPECIFIC BINDING SITES FOR ATRIAL NATRIURETIC PEPTIDE IN HUMAN BRAIN.

Fiona Lyall, Tony J. Balmforth and James J. Morton.
MRC Blood Pressure Unit, Western Infirmary, Glasgow, G11 6NT, Scotland.

Specific binding sites for atrial natriuretic peptide have been identified in a membrane preparation obtained from cultures of the human astrocytome cell line G-CCM. Scatchard transformation of the binding data showed marked curvature consistent with the presence of high and low affinity binding sites. Estimation of the Kd (<250 pM) for the high affinity binding site is in the range expected of a physiological receptor. HPLC showed that there was significant breakdown of the radiolabelled ANP even at 0°C.

Neither angiotensin II arginine-8-vasopressin or bradykinin competed for the binding sites, demonstrating further the specificity of binding. Binding affinity of ANP-related peptides was ANP (human 28aa) > ANF (rat 8-33) > atriopeptin III (rat) > atriopeptin II (rat > atriopeptin I (rat) > ANP fragment (1-11 rat).

Our preliminary data also suggests that ANP stimulates cGMP formation in this cell line, confirming recent work which has shown that high concentrations of ANP stimulated a 100-fold increase in cGMP in primary astroglia rich brain cell cultures.

These results suggest that ANP receptors are exposed on the cell membrane of neuroglia. The implications of this finding will be discussed in terms of possible regulatory roles for glia.

19 CYCLIC AMP AND CYCLIC GMP STIMULATION IN PRIMARY CULTURES OF CEREBELLAR GRANULE CELLS.

A. Novelli and R. C. Henneberry, Laboratory of Molecular Biology, NINCDS, National Institutes of Health, Bethesda, MD 20892.

Primary cultures from the cerebellum of the 8-day old rat can be highly enriched for glutamatergic neurons (granule cells) and can serve as a useful model for studying responses to neurotransmitters. Inositol phospholipid turnover, calcium influx, and guanylate cyclase activation follow stimulation by excitatory amino acids in these neurons. We now report that the calcium ionophore A23187 stimulated cGMP synthesis in a calcium-dependent manner; furthermore, a 3-fold greater response was seen when 1 mM $MgCl_2$ was also present. Other divalent cations such as Mn^{++}, Co^{++}, Ni^{++}, and Cd^{++} also modulated cGMP synthesis, but to varying degrees. Although glutamate and kainate strongly stimulated cGMP production, they had a negligible effect on cAMP synthesis in cultures enriched for neurons. However, kainate caused a significant increase in the intracellular content of cAMP in mixed cultures of astrocytes and neurons. In support of the hypothesis that the increase in cAMP following addition of kainate was due to kainate-induced release of adenosine, the adenosine analogue cyclohexyladenosine strongly stimulated cAMP synthesis in mixed cultures but had very little effect in cultures enriched for neurons. Veratrine, which stimulates cGMP production as strongly as kainate, did not elevate cAMP levels in these mixed cultures. These results suggest that (1) elevation of cAMP levels in response to kainate in mixed cultures is due to the release from neurons of adenosine which then acts at an A_2 receptor on astrocytes; and (2) the increase in cGMP levels is not sufficient to promote the release of adenosine.

20 ALPHA$_2$-ADRENERGIC RECEPTOR - LIGAND INTERACTIONS RETAIN THEIR SENSITIVITY TO Na^+ FOLLOWING PARTIAL PURIFICATION USING AFFINITY CHROMATOGRAPHY. Jodi M. Nunnari, Mary G. Repaske and Lee E. Limbird, Department of Pharmacology, Vanderbilt University, Nashville, Tennessee.

Alpha$_2$-adrenergic receptors (α_2AR) inhibit adenylate cyclase and interact with ligands in a manner that is regulated by Na^+. Na^+ sensitivity of α_2AR - ligand interactions is retained in digitonin-solubilized preparations of porcine brain: α_2AR affinity for agonists is decreased 20-fold by Na^+ with an EC_{50} of 3mM and a cation selectivity of $Na^+ > Li^+ >> K^+$; Na^+ increases α_2AR affinity for antagonists two-fold with a similar potency and selectivity for monovalent cations. Interestingly, both H^+ and amiloride analogs also influence α_2AR - ligand interactions in an allosteric manner, raising the possibility that the effects of Na^+ may relate to our observation that α_2AR can activate Na^+/H^+ exchange in the NG108-15 neuroblastoma x glioma cell line and that α_2AR - provoked platelet secretion occurs via a pathway involving Na^+/H^+ exchange.

α_2AR purified to a specific activity of ~ 1400 pmol/mg protein using yohimbine - agarose chromatography retain the effects of Na^+ on α_2AR - ligand interactions. Similarly, Na^+ effects are retained after further purification to >6800 pmol α_2AR/mg protein using wheat germ agglutinin agarose chromatography (A homogeneous α_2AR preparation would contain ~ 16,000 pmol/mg protein.) The data suggest that either the Na^+ regulatory site is on the α_2AR binding subunit or that a distinct Na^+ regulatory component is co-purifying with the α_2AR. If the latter interpretation is true, then the component can be isolated and characterized by virtue of its co-purification with porcine brain α_2AR.

21 HETEROGENEITY OF THE ALPHA$_1$ RECEPTOR ASSOCIATED WITH VASCULAR SMOOTH MUSCLE: EVIDENCE FROM FUNCTIONAL AND LIGAND BINDING STUDIES. M. T. Piascik, N. W. Pedigo, K. A. King and M. Babich, Department of Pharmacology, University of Kentucky Medical Center, Lexington, Kentucky 40536

We have examined the alpha$_1$ receptor associated with rabbit aorta in functional and receptor binding studies. In isolated aortic rings, the dose-response curve for (-)metaraminol was not parallel to that of other alpha agonists and was best fit to a quadratic rather than a sigmoid function. The occupancy versus response relationship for (-)norepinephrine, (-)epinephrine and phenylephrine was hyperbolic, whereas the metaraminol occupancy versus response relationship was not. The possibility that metaraminol interacts with different functional groups on the alpha receptor than other agonists was studied in receptor binding studies. In microsomes prepared from frozen aorta, [^{3}H]prazosin bound to a single class of sites (K_D=150.8±7.7 pM; B_{max}=168±39 fmol/mg, and this site exhibited the characteristics of an alpha$_1$ receptor. In microsomes prepared from frozen aorta, aorta shipped in serum on ice (Pel-Freeze Biol.) or aorta from animals killed in our laboratory, metaraminol bound to two sites or affinity states (estimates from fresh tissue: $K_H \sim 0.52$ µM, $K_L \sim 25.7$ µM; $R_H \sim 24.1\%$ $R_L \sim 76\%$). Similarly, norepinephrine bound to two classes of sites in the three microsomal preparations ($K_H \sim 0.02$ µM, $K_L \sim 3.67$ µM; $R_H \sim 50.7\%$, $R_L \sim 49.2\%$). Epinephrine bound to only one site in the three microsomal preparations. Our results from two independent lines of investigation support the idea that there are subtypes of the vascular alpha$_1$ receptor. Furthermore, metaraminol and norepinephrine interact with both classes of sites while epinephrine interacts with only one of these sites.

22 EFFECTS OF HORMONE-INDUCED DESENSITIZATION ON THE HYDRODYNAMIC PROPERTIES OF GONADOTROPIN RECEPTORS FROM A MURINE LEYDIG TUMOR CELL LINE. *R. Victor Rebois and Roy M. Bradley, Membrane Biochemistry Section, Developmental & Metabolic Neurology Branch, NINCDS, NIH, Bethesda MD 20892.*

The hydrodynamic properties of gonadotropin receptors (GR) from the murine Leydig tumor cell line, MLTC-1, were studied. Sucrose density gradient sedimentation in H_2O and D_2O, and gel filtration chromatography were used to estimate the molecular weight of the detergent-solubilized hormone-receptor complex. [^{125}I]-human chorionic gonadotropin (hCG) was bound to MLTC-1 cells and the hCG-receptor complex (hCG-GR) solubilized in Triton X-100. hCG was bound under conditions that allow (37°C) or prevent (0°C) hCG-induced refractoriness of the adenylate cyclase response (i.e. desensitization). In the absence of desensitization, "control" hCG-GR had a estimated molecular weight of 210 kDa whereas the estimated molecular weight of "desensitized" hCG-GR was 160 kDa. Deglycosylated hCG (DGhCG) is an antagonist that binds with high affinity to GR, but fails to stimulate adenylate cyclase or cause desensitization. [^{125}I]-DGhCG was bound to MLTC-1 cells at 37°C and DGhCG-GR solubilized in Triton X-100. The estimated molecular weight of DGhCG-GR was the same as that for the "control" hCG-GR. There was no association of the regulatory component of adenylate cyclase with the soluble "control" hCG-GR. When hCG was cross-linked to GR and solubilized with sodium dodecylsulfate (SDS) the estimated molecular weight was similar to that determined by SDS-polyacrylamide gel electrophoresis, and only half that of the Triton X-100 solubilized "control" hCG-GR.

23 CALMODULIN STIMULATED PARTICULATE GUANYLATE CYCLASE IN CRAYFISH.

W. Riediger and D. Sedlmeier; Universität Bonn, Institut für Zoophysiologie, Endenicher Allee 11-13, 5300 Bonn 1, FRG.

Guanylate cyclases (E.C.4.6.1.2.) from vertebrate tissues exist in a soluble form in the cytosol and a particulate form bound to the membrane, whereas in invertebrate tissues the enzyme seems to be essentially particulate. The particulate guanylate cyclase from crayfish hepatopancreas membranes was investigated with respect to its dependence on Ca^{2+} and calmodulin. The enzyme was almost completely blocked by EGTA and full activity was regained by the addition of Ca^{2+}. Calmodulin stimulated the activity about five-fold. This effect could be abolished by the calmodulin antagonist compound 48/80. These results give evidence that the particulate guanylate cyclase of crayfish hepatopancreas is a Ca^{2+} and calmodulin-dependent enzyme.

24 SEPARATION OF LYMPHOCYTE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES BY ION EXCHANGE HIGH PRESSURE LIQUID CHROMATOGRAPHY. Steven Robicsek, James B. Polson, Joseph J. Krzanowski, Jr., Richard F. Lockey, and Andor Szentivanyi. Departments of Pharmacology and Therapeutics and Internal Medicine, University of South Florida College of Medicine and Section of Allergy and Immunology, James A. Haley Veterans Administration Hospital, Tampa, Florida 33612.

Experiments were carried out to develop a rapid method for the separation of different forms of cyclic nucleotide phosphodiesterase (PDE) from human peripheral blood lymphocytes using high pressure liquid chromatography (HPLC). A Waters HPLC system with a DEAE Protein Pak column packed in ice to reduce loss of enzyme activity was employed. Lymphocytes were purified on Ficoll-Hypaque, then plastic plate adherence was utilized to remove monocytes. The nonadherent cells were repeatedly washed to remove platelets. After Dounce homogenization, 0.75 to 1.0 ml volumes of supernatant extract (from 1.5 to 2 X 10^8 cells) were injected into the HPLC system. Using a linear gradient of 0 to 1M NaCl over a period of 1 hr and a 1 ml/min flow rate, cyclic GMP hydrolyzing activity eluted about 20 mins after injection (corresponding to approximately 0.3M NaCl). Cyclic AMP hydrolyzing activity eluted in a broad peak between 22 and 30 mins (0.38 to 0.48M NaCl). The latter peak appeared to contain two or more partially resolved forms of the enzyme. This finding suggests that the multiple forms of lymphocyte cAMP-PDE that have been separated by density gradient sedimentation (Thompson et al, J. Biol. Chem. 251: 4922-4929, 1976) and isoelectric focusing (Wedner et al, J. Immunol. 123: 725-732, 1979) cannot be entirely attributed to modification of a single molecular species during the relatively long time-periods required for these separation procedures (15-18 hrs for sucrose gradients, 20 hrs for isoelectric focusing).

25 CHARACTERIZATION OF RABBIT RETINAL DOPAMINE SENSITIVE ADENYLATE CYCLASE WITH NEW TYPES OF D_1-AGONISTS (e.g. SKF 38393-A) OR -ANTAGONISTS (e.g. SCH 23390). M. Schorderet, School of Pharmacy, Lausanne and Department of Pharmacology, CMU, CH 1211 Geneva 4, Switzerland.

Homogenates of rabbit retina were used to study the effect of new dopamine D_1-agonists and -antagonists on the generation of cAMP in absence or presence of dopamine (DA) or of various agents acting on the N_s regulatory protein and/or on the catalytic site of adenylate cyclase (NaF, GTP, forskolin). SKF 38393-A was found to stimulate cAMP generation in a dose-dependent manner and to be more potent than DA (ED_{50} = 0.1 µM compared to 1 µM for DA). Maximal effects were already obtained at 1 µM. However, SKF 38393-A was less efficacious than DA (respectively 160 % and 210 % stimulation over controls). The SKF-induced accumulation of cAMP was blocked by (+)-butaclamol, and not by (-)-butaclamol, although the active concentration of the antagonist was larger than that used for DA blockade, reflecting a higher affinity of SKF for D_1-receptors. On the other hand, the agonist actions of DA or SKF 38393-A were inhibited by a new D_1-receptor antagonist, SCH 23390, in a dose-dependent manner. Other studies with various drugs (including new D_2-receptors-agonists, e.g. LY 171555 or RU 24926, and classical D_2-antagonists, e.g. domperidone or sulpiride) indicate that rabbit retinal homogenates are very selective for the screening of agents acting at D_1-receptors and the study of their possible interaction(s) with other factors acting at regulatory or catalytic subunits of the enzyme. Supported by SNSF Grant No. 3.969.084.

26 SHALLOW AGONIST COMPETITION BINDING CURVES FOR β-ADRENERGIC RECEPTORS : THE ROLE OF TIGHT AGONIST BINDING.

Y. SEVERNE[1], A. IJZERMAN[2], V. NERME[3], T. ABRAHAMSSON[3], H. TIMMERMAN[2], G. VAUQUELIN[1],
[1]Inst. of Mol. Biol., Free Univ. of Brussels (VUB), 65 Paardenstraat, B-1640 St.-Genesius-Rode, Belgium, [2]Dept. of Pharmacochem., Free University, De Boelelaan 1083, 1081HV Amsterdam, Netherlands, [3]Dept. of Pharmacol. and Biochem., Hässle Cardiovascular Res. Lab., S-3183 Mölndal, Sweden

The β_2-adrenergic receptors of bovine trapezius muscle membranes demonstrate tight agonist binding resulting from the formation of H.R.Ns complexes. Preincubation of the membranes with (-)-isoproterenol (followed by washing) causes a time- and concentration-dependent decrease in the number of (-)-(^{3}H)-dihydroalprenolol binding sites to a plateau value of 41.5 %. This decrease is quasi-irreversible under radioligand binding conditions but is readily reversed in the presence of GTP. The isoproterenol/(-)-(^{3}H)-dihydroalprenolol competition binding curves are shallow. This is usually interpreted in terms of two interconvertible affinity states of the receptor : the high affinity state reflecting the coupling of the H.R complex to Ns and the low affinity state not interacting with Ns. However, with increasing incubation time the competition curves are shifted to the left. This apparent non-equilibrium can be computer simulated by models in which tight agonist binding to part of the receptor population is included. The usual computerized interpretation of the competition binding curves do not allow the correct evaluation of agonist binding parameters in the presence of tight agonist binding.

27 SOLUBILIZATION AND RECONSTITUTION OF THE D-1 DOPAMINE RECEPTOR FROM RAT STRIATUM.

Anita Sidhu and Peter H. Fishman, Membrane Biochemistry Section, Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, The National Institutes of Health, Bethesda, Maryland 20892.

The D-1 dopamine receptor was extracted from rat striatal membranes with 0.7% sodium cholate and 1.M NaCl. Pretreatment of the membranes with a D-1 specific agonist (SK & F R-38393), inclusion of crude phospholipids in the solubilization buffer, and subsequent removal of the detergent led to a maximal extraction of 48% of the receptor binding sites. The D-1 antagonist, (^{125}I)SCH 23982, bound to a single class of sites with a K_d of 1.8 nM and a B_{max} of 1.65 pmol/mg protein. The solubilized receptors retained the ability to discriminate between active and inactive enantiomers of agonists and antagonists selective for the D-1 receptor. Approximately 70% of the solubilized receptors were reconstituted into phospholipid vesicles. The reconstituted receptors retained the pharmacological properties typical of the D-1 dopamine receptor.

28 CHARACTERIZATION OF β-ADRENERGIC RECEPTORS FROM ISOPROTERENOL-DESENSITIZED TURKEY ERYTHROCYTES. J. M. Stadel, R. Rebar, S. T. Crooke. Smith Kline and French Labs., Phila., Pa. 19101

Preincubation of turkey erythrocytes (TE) with 10^{-6}M isoproterenol (ISO) resulted in a 50-60% decrease in agonist stimulated adenylate cyclase activity. Desensitization was accompanied by 1) decreased mobility of β-adrenergic receptor (BAR) proteins, specifically photoaffinity labeled with ^{125}I-p-azidobenzylcarazolol (IPABC), as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and 2) a 2-3 fold increase in phosphate incorporation into BAR (Stadel et al, PNAS, 80:3137, 1983). Limited-digestion peptide maps of IPABC-labeled BAR from control and desensitized TE showed different sensitivities of the two BAR to cleavage by chymotrypsin and S. aureus protease. The altered mobility of IPABC-labeled BAR from desensitized TE was eliminated when 5M urea was included during SDS-PAGE. Using a low crosslinked polyacrylamide gel (Dreyfuss et al., Mol. Cell. Biol., 4:415, 1984) the 42,000M_r BAR protein was resolved into a doublet compared to the single 38,000M_r BAR protein of control. Appearance of the doublet was agonist dependent since incubation of TE with dibutyrylcAMP (5mM) did not promote formation of the doublet but decreased agonist stimulated adenylate cyclase activity 40-50%. Exposure of TE for 20 hr at 37°C to $^{32}P_i$ labels BAR. ^{32}P-BAR was partially purified by affinity chromatography over alprenolol-Sepharose. The 42,000M_r protein of ^{32}P-BAR from ISO-desensitized TE also revealed a doublet on SDS-PAGE. Limited-digest peptide maps of ^{32}P-BAR from ISO-desensitized TE using papain identified a unique peptide (28000M_r) absent in control. This unique ^{32}P-peptide was found only in the upper BAR protein band of the doublet. These data provide evidence that agonist promoted phosphorylation results in a conformational change in BAR and suggest that agonists induce desensitization of BAR in TE by a multistep mechanism.

29 ACTIVATION OF ALPHA ADRENOCEPTORS AND THE cGMP RESPONSE IN MOUSE HEART. Y. Vulliemoz, M. Verosky, Dept. of Anesthesiology, Col. of Physicians & Surgeons, Columbia Univ., New York, NY, 10032, U.S.A.

It was previously shown that stimulation of alpha adrenoceptors increases cGMP content in the heart. In an attempt to characterize the alpha adrenoceptor associated with this effect, mice were given i.p. alpha adrenergic agonists and antagonists with different alpha-2/alpha-1 activity ratios and were sacrificed by microwave radiation at an optimal time after treatment. Cyclic GMP was measured in myocardial tissue by radioimmunoassay. Oxymetazoline (OX), St-91 [(2,6-diethylphenylamine)-2-imidazoline], phenylephrine (PE) and methoxamine (ME) increased myocardial cGMP content. Their dose-response curves were parallel (slope: 801 ± 54, 858 ± 78, 857 ± 53 for OX, St-91, PE, resp.) and the order of potency corresponded to their affinity for the alpha-2 adrenoceptor; the dose of agonist required to increase cGMP by 500 fmole/mg protein (ED_{+500}) from a control value of 169 ± 13 was 0.94 ± 0.08, 1.19 ± 0.11, 4.96 ± 0.07 and > 12 µmole/kg, resp. for OX, St-91, PE and ME. The alpha-2 adrenergic antagonists yohimbine and rauwolscine (5 and 0.2 mg/kg, resp.) inhibited the increase in cGMP induced by equieffective doses of all four agonists by 50 to 60%. In contrast, the alpha-1 adrenergic antagonists prazosin and corynanthine (1 and 2.5 mg/kg, resp.) inhibited the cGMP response to PE and ME, phenylethylamine derivatives, but did not affect that to OX and St-91, imidazoline derivatives. Furthermore, the cGMP response to St-91 was not altered by chemical sympathectomy; the slopes of the dose-response curves were 858 ± 78 and 924 ± 85 and the ED_{+500} were 1.19 ± 0.11 and 1.28 ± 0.11 µmole/kg in mice with intact adrenergic nerve endings and treated with 6-hydroxydopamine, resp. The results suggest that the increase in myocardial cGMP content induced by the imidazoline derivatives may be related to activation of alpha-2 adrenoceptors distal, at least in part, to the adrenergic nerve endings while the response to the phenylethylamines may include both an alpha-1 and an alpha-2 mediated component, the latter determining the potency of the agonist.

30 EVIDENCE FOR TWO DIFFERENT STIMULATORY ADENYLATE CYCLASE COUPLING MECHANISMS IN RAT RENAL PAPILLA.

Elizabeth A. Woodcock. Monash University Department of Medicine, and Medical Research Centre, Prince Henry's Hospital, St. Kilda Road, Melbourne. 3004. Australia.

Adenylate cyclase (AC) activity in renal papillary membranes was stimulated by both vasopressin (VP) and the adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA). The two stimulations interacted differently with other AC-stimulatory factors. Treatment of papillary tubules with cholera toxin increased AC activity from 4.5 ± 1.5 to 110 ± 9.1 (SE, n=5) pmol/min/mg protein. Maximally effective concentrations of VP increased activity in control preparations to 10.3 ± 2.8 (an increase of 5.8 ± 1.3) and in cholera toxin treated preparations to 138.9 ± 14.5 (an increase of 28.9 ± 5.4, n=5; $p<.01$). Pertussis toxin increased activity to 9.1 ± 3.0. The response to VP was enhanced to 12.6 ± 3.9 (n=5; $p<.01$ relative to control tubules). Addition of the two toxins together produced a greater than additive stimulation of AC to 145 ± 36. Maximum increase in activity caused by VP was 48 ± 13 (n=5; $p<.01$). In contrast AC stimulation by NECA was additive with stimulations by the two toxins, separately and in combination. The NECA stimulation however, was enhanced in the presence of fluoride ion while the VP stimulation was additive at all concentrations.

Papillary membranes contained two different cyclase-stimulatory coupling proteins with α-subunits of MW's 46,600 ± 450 (SE, n=6) and 41,500 ± 480 (SE, n=6) as identified on SDS-polyacrylamide gel electrophoresis following cholera toxin labeling.

Taken together, these data suggest that two adenylate cyclase-stimulatory coupling mechanisms with different properties are operative in renal papillary membranes.

31 CHARACTERIZATION OF THE GLUTAMATE-INDUCED STIMULATION OF PHOSPHOINOSITIDE METABOLISM IN HIPPOCAMPAL SLICES OF THE ADULT RAT

P.A. Baumann and P.C. Waldmeier, Research Department, Pharmaceuticals Division, CIBA-GEIGY Ltd., CH-4002 Basle, Switzerland

In hippocampal slices of the adult male rat glutamate (glu) dose-dependently increased the formation of ^{3}H-inositol-1-phosphate (3 to 5 fold at 5 mM). This effect appeared to be specific, since it was still present when α_1-adrenergic, cholinergic, serotoninergic and histaminergic receptors were blocked. Aspartate also increased PI-turnover to a comparable extent. With respect to the stereoselectivity, L-glu was about twice as active as D-glu. With aspartate no stereoselectivity was observed. It seems that the receptor involved in the glu-induced PI-turnover is of the quisqualate type. Of the selective exitatory amino acid agonists quisqualate and AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) were most effective (quisqualate beeing approx. equipotent with glu). The effects of NMDA and kainate were weaker. Ibotenate also proved to very potently induce PI-turnover (7-fold at 1 mM). The effect of carbachol was additive to those of noradrenaline and histamine suggesting that the involved receptors do not belong to the same PI-metabolic unit. On the other hand, glu-induced PI-turnover was not additive to that induced by carbachol, noradrenaline and histamine. Therefore, the glu receptors may occur linked to common PI-metabolic units in combination with other receptor types.

32 β-ADRENERGIC RECEPTOR KINASE: ROLE IN MEDIATING HORMONE INDUCED DESENSITIZATION.
J.L. Benovic, F. Mayor, Jr., R.H. Strasser, C. Staniszewski, K.L. Somers, M.G. Caron and R.J. Lefkowitz. Duke Univ. Med. Ctr., Durham, NC and NIH, Bethesda, MD

The actions of many hormones and drugs which act through the adenylate cyclase system, as well as many other systems, are ultimately blunted by desensitization. Agonist-specific or homologous desensitization has been extensively characterized using the β-adrenergic receptor-coupled adenylate cyclase system. Homologous desensitization is cAMP-independent and intimately associated with phosphorylation of the β-receptor. We have identified a novel protein kinase, termed the β-adrenergic receptor kinase (βARK), which specifically phosphorylates only the agonist-occupied form of the receptor. β-agonists induce an ∿ 10 fold increase in β-receptor phosphorylation which can be completely blocked by β-antagonists. Moreover, partial agonists appear to induce only a submaximal increase in receptor phosphorylation. Receptor phosphorylated by βARK has a greatly diminished ability (∿ 80%) to interact with N_s suggesting that phosphorylation can directly uncouple receptor and N_s. βARK has been purified to homogeneity from bovine cerebral cortex via sequential chromatography on gel permeation, ion exchange and hydroxylapatite columns and appears to consist of a single polypeptide chain of M_r ∿ 80,000. βARK does not phosphorylate general kinase substrates such as casein and histones; however, rhodopsin, the retinal receptor for light, serves as a good substrate for βARK in a light (agonist) dependent fashion. Furthermore, rhodopsin kinase, which is involved in regulating cGMP phosphodiesterase activation by rhodopsin in the visual transduction system, can phosphorylate the β-receptor in a totally agonist dependent fashion. Thus the mechanisms which regulate these disparate signaling systems appear to be quite similar.

33 PROTEIN KINASE A AND C CATALYZE PHOSPHORYLATION OF β_2- and α_1-ADRENERGIC RECEPTORS.
M. Bouvier, L.M.F. Leeb-Lundberg, J.L. Benovic, M.G. Caron and R.J. Lefkowitz, Duke University Medical Center, Durham, NC

Phosphorylation of the β_2-adrenergic receptor (β_2-AR) and of the α_1-adrenergic receptor (α_1-AR) is associated with desensitization of their respective transmission pathways. This study was therefore undertaken to explore and document the ability of protein kinase A (PKA) and C (PKC) to directly phosphorylate the purified β_2- and α_1-AR. Both the catalytic subunit of PKA and the PKC, purified from bovine heart and pig brain respectively, were able to phosphorylate the affinity purified hamster lung β_2-AR (1 mol and 0.4 mol phosphate/mol receptor respectively). The addition of a β_2-AR agonist, isoproterenol (20 μM), to the phosphorylation mixture increased the rate but not the extent of phosphorylation of the β_2-AR by PKA but not by PKC. Phosphopeptide separation, by reverse phase HPLC, of the trypsinized receptor previously phosphorylated by either kinase revealed identical peptide map profiles suggesting similar sites of phosphorylation for the two kinases. The α_1-AR purified from DDT$_1$ smooth muscle cells could also be phosphorylated by the two kinases (3 mol PO_4/mol PKC, 1 mol PO_4/mol PKA). In contrast with the β_2-AR the addition of an α_1 agonist, norepinephrine (100 μM), increased the rate but not the extent of phosphorylation of the α_1-AR by the PKC only. The phosphopeptide map of the trypsinized α_1-AR phosphorylated by PKC drastically differed from the map obtained for the receptor phosphorylated by PKA. Therefore the β_2-AR and the α_1-AR are both substrates for PKA and PKC. However, agonist occupancy of the receptors facilitates their phosphorylation only by the protein kinase coupled to their own transmission pathway. "Feedback" and "cross system" phosphorylation may represent distinct and differently regulated mechanisms of receptor modulation.

34 ADENYLATE CYCLASE OF PERMEABILIZED REFRACTORY C6-2B CELLS REVEAL DIFFERENCES IN HETEROLOGOUS DESENSITIZATION INDUCED BY ISOPROTERENOL OR DIBUTYRYL CYCLIC AMP.
Gary Brooker, Department of Biochemistry, Georgetown University Medical Center, Washington, D.C. 20007.
The adenylate cyclase of permeabilized cells is stimulated more than 100 fold by isoproterenol (ISO) or forskolin and responds in a manner analogous to whole cells (J.Cyc.Nucleo.Protein Phos.Res., 11,113-122,1986). C6-2B cells were grown in 16mm plastic cell culture wells and cyclic AMP determined in whole cells or adenylate cyclase activity determined after incubation of permeabilized cells with 10 uM ISO, 0.1 mM ATP and 1 uM GTP. In intact cells preincubated with 10 uM ISO or 1mM dibutyryl cyclic AMP for 4 hrs, the 30 min ISO rechallenge response in whole cells was 1.5% and 12% of the control response respectively. The cyclase activity from cells preincubated with ISO was 24% of control. In contrast, permeabilized cell cyclase from cells made refractory by dibutyryl cyclic AMP was 94% of control activity and thus permeabilized adenylate cyclase was not refractory. The subsequent ISO rechallenge response in whole cells was greater if the protein synthesis inhibitor, cycloheximide (CH) (5 ug/ml) was included during the induction of refractoriness with ISO (12% of the control response vs 1.5% without CH) or dibutyryl cyclic AMP (85% of the control response vs 12% without CH). While CH did not totally prevent ISO induced refractoriness, the subsequent ISO response was increased 8 fold by CH. CH almost completely prevented dibutyryl cyclic AMP induced refractoriness in whole cells. The protective effect of CH on ISO induced refractoriness was evident in permeabilized cyclase assays from isoproternol and CH treated cells (24% of the control response vs 12% without CH). These experiments demonstrate that ISO induced refractoriness causes a protein synthesis dependent stable change in hormone stimulated adenylate cyclase associated with the cell membrane. In contrast, dibutyryl cyclic AMP induced refractoriness to ISO is almost totally dependent upon protein synthesis and the refractoriness is lost upon permeabilization of the cells, suggesting that the refractoriness induced by dibutyryl cyclic AMP is mediated by factors which are loosely associated with the cell membrane and are lost during permeabilization of the cells. Supported by NIH Grant 28940.

35 **B lymphocyte antigen receptors (mIg) BSF1 receptors and Ia tranduce signals by distinct but interactive second messenger cascades.** J.C. Cambier, K.M. Newell, L.B. Justement, Z.Z. Chen, M.J. Klemz, J.T. Ransom and L.K. Harris. Division of Immunology, National Jewish Center for Immunology, Denver, CO 80206

Quiescient B lymphocytes stimulated with antigen or surrogate anti-immunoglobulin (Ig) antibodies, or B cell stimulatory factor 1 (BSF1) undergo a rapid increase in expression of genes encoding c-fos, c-myc, and MHC class II molecules. Ligands which bind type II major histocompatibility antigens (Ia) antagonize signaling via both mIg and BSF1 receptors and also block lipopolysaccharide induced B cell mitogenesis. We report findings which indicate that B cell antigen receptors (mIg) transduce signals via activation of polyphosphoinositide hydrolysis, Ca^{++} mobilization, protein kinase C translocation to the plasma membrane, and membrane depolarization. BSF1 receptor utilize a cascade involving phosphorylation of a 44,000 Da membrane protein, possibly the BSF1 receptor, but none of the above mentioned PIP2 hydrolysis attendant events. Ia molecules appear to transduce signals utilizing a ß adrenergic-type mechanisms involving activation of adenylate cyclase, elevation of cellular CAMP levels and translocation of protein kinase C to the nuclear fraction. The biologic effects (including PKC translocation) of mIa ligation are mimicked by elevation CAMP using db-CAMP, 8br-CAMP, forskolin, PGE_2 and Cholera toxin. Results suggest a novel mechanism by which α1, and ß adrenergic signal transduction cascades may interact antagonistically, which involves competition for the cellular pool of protein kinase C. Research supported by PHS grants AI 20519 and AI 21768.

36 **RELATIONSHIP OF OPIATE RECEPTOR BINDING SITES AND OPIATE-INHIBITED ADENYLATE CYCLASE IN BRAIN MEMBRANES.** S.R. Childers, P. Nijssen and J. Harris, Dept. of Pharmacology, Univ. of Florida College of Med., Gainesville, FL 32610.

Adenylate cyclase appears to be coupled to delta opiate receptors in NG108-15 cells, but the pharmacological nature of the adenylate cyclase-linked opiate receptor in mammalian brain has yet to be established. To determine the relationship between high affinity opiate receptor binding sites and adenylate cyclase in brain, we assayed membranes after treatment with agents that block high affinity opiate agonist binding. Incubation of membranes with phospholipase A (500 ng/ml) inhibited 93-100% of the binding of [^{3}H]-DAGO, [^{3}H]-DSTLE and [^{3}H]-EKC to mu, delta, and kappa sites, respectively. However, adenylate cyclase in treated membranes was still inhibited by D-Ala enk; moreover, the D-Ala enk inhibition curve was not affected by phospholipase treatment. To obtain more specific receptor blockade, membranes were incubated with irreversible opiates, then washed to remove reversibly-acting drugs; control membranes contained equal concentrations of naloxone to control for washing. Incubation with the irreversible antagonists naloxone azine and beta-FNA, and the irreversible agonist p-nitro-phenyl-oxymorphone, did not affect adenylate cyclase inhibition curves, but blocked high affinity receptor binding by 75-100%. Incubation with beta-CNA (10 μM), which blocked binding by 95-100%, shifted the adenylate cyclase dose response curve for D-Ala-enk by approximately 10-fold. These results indicate an irreversible loss in adenylate cyclase-linked receptors by beta-CNA, but not by other irreversible opiates, and suggest that opiate receptors linked to adenylate cyclase in brain do not correspond to any of the sites labeled with nM concentrations of [^{3}H]-agonists.

Supported by grant DA-02904 from the National Institute on Drug Abuse.

37 PHOSPHORYLATION OF THE ß-ADRENERGIC RECEPTOR IN LYMPHOMA CELLS IN RESPONSE TO PMA.

R.B. Clark, J. Friedman, M.W. Kunkel, N. Prashad, and J.A. Johnson. University of Texas Graduate School of Biomedical Sciences, Laboratories of Cyclic Nucleotide Research, P.O. Box 20334, Houston, Texas, 77225, USA.

Treatment of S49 WT lymphoma cells with phorbol 12-myristate, 13-acetate (PMA) caused a 3-5 fold increase in the K_{act} for epinephrine (epi) stimulation of adenylate cyclase (AC) with no change in ß-adrenergic receptor (ßR) levels or any evidence of internalization. It also resulted in a 60-80% loss of GppNHp inhibition of AC. The increased K_{act}, which resulted in considerable inhibition of epi stimulation of AC, closely resembled the increased K_{act} induced by epi treatment of cells. Low free Mg^{++} (<1.0 mM) was required to observe these inhibitory effects of PMA reflecting about a 6-fold increase in the K_{act} for Mg^{++} stimulation of AC. To test the hypothesis that the PMA-induced inhibition of epi stimulation of AC involved protein kinase C (PKC)-mediated phosphorylation of the ßR, cyc^- cells were labeled with [^{32}P]PO and then treated with either 0.5 μM PMA or 1.0 μM epi for 5 min. Incorporation of the ^{32}P into the ßR was determined following purification of the ßR by alprenolol affinity chromatography, SDS-PAGE and autoradiography. The mobility of the purified ßR on SDS-PAGE was identified by photolabeling with [^{125}I] iodoazidobenzylpindolol (IABP). The M_r of control and PMA-treated ßR was 65 kDal. PMA caused a significant increase in the phosphorylation of the ßR which exceeded that induced by epi by about 2-fold. There was no significant phosphorylation of the ßR in untreated cells; thus, the PMA-induced phosphorylation could not be expressed as a fold increase over control. Our results suggested that PMA activation of PKC could result in phosphorylation and desensitization of the ßR. However, analysis of the functions of the purified receptor following intact cell phosphorylation will be necessary before any firm conclusions can be drawn.

38 ROLE OF PERTUSSIS TOXIN (PT) SENSITIVE GTP BINDING PROTEINS IN THE REGULATION OF FRTL-5 RAT THYROID CELLS. Daniela Corda and Leonard D. Kohn, LBM, NIDDK, NIH, Bethesda, MD. 20892

PT-sensitive GTP binding proteins regulate cAMP dependent and independent mechanisms leading to the iodination of thyroglobulin and the formation of thyroid hormones. The inhibitory regulatory protein of the adenylate cyclase, N_I, is present in FRTL-5 cells, as shown by PT induced ADP ribosylation of a 40K protein identified as α_I of N_I. Pretreatment of FRTL-5 cells with TSH (1 h) as well as PT (18 h) causes the disappearance of α_I. This can be demonstrated by the loss of 40K substrate available for PT induced ADP ribosylation in the membrane preparations. PT and TSH individually stimulate cAMP accumulation in FRTL-5 cells; however when PT is added to TSH stimulated cells, a small decrease of the cAMP level is observed. In contrast the PT and TSH effects are additive to forskolin and cholera toxin stimulation of adenylate cyclase. Coordinate TSH modulation of PT induced ADP ribosylation and cAMP accumulation, suggests a role for N_I in the TSH stimulation of adenylate cyclase activity. Both TSH and α_1 adrenergic agents utilize the IP_3-Ca^{++}-arachidonic acid signal system to regulate iodide efflux in cells chronically exposed to TSH; these cells have a desensitized adenylate cyclase response to TSH but maximal iodide uptake. Pretreatment of FRTL-5 cells with 10^{-10} M PT for 4 h inhibits the increase in iodide efflux induced by norepinephrine (NE). Under these conditions there is no effect of PT on NE induced increase in IP_3 or cytosolic Ca^{++} but there is complete inhibition of arachidonic acid release. There is no effect of PT on the ability of TSH to increase any of these activities. Therefore, although both TSH and NE use the IP_3-Ca^{++}-arachidonic acid signal system, the coupling of α_1 adrenergic, but not TSH receptor to phospholipase A_2 is mediated by a PT sensitive GTP binding protein.

39 DIPHTHERIA TOXIN AND CYCLOHEXIMIDE PREVENT CATECHOLAMINE DESENSITIZATION OF A431 CELLS.
Mariadele DeBernardi and Gary Brooker, Department of Biochemistry, Georgetown University Medical Center, Washington, D.C. 20007.
Our laboratory has previously shown in C6-2B rat astrocytoma cells that inhibitors of RNA and protein synthesis can reduce or prevent catecholamine refractoriness. Thus our working hypothesis is that induction of protein synthesis, after stimulation of cyclic AMP accumulation in C6-2B cells, produces an inhibitory protein which inhibits subsequent catecholamine stimulation of adenylate cyclase. Monolayer cultures of A431 cells, a human epidermoid carcinoma cell line, responded to 10 uM isoproterenol (ISO), increasing intracellular cyclic AMP up to 1000 fold above basal after 30 minutes depending upon passage number. Basal values ranged from 9-35 pmol/mg protein. After 4 hours of ISO pretreatment the cells became desensitized to subsequent ISO treatment. Cycloheximide co-incubation with ISO during this first 4 hour incubation prevented the decline in cyclic AMP, and enhanced, more than seven fold, the accumulation of cyclic AMP after a subsequent 30 minute rechallenge with ISO. Diphtheria toxin, which inhibits protein synthesis by ADP-ribosylation of elongation factor II was found to prevent catecholamine refractoriness in A431 cells. A431 cells were intoxicated with 1 ng/ml of the toxin for 24 hours. Protein synthesis was inhibited more than 88% however the cells were still viable since they excluded the vital stain, trypan blue. These toxin treated cells still responded well to ISO yielding 9108 pmol/mg protein in 30 minutes compared to control cells which produced 11,292. After 4 hours of ISO treatment, in control cells cyclic AMP had declined to 2158, and strikingly, in diphtheria toxin treated cells, cyclic AMP was 6286 thus demonstrating the protective effect of diphtheria toxin upon catecholamine desensitization. Supported by NIH Grant No. 28940

40 DIFFERENTIAL COUPLING OF MUSCARINIC RECEPTORS TO HYDROLYSIS OF PHOSPHOINOSITIDES IN GUINEA-PIG CORTEX AND PAROTID GLAND. [1]Ek B, [2]Keen M, Nahorski S. [1]Dept of Pharmacol and Biochem, AB Hässle, S-431 83 Mölndal, Sweden. [2]Dept of Pharmacol and Med Ther, Univ of Leicester, UK.

Muscarinic receptor activation induces a rapid hydrolysis of phosphoinositides in several tissues and allows examination of the relationship between agonist receptor occupancy and phosphoinositide response. In the present experiments, guinea-pig parotid gland and cerebral cortex were labeled with 3-H-myoinositol or 3-H-N-methylscopolamine and stimulated with the full agonist carbachol and the partial agonist oxotremorine in the presence of 5mM of Li^+.

Carbachol showed a 10-fold lower EC_{50}-value (3.7μM) and oxotremorine a 3-fold higher intrinsic activity (40%) in the parotid gland than in the cortex, suggesting possible differences in receptor coupling to the phosphoinositide response in the two tissues. This possibility was examined by incubating parotid gland and cerebral cortex slices with the alkylating agent benzylcholine mustard, 0.1μM, in order to inactivate muscarinic receptors. Reduction in specific muscarinic binding sites was accompanied in the parotid gland by a rightward shift of the carbachol dose-response curve and, at higher receptor loss, by a suppression of the maximal response. In cerebral cortex, however, only a decrease in the maximal response was seen. In both tissues, the maximal response of the partial agonist oxotremorine was suppressed without changing the position of the dose-response curves. The binding affinity of the agonists, in the presence of GTP (100μM), corresponds well with the estimated agonist potency regarding hydrolysis of phosphoinositides in the benzylcholine treated slices.

These results suggest a difference in the extent of receptor reserve in the parotid gland and cerebral cortex. This may suggest a difference in coupling of the muscarinic receptors to the phosphoinositide response in the two tissues.

41 P_2-PURINERGIC RECEPTORS MEDIATE INOSITOL PHOSPHATE AND PROSTACYCLIN FORMATION IN BOVINE ADRENAL MEDULLARY ENDOTHELIAL CELLS. E. Forsberg, G. Feuerstein, E. Shohami and H. Pollard, LCBG, NIDDK, NIH, Bethesda, MD 20892.

Adrenal medullary endothelial cells are closely juxtaposed to adrenal chromaffin cells but the nature of their interaction is for the most part unknown. Since high concentrations of products released from chromaffin cells certainly bathe the endothelial cells, we reasoned that these products may affect endothelial cell metabolism. We have found that one component secreted from chromaffin cells, ATP, stimulates inositol phospholipid metabolism and prostacyclin formation in cultured adrenal medullary endothelial cells. Within 15 sec after the addition of 1 mM ATP, inositol trisphosphate (IP_3) and inositol bisphosphate (IP_2) levels peak at greater than twice control levels. Half maximal stimulation occurs at about 30 uM ATP if IP_3 and IP_2 are measured at 15 sec. ADP is almost as potent and efficacious as ATP but AMP and adenosine have no effect. Other nucleotides such as GTP, CTP, TTP and UTP are less effective in stimulating inositol phosphate production. ATP also more than doubles prostacyclin formation with half-maximal effects occurring at about 20 uM ATP. The nucleotide specificity for prostacyclin formation is similar to that for inositol phosphate production. We hypothesize that ATP released from chromaffin cells interacts with P_2-purinergic receptors on endothelial cells and stimulates the formation of prostacyclin via the generation of the second messenger IP_3 and the subsequent increase in $[Ca^{++}]_{in}$. Prostacyclin-induced vasodilation of the adrenal medulla may enhance the distribution of chromaffin cell products to target organs.

42 GTP-INDEPENDENT STIMULATION OF RABBIT HEART ADENYLATE CYCLASE BY ISOPROTERENOL S.E. Harding and P. Harris, The Cardiothoracic Institute, 2 Beaumont Street, London, W1N 2DX, U.K.

Isoproterenol stimulates adenylate cyclase activity of rabbit heart sarcolemmal preparations by the same proportion (x2) whether or not GTP is added to the assay medium. This GTP-independent stimulation by isoproterenol requires the presence of physiological concentrations of ATP in the assay medium. Lowering ATP from 5 to 0.5mM almost completely abolished stimulation by 0.1mM isoproterenol, while basal cyclase activity was unaffected. Reduction of ATP concentration from 5 to 0.5mM decreased activation by 10 μM GppNHp from x15 to x9 but had no effect on stimulation by 200mM KCl (x5). GTP-independent stimulation by isoproterenol was similar with ATP from equine muscle (Sigma), ATP synthesised from adenosine (Sigma), special quality ATP(BCL) or commercial ATP further purified in our laboratory. ATP does not therefore appear to be a source of contaminating GTP. No ATP regenerating system was present in the experiments because of possible GTP contamination of the reagents. From experiments where 3H-GTP was added to the initial rabbit heart homogenate, it was estimated that the sarcolemmal preparation would contribute about 0.1nM free GTP to the final assay medium. Greater than 10nM added GTP was necessary to mimmick the effect on isoproterenol stimulation of raising ATP from 0.5 to 5mM. The concentration of GDP in cardiac tissue is lower than that of GTP, and GDP is easily removed from cardiac sarcolemma by washing. It is therefore unlikely that transphosphorylation of GDP to GTP by ATP and nucleoside diphosphate kinase could produce sufficient GTP to account for the effects observed. GTP displaced GppNHp when the two were added to the sarcolemmal preparation simultaneously. ATP did not displace GppNHp under the same conditions. We conclude that the effect of ATP on isoproterenol stimulation may be mediated by a site separate from that at which GTP acts.

43 RAT PAROTID ADENYLATE CYCLASE CATALYST ACTIVITY DESENSITIZATION IS COUNTERACTED BY FORSKOLIN. Jeffrey F. Harper, Depts Internal Medicine and Pharmacology, University of Texas Medical School, Houston, TX 77225.

Desensitized adenylate cyclase (AC) reflects intact cell desensitization to isoproterenol (INE), both in its development and its counteraction by 1 μM but not 0.1 μM forskolin. Only 53±7% (n=12) of control activity to stimulation by INE is retained in tissue desensitized by INE prior to membrane preparation ($p<0.01$). 73±5% of normal response ($p<.025$) is retained in desensitized tissue to stimulation by 100 μM forskolin plus 30 mM $MnCl_2$. Forskolin therefore doesn't counteract desensitization in broken cells. Forskolin does counteract desensitization development when included with isoproterenol during initial intact cell incubation with the agonist. Membranes prepared after tissue incubation in which 1 μM forskolin is included with INE during the desensitizing incubation contain fully active AC (measured as response to 100 μM forskolin with $MnCl_2$). Inclusion of 0.1 μM forskolin, on the other hand, does not counteract subsequent AC desensitization ($p>0.1$ vs. control and $p<0.05$ different from effect of 1 μM forskolin), in agreement with intact cell cyclic AMP accumulation studies (Harper, JCNPPR 9,401-414, 1984). AC desensitization is maintained in solubilized preparations. Parotid slices were desensitized to INE, then membranes treated with CHAPS. The solubilized AC retained its desensitization to 100 μM forskolin stimulation during this process, showing reduction to 81±8% of control ($p<.025$, n=18). These data show that desensitization is stable to the effects of CHAPS. $MnCl_2$ uncouples G_s-mediated AC stimulation (by INE or GTP-γ-S), while desensitization to AC stimulation by forskolin is observed. Whatever produces desensitization is intimately associated with AC. INE produces concomitant secretion desensitization. α-Amylase secretion was measured continually on-line to determine secretion rate. With continual stimulation by 2.4 nM INE the secretion rate peaks, then declines so that within 60 min basal secretion rate prevails despite continued INE stimulation. Forskolin counteracts this secretion desensitization essentially immediately, mirroring its ability to do so for cyclic AMP desensitization. Other experiments show that secretion stimulated initially by forskolin plus INE is maintained significantly longer (140 ± 20 min decline time to half of the peak, n=3) than with INE alone (17 ± 2 min, n=4). Forskolin thus also delays secretion desensitization to INE. There is a close correlation between cyclic AMP and secretion desensitization not only during development but also with forskolin's ability to overcome desensitization. *Supported by grants from NIH and CFF.*

44 MECHANISM OF ENHANCED ISOPROTERENOL-STIMULATED CAMP ACCUMULATION IN ADIPOCYTES EXPOSED CHRONICALLY TO AN INHIBITORY A_1 RECEPTOR AGONIST. BB Hoffman, JM Thomas, H Chang and GM Reaven. Stanford University and VA Medical Center, Palo Alto, CA.

Adipocytes contain A_1 receptors which inhibit lipolysis by suppressing intracellular cAMP accumulation. We have found that rats become tolerant to the antilipolytic effects of the A_1 agonist phenylisopropyladenosine (PIA) during 6 days of continuous infusion. Tolerance in adipocytes isolated from PIA-infused rats is likely explained by the 10 fold greater isoproterenol-stimulated cAMP accumulation in these cells than in controls (J. Clin. Invest, in press). The present studies examine the mechanism for the enhanced steady-state cAMP accumulation in response to isoproterenol. Isoproterenol-stimulated cAMP production rate (pmol/10^5 cells/min) was increased in cells from PIA-infused rats: 291 ± 13 vs 77 ± 16 in controls. Isoproterenol-stimulated adenylate cyclase activity was increased 2 fold in membranes from treated cells. Also, the cAMP degredation rate was decreased in cells from PIA-infused rats ($0.18\pm.03$ min^{-1}) compared with controls ($0.34\pm.03$ min^{-1}). The ability of PIA, PGE_1 and nicotinic acid to suppress isoproterenol-stimulated cAMP accumulation was examined. While these agonists each inhibited cAMP accumulation >95% in both groups, EC_{50}'s were increased 100 fold (PIA), 10 fold (PGE_1) and 3 fold (nicotinic acid) in cells from PIA-infused rats. These results indicate that prolonged exposure of adipocytes to PIA leads to greater isoproterenol-stimulated cAMP accumulation due to enhanced production and decreased metabolism of cAMP. Also, these cells are heterologously desensitized to receptor-mediated cAMP inhibition but the largest change occured at A_1 receptors. Some of the results have analogies to adaptations to narcotics that occur in nerve cells.

45 MODULATION OF ADENYLATE CYCLASE ACTIVITY IN NCB-20 CELLS BY THE PROTEIN KINASE C ACTIVATOR, PHORBAL-12-MYRISTATE-13-ACETATE. Elizabeth B. Hollingsworth and John W. Daly, Laboratory of Bioorganic Chemistry, NIH, Bethesda, MD, 20892

The activation of protein kinase C through the addition of phorbal esters, such as phorbal-12-myristate-13-acetate (PMA) modulates responsiveness of the cyclase system, in many cell types. In the neuroblastoma x Chinese hamster embryonic brain explant hybrid cell line (NCB-20) addition of PMA results in a down regulation of receptor-mediated accumulation of cyclic AMP. Down regulation by PMA occurs within 5 minutes and is still apparent at 45 minutes. This occurs in a concentration dependent manner with an EC50 for PMA of 3 x 10-8M. Accumulations of cyclic AMP that are elicited by prostaglandin E_1, vasoactive intestinal peptide or 2-chloroadenosine are decreased in the presence of PMA. Responses to a prostaglandin-forskolin combination are also reduced by PMA. But accumulations of cyclic AMP that are elicited by forskolin in the absence of a receptor agonist are unaffected by the presence of PMA for the first 10 minutes. After 10 minutes the accumulation of cyclic AMP elicited by forskolin alone decreases steadily to a value about 50% of the maximal level attained at about 5 minutes. PMA completely prevents the decline in the forskolin response. Inhibition of cyclic AMP generation by dopamine is not diminished by PMA suggesting the receptor-input through the inhibitory (Ni) guanylnucleotide binding protein is still functional after PMA treatment. The inhibition of receptor-mediated responses by PMA may represent a protein kinase C-mediated enhancement of desensitization, although other mechanisms are possible.

46 ALPHA2-ADRENERGIC RECEPTORS ACTIVATE Na^+/H^+ EXCHANGE IN NEUROBLASTOMA X GLIOMA CELLS. Lori L. Isom and Lee E. Limbird, Vanderbilt University, Nashville, Tennessee.

Alpha2-adrenergic receptors (α_2AR) are one of a population of receptors linked to the inhibition of adenylate cyclase through a GTP binding protein (Gi). However, in many systems, the physiological effects elicited by α_2AR cannot be explained solely by decreases in cAMP, suggesting the existence of other regulatory pathways that are activated by α_2AR occupation. Recent data from our laboratory show that α_2AR on the human platelet activate a phosphatidylinositol-hydrolyzing phospholipase A_2 via a mechanism involving Na^+/H^+ exchange in order to evoke, ultimately, platelet secretion.

We have examined whether α_2AR might activate Na^+/H^+ exchange in NG108-15 neuroblastoma x glioma hybrid cells using the pH-sensitive probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein. When the intracellular pH (pHi) of NG108-15 cells in HCO_3-free medium is reduced by the external application of acetate, the recovery of pH_i to its resting value is blocked by the removal of extracellular Na^+, by the addition of extracellular H^+ and by various amiloride analogs. The recovery of pH_i exhibits an ionic selectivity of $Na^+ > Li^+ > K^+$, with no recovery observed in N-methyl-d-glucamine. These data indicate that the regulation of pH_i in NG108-15 cells under these experimental conditions is due primarily to the functioning of a Na^+/H^+ antiporter.

Na^+/H^+ exchange in NG108-15 cells is accelerated by α_2AR. Thus, (l)-epinephrine, but not (d)-epinephrine, elicits an intracellular alkalinization that is blocked by yohimbine but not prazosin or propranolol. The α_2AR agonists norepinephrine, clonidine and UK-14304 also cause alkalinization of NG108-15 cells, whereas isoproterenol and phenylephrine do not. Furthermore, the effect of α_2AR agonists to alkalinize the interior of NG108-15 cells is blocked by the manipulations utilized above to block Na^+/H^+ exchange. These data indicate that α_2AR in NG108-15 cells activate Na^+/H^+ exchange, and further suggest that the acceleration of Na^+/H^+ exchange may be a mechanism common to α_2AR in many cellular systems.

47 PHOSPHATIDYLINOSITOL HYDROLYSIS IN PERMEABILIZED EMBRYONIC CHICK HEART CELLS. Linda G. Jones and Joan Heller Brown. Department of Medicine, U.C. San Diego, La Jolla, CA 92093

We have shown that stimulation of muscarinic receptors in dissociated embryonic chick heart cells promotes the hydrolysis of the phosphoinositides resulting in accumulation of the breakdown products inositol tris-, bis-, and mono-phosphate (IP, IP_2, and IP_3). In order to address the question of whether a guanine nucleotide-binding protein couples receptor stimulation to PI hydrolysis in the chick heart cell, we developed a saponin-permeabilized cell preparation. The extent of permeabilization was assessed by trypan blue exclusion and the release of lactate dehydrogenase. In the permeabilized cell preparation GTPγS alone was found to increase the accumulation of IP, IP_2 and IP_3, suggesting that a polyphosphatidylinositol phospholipase C was activated. This effect was found to be specific for guanine nucleotides, as Gpp(NH)p and GTP were also effective while App(NH)p was not. The response to GTPγS occurred in a dose-dependent fashion with half-maximal stimulation obtained at 1 μM GTPγS. GTPγS was ineffective in promoting PI hydrolysis in the intact (nonpermeabilized) chick heart cell except at high concentrations (30-100 μM). While carbachol has been shown to stimulate PI hydrolysis in the intact cell, it was ineffective in promoting this response, either alone or in combination with GTPγS, in the permeabilized cell preparation. Muscarinic receptor binding studies demonstrated the same number of receptors on permeabilized as on intact cells. In addition, carbachol was found to effectively compete for [^{3}H]NMS binding sites with the same K_D in both cell preparations, suggesting no change in receptor binding affinity in the permeabilized cell. The loss of the PI response to hormone may reflect an uncoupling of receptor stimulation to PI hydrolysis due to the saponin treatment. That GTPγS alone stimulates this response provides evidence for guanine nucleotide regulation of phosphoinositide turnover in the heart.

48 AGONIST INDUCED PHOSPHORYLATION OF THE CARDIAC MUSCARINIC RECEPTOR. Madan M. Kwatra, Arie Maan, Katharyn K. McMahon, and M. Marlene Hosey. Dept. of Biol. Chemistry & Structure, The Chicago Medical School, 3333 Green Bay Road, N. Chicago, IL 60064.

We have tested the possibility that regulation of cardiac muscarinic receptor function may involve receptor phosphorylation. Chick heart muscarinic receptors were purified from relatively small amounts of tissue to near homogeneity using a three-step chromatographic procedure that utilized the affinity chromatography procedure of Haga and Haga (J. Biol. Chem. 258, 13575-13579, 1983). The purified preparations contained a single major peptide which migrated on $NaDodSO_4$ gels with an apparent M_r of 79,000. When receptors were purified from ^{32}P-bathed hearts, a single major phosphopeptide eluted from the affinity column and comigrated on $NaDodSO_4$ gels with the band of stained receptor. Treatment of hearts with the agonist carbachol led to marked increases (10-12 fold) in the phosphorylation of the receptor. Ligand binding assays performed with membranes isolated from control and carbachol-treated preparations indicated that the carbachol treatment led to decreases in the ability of the receptor to recognize agonist with high affinity. The results show that the muscarinic receptor is a phosphoprotein in cardiac tissue, and that treatment with a receptor agonist regulates its phosphorylation in the intact cell. Phosphorylation of the receptor may be correlated with the process of receptor desensitization. (Supported by NIH grant HL 31601 and the American Heart Association.)

49 CYCLIC GMP AND ALPHA-2 ADRENERGIC RECEPTORS MODULATE TOTAL GLUCOSE UTILIZATION (GLU) AND INSULIN RELEASE IN PANCREATIC ISLETS OF LANGERHANS OF THE RAT. S.G. Laychock, Department of Pharmacology and Toxicology, Medical College of Virginia, Richmond, Virginia 23298.

Dynamic changes in GLU in isolated islets were determined by quantitation of the formation of ^3H_2O from D-[5-^{3}H]glucose. The addition of 8-bromo (Br)-cGMP (0.02-2 mM) or monobutyryl-cGMP to the islets during a linear phase of glucose utilization resulted in concentration- and time-dependent increases in GLU. The effects of Br-GMP occurred within 5 min with 10 mM glucose. Stimulatory effects of Br-cGMP were observed in the presence of cycloheximide. Analogues of cAMP, cIMP, or 5'-GMP did not mimic the effects of Br-cGMP. Insulin release from islets incubated in the absence of glucose was not changed by addition of Br-cGMP (2 mM), however, glucose (10 mM)-stimulated insulin release was potentiated by Br-cGMP. Br-cGMP did not alter insulin secretion in response to glyceraldehyde or 2-ketoisocaproic acid. Islet beta cells are regulated by alpha-2 adrenergic receptors, and GLU and insulin release were inhibited in islets incubated with clonidine or epinephrine; yohimbine antagonized this effect. Br-cGMP increased GLU and insulin release in clonidine- or catecholamine-treated islets. Carbamylcholine, which stimulates cGMP production in islets, reversed the inhibition of GLU by clonidine, and 3-isobutyl-1-methylxanthine enhanced the stimulatory response to carbamylcholine. Carbamylcholine alone did not alter GLU. In conclusion, GLU in the islet is modulated by cGMP. Alterations in GLU probably mediate the effects of cGMP on insulin release. In addition, cGMP antagonizes the inhibitory effects of alpha-2 adrenergic receptor agonism on GLU and insulin release. Supported by NIH grant AM25705.

50 DESENSITIZATION AND PHOSPHORYLATION OF α_1-ADRENERGIC RECEPTORS COUPLED TO INOSITOL PHOSPHOLIPID METABOLISM. L.M.F. Leeb-Lundberg, S. Cotecchia, M.G. Caron and R.J. Lefkowitz. Duke Univ. Med. Ctr., Durham, N.C.

Norepinephrine (NE) acting through α_1-adrenergic receptors (α_1AR) influence cells via inositol phospholipid hydrolysis and generation of the two second messengers inositol trisphosphate and diacylglycerol. Continuous exposure of DDT$_1$ MF-2 smooth muscle cells to NE results in a dramatic attenuation of α_1AR function as measured by NE-stimulated incorporation of $^{32}P_i$ into phosphatidylinositol and phosphatidic acid. In addition, NE exposure reduces the number of accessible cell surface α_1AR as assayed by [^{3}H]prazosin binding to intact cells at 4°C. Purification of α_1AR from ^{32}P-labeled cells desensitized with NE yields the phosphorylated M_r 80K ligand binding peptide. The time courses of receptor phosphorylation and attenuation of receptor number and receptor function are very comparable being half-maximal within 1-2 min. The tumor-promoting esters (PE) (Leeb-Lundberg et al., P.N.A.S. 82, 5651-5655, 1985) and the vasoactive peptide bradykinin also induce desensitization and phosphorylation of α_1AR. However, unlike NE neither PE nor bradykinin reduce the number of cell surface α_1AR. Virtually the same tryptic phosphopeptides are obtained from phosphorylated α_1AR derived from cells treated with NE, PE and bradykinin. The various agents induce phosphorylation on serine and threonine. These results indicate that desensitization of α_1AR is paralleled by an increase in phosphorylation at specific sites in the M_r 80K α_1AR peptide. α_1AR agonists, in addition, promote a decrease in accessible cell surface α_1AR receptors. Thus, phosphorylation of receptors linked to the inositol phospholipid/Ca^{2+} signal transduction pathway may represent an important mechanism of regulation of receptor responsiveness.

51 SYNERGISTIC ACTION OF ADENOSINE (ADO) AND fMET-LEU-PHE IN RAISING cAMP CONTENT OF HUMAN MONOCYTES. E.J. Leonard and K.R.F. Elliott, National Cancer Institute, Frederick, MD 21701.

The release of superoxide by human monocytes in response to fMet-Leu-Phe was inhibited by ADO. Comparison of the potency order of ADO analogues with published data suggested that ADO acts on the stimulatory receptor for adenylate cyclase. Since elevations of cAMP inhibit superoxide release, we determined cAMP content of ADO treated cells, and the effect of stimulation by fMet-Leu-Phe. The Table shows increases of cAMP above the basal level of 1.3 ± 0.2 in 5 experiments.

Stimulus		Increase in cAMP, pmols/10^6 monocytes
A.	fMet-Leu-Phe, 1 min.	1.3 ± 0.2
B.	Adenosine, 3 min.	0.4 ± 0.3
	Sum of A and B	1.7 ± 0.4
C.	Adenosine, 3 min; fMet-Leu-Phe added after 2 min of adenosine	3.5 ± 0.7
	C/(A + B)	*2.2 ± 0.3*

Stimulation of monocytes with fMet-Leu-Phe increased cAMP. This was previously shown for neutrophils, in which the rise in cAMP was attributed to inhibition of phosphodiesterase (PDE), since adenylate cyclase did not increase. When ADO-treated monocytes were stimulated by fMet-Leu-Phe, the increase in cAMP was twice the sum of the increases induced by the 2 agents alone. These data suggest that the increase in cAMP by ADO-induced cyclase activation is limited by the activity of PDE, and that the latter can be inhibited by fMet-Leu-Phe.

52 **A GTP-BINDING PROTEIN MEDIATES SOMATOSTATIN INHIBITION OF CALCIUM CURRENT.** Deborah L. Lewis, Alberto Luini, and Forrest F. Weight. NIAAA, Rockville, MD 20852 and NIMH, NIH, Bethesda, MD 20892.

Somatostatin inhibits the voltage-dependent calcium current measured by the whole-cell patch-clamp technique, in the neurosecretory pituitary cell line AtT-20/D16-16. We tested the involvement of guanine nucleotide-binding proteins, G proteins, in the signal transduction mechanism between the somatostatin receptor and the voltage-gated calcium channel.

The patch-clamp method was used to record the calcium current in the whole-cell voltage-clamp mode. The extracellular solution contained: 150mM TEA-Cl, 0.8mM $MgCl_2$, 5.4mM KCl, 10mM $CaCl_2$, 10mM HEPES/CsOH (pH 7.4), and 1µM tetrodotoxin with an osmolarity of 340 mosmol/kg. The intracellular patch pipette solution contained: 120mM CsCl, 11mM EGTA, 2mM TEA-Cl, 2mM $MgCl_2$, 10mM HEPES/CsOH (pH 7.4), 4mM MgATP, 20mM creatine phosphate, and 50 U/ml creatine kinase with an osmolarity of 318 mosmol/kg. The nonhydrolyzable GTP analog, guanosine 5'-(3-*O*-*thio*)triphosphate (GTPγS), was added to the patch pipette solution at a concentration of 100µM. Some patch pipettes had an additional 100µM cAMP and 1mM IBMX. Cells were voltage clamped to a holding potential of –80mV and stepped to +10mV, the peak of the current-voltage relationship for calcium current. All recording was at room temperature, 20-22°C. Somatostatin was applied via a macropipette lowered into the recording bath near the surface of the cell under study. The macropipette was withdrawn from the bath to terminate somatostatin application.

Pertussis toxin treatment (100ng/ml, overnight), which blocks the function of the GTP-binding proteins Gi and Go, completely abolished the action of somatostatin on calcium current. Intracellular application of 100µM GTPγS via the patch pipette renendered irreversible the somatostatin-induced inhibition of calcium current. In addition, GTPγS alone caused a slow decline in the calcium current amplitude over 500 s. When calcium current was inhibited by GTPγS (alone or with somatostatin), there was little or no response of calcium current to subsequent application of somatostatin. The affect of somatostatin on calcium current was not altered by intracellular application of cAMP (100µM) and IBMX (1mM) alone or in the presence of GTPγS. The results suggest that the somatostatin receptor is coupled to a GTP-binding protein which mediates inhibition of voltage-dependent calcium channels in a cAMP-independent manner.

53 INCREASED CYCLIC NUCLEOTIDE SYNTHESIS IN MONOCYTES FROM PATIENTS WITH ATOPIC DERMATITIS MAY BE RELATED TO ABNORMAL GTP-BINDING PROTEINS. R. Lloyd, S.C. Chan, J.M. Hanifin. Oregon Health Sciences University, Portland, Oregon 97201.

Mononuclear leukocytes (MNL) from patients with atopic dermatitis (AD) have higher basal adenylate cyclase (AC) activity but a blunted response to a variety of hormones including beta-adrenergic agonists and prostaglandins. Phosphoinositide (PI) pathway abnormalities include increased incorporation of tritiated inositol into phosphoinositides in AD MNLs. The signal transduction system involving GTP-binding proteins is associated with both AC and PI pathways, and may be the site of a common defect in AD. We tested AC activity of MNL membranes with GTP and Gpp(NH)p, as non-hormonal stimulators, to probe for a defect in the AC receptor-coupling system. MNLs were isolated from peripheral blood on Hypaque/Ficoll gradients, and monocytes separated from platelets and lymphocytes by adherence. Membranes were prepared by freeze-fracture of cells, Dounce homogenization, centrifugation at 45,000g and resuspension of the pellet by sonication. Membranes were assayed for AC activity by Solomon's method using 32P-labeled ATP as substrate for adenylate cyclase. Forskolin (FSK) and Mn^{2+} were used as catalytic-unit activators. Basal AC activity of monocyte membranes of normals was 0.22 ± 0.09 (S.E.M.)$_5$ nmol cAMP/mg. protein /30min., compared to 0.60 ± 0.08 nmol in AD monocytes (n=6,p<0.025). Stimulating with Gpp(NH)p 10^{-5}M caused a net increase of 2.42 ± 0.68 nmol cAMP in normal membranes and 4.67 ± 0.44 nmol in AD preparations, i.e., a 2-fold greater increase in AD compared to normal AC activity. FSK also caused a greater increase in AC activity in AD monocytes. The greater cAMP production by AD monocytes in response to the GTP analog suggests that the defective regulation of cAMP synthesis may involve one or both G proteins, with an increased stimulatory (Gs) effect and/or less inhibitory (Gi) influence on AC catalysis.

54 ADENYLATE CYCLASE (AC) OF DIFFERENTIATED PREADIPOCYTES IN PRIMARY CULTURE: RELATIVE LACK OF N_i MAY DETERMINE THE DIFFERENCE FROM ADIPOCYTES OF PROSTAGLANDIN E_1 (PGE_1) AND CATECHOLAMINE RESPONSES

Z.D. LU, M.A. PIÑEYRO, J. KIRKLAND, Z.H. LI, and R.I. GREGERMAN, Gerontology Res. Ctr., NIA, NIH, F.S.K. Med. Ctr., Baltimore, MD 21224

Preadipocytes derived from the stromal-vascular fraction of collagenase digested perirenal fat pads of Fisher 344 rats grow in α-MEM containing 10% FCS to confluence and accumulate lipid by 12 days. The cells show AC activity stimulated by GTP, Gpp(NH)p, NaF and forskolin, but response to isoproterenol and epinephrine is minimal (1.88 ± .75 x basal, n = 12, and 2.06 ± .75 x basal, n = 10, respectively). In contrast, GTP-dependent activation of AC by PGE_1 (10^{-5}M) is 7.55 ± 1.11 x basal (n = 8). This activation occurs even in the presence of 140 mM Na^+, a condition which leads to PGE_1 inhibition of AC in mature adipocytes. Also unlike the AC of mature adipocytes, forskolin-activated preadipocyte AC does not show a frank inhibition by GTP, and pertussis toxin treatment raises basal and enhances stimulation by GTP and PGE_1. Pertussis toxin labelling of N_i with ^{32}P-NAD (25 μM) shows about 25% of the N_i of adipocytes. The results indicate that, unlike adipocytes, the differentiated preadipocytes have not developed a fully functional N_i pathway. A lowered ratio of N_i:N_s may also relate to failure of activation by catecholamines.

55 DOWN REGULATION OF INTERLEUKIN 1 (IL 1) RECEPTOR EXPRESSION BY IL 1 AND FATE OF INTERNALIZED ^{125}I-LABELED IL 1-β IN HUMAN LARGE GRANULAR LYMPHOCYTE CELL LINE. Kouji Matsushima and Joost J. Oppenheim. Laboratory of Molecular Immunoregulation, Biological Response modifiers Program, Division of Cancer Treatment, NCI, NIH, Frederick Cancer Research Facility, Frederick, MD 21701

The regulation of interleukin 1 (IL 1) receptor expression on a human large granular lymphocyte cell line, YT, and fate of internalized ^{125}I-labeled IL 1-β (^{125}I-IL 1-β) were studied. YT cells respond exogenously added IL 1 by expressing high affinity IL 2 receptors, and do not produce IL 1. In addition, YT cells constitutively express about 7 x 10^3 IL 1 receptor per cell with a KD of ~10^{-10}M. Neither IL 2, phorbol myristic acid, nor lipopolysaccharide affected the total binding of ^{125}I-IL 1-β by YT cells. In contrast, the capacity of YT cells to bind ^{125}I-IL 1-β when incubated at 37°C for 3 to 16 hr with a low dose of purified IL 1-β (~6 U/ml) was reduced by >80%. The binding capability of YT cells recovered by 16 hr after removal of IL 1-β. Acid treatment of YT cells to remove bound ^{125}I1-β showed that 50% of the ^{125}I-IL 1-β bound to cells could no longer be recovered after 30 min at 37°C and and this increased to 80% after 3 hr at 37°C. Fractionation of cell extracts on Percoll gradient further showed that after receptor binding on plasma membranes, ^{125}I-IL 1-β could be successively recovered from intracellular sites associated with some membranous organelles (d=1.037), with an intermediate density organelle (d=1.050), and to finally end with the lysosomal cell fractions (d=1.05 to 1.08) at about 3 hr at 37°C. Only ~ 5% of internalized ^{125}I-IL 1-β was released into culture media by 6 hr of incubation at 37°C. This observation suggests that the low number of detectible receptors for IL 1 on many cell types may be attributable to persistent down-regulation by low levels of exogeneous IL 1.

56 UNCOUPLING OF THE MITOGENIC RESPONSE OF INSULIN AND IGF-I STIMULATION ON DIFFERENTIATED HUMAN SH-SY5Y NEUROBLASTOMA CELLS. M.E.K. Mattsson[a], G. Enberg[b], S. Gammeltoft[c], K. Hall[b] and S. Påhlman[a], [a]Department of Pathology, University of Uppsala, Sweden, [b]Department of Endocrinology, Karolinska Institute, Stockholm, Sweden, [c]Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark.

Insulin and insulin-like growth factor I (IGF-I) induce at physiological concentrations ornithine decarboxylase activity, ^{3}H-thymidine incorporation and cell division in SH-SY5Y neuroblastoma cells. The cross-reactivity pattern in binding studies using ^{125}I-labelled ligands and cross-linking experiments followed by analysis of the ligand-receptor complexes by SDS-electroforeses, have shown that the SH-SY5Y cells express insulin, IGF-I and IGF-II receptors. The molecular weights of the insulin/IGF-I receptors and the IGF-II receptor were 130 kD and 250 kD, respectively. The biologically active phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induces growth inhibition, morphological and functional differentiation in SH-SY5Y cells. The mitogenic response (induction of ornithine decarboxylase activity and ^{3}H-thymidine incorporation) to insulin and IGF-I is lost in cells treated by TPA for 4 days. However, TPA-treated cells still bind insulin and IGF-I. In contrast, the IGF-II binding disappears, during TPA treatment. The insulin and IGF-I receptor-mediated mitogenic signals are apparently uncoupled at an unknown stage of the intracellular mitogenic pathway. In an attempt to localize the step at which TPA differentiation interferes with the mitogenic signal transduction, we have analysed whether or not early effects of insulin and IGF-I stimulation in non-treated cells still are induced after TPA-treatment. This includes receptor autophosphorylation, actin reorganization and c-fos expression.

57 ENHANCEMENT OF HORMONE-STIMULATED CYCLIC AMP ACCUMULATION IN HUMAN MONONUCLEAR LEUKOCYTES BY A FACTOR IN SERUM

Harvey Motulsky, Div. Pharmacology, Univ. Cal. San Diego.

Human mononuclear leukocytes (MNL) contain beta-adrenergic and histamine receptors coupled to a stimulation of adenylate cyclase, and are a popular model system for studying the regulation of receptors and adenylate cyclase in humans. We have found that MNL prepared at 4° accumulate fivefold more cAMP in response to isoproterenol than do MNL prepared at ambient temperature. Warming these MNL prepared at 4° did not decrease cellular ATP levels, but lowered cAMP accumulation stimulated by isoproterenol, histamine, and prostaglandin $E_1$1, but not forskolin. Similar results were obtained in cells pretreated with pertussis toxin to inactivate the inhibitory guanine nucleotide binding protein. The difference between the two batches of MNL could only be observed in intact cells; no difference was observed in isoproterenol-stimulated adenylate cyclase activities in membranes or in phosphodiesterase activities in homogenates. cAMP accumulation in warmed MNL was increased by preincubation with plasma. Serum (human, horse, or fetal calf) also enhanced cAMP accumulation, and this activity remained in the supernatant after heating human serum to 100° for 10 min and pelleting the protein flocculate. Preliminary data indicate that the ability of this serum extract to enhance cAMP accumulation remains after dialysis, has nearly full potency after a 1:16 dilution, and enhances cAMP accumulation in S49 lymphoma cells as well as in MNL. These data suggest that blood serum contains one or more factor(s) that elevate hormone-stimulated cAMP accumulation in intact cells.

58 ACTIVATION OF FAT CELL ADENYLATE CYCLASE BY PROTEIN KINASE C.

S. Naghshineh, M. Noguchi, K.P. Huang, and C. Londos, NIDDK, NIH, Bethesda, MD 20892.

Highly purified protein kinase C (C-kinase) from rat brain and partially purified C-kinase from guinea pig pancreas stimulate adenylate cyclase activity in purified rat adipocyte membranes. Cyclase stimulation occurs over 100 to 1000 mU/ml of C-kinase activity, requires 1-10 μM calcium, but does not require exogenous phospholipid. C-kinase inhibitors, such as chlorpromazine, palmitoyl carnitine, and polymyxin B inhibit selectively adenylate cyclase which is activated by C-kinase and calcium. Depending on assay conditions, 10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) either enhances or is required for C-kinase action on cyclase. Also, TPA plus calcium promote the quantitative association of C-kinase with membranes. Adenylate cyclase activation by C-kinase is seen both in the presence and absence of exogenous GTP and in membranes from cells that have been treated with pertussis toxin, suggesting that the kinase effect does not result from an action on the GTP-binding, inhibitory regulatory component (N_i) of the cyclase system. Similarly, treatment with cholera toxin does not alter adenylate cyclase stimulation by C-kinase. Finally, the kinase effect is seen in the presence of non-phosphorylating ATP analogs, such as AppNHp and $AppCH_2p$, which have been treated with glucose and hexokinase to remove residual ATP. Such data indicate that the effects of C-kinase described herein may result from associatn with, rather than phosphorylation of, adenylate cyclase.

59

DEXAMETHASONE AND SODIUM BUTYRATE MODULATE COMPONENTS OF THE β-ADRENERGIC RECEPTOR-ADENYLATE CYCLASE COMPLEX IN 3T3-L1 PREADIPOCYTES. M.T. Nakada, J.M. Stadel, K.S. Poksay, S.T. Crooke. Smith Kline & French Labs, Philadelphia, PA 19101.

In mouse 3T3-L1 preadipocytes, the glucocorticoid dexamethasone (dex) effects a switch in β-adrenergic receptor (βAR) subtype expression from β_1AR to β_2AR and increases total βAR number. These effects are dose and time dependent. The ability of other steroids to cause the subtype switch correlates with their glucocorticoid activity. Compounds most effective in regulating βAR are also the most potent competitors for [^{3}H]-dex binding to the 3T3-L1 glucocorticoid receptor. RNA synthesis, protein synthesis, and N-linked glycosylation appear to be required for the dex-induced βAR subtype switch and increase since these changes are not observed in the presence of inhibitors for these processes. Another gene activating agent, sodium butyrate, mimics the effects of dex in altering βAR subtype and number. Butyrate's effects are dose and time dependent. Other short chain acids are not as effective as butyrate in altering βAR. Cyclohexamide inhibits butyrate's effects on βAR, suggesting that protein synthesis is required. The combined effect of dex and butyrate in regulating βAR is greater than additive suggesting that these two compounds may be acting through different mechanisms. Butyrate, and to a lesser extent dex, promote a 2-3 fold increase in labeling of G_s and G_i using cholera toxin and pertussis toxin respectively. These data suggest that both butyrate and dex coordinately regulate components of the βAR-adenylate cyclase complex in 3T3-L1 preadipocytes.

60 THE BINDING OF [3H]FORSKOLIN TO PROTEINS SOLUBILIZED FROM BOVINE BRAIN MEMBRANES.

C.A. Nelson and K.B. Seamon Center for Drugs and Biologics/FDA Bethesda MD 20892

The binding of ^{3}H-forskolin (^{3}H-FSK) to proteins solubilized from bovine brain membranes was studied using a precipitation assay. The K_d for ^{3}H-FSK binding to solubilized proteins was 14 nM which is similar to that reported for binding sites in membranes from rat brain and human platelets. The rank potency of forskolin analogues to compete for ^{3}H-FSK binding sites was the same in brain membranes and solubilized preparations. Proteins were solubilized from membranes preactivated with GppNHp. ^{3}H-FSK bound to proteins solubilized from preactivated membranes with a Bmax of 106 fmol/mg protein. Solubilized proteins from non-preactivated membranes had a Bmax of only 38 fmol/mg protein which was increased to 94 fmol/mg protein when GppNHp was included in the binding assay. In contrast, GppNHp had no effect on ^{3}H-FSK binding to proteins solubilized from preactivated membranes. Solubilized adenylate cyclase from non-preactivated membranes had a basal activity of 130 pmol/mg/min which was stimulated 7-fold by GppNHp. In contrast, adenylate cyclase from preactivated membranes had a basal activity of 850 pmol/mg/min and was not stimulated by GppNHp or forskolin. Thus, the formation of the high affinity binding sites for ^{3}H-FSK was coincident with the activation of adenylate cyclase via the guanine nucleotide binding protein. ^{3}H-FSK binding comigrated with adenylate cyclase activity from preactivated membranes on a GF-450 molecular exclusion matrix indicating that the activated catalytic unit of adenylate cyclase and the ^{3}H-FSK binding site have similar molecular weights and may be the same complex.

61 SPONTANEOUS COUPLING OF THE β-ADRENERGIC RECEPTOR TO N_S IN CARDIAC MEMBRANES

V. Nerme, Y. Severne, T. Abrahamsson, G. Vauquelin. Department of Pharmacology and Biochemistry, Hässle Cardiovascular Research Laboratories, S-431 83 Mölndal, Sweden and Institute of Molecular Biology, Free University of Brussels, 65 Paardenstraat, B-1640, St. Genesius-Rode, Belgium

The alkylating reagent N-ethylmaleimide (NEM) does not affect the number of β-adrenergic receptors in membranes from turkey erythrocytes, S49 lymphoma cells and rat lung. However, in the presence of β-agonist, NEM provokes a decrease in the receptor number by 50 to 65%. This has been ascribed to the ability of NEM to alkylate a sulfhydryl group which becomes exposed at the surface of the adenylate cyclase stimulatory protein N_s, upon interacting with the agonist-occupied receptor.

In the present study, we show that NEM alone can cause a time and concentration dependent decrease in the β-receptor number by approximately 20% (at 0.1 mM NEM) in cardiac membranes from reserpinized cat, rat and guinea pig. The noradrenaline concentration is less than 0.1 nM in these preparations. The NEM-mediated decrease occurs also in the absence of Mg^{++} but is effectively prevented and even reversed by GTP. Our data are compatible with a model in which part of the β-adrenergic receptor population (20%) are able to undergo agonist independent coupling to N_s in cardiac membranes.

62 RELATIONSHIP BETWEEN β-ADRENOCEPTOR REGULATION AND β-ADRENERGIC RESPONSIVENESS IN HEPATOCYTES. M. Refsnes, D. Sandnes and T. Christoffersen. Department of Pharmacology, University of Oslo, P.O.Box 1057 Blindern, 0316 Oslo 3, Norway.

Mechanisms regulating the responsiveness of the adenylate cyclase (AC) to catecholamine stimulation were studied in cultured rat hepatocytes. These cells acquire a several-fold increase in the β-adrenergic responsiveness when plated as monolayer cultures. Comparison of the emergence of isoproterenol (ISO)-sensitive AC and binding of [^{125}I]iodocyanopindolol (^{125}ICYP) showed roughly parallel rises after the plating. The increase in ^{125}ICYP binding (starting at ~1 h after plating) slightly preceded the rise in ISO response, but the response thereafter rose steeply and transitorily (~2 h - 5 h) exceeded the increase in binding. Pertussis toxin (IAP) added at 0 h increased the ISO-sensitive AC activity measured at 7 h and 24 h, without affecting the number of ^{125}ICYP binding sites; this effect was non-selective, since the glucagon-sensitive activity was similarly potentiated by IAP. Addition of ISO (10 μM) to cells after 20 h caused a rapid homologous desensitization of the AC (50% after~ 5 min). This was paralleled by a down-regulation of β-adrenoceptors. Removal of ISO led to resensitization of the AC, which was rapid and protein synthesis-independent after a brief (10 min) desensitization, or slow and cycloheximide-sensitive after prolonged (4 h) exposure to the agonist. In both cases an up-regulation of the ^{125}ICYP binding paralleled the recovery from refractoriness. Glucagon desensitized (partly) the AC to ISO and glucagon, with no effect on ^{125}ICYP binding. The results suggest that, although superimposed mechanisms operate, regulation at the β-adrenoceptor level is a major determinant for both short- and long-term changes of the β-adrenergic responsiveness in hepatocytes.

63 **DIFFERENTIAL INHIBITION, BY METHACHOLINE (MECH) AND HISTAMINE (HIS), OF ISOPROTERENOL (ISO)-INDUCED PROTEIN KINASE (PK) ACTIVATION AND RELAXATION OF TRACHEAL SMOOTH MUSCLE (TSM).** **G.A. Rinard, T.J. Torphy, and A.M. Puckett, Emory Univ., Atlanta, GA & Smith, Kline & French, Phila, PA.**
Maximally effective doses of MECH and HIS contract canine TSM to the same force per cross sectional area. We used functionally equivalent contractile doses (EC_{33}, EC_{66}, EC_{100}) of MECH & HIS to precontract TSM prior to ISO relaxation dose-response studies. MECH was a stronger functional antagonist than HIS. Maximal (EC_{100}) MECH prevented ISO relaxation and inhibited PK activation. Equivalent HIS contractions were readily relaxed by ISO and PK activation was not inhibited. Increasing doses of HIS gave ISO EC_{50} values of 1.6 nM, 2.5 nM 5.5 nM in cervical TSM and 6.3 nM, 10 nM & 12.6 nM in thoracic TSM. Increasing doses of MECH gave ISO EC_{50} values of 2.5 nM, 20 nM & no relaxation in cervical TSM and 25 nM, 251 nM & no relaxation in thoracic TSM. We suggest that methacholine has an effect to inhibit beta adrenergic activation of the cyclic AMP-dependent protein kinase pathway in TSM but that histamine, even though it produces the same contractile force lacks such an effect. Thus ISO readily relaxes the complete range of HIS-induced contractions but as increasing doses of MECH increase the force of contraction, ISO is increasingly ineffective in relaxing MECH-induced contractions in canine tracheal smooth muscle.
Supported by NIH, HL-32770.

64 **DUAL REGULATION BY CALCIUM AND GUANOSINE NUCLEOTIDES OF β-MSH RECEPTOR FUNCTION IN THE MZR MELANOMA CELL LINE.** Yoram Salomon, Josepha Sole and Jeffrey E. Garst, Department of Hormone Research, The Weizmann Institute of Science, 76100 Rehovot, Israel.

Calcium has been implicated as a potential regulator of both melanotropin (α and β) and corticotropin-sensitive adenylate cyclase. We have examined the melanotropin (MSH) receptor-adenylate cyclase system in the MZR mouse melanoma clone. Our results demonstrate that both β-MSH binding and subsequent activation of adenylate cyclase is dependent on concentrations of extracellular calcium in the micromolar range. This effect can be shown both in the intact cell and in a plasma membrane preparation derived thereof, using an EGTA buffer system. In contrast, stimulation of adenylate cyclase by prostaglandin E_1, forskolin, or GTPγS is calcium-insensitive. Calcium appears to increase the binding affinity of β-MSH to its receptor by a factor of 20 (from 400 to 20 nM), without affecting maximal hormone binding. Hormone-receptor complexes formed in the presence of calcium dissociate rapidly (<2 min) and reversibly upon the elimination of calcium by excess EGTA. However, above saturating concentrations of β-MSH (>200 nM) hormone binding and stimulation of adenylate cyclase becomes calcium-independent. Under physiological concentrations of hormone and calcium, binding of β-MSH is entirely calcium-dependent. While calcium promotes β-MSH binding, GTP and its stable nucleotide analogs lead to a reduction both in affinity (2-fold) and maximal binding (65%). Guanosine nucleotide effects are evident in both a time- and concentration-dependent manner. However, these effects appear to be calcium-independent, suggesting that the dual control of β-MSH binding by calcium and guanosine nucleotides is mediated by two separate and independent mechanisms.

65 THE GUANINE NUCLEOTIDE BINDING PROTEIN N_o CO-PURIFIES WITH THE D_2 DOPAMINE RECEPTOR OF ANTERIOR PITUITARY. S.E. Senogles, J.L. Benovic, N. Amlaiky, R. Vinitsky, A.M. Spiegel and M.G. Caron. Duke Univ. Med. Ctr., Durham, N.C. and NIADDK, NIH, Bethesda, MD

The D_2 dopamine receptor (D_2R) of the anterior pituitary gland mediates the dopaminergic inhibition of prolactin release. Many lines of evidence suggest this process may be mediated through both adenylate cyclase and phosphoinositide metabolism. D_2R purified by affinity chromatography on CHOS Sepharose (spiperone derivative) appears to be functionally coupled to a guanine nucleotide regulatory protein (N) as evidenced by the presence of agonist high affinity binding to the partially purified D_2R after re-insertion into phospholipid vesicles. The endogenous N protein is a substrate for pertussis toxin yielding a ^{32}P-ADP ribosylated product of M. γ39,000 on SDS-PAGE, which co-migrates with brain N_o. Peptide maps generated using elastase of the ^{32}P-ADP ribosylated endogenous N protein, N_1, N_o and transducin, revealed strong homology with brain N_o. Immunoblotting of the partially purified D_2R with RV3 (an N_o specific antibody) and CW6 (an N_1 specific antibody) showed crossreactivity only with RV3. Functional interaction between the D_2R and the endogenous N_o was demonstrated by an agonist promoted 2-3 fold stimulation in GTPase activity or [^{35}S]GTPγS binding. Association of endogenous N_o with D_2R was increased by agonist pretreatment and decreased by guanine nucleotides. These results suggest a specific association and preferential coupling of N_o with the D_2R of pituitary. Reconstitution of agonist stimulated GTPase activity could be obtained using purified brain N_o and partially purified resolved D_2R. These results constitute the first documentation of an endogenous association of N_o with a receptor system. Further evaluation of this system may elucidate the processes determining the specificity of the association of N proteins with receptor systems.

66 DIFFERENTIAL EFFECTS OF PERTUSSIS TOXIN AND PHORBOL ESTERS ON SIGNAL TRANSDUCTION VIA THE CHEMOATTRACTANT RECEPTOR-N PROTEIN-PHOSPHOLIPASE C SYSTEM OF HUMAN NEUTROPHILS. Charles D. Smith and Ralph Snyderman, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710.

Treatment of PMNs with pertussis toxin (PT) inhibits inositol trisphosphate (IP_3) generation induced by the chemoattractant fMet-Leu-Phe (fMLP), indicating that its receptors activate a phospholipase C (PLC) via a PT-sensitive N protein. FMLP-induced IP_3 production is also inhibited by phorbol myristate acetate (PMA). Receptor interaction with the N protein can be measured as fMLP-stimulated GTPγ[^{35}S] binding to membranes. This stimulated binding is absent in membranes from PT-treated PMNs, but remains in membranes from PMA-treated cells. Phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis via PLC can be activated in isolated plasma membranes at low concentrations of Ca^{2+} (1μM) with either fMLP plus GTP or GTPγS alone. Membranes from PT-treated PMNs degrade PIP_2 upon exposure to GTPγS, but not fMLP plus GTP. Membranes from PMA-treated PMNs do not hydrolyze PIP_2 when incubated with GTPγS, but do degrade PIP_2 in the presence of 1 mM Ca^{2+}. Therefore, PMA-treatment does not directly inactivate PLC. Incubation of PMNs with phorbol dibutyrate, but not an inactive ester, 4-α-phorbol didecanoate, causes a similar loss of GTPγS-activated PIP_2 hydrolysis. In summary, PT appears to block the ability of occupied receptors to activate the N protein by stimulating nucleotide binding, but does prevent the activated N from stimulating PLC. In contrast, phorbol esters appear to induce a protein kinase C-catalyzed reaction which does not affect receptor-N protein interactions, but does prevent coupling of the activated N protein to PLC. Similar activation of protein kinase C by diacylglycerol produced upon receptor occupancy may provide a negative feedback signal for chemoattractant receptors.

67 GTP MODULATION OF THE MEMBRANE-BOUND ADENYLATE CYCLASE (AC) ACTIVITY IN RAT THYROID EPITHELIAL CELLS INFECTED BY RAS GENES EXPRESSING VIRUS.

A. Spina, A.Di Donato, F. Valente, G. Illiano- III Cattedra di Chimica Biologica, I Facolta' di Medicina e Chirurgia, Napoli, Italia; M.D. Berlingieri A. Fusco, M. Grieco-Centro di Endocrinologia e Oncologia Sperimentale del CNR- II Facolta' di Medicina e Chirurgia, Napoli, Italia.

We are studying the influence on the membrane bound AC activity of the ras-genes expressing virus infection in a Fisher rat thyroid differentiated epithelial cell line (PC-CL3), which maintains in vitro the typical markers of thyroid differentiation. After infection by Harvey murine sarcoma virus carrying V-ras Ha, PC-CL3 cells change their morphology and became indipendent from TSH for the growth but they are not tumorigenic. We have found that the basal activity in PC-CL3 cells is very low (.46 pmoles cAMP/min/mg protein), whereas it is 10 times higher in PC-CL3 Ha MSV cells. The stimulation of the AC catalytic subunit by Mn^{2+} is equally efficient in control as well as in infected cells, whereas the modulation by GTP-binding protein either in the presence of Gpp(NH)p or NaF is impaired in the infected cells. We have carried out kinetic studies in the presence of increasing concentrations of Gpp(NH)p. The obtained results indicate that in both cell systems the maximal stimulation occurs at 10^{-5} M Gpp(NH)p. The increasing nucleotide concentration by 100 times doesn't allow the infected cells to get a stimulation of the basal activity to an extent comparable to that one obtained in control cells. These results exclude that the less efficient Gpp(NH)p modulation in the infected cells can be due to a competition for the GTP between the AC G-binding protein and the virally expressed p21 protein in the infected cells. Alternative molecular mechanisms consistent with our kinetic data will be presented.

68 FLUORIDE ION AS A PROBE FOR THE GUANINE-NUCLEOTIDE BINDING PROTEIN INVOLVED IN NEUTROPHIL ACTIVATION. Colette F. Strnad, Janice E. Parente, and Kenneth Wong. Departments of Pharmacology and Medicine, University of Alberta, Edmonton, Alberta, T6G 2G3.

Activation of human neutrophils with 18 mM sodium fluoride was found to be associated with phosphoinositide turnover as monitored by the accumulation of inositol 1-phosphate (IP_1), inositol 1,4-bisphosphate (IP_2), and inositol 1,4,5-trisphosphate (IP_3). Fluoride-induced phosphoinositide degradation was characterized by a 5 to 10 min lag period after which inositol phosphate levels increased and remained elevated over a 25 min time interval in lithium-treated cells. Phosphoinositide turnover was accompanied by the elevation of cytosolic calcium concentrations and the translocation of protein kinase C from the cytosolic to the membrane compartment. Unlike phosphoinositide turnover induced by the chemotactic peptide, formylmethionyl-leucyl-phenylalanine, that induced by fluoride was not inhibited by either pertussis toxin or dibutyryl cyclic AMP. Fluoride-induced phosphoinositide turnover was, however, subject to inhibition following pretreatment with the phorbol ester tumor promotor, phorbol myristate acetate. We have proposed that the mode of action of fluoride as an inflammatory stimulus involves interaction with a guanine nucleotide-binding protein which serves as an intermediary unit between cell surface receptors and the phosphoinositide-specific phosphodiesterase. The differential inhibitor effects suggest that ADP-ribosylation by pertussis toxin, as well as cyclic AMP-dependent phosphorylation reactions, do not interfere with fluoride's ability to occupy its active site or to activate the protein. Conversely, protein kinase C-dependent phosphorylation appears to render the activation site either inaccessible or insensitive to fluoride.

69 DESENSITIZATION OF THE SUBSTANCE P-INDUCED FORMATION OF INOSITOL TRISPHOSPHATE IN PAROTID ACINAR CELLS. H. Sugiya and J.W. Putney, Jr. Division of Cellular Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298.

Substance P (SP) is a putative neurotransmitter widely distributed in the mammalian central and peripheral nervous systems. The parotid gland is considered a target organ of SP, and the receptor is coupled to stimulation of inositol phospholipid metabolism and initiates secretion. After prolonged incubation of target cells with hormones or neurotransmitters, the cellular responses are often blunted or desensitized. We have evaluated desensitization of the SP-induced formation of inositol trisphosphate (IP_3) in rat parotid acinar cells. Results were as follows: (1) Exposure of parotid cells to SP (0.1 μM) which were prelabelled with [^{3}H]inositol caused a rapid increase in IP_3 from 2 sec up to 1 min, after which the level of IP_3 reached a plateau. (2) Methacholine (Mec, 0.1 mM) caused a sustained increase in IP_3 from 2 sec up to 5 min. (3) The response to SP and Mec applied simultaneously was not greater than the response of either agonist given alone. (4) After the level of IP_3 reached a plateau in the presence of SP, addition of Mec induced a further increase in IP_3 but a second addition of SP had no effect. (5) After exposure to SP for 5 min, although cells were washed and resuspended in fresh medium, desensitization of the SP-induced formation of IP_3 remained; desensitization did not occur in cells pre-exposed to Mec. (6) Following prolonged incubation in fresh medium, gradual resensitization occurred, and during this process there was a good correlation between the SP-induced formation of IP_3 and [^{3}H]-SP binding sites. These results suggest that desensitization of the SP-induced formation of IP_3 results from homologous down-regulation of SP receptors. (Supported by NIH Grant DE-05764).

70 PROTEIN KINASE C PREVENTS INHIBITION OF ADENYLATE CYCLASE IN NG 108-15 CELLS
B. TOCQUE - RHONE-POULENC SANTE - Cancer Research Department - 13, quai Jules Guesde 94400 VITRY SUR SEINE - FRANCE

The adenylate cyclase of NG 108-15 membranes is stimulated and inhibited, respectively, by PGE_1 and opiates acting via the stimulating (Ns) and inhibitory (Ni) guanine nucleotide-binding regulatory proteins.

GTP-dependent Dalamid induced inhibition of basal, PGE_1 and Forskolin stimulated activities were reduced in membrane of TPA-pretreated NG 108-15.

To substantiate this observation we studied whether addition of purified protein kinase C to NG 108-15 membranes causes similar changes in regulation of adenylate cyclase activity as observed in membranes of TPA pretreated NG 108-15 cells. Hormonal inhibition of the NG 108-15 adenylate cyclase by opiate was largely impaired after incubation of NG 108-15 membranes (prepared with EDTA, EGTA buffer) with partially purified kinase C stimulated by TPA.

Bradykinin has been reported to inhibit adenylate cyclase in NG 108-15 cells in a manner which is additive to the opiate inhibition suggesting that Bradykinin was acting through a different pathway. We asked the question whether or not, kinase C would inhibit Bradykinin effect on cyclase. We found that kinase C also affects Bradykinin induced inhibition of cyclase. The results indicate that the pathway for Bradykinin inhibition of cyclase is, at some stage, a target for kinase C which might be Ni or No.

71 A PERTUSSIS/CHOLERATOXIN-SENSITIVE N PROTEIN MEDIATES CHEMOATTRACTANT RECEPTOR SIGNAL TRANSDUCTION. Margrith Verghese, Ronald J. Uhing and Ralph Snyderman, Howard Hughes Med. Inst., Div. Rheum. and Immunol., Dept. Med., Duke Univ. Med. Ctr., Durham, N.C. 27710.

Affinities of chemoattractant receptors (CTX-R) on phagocytic leukocytes are regulated by GDP and GTP. These receptors are not coupled to adenylate cyclase but utilize a guanine nucleotide regulatory (N) protein to activate phospholipase C and subsequent biological responses. Since pertussis toxin (PT) ribosylates a 40-41 kD protein in leukocytes and thereby inhibits their activation by chemoattractants, it had been assumed that CTX-R are coupled to N_i. We now report that human polymorphonuclear leukocytes (PMNs), monocytes, and the myeloid HL-60 and U937 cell lines, but not erythrocytes nor bovine brain contain a ca. 40 kD protein which is a substrate for ADP ribosylation by choleratoxin (CT). In contrast to N_s, ribosylation of the 40 kD substrate by CT is inhibited by GDP or GTP. The 40 kD CT substrate, termed N_c for chemotaxis-related N protein, comigrates with the ca. 40 kD PT substrate in one-dimensional gel electrophoresis. Heptylamine-sepharose chromatography of cholate-solubilized HL-60 membranes prelabelled with CT and ^{32}P-NAD show that a 40 kD ^{32}P-labelled protein comigrates with the major peak of GTPγS-binding activity. This peak is partially resolved from the 43 kD (N_s) protein labelled under the same conditions. A 40 kD PT substrate also coelutes with the GTPγS-binding activity suggesting that N_c is a single protein which is a substrate for PT and CT. Additionally, treatment of intact PMNs with PT or CT reduced GTP regulated high affinity binding of formylpeptide chemoattractants to membrane preparations from the cells. This further implies that CTX-R are coupled to an N protein which is a substrate for both PT and CT. We suggest that N_c regulates chemoattractant receptor signal transduction.

72 NAD INHIBITS WHILE AppNHp AND GppNHp STIMULATE PHOSPHOLIPASE C IN THE CYTOSOL OF WHITE ADIPOCYTES. M.A. Wallace, R.J.H. Wojcikiewicz, D. Bojanic, L.S. Bradham and J.N. Fain. Department of Biochemistry, University of Tennessee, Memphis; Memphis, TN 38163.

Cytosol preparations from rat white adipocytes contain phospholipase C activities which can be assayed using ^{3}H-PIP_2, ^{3}H-PIP, or ^{3}H-PI as substrates. Two major observations have been made concerning these activities. First, 5'-adenylylimidodiphosphate (AppNHp) or 5'-guanylylimidodiphosphate (GppNHp) stimulate the hydrolysis of ^{3}H-PIP_2 with identical potency and efficacy. Second, NAD, NADP, or adenosine 5'-diphosphoribose, but not nicotinamide, inhibit phospholipase C. This inhibition can be overcome by high doses of GppNHp or AppNHp (0.1mM) which, in the presence of an otherwise fully inhibitory concentration of NAD (1.0mM), give complete or even supramaximal stimulatory effects.

Thus, the apparent guanine nucleotide specificity of phospholipase C activation in the presence or absence of hormones, as demonstrated in other studies, may need to be reassessed. Identification of the nucleotide regulatory components of phospholipase C must take into account a lack of guanine nucleotide specificity. Finally, the inhibitory effects of NAD may provide a clue as to how the activity of cytosolic phospholipase C is tonically suppressed in the cell.

73 GTP-SENSITIVE AGONIST BINDING IN MEMBRANES CAN EXPLAIN THE RECEPTOR RESERVE IN INTACT RAT RETICULOCYTES.

Anton Wellstein and Dieter Palm, Z.Pharmakologie, J.W.Goethe-Universität, D-6000 Frankfurt 70; FRG.

Rat reticulocytes are a good tool for studying the modulation of the ß-adrenoceptor mediated cAMP response (Naunyn-Schmiedeberg's Arch Pharmacol 317:294-301; 1981). We have used this model to investigate aspects of the receptor reserve. Cells were prepared and monitored for cAMP-accumulation as described earlier (see above). ß-Adrenoceptors in intact cells as well as membranes were detected by the radioligand ^{3}H-CGP 12177 in a filtration asssay. RESULTS: Intact cells: For antagonist ligands monophasic inhibition isotherms of agonist induced cAMP-accumulation as well as radioligand binding were observed with identical Kd-values in both measurement systems. Agonists (e.g. (-)-isoprenaline = ISO) also showed monophasic concentration- effect curves of cAMP-accumulation and inhibition of radioligand binding. Their potency, however, differed by more than 1 decade in favour of cAMP-accumulation (e.g. ISO: EC50 cAMP = 58 ± 11 nM; Kd receptor binding = 1,040 ± 400 nM; n= 17) indicating a receptor reserve of 60-70%. Membranes: After lysis of the cells, the agonist competition isotherms for the radioligand became biphasic. The GTP-sensitive high affinity component showed Kd-values not different from the EC50 of cAMP accumulation in intact cells. The portion of this component (33 ± 5 %) is in agreement with the above receptor reserve and seems to reflect the portion of receptors participating in the transient coupling in the intact system.

74 MECHANISM OF REGULATION OF SOLUBLE GUANYLATE CYCLASE IN THE PULMONARY ARTERY BY HYDROGEN PEROXIDE. Michael S. Wolin and Theresa M. Burke, Dept. of Physiology, New York Medical College, Valhalla, NY 10595

Hydrogen peroxide generation by glucose oxidase causes up to a four-fold activation of unpurified soluble guanylate cyclase (GC) from bovine pulmonary arteries which is potentiated to over 25-fold by the addition of purified bovine catalase (CAT). Compound I (Cpd I), a species of CAT formed during the metabolism of peroxide, appears to mediate GC activation based on the following observations: 1) Only peroxides that form Cpd I activate GC. 2) Quenchers of Cpd I (alcohols and formate) block GC activation. 3) Inhibition of CAT (by aminotriazole, superoxide anion, boiling) block GC activation. 4) Other peroxide metabolizing enzymes (peroxidase, fungal catalase) do not mimic catalase. 5) Scavangers of hydroxyl radical (mannitol, DMSO, desferoxamine) do not inhibit GC activation. In the pulmonary artery, micromolar concentrations of H_2O_2 elicit increases in tissue cycle GMP levels that are closely associated with decreases in force in a time and concentration dependent manner. Since Cpd I is formed in tissues, this is a physiologically relevant mechanism of regulation of GC and an attractive pathway for sensing of O_2 tension. (Funded by USPHS HL 31069)

75 PROTEOLYTIC ACTIVATION OF MEMBRANE-BOUND PROTEIN KINASE C BY TRYPSIN-LIKE PROTEASE. Hirohei Yamamura, Eikichi Hashimoto, Keiko Mizuta, Youichirou Sakanoue, Shun-ichi Nakamura, Tomoko Kobayashi and Keiko Sakai. Dept. Biochem. Fukui Medical School, Fukui 910-11 Japan

It has been well established that phosphorylation of ribosomal protein S6 is increased in response to growth-promoting stimuli. In an attempt to identify the cAMP- and Ca^{2+}-independent S6 kinase, we examined a possible role of protease-activated form of membrane-bound protein kinase C. When rat liver plasma membrane was digested with pancreatic trypsin, a cAMP- and Ca^{2+}-independent protein kinase having a molecular weight of 5×10^4 was produced. This enzyme phosphorylated ribosomal protein with a molecular weight of 3×10^4 which may correspond to S6 protein. By extraction of membrane with a buffer containing Triton X-100, proenzyme was detected and showed Ca^{2+}-phospholipid dependency in H1 histone phosphorylation. These results suggest that protease-activated form of protein kinase C is one of the candidates of S6 kinase. Protein kinase C was also activated by membrane-bound, trypsin-like protease discovered by Tanaka et al. (J. Biol. Chem. 261, 2610-2615 (1986)). Another experiment showed that proteolytic activation of protein kinase C is stimulated in the presence of salt such as NaCl. When rat liver plasma membrane was incubated in the presence of increasing concentration of NaCl, the production of activated form of protein kinase C was greatly increased. This reaction was inhibited by leupeptin and pancreatic trypsin inhibitor. These results may suggest the possibility that protein kinase C is activated by membrane-bound protease coupling with an increase in ionic strength elicited by growth factor-induced Na^+ influx.

76 PHOSPHORYLATION OF THE CATALYTIC UNIT OF ADENYLATE CYCLASE BY CYCLIC AMP-DEPENDENT PROTEIN KINASE AND PROTEIN KINASE C. T. Yoshimasa, M. Bouvier, J.L. Benovic, N. Amlaiky, R.J. Lefkowitz and M.G. Caron. Duke Univ. Med. Ctr., Durham, NC 27710

Regulation of the responsiveness of β-adrenergic receptor-coupled adenylate cyclase (C) systems such as in some forms of desensitization to hormones or during the general sensitization observed after treatment of some cells with phorbol esters might involve covalent modification (phosphorylation) of C itself. To test this hypothesis, we examined whether C is a substrate for the cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) and investigated whether such phosphorylation regulates the function of the enzyme. C was purified from bovine caudate ~17,000 fold to a specific activity of 1.5 $\mu mol.mg^{-1}.min^{-1}$ by Lubrol PX solubilization, forskolin-Sepharose and wheat germ agglutinin-agarose chromatography. C preparations contained a single polypeptide of M_r ~160,000 revealed by radioiodination, SDS-PAGE and autoradiography. The M_r ~160,000 band correlated well with enzymatic activity on gel permeation HPLC. The pure C could be reconstituted with the purified β_2-adrenergic receptor and the stimulatory guanine nucleotide regulatory protein (N_s) to form a hormone-stimulated adenylate cyclase system. PKA and PKC were able to phosphorylate C in a stoichiometric fashion (~0.8 mol PO_4/mol C). Ability of control and phosphorylated C to couple to N_s was compared. When control and phosphorylated C were reconstituted with N_s, the Gpp(NH)p stimulated activity of PKA phosphorylated C was reduced by ~42% while that of PKC phosphorylated C was enhanced by ~19% relative to control values. These results suggest that hormone sensitive adenylate cyclase systems might be regulated both by feedback inhibition and by "cross talk" from the phosphatidylinositol pathway through cAMP-and PKC-dependent phosphorylation of C, respectively.

77 PROTEIN KINASE C MODULATES ADENYLATE CYCLASE ACTIVITY IN RAT BRAIN STRIATUM: POSSIBLE INVOLVEMENT OF Gi-GUANINE NUCLEOTIDE REGULATORY PROTEIN

Madhu B. Anand-Srivastava and Ashok K. Srivastava, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7, Canada

The effects of partially purified Ca^{2+}, phospholipid-dependent protein kinase (C-kinase) were studied on adenylate cyclase activity from rat brain striatum. C-kinase treatment of the membranes stimulated adenylate cyclase activity, the maximal stimulation between 50-80% was observed at 3.5 U/ml. The inclusion of Ca^{2+} and phosphatidyl serine did not enhance the percent stimulation of adenylate cyclase by C-kinase, however, the stimulatory effect of C-kinase was inhibited by EGTA. In addition, in the presence of GTP, the stimulatory effect of C-kinase was potentiated. Furthermore, C-kinase also potentiated the stimulatory effects of N-Ethylcarboxamide adenosine (NECA), dopamine (DA) and forskolin (Fsk) on adenylate cyclase activity and attenuated the GTP and oxotremorine-mediated inhibition of enzyme activities. On the other hand, the catalytic subunit of cAMP-dependent protein kinase did not show any effect on adenylate cyclase activity. These data suggest that the stimulation of adenylate cyclase and inhibition of inhibitory influence of oxotremorine and GTP by C-kinase may be mediated through the inactivation (phosphorylation) of Gi-guanine nucleotide regulatory protein. (Supported by grants from MRC and OHF).

78 GUANINE NUCLEOTIDE REGULATION OF RECEPTOR BINDING AND IP3 FORMATION. D.L.Aub and T.E.Cote Dept. of Pharmacology, USUHS, Bethesda, MD 20814-4799. A novel GTP binding protein has been suggested to couple those receptors which stimulate PIP2 hydrolysis to phospholipase C. In 7315c cell lysates GTPγS and Gpp(NH)p, nonhydrolyzable analogs of GTP, stimulated a time and dose dependent formation of IP3 in the absence of agonist. GTPγS was more potent and more efficacious than Gpp(NH)p in stimulating IP3 formation. GTP was able to decrease the potency but not the efficacy of GTPγS-stimulated IP3 formation,as evidenced by a rightward shift in the GTPγS concentration response curve, suggesting that a GTPase activity may be associated with guanine nucleotide regulation of PIP2 turnover. In addition, TRH stimulation of IP3 formation was enhanced by the addition of GTP. In 7315c cell membranes Me-TRH binds to a single high affinity site (Kd = 2 nM).GTPγS and Gpp(NH)p decreased specific Me-TRH binding in a concentration dependent manner with a half-maximal potency of 0.092 μM and 1 μM respectively; their potency for stimulation of IP3 formation was the same. In membranes made from cells which had been treated for 24hours with 30 ng/ml pertussis toxin, which is known to uncouple receptors from Ni, GTPγS was still able to decrease specific TRH binding (half-maximal potency for control and pertussis toxin treatment, 61 nM and 38 nM, respectively). Therefore, pertussis toxin does not interfere with the interaction between the TRH receptor and its guanine nucleotide binding protein. GTPγS appears to decrease specific Me-TRH binding by decreasing the affinity of the TRH receptor for its ligand without altering the number of binding sites (control: Kd = 2 nM, Bmax = 26 fmol/mg protein; 10-7 M GTP S: Kd = 7.3 nM, Bmax = 26 fmol/mg protein). These results indicate that a guanine nucleotide binding protein may be involved in both the regulation of TRH binding and subsequent stimulation of IP3 formation.

79 EVIDENCE FOR AN ALTERED ADENYLATE CYCLASE CATALYTIC UNIT IN A PATIENT WITH MULTIPLE HORMONE RESISTANCE. D. Barrett, A. Spiegel, M. Wax, P.B. Molinoff, R.W. Downs, Medical College of Virginia, Richmond, Virginia 23298

Deficiency of G_s is the putative lesion in pseudohypoparathyroidism Type Ia (PHP 1a). However, a small group of atypical patients who have PHP and resistance to multiple hormones have normal red blood cell membrane G_s. We have found that cultured skin fibroblasts from one such patient (A-PHP) generate 50-70% less cAMP in response to parathyroid hormone, isoproterenol or prostaglandin E_1, when compared to six normal fibroblast lines.

We now report that the skin fibroblast G_s of A-PHP has normal activity as measured by reconstitution into S49 cyc$^-$ membranes. Computer-assisted analysis demonstrates that isoproterenol displacement of I^{125}-iodopindolol binding in the absence of GTP defines a population of high affinity binding sites in 4 control and A-PHP fibroblast membranes which represent β-adrenergic receptors coupled to N_s. No significant difference is apparent between the percent of receptors in this ternary complex state for A-PHP (43±5%) vs the average of controls (53±13%).

Stimulation of A-PHP fibroblast membranes with fluoride ion (10 mM) and GTPγS (100 μM) produces only 27±5% and 33±6% of control adenylate cyclase activity respectively. Direct stimulation of catalytic unit with manganese ion (Mn^{-2} 4 mM) is 36±6% of controls and stimulation with Mn^{-2} plus forskolin (100 μM) is 36±4% of controls. A defect in the catalytic unit can account for all these results and may be a mechanism in this group of patients for their resistance to multiple hormones which act through adenylate cyclase.

80 DETECTION OF NOVEL GTP-BINDING PROTEINS ON NITROCELLULOSE BLOTS OF MEMBRANE PROTEINS SEPARATED BY SDS-PAGE. R.P. Bhullar and R.J. Haslam. Dept. of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5.

Human and rabbit platelets were lysed by freezing and thawing and the membrane fraction isolated. Membrane protein was precipitated with trichloroacetic acid, dissolved in Laemmli buffer and analysed by SDS-PAGE. Resolved polypeptides were then transferred electrophoretically to nitrocellulose and the blots incubated for 30 min at 20°C with 0.5 nM [α-^{32}P]GTP in buffer containing 50 mM Tris-HCl, pH 7.5, and 0.3% Tween 20 (0.2 μCi/ml). After washing in buffer without [^{32}P]GTP, the blots were dried and bound ^{32}P detected by autoradiography. Major labelled bands were observed corresponding to proteins of 27 kDa and 24 kDa. Additional minor bands were detected in the 23-26 kDa range. Addition of 1 mM PMSF, 0.2 mM leupeptin and 5 mM EDTA during preparation of the membranes had no effect on the pattern of labelling observed. SDS released the ^{32}P from the blots, indicating non-covalent binding of [^{32}P]GTP. Binding of [^{32}P]GTP was specific in that it was prevented by simultaneous addition of 0.5 μM GTP or GTPγS or by 10 μM GDP, but was unaffected by 50 μM ATP or 10 μM GMP. No GTP-binding protein was detected in the platelet soluble fraction. Human and rabbit red cell ghosts contained the 27 kDa but not the 24 kDa GTP-binding protein. Analysis of rat tissues by the blotting technique demonstrated high concentrations of 27 kDa and 26 kDa GTP-binding proteins in brain, lesser amounts of 27 kDa protein in adrenal and lung tissue and small amounts of 27 kDa protein in liver, kidney, spleen, heart and skeletal muscle. The results demonstrate the existence of a new family of GTP-binding proteins distributed widely in mammalian tissues.

Supported by the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario.

81 NUCLEOTIDE ACTIVATION OF RAT BRAIN PHOSPHOLIPASE C. L.S. Brandham, M.A. Wallace, R.J.H. Wojcikiewicz, D. Bojanic and J.N. Fain. Department of Biochemistry, University of Tennessee, Memphis; Memphis, TN 38163.

Phospholipase C activity was isolated from brain membranes by cholate extraction followed by centrifugation at 100,000 x g for 1 hr. The solubilized enzyme catalyzed the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) but not phosphatidylinositol (PI) under our assay conditions. Enzymatic activity did not require the addition of divalent cations but was increased in the presence of 3 mM sodium deoxycholate.

Hydrolysis of PIP_2 and PIP was stimulated by 5'-guanylyl-imidodiphosphate with half maximal activation at a nucleotide concentration of approximately 10 μM. Nucleotide activation was inhibited by either 1 mM Ca^{2+} or 1 mM Mg^{2+}. These data suggest a role for nucleotides in the regulation of phospholipase C in brain membranes. Whether this regulation is mediated by a guanine nucleotide binding protein remains to be determined.

82

LOCALIZATION OF G-PROTEIN mRNAS (Tα,Tβ,Gs,Gi) IN BOVINE RETINA BY *IN SITU* HYBRIDIZATION HISTOCHEMISTRY: M.R. Brann, Laboratory of Cell Biology, NIMH, Metabolic Diseases Branch, NIDDK, NIH, Bethesda, MD.

In addition to photosensitive rods and cones, the retina contains a diversity of neurons which synthesize distinct neurotransmitters and neurotransmitter receptors. Recent evidence indicates that the response of rods to light, and the response of receptors to neurotransmitters are mediated by a family of closely related G-proteins. We prepared synthetic 48 base oligodeoxynucleotide probes which are complementary to the published sequences of mRNAs encoding the alpha (Tα) and beta (Tβ) subunits of transducin, the G-protein of rods; the alpha subunits of Gs and Gi, the G-proteins associated with neurotransmitter control of adenylate cyclase. These probes were used for localization of mRNAs in bovine retina by *in situ* hybridization histochemistry as previously described (Brann and Young, *FEBS Letters* (1986) 200:275).

mRNAs encoding Tα and Tβ were present within rods, and there both of the mRNAs were present in the outer nuclear layer (region of rod nuclei), both mRNAs were most abundant in the rod inner segments, and neither mRNA was present in the rod outer segments. Tα mRNA was not present in the inner layers of the retina. On the other hand, mRNAs encoding Gi and Gs were most abundant in the inner layers of the retina, and both of the mRNAs were most abundant in ganglion cells and in the inner nuclear layer. Gi mRNA was also present in the outer nuclear layer, suggesting rods express both Tα and Gi. These results illustrate the utility of *in situ* hybridization histochemistry in the study of the expression of mRNAs encoding G-proteins by individual neurons within the retina.

83 HUMAN cDNA CLONES FOR FOUR SPECIES OF $G\alpha_s$ mRNA AND FOR $G\alpha_i$ mRNA.
Bray, P., Carter, A., Simons, C., Guo, V. Puckett, C., Kamholtz, J., Spiegel, A., and Nirenberg, M. NIH, Bethesda, MD.

A λgtll cDNA library prepared from human basal ganglia was screened with 3 oligodeoxynucleotide probes for recombinants coding for α subunits of G signal transducing proteins. Fourteen of the 575,000 recombinant clones screened formed hybrids with 2 or 3 probes. Nucleotide sequences of two clones, BG-4 and BG-21-2, are highly homologous to bovine brain α_i cDNA (Nukada, T. et. al., FEBS Letters 197, 305, 1986). The nucleotide sequences of both strands of BG-4 α_i cDNA were determined. The sequence begins at the 14th nucleotide residue of the bovine α_i coding sequence and ends at 1261 in the 3' untranslated region. Nucleotide sequences of 11 clones are highly homologous to the sequence of bovine α_s (Nukada, T. et al., FEBS Letters 195, 220, 1986; Robishaw, J. et al., Proc. Natl. Acad. Sci. USA 83, 1251, 1986). Both strands of BG-3 α_s cDNA were sequenced completely. The first nucleotide residue corresponds to the 34th residue of the bovine α_s coding sequence and the last, 1278, in the 3'-untranslated region. cDNA clones for 3 additional species of α_s mRNA were obtained that differ in the region coding for amino acid residues 71-87; α_s-1 (clones BG-1, HB-2, BG-8, and BG-21-5) code for the same amino acid residues, 71-87, that are present in bovine α_s; α_s-2, (BG-3) contains 3 additional bases which code for Ser^{87}; α_s-3 (BG-6, BG-20) contain Asp^{71} in place of Glu^{71} and lacks residues 72-86; and α_s-4 (BG-13, BG-21-1) contain Asp^{71} in place of Glu^{71}, lack α_s-1 residues 72-86 and contains 3 additional bases which code for a serine following Glu^{71}. Hybridization of cloned cDNA to total RNA followed by digestion with S_1 nuclease revealed multiple forms of α_s mRNA. We propose that the 4 species of α_s mRNA are formed by alternative splicing of an α_s RNA transcript.

84 MULTI-SITE MODULATION OF HORMONE-SENSITIVE ADENYLATE CYCLASE BY PHORBOL ESTERS/C-KINASE IN S49 LYMPHOMA CELLS. LL Brunton, LA Speizer, JR Kanter, MJ Watson and JD Bell. UCSD, La Jolla, CA

12-o-tetradecanoylphorbol-13-acetate (TPA) has at least three functionally distinct effects on hormone-sensitive adenylate cyclase (AC) in S49 lymphoma cells: 1) a 50% reduction in agonist affinity for both isoproterenol (INE) and PGE_1 with no detectable reduction in β-receptor density; 2) attenuation of functional coupling of hormone receptor and transducer protein (G_s). The magnitude of this effect is agonist-specific and is exaggerated by lowering experimental temperature to 27°C, where TPA inhibits the maximum response to PGE_1 by 50% and the response to INE by 25%; 3) TPA enhances GTP-dependent AC activity. This enhancement seems to involve a GTP-binding protein since TPA enhances forskolin-stimulated AC activity by 35% in the presence of guanine nucleotide but only minimally in its absence in wild type (WT) membranes. We obtain comparable results in the cyc- variant which lacks the α subunit of G_s, suggesting the involvement of the inhibitory GTP-binding protein, G_i. Blockade of G_i activity by high concentrations of Mg^{++} (~100 mM) or Mn^{++} (>300 μm) abolishes the effect of TPA to enhance AC activity in WT membranes. Kinetic studies with a non-hydrolyzable GTP analog suggest that TPA treatment reduces both the rate and extent of activation of G_i by guanine nucleotide. Appropriate analog specificity, binding of [^{3}H]phorbol dibutyrate (K_D = 31 nM; B_{max} = 4.3 pmol/mg protein), TPA-induced translocation of C-kinase activity from cytosol to membrane, and the phosphorylation of a discrete group of membrane proteins in response to TPA all support the effects of TPA being mediated by activation of protein kinase C. (Supported by NSF PCM 81-10116, NIH HL 17682, RCDA HL 00935 and a PMA fellowship to JD Bell)

85

WITHDRAWN

86 DESENSITIZATION IS VERY EXTENSIVE IN AN S49 MOUSE LYMPHOMA VARIANT WITH LOW PDE ACTIVITY. R. W. Butcher, R. Barber, and T. Goka. University of Texas Graduate School of Biomedical Sciences, Laboratories of Cyclic Nucleotide Research, P.O. Box 20334, Houston, Texas 77225.

A variant of S49 cultured lymphoma cells (Blr) deficient in cAMP dependent protein kinase activity and with low phosphodiesterase (PDE) activities has been isolated and partially characterized by Bourne et al. (J. Cell. Physiol. 85: 611-620). This low PDE activity in Blr cells made the variant an attractive one for detailed studies of intracellular cAMP metabolism. We have found that, as expected from the low phosphodiesterase activity previously reported, the cAMP turnover rate in Blr cells is relatively slow ($t_{\frac{1}{2}}$ = 18-23 min at 37°C). Further, cAMP levels in unstimulated cells ranged from about 1% to 5% of cellular ATP (i.e., 20-100μM or 100-500 pmol/mg protein). These levels are many times higher than most S49 WT cells, and they made measurements of turnover in the absence of a hormone possible. The turnover constant for cAMP in unstimulated cells rate is 0.030 ± 0.0031/min. This is not significantly different than the value measured during epinephrine stimulation, which is 0.035 ± 0.004/min. The turnover values were used to determine precise levels of adenylate cyclase activity throughout the time course of epinephrine stimulation. Desensitization was both rapid and profound, with the level of adenylate cyclase activity falling by 70% within the first 4 min of stimulation. This suggests that desensitization may be a very major factor in the attenuation of catecholamine action, at least in some cell types. Further, epinephrine induced desensitization in Blr cells is manifested by an experimentally demonstrable left shift of the EC_{50}. Conversely, S49 WT cells responded to desensitization such that EC_{50} was shifted to the right. We hypothesize that these phenomena may, at least in part, be secondary to the differences in the speed and extent of desensitization in the two cell types.

87 PSEUDOHYPOPARATHYROIDISM TYPE Ia - DECREASED TRANSCRIPTION OF MULTIPLE FORMS OF G_s α: A. Carter, C. Bardin, R. Collins, P. Bray*, C. Simons, and A. Spiegel, Metabolic Diseases Branch, NIDDK, and *Laboratory of Biochemical Genetics, NHLBI, NIH, Bethesda, MD

Pseudohypoparathyroidism (PHP) Type Ia is a genetic disorder characterized by target organ resistance to multiple hormones acting via stimulation of cAMP. Several laboratories have demonstrated that plasma membranes from subjects with PHP Ia have decreased activity of the stimulatory guanine nucleotide binding protein (G_s) associated with adenylate cyclase. Cholera-toxin labeling of the α subunit of G_s is also reduced in plasma membranes from subjects with PHP Ia. Using bovine and human cDNA probes for G_s-α we have performed Northern blot and S1 analysis of total RNA extracted from fibroblasts of normal and PHP individuals. Of 5 patients studied, four show decreased steady-state levels of G_s-α mRNA. G_s-α mRNA exists in at least two forms (long and short), differing by approximately 45 bp. These are not distinguished by Northern blot hybridization but can be distinguished by S1 nuclease protection assay. S1 analysis indicates that both long and short forms are decreased in subjects with PHP. Moreover, there appears to be no significant change in the ratio of long to short forms in PHP compared with normal individuals. It appears likely that in some cases of PHP Type Ia, the genetic lesion affects some component of the transcription of multiple forms of the G_s-α subunit.

88 The Effects of $MgCl_2$, $B\gamma_T$, and the Cyclic GMP-Dependent Phosphodiesterase on the Rhodopsin-promoted GTPase of Transducin. R. Cerione, M. Lakonishok, R. Somers, and A. Spiegel. Cornell U., Ithaca, NY 14853, NIH, Bethesda, MD 20205.

The GTPase cycle of the retinal nucleotide regulatory protein, transducin (TD), has been examined after reconstituting the pure components of the phototransduction system into lipid vesicles. Transducin, alone, has little or no GTPase activity; however, the addition of TD to lipid vesicles containing RHO results in the activation of multiple TD molecules (per molecule of RHO) and a significant GTPase (≈ 1 mol Pi released per min. per mol TD). Dose response curves for the effects of $MgCl_2$ on the RHO-stimulated GTPase are complex and include both stimulatory and inhibitory phases. Other salts such as KCl only partially mimic the stimulatory phase, suggesting that the effects by $MgCl_2$ reflect specific interactions between the divalent metal and TD and/or RHO. The addition of the pure $B\gamma_T$ complex to RHO/TD vesicles can also increase the RHO-stimulated GTPase activity. This stimulation is dependent on both $[MgCl_2]$ and $[B\gamma_T]$ and appears to reflect the promotion by $B\gamma_T$ of the RHO-α_T GDP interactions which occur at the outset of each new GTPase cycle. The inclusion of the pure cyclic GMP-dependent phosphodiesterase (PDE) in the reconstituted RHO/TD vesicle systems has no significant effect on the RHO-stimulated GTPase activity, either in the absence or presence of the substrate cyclic GMP, or its 8-bromo-analog. Overall the results obtained from these studies now suggest 1) subunit dissociation of TD must occur within the time frame of the activation-deactivation cycle of this protein and 2) the interaction of the effector enzyme (PDE) with TD has no significant effect on the rate of deactivation (GTPase) of TD.

89 **IDENTIFICATION OF A CONSERVED DOMAIN AMONG CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM DIVERSE SPECIES.** H. Charbonneau, N. Beier*, K.A. Walsh, and J.A. Beavo*. Departments of Biochemistry and *Pharmacology, University of Washington, Seattle, WA 98195. Multiple forms of cyclic nucleotide phosphodiesterase (PDE) differing in apparent molecular weight, substrate specificity, immunologic reactivity, and mode of regulation have been identified within the same tissues and in many cases the same cell. In order to understand the structural basis for the diverse catalytic and regulatory properties of these PDE isozymes, we have determined the partial amino acid sequences of two bovine isozymes, the 61 KDa calmodulin-dependent PDE and the cGMP-stimulated PDE. The partial amino acid sequence of the bovine enzymes have been examined for homologous segments and have been compared to the predicted amino acid sequences derived from the recently determined nucleotide sequences of the yeast PDE2 gene (P. Sass, J. Field, J. Nikawa, T. Toda, and M. Wigler, Proc. Natl. Acad. Sci. USA, manuscript submitted) and the Drosophila dunce$^+$ gene (C.-N. Chen, S. Denome, and R.L. Davis, Proc. Natl. Acad. Sci. USA, manuscript submitted). These comparisons show that despite their distinct sizes, diverse phylogeny, and different regulatory properties all four enzymes display structural homology within a single domain of about 270 residues. For example, there is 41% sequence identity between the domains of the dunce$^+$ gene product and the calmodulin-dependent PDE. The absence of absolute sequence identity between the two bovine enzymes shows that they are unique gene products that are not produced by alternative processing of either a single mRNA precursor or a larger polypeptide chain. These homologous domains may comprise the catalytic site in these PDEs. Limited proteolytic studies are being conducted in an attempt to isolate and sequence fragments of the bovine isozymes that retain catalytic activity.

90 **REGULATION OF ROS cGMP PDE BY RHODOPSIN PHOSPHORYLATION AND 48K PROTEIN,** Deborah A. Fox, James L. Miller, and Burton J. Litman, Department of Biochemistry, University of Virginia, School of Medicine, Charlottesville, Va. 22908.

Rhodopsin species containing 0, 2 and ≥4 (high) phosphates per rhodopsin (P/R) were purified and reconstituted into phosphatidylcholine vesicles. These vesicle preparations were used to investigate the light-dependent activation of the rod outer segment cGMP phosphodiesterase (PDE) in the absence and presence of purified 48kd protein (48K). In the absence of 48K, a graded reduction in the maximum velocity (V_{max}) of light-stimulated PDE activity was observed with increasing rhodopsin phosphorylation. The inhibition of PDE V_{max} was greatest following low fractional rhodopsin bleaches and became less pronounced at higher fractional rhodopsin bleaches. Following bleaches of 0.01 and 1%, the V_{max} of the PDE in the high P/R vesicles was reduced by 85% and 30% respectively, relative to the 0 P/R control vesicles. Phosphorylation alone had no significant effect on the lifetime of the active rhodopsin species (Rho*) ($\tau_{off}(1/e) \sim 400$sec).

The effect of adding 48K to the assay mixture was investigated at several bleach levels. The molar ratios of 48K:Rho* ranged between 20:1 and 2:1. Addition of 48K did not change τ_{off} for 0 P/R vesicles, however, it did produce a slight reduction in the PDE V_{max}. In contrast, τ_{off} for high P/R vesicle samples was reduced by ~85% in the presence of 48K (from >400sec to <70sec), whereas, the V_{max} was reduced by an additional 20% beyond that produced by phosphorylation in the absence of 48K (0.1% bleach). When ATP was included in the reaction mixture, there was no further effect on τ_{off} or V_{max}. These results demonstrate that rhodopsin phosphorylation is a necessary requirement for 48K to effect PDE V_{max} and τ_{off} of Rho*. Supported by NIH grant EY00548.

91 Cr(III) GUANINE NUCLEOTIDE COMPLEXES AS PROBES FOR GTP-BINDING IN BOVINE TRANSDUCIN. Suzanne E. Frey and Yee-Kin Ho, Department of Biological Chemistry, University of Illinois at Chicago, Health Sciences Center, Chicago, Illinois 60612.

We have synthesized Cr(III)-β,γ bidentate GTP and Gpp(NH)p complexes to probe the GTP binding activities of transducin. Cr(III)·GTP at ten-fold higher concentration of Mg·GTP promoted the light-activation of the PDE. The hydrolysis of the bound GTP as monitored by the decrease of the activated PDE activity indicated that both complexes were hydolyzed at the same rate. However, using [γ-^{32}P]Cr(III)·GTP as substrate, it was shown that Cr(III)·GTP was degraded five times slower than MgGTP in the photo-activating cycle. Unlike the transducin-Mg·GTP which dissociated from the rhodopsin membrane, the transducin-Cr(III)·GTP complexes were found tightly associated with the photolyzed rhodopsin. The different Cr(III)·GTP hydrolysis rates could be explained in the following terms. In the radioactive tracer assay, the lack of free photolyzed-rhodopsin for repeated activation of transducin could lead to a slower hydrolysis of the [^{32}P]Cr(III)·GTP. However, in the PDE assay the membrane bound PDE only interacts with the membrane-associated transducin-Mg·GTP or -Cr(III)·GTP complexes for its activation. The measure of the decrease of the PDE activity could not sense the soluble transducin-Mg·GTP which could remain active in hydrolyzing GTP. Therefore, a similar turnover rate was observed. These results suggest that the activation of the cGMP cascade remains a membrane-associated event. Cr(III)·Gpp(NH)p was found to behave similarly to Mg·Gpp(NH)p which bound tightly to transducin. The stereo-isomers of the Cr(III)·Gpp(NH)p complex were separated by reverse phase HPLC. All isomers were found capable in activating the PDE. No significant stereospecificity of the Cr(III)·Gpp(NH)p complexes was observed.

92 PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION OF THE MAJOR PERTUSSIS TOXIN SUBSTRATE OF BOVINE NEUTROPHILS: P. Gierschik*, D. Sidiropoulos*, A. Spiegel+, and K.H. Jakobs*, *Pharmakologisches Institut der Universität Heidelberg, FRG, and +Metabolic Diseases Branch, NIADDK, NIH, Bethesda, MD.

Bovine peripheral neutrophils contain high levels of a 40 kDal pertussis toxin (PT) substrate, which was found in both plasma membrane and cytosolic, but not in granular fractions upon subcellular fractionation. The 36 kDal beta subunit, common to all known PT substrates, was exclusively found in plasma membrane fractions. Both the 40 kDal protein and beta subunits were purified from plasma membranes to near homogeneity by sequential ion exchange, gel filtration, and hydrophobic chromatography. The purified 40 kDal protein was shown to interact with homologous and heterologous beta/gamma complexes, undergo ADP-ribosylation by PT and bind guanine nucleotides. The mobility of the protein on SDS polyacrylamide gels differs slightly from those of the alpha subunits of transducin (G_t), G_o and G_i, purified from bovine retinas and brain, respectively. Several polyclonal antisera against the alpha subunits of bovine G_t and G_o did not react with the 40 kDal neutrophil protein on immunoblots. CW 6, a polyclonal antiserum reactive against bovine G_i alpha, reacted only minimally with the 40 kDal neutrophil protein. These results suggest that the major PT substrate of bovine neutrophils is different from previously identified substrates and may represent a novel guanine nucleotide binding protein.

93 BOVINE PHOTORECEPTOR PHOSPHODIESTERASE NONCATALYTIC CYCLIC GMP BINDING SITES ARE ENRICHED IN A DISTANT ISOZYME. Peter G. Gillespie and Joe A. Beavo, Department of Pharmacology SJ-30, University of Washington, Seattle, Washington 98195.

It has been reported that frog rod outer segment phosphodiesterase (PDE) contains high-affinity, non-catalytic cyclic GMP (cGMP) binding sites (Yamazaki et al., J. Biol. Chem., 255:11619, 1980). We have begun to characterize the binding sites of outer segment phosphodiesterases from bovine retinas, where greater amounts of protein can be easily obtained.

Bovine photoreceptor phosphodiesterase isozymes were separated into two major peaks by anion exchange chromatography of hypotonic retinal extracts. The peak of activity eluting at lower conductivity (peak I) is the predominant form in cones, while the bulk of the PDE activity (peak II) is derived from rods (Hurwitz et al., J. Biol. Chem., 260:568, 1985). [^{3}H]cGMP binding to PDE is substantially greater in peak I than in peak II. Scatchard analysis of peak I binding indicated a single class of binding sites, with K_D = 12 nM. The maximal binding (B_{max}) was 10.1 pmoles cGMP bound per unit of PDE activity. Scatchard plots of peak II cGMP binding were curved; analysis of the data, assuming two noninteracting classes of binding sites, indicated K_D values of 16 and 234 nM. The B_{max} for peak II per unit activity was 10- to 20-fold less than for peak I. Peak I cGMP binding was stimulated by IBMX and inhibited by cGMP analogs with order of potency (Sp)cGMPS>(Rp)cGMPS>cGMP> 8-Br-cGMP. Several criterea suggest that the binding site is distinct from the catalytic site. These experiments indicate significant differences in cyclic GMP binding between the forms of photoreceptor phosphodiesterase.

94 NEURAL REGULATION OF ADENYLATE CYCLASE IN RAT BROWN ADIPOSE TISSUE. J.G. Granneman, Center for Cell Biology, Sinai Hospital of Detroit, Detroit, Mi 48235.

Many tissues decrease their responsiveness after exposure to increased levels of stimulation. In contrast, we have reported that cold exposure, which increased sympathetic nerve stimulation of brown adipose tissue (BAT) in rats, increased the responsiveness of adenylate cyclase to norepinephrine and fluoride. We are examining the molecular basis of the increase in adenylate cyclase responsiveness and have found that exposing rats to 4°C for 3 days increased cholera toxin-catalyzed [^{32}P]-ADP ribosylation of the stimulatory regulatory protein of adenylate cyclase (G_s) in BAT membranes by 2-3 times. The increase in labelling of G_s corresponded to the observed increase in adenylate cyclase activity. Furthermore, surgical denervation of BAT prevented both the increased labelling of G_s and the sensitization of adenylate cyclase, indicating that both result from increased neural stimulation of the tissue. In contrast, cold exposure did not alter pertussis toxin-catalyzed ADP-ribosylation of the inhibitory regulatory protein. Additional studies demonstrated that detergent extraction normalized adenylate cyclase activity to guanylyl-5'-imidodiphosphate stimulation without affecting the supersensitive response to fluoride, and that the supersensitive response to fluoride was reversed after reconstitution of detergent extracts into membranes of S49 cyc$^-$ lymphoma cells. We conclude that neural activation of BAT sensitizes adenylate cyclase by altering G_s. The exact nature of this change is not certain, but it may result from the interaction of G_s with membrane components that are unique to membranes of cold-exposed rats. Supported by NIH grant AM 37006.

95 POSSIBLE MECHANISM FOR INTRANEURONAL REGULATION OF ADENYLATE CYCLASE: NUCLEOTIDE TRANSFER BETWEEN GTP-BINDING PROTEINS. Shinichi Hatta, Marietta M. Marcus, Yuko Hatta and Mark M. Rasenick Department of Physiology and Biophysics, University of Illinois College of Medicine. Chicago, IL 60680

P^3-azidoanilido-P^1-5'GTP (AAGTP) is an hydrolysis-resistant, photoaffinity GTP analog which is capable of supporting activation or inhibition of adenylate cyclase which is sustained subsequent to buffer washing of membranes. Under conditions where adenylate cyclase inhibition is seen, photoaffinity analysis of synaptic membranes indicates ^{32}P AAGTP bound to the inhibitory, 40 KDa GTP-binding protein [GNi]. If these membranes are then incubated under conditions which reverse the inhibition of adenylate cyclase, and the membranes are subsequently subjected to UV irradiation, AAGTP appears bound to the stimulatory GTP-binding protein [GNs]. The degree of AAGTP appearing on GNs is proportional to the reversal of adenylate cyclase inhibition, and the sum of AAGTP bound to GNs and GNi is constant. These results are consistent with direct transfer of nucleotide between GNi and GNs. Whereas Mg^{++} is required for AAGTP binding to GNi and for the transfer of AAGTP between GNi and GNs, it is not required for AAGTP binding to GNs. The phenomenon of AAGTP transfer between GNi and GNs as well as the existence of a novel 32 KDa AAGTP-binding protein has been observed, thus far, only in membranes from neural crest cells. Due to the slow GTPase activity expressed by GNs and GNi, exchange of GTP between GN proteins is a possibility. We believe that the phenomenon of nucleotide transfer between GNs and GNi may represent a mechanism for the intracellular regulation of adenylate cyclase in nervous tissue.

96 GUANINE NUCLEOTIDE REGULATORY PROTEINS IN THE ADRENAL CORTEX
William P. Hausdorff, Greti Aguilera, and Kevin J. Catt, NICHD, NIH, Bethesda, MD 20892

We examined the effects of pertussis toxin (PT) on glomerulosa cell responses to angiotensin II (AII) and somatostatin (SRIF), which respectively stimulate and inhibit aldosterone production by the adrenal gland. Both hormones inhibit cAMP accumulation in isolated glomerulosa cells, and this effect was attenuated by prior exposure to PT. The toxin also blocked the ability of SRIF to inhibit the aldosterone response to 1 nM AII. However, PT effects on the stimulation of steroid production by AII alone were more complex. AII elicits a biphasic steroid response in which AII concentrations up to 10 nM caused dose-dependent increases in aldosterone release; this response was unaffected by PT treatment. At higher hormone concentrations steroid output begins to decline, and this response was blocked by the toxin. In studies with radiolabeled ligands, ^{125}I-Tyr^0-SRIF binding to permeabilized cells became almost undetectable following PT pretreatment. Under the same conditions, ^{125}I-AII binding, and the inhibitory effects of guanine nucleotides thereupon, were apparently unperturbed. We also detected a low K_m GTPase activity in permeabilized cells that is specifically stimulated by AII, and this activity was found to be refractory to PT pretreatment. Studies with [^{32}P]NAD revealed a 40-41 kDa band that was specifically ADP-ribosylated in the presence of the toxin; immunoblot studies indicated that this protein was not the α subunit of N_o. These results are consistent with a model in which low concentrations of AII (< 10 nM) couple the AII receptor to a PT-insensitive guanine nucleotide binding protein (N_p) that activates polyphosphoinositide hydrolysis and stimulates steroid production. Furthermore, SRIF or high concentrations (> 10 nM) of AII activate N_i and cause partial inhibition of the aldosterone response elicited by physiological AII concentrations.

97 N-PROTEIN-MEDIATED INHIBITORY EFFECT OF OPIATES ON VOLTAGE-DEPENDENT CALCIUM CHANNELS IN NEUROBLASTOMA X GLIOMA HYBRIDS. J.Hescheler, W.Rosenthal, W.Trautwein, and G.Schultz. II. Physiologisches Institut, Universität des Saarlandes, D-6650 Homburg/Saar, F.R.G., and Institut für Pharmakologie, Freie Universität Berlin, D-1000 Berlin 33, F.R.G.

In neuroblastoma x glioma hybrid cells (108CC15, NxG cells), opiates and α_2-adrenergic agonists cause adenylate cyclase inhibition, presumably via N_i, the inhibitory N-protein. N_i and N_o, another N-protein with unknown function, are substrates of B. pertussis toxin (PT) in NxG cells. Effects of opiates and α_2-adrenergic agonists on voltage-dependent calcium channels were studied in differentiated NxG cells, using the whole cell clamp technique with 10.8 mM Ba^{2+} as charge carrier. D-Ala^2,D-Leu^5-enkephalin (DADLE) concentration-dependently reduced the calcium current (I_{Ca}). Maximal and almost complete inhibition of I_{Ca} was seen with 1 µM DADLE, whose effect was half-maximal between 10 and 100 µM. Morphine (10-100 µM) and adrenaline (10-100 µM) plus propranolol (10 µM) had smaller inhibitory effects on I_{Ca}, carbachol (10-100 µM) was without effect. The DADLE effect was rapidly reversed by washing or by addition of naloxone (10 µM). Intracellular application of GTPγS (10-100 µM) through the patch electrode mimicked the DADLE effect. The effect of DADLE but not that of GTPγS was blocked by about 90% by pretreatment with PT (200 ng/ml) for several hours. The DADLE effect on I_{Ca} in PT-treated cells was restored by intracellular application of N_i and N_o purified from porcine brain; the α-subunit of N_o was more potent than N_i in restoring the DADLE effect on I_{Ca}. These data indicate that PT-sensitive N-proteins, most likely N_o, are involved in functional coupling of opiate receptors to voltage-dependent neuronal calcium channels.

98 ACTIVATION OF PHOSPHOLIPASE C IN RABBIT PLATELET MEMBRANES BY GTPγS OR BY THROMBIN AND GTP. J. K. Hrboticky, M. Culty and R. J. Haslam. Dept. of Pathology, McMaster Univ., Hamilton, Ont., Canada.

Rabbit platelets were labelled with [^{3}H]inositol and lysed by freezing and thawing in 100 mM KCl, 25 mM Hepes (pH 7.4), 2.5 mM EGTA, 1 mM MgATP and 0.5 mM Mg^{2+}_{free}. The membrane fraction was washed twice at 4°C in this medium minus KCl and was finally resuspended in medium with KCl. Samples were incubated for 10 min at 25°C. Addition of $CaCl_2$ to give 0.1 μM Ca^{2+}_{free} led to the formation of [^{3}H]inositol monophosphate (IP) and bisphosphate (IP_2) in a ratio of about 1:5. Further addition of 10 μM GTPγS increased IP about 3-fold and IP_2 about 5-fold. Little [^{3}H]inositol trisphosphate (IP_3) accumulated (<10% of IP_2), even when unlabelled IP_3 was added. GTPγS was ineffective in the absence of Ca^{2+}. Half-maximal effects were observed with 0.3 μM GTPγS. Measurement of the ^{3}H in membrane phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) gave initial ratios of 9:3:1 and demonstrated a 30% increase in PIP_2 in control incubations. Addition of 10 μM GTPγS prevented this increase in PIP_2 and led to a 20% decrease in PIP. GTP (1-100 μM) also increased IP_2 formation but only to the level observed with 0.2 μM GTPγS. Thrombin (2 units/ml) induced only small increases in IP_2 in the absence of added GTP but, in the presence of ≥10 μM GTP increased IP and IP_2 to the same extent as 10 μM GTPγS. Synergism was also observed between thrombin and 0.1 μM GTPγS. PIP was again the major source of IP_2; little IP_3 was formed. The effects of thrombin in the presence or absence of GTP and of GTPγS were inhibited by 400 μM GDPβS. The results indicate that a G-protein mediates the activation of phospholipase C by thrombin in the platelet and suggest that PIP is a major substrate of this enzyme.

Supported by the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario.

99 THE βγ SUBUNITS OF TRANSDUCIN ACTIVATE AND THE α SUBUNIT INHIBITS PHOSPHOLIPASE A_2. C.L. Jelsema and J. Axelrod. NIMH, Bethesda MD 20892.

Transducin (Td), the major GTP-binding protein (G protein) of the retina, has been demonstrated to couple photoexcitation of rhodopsin to activation of phospholipase A_2 (PLA_2) in rod outer segments (ROS) of bovine retina (Jelsema, Fed. Proc. 45, 1560), as measured by liberation of arachidonate from phosphatidylcholine in in vitro assays of PLA_2. Loss of Td from dark-adapted ROS membranes was accompanied by loss of light-activated PLA_2 whereas addition of purified Td to Td-depleted ROS partially restored the light-stimulated PLA_2 activity. Addition of GTPγS, which mimics the light-induced dissociation of Td into subunits, also increased PLA_2 activation associated with the addition of exogenous Td to Td-depleted ROS. Pertussis toxin pretreatment of Td, which prevents subunit dissociation, also prevented PLA_2 activation by Td. These results indicate a requirement for subunit dissociation in the Td-mediated activation of PLA_2. To examine the role of the individual Td subunits in modulation of PLA_2, purified Td was obtained by GTP-extraction of purified, light-activated ROS and the α and βγ subunits were separated by two different methods following activation of pure Td with Al^{+++}, Mg^{++}, and F^-. The isolated subunits were then added to either Td-depleted, dark-adapted ROS or to soluble snake venom PLA_2. Addition of the isolated α or βγ subunits, prepared by either method, were each found to stimulate the PLA_2 activity of both Td-depleted ROS and the soluble enzyme. The activation associated with the βγ subunits, however, was consistently 3 to 5 times the activation associated with the isolated α subunit. Addition of the α and βγ subunits together inhibited the stimulation observed with the individual subunits. This inhibition was prevented by addition of GTPγS and enhanced by pretreatment with pertussis toxin. These results suggest a stimulatory role for the βγ subunits of Td and an inhibitory role for the α subunit in the modulation of PLA_2.

100 IMMUNOCYTOCHEMICAL DISTRIBUTION OF CaM-DEPENDENT PHOSPHODIESTERASE IN RAT BRAIN: EVIDENCE FOR SELECTIVE EXPRESSION AND TRANS-SYNAPTIC REGULATION. R. L. Kincaid+, C. D. Balaban*, M. L. Billingsley**. +NHLBI, NIH, Bethesda, MD., Depts. of *Anatomy and **Pharmacology, Pennsylvania State University, Hershey, PA.

Antibodies to bovine brain CaM-dependent cyclic nucleotide phosphodiesterase (PDE) were raised in rabbits and further purified by affinity chromatography on Protein-A Sepharose and PDE-Sepharose. These were characterized by immunoprecipitation, solid-phase assays (ELISA), Western blot and immuno-affinity chromatography analysis to be specific for the CaM-dependent form of PDE; no cross-reactivity to other phosphodiesterases, CaM-binding proteins or cytosolic brain proteins was seen. Enzyme activity could be removed from brain supernatant by immunoadsorption and quantitatively recovered in the pellet. Immunolocalization in rat brain demonstrated very high concentrations of PDE in dendritic fields of cerebellar Purkinje cells, as well as pyramidal cells in cerebral cortex and hippocampus; some areas such as hypothalamus showed very little immunoreactivity. These data suggest selective expression of this enzyme in major output neurons of the central nervous system, many of which exhibit Ca and cyclic nucleotide-dependent electrophysiologic responses. Furthermore, selective pharmacologic lesions of climbing fiber input to Purkinje cells result in a virtually complete loss of immunoreactivity in the dendrites, while not affecting that in other brain regions. Immunoblot analysis of whole cerebellum after such treatment indicated a decrease of > 75% in PDE content. These findings are consistent with trans-synaptic control of PDE expression in these neurons and suggest a role for the enzyme in intraneuronal integration.

101 CHARACTERIZATION OF N-PROTEIN SUBUNITS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS (2D-PAGE). D. Koesling, M. Pallast, C. Kleuss and W. Rosenthal.
Institut für Pharmakologie, Freie Universität Berlin, D-1000 Berlin 33, F.R.G.

In human neutrophil leukocytes (PMNs), pertussis toxin (PT)-sensitive N-proteins are involved in transmembranous signalling between the receptor for chemotactic peptides and various effector systems including phospholipase C (PLC). The PT-sensitivity of the PMN N-protein involved in PLC stimulation is in contrast to other tissues where PLC is apparently regulated by a PT-insensitive N-protein. We compared the PT substrates in membranes of PMNs, human erythrocytes (RBCs) and human platelets. Membranes were incubated with ^{32}P-NAD and PT and consequently analyzed by 2D-PAGE. Radiolabelled proteins were visualized by autoradiography. In all 3 tissues, a major PT substrate of 40 kDa and a pI value of about 5.3 was identified. Only in PMN membranes, a second major PT substrate of 40 kDa with a pI value of about 5.4 was observed. Compared to the more acidic substrate, Lubrol PX and $\beta\gamma$-complex overproportionally increased incorporation of radioactivity into the PMN-specific peptide. We conclude that PMN membranes contain a cell-specific PT-sensitive N-protein. - We also used 2D-PAGE for the characterization of N-protein β-subunits. β-Subunits were detected by immunoblotting, using an antiserum against transducin (TD), which recognized all TD subunits and cross-reacted with the 36 kDa β-subunit of N_s, N_i and N_o. N-proteins purified from porcine brain and crude cholate extracts of brain and RBC membranes were tested. In preparations of purified N_i or N_o and in the crude extracts from both tissues, two immunoreactive peptides of 36 kDa were identified which differed in their pI values by 0.15 pH units. The occurence of different 36 kDa β-subunits may be explained by posttranslational modification.

102 PROTEIN KINASES AND PROTEIN PHOSPHATASES AS REGULATORY ENZYMES FOR CALCIUM TRANSPORT IN CARDIAC SARCOPLASMIC RETICULUM. E.G. Kranias, B.A. Davis and J. Di Salvo, Univ. of Cincinnati College of Medicine, Cincinnati, OH 45267-0575.

Calcium transport by cardiac sarcoplasmic reticulum (SR) appears to be regulated through phosphorylation of phospholamban, a 24,000 dalton polymeric proteolipid. Phospholamban is phosphorylated by cAMP-dependent protein kinase and by an endogenous Ca^{2+}-calmodulin(CAM)-dependent protein kinase which is regulated by calcium concentrations similar to those occurring intracellularly during contraction and relaxation of the cardiac muscle. Phosphorylation of phospholamban by cAMP-dependent or by Ca^{2+}-CAM-dependent protein kinase is associated with increased rates of calcium transport, which reflects an increased affinity of the transport protein for calcium. Phosphorylation of phospholamban by cAMP-dependent and Ca^{2+}-CAM-dependent protein kinases occurs in an independent manner. The stimulatory effects of the two protein kinases on the calcium pump may be reversed by an endogenous protein phosphatase activity. Dephosphorylation of phospholamban results in a reduction of the stimulation on calcium transport produced by the protein kinases. Rephosphorylation of phospholamban by protein kinases causes full recovery of the stimulation on calcium transport and reversal of the effects mediated by the protein phosphatase. The phospholamban protein phosphatase may be solubilized from cardiac SR membranes by Triton X-100 and subsequently purified by chromatography on Mono Q HR 5/5 and polylysine agarose columns. When subjected to sucrose density centrifugation the SR protein phosphatase appears to have a M_r of 46,000. These findings indicate that the calcium pump in cardiac SR is under reversible regulation by protein kinase(s) and protein phosphatase(s). Such regulation may represent an important control mechanism for the myocardium. (Supported by NIH grants HL 26057, HL 22619 and HL 20196).

103 INSULIN-DEPENDENT PHOSPHORYLATION OF G PROTEINS IN PHOSPHOLIPID VESICLES by J. Krupinski, M. Lakonishok, J. Benovic, and R. Cerione, Cornell U., Ithaca, NY 14853, Duke U., Durham, NC 27710.

We have investigated the involvement of GTP binding proteins (G proteins) in insulin action by co-reconstituting tyrosine agarose-purified, or insulin-sepharose purified, insulin receptors and G proteins into phospholipid vesicles. When pertussis toxin sensitive G proteins from bovine brain (G_i and G_o) are used in the co-reconstitution, an insulin-stimulated phosphorylation of the α (α_i and α_o) and γ subunits of the heterotrimeric G proteins results. Under optimal conditions this phosphorylation is equal in extent to the insulin-stimulated phosphorylation of the receptor itself. However, while most of the receptor phosphorylation occurs at a tyrosine residue, this is not the case for the phosphorylation of the G proteins, suggesting the involvement of another kinase which is present in the tyrosine agarose preparations. The insulin-promoted phosphorylation of the G proteins requires their co-insertion with insulin receptors into lipid vesicles, i.e. little or no phosphorylation occurs when these proteins are simply mixed in detergent solution. Moreover, the insulin-promoted phosphorylation appears to be specific for G_i or G_o; other heterotrimeric GTP binding proteins such as G_s or transducin are poor substitutes in the vesicles. The G proteins cause an enhancement of the insulin-stimulated autophosphorylation of the purified receptor. This enhancement is in part a reflection of an increased incorporation of the insulin receptor into lipid vesicles (induced by the G proteins). Taken together, these studies provide evidence for the interactions of GTP binding proteins with the insulin receptor in a lipid milieu.

104 EFFECT OF PERTUSSIS TOXIN ON THE PHOSPHODIESTERATIC CLEAVAGE OF THE POLYPHOSPHOINOSITIDES BY GUANOSINE 5'-O-THIOTRIPHOSPHATE AND THROMBIN IN PERMEABILIZED HUMAN PLATELETS. Eduardo G. Lapetina. Department of Molecular Biology, The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, North Carolina 17709, U.S.A.

Permeabilization of human platelets with 15 to 25 μg/ml saponin allows ADP-ribosylation by pertussis toxin of the α_i-subunit of G_i (N_i), a guanine nucleotide-binding regulatory protein. We have used the same assay conditions to determine phospholipase C in permeabilized platelets. Guanosine 5'-O-thiotriphosphate ($GTP\gamma S$) activated phospholipase C in permeabilized platelets whose inositol phospholipids were prelabeled with [^{3}H]inositol. Phospholipase C activity was measured by [^{3}H]polyphosphoinositide decreases and formation of [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate. Prostacyclin, cyclic AMP or pretreatment of permeabilized platelets with pertussis toxin did not alter this effect under conditions in which the α_i-subunit was effectively ADP-ribosylated by pertussis toxin. This information indicated that ADP-ribosylation and inactivation of G_i-protein was not directly related to activation or inhibition of platelet phospholipase C by $GTP\gamma S$. Thrombin also activated phospholipase C in permeabilized platelets and, surprisingly, this action was enhanced by enhanced by pertussis toxin pretreatment. This indicates that inactivation of G_i-protein facilitates the action of thrombin on phospholipase C.

105 STIMULATION OF HUMAN PLATELETS WITH TRYPSIN, THROMBIN AND COLLAGEN INHIBIT THE PERTUSSIS TOXIN-INDUCED ADP-RIBOSYLATION OF A 41,000 DALTON PROTEIN. Eduardo G. Lapetina, Bryan Reep, and Kwen-Jen Chang. Molecular Biology Department, Burroughs Wellcome Co., Research Triangle Park, NC 27709

Permeabilization of human platelets with 15-25 μg/ml saponin allows the determination of the ADP-ribosylation of a 41,000 dalton protein by pertussis toxin. The ADP-ribosylated protein is present in the particulate fraction. ADP-ribosylation of the 41,000 dalton protein increases for 20 min; it is not affected by indomethacin, prostacyclin, and 1,2-diacylglycerols, and is inhibited by 1 mM Ca^{2+} and phorbol esters. The stimulation of platelets with TPCK-trypsin, thrombin or collagen before saponin addition precludes subsequent pertussis toxin-induced ADP-ribosylation of the 41,000 dalton protein. The effect of TPCK- trypsin and thrombin is blocked by protease inhibitors such as soybean trypsin inhibitor and leupeptin. TPCK-trypsin proteolytically cleaves the ADP-ribosylated 41000 dalton protein to an ADP-ribosylated fragment slightly smaller than 20000 dalton. The results suggest that a modification of a guanine nucleotide-binding regulatory protein is associated with the action of TPCK-trypsin, thrombin and collagen on platelet activation.

106 G PROTEINS MEDIATE MULTIPLE INHIBITORY ACTIONS OF SOMATOSTATIN IN AtT-20 CELLS. Luini, A. Axelrod, J. and Lewis, D. NIAA, Rockville, MD 20852 and NIMH, Bethesda, MD 20892.

Opiates, GABA, noradrenaline, acetylcholine and somatostatin all act on receptors that inhibit neurotransmitter or hormone secretion. To study the mechanism of action of somatostatin , the use of a secretory clonal cell line (AtT-20) amenable to biochemical and electrophysiological studies, has been advantageous. AtT-20 cell secrete ACTH in response to an intracellular increase of cAMP and/or calcium. The inhibition of adenylate cyclase via a GTP binding protein (Gi) is an already known action of somatostatin. Here, we show two other separate mechanisms of somatostatin, both involving a G protein. One is the inhibition of voltage dependent calcium channels: In whole cell patch clamp experiments, somatostatin reduced the voltage-dependent calcium current. The intracellular free calcium concentrations (measured by the Quin-2 technique) were also reduced. Treatment of the cells with pertussis toxin, which ADP ribosylates the GTP binding proteins Gi and Go, thereby presumably inactivating them, abolished the action of somatostatin on both calcium current and intracellular free calcium. Intracellular application of the nonhydrolyzable guanine nucleotide analog GTPS, which irreversibly activates G proteins, changed the somatostatin effect on calcium current from a reversible to an irreversible inhibition. Intracellular GTPS alone caused a very slowly developing inhibition of calcium current. The effect of GTPS on the inhibition of calcium current by somatostatin was not altered by the intracellular application of cAMP and IBMX. These results suggest that a GTP-binding protein is directly involved in the cAMP-independent, receptor-mediated inhibition of voltage-dependent calcium channels. A second mechanism is inhibition of the ACTH exocytotic machinery: Somatostatin reduced secretion caused by calcium ionophores without modifying their effect on cytosolic calcium. This action of somatostatin was not changed by large concentrations of cAMP analogs and it was abolished by pertussis toxin. In cells permabilized with digitonin, calcium dependent secretion was inhibited by somatostatin and the inhibition was GTP dependent. In summary, somatostatin receptors inhibit adenylate cyclase, voltage dependent calcium channels and an as yet unknown step of the secretion process, all via a GTP binding protein.

107 IMMUNOLOGICAL IDENTIFICATION OF THE MAJOR PLATELET LOW Km cAMP PHOSPHODIESTERASE: A TARGET FOR ANTI-THROMBOTIC AGENTS AND cAMP-DEPENDENT PHOSPHORYLATION. C.H. Macphee, S.A. Harrison, D.H. Reifsnyder, and J.A. Beavo. Department of Pharmacology, University of Washington, Seattle, WA 98195

A novel low Km cAMP phosphodiesterase (PDE) has recently been purified in our laboratory from bovine heart (Harrison et. al., Mol. Pharmacol. 29, 506-514, 1986). This enzyme has been designated as cGMP-inhibited PDE since low concentrations of cGMP inhibit (Ki=60nM) cAMP hydrolysis. Western blot and enzyme activity analyses, using mouse antiserum and monoclonal antibodies, indicated that more than 90% of the total low Km cAMP PDE activity in bovine and human platelets resided in a single 110KDa peptide which was immunologically and structurally indistinguishable from the recently purified heart enzyme. This platelet PDE was found to be extremely susceptible to proteolytic activation resulting in the formation of 80KDa and 60KDa fragments. In addition, several known PDE inhibitor compounds which have been found useful in inhibiting platelet aggregation also inhibited the platelet low Km cAMP PDE with potencies very similar to their anti-thrombotic effects. Cilostamide, Ro 15-2041, milrinone, papaverine, IBMX, and theophylline inhibited the 110KDa platelet enzyme with I.C.$_{50}$ values of 0.04, 0.13, 0.46,1.4, 2.6, and 110μM, respectively. Since both the heart and platelet 110KDa PDE can be phosphorylated in vitro by cAMP-dependent kinase we attempted to phosphorylate the enzyme in fresh intact human platelets prelabelled with[^{32}P]-PO$_4$. Specific immunoadsorbtion of the platelet PDE using solid-phase antibody reagent demonstrated that PGE$_1$, an agent which elevates platelet cytosolic cAMP levels, promoted the rapid (<1Min.) and dose-dependent phosphorylation of the 110KDa PDE in intact human platelets.

108 PURIFICATION OF A NEW GTP-BINDING PROTEIN FROM BOVINE BRAIN. M.W. Martin, G.L. Waldo, T. Evans, J.K. Northup, and T.K. Harden. Dept. of Pharmacology, Univ. of North Carolina School of Medicine, Chapel Hill, N.C. and Dept. of Pharmacology and Experimental Therapeutics, Univ. of Calgary, Calgary, Alberta, Canada.

A protein that binds guanine nucleotides with high affinity was purified from cholate extracts of bovine brain membranes by sequential DEAE-Sephacel, Ultrogel AcA-34, heptylamine-Sepharose, and Sephadex G-150 chromatography. Photoaffinity labelling with (γ-^{32}P)-8-azido-GTP indicated that the GTP-binding activity of the protein is associated with a 25,000 dalton peptide alpha subunit. The alpha subunit copurified with 35 and 36 kilodalton peptides that were immunologically and electrophoretically indistinquishable from the β subunits of known GTP-binding proteins. The purified protein specifically bound 17.2 nmol of ^{35}S-GTPγS per mg protein with high affinity (Kd = 15 nM), i.e. one mole of GTPγS was bound per mole of native protein, assuming an Mr of 67,000. The protein is not a substrate for ADP-ribosylation by either pertussis or cholera toxins. The protein purified from brain comigrated on SDS-polyacrylamide gels with the GTP-binding protein, Gp, recently purified from human placental membranes by Evans, et al. (J.Biol.Chem. 261:7052, 1986) and cross-reacted on Western blots with polyclonal antibodies raised against platelet Gp, but not with antibodies raised to brain Go. The functional activity of this protein is under investigation.
Supported by USPHS Grants GM29536 and NS23019.

109 cDNA CLONING OF THE GTP BINDING PROTEIN, α_i, AND OF A NOVEL G PROTEIN, α_h. T. Michel[1], J.W. Winslow[1], J.A. Smith[2], J.G. Seidman[3] and E.J. Neer[1], [1]Cardiovascular Division, Brigham and Women's Hospital; [2]Dept. Molecular Biology and Pathology, Massachusetts General Hospital; and Depts. of [1]Medicine, [2]Pathology and [3]Genetics, Harvard Medical School, Boston, Massachusetts.

Signal transduction by receptors in biological membranes is modulated by a family of related GTP binding proteins (G proteins). A 41 kDa G protein, α_i, is implicated in signal transduction by receptors coupled to adenylate cyclase inhibition and by receptors which modulate Ca^{++} flux and phosphoinositide metabolism. We have determined the amino acid sequence of peptides isolated from the α_i protein and from a related 39 kDa protein, α_o, both proteins purified from bovine brain. The amino acid sequences of these peptides show homology with one another, and show 75% sequence identity with the reported sequences of the G protein transducin (α_t). Based upon this striking sequence homology, we used the cDNA for α_t to screen a bovine pituitary library, at low hybridization stringency, and we have isolated and characterized a cDNA clone which encodes α_i. The deduced amino acid sequence of the α_i clone demonstrates marked homology to several other GTP binding proteins. Southern blot analysis of the cloned α_i cDNA suggests the presence of two genes encoding this protein, which, in turn, are distinct from those encoding other G proteins. In addition, we identified a cDNA clone for a novel, putative G protein, α_h, which demonstrates marked sequence homology with other G proteins, but which represents a clearly distinct gene product. Taken together, these data suggest a new level of complexity in the organization of the G protein supergene family, with multiple G proteins of similar overall structural and mechanistic properties likely to be identified as products of distinct genes.

110 MEMBRANE FLUIDITY GOVERNS THE RECONSTITUTION OF GTP SENSITIVE ADENYLATE CYCLASE ACTIVITY. Dianne L. Newton and Werner A. Klee, Laboratory of Molecular Biology, NIMH, Bethesda, MD

The role of membrane fluidity in the interactions among adenylate cyclase, the GTP-binding regulatory proteins, and the opiate receptor has been studied by reconstitution of these proteins in phospholipid vesicles of varying composition. Adenylate cyclase was purified from rat brain membranes by a combination of forskolin or calmodulin-agarose and wheat germ agglutinin-agarose affinity chromatography. The GTP-binding regulatory proteins were purified from rabbit liver and bovine brain by published procedures (Sternweis et al., J. Biol. Chem. 256, 11517 (1981); Milligan and Klee, J. Biol. Chem. 260, 2057 (1985). Reconstitution into phospholipid vesicles was accomplished by dialysis. GTP insensitive stimulation of adenylate cyclase by G_s and its inhibition by G_i was observed with lipids of relatively low fluidity, such as asolectin and dipalmitoylphosphatidyl choline (DPPC). The GTP insensitive stimulation by G_s is enhanced by aluminum fluoride and is inhibited by G_i and the $\beta\gamma$ subunit of transducin. Thus, the activity of G_s in lipids of low fluidity reflects the presence of bound GDP which is not readily exchangeable with GTP under these conditions. However, GTP analogues do stimulate G_s and G_i activities when lipids of greater fluidity are used in the reconstitution. Thus, dimyristoylphosphatidyl choline (DMPC) and lecithins containing fatty acid moieties of 10 and 12 carbons in length support G_s and G_i activities which are stimulated by GTP analogues. Addition of cholesterol, which is expected to decrease the fluidity of the C_{10}-C_{14} lecithins and increase that of DPPC, decreased GppNHp stimulated activity with the short chain lipids and did not affect activity with DPPC. Cholesterol increases the GTP independent activity of G_s when the C_{12} lecithin is used. Thus, the GppNHp sensitive activity of G_s requires a highly fluid membrane environment, whereas the activity of the GDP-G_s complex is optimal with lipids of intermediate fluidity.

111 BINDING SITES FOR [^{3}H]FORSKOLIN IN MEMBRANES FROM RAT HEART, LUNG, KIDNEY AND LIVER: Their Regulation by GppNHp. K.B. Seamon and C.A. Nelson Center for Drugs and Biologics FDA Bethesda, MD 20892

A survey of the characteristics of ^{3}H-forskolin (^{3}H-FSK) binding to membranes from various rat tissues was carried out using a filtration assay. Binding sites have been reported in membranes from rat brain and human platelets which have a K_d of 20 nM and are increased up to 4-fold in the presence of GppNHp. ^{3}H-FSK binding sites were detected in membranes from rat lung which had a Bmax of 13 fmol/mg protein which was increased to 113 fmol/mg protein in the presence of GppNHp. Similarly, binding sites for ^{3}H-FSK were detected in membranes from rat heart which had a Bmax of 127 fmol/mg protein which was increased to 193 fmol/mg protein in the presence of GppNHp. The binding of ^{3}H-FSK to rat kidney membranes could only be detected in the presence of GppNHp with a Bmax of 31 fmol/mg protein. The Kd values for ^{3}H-FSK binding in heart, lung, and kidney were 34 nM, 29 nM, and 15 nM, respectively which is similar to values reported for membranes from rat brain and human platelets. In contrast, liver membranes had a K_d value of 329 nM for ^{3}H-FSK binding. This low affinity binding of ^{3}H-FSK had a Bmax of 3600 fmol/mg protein and was not regulated by GppNHp. There was a linear correlation between adenylate cyclase activity and the number of ^{3}H-FSK binding sites in all tissues except for liver. It was determined by HPLC chromatography that no degradation of the ^{3}H-FSK ligand occurred during incubations in any of the tissues studied. Current evidence indicates that the formation of the high affinity binding site for ^{3}H-FSK is coincident with the activation of adenylate cyclase via the guanine nucleotide binding protein. The nature of the low affinity binding site in liver is not known.

112 GUANINE NUCLEOTIDES STIMULATE NADPH OXIDASE IN PLASMA MEMBRANES OF HUMAN NEUTROPHILS: EVIDENCE FOR AN INVOLVEMENT OF A GUANINE NUCLEOTIDE-BINDING PROTEIN. R. Seifert, W. Rosenthal, and G. Schultz. Pharmakologisches Institut, Freie Universität Berlin, D-1000 Berlin 33, F.R.G.

The chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP), binds to specific membrane receptors and stimulates superoxide formation by NADPH oxidase (NO) in intact human neutrophils. The effect of FMLP involves a pertussis toxin(PT)-sensitive guanine nucleotide-binding protein (N-protein). As phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PK-C), also stimulates NO in intact cells, it is widely believed that PK-C-catalyzed phosphorylation of NO or of a regulatory component is involved in NO stimulation. We studied NO regulation in plasma membranes purified from unstimulated human neutrophils. In the presence of neutrophil cytosol, arachidonic acid (AA, 10 - 200 μM, peak at 50 μM) caused stimulation of NO (up to 2 μmoles x mg^{-1} x min^{-1}). Other long chain unsaturated cis- and trans-fatty acids had similar effects; saturated fatty acids and PMA did not activate NO. Addition of the stable GTP analogs, GTPγS (10 μM) and GppNHp (100 μM), or NaF (10mM), which activate N-proteins, prior to or with AA, enhanced NO activity up to 4-fold. The GTP analogs were without effect when added several minutes after AA. GDPβS inhibited the effect of GTPγS. In membranes from PT-treated neutrophils, the effects of GTPγS and GppNHp were not changed. Removal of cytosolic ATP by hexokinase and glucose suppressed the stimulatory effect of AA but not that of GTPγS. As trans-fatty acids are not known to stimulate PK-C and PK-C purified from chicken gizzard was no substitute for neutrophil cytosol, the cytosolic cofactor required for NO activation may not represent PK-C. The data suggest that NO is regulated by an N-protein, independent of activation of PK-C or elevation of cytoplasmic calcium.

113 ADENYLATE CYCLASE-LINKED STIMULATORY (R_s) AND INHIBITORY (R_i) RECEPTORS AND ASSOCIATED GTP-BINDING PROTEINS REGULATE INSULIN-STIMULATED PROCESSES IN RAT ADIPOCYTES BY cAMP-INDEPENDENT MECHANISMS. IA Simpson, M Kuroda, RC Honnor, SW Cushman, & C Londos, NIH, NIDDK, Bethesda, MD 20892

Rat adipocytes contain receptors whose actions, as defined by effects on adenylate cyclase, are either stimulatory (R_s) or inhibitory (R_i), and regulated by their respective GTP-binding complexes, N_s or N_i. In the absence of insulin ligands for R_s (eg. isoproterenol, ACTH) and R_i receptors (eg. adenosine, PGE_1) control lipolysis exclusively by modulating cAMP concentration and cAMP-dependent protein kinase (A-kinase) activity. However, in the presence of insulin, ligands for R_s and R_i receptors are shown to control several processes by mechanisms unrelated to cAMP or A-kinase activity. For example, R_s ligands increase and R_i ligands decrease insulin concentrations required for initiation of insulin-mediated antilipolysis and insulin-stimulated glucose transport activity. In insulin-stimulated cells, the intrinsic activity of the glucose transporters in the plasma membrane is increased by R_i ligands and decreased by R_s ligands. In the absence of R_s or R_i ligands, treatment of cells with cholera or pertussis toxin does not change transport activity. However, treatment with cholera toxin eliminates the effects of Rs ligands, and treatment with pertussis toxin eliminates R_i effects on transport. Thus, these cAMP-independent actions of R_s and R_i ligands on the glucose transporter are mediated by the N_s and N_i proteins, respectively. Moreover, the entire receptor package, both R_s and R_i, that is linked to adenylate cyclase is linked also to these cAMP-independent processes. It is concluded that adenylate cyclase is but one of a number of proteins which is regulated by a common $R_sN_s{\cdot}R_iN_i$ package in the adipocyte plasma membrane.

114 INSULIN REGULATION OF LIPOLYSIS AND PARTICULATE LOW K_m cAMP PHOSPHODIESTERASE (PDE) ACTIVITY IN RAT ADIPOCYTES. Carolyn J. Smith and Vincent C. Manganiello. Laboratory of Cellular Metabolism, NHLBI, NIH, Bethesda, MD. 20892

Lipolysis and particulate "low K_m" cAMP PDE were studied under conditions "defined" by incubation of adipocytes with adenosine (Ado) or adenosine deaminase (ADA) without or with N^6-phenylisopropyl-adenosine (PIA). ADA increased both lipolysis and particulate (not soluble) PDE activity (K act $\sim$ .02 U/ml), with maximal effects at 0.5-1 U/ml. PIA inhibited ADA-stimulated lipolysis and PDE ($K_i \sim$ 4 nM). Isoproterenol (Iso) increased lipolysis to the same extent with Ado, ADA, or ADA + PIA (10^{-7}M), but sensitivity to Iso was greater with ADA (K act $\sim$ 1 nM) than Ado or ADA + PIA (K act $\sim$ 15 nM). Maximal lipolysis with ADA alone was 65% that with Iso. Maximal Iso increased PDE activity by $\sim$ 190% with either Ado or ADA + PIA (K act $\sim$ 4 nM), but only increased PDE activity by $\sim$ 20% with ADA which itself increased PDE activity by $\sim$ 165%. Insulin (Ins) inhibited lipolysis stimulated by Iso + Ado (K_I $\sim$10 pM) and increased PDE activity $\sim$ 165% with Ado (K act $\sim$ 17 pM). With Ado, maximal Ins and Iso together increased PDE to a greater extent than either alone. Ins inhibited lipolysis stimulated by ADA alone, but effects of ADA and Ins on PDE were not > ADA alone. With ADA, Ins increased PDE in the presence of PIA, PGE_1, and nicotinic acid, agonists which activate N_i - coupled receptors and inhibited ADA stimulation of lipolysis and PDE. With ADA plus low Iso (< 10 nM) insulin alone inhibited lipolysis; at higher Iso, > 4 nM PIA was required for insulin inhibition. These studies point out a close relationship between presumed effector-induced activation of cAMP dependent protein kinase and activation of particulate (not soluble) cAMP PDE as well as hormone sensitive lipase. In addition they suggest a possible role for N_i-activation in the regulation of particulate cAMP PDE by insulin.

115 DISTINCT PROPERTIES OF PURIFIED KIDNEY HIGH AFFINITY CYCLIC AMP(cA) PHOSPHODIESTERASE (Type IV PDE) S.J. Strada, C.-C. Shen and N.J. Thompson, Dept. Pharm., U.of So. Alabama, Mobile, AL 36688.

High affinity forms of cA PDE from rat heart, kidney and liver tissues were compared to a dog kidney enzyme purified to a 50-fold higher specific activity than previously reported (Biochem.18: 5228, 1979). Type IV kidney PDE is separated from a 60 kD binding protein identified with polyclonal antisera raised against the enzyme complexed with the binding protein which appeared homogeneous by classical criteria. Purification has been improved by including Affi-gel blue chromatography between DEAE fractionation and S200 gel filtration. The enzyme binds avidly to Affi-gel blue allowing removal of contaminants rapidly and in large quantity. The fold purification of the enzyme from kidney homogenates is 6000. The Km of the high specific activity enzyme(1.6 uM) and pharmacological specificity are the same as those previously reported. The maximum velocity of the enzyme is 8-20 umoles/min/mg in various preparations. 10% SDS gels show ca. 80% of the protein at 81 kD, with contaminats evident at 63, 47, 34 and 32 kD. DEAE-cellulose fractionation of rat tissue supernatants resolves Type IV activities from other enzyme forms. These activities can be distinguished by cG inhibition of cA hydrolysis and by pharmacological specificity. Heart is predominantly cG inhibited, liver contains both cG inhibited and non-inhibited Type IV activities, whereas kidney contains soley non-cG inhibited activity. All cG inhibited activities are markedly inhibited by cilostamide and milrinone whilst kidney-like forms are much more sensitive to inhibition by SQ-65442. These studies indicate that an activity similar to that purified from dog kidney exists in other tissues and that Type IV enzyme activities can be distinguished on the basis of their sensitivities to inhibition by cG and by pharmacological agents. This research is supported by GM 33538.

116 ONTOGENY OF GUANINE NUCLEOTIDE AND IONIC REGULATORY FACTORS OF OPIOID RECEPTORS. M. Szűcs, G.M. Oetting, J.W. Spain and C.J. Coscia, E.A. Doisy Department of Biochemistry, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104.

Having established differential ontogeny for μ and δ opioid receptors in rat brain, we have examined their regulation by mono- and divalent cations and guanine nucleotides (GN) during postnatal development. These agents are known not only to modulate the receptor binding directly, but also be required for coupling of opioid receptors to the secondary messenger system. The binding of ^{3}H-DADLE (in the presence of 10 nM DAGO) was most sensitive to divalent cations during the first week postnatal with increases over control values as high as 100% by Mn^{++}, and 70% by Mg^{++} or Ca^{++}. In contrast, μ binding (measured with 1 nM ^{3}H-DAGO) was inhibited by cations and the effect did not change during development. GTP, Gpp(NH)p, and GDP inhibited μ by 40-60% and δ binding by 15-30%, an effect that again remained constant throughout development. Na^+ substantially increased the potency of GNs in all ages. The inhibition of binding by Gpp(NH)p+Na^+ was increasingly reversed at both sites by the addition of Mn^{++}, with adult levels of reversal being achieved by the second week postnatal. In order to assess whether the GN binding proteins undergo a time-dependent development, membranes from 5 day old and adult rat brains were labeled with [^{32}P]NAD in the presence of pertussis toxin. For neonates, an intense labeling of a doublet with molecular weights of 39-41 kDa was observed corresponding to bands labeled in adults. We conclude that: (1) divalent cations bind to a site distinct from the GN site; and (2) GN binding proteins appear early in development, but further work is required to assess whether they are functionally coupled to μ/δ opioid receptors in neonates.

117 CORRELATION OF VANADATE/GLUTATHIONE(V/GSH) ACTIVATION AND CYCLIC GMP(cG) INHIBITION OF RABBIT LIVER PARTICULATE HIGH AFFINITY CYCLIC AMP(cA) PHOSPHODIESTERASE (TYPE IV PDE). M.J. Thompson, B.H. Tan and S.J. Strada, Dept. Pharm., U.So.Alabama, Mobile, AL 36688.

We previously reported activation of particulate Type IV PDE activity in rat adipocytes (Souness et al., JCNPPR 10:483,1985) by a complex of sodium vanadate and glutathione (V/GSH). Similarly, the maximum velocity of high affinity liver particulate activity (Ka=0.7) is increased (280% above basal) by vanadyl ion complexed with glutathione (1:2). Other elements capable of the V-IV valence transition states with GSH, eg., tungsten, tantallium and niobium will stubstitute for vanadium. Mixtures of GSH and manganese, chromium or molybdenum are inactive. L-cysteine, cysteic acid, cysteamine or N-acetyl-cysteine, but not cystamine, cystine, homocysteine or taurine, can substitute for GSH. These results indicate the critical role of the -SH as an electron donor in the complex activation. Particulate cG PDE hydrolysis (Km=12 uM) is not affected by V/GSH, however, the sensitivity of Type IV PDE to cG inhibition is increased 20-fold by V/GSH activation. Type IV PDE influenced by these effectors is solubilized with 0.2% Triton X-100 but not by 0.4M NaCl or hypotonic lysis. Differential centrifugation shows effector localized to light microsomal membranes. cG inhibits both basal and V/GSH activated enzyme activity to the same extent, suggesting an effect on the same enzyme activity. In addition, drug inhibition profiles of cG inhibited and V/GSH activated activity are parallel. These data suggest that V/GSH complexes in vitro mimic insulin activation of Type IV PDE in intact cells. Further, both agents affect the cG inhibited form of high affinity in hepatic membranes, perhaps via an oxidation-reduction mechanism. Research supported by USPHS grant GM 33538.

118 DEDUCED AMINO ACID SEQUENCE OF A cDNA CLONE FOR BOVINE $G_{o\alpha}$: POTENTIAL CHOLERAGEN AND PERTUSSIS TOXIN MODIFICATION SITES AND SIMILARITIES TO OTHER GUANYL NUCLEOTIDE-BINDING PROTEINS. K.P. Van Meurs*, C.W. Angus*, S.R. Price*, S.-C. Tsai*, R. Adamik*, S. Lavu+, H.-F. Kung++, J. Moss*, and M. Vaughan*. *National Heart, Lung, and Blood Institute, NIH, Bethesda, MD; +PRI and ++BRMP, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD.

Guanyl nucleotide-binding proteins (GNPs) are critical in the regulation of many receptor-mediated events. G_o is a GNP which interacts functionally with rhodopsin and muscarinic receptors, but is apparently not involved in regulation of adenylate cyclase. G_o is similar to other GNPs in that it is a heterotrimer of α, β, and γ subunits. A cDNA clone (λGO9, ~1.7 kb in length) was isolated from a bovine retinal λgt10 cDNA library using oligonucleotide probes complementary to sequences found in two cDNA clones for the alpha subunit of transducin (Angus et al. (1986) Proc. Natl. Acad. Sci. USA in press). A comparison of amino acid sequences of tryptic fragments from bovine brain $G_{o\alpha}$ with the deduced amino acid sequences for λGO9 revealed complete identity, consistent with the conclusion that this clone was derived from a $G_{o\alpha}$ mRNA. Analysis of nucleotide and deduced amino acid sequences of λGO9 also revealed significant structural similarities to the α subunits of bovine G_t, G_s and G_i and rat brain $G_{o\alpha}$. λGO9 encodes for an amino acid sequence highly homologous to the region surrounding the arginine that is the choleragen ADP-ribosylation site in $G_{t\alpha}$, and for a carboxy terminal sequence which contains the cysteine modified by pertussis toxin. The levels of $G_{o\alpha}$ mRNA determined by Northern analysis were consistent with the observed tissue distribution of the $G_{o\alpha}$ protein. λGO9 exhibits characteristics indistinguishable from those observed for $G_{o\alpha}$ and demonstrates substantial similarities to the alpha subunits of bovine G_t, G_s and G_i and to rat brain G_o.

119 *INSULIN STIMULATION OF ADIPOCYTE CYCLIC AMP PHOSPHODIESTERASE IS INDEPENDENT OF PERTUSSIS OR CHOLERA TOXIN SENSITIVE INTERMEDIATES.* Heather W. Weber, Kirsten Day, Fu-Zon Chung and M. Michael Appleman, Molecular Biology, University of Southern California, Los Angeles, CA.

Insulin can rapidly reduce cyclic AMP levels in isolated rat adipocytes due to a glucose-independent stimulation of the degradative enzyme cyclic AMP phosphodiesterase. Adenylate cyclase activity is stimulated by β-adrenergic agents and inhibited by adenosine agonists. Action of these stimulatory and inhibitory agonists is mediated by GTP-binding proteins (N_s and N_i, respectively). It has been suggested that these or related proteins may be involved in the insulin action on the cAMP phosphodiesterase. Using agonists and antagonists of the adenosine receptor, it can be shown that the cAMP phosphodiesterase activation is independent of the regulatory status of the adenylate cyclase inhibitory receptor. Pertussis toxin specifically modifies N_i, so that adenylate cyclase activity is no longer inhibited by adenosine agonists. Using pertussis toxin-treated adipocytes, it can be shown that insulin can still activate the phosphodiesterase. Cholera toxin modifies N_s, so that adenylate cyclase activity is fully stimulated. Treatment of adipocytes with cholera toxin does not alter the ability of insulin to stimulate cAMP phosphodiesterase activity. Treatment of adipocytes with either toxin causes an increase in the cAMP phosphodiesterase activity over the control. The ability of isoproterenol to stimulate this enzyme is inhibited by either toxin treatment. While these results indicate that in adipocytes the stimulation of cAMP phosphodiesterase by isoproterenol may involve the adenylate cyclase regulatory subunits, the regulation of cAMP phosphodiesterase by insulin is independent of N_s or N_i. Supported by American Diabetes Assoc., So. Cal. Affil., the Ray Fdn. and the USC Faculty Innovation Fund.

120 SPECIES DIFFERENCES IN VENTRICULAR LOW K_m, CYCLIC AMP PHOSPHODIESTERASE AND THE IN VIVO CARDIOTONIC RESPONSE TO CI-914. Ronald E. Weishaar, Dianne C. Kobylarz-Singer, Robert P. Steffen, and Dale B. Evans. Warner-Lambert/Parke-Davis Pharm. Res., Ann Arbor, MI 48105.

Multiple molecular forms of phosphodiesterase (PDE) were isolated from guinea pig (GP), hamster (H), rat (R), and rhesus monkey (RM) left ventricular muscle, and the inhibitory effects of CI-914 and other selective inhibitors on each form of PDE were evaluated. In addition, the relationship between in vitro inhibition of the low K_m, cyclic AMP-specific form of PDE (PDE III) by CI-914, and the in vivo cardiotonic response to CI-914 was also determined. Three molecular forms of PDE were isolated from each species. PDE I is stimulated by calmodulin/calcium and, in the GP, H, and RM, hydrolyzes both cyclic AMP and cyclic GMP (K_m=1.0 uM). PDE II also hydrolyzes both cyclic AMP and cyclic GMP (K_m=20-30 uM). In addition, the cyclic AMP hydrolytic activity PDE II is stimulated by low concentrations of cyclic GMP. PDE III selectively hydrolyzes cyclic AMP (K_m=0.5-1.5 uM). MMB 22,948 (a selective PDE I inhibitor), AR-L 57 (a selective PDE II inhibitor) and theophylline (a non-selective PDE inhibitor) exerted a comparable inhibitory profile on PDE I, PDE II, PDE III in all four species. CI-914 exerted a potent and selective inhibitory effect on GP and RM left ventricle PDE III (IC_{50}=8.2 and 5.0 uM, respectively), but only modestly inhibited PDE III from H and R left ventricle (IC_{50}=180 and 110 uM, respectively). CI-914 also exerted a greater in vivo positive inotropic effect in the RM than in H and R (ED_{50}=30 ugm/kg, i.v. for RM, with little or no effect in the H or R). Although CI-914 potently inhibited GP left ventricular PDE III, CI-914 exerted only a modest in vivo positive inotropic effect in the GP. These results demonstrate that i) species differences exist regarding the inhibitory effect of CI-914 on left ventricle PDE III, and ii) for the RM, H and R, but not for the GP, these differences are predictive of the in vivo cardiotonic effect of CI-914. The lack of correlation in the GP implies the link between inhibition of left ventricular PDE III and increased contractility does not apply to all species. One possible explanation for his lack of correlation may be a different extracellular localization for PDE III in the GP compared to the RM.

121 *PURIFICATION OF MEMBRANE-ASSOCIATED CGMP-STIMULATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE (TYPE II PDE) FROM RABBIT BRAIN.* M.E. Whalin, S.J. Strada and W.J. Thompson. Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL 36688.

Rabbit brain Type II PDE has been purified to near homogeneity. Activity of cortex homogenates measured as the hydrolysis of 5 uM [3H]-cAMP in the presence of 2 uM cGMP is greater than 75% particulate. This activity or cGMP hydrolysis at 40 uM substrate can be solubilized with Triton X-100, but not with high ionic strength buffers indicating an integral membrane nature. Cyclic GMP acitivation of cAMP hydrolysis is lost by detergent solubilization. However, cleavage of the enzyme from the 13,000 x g particulate fraction by TPCK-trypsin proteolysis (1:600) retains full cGMP regulation. The enzyme was purified with a 3500 fold increase in specific activity from the particulate fraction using DE 52 anion-exchange chromatography, epoxy-Sepharose-cGMP affinity chromatography and Sephacryl S200 gel filtration. Particulate Type II PDE has an apparent native size of 240 kD. 8% acrylamide gel electrophoresis under denaturing conditions shows a major protein band at 110 kD. Minor bands of 36 and 38 kD are evident. Positive homotropic cooperative kinetic behavior of the enzyme is observed with either cAMP or cGMP as substrate. Extrapolated maximum velocities are 122 U/mg and 194 U/mg for cAMP and cGMP substrates, respectively. Substrate concentrations required to achieve half-maximal velocities are 28 uM for cAMP and 16 uM for cGMP. The Kact for cGMP stimulation of cAMP hydrolysis at 5 uM substrate is 0.4 uM and maximal stimulation (5 fold) is achieved with 2 uM cGMP. Purified Type II PDE cleaved from rabbit brain particulate fractions shows many properties similar to other Type II PDE activities purified from cytosolic heart, liver and adrenal tissues sources. This research was supported by USPHS GM 33538.

122 REACTIVE SULFHYDRYL GROUPS OF THE 39 kDa GUANINE NUCLEOTIDE BINDING PROTEIN (α_{39}) FROM BOVINE BRAIN. J.W. Winslow, J. Bradley, J.A. Smith, E. Neer, Cardiovascular Division, Brigham and Women's Hospital; Dept. Molecular Biology and Pathology, Massachusetts General Hospital; and Depts. Medicine and Pathology, Harvard Medical School, Boston, Massachusetts.

The guanine nucleotide binding proteins which mediate hormonal inhibition of adenylate cyclase as well as hormonal regulation of other membrane functions are α, β, γ heterotrimers which are structurally homologous to each other. In brain, the predominant guanine nucleotide-binding component is a 39 kDa protein whose physiological role is as yet unknown.

We have used N-ethylmaleimide to define functionally important sulfhydryl groups on α_{39}. Three cysteine residues in the molecule are reactive in unliganded α_{39}. Alkylation of two of these is reduced when GTPγS is bound. We have isolated and sequenced tryptic peptides containing the three reactive cysteines. The octapeptide containing the GTPγS-insensitive cysteine is at a position equivalent to amino acids 106-113 of the transducin αsubunit (Lochrie et al. (1985) Science 228:96-99). Alkylation of this cysteine blocks ADP-ribosylation of cysteine 351 by pertussis toxin. However, alkylation does not prevent association of α with the $\beta.\gamma$ subunits nor does it inhibit GTPase activity. The two GTPγS-sensitive cysteines are at positions equivalent to 139 and 286 of the transducin αsubunit. Alkylation of these residues inhibits GTPase activity. Neither of these GTPγS-sensitive cysteines are in those regions of α_{39} which are highly homologous to the GTP binding site of elongation factor Tu (Jurnak (1985) Science 230:32-36). However, both are present in the brain 41 kDa guanine nucleotide-binding protein and in the two transducins. The conservation of these cysteine residues suggests that they are important for the function of the subunits.

123 MULTI-SITE PHOSPHORYLATION OF THE ALPHA SUBUNIT OF TRANSDUCIN BY THE INSULIN RECEPTOR KINASE AND PROTEIN KINASE C by Y. Zick and R. Sagi-Eisenberg, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel, and M. Pines, P. Gierschick, and A. Spiegel, Section of Molecular Pathophysiology, Metabolic Diseases Branch, Bethesda, MD,

The GDP-bound α-subunit of transducin but not the GTP-gamma-S bound one undergoes phosphorylation on tyrosine residues by the insulin receptor kinase and on serine residues by protein kinase C. Holotransducin is poorly phosphorylated by the insulin receptor kinase and is not phosphorylated by protein kinase C. Neither holotransducin nor any of its subunits were phosphorylated by the cAMP-dependent protein kinase. That a given subunit of transducin undergoes multisite phosphorylation depending on the type of nucleotide bound to it or the nature of the kinase suggests that hormone-dependent phosphorylation could provide a versatile mode for regulation of G-proteins function. In particular, the findings that certain G-proteins serve as substrates for both the insulin receptor kinase and protein kinase C implicate G-proteins in playing a key role in mediating the action of insulin and liganda that act to activate protein kinase C.

124 THE EFFECTS OF THE CHARGED DIHYDROPYRIDINE PN 207-180 ON RECONSTITUTED SINGLE CHANNELS FROM PURIFIED MUSCLE TRANSVERSE TUBULES. Hubert Affolter and Roberto Coronado, Department of Physiology, Yale University, New Haven, CT 06510, and Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

The charged dihydropyridine derivative PN 207-180 (gift from Sandoz AG, Basel, Switzerland) was used to assess the site of dihydropyridine blockade in single calcium channels reconstituted from purified t-tubules from rat sceletal muscle. Spontaneous single channel openings were induced with the agonist Bay-K8644 and recordings were made in a two chamber planar bilayer setup so that drugs could be delivered to either site of the channel. It has been previously shown by the asymetric blockade by the membrane impermeable verapamile derivative D890 (Affolter and Coronado, Biophys. J. 49:767-771, 1986) that under the chosen conditions, calcium channels are incorporated into the membrane leaving the cytoplasmic end on the cis side of the bilayer. Nanomolar additions of PN 207-180 on either side of the bilayer resulted in >10 fold decrease in probability of open channel events as well as in mean open time. The lack of sidedness of PN 207-180 blockade can be explained by the fact that the long spacer $-(CH_2)_{10}-$ which links the charged quaternary amine to the dihydropyridine ring allows the active dihydropyridine moiety to reach the center of the bilayer from either side. This fact supports the view that the side of dihydropyridine action resides in the center of the lipid phase. Supported by a fellowship from Swiss National Funds and by NIH grant RO1-GM-32824.

125 BAY K 8644 ONLY MODIFIES THE GATING OF PHOSPHORYLATED CALCIUM CHANNELS. David Armstrong and Christian Erxleben, Department of Biology, UCLA, Los Angeles, CA 90024.

Dihydropyridines, calcium ions and agents that promote cyclic AMP-dependent protein phosphorylation all modulate the same class of voltage-activated Ca channels that have a conductance near 23 pS in 90mM Ba^{2+}, activate at voltages more positive than -20 mV, and do not inactivate at -40 mV. We have studied the modulation of those channels by BAY K 8644 (BAY K) in cell-free membrane patches from a mammalian cell line (GH_3). The cytoplasmic sides of the patches were bathed in a minimal CsCl solution that contained 40 mM HEPES (pH 7.2), 5 mM EGTA (pCa 8) and 0.1 mM leupeptin, a protease inhibitor, and were voltage clamped to -40 mV. The Ca channels in such patches quickly stop responding to membrane depolarization unless ATP-Mg and the catalytic subunit (CS) of cyclic AMP dependent protein kinase are added to the cytoplasmic side of the patch (Armstrong & Eckert, 1985, J. Gen. Physiol. 86:25a). BAY K alone (0.1 - 10 μM) did not restore Ca channel activity once it had disappeared from patches exposed to the minimal solution, but when it was applied in the presence of CS plus ATP, BAY K produced a dramatic increase in the mean lifetime of the open channel during depolarizing steps to 0 mV. However, if intact cells were exposed to BAY K before the patches were excised, BAY K not only modified Ca channel gating, it substantially delayed the loss of activity in the minimal solution. Since BAY K only modulates Ca channel gating when it is applied in the presence of phosphorylating agents, we suggest that BAY K produces its effect on gating by inhibiting dephosphorylation of the channel. That would explain why successful reconstitution of these phosphorylation-dependent Ca channels in the absence of ATP has only been achieved in the presence of BAY K. (Supported by USPHS NS-8364 to Roger Eckert.)

126 K^+ CHANNEL BLOCKADE ENHANCES ELECTRICALLY STIMULATED VASOPRESSIN RELEASE FROM RAT NEUROHYPOPHYSES

Frequency dependent facilitation of neurosecretion has been described in both mouse and rat neural lobes and in a variety of other vertebrate and invertebrate neuronal preparations. Associated with increased stimulus frequency is a prolongation of the action potential duration which has been demonstrated by potentiometric optical probes in the mouse neurohypophysis and by intracellular recording from crab sinus gland nerve terminals. Focusing on the possibility that frequency dependent modulation of K^+ channel activity could be the mechanism of spike broadening resulting in greater Ca^{++} entry and, therefore, peptide release, we have examined the effect of K^+ channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4AP) on vasopressin (VP) release from rat neurointermediate lobes (NIL's) in vitro. NIL's were impaled on platinum electrodes suspended in Ringer's solution with 2 mM $CaCl_2$ at 37°C, and stimulated with 600 negative square wave pulses of 0.5 ms and -3V at various frequencies. While neither agent effected basal release as measured by RIA, both enhanced electrically evoked VP release. Compared with control (n=14), 4AP (100μM, n=6) caused a 2.7 fold increase in VP release at 4 Hz, and a 1.8 fold increase at 12 Hz. TEA (10mM, n=5) increased the release 2.2 and 1.2 fold at 4 and 12 Hz respectively. TEA and 4AP together did not produce greater release than 4AP alone. These data suggest that K^+ channel inactivation by 4AP and TEA leads to a prolonged delay in rectification of membrane potential allowing for more Ca^{++} entry through voltage sensitive channels thus enhancing secretion.

127 ANTIBODIES TO THE SUBUNITS OF VOLTAGE-SENSITIVE CALCIUM CHANNELS.
Hemin Chin, Karl Krueger, Troy Beeler*, and Marshall Nirenberg, NIH and USUHS*, Bethesda, MD.

Rat skeletal muscle T-tubule membranes were solubilized and voltage-sensitive calcium channel proteins were purified by a modification of the procedure reported by Curtis and Catterall (Biochemistry, 23, 2113, 1984). Three major proteins (apparent M_r=160,000, 36,000 and 30,000 on non-reducing SDS-PAGE), which cosediment with specifically bound ^{3}H-nitrendipine in sucrose density gradient centrifugation fractions, were used to immunize rabbits. Antibodies monospecific for the 160,000, 36,000, or 30,000 M_r proteins were obtained by affinity purification against immobilized essentially homogeneous preparations of each protein. A λgt11 cDNA library prepared from 3 day-old rat skeletal muscle poly A^+ mRNA was screened with antibodies for recombinants that synthesize the α or γ subunits of voltage-sensitive Ca^{2+} channels fused with β-galactosidase. Twenty positive plaques were detected with antibody HK-6, which recognizes the 160,000 M_r α-subunit, out of 550,000 recombinants tested. HK-6 antibody was incubated with 10 immobilized fusion protein preparations. The [antibody·antigen] complexes were washed, antibody was dissociated from the antigen, eluted, and tested on Western blots of protein solubilized from rat T-tubules. Eluted antibodies from the fusion protein coded by λCChα2 cDNA bound only to the 160,000 M_r α-subunit, but not antibodies from 9 other fusion proteins. In addition, we have screened for recombinants producing fusion proteins that contain the 30,000 M_r subunit of the calcium channel with affinity purified antibody HK-2. Twenty nine of the 850,000 recombinant phage plaques screened were positive. Further work is needed to confirm the identity of the putative clones for voltage-sensitive calcium channel subunits.

128 CYCLIC-AMP-MEDIATED, NOREPINEPHRINE-INDUCED SODIUM ION INFLUX IN ISOLATED BROWN ADIPOCYTES. Eamonn Connolly, Department of Metabolic Research, The Wenner-Gren Institute, Biologihus F3, University of Stockholm, S-106 91, Stockholm, Sweden.

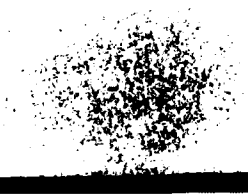
To examine the involvement of Na^+ ions in adrenergic responses in brown fat, an assay to measure Na^+ influx into isolated brown adipocytes was developed, which involved short (5-30 sec) incubations with $^{22}Na^+$, followed by a two-step centrifugation recovery procedure. Using this method, a clear, norepinephrine-mediated accumulation of intracellular $^{22}Na^+$ was demonstrated, which was enhanced by the addition of ouabain (the Na^+/K^+-ATPase inhibitor), was not inhibited by amiloride (a Na^+/H^+ exchange blocker) and could not be mimicked by the total removal of oxygen from the medium. This dose-dependent response to norepinephrine (EC_{50}, 250 nM) could be completely blocked by propranolol (beta-blocker), but not by prazosin (alpha$_1$-blocker). The response was cAMP-mediated since both theophylline (a phosphodiesterase inhibitor) and forskolin (an adenylate cyclase activator) could induce Na^+ influx. However, the Na^+ influx was not linked to other known cAMP-stimulated cellular responses, such as fatty acid mobilization, mitochondrial uncoupling or elevated respiration, since neither added free fatty acid substrates nor FCCP could induce the Na^+ entry. Furthermore, total respiratory inhibition with rotenone was unable to affect the norepinephrine-stimulated Na^+ influx. Could this beta-stimulated, cAMP-dependent Na^+ ion entry be (one of) the signalling factor(s) initiating DNA replication, protein synthesis and tissue recruitment in the chronically beta-stimulated brown fat of animals facing increased thermogenic demands?

129 CALCIUM SPECIFICITY OF THE Fc_ϵ RECEPTOR ACTIVATED ION CHANNELS IN MAST-CELL MEMBRANES. Ayus Corcia, Benjamin Rivnay and Israel Pecht, Depts. of Membrane Research and Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel.

A transient rise in cytosolic free Ca^{+2} occurs in mast cells and basophils upon specific aggregation of their Fc_ϵ receptors ($Fc_\epsilon R$) and leads to degranulation. We have shown, in model membranes, that ion channel activity can be induced by aggregating $Fc_\epsilon R$ and that this channel activity can be attained by reconstituting, into lipid bilayers, two components isolated from membranes of rat basophilic leukemia cells (line RBL-2H3). These components are the $Fc_\epsilon R$ complex and the cromolyn binding protein (CBP). To examine these channels' cation specificity, we have studied conductance and open-time characteristics of micropipette supported bilayers exposed to solutions containing: 1) only $CaCl_2$ (100 mM) or 2) only NaCl (0,5 M) on both sides of the membranes (i.e. symmetric conditions) and 3) NaCl (150 mM) on both sides and a calcium gradient namely, 1.8 mM $CaCl_2$ in the bath and ~ 0.1 μM in the pipette interior. Under these conditions, two populations of antigen-induced channels are observed: one, accounting for ~90% of all the events, has a small conductance (4-5 pS in symmetrical Ca; 8 pS in symmetrical Na and 3 pS in the presence of the Ca gradient), is Ca^{2+} specific (reversal potential 130 mV with the Ca gradient) but allows passage of Na^+ when it is the only cation present. The second population, 5-10% of the events, has a higher conductance (10-12 pS/25 pS/21 pS) and allows the passage of both Ca^{2+} and Na^+ with a clear preference for the former (reversal potential 16 mV). Both types of events have short open-times, appear mainly isolated and with relatively long periods of non-conducting state in between, and are all blocked by cromolyn. Thus antigen-induced Ca^{2+}-specific channels are activated in this reconstituted system.

130 RESERPINES: A NEW CLASS OF COMPETITIVE LIGANDS FOR THE BATRACHOTOXIN-BINDING SITE ON SODIUM CHANNELS. C.R. Creveling, M.E. Bell, R. del la Cruz, D. Rossignol* and J.W. Daly. LBC, NIDDK, NIH, Bethesda, Maryland 20892 and Dupont Exptl. Station, Wilmington, DE 19898*.

The steroidal alkaloid, batrachotoxin (BTX), and an equipotent, benzoate analog (BTX-B) modify the properties of voltage-dependent sodium channels by binding specifically with high affinity to a single site associated with the channel. High affinity binding of [^{3}H]BTX-B is optimally achieved in the presence of a scorpion toxin-induced shift of the channel to an open or conducting configuration. Other sodium channel specific toxins, such as veratridine, aconitine, and grayanotoxin, competitively inhibit the specific binding of [^{3}H]BTX-B, as do a large number of local anesthetics. The inhibition by local anesthetics is, however, not simple competition for the BTX-binding site, but is due to an allosteric change in the site. The consequence of this change is a reduction in the affinity of the site for BTX-B as evidenced by a marked increase in the dissociation rate of [^{3}H]BTX-B in the presence of local anesthetics. The dissociation rate for [^{3}H]BTX from sodium channels in synaptoneurosomes from guinea pig cerebral cortex has a $T_{1/2}$ = 31.2 min. measured in the presence of excess unlabeled BTX-B. Unlike the action of local anesthetics, true competitive ligands, such as veratridine or aconitine, do not increase the $T_{1/2}$ for [^{3}H]BTX-B beyond the rate achieved with BTX-B alone. By this criteria, reserpine is a true competitive ligand for the [^{3}H]-BTX-B binding site (K_i = 1.2 μM). A structure-activity study of a series of reserpines defines some structural features essential to binding to the [^{3}H]BTX-B site. Analysis of favored conformations of BTX-B, veratridine, aconitine, and reserpine suggest that these competitive ligands have certain structural elements in common, including a triad of oxygens, which when matched for these four compounds produce similar alignments of the phenyl ring of the aryl ester function in each compound.



131 CONTROL OF PTH SECRETION IS MEDIATED THROUGH CALCIUM CHANNELS VIA A G-PROTEIN: L.A. Fitspatrick, M.L. Brandi, and G.D. Aurbach, Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

Calcium negatively regulates parathyroid hormone (PTH) secretion through mechanisms yet to be elucidated. Pertussis toxin which inactivates a guanine nucleotide regulatory protein, blocks the calcium inhibition of PTH release. We tested voltage sensitive calcium channel agonists and antagonists to evaluate the role of calcium channels in PTH secretion. Maitotoxin (calcium channel activator) inhibited PTH release with maximal effect at 5 ng/ml. Enantiomers of the dihydropyridines (-)202-791 and (+)202-791 (Sandoz) were incubated with dispersed bovine parathyroid cells. PTH was assayed in the cell supernates by mid-region radioimmunoassay. Calcium channel agonist +202-791 (10^{-11}-10^{-6}M) caused a dose-dependent inhibition of PTH release. Similar effects were found with the agonist Bay K 8644. Pre-treatment of cells with pertussis toxin blocked the agonist inhibition and catalyzed ADP-ribosylation of a 40 kDa protein in a time-dependent manner in parathyroid cell membranes. Calcium channel antagonists (-202-791 and to a lesser extent nifedipine and verapamil) stimulated PTH release. We conclude: 1) calcium inhibition of PTH release is blocked by pertussis toxin; 2) calcium channel agonists inhibit while calcium channel antagonists stimulate PTH release; 3) calcium channels are involved in PTH regulation; 4) a G-protein is interposed between the calcium channel and the "intracellular" effector mediating PTH release.

132 THE PURIFIED RECEPTOR FOR CA CHANNEL BLOCKERS (CCBR) IS A FUNCTIONAL CA CHANNEL

Veit Flockerzi*, Franz Hofmann

The CCBR was purified from rabbit skeletal muscle microsomes to an app. density of 1.5 to 2nmol/mg protein. SDS-PAGE -DTT yielded 3 peptide bands of 142,56 and 30kDa, whereas +DTT bands of M_r 142,122,56,31, 26 and 22 were obtained. This gel pattern was obtained regardless if the receptor was purified as nitrendipine plus diltiazem (DIL) or as PN200/110(PN) complex or ± DTT. cA-kinase phosphorylated preferentially the 142kDa band (.82 mol phosphate/mol peptide) whereas the 56 kDa peptide was phosphorylated only in substoichiometric amounts. PN bound at 4°C to one site with app. K_D and B_{Max} values of 9.3nM and 2.2nmol/mg protein, respectively. The binding was stereospecific and was not observed in the presence of 1mM EGTA. (-)Desmethoxyverapamil (D) inhibited the binding of PN at 37°C incompletely and stimulated it at 18°C, whereas (+)D inhibited PN binding at all temperatures tested. D-cis-DIL stimulated the binding of PN only above 18°C (app. EC_{50}=1.4uM at 37°C). The purified CCBR was incorporated into phospholipid bilayer membranes. When a potential gradient was applied across the membranes single channel openings occurred spontaneously (single channel conductance 20pS). This current was blocked by the CCB gallopamil, whereas the Ca-agonist BayK8644 induced long channel openings. Phosphorylation of the CCBR prolonged the open channel life times. These results show: 1. The purified CCBR contains at least different sites for dihydropyridines and phenylalkylamines. 2. It can be reconstituted to a functional Ca channel, which retains the principal regulating, biochemical and pharmacological properties of membrane bound l-type Ca channels.

Physiologisch Chemie, Universität des Saarlandes, D 6650 Homburg, Germany.
*present adress: Department of Medical Chemistry, Kyoto University, Kyoto, Japan.

133 EFFECTS OF ION CHANNEL ANTAGONISTS AND DIVALENT CATIONS ON STIMULATION OF PHOSPHOINOSITIDE BREAKDOWN INDUCED BY AGENTS THAT INCREASE INTRACELLULAR SODIUM IN GUINEA PIG BRAIN SYNAPTONEUROSOMES. F. Gusovsky and J.W. Daly. NIDDK, National Institutes of Health, Bethesda, Maryland 20892.

Agents, such as batrachotoxin (BTX), veratridine (VT), scorpion toxins and pumiliotoxin B that activate sodium channels, induce the breakdown of phosphoinositides in guinea pig brain synaptoneurosomes as assessed by accumulation of [^{3}H]inositol phosphates in the presence of Li^+. The responses can occur without membrane depolarization with scorpion venoms and pumiliotoxin B. Monensin, a sodium ionophore, also elicits phosphoinositide breakdown. Tetrodotoxin, a specific antagonist for sodium channels, completely blocks the response to scorpion venom and to pumiliotoxin B, but not the response to BTX or VT. The potencies of BTX and VT in causing phosphoinositide breakdown are consonant with their potencies for activation of sodium channels. Activation of voltage-dependent calcium channels does not appear to be significantly involved since nifedipine and methoxyverapamil do not significantly decrease responses to BTX and scorpion venom. Tetrodotoxin-resistant channels in some neural preparations are blocked by the divalent cation cadmium and Cd^{2+} at 200 µM completely blocks BTX and scorpion venom-induced phosphoinositide breakdown. At this concentration Cd^{2+} does not reduce phosphoinositide breakdown induced by monensin, carbamylcholine or the calcium ionophore ionomycin. However, responses to norepinephrine and histamine are significantly reduced in the presence of Cd^{2+}. Zn^{2+} at 200 µM induces a smaller inhibition of BTX response than that caused by Cd^{2+}. Other divalent cations, Ni^{2+} and Sn^{2+} at 200 µM do not affect the BTX response. Thus, BTX-induced phosphoinositide breakdown may involve Cd^{2+}-sensitive sodium channels and activation of calcium channels has a minimal role in the response to BTX and other sodium channel agents.

134 TWO POLYPHOSPHATIDYLINOSITIDE METABOLITES CONTROL TWO K-CURRENTS IN NEUROBLASTOMA HYBRID CELLS. H. HIGASHIDA and D. A. Brown. NIH, Bethesda, MD20205, U.S.A.

The role of second messengers (inositol 1,4,5-trisphosphate and diacylglycerol) on membrane conductance was examined in voltage-clamped NG108-15 neuroblastoma x glioma hybrid cells. A brief local application of bradykinin (BK) induced an immediate outward current lasting 10-20 sec, which then gave way to a more sustained inward current. The initial outward current was a Ca-dependent K-current, which is generated indirectly through the conversion of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) following BK receptor activation and then by the subsequent release of Ca from intracellular stores by the IP3. The evidence for this is as follows: (1) Intracellular iontophoretic injection of Ca or of IP3 produced a very similar outward current to that evoked by BK. (2) Outward currents induced by BK, IP3 and Ca were also pharmacologically identical in that all three were very sensitive to the Ca-dependent K-current blockers, apamin and d-tubocurarine, and insensitive to the low concentration of tetraethylammonium. (3) The outward currents produced by BK and IP3 dissipated after a few minutes' perfusion with a Ca-free solution. The secondary inward current induced by BK resulted primarily from the inhibition of a voltage-dependent K-current closely resembling the M-current originally described in sympathetic ganglion cells. Application of phorbol dibutyrate produced a voltage-dependent inward current and inhibited the M-current, and thereafter occluded the inward current response to BK. The result suggests a dual, time-dependent role for these two intracellular messengers in the control of neuronal signalling by BK.

135 BORDATELLA PERTUSSIS TOXIN-SENSITIVE GTP-BINDING PROTEINS COUPLE ALPHA-2 ADRENERGIC AND GABA-B RECEPTORS TO INHIBITION OF NEUROSECRETION IN CHICK DORSAL ROOT GANGLION NEURONS. G. G. Holz IV, R. M. Kream, and K. Dunlap, Depts. of Physiol. and Anesthesia Res., Tufts Univ. Med. Sch., Boston, MA.

Norepinephrine (NE) and gamma aminobutyric acid (GABA) inhibit current flow through voltage-sensitive L-type (long lasting) Ca channels in chick dorsal root ganglion (DRG) neurons. This inhibition of Ca channel function results from the activation of alpha-2 adrenergic and GABA-B receptors coupled to a cAMP-independent second messenger pathway. Whole cell patch clamp recordings from DRG cells demonstrated a role for GTP-binding proteins as signal transducers in the NE and GABA receptor-mediated inhibition of L-type Ca channels. The action of the transmitters was blocked by intracellular dialysis of DRG cells with guanosine 5'-O-(2-thiodiphosphate), or by pretreatment with Bordatella pertussis toxin (PTX, Holz et al., Nature 319: 670-672, 1986). We now demonstrate that receptors with similar pharmacological and biochemical properties mediate inhibition of neuropeptide secretion from DRG cells. NE (50 uM) and GABA (10 uM) inhibited the electrically-evoked, Ca-dependent release of substance P (SP, as measured by RIA and authenticated by HPLC) from DRG cell cultures by 46±5% and 31±5%, respectively (mean ± s.e.m., n=81 cultures). The action of NE was blocked by 10 uM yohimbine but not 10 uM prazosin or 10 uM propranolol, whereas the action of GABA was not blocked by 50 uM (+) bicuculline, but was mimiced by 10 uM (-) baclofen. Furthermore, pretreatment of cultures with 140 ng/ml PTX completely blocked the ability of NE and GABA to inhibit SP release. We conclude that PTX-sensitive GTP-binding proteins couple alpha-2 and GABA-B receptors to inhibition of L-type Ca channels and inhibition of Ca-dependent neurosecretion. Such a presynaptic mechanism of inhibition may explain, at least in part, the spinal antinociceptive actions of alpha-2 and GABA-B receptor agonists.

136 IN VITRO AND IN SITU PHOSPHORYLATION OF A PROTEIN ASSOCIATED WITH VOLTAGE-DEPENDENT CALCIUM CHANNELS FROM SKELETAL MUSCLE. M. Marlene Hosey, Christy Cooper, Cliff O'Callahan, Mark Borsotto, and Michele Lazdunski. Dept. of Biol. Chem. & Structure, The Chicago Medical School, 3333 Green Bay Road, N. Chicago, IL 60064 and Centre de Biochemie, Universite de Nice, Nice, France.

The phosphorylation and dephosphorylation of a protein associated with the dihydropyridine-sensitive Ca^{2+}-channel was studied in vitro in transverse-tubule membranes isolated from rabbit skeletal muscle, and in situ in intact rabbit diaphragms. Exposure of T-tubule membranes in vitro to either cAMP-dependent or a Ca^{2+}/calmodulin-dependent protein kinase resulted in phosphorylation of a protein with properties similar to the major component of the skeletal muscle calcium channel. The molecular weight of the phosphoprotein was 140 KDa or 160KDa, depending on the electrophoretic conditions. The stoichiometry of the phosphorylation was calculated to be 0.4-1.0 mol phosphate/mol protein. Limited proteolytic digestion of the 140-160 kDa protein that had been phosphorylated by either or both protein kinases yielded a single phosphopeptide of approximately 5,400 daltons. The Ca^{2+}-dependent phosphatase calcineurin dephosphorylated the 140-160 kDa protein that had been previously phosphorylated by either protein kinase. In situ experiments were performed in which calcium channels were isolated from diaphragms which had been bathed in ^{32}P-containing Krebs-Hensleit solution. A protein with properties similar to those described above and which co-purified with the calcium channel protein was phosphorylated in the intact muscle. The results suggest that the well known regulation of calcium channel activity by cAMP and Ca^{2+}-dependent processes may occur via the phosphorylation and dephosphorylation of the 140-160 kDa protein associated with the calcium channel.

137 DIFFERENTIAL REGULATION OF Na^+/H^+ EXCHANGE ACTIVITY BY PROTEIN KINASE C. G.A. Jamieson, Jr., L.L. Muldoon and M.L. Villereal. Univ. of Chicago, Chicago, IL 60637.

Mitogenic stimulation of quiescent human fibroblasts (HSWP and WI-38 cells) stimulates the release of inositol phosphates, mobilization of intracellular calcium and activation of Na^+/H^+ exchange. In HSWP cells, TPA pretreatment substantially inhibits mitogen-stimulated inositol phosphate release and Na^+ influx (half-maximal effect at 1-2 nM TPA). In digitonin-permeabilized HSWP cells, maximal release of inositol phosphates (or release of ^{45}Ca from non-mitochondrial stores) is mitogen- and GTP-dependent. Pretreatment with TPA prior to permeabilization results in substantial reduction of the mitogen-induced release of inositol phosphates and the subsequent release of ^{45}Ca from non-mitochondrial stores. In WI-38 cells, TPA treatment stimulates Na^+ influx, without stimulating inositol phosphate release, presumably through the activation of an endogenous protein kinase C activity. In both HSWP and WI-38 cells, down regulation of protein kinase C activity by long term pretreatment with TPA removes the normal effector function of TPA on Na^+ influx (i.e. the inhibitory effect in HSWP cells and the stimulatory effect in WI-38 cells). Other cell types have been characterized as being responsive or unresponsive with regard to TPA's ability to directly stimulate Na^+ influx. Measurement of the resting intracellular Ca activity in several cell types has determined that cells which are responders have a significantly higher intracellular Ca activity (WI-38; 137 $\pm$ 17 nM) vs. that determined in non-responders (HSWP; 57 $\pm$ 5 nM). These data suggest that in different cell types the activation of Na^+/H^+ exchange is regulated differently, even in closely related cell types, and that one aspect of this regulation may be determined by the resting Ca activity of a particular cell type. (Supported by NIH grants GM-28359 and RCDA AM-01182 (MLV) and 5T32 GM-07151 (LLM)).

138 POSSIBLE INVOLVEMENT OF PHOSPHORYLATION/DEPHOSPHORYLATION MECHANISMS IN THE REGULATION OF EPITHELIAL Na^+ CHANNELS. David S. Lester, Carol Asher and Haim Garty. Dept. of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel.

Recent studies indicate that epithelial Na^+ channels are down-regulated by a Ca^{2+} -dependent process [H.Garty and C.Asher (1986) J.Biol.Chem. 261:7400]. Channel mediated flux is low in membranes from Ca^{2+} ($>10^{-5}$ M)- preincubated cells, and high in membranes derived from EGTA-preincubated cells. Involvement of Ca^{2+} - dependent protein kinases in this process was excluded on the basis of the following results: (1) Incubating cells with phorbol ester had no effect on the Ca^{2+} inhibition constant. (2) Trapping purified protein kinase C, ATP, Ca^{2+} and phorbol ester in the isolated vesicles did not mimic the inhibition induced by incubating whole cells with Ca^{2+} (3) The calmodulin inhibitor, trifluoroperazine, had no effect on transport in the presence or absence of Ca^{2+}. Preliminary data indicate that Ca^{2+} inhibition of transport may act via a phosphatase mediated pathway. Investigation of membrane-associated phosphatases indicated the presence of two Ca^{2+} -dependent phosphatases: (i) a Ca^{2+} -activated, calmodulin-independent, vanadate-insensitive pNPPase, (ii) a Ca^{2+} - inhibited (IC_{50} ~0.5uM), vanadate-sensitive pNPPase. In addition, we observed Ca^{2+} -dependent dephosphorylation of a membranal 51 KD phosphoprotein which coresponds to the phosphoprotein reported by Lorenzo et.al. [(1975). J.Gen.Physiol. 65: 153]. This protein was tentatively identified as the regulatory subunit of cAMP -dependent protein kinase. Preincubation of whole bladders with 8Br-cAMP was shown to double the channel mediated fluxes in EGTA-activated vesicles. Conditions of preincubation (Ca^{2+} or EGTA) also affected the potency of membranes to phosphorylate histone fragment 2b, a specific cAMP-dependent protein kinase substrate. [Supported by NIH grant AM36328 and US-Israel BSF grant 84-00066].

139 cAMP-SENSITIVE Ca^{2+}-DEPENDENT K^+ CHANNELS IN PATCH CLAMP STUDIES OF SHEEP GONADOTROPHS.

R.P. McIntosh*, W.T. Mason, and S.K. Sikdar. AFRC Institute of Animal Physiology, Cambridge, U.K.

Single K^+ channels of large conductance (~100 pS) were observed in patch clamp studies on dispersed, cultured gonadotrophs from sheep *pars tuberalis* as described by Mason and Waring† after treatment with GnRH (160 nM) or A23187 (10 µM) (pipette solution, 125 mM K^+, 5 mM Ca^{2+}; bath solution, 130 mM Na^+, 5 mM Ca^{2+}, 5 mM K^+). The open time probability of these channels in cell attached recordings was reduced in a dose dependent manner on addition of dbcAMP. This negative effect of dbcAMP declined with time. Addition of EGTA to the bath also reduced opening probability which could be restored by Ca^{2+}. In a particular example, 10 mM dbcAMP reduced channel open probability from 0.56 to 0.33 (restored in 8 min). 15 mM EGTA reduced open probability to 0.02 (restored by Ca^{2+}:EGTA in the ratio 3:1). Similar effects of cAMP (2 mM) were observed less reliably with Ca^{2+}-dependent K^+ channels in inside-out patches of gonadotroph plasma membrane (bath solution containing 120 mM K^+), indicating a possible requirement for intracellular components. cAMP action on GnRH-activated K^+ channels might be expected to influence intracellular Ca^{2+} levels. Its possible role in secretory behaviour of gonadotrophs is being investigated. Such channels are a possible point of interaction of IP3- and cAMP-mediated hormone actions.

†*Neuroendocrinology* 43, 205 (1986). *Permanent address: Wellington Clinical School, New Zealand.

140 TRH AND THE CALCIUM CHANNEL AGONIST BAY K 8644 SYNERGISTICALLY ELEVATE PROLACTIN SECRETION FROM PERIFUSED ANTERIOR PITUITARY CELLS. Jonathan A. Pachter, Greg J. Law, Howard Rasmussen, and Priscilla S. Dannies, Yale University School of Medicine, New Haven, CT 06510.

TRH (thyrotropin releasing hormone) stimulates prolactin secretion from anterior pituitary lactotrophs by a mechanism which is thought to involve stimulation of phosphoinositide metabolism and opening of membrane Ca^{2+} channels to cause elevation of cytoplasmic calcium levels (Rebecchi et al., J. Biol. Chem. 258, 227). In perifusion, TRH (1 μM) elicited a rapid 11.5-fold stimulation of prolactin secretion from isolated rat anterior pituitary cells which decayed to a sustained level only 2-fold above basal secretion within 6 minutes. The calcium channel agonist BAY K 8644 (1 μM) also stimulated prolactin secretion (7-fold over basal) with a similar decay. When added simultaneously, TRH and BAY K 8644 were synergistic in the stimulation of prolactin secretion. When BAY K 8644 was added during the low sustained phase of TRH-mediated stimulation, its effect on prolactin secretion was approximately twice as large as its effect on previously untreated cells. In contrast to the synergism between TRH and BAY K 8644, the calcium ionophore A23187 (20 μM) stimulated no additional prolactin secretion when added simultaneously with 1 μM TRH. Furthermore, A23187 was no more efficacious when added during the low sustained phase of TRH-stimulated secretion than it was when added to untreated cells. These results indicate that although the prolactin response to TRH is transient, TRH also produces a sustained increase in the sensitivity of the prolactin secretory response to subsequent stimulation of Ca^{2+} channels. The differential effects of BAY K 8644 and A23187 suggest that TRH increases the responsiveness of Ca^{2+} channels or the responsiveness of prolactin secretion to Ca^{2+} influxes, rather than increasing cellular responsiveness to a general rise in cytoplasmic Ca^{2+} concentration.

141 CYCLIC AMP-MEDIATED PHOSPHORYLATION OF VOLTAGE SENSITIVE SODIUM CHANNELS IN PRIMARY CULTURES OF RAT BRAIN CELLS. S. Rossie & W.A. Catterall. Dept. of Pharmacology, University of Washington, Seattle, WA 98195. The α subunit of the rat brain voltage-sensitive Na channel is phosphorylated by cAMP-dependent protein kinase in purified preparations and in synaptosomes (Costa et al, J. Biol. Chem. 257, 7918, 1982; 259, 8210, 1984). We are studying phosphorylation of Na channels in primary culture of rat brain cells. In back-phosphorylation studies, cells were treated with drugs to increase intracellular cAMP, solubilized, and Na channels isolated by immunoprecipitation. Purified Na channels were then phosphorylated by the catalytic subunit of cAMP-dependent protein kinase with ^{32}P-ATP to incorporate ^{32}P into available cAMP-dependent phosphorylation sites. The amount of ^{32}P incorporated is inversely proportional to the extent of endogenous phosphorylation. Incubation of cells with forskolin, 8-Br-cAMP, or isobutylmethylxanthine inhibited subsequent incorporation of ^{32}P into isolated Na channels, indicating that treatment of cells with these drugs had increased endogenous phosphorylation. To examine the magnitude of basal Na channel phosphorylation and the extent of stimulation, the amount of ^{32}P incorporated into Na channels from control and stimulated cells was compared to that from matched samples which had been dephosphorylated with calcineurin. Na channels from control cells incorporated 2-fold more ^{32}P after dephosphorylation, indicating that cAMP-dependent phosphorylation sites on Na channels are approximately 50% phosphorylated in the basal state. Na channels from forskolin-treated cells incorporated 5-fold more ^{32}P after dephosphorylation, indicating that sites are approximately 80% phosphorylated after stimulation. When cells were metabolically labeled with ^{32}P and stimulated with forskolin, ^{32}P incorporation into Na channels was increased 1.5-fold, in agreement with an increase in overall phosphorylation from 50% to 80%. Thus, Na channels in rat brain cells in primary culture are phosphorylated, and the extent of phosphorylation can be modified in a cAMP-dependent manner.

142 VOLTAGE-GATED CALCIUM ENTRY INTO PARAMECIUM LINKED TO cGMP INCREASE.

Joachim E. Schultz, Pharmazeutisches Institut, 7400 Tübingen, West-Germany

Inward calcium currents exist in many excitable tissues and are linked to regulation of cellular processes such as cGMP production. In Paramecium, a graded calcium/potassium action potential regulates swimming behavior. The transient inwardly directed calcium signal triggers ciliary reversal, i.e. backward swimming. A guanylate cyclase in Paramecium is activated specifically by μmolar concentrations of calcium. The ED_{50} of 9 μM calcium is not affected by the presence of 1 mM barium. Barium ions were used to increase the excitability of the calcium channels by decreasing their rate of inactivation, thereby producing a more sustained calcium entry. 3 mM Barium evoked a rapid, large, and transient increase in cGMP in wildtype cells. Further evidence for a link between the inward calcium current and cGMP formation was obtained by studying mutants with reduced ('PAWN') or exaggerated ('DANCER') calcium currents. Addition of barium to pawns did not stimulate cGMP biosynthesis as in wildtype cells. In dancer cells, addition of potassium elicited a sustained elevation of cGMP levels correlated with a large behavioral response. Potassium ions as a weak excitatory agent are almost inactive with wildtype cells. We demonstrate here for the first time an unequivocal link between an inward calcium current and an enhanced cGMP production, i.e. an electrical signal is converted and amplified to a biochemical one. The physiological role of cGMP in Paramecium is currently under investigation.

143 RECONSTITUTION OF ISOPROTERENOL STIMULATED ADENYLATE CYCLASE ACTIVITY AND NOVEL ELECTROPHYSIOLOGICAL RESPONSES IN XENOPUS LAEVIS OOCYTES AFTER MICROINJECTION OF RAT ASTROCYTOMA C6-2B mRNA.

Angel Anne Smith and Gary Brooker, Department of Biochemistry, Georgetown University Medical Center, Washington, D.C. 20007.

We have purified mRNA from the rat astrocytoma C6-2B cell line, which exhibits high levels of beta adrenergic receptor (BAR) mediated adenylate cyclase activity. Microinjection of this mRNA into Xenopus laevis oocytes yielded evidence of C6-2B BAR mRNA translation and functional integration into the oocyte membrane. Surgically removed ovarian oocytes are encapsulated by follicle cells which exhibit BAR coupled adenylate cyclase activity. This complicated our use of such ovarian oocytes for demonstration of new receptor synthesis following the injection of exogenous mRNA. A method was developed to completely remove the follicle cells from the ovarian oocytes. The efficacy of the procedure was confirmed morphologically by transmission electron microscopy, biochemically because the oocytes were free of BAR coupled cyclase activity and electrophysiologically because they no longer hyperpolarized in response to isoproterenol. Microinjection of mRNA purified from log phase C6 cells into oocytes followed by defolliculation resulted in the reconstitution of receptor mediated adenylate cyclase activity in oocyte membranes previously devoid of such activity. Transmembrane electrical potentials were measured in ovarian and defolliculated oocytes before and after the injection of the mRNA. While ovarian oocytes were hyperpolarized by isoproterenol, defolliculated oocytes were unresponsive. In defolliculated oocytes which had been injected with C6-2B mRNA, a novel depolarizing response to isoproterenol was observed suggesting that in addition to incorporation of beta receptor coupled cyclase activity, the C6-2B mRNA directed the oocyte to synthesize an ion channel which could be activated by isoproterenol. Supported by NIH Grant No. 28940.

144 MECHANISMS OF INTERACTION OF VASOPRESSIN (VP) AND CRF ON PITUITARY ADENYLATE CYCLASE.

A.B. Abou-Samra, J.P. Harwood, K.J. Catt, G. Aguilera, NICHD, NIH, Bethesda, MD 20892

The synergistic action of VP and corticotropin releasing factor (CRF) on cAMP production involves inhibition of phosphodiesterase and stimulation of adenylate cyclase (AC) by VP, an action which is mimicked by the protein kinase C (PKC) activator, PMA. To determine the mechanisms by which VP potentiates CRF action we analyzed the interactions of PMA, VP and pertussis toxin (PT) with CRF upon cellular cAMP and adenylate cyclase (AC) activities in cultured rat anterior pituitary cells. VP or PMA (0.01-100 nM) alone did not alter cellular cAMP content, but markedly increased up to 9-fold the effect of 10 nM CRF on cAMP with ED_{50} values of 1 nM VP and 1.7 nM PMA. With increasing CRF concentrations, 10 nM VP or PMA increased the maximum cAMP production without altering the ED_{50} for CRF (1 nM). Measurement of AC in pituitary homogenates using [α-^{32}P]ATP showed a paradoxical inhibitory effect of VP but not of PMA on basal and CRF-stimulated AC activity, suggesting that the VP receptor is coupled to an inhibitory guanyl nucleotide binding protein. However, in intact cells, following [^{3}H]adenine labeling of endogenous ATP pools, measurement of AC in the presence of phosphodiesterase inhibitors showed no effect of VP and PMA alone, but a 2-fold potentiation of the effect of CRF. Similar to PMA, PT potentiated the effect of CRF on cAMP and ACTH secretion, an effect which was not additive to VP, suggesting that the potentiating mechanism involves suppression of the inhibitory influence of Ni on AC. The data suggest that VP has a dual interaction with the pituitary AC system: a direct inhibitory effect, manifested only in broken cells, mediated by a receptor-coupled guanyl nucleotide binding protein; and a physiologically predominant indirect stimulatory effect in the intact cell, mediated by PKC phosphorylation of Ni or other components of the CRF-activated AC system.

145 ISOLATION OF A CHO CELL LINE WITH INCREASED cAMP DEPENDENT PROTEIN KINASE ACTIVITY.

Irene Abraham, John Hui and Judy Mayo. Cell Biology Department, The Upjohn Company, Kalamazoo, MI 49001.

Through the selection of revertants from a Chinese Hamster ovary (CHO) cell line with a mutation in the gene for the RI subunit of the cAMP dependent protein kinase (PK A) we have recovered a cell line with PK A activity higher than either the mutant or wildtype cell. The original dominant mutant was isolated on the basis of its resistance to the growth inhibitory effects of cAMP (Gottesman et al., 1980, Somat. Cell Genet. 6:45-61; Singh et al., J. Biol. Chem. 260:13927-13933) and has PK A type I but only very low amounts of PK A type II. Wildtype cells have both PK A type I and PK A type II activity in close to equal amounts. We have transfected DNA from this mutant into wildtype cells. One transfectant, 11564, has been extensively studied and exhibits the characteristics of the original mutant (Abraham et al., 1985, J. Biol. Chem. 260:13934-13940). Both the mutant and transfectant are super-sensitive to some anti-mitotic drugs such as vinblastine, colcemid and colchicine, as well as other drugs such as puromycin. We have used this characteristic of drug sensitivity to isolate revertants from the transfectant. Five revertants have been selected and characterized that have simultaneously regained their cAMP sensitivity along with losing their super-sensitivity to colcemid. One of the revertants, 11564 R3, has an increased total PK A activity as compared to either the mutant, transfectant or wildtype cells. The increase in activity co-elutes with PK A type I after DEAE-Sephacel chromatography. This revertant is even more sensitive to the growth inhibitory effects of cAMP than is the wildtype, which is consistent with its having a higher level of PK A. We are currently studying the genetic basis of this revertant.

146

DYNORPHIN A INHIBITS B50 PROTEIN PHOSPHORYLATION AND PROTEIN KINASE C ACTIVITY.
Aloyo, V.J. and Walker, R.F. Dept. of Pharmacology, Medical College of Pennsylvania, Philadelphia, PA 19129.

Dynorphin A (Dyn) is a potent opioid receptor agonist that is highly selective for the kappa-receptor subtype. Since some behavioral affects of Dyn are not reversed by naloxone and removal of the N-terminal tyrosine (which is necessary for opioid receptor binding) still results in a behaviorally active peptide, Dyn may be acting by multiple mechanisms. Other neuronally active peptides such as ACTH and somatostatin have as part of their mechanism of action, the inhibition of the phosphorylation of the brain specific, kinase C substrate, B50 protein. The present experiments were designed to investigate the possibility that Dyn inhibits B50 protein phosphorylation. First, the effects of Dyn on the endogenous phosphorylation of B50 protein in synaptosomal plasma membrane (SPM) was studied. In this system, Dyn inhibited B50 phosphorylation with a potency approximately equal to ACTH-(1-24). This inhibition was not reversed by the specific opioid antagonist, naloxone, even at 100 uM. Furthermore, the nonopioid Dyn fragment, des-tyrosine^1-Dynorphin A, also inhibited B50 phosphorylation in SPM. Second, Dyn was tested for its ability to inhibit histone phosphorylation using a partially purified kinase C preparation. Dyn, like ACTH-(1-24), inhibited the calcium-dependent, phospholipid-stimulated kinase C activity. These results suggest that inhibition of protein kinase C activity and inhibition of B50 phosphorylation are possible mechanisms by which Dyn exhibits its nonopioid effects.

147 PHOSPHORYLATION OF TYROSINE HYDROXYLASE PURIFIED FROM RAT STRIATUM BY CALCIUM, CALMODULIN-DEPENDENT PROTEIN KINASE II AND THE EFFECTS OF A PROTEIN ACTIVATOR.
J.L. Atkinson, N.M. Richtand, C.M. Schworer, R.T. Kuczenski, and T.R. Soderling. Howard Hughes Medical Institute, Vanderbilt University, Nashville, TN 37232.

Phosphorylation of purified rat striatal tyrosine hydroxylase by purified calcium, calmodulin-dependent protein kinase II has been investigated. The calcium and calmodulin-dependent phosphorylation was additive to the phosphorylation catalyzed by the cAMP-dependent protein kinase, suggesting different sites of phosphorylation. Reverse phase high performance liquid chromatography of tryptic $^{32}PO_4$-peptides derived from tyrosine hydroxylase established that these two kinases phosphorylate different primary sites. Limited tryptic digestion sequentially reduced the subunit M_r from 62,000 Da to 59,000 Da and finally to 57,000 Da which resulted in the loss of the site phosphorylated by the CaM-dependent protein kinase but not the site phosphorylated by the cAMP-dependent protein kinase. Analysis of partial acid hydrolysates of $^{32}PO_4$-labeled tyrosine hydroxylase indicated that serine was the only residue phosphorylated by either kinase.

Phosphorylation by the cAMP-dependent protein kinase directly activated tyrosine hydroxylase by decreasing the K_m for the cofactor 6-methyltetrahydropterin. Phosphorylation of the primary site by Ca^{++}(CAM)-dependent protein kinase II had little direct kinetic effect. However, tyrosine hydroxylase phosphorylated by the Ca^{++}(CAM)-dependent protein kinase II was activated 2-fold by addition of a partially-purified activator protein. This heat-labile activator protein increased V_{max} without affecting the K_m for the cofactor. The activator protein had no effect on nonphosphorylated tyrosine hydroxylase or on tyrosine hydroxylase phosphorylated by the cAMP-dependent protein kinase. The effect of phosphorylation of the activator protein on its ability to activate tyrosine hydroxylase has also been investigated.

148 BOTH cAMP-DEPENDENT PROTEIN KINASE ISOZYMES MEDIATE PHOSPHORYLASE ACTIVATION.
S.J. Beebe, P.F. Blackmore, T.D. Chrisman, and J.D. Corbin. Howard Hughes Medical Institute, Dept. Molecular Physiology and Biophysics, Vanderbilt Medical School, Nashville, TN 37232

The effects of cAMP analogs on phosphorylase activation in the absence and presence of insulin or EGTA were studied in intact cells. Because type I and type II isozymes of cAMP-dependent protein kinase (PK) differ in each of the two cooperative intrasubunit cAMP binding sites, it is possible to cause a synergistic activation of one or the other PK isozyme by using isozyme-directed analog pairs. In bovine neutrophils (80-85% type I), rat adipocytes ($\geq$ 95% type II) and rat hepatocytes (type I $\approx$ type II), type I-directed and type II-directed analog pairs caused a synergism of phosphorylase activation in proportion to the relative ratios of the isozymes present in the cells. However, these typical responses could be modified. The extent of synergism in hepatocytes and neutrophils with both isozyme-directed pairs was greater when cells were preincubated with EGTA to reduce intracellular Ca^{2+}. In one series of hepatocyte experiments, preincubation with 1 nM insulin, which lowers elevated cAMP levels, was required to demonstrate synergism with type II pairs, while responses to type I pairs were unaffected. When typical hepatocyte preparations were preincubated with 10 pM glucagon, so that phosphorylase was slightly activated and then returned to the basal level, synergism was observed only with type I pairs, in contrast with the usual response. These results do not reveal specific functions for the two PK isozymes, but indicate that previous hormone stimulation and intracellular Ca^{2+} levels may modify the isozyme responses for phosphorylase activation.

149 IMMUNOLOGICAL DIFFERENCES IN TYPE IIA AND TYPE IIB FORMS OF THE TYPE II REGULATORY SUBUNIT
S.J. Beebe, V.S. Sheorain, S. R. Cordle, L. Wolfe, and J.D. Corbin. Howard Hughes Medical Institute, Molecular Physiology and Biophysics, Vanderbilt Medical School, Nashville, TN 37232

The type II regulatory subunit (RII) of the cAMP-dependent protein kinase is known to be microheterogeneous. One nomenclature describing the forms is based on an operational classification (Robinson-Steiner, A.M., Beebe, S.J., Rannels, S.R., and Corbin, J.D. (1984) J. Biol. Chem. 259, 10595). RIIA forms shifted after autophosphoylation to higher apparent molecular weight upon SDS-PAGE but RIIB forms did not. RIIA forms were present in the hearts of ungulates (bovine and porcine) while RIIB forms were present in the hearts of rodents (rat), legomorphs (rabbits) and primates. These two diverse groups of species diverged about 70 million years ago. In order to determine if there was an immunological difference between RIIA and RIIB, several polyclonal antibodies directed against RII were purified from the serum of rabbits or guinea pigs and characterized by enzyme-linked immunosorbant assays [ELISA], immunoprecipitation and immunoblot analysis. A rabbit antibody against the bovine heart RII detected RIIA forms but did not detect RIIB forms. Guinea pig antibodies directed against either bovine heart RII or rabbit skeletal muscle RII detected both RIIA and RIIB forms. None of the antibodies detected RI or the cGMP-dependent protein kinase. These antibodies were used to quantitate RII from crude extracts of several tissues and cells in culture. These results establish an immunological basis for the classification of RIIA and RIIB forms.

150 MODULATION OF ENZYMIC ACTIVITY OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A (HMG-CoA) REDUCTASE BY MULTIPLE PROTEIN KINASES INVOLVING REVERSIBLE PHOSPHORYLATION. Z.H. BEG, J.A. Stonik and H.B. Brewer, Jr., Molecular Disease Branch, NHLBI, NIH, Bethesda, MD 20892.

HMG-CoA reductase is the rate limiting enzyme that regulates the synthesis of cholesterol and other polyisoprenoids. Proteolysis of the native insoluble enzyme (MW 100,000) results in a soluble active fragment (MW 53,000). We have previously demonstrated that the enzymic activity of HMG-CoA reductase is modulated in a bicyclic cascade system involving reversible phosphorylation of both HMG-CoA reductase and reductase kinase. In addition to the reductase kinase system, we have recently identified two new systems for the short-term regulation of HMG-CoA reductase. Protein kinase C and a calmodulin-dependent kinase, purified from rat brain cytosol, were able to phosphorylate and concomitantly inactivate both native and soluble purified HMG-CoA reductase. Incubation of phosphorylated ^{32}P-HMG-CoA reductase with phosphatase was associated with the loss of ^{32}P and reactivation of inactive enzyme. Maximal phosphorylation of purified HMG-CoA reductase by two kinases involved the introduction of approximately one mol of PO_4/mol of native enzyme (MW 100,000). Ca^{2+}, calmodulin-dependent kinase is able to phosphorylate two different sites in the HMG-CoA reductase molecule. The elucidation of multiple kinase systems for the modulation of HMG-CoA reductase by covalent phosphorylation provides new insights into the molecular mechanisms involved in the short-term regulation of cholesterol synthesis.

151 PROTEIN PHOSPHORYLATION IN PRIMARY ASTROCYTES D. Benzil and J.P. Bressler, NIH, Beth, MD

Astrocytes are known to respond to epidermal growth factor (EGF), fibroblast growth factor (FGF), phorbol myristate acetate (PMA), and cAMP stimulators such as forskolin. This response includes a phosphorylation cascade which may then be involved in growth regulation. The objectives of this study are to determine the phorphorylation substrates of PMA, EGF, FGF, and forskolin in primary rat astrocytes and to determine if these substrate sets are unique for each factor. Primary rat astrocytes were prepared from 1-2 day old rat cerebral cortex and used 7-10 days later after shaking. Miniwell cultures were incubated with [32]Pi and then stimulated with PMA, EGF, FGF, forskolin, or solvent. The reaction was quenched with cold PBS washes and the proteins precipitated with TCA. Samples were resuspended in SDS buffer and subjected to SDS-polyacrylamide gel electrophoresis. Gels were silver stained and prepared for autoradiography. Autoradiographs were analyzed by cutting out bands followed by liquid scintillation spectroscopy. Induction of protein phosphorylation for the various factors is shown in the figure. The protein substrates identified in these studies may play a role in regulation of glial growth. Three of these, PMA, EGF and FGF, stimulate astrocyte growth though the former two act primarily through different kinases. The 25K protein is induced by all three of these factors but not by forskolin suggesting this protein may have a function in glial growth regulation but phosphorylation is distal to the primary effect of the receptor kinase.

	MOLECULAR WEIGHT (k Dalton)							
	90	80	70	50	40	30	25	20
FACTOR								
PMA	*	*			*		*	
EGF			*		*		*	
Forskolin	*		*		*	*		
FGF			*	*			*	*

152 A protein kinase which phosphorylates aminoacyl-tRNA synthetase protein has been purified by affinity chromatography and gel electrophoresis. The protein kinase phosphorylates 21 of 28 (Coomassie, Ag) bands obtained by purifying aminoacyl-tRNA synthetases from mouse liver by gel filtration and affinity chromatography. The individual synthetases were located on the gel by zymogram technique. Purity of bands were controlled by two dimension electrophoresis. The activities of alanyl-, arginyl-, glutamyl-, histidyl-, iso-leucyl-, leucyl-, lysyl-, methionyl-, phenyl-, alanyl-, tyrosyl-, and valyl-tRNA synthetases decreased up to 80% after phosphorylation, whereas the activities of asparaginyl-, aspartyl-, prolyl-, seryl-, and threonyl-tRNA synthetases increased.

Berg, Berit Helene

153 ALTERED SUBCELLULAR DISTRUBUTION OF PULMONARY PROTEOLYTIC ACTIVITY FOLLOWING TREATMENT OF MICE WITH BUTYLATED HYDROXYTOLUENE, Elliott J. Blumenthal and Alvin M. Malkinson, University of Colorado, School of Pharmacy, Boulder, Colorado 80309.

The antioxidant, butylated hydroxytoluene (BHT), causes lung toxicity in mice and subsequent regenerative repair. Under certain injection regimes it can modulate lung adenoma development. BHT treatment lowers pulmonary cytosolic Ca^{++}-activated neutral protease (calpain) activity and increases the activity of an endogenous inhibitor, calpastatin, both in a dose-dependent manner. The decrease in calpain activity cannot be entirely accounted for by the increased calpastatin activity since upon separation of these proteins by DEAE column chromatography the amount of calpain activity from BHT-treated mice remains lower than the corresponding peak from control mice. Decreased calpain activity occurs 1 day following BHT administration, is maximally low level at 4-7 days, and returns to normal levels by day 21. BHT also increases formation of a 37 K degradative product of cAMP-dependent protein kinase regulatory (R) subunit. This alteration is also dose- and time-dependent, exhibiting changes at a dose that does not cause any observable toxic effects. Dissociated R-subunits are more suseptible to degradation by calpain, but BHT treatment does not enhance R-subunit dissociation from the holoenzyme. Rather, there appears to be increased R-subunit proteolysis associated with the membrane fraction of BHT treated mouse lung tissue homogenates. Additionally, increased Ca^{++}-independent proteolytic activity was observed in the particulate fraction of lung extracts after BHT treatment. This protease is inhibited by calpastatin, leupeptin and E-64, which are known to inhibit calpain. We hypothesize that BHT treatment stimulates calpain translocation, and that in this process Ca^{++} is no longer required for calpain activation. (Supported by USPHS grants ES02370, CA33497 and RCDA CA00939.)

154 MUTATIONS AFFECTING THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE
S.Botterell, D.A.Jans and B.A.Hemmings, Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland.

Using forskolin and isobutylmethylxanthine as selective agents, a number of mutants, resistant to the cytotoxic effects of cAMP, were isolated from the porcine kidney epithelial cell line, LLC-PK_1. Two of the cAMP-resistant mutants, FIB4 and FIB6, had altered cAMP-dependent protein kinase (cAMP-PK), resulting in a 90% reduction of activity compared to wild type. The residual cAMP-PK activity was associated almost entirely with the type II holoenzyme, and showed normal affinity for cAMP, normal affinity for the substrates ATP and Kemptide, and normal in vivo regulation. Analysis of C subunit levels using a polyclonal antibody revealed that the mutants produced approximately normal amounts of immunoreactive protein. The results suggest that the low kinase activity in the mutants is due to either a mutation in the gene for the C subunit or in a gene encoding an enzyme involved in posttranslational modification of the C subunit. Somatic cell hybrids were formed between wild type and mutant cells and analyzed for complementation of the mutation. The hybrids possessed cAMP-PK activity intermediate between mutant and normal parents, associated with both type I and type II cAMP-PK. The mutation was thus expressed codominantly in the presence of the normal allele. The nature of the mutation is currently being investigated at the molecular level using cDNA clones for the porcine C subunit.

155 EXPRESSION AND MUTAGENESIS OF THE TYPE I REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE. José Bubis, Lakshmi D. Saraswat, and Susan Taylor. Department of Chemistry, University of California, San Diego, La Jolla, CA 92093.

A cDNA clone containing the complete coding sequence of the bovine type I regulatory subunit (R^I) of cAMP dependent protein kinase has been ligated into the expression vector, pUC7. The expressed R-subunit was visualized by photolabeling with 8-N_3[^{32}P]cAMP following electrotransfer from SDS-polyacrylamide gels to nitrocellulose or by immunoblotting using anti-R^I serum antibodies. The expressed R-subunit was functionally indistinguishable from R^I purified from porcine skeletal muscle even though the expressed R-subunit represents a fusion protein that contains 10 additional amino acids at the NH_2-terminus that are provided by the lacZ gene of the vector. Maximum expression of R-subunit (20 mg/liter of culture) was seen in E. coli 222. Oligonucleotide-directed mutagenesis of the R-subunit was initiated in order to better understand structural changes that are induced as a consequence of cAMP binding. The site that was targeted for mutagenesis was identified initially by photolabeling with 8-N_3cAMP which leads to nearly stoichiometric modification of Tyr 371. In order to establish whether there exists a dipole-dipole interaction between the adenine ring of cAMP bound to the cAMP-binding domain B and Tyr 371, a single base change was introduced so that the mRNA now codes for a phenylalanine instead of a tyrosine at position 371. Introduction of this change yields a stable protein with altered cAMP binding properties: 1) the apparent Kd (cAMP) was shifted from 16 nM in the wild type to 60 nM in the mutant; 2) Scatchard plots showed no cooperativity between the cAMP binding sites in the mutant, in contrast to the positive cooperativity that is observed for the wild type R^I; and 3) the Hill coefficient of 1.6 for the wild type R-subunit was reduced to 0.99.
(Supported by Public Health Service Grant GM 34921).

156 INHIBITION OF THE BETA-ADRENERGIC RESPONSE AND FORSKOLIN RESPONSE BY PROTEIN KINASE C ACTIVATORS IN C6 RAT GLIOMA CELLS J.P. Bressler and P. Tinsley, NIH, Bethesda, Maryland

In the C6 rat glioma cell line, cAMP is an important intracellular signal for the expression of differentiated properties. Glial proteins such as S-100 proteins, glial fibrous acidic protein, and to some extent, glycerol phosphate dehydrogenase (GPDH), are inducible when intracellular cAMP levels are raised. Phorbol ester (PE) tumor promoters and diacylglycerols (DG) inhibit the expression of GPDH induction. PE and DG both activate the protein kinase C and bind to the same site on the protein. PE also inhibits the beta-adrenergic response in C6 cells and this ability to attentuate cAMP levels may be responsible for inhibiting GPDH induction. Since PE may also modulate cellular functions by protein kinase C independent mechanisms, we have asked whether elevated DG levels lead to an inhibition in the beta-adrenergic response. We have also tried to localize the site at which the inhibiton is mediated. In C6 cells DG can be elevated 2-fold by incubating the cells with either phospholipase C or 2-bromooctanoate. Under these conditions, the beta-adrenergic mediated increase in cAMP levels was inhibited. 2-bromooctanoate and phospholipase C exhibited an ED 50 of 1mM and 0.03 units/ml, respectively. The forskolin response, which is distal to the receptor, was inhibited 50% when cells were incubated with phospholipase C at 0.02 units/ml and 40% when incubated with phorbol 12,13, myristate acetate at 20 nM. The attenuated response to these agonists was not due to elevated phosphodiesterase levels. From these studies we conclude that the protein kinase C is involved in the inhibition of the beta-adrenergic response in C6 rat glioma cells. In addition, the site of inhibition is probably localized at the regulatory proteins and/or catalytic subunit of adenylate cyclase.

157 CAUDATE NUCLEUS PROTEIN PHOSPHORYLATION: ACTIONS OF NEUROTENSIN. Scott T. Cain and Charles B. Nemeroff, Departments of Psychiatry and Pharmacology, Duke University Medical Center, Durham, N. C., 27710.

The brain-gut tridecapeptide, neurotensin (NT) (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) is heterogeneously distributed in the brain. NT has many of the properties of a neurotransmitter/neuromodulator such as synaptosomal localization, calcium-dependent release and specific, saturable, high affinity binding to brain membranes. However, details of the processes underlying transduction of the NT signal are sparse. Therefore, in view of its ubiquitous regulatory role in cellular function, we have initiated a series of studies designed to elucidate the role of protein phosphorylation/dephosphorylation in mediating the coupling of NT receptor binding and function.

We have reported that after phosphorylation of rat caudate nucleus synaptosomes with 500 uM ATP, NT alters the amount of phosphate incorporated into a TCA-precipitatable protein fraction (Cain and Nemeroff, N.Y. Acad. Sci., in press). We have now studied the effects of NT (1.8 uM) on calcium (0.5 mM)-stimulated phosphorylation in synaptosomal membranes alone and synaptosomal membranes reconstituted with synaptosomal cytosol. NT caused no significant change in the magnitude of calcium-stimulated phosphorylation in either fraction. In synaptosomal membranes, Ca^{2+} alone stimulated phosphorylation 222% while in combination with NT, Ca^{2+} stimulation was 203% of basal. In the reconstituted fraction, Ca^{2+}-stimulated phosphorylation was 295% of control vs. 312% in combination with NT. Work is in progress to determine and characterize individual phosphoproteins which are responsive to NT. Supported by NIMH-39415 and NIMH-15177-09.

158 ROLE OF PROSTAGLANDINS IN THE INTERCELLULAR MODULATION OF PROTEIN PHOSPHORYLATION IN THE LIVER.

Eric Casteleijn, Johan Kuiper, Theo J.C. van Berkel and Johan F. Koster, Dept. of Biochemistry I, Erasmus University, P.O.Box 1738, 3000 DR Rotterdam, The Netherlands.

In different liver cell types, the hormonal control of protein phosphorylation and its role in intercellular communication was studied. Different rat liver cell types i.e. parenchymal, Kupffer and endothelial cells were isolated (1). After 4 h incubation, media with Kupffer or endothelial cell secretory products were collected. In parenchymal cells loaded with $^{32}PO_4$, a 5 min challenge with conditioned media from Kupffer or endothelial cells resulted in a decreased phosphorylation state of a Mw 67000 phosphoprotein and an increased phosphorylation state of a Mw 45000 phosphoprotein. These changes could also be brought about by prostaglandins E_1 and E_2, both known to be produced by non-parenchymal liver cells. Non-parenchymal cell media and prostaglandins E_1, E_2 and D_2 also decreased the amount of ^{32}P detected in a Mw 65000 phosphoprotein, secreted by parenchymal liver cells (2). It is concluded that changes in the phosphorylation state of parenchymal cell proteins can be brought about by secretory products from Kupffer and endothelial liver cells. This raises the possibility that hormonal signals on liver parenchymal cells may be mediated second signals from Kupffer and endothelial cells, leading to the concept of cellular communication in hormonal signal transduction.

1. Nagelkerke, J.F., Barto, K.P. and Van Berkel, Th.J.C. (1983) J. Biol. Chem. 258: 12221-12227.
2. Le Cam, A., Magnaldo, I., Le Cam, G. and Auberger, P. (1985) J. Biol. Chem. 260: 15965-15971.

159 AUTOPHOSPHORYLATION SITES AND THE Ca^{2+}-INDEPENDENT ACTIVITY OF Ca^{2+}(CALMODULIN)-DEPENDENT PROTEIN KINASE II

- R.J. Colbran, C.M. Schworer, and T.R. Soderling, Howard Hughes Medical Institute, Department of Molecular Biology and Biophysics, Vanderbilt University, Nashville, TN 37232.

Incubation of Ca^{2+}(Calmodulin)-dependent protein kinase II purified from rabbit liver with Ca^{2+}, calmodulin (CaM), [γ^{32}P]ATP and magnesium acetate leads to the incorporation of phosphate into both 51kDa and 53kDa subunits of the enzyme to similar extents. The maximum incorporation observed was 3-4 moles phosphate / mole protein kinase. During this incubation the kinase is converted from its native Ca^{2+}- and CaM-dependent form, to a form which exhibits as much as 70% of the maximum activity even in the presence of excess EGTA. Several lines of evidence indicate that this Ca^{2+}-independent form is generated by autophosphorylation of the kinase (Schworer et al., 1986 J. Biol. Chem "in press").

We were interested in identifying specific autophosphorylation sites that might be responsible for this transition. The maximally phosphorylated kinase was subjected to SDS-polyacrylamide gel electrophoresis to isolate the two subunits. Following extraction of the protein from the gel, each subunit was subjected to tryptic digestion followed by reverse-phase HPLC. Four major phosphopeptides were detected in digests of the 51kDa subunit. Tryptic phosphopeptides with identical retention times were also observed in digests of the 53kDa subunit and an additional phosphopeptide was also present in these digests. Generation of the Ca^{2+}-independent form of the kinase was essentially complete within 1 min, when approximately 1 mole phosphate / mole kinase was incorporated. Maximum phosphate incorporation was achieved only after an additional 15 min of incubation. Examination of the time course of appearance of ^{32}P-peptides in each subunit revealed that phosphorylation of one of the peptides found in digests of both subunits was essentially complete by 1 min, a time that correlated closely with the observed change in enzyme activity. Appearance of the other ^{32}P-peptides occured over a much longer time interval. These data suggest that this one rapidly autophosphorylated site may be very important in transition of the kinase to the Ca^{2+}-independent form. (Supported in part by NIH Grant AM17808.)

160 A UNIFYING MODEL FOR THE ROLE OF RETINOIDS AND THEIR RECEPTORS AS DIRECT KINASE CONTROL FACTORS IN VISION AND CYTODIFFERENTIATION. F.O. Cope. Southern Research Institute, Birmingham, AL 35255-5305, U.S.A.

Retinoids, including retinoic acid, have been shown to have strong antitumorigenic potential in vivo, particularly when the tumor model system is dependent upon the activation protein kinase C (PK-C). We have demonstrated that the antitumorigenic potential of retinoids is closely linked to a fast-acting membrane-associated signal transduction system (like the PK-C/phosphoinositol system) and that retinoid efficacy is time- and concentration-dependent with regard to activation of the PK-C enzyme. Moreover, we have found that numerous holo-form retinoid-binding proteins can inactivate PK-C while the apo-forms are substrates for the kinase; 2) that retinoids can induce a translocation of PK-C in neural cells; 3) that retinoids can inhibit PK-C in vivo; and 4) that holo-form retinoid receptors affect the affinity of PK-C for its ligands (Ca^{2+} and phosphatidylserine) and vice versa. Based on these data and others, we will present a unifying model for retinoid function in vision and cytodifferentiation which accounts for the antagonistic function of retinoids to PK-C in the cell cycle and also demonstrate that this antagonism is the basis for the retinaldehyde and retinol/PK-C/Ca^{2+}-dependent dark adaptation in the visual cycle. This model also suggests that the chromophore, rhodopsin, evolved not only because of Schiff base potential but also because of the ability of retinaldehyde to regulate PK-C and the concomitant translocation of PK-C with Ca^{2+}.

Likewise, the other retinoids, retinol and retinoic acid (the reduced and oxidized products of retinaldehyde, respectively), as well as their holo-receptors function to modulate PK-C activity as this activity is necessary or not in cell cycle and cytodifferentiation. (This research is supported by NIH Grants CA34968 and CA40894.)

161 PROPERTIES OF PROTEIN KINASE C FROM AORTA. K.R. Dell and D.L. Severson, Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta T2N 4N1.

The objective of our work is to purify the Ca^{2+}- and phospholipid-dependent protein kinase (kinase C) from the media of aortas because kinase C may have a role in the proliferation of smooth muscle cells seen in atherosclerosis. Kinase C has been partially purified from the media of bovine aortas by chromatography on DEAE-Sephacel and Ultrogel AcA-34. Enzyme activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine-rich histone or chicken gizzard vinculin, a cytoskeletal protein that is a physiological substrate for kinase C. With vinculin as substrate, specific activities of 66-267 pmol P_i incorporated/min/mg were obtained, an increase of 14- to 20-fold over activity in the high speed supernatant. With histone, specific activities of up to 1.57 nmol/min/mg were measured. The enzyme was both phosphatidylserine (PS)- and Ca^{2+}-dependent with both substrates. The K_a for PS in the presence of Ca^{2+} was 20 µg/ml and enzyme activity increased over the concentration range of 10^{-6}-10^{-4} M Ca^{2+}. The addition of diolein (3.2 µg/ml) increased enzyme activity 3.4-fold at 10^{-7} M Ca^{2+} with histone; the diolein effect at 10^{-5} M Ca^{2+} was less. Using vinculin as substrate, diolein increased kinase C activity 1.5-fold at 10^{-7} M Ca^{2+}. In conclusion, kinase C from bovine aorta phosphorylated the physiological substrate vinculin in a PS- and Ca^{2+}-dependent manner. The addition of diolein further stimulated this phosphorylation. Platelet-derived growth factor (PDGF) stimulates the metabolism of phosphatidylinositol and the production of diacylglycerol. This suggests a link between kinase C activation and the stimulatory effect of PDGF on vascular smooth muscle cell proliferation. This work was supported by a grant from the Alberta Heart Foundation.

162 PRIMARY SCREENING FOR EVALUATION OF POTENTIAL CALMODULIN ANTAGONIST: COMPARISON OF METHODS
P.R.M. Dobson, S.F. Pong*, S.M. Demuth*, G. Burns & B.L. Brown *Norwich Eaton Pharmaceuticals, Norwich, NY and Dept. of Human Metabolism & Clinical Biochemistry, Univ. of Sheffield, Sheffield, U.K.

The ubiquitous calcium binding protein, calmodulin (CaM) regulates may calcium-medicated events. Thus, modification of the activity of CaM would have profound biological consequences. Moveover the possibility exists that specific CaM antagonists might have therapeutic potential in various disorders. Most CaM "antagonists" currently available also interact with other calcium binding proteins including protein kinase C. In an attempt to develop a simple screening test for CaM antagonists, prior to determining their specificity, we have evaluated two distinct assay systems; a ^{3}H-CaM binding and CaM-dependent phosphodiesterase assays. The binding assay utilized rat brain synaptosomal membranes(BBA 714 84-92 1982). The CaM-dependent phosphodiestrase (PDE) was prepared from pig brain (Adv. Cyclic Nucl. Res. 10 69-92 1979). Assay of known CaM antagonists in both systems produced results which correlated significantly with those in the literature. A total of 126 compounds were screened in the binding assay and 32 inhibited binding by more than 50% (at 50 uM). Of the 32 active compounds, 24 were subjected to the CaM-dependent PDE assay. All compounds active in the binding assay were also active in the PDE assay. The PDE assay also detected several agents which inhibited basal activity (ie without CaM); the results with these agents did not correlate well between assays. We conclude that both assays are reliable and suitable tests for the screening of potential CaM antagonists. The binding assay is simpler, however, the PDE assay can identify PDE inhibitors in addition to CaM antagonists.

163 A SIMPLE, RAPID, AND SENSITIVE COLUMN METHOD FOR ASSAYING PROTEIN KINASE ACTIVITY
John J. Egan, Min-Kun Chang, and Constantine Londos. NIDDK, NIH Bethesda, MD 20892

The measurement of cyclic AMP-dependent protein kinase (A-kinase) activity in cellular preparations often depends upon the use of time-consuming and cumbersome assay methods (filtration). Alternatively, assays in which binding of a basic heptapeptide substrate, Kemptide, to acidic paper (P-81) yield varying results dependent upon the quality of the paper. In both cases, background ^{32}P-ATP lies within the range of 350-1000 cpm per 10^6 cpm ^{32}P-ATP added per assay tube. Attempts to lower this background by increased washing (the latter method) results in reduced product yields. The following method reduces the labor and the background radioactivity levels in the assay of A-kinase activity. The kinase reaction is stopped with 30 % acetic acid, and this mixture is dumped directly into a column of CM Sephadex. Kemptide quantitatively binds to the resin, but the bulk of the ATP passes directly through the column. Sephadex columns are then mounted atop columns containing the anion exchange resin, AG1-X8, and eluted with 30 % acetic acid. The effluent is passed directly into vials for liquid scintillation counting. Residual ATP is retained on the anion exchange resin, but at least 95 % of the Kemptide is eluted. Background levels of ATP are routinely 10 cpm per 10^6 cpm ^{32}P-ATP added per assay tube. Columns are rapidly regenerated and have been re-used for more than 50 assays with no loss in Kemptide recovery nor any increase in background radioactivity. Typically, processing of 100 samples is completed in 30 min and requires less than 7 min operator attendance time. It is anticipated that with appropriate oligopeptide substrates other protein kinases may be assayed with this simple method.

164 ACTIVATION OF PROTEIN KINASE C INCREASES MULTIDRUG RESISTANCE IN HUMAN MCF-7 BREAST CANCER CELLS. R. L. Pine, J. Patel, J. Carmichael, K. H. Cowan and B. A. Chabner. National Cancer Institute and National Institute of Mental Health, Bethesda, Maryland 20852.

Multidrug resistance (MDR) in mammalian cancer cells can occur after exposure to a single drug from a natural product source such as vincristine (VCR) or adriamycin (ADR). MDR is associated with a decreased net intracellular accumulation of drug due to an increased drug efflux mechanism. Calmodulin antagonists and calcium channel blockers, such as verapamil, can reverse the resistant phenotypes, and they also are known to inhibit protein kinase C (PKC) and calmodulin-associated kinases. Thus, we investigated the relationship of these kinases to changes in drug resistance in 21 human breast and 17 human small-cell lung cancer lines. MDR was determined in the lines by clonogenic assay and by clinical history. Increased phosphorylation of a 20-kD protein was found in all of the above resistant lines and it was absent or minimal in sensitive lines by an in vitro ^{32}P-ATP phosphorylation assay. PKC activity was increased 7-fold in some resistant MCF-7 breast cancer lines. Activation of PKC by phorbol esters in both sensitive and resistant breast cancer lines was associated with increases in the following: a) phosphorylation of the 20-kD protein, b) MDR in clonogenic assay, and c) decreased net intracellular accumulation of VCR and ADR. Verapamil decreased the phosphorylation of the 20-kD protein and drug resistance in these cells, while it increased intracellular drug accumulation of VCR and ADR. These experiments indicate that PKC activation is associated with the MDR phenotype via decreased accumulation and that verapamil may be reversing resistance by unknown mechanisms.

165 A COMPARATIVE ANALYSIS OF PROTEIN KINASE-C ACTIVATORS

Garland, L.G., Dawson, J., Bonser, R.W., Thompson, N.T., Blackwell, G.J.* & Hodson, H.F.†, Departments of Biochemistry, Mediator Pharmacology* & Medicinal Chemistry†, Wellcome Research Laboratories, Beckenham, Kent.

It is believed that synthetic diglycerides and phorbol esters stimulate cellular responses through the same "receptor" un protein kinase-C (PKC), but such responses have not been rigorously compared. We have studied superoxide (O_2^-) production by HL60 granulocytes stimulated by 1,2dihexanoyl-*sn*-glycerol (DiHex), phorbol dibutyrate (PDBu) and phorbol myristyl acetate (PMA). From full dose-response curves their negative log ED_{50} values were 4.8, 7.2 and 9.4, respectively. However, the maximum response to DiHex was only 45% of that produced by PMA or PDBu. This is not due to poor cell penetration since DiHex displaced 100% of ^{3}H-PDBu bound specifically by intact HL60 cells: this displacement showed enantiomeric specificity. Thus, DiHex may have been a "partial" activator of PKC, with low efficacy at the phorbol receptor. If this were so, DiHex should have competitively antagonised O_2^- production stimulated by PDBu. In such interaction experiments, DiHex blocked PDBu responses, but not competitively. Therefore, we conclude that DiHex has two properties in HL60 granulocytes, one being activation of PKC, the other being an effect which opposes cell activation through PKC. This is supported by observation of bell-shaped dose-response curves for HL60 degranulation activated by DiHex.

166 A CORRELATION BETWEEN PHORBOL DIESTER-INDUCED PROTEIN PHOSPHORYLATION AND SUPEROXIDE ANION GENERATION IN HL-60 CELLS DURING GRANULOCYTIC MATURATION. J.R. GAUT, C.M. KRAMER & R.A. CARCHMAN DEPT. OF PHARMACOLOGY & TOXICOLOGY, MEDICAL COLLEGE OF VA., V.C.U. RICHMOND, VA.23298

Phorbol diester-induced superoxide anion production was compared to phorbol diester-induced protein phosphorylation as HL-60 cells matured along the granulocytic pathway. Maturation was induced by 0,2,4, or 6 days incubation with dimethylsulfoxide (DMSO). In 0 day DMSO HL-60 cells, phorbol 12-myristate, 13-acetate (PMA) induced phosphorylation in the 100,000xg supernatant of protein pp29 (Mr=28,600) and to a lesser extent protein pp76 (Mr=76,300). With increased time of DMSO incubation, PMA-induced phosphorylation of pp212 (Mr=211,800), pp134 (Mr=134,200) and pp76, while the phosphorylation of pp29 did not change appreciably. In close agreement with this increase in protein phosphorylation was the observed increase in phorbol diester-induced superoxide anion formation. Both responses increased markedly between days 2 & 4 of DMSO exposure. Morphological characterization of cells during DMSO induced differentiation using Wright Giemsa staining revealed that these increases in phorbol diester responses were probably attributable to the parallel rise in metamyelocytes, band and segmented neutrophils. The proportional increase in pp76 phosphorylation was strongly correlated to the increase in superoxide anion formation (r=0.99). Similarly, the structure activity relationship of six phorbol diester derivatives for protein phosphorylation was also correlated to their ability to increase superoxide anion generation. Thus, we propose that phorbol diester-induced phosphorylation of pp212, pp134, and pp76, (but not pp29) may play a role in mediating the functional response of phorbol diester-induced superoxide anion generation. (funded by NIH ES03572)

167 EFFECT OF β-BLOCKADE ON SKELETAL MUSCLE GLYCOGENOLYSIS AND CYCLIC AMP DURING EXERCISE. A.H. Goldfarb and J. DiVetta. Exercise Science Laboratory, University of Maryland, College Park, Maryland 20742.

Twenty-eight, male Sprague-Dawley rats (200-250 gms) were randomly assigned to either a resting-saline (RS), a resting-propranolol (RP), an exercise-saline (ES) or an exercise-propranolol (EP) group to determine the effects of Beta-receptor blockage on red fast twitch (R), white fast twitch (W), and red slow twitch (S) skeletal muscle glycogenolysis and cyclic AMP during exercise. The exercise consisted of running at 20 meters/min for 30 min on a motorized rodent treadmill. Administration of propranolol (6 mg/kg) did not alter resting values for glycogen or cyclic AMP in R, W, or S skeletal muscle. The acute exercise resulted in a 40% and 42% decrease in glycogen in R and S skeletal muscle respectively, with a non-significant degradation in W muscle. Propanolol administration completely prevented the degradation of glycogen in S muscle (ES = 2.84 ± .23, EP = 5.17 ± .24 mg/gm wet weight) while only partially preventing the decline in glycogen in R muscle (ES = 3.93 ± .34, EP = 4.82 + .12 mg/gm) compared to resting values (RS = 6.53 + .38, RP = 6.19 ± .29 mg/gm). The acute exercise significantly increased cyclic AMP in R (RS = 302.8 ± 16.3, ES = 481.6 ± 30.8 pMol/mg wet weight), W (RS = 268.3 ± 21.3, ES = 430.9 ± 25.4 pMol/mg wet weight), and S (RS = 304.8 ± 24.6, ES = 510.8 ± 33.9 pMol/mg wet weight). Beta-blockade prevented the exercise-induced increase in cyclic AMP in both W (EP = 291.4 ± 29.6 pMol/mg wet weight) and S (EP = 340.0 ± 21.8 pMol/mg wet weight) but only attenuated the cyclic AMP rise in R muscle (EP = 367.0 ± 15.9 pMol/mg wet weight). These results suggest that Beta-receptor blockade in skeletal muscle is fiber dependent in inhibiting glycogen degradation and cyclic AMP accumulation during exercise.

168 THE MR 43,000 POLYPEPTIDE, ν_1, OF ACHR-ENRICHED MEMBRANES IS A PROTEIN KINASE. A.S. Gordon, Dept. of Neurology, Univ. of Cal. Sch. of Med., San Francisco, CA 94143.

AChR-enriched membranes have been shown to contain 3 alkali-extractable polypeptides of Mr 43,000, ν_1, ν_2, and ν_3 which can be separated by 2-dimensional electrophoresis. ν_1 is a membrane-bound polypeptide having a pI between 7.0 and 7.4 which copurifies with the AChR. Immunochemical studies show that ν_1 is exclusively a post-synaptic membrane protein. We have found that an alkaline-extractable Mr 43,000 polypeptide has an ATP binding site and that the alkaline extract has protein kinase activity. Our results suggest that ν_1 may be a protein kinase. We have used monoclonal antibody (mcAb) to ν_1 to address this question. AChR-enriched membranes were covalently labeled with [α-^{32}P]ATP. When these membranes were solubilized and immunoprecipitated with anti-ν_1 mcAb, we found a covalently labeled polypeptide of Mr 43,000 on autoradiograms of SDS gels which was not present when control IgG was used. Therefore, ν_1 is an ATP binding protein.

In order to demonstrate that the ATP binding protein precipitated by the mcAb is a protein kinase, we show that protein kinase activity is also precipitated from solution by mcAb. pH 11 extract is neutralized and incubated with either mcAb or control mouse IgG and Staph. Aureus is added to precipitate the immune complexes. After centrifugation, the supernatant is assayed for protein kinase activity. We find that pH 11 extract incubated with anti-ν_1 mcAb shows no phosphorylation activity. In contrast, protein kinase activity is observed in the pH 11 extract incubated with control mouse IgG. Therefore, the mcAb must have precipitated the protein kinase and we can conclude that ν_1 is a protein kinase. Since there is only one ATP binding protein present in AChR-enriched membranes, ν_1 is probably the receptor kinase which phosphorylates the AChR.

169 Ca^{++}. CALMODULIN-DEPENDENT PROTEIN KINASE II AND Ca^{++}.PHOSPHOLIPID-DEPENDENT PROTEIN KINASE ACTIVITIES IN RAT TISSUES ASSAYED WITH A SYNTHETIC PEPTIDE. Y. Hashimoto and T.R. Soderling, Howard Hughes Med. Inst., and Dept. of Molecular Physiology and Biophysics, Nashville, TN 37232.

Rat tissue levels of Ca^{++}. calmodulin-dependent protein kinase II (protein kinase II) and Ca^{++}. phospholipid-dependent protein kinase (protein kinase C) were selectively assayed using the synthetic peptide syntide-2 as substrate. The sequence of syntide-2 (pro-leu-ala-arg-thr-leu-ser-val-ala-gly-leu-pro-gly-lys-lys) is homologous to phosphorylation site 2 in glycogen synthase. The relative Vmax/Km ratios of the known Ca^{++}-dependent protein kinases for syntide-2 were determined to be as follows: protein kinase II, 100; protein kinase C, 22; phosphorylase kinase, 2; myosin light chain kinase, 0.05. Levels of protein kinase II were highest in cerebrum (3.36 units/g tissue) and spleen (0.85 units/g) and lowest in testis (0.05 units/g) and kidney (0.04 units/g). Protein kinase II activity was localized predominantly in the 100,000g particulate fraction of cerebrum and testis; in the cytosolic fraction of heart, liver, adrenal, and kidney; and about equally distributed between the particulate and cytolic fractions in spleen and lung. On the other hand, protein kinase C was highest in cerebrum (0.56 units/g) and spleen, (0.47 units/g), and the majority of activity was present in the cytosolic fraction for all tissues measured except for cerebrum and testis in which the kinase activity was equal in both fractions. Lastly, the ratios of protein kinase II to protein kinase C were different in various rat tissues and between the particulate and cytosolic fractions. These results suggest somewhat different functions for these two Ca^{++}-regulated, multifunctional protein kinases. (Supported in part by NIH grant AM17808.)

170 MOLECULAR CLONING OF THE cAMP-DEPENDENT PROTEIN KINASE FROM LLC-PK$_1$ CELLS
B.A.Hemmings, M.Schwarz, S.R.Adavani, D.A.Jans, I.Nowak, M.O.Showers* and R.A.Maurer*, Friedrich Miescher-Institut, P.O.Box 2543, CH-4002 Basel, Switzerland, *Dept. of Physiology & Biophysics, University of Iowa, IA, USA

The cAMP-dependent protein kinase (cAMP-PK) is thought to play an integral role in the regulation of transcription of specific genes. In the porcine kidney cell line LLC-PK$_1$, elevation of cAMP mediated by the hormone calcitonin leads to a 100-fold increase of the rate of transcription of the plasminogen activator gene. As a step towards understanding the role of the kinase in the induction process we undertook the molecular cloning of the subunits of the cAMP-PK from the LLC-PK$_1$ cell line. Using the λgt 11 system, cDNAs were isolated for both the porcine R_I and R_{II} using a bovine R_I cDNA and antibody to bovine R_{II}, respectively. The LLC-PK$_1$ cDNA library was also screened using a bovine cDNA for the C subunit and two distinct cDNAs were isolated for the porcine C subunit. DNA sequence analysis confirmed the existence of two mRNA species (C_α and C_β) coding for the C subunit, most likely encoded by different genes. The two types of C subunit, with presumably somewhat different activities, provide the possibility of fine control over cellular activities. Comparison of deduced amino acid sequences of the porcine R_I, R_{II} and $C_{\alpha/\beta}$ with the bovine sequences revealed very high homology for all subunits, indicating high conservation of structure and function in this important regulatory enzyme.

171 DISTRIBUTION OF PHOSPHOPROTEINS (PP) IN DIFFERENT BRAIN AREAS STUDIED BY TWO-DIMENSIONAL GEL ELECTROPHORESIS (2DE). W.E. Heydorn, J. Patel, G.J. Creed and D.M. Jacobowitz, FDA, Rockville, MD 20857 and Laboratory of Clinical Science, NIMH, Bethesda, MD 20892.

The distribution of PP was studied in 7 brain areas: caudate nucleus (CN), olfactory tubercle (OT), prefrontal cortex (PRE), hippocampus (HIP), habenula (HAB), arcuate-median eminence (ARC-ME) and ventromedial nucleus (VMN). Under basal conditions, approximately 40 phosphoproteins could be seen, with 12 being visible on silver-stained 2D gels of rat brain. Three distinct regional differences were noted. First, a 94 kDa (pI 5.6-5.8) PP was found primarily in the CN, HIP and OT. Second, a pair of high MW (95-100 kDa) PP was consistently visible only in the CN, PRE, HAB and HIP. Third, a group of proteins of MW 32-37 kDa, pI 4.9-5.4 consistently phosphorylated only in the CN and the ARC-ME. In the presence of Ca/calmodulin, the phosphorylation of 3 acidic proteins (pI < 5.0) was markedly elevated in all 7 brain areas examined. Cyclic AMP produced an increase in the phosphorylation of a number of different proteins. The 94 kDa protein showed a marked increase in phosphorylation only in the CN and OT. Cyclic AMP also stimulated the phosphorylation of a poorly focusing 83 kDa protein in the CN, OT and, to a lesser degree, in the HIP, HAB and ARC-ME. Phosphorylation of an acidic protein that appears to correspond to DARPP-32 was stimulated by cAMP in the CN, OT, HAB and ARC-ME. A group of basic proteins that appears to correspond with Protein IIIb was phosphorylated in the presence of cAMP in all 7 brain areas studied. Finally, 2 PP of MW 45-46 kDa, pI 5.5 that were not apparent under basal conditions were phosphorylated in all 7 brain areas. These results demonstrate: 1) A number of silver-stained proteins visible on 2D gels of rat brain are phosphoproteins, 2) Cyclic AMP has region-specific effects on the phosphorylation of individual proteins, and 3) These effects can easily be visualized using 2DE.

172 INHIBITION BY CALMODULIN ANTAGONISTS, H-7 AND H-8 OF FORMYL PEPTIDE, LEUKOTRIENE B_4, PHORBOL DIESTER AND DIACYLGLYCEROL STIMULATION OF MACROPHAGES. A. Holian, M. K. Jordan and H. V. Nguyen. Univ. of Texas Sch. of Med., Houston, TX, USA 77030.

The proposed roles of protein kinase C (PKC) in the activation sequence of the stimulated production of O_2 by guinea pig alveolar macrophages was examined using trifluoperazine (TFP), fluphenazine (FLU), W-7, H-7, and H-8. All five agents inhibited O_2 production stimulated by f-nle-leu-phe (FNLLP), leukotriene B_4 (LTB_4), phorbol-12,13-dibutyrate (PDB) and sn-1,2-dioctanoylglycerol. The order of potency was TFP > FLU > W-7 = H-7 > H-8. The inhibition by TFP and FLU could be partially reversed by the addition of 1mM Ca^{+2} to medium with Ca^{+2} < 10 uM. TFP, FLU and W-7 partially enhanced $45Ca^{+2}$ efflux. W-7 enhanced $45Ca^{+2}$ efflux to a similar extent as that achieved with FNLLP. FNLLP-stimulated $45Ca^{+2}$ efflux in the presence of FLU or TFP was diminished to the same extent which FLU or TFP enhanced $45Ca^{+2}$ efflux resulting in the same total net $45Ca^{+2}$ efflux. The normally short-lived phosphatidyl inositol (PI) turnover stimulated by either FNLLP or LTB_4 was not markedly affected by FLU and still terminated when measured either as changes in 32P incorporation into phosphatidic acid or as 3H-diacylglycerol production. FLU and, more markedly, W-7 enhanced the incorporation of 32Pi into the phospholipids of the PI cycle. W-7 also stimulated a weak and short-lived "burst" of O_2 production. These results suggest: 1) The calmodulin antagonists (FLU, TFP and W-7) have a number of sites of action which appear to include PKC (where H-7 and H-8 inhibit) and possibly phospholipase C. 2) FNLLP and LTB_4 can still stimulate those events preceeding the site of inhibition (i.e., PKC); and 3) Activation of PKC may not be involved in the termination of FNLLP and LTB_4 stimulated PI turnover. This work was supported in part by HL-33953.

173 PROPERTIES OF THE ENDOGENOUS TRIMERIC FORM OF A-KINASE PRESENT IN RAT OVARIAN EXTRACTS. M. Hunzicker-Dunn, L. Lynch and R.C. Ekstrom, Dept. Molec. Biol, Northwestern U Med Sch, Chicago IL.

Separation of A-kinase holoenzymes by DEAE-cellulose chromatography has shown that soluble extracts of immature rat ovaries contain 2 holoenzyme forms of the type II enzyme: a tetrameric form composed of a regulatory (R) subunit dimer and 2 catalytic (C) subunit monomers; and an apparent trimeric form (Hunzicker-Dunn et al., J Biol Chem 260:13360,1985). The apparent trimeric A-kinase was stimulated by cAMP, contained authentic R^{II} and exhibited a $s_{20,w}$ (6.08± 0.19, n=19) lower than that of the tetramer (7.5±0.19, n=19). The purpose of the following studies was to confirm the MW of the apparent trimeric A-kinase and to establish its subunit composition (as R_2C or RC_2). To determine its MW, autophosphorylated tetrameric and apparent trimeric holoenzymes were separated by nondenaturing PAGE. MW estimates for the tetrameric and trimeric forms of 215000 and 175000, respectively, were obtained. The 2 enzyme forms were also separated on Sephacryl S-300.MW estimates from gel filtration were 245000 and 195000, respectively, for the tetrameric and trimeric forms. To determine the subunit composition of the trimer, the extent of autophosphorylation of R^{II} associated with sucrose gradient-purified trimeric and tetrameric A-kinase forms, in the presence of equal C activity, were compared. There was no reduction in the incorporation of label into the trimeric R^{II} compared to tetrameric R^{II}. A comparison of relative cAMP-binding to phosphotransferase (R:C) activities of the 2 enzyme forms resulted in values of 0.62±0.09 (n=23) for the tetrameric enzyme and 2.1±0.42 (n=23) for the trimeric enzyme, consistent with reduced phosphotransferase activity associated with the trimer. From these results, an R_2C subunit composition can be predicted for the trimer. We conclude that rat ovaries contain a trimeric type II A-kinase with a subunit composition of R_2C. Supported by NIH HD 11356 to MHD.

174 PHOSPHORYLATION AND DEPHOSPHORYLATION IN *LEISHMANIA MAJOR*
Charles L. Jaffe and David S. Lester Departments of Biophysics and Membrane Research Weizmann Institute of Science, Rehovot, Israel

Labelled phosphoproteins and phosphatase activity were investigated in logarithmic stage promastigotes of *L. major*. Whole cells incubated in [^{32}P]-orthophosphate revealed 13 bands. Three bands (105, 70 and 16 kd) were found in both membrane and cytosol fractions. The 16 kd band was the major phosphoprotein. Incubation of cells with calcium intensified labelling of the 70 kd protein. This calcium stimulation was also observed with three additional proteins in the membrane fraction (48, 42 and 18 kd). Incubation with the calcium ionophore A23187 drastically reduced labelling of the 16 kd protein. [γ-^{32}P]-ATP labelling of lysed parasites identified 7 phosphoproteins; the predominant band migrated at 16 kd. The pattern of labelling was similar to that observed for membranes of [^{32}P]-labelled whole cells. Calcium specifically decreased labelling of two phosphoproteins (90 and 56 kd). Phorbol ester and 8-BrcAMP had no effect. pNPP-alkaline phosphatase activity was examined in whole cells, membrane and cytosol fractions. The membrane activity was externally located and had a pH optimum of 7.0-7.5. The specific activity of the cytosol fraction was at least 4-fold greater than the membrane fraction and preincubation of whole cells in calcium shifted the pH optimum of the cytosol fraction from 7.0 to 6.5. All phosphatase activity was inhibitable by calcium and vanadate. Developmental studies of these processes are under investigation.

Funding was provided by the John and Catterine T. MacArthur Foundation.

175 PHOSPHORYLATING SYSTEM CONTROLLING ASPARAGINASE CYCLIC ACTIVITY. S. Jerebzoff-Quintin and S. Jerebzoff, Biorhythm Lab. and UA 241 CNRS, P. Sabatier Univ., TOULOUSE, France.

Asparaginase cyclic activity in Leptosphaeria michotii has been shown to be dependent on a reversible phosphorylating process. Asparaginase has been isolated as a purified protein complex, which possessed autophosphorylating and autodephosphorylating capacities (1)(2).

A protein kinase was present in this complex, as demonstrated by time course of Mg^{2+}-dependent phosphorylation by [γ-^{32}P]ATP, whereas [α-^{32}P]ATP was not incorporated, and identification of the phosphoaminoacid as threo(P) ; it was cAMP non dependent and active at pH from 5 to 9. A rhythm of protein kinase activity synchronous with the rhythm of asparaginase activity was observed.

Autodephosphorylation of asparaginase complex was controlled by a Mg^{2+}-Zn^{2+}-dependent protein alkaline phosphatase (active at pH from 7 to 9), Mr 60 000, phosphorylable, which could hydrolyze non protein phosphoesters. In the time course of asparaginase activity cycle, Zn^{2+} addition in the presence of [γ-^{32}P]ATP-Mg^{2+}, stimulated dephosphorylation of the protein complex when asparaginase activity is decreasing, whereas it stimulated ^{32}P incorporation when asparaginase activity is increasing.

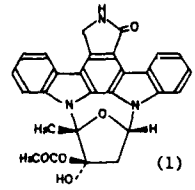
Opposite effects of protein phosphatase activation on asparaginase activity, together with the fact that protein phosphatase was phosphorylable, give strong support for an important regulatory role of this enzyme in the control of asparaginase oscillatory states.

(1) FEBS Lett., 1984, 171, 67. (2) Physiol. Plant., 1985, 64, 74.

176 K-252a; A POTENT, NEW INHIBITOR OF PROTEIN KINASES FROM MICROBIAL ORIGIN

H. Kase, K. Iwahashi, Y. Matsuda, S. Nakanishi and K. Yamada
Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.
Machida-shi, Tokyo, Japan

K-252a(1), a novel compound isolated from the culture broth of Nocardiopsis, sp., was found to potently inhibit protein kinase C (Kase et al. J. Antibiotics, in press). The compound also inhibited cyclic nucleotide-dependent protein kinases. Apparent Ki values of K-252a for protein kinase C, and cAMP-dependent and cGMP-dependent protein kinases were 25, 7, and 20 nM, respectively. The inhibitions were not reversed by the phosphate acceptors, or the enzyme activators such as cAMP, cGMP and phospholipid. Alternatively, ATP reversed the inhibition of K-252a in competitive manners. The compound also inhibited calmodulin, but its effect (IC_{50}; 2.9 μM) was much less than that on protein kinases. K-252c, the aglicon of K-252a, and K-252b and d, which contained K-252a aglicon in their structures, were also isolated from the culture broth and inhibited the protein kinases to various degrees.



(1)

In rabbit platelet, K-252a inhibited ATP secretion induced by thrombin, platelet activating factor, and phorbol myristate acetate. These newly developed compounds should facilitate studies on the physiological roles of protein kinases.

177 THE RELATIONSHIP BETWEEN PHORBOL ESTER - STIMULATED LYSOSOMAL ENZYME RELEASE AND PROTEIN PHOSPHORYLATION IN HUMAN MONOCYTES. Barbara A. Kelly and Richard A. Carchman, Dept. of Pharmacology and Toxicology, Medical College of Virginia, V.C.U., Richmond, Va. 23298.

PMA (Phorbol-12-myristate-13-acetate), a potent tumor promoter, stimulated the secretion of the lysosomal enzyme N-acetyl-B-D-glucosaminidase (NAGA) from human monocytes. PMA elicited a concentration-dependent (EC50=53 nM) increase in NAGA secretion over a tested range of 0.1 nM to 1 uM. This time-dependent release (22% of total enzyme at 10 uM PMA) reached a near maximal response at 60 minutes. In addition, PMA stimulated the phosphorylation of several endogenous protein substrates in broken cell preparations as well as intact cell preparations in a concentration-dependent manner over a tested range of 0.3 nM to 3 uM. Protein substrates specifically phosphorylated by PMA had apparent molecular weights of 20K, 30K, 41K, and 75K in broken cell preparations and 20K, 55K, 61K and 66K in whole cell preparations. Further studies of lysosomal enzyme release and protein phosphorylation stimulated by various diterpene derivatives relealed similar structure activity relationships (SAR) to the SAR characterized for phorbol ester-binding to human monocytes (J. Clin. Invest. 70:669-706, 1982) These findings support the hypothesis that phorbol ester-elicited events are mediated by the activation of a receptor enzyme known as calcium-dependent, phospholipid-sensitive protein kinase (PK-C); PK-C phosphorylates specific protein substrates purportedly resulting in various functional responses. Finally, the effects of opsonized zymosan to stimulate protein phosphorylation were investigated in an attempt to further identify those protein substrates key to the regulation of lysosomal enzyme release. (Supported by NIH 5 T32 ES07087 and NIH ES03572).

178 FORSKOLIN ALSO INHIBITS ADENYLATE CYCLASE ACTIVITY AND cAMP PRODUCTION Azra Khanum and Maria L. Dufau, NICHD, NIH, Bethesda, MD 20892

In testicular Leydig cells (LC), forskolin (F) causes stimulation of all cAMP pools (extra, intracellular & receptor-bound) and testosterone production, and potentiates gonadotropin-induced responses, when present in concentrations of 1-10 μM. In addition, when added at lower doses (100 nM) that did not affect cAMP generation, F caused an increase in sensitivity to hormonal stimulation for all cAMP pools and testosterone production. Minor increases in bound cAMP (25%) elicited by gonadotropin caused maximal testosterone production, while with comparable increases by F (10^{-6}), testosterone increases were only 36% of maximal. In contrast to the known stimulatory actions of F, low doses (in the pM-nM range) markedly inhibited basal and gonadotropin stimulated cAMP and testosterone production. Furthermore, F significantly decreased adenylate cyclase activation by GTP and luteinizing hormone. This inhibitory action, was prevented by preincubating the cells and membranes with pertussis toxin before addition of F. A dose-dependent reversal of F inhibition was observed over the range of 0.1-30 ng toxin. These studies have shown the diverse effects of F: first the stimulatory effects on LC cAMP pools, including potentiation of the hormonal increase in receptor-bound cAMP by F; and have provided additional evidence for the functional importance of cAMP compartmentalization during hormonal stimulation of steroidogenesis. Second, we have demonstrated a novel, high-affinity inhibitory action of F upon adenylate cyclase activity and cAMP generation, an effect that seems to be mediated by the Ni unit. This finding provides a new approach for direct evaluation of the functional inhibitory influence of Ni unit in the intact LC and cell membrane and has potential general application to other systems.

179 PROTEIN KINASE ACTIVITY IN CLONED MURINE T CELL LINES. D-K Kim and F.W. Fitch. Dept. of Path., Box 332, Univ. of Chicago

We have observed that our typical cloned cytolytic T cell lines, exemplified by L3, proliferate in response to phorbol 12-myristate 13-acetate (PMA); in contrast, our typical cloned helper T cell lines, exemplified by L2, do not proliferate in response to PMA. A number of recent reports have suggested that translocation of protein kinase C (PKC) from the cytosol to the microsomal fraction (membrane) is associated with the induction of the characteristic functions of a variety of cell types. We now have obtained evidence that translocation of a Ca++, phospholipid-dependent kinase activity (PKC activity) from the cytosol to the membrane occurs after a ten minute PMA treatment both in the cloned cytolytic T cell line, L3, and in the cloned helper T cell line, L2. Thus the translocation of PKC activity itself is not necessarily associated with the induction of proliferation of T cells, because translocation of PKC activity occurs after PMA treatment in a T cell line, L2, that does not proliferate in response to PMA. The level of PKC activity found in the membrane after PMA treatment, however, differs between the two cell lines: only 15-25% of the PKC activity found in the cytosol of untreated L3 cells is translocated to the membrane of PMA-treated L3 cells; in contrast, nearly the entire PKC activity found in the cytosol of untreated L2 cells is translocated to the membrane of PMA-treated L2 cells. In both L3 and L2 cells, PKC activity in the cytosol is completely depleted after PMA treatment. Furthermore, preliminary evidence suggests that in PMA-treated L3 cells, but not in untreated L3 cells, a substantial level of the PKC activity is translocated to the nuclear fraction.

180 THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASES: A HETEROGENEOUS ENZYME
V. Kinzel, A. Hotz, N. Koenig, W. Pyerin and J. Reed, German Cancer Research Center, Institute of Experimental Pathology, Im Neuenheimer Feld 280, D-6900 Heidelberg 1, FRG

Electrophoretically homogeneous preparations of catalytic subunit (C) of cAMP-dependent protein kinase (type I and II) from striated muscle of different species (rat, rabbit, cattle) can be separated by cation exchange chromatography into 2 major stable forms designated C_A and C_B (according to their elution). They may be expressed simultaneously in the tissue. Both forms differ by approx. 0.5 units in their isoelectric values. No differences between C_A and C_B were detectable by a number of criteria including (i) phosphate content, (ii) kinetic behaviour, (iii) interaction with regulatory subunit type I and II (in cooperation with F. Hofmann, Homburg, FRG), (iv) heat stable inhibitor. However, the protease specific for native C (E. Alhanaty et al., PNAS 78, 3492) cuts C_B consistently faster than C_A, a phenomenon which is enhanced in both cases by the substrate Kemptide (in cooperation with S. Shaltiel, Rehovot, Israel). Circular dichroic (CD) measurements using Blue Dextran as a sensor for the nucleotide binding site (J. Reed, V. Kinzel, Biochemistry 23, 968) exhibit a differential induced CD spectrum of C_A and C_B on binding of Kemptide thus indicating that the forms differ in the conformational response of their ATP-binding site to substrate binding. The introduction of a high resolution isoelectric focusing system revealed that C_A as well as C_B consists of 2 major isoelectric variants ($\Delta pH \leq 0.1$ units) and of 1-2 minor variants. Whether these forms are products of different genes (M.D. Uhler et al., PNAS 83, 1300) or/and posttranslational modification(s) is being determined through studies which are in progress.

181 cAMP-DEPENDENT PROTEIN KINASE FROM DICTYOSTELIUM DISCOIDEUM: CLONING AND cDNA SEQUENCE OF REGULATORY SUBUNIT.
Lacombe,M.L., Simon,M.N., Mutzel,R., de Gunzburg,J. and Véron,M. - Unité de Biochimie Cellulaire - 28, rue du Docteur Roux - 75724 Paris Cedex 15, France

Dictyostelium discoideum contains only one type of cAMP-dependent protein kinase (cAK) which is highly developmentally regulated. The enzyme composed of regulatory (R) and catalytic (C) subunits is organized as an inactive (RC) dimer at the difference of the R_2C_2 complex found in other organisms. Moreover, the R monomer contains only one cAMP binding site instead of the two found in mammalian R.

cDNA clones encoding the R subunit of the Dictyostelium cAK were isolated by immunoscreening of a cDNA library constructed in the expression vector λgt11. Presence of high affinity cAMP binding activity was demonstrated in extracts from bacteria lysogenized with these clones. Nucleotide sequence analysis of three overlapping clones allowed the determination of a 1149 bp cDNA sequence coding for the entire R subunit. The open reading frame codes for a protein of 327 amino acids corresponding to a molecular weight of 36,794. DNA blot analysis demonstrates that the R subunit gene exists as a single copy in the genome. The R subunit from Dictyostelium shares a high degree of homology with RI, RII and cGMP-dependent protein kinase from mammals. However, it is lacking the N-terminal domain required in other eukaryotes for the dimerization of R subunits. The sequence of the R from Dictyostelium reveals 2 cAMP binding domains, each one homologous to the catabolite gene activator protein (CAP) from E.coli and to the corresponding domains in the mammalian R subunits.

182 CHARACTERIZATION OF A POLYCLONAL ANTIBODY TO PROTEIN KINASE C. Karen L. Leach, James C. McGuire and Elaine A. Powers. Dept. of Cell Biology, The Upjohn Company, Kalamazoo, MI 49001

We have used purified protein kinase C (PKC), prepared from rat brain using DEAE chromatography, ammonium sulfate precipitation and phosphatidylserine affinity chromatography to produce a polyclonal antibody in sheep. Using Western blotting techniques, the immune sera recognizes the 82 kD PKC at concentrations less than 500 ng. In crude preparations from rat brain, and in cell extracts from CHO, GH_3, C6 and S49 cells the antibody recognizes the 82 kD protein as well as several lower molecular weight proteins, presumed to be proteolytic products of PKC. The immune sera immunoprecipitates autophosphorylated and [^{35}S]methionine-labeled PKC from intact cells, suggesting that the native protein is recognized by the antibody. This antibody should be a valuable tool in our continuing studies of the regulation of PKC.

183 AUTONOMOUS ACTIVITY OF THE MULTIFUNCTIONAL Ca^{2+}/CALMODULIN DEPENDENT KINASE AFTER AUTOPHOSPHORYLATION. By Lillian L. Lou and Howard Schulman. Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

ATP modulates the effect of autophosphorylation on the activity of the rat brain Ca^{2+}/calmodulin-dependent protein kinase. At low, but saturating, levels of ATP (<20 μM) autophosphorylation inhibits activity towards exogenous substrates by 75%. By contrast, at high (and more physiological) levels of ATP (500 μM) autophosphorylation converts the kinase to a form which is autonomous of Ca^{2+} and calmodulin. Aside from serving as a phosphate donor during catalysis, ATP appears to regulate the site(s) which become autophosphorylated and thereby modulates the dual effects of autophosphorylation. Complete autonomy is generated at a threshold level of autophosphorylation which is 10-20% of maximal phosphate incorporation. Once the threshold is reached, additional autophosphorylation occurs independent of the presence of Ca^{2+} and calmodulin. Despite this independence from control by Ca^{2+} and calmodulin, the autophosphorylation is modified in a qualitiative way by the presence of these agents. Ca^{2+}/calmodulin regulate the site(s) of this additional self-phosphorylation by the autonomous kinase. Furthermore, they regulate the effect of this autophosphorylation on substrate phosphorylation. In the absence of Ca^{2+}/calmodulin, additional autophosphorylation (beyond threshold) inhibits kinase activity towards exogenous substrates; in the presence of Ca^{2+}/calmodulin, no inhibition is seen. Extending the initial phosphate incorporation beyond the threshold, prior to removal of Ca^{2+}/calmodulin, will protect the enzyme from this subsequent inhibition by autophosphorylation. ATP and Ca^{2+}/calmodulin regulate the sites of autophosphorylation which may cause distinct modifications in protein conformation and thereby elicit the profound changes in the functional characteristics of the Ca^{2+}/calmodulin-dependent protein kinase.

184 LOCALIZATION OF A CYCLIC GMP-STIMULATED PHOSPHODIESTERASE IN THE BOVINE ADRENAL GLAND R.T. MacFarland, B.D. Zelus, J.P. Novack and J.A. Beavo. Department of Pharmacology SJ-30, School of Medicine, University of Washington, Seattle WA 98195.

Previous kinetic and immunological studies from this laboratory have shown the cyclic GMP-stimulated phosphodiesterase isozyme (cGS-PDE) to be the predominant isozyme present in the bovine adrenal gland. Immunoblotting experiments now indicate that the bulk of this activity is concentrated in the outermost layer of cells of the adrenal cortex, corresponding to the capsule and zona glomerulosa regions. Fresh bovine adrenal glands were sectioned using a freezing microtome. Frozen sections (60 μm) were then extracted overnight in 1% SDS. Extracts from individual sections were separated by SDS-PAGE followed by transfer to nitrocellulose. Protein blotting using a monospecific antisera directed against the cGS-PDE indicated that most of this isozyme was localized to the outermost 1.5mm of the adrenal cortex.

These findings support the suggestion for a role of cyclic GMP in the regulation of aldosterone secretion. Elevation of cyclic GMP within the glomerulosa cells, in response to stimulation by atrial natriuretic factor for example, may thus lead to a corresponding decrease in cyclic AMP levels via increases in the activity of the cGS-PDE.

185 PHOSPHOTYROSINE IN PROTEINS: CHEMICAL AND IMMUNOLOGICAL IDENTIFICATION AND ISOLATION OF PHOSPHOTYROSYL PROTEINS. T. M. Martensen, Laboratory of Biochemistry, NHLBI, NIH, Bethesda, Maryland 20892.

Proteins which contain phosphotyrosine (Tyr-P) residues can be identified and purified by utilizing the chemical and immunological properties of the Tyr-P moiety. The stability of Tyr-P to basic conditions which cleave most ser/thr phosphoryl bonds enabled the analysis of cellular phosphoproteins after Na dodecyl SO_4 gel electrophoresis. Analyses were simplified using a nylon electroblot of the gel which is stable to 1 hr incubation in 1 N NaOH at 65°C. Autoradiograms of nylon blots before and after base treatment were performed on ^{32}P-labeled phosphoproteins of normal and retrovirus (Rous, Kirsten, and Moloney) transformed fibroblasts. Conditions for optimal labeling of cellular proteins were studied by protein tyrosine kinase activity measurements using poly glu_4tyr. Differences in base resistant phosphoprotein patterns could be seen for each transformed cell line. Confirmation that certain base resistant phosphoproteins contained Tyr-P was achieved by immunostaining with affinity-purified sheep anti•Tyr-P antibodies (Ab). These Ab served as useful reagents to study Tyr-P proteins since they inhibit their dephosphorylation by phosphatases, and precipitate them in the presence of rabbit anti•sheep IgG Ab. Tyr-P protein was immobilized when mixed with anti•Tyr-P Ab and incubated with immobilized protein A sepharose rabbit anti•sheep IgG Ab complex. Selective elution of the Tyr-P protein was achieved with free Tyr-P.

186 IMMUNOGOLD ELECTRON MICROSCOPIC LOCALIZATION OF cAMP-DEPENDENT PROTEIN KINASE SUBUNITS IN SECRETORY CELLS. M.I. Mednieks, R.P. Siraganian, S. Bae, L. Schaake and A.R. Hand. National Institute of Dental Research, NIH, Bethesda, MD.

Hormonally regulated protein secretion from the parotid gland is mediated by cyclic AMP. Stimulation by norepinephrine or its pharmacologic analogs involves changes in protein phosphorylation cAMP-dependent protein kinase (cAPK) and an intracellular redistribution of its subunits (Mednieks and Hand, Eur. J. Cell Biol., 28, 264, 1982). Biochemical findings indicate that cAPK subunits are present in the nucleus, cytoplasm and secretory granules of acinar cells of the parotid as well as in ductal saliva (Mednieks and Hand, Experientia, 40, 945, 1984). Immunogold electron microscopic studies were carried out to determine the localization of cyclic AMP-dependent protein kinase (cAPK) subunits in hormonally responsive protein secreting cells. Specific polyclonal antibodies to regulatory subunits of cAPK type I and type II were used to determine their distribution by gold labeling: approximately 60% were distributed over the cytoplasm, 27% in the secretory granules and 12% in the nucleus (primarily associated with heterochromatin). Similar findings were obtained using a monoclonal IgM that reacted on ELISA with R subunits in saliva, parotid extract, and with bovine heart RII. This hybridoma reagent (MAb BB1) immunoprecipitated photoaffinity labeled bovine RII, a photoaffinity labeled (40 Kdal) fragment from both cAPK I and cAPK II preparations, but not rabbit muscle RI. Immunogold localization using MAb BB1 was also studied in pancreatic and seminal vesicles, since photoaffinity labeled R subunits were found in ductal secretions of both tissues. Secretory granules of the pancreas and of seminal vesicles were also labeled, in addition to the nucleus and cytoplasm. Our results indicate that cAPK R subunits are secretory proteins of several hormonally responsive cell types, their physiologic function, however, is not known.

187 *GANGLIOSIDE INHIBITION OF CELLULAR EVENTS INDUCED* BY PHORBOL ESTERS: PROTEIN KINASE C AS A POTENTIAL TARGET FOR GANGLIOSIDE MODULATION. W.D. Merritt, C. Drogula and J.M. Bailey. Dept. of Biochemistry, The George Washington University Medical Center, Washington, D.C. 20037.

We have shown that exogenous gangliosides inhibit proliferation of lymphocytes stimulated by interleukin 2 (IL-2). To assess whether a protein kinase C (PKC)-activation pathway is a site for ganglioside modulation, CT6 murine T cells were cultured with phorbol-13-myristate-12-acetate (PMA), IL-2, or both and in the presence or absence of gangliosides. PMA (1-20 nM) stimulated CT6 proliferation in IL-2-free media, but mitogenesis was 40% that induced by IL-2. Mixed brain gangliosides inhibited PMA-induced growth in a dose-dependent fashion, and the I_{50} for IL-2 and PMA-induced growth were similar. Polysialogangliosides and ganglio series glycolipids were potent inhibitors, while other neutral glycolipids were ineffective. PMA inhibited IL-2-induced proliferation, and ganglioside and PMA inhibition of IL-2-induced growth was additive. To examine ganglioside modulation of other protein kinase C-mediated T-cell events, the influence of gangliosides on induction of the IL-2 receptor in JURKAT acute T-lymphocytic leukemia cells treated with PMA was studied. Receptor expression, measured by binding of ^3H-anti-Tac antibody, was depressed in a dose-dependent manner by mixed brain gangliosides, with no effect on cell viability. To examine the potential for direct ganglioside modulation of PKC as a mechanism for these effects, the influence of gangliosides on the binding of ^3H-phorbol-12,13-dibutyrate to JURKAT cells was tested. A dose and time-dependent inhibition of binding was observed when cells were pretreated with gangliosides, whereas slight inhibition was observed if gangliosides were present only in the binding assay. These studies indicate that cellular events linked to activation of protein kinase C are particularly sensitive to the inhibitory effects of gangliosides and suggest this enzyme is a primary target for ganglioside modulation.

188 THE DISTRIBUTION OF THE MULTIFUNCTIONAL CALMODULIN-DEPENDENT PROTEIN KINASE IN VARIOUS TISSUES AND THE SUBSTRATE FOR THE BRAIN ENZYME.

E. Miyamoto, K. Fukunaga, H. Yamamoto, E. Tanaka and T. Iwasa.
Department of Pharmacology, Kumamoto University Medical School, Kumamoto 860, JAPAN.

We have purified a multifunctional calmodulin-dependent protein kinase (CaM-kinase) from the brain which has a high molecular weight of about 560,000 (J. Neurochem. 39, 1607-1617, 1982). In contrast to muscle myosin light chain kinase, the enzyme had the multiple substrate specificity.

We have prepared hybridoma cells that produce monoclonal antibody against CaM-kinase from rat brain. The monoclonal antibody was of the IgM class of immunoglobulins and crossreacted with a subunit of the enzyme with Mr. 49,000, but not with a subunit with Mr. 60,000. There was no cross-reaction with the Ca^{2+}, calmodulin-dependent phosphodiesterase, calcineurin and other calmodulin-binding proteins from the brain. The analysis of various tissues by immunoblotting showed the presence of immunoreactive proteins with Mr. 52,000 in the spleen, stomach, pancreas, lung and adipose tissue and with Mr. 55,000 in the skeletal muscle and heart.

The endogenous substrate in the brain was examined. We found that CaM-kinase could phosphorylate myosin isolated from the brain. The phosphorylated proteins were two light chains and the heavy chain of myosin. Only the seryl residue in the light chains and heavy chain was phosphorylated. The phosphorylation of brain myosin by the enzyme led to an increase in actin-activated Mg-ATPase activity. The results suggest that brain myosin is regulated by brain CaM-kinase in a similar manner to smooth muscle except for the phosphorylation of the heavy chain.

189 MONOCLONAL ANTIBODIES TO PROTEIN KINASE C

*D. Mochly-Rosen and +D.E. Koshland, Jr.,
*University of California, San Francisco, Department of Neurology
+University of California, Berkeley, Department of Biochemistry

The phospholipid and calcium dependent protein kinase C autophosphorylates both on the catalytic and the regulatory domains. The autophosphorylation increases the activity of the enzyme by changing the Km for its protein substrates. We have prepared monoclonal antibodies against the purified enzyme which inhibit the activity of protein kinase C. One monoclonal antibody inhibits both the intact enzyme and a catalytic fragment obtained by limited proteolysis. The activity of this catalytic fragment is independent of phospholipids and calcium and the fragment can no longer autophosphorylate. Another monoclonal antibody is an effective inhibitor of only the intact enzyme. A third antibody inhibits the autophosphorylation of the enzyme, but increases the phosphorylation activity of the enzyme towards its protein substrate. Therefore these antibodies can be used as markers for the various domains of the enzyme.

190 NERVE GROWTH FACTOR (NGF) OR FORSKOLIN REDUCES Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE III (CaM KINASE III) ACTIVITY IN CELL-FREE EXTRACTS OF PC 12 CELLS.

A.C.Nairn, R.A.Nichols, & H.C. Palfrey*, Lab. of Molec. & Cell. Neurosci., Rockefeller Univ., New York, NY 10021, and *Dept. of Pharmacol. & Physiol. Sci., Univ. Chicago, Chicago, Il 60637.

CaM kinase III phosphorylates a protein of Mr 100,000 (100k), a major substrate for CaM-dependent protein phosphorylation found in many mammalian tissues and cell lines (Nairn et al, PNAS 82, 7939 1985). A number of recent studies have shown that treatment of PC 12 cells with NGF or forskolin leads to a decrease in the CaM-dependent phosphorylation of 100k in cytosolic extracts. The effect of NGF on the phosphorylation of 100k was observed after 1 hour, the effect was maximal (30% of control) after 12 hours, and the effect began to reverse after 24 hours. The effect of forskolin was more rapid, the maximal effect was greater (5-10% of control), and the effect did not reverse unless forskolin was removed. Studies in which purified 100k and catalytic levels of cell extracts were used suggest that the effects of NGF and forskolin was to reduce the activity of CaM kinase III in cell extracts. The levels of 100k was not changed by these treatments. Addition of cell extracts to partially purified CaM kinase III did not affect its activity, suggesting the absence of an inhibitor. In addition, the effect of NGF or forskolin on CaM kinase III was stable following extraction of the enzyme from PC 12 cells. These results suggest, therefore, that the effect of NGF or forskolin is to lower the total amount of CaM kinase III per se, not to alter its activity. Similar results were obtained with NG108-15 and NCB20 cells, two other neural cell lines. (Supported by AM-20595 (HCP)).

191 GONADOTROPIN RELEASING HORMONE INDUCES THE PHOSPHORYLATION OF SPECIFIC PITUITARY PROTEINS: DIRECT INVOLVEMENT OF PROTEIN KINASE C. John Nestor, Jr. and Berta Strulovici, Institutes of Bioorganic Chemistry and Biological Sciences, Syntex Research, Palo Alto, CA 94043.

The object of the present study was to demonstrate the involvement of protein kinase C (PKC) in the signal transduction of Gn-RH action by a) testing the ability of potent Gn-RH analogs to induce selective phosphorylation of protein substrates in intact rat pituitary cells, and b) quantitating the number of [^{3}H] PDBu binding sites in the cytosol and particulate fraction of pituitary cells upon stimulation with Gn-RH agonists. Exposure of ^{32}P-labeled rat pituitary cells to PMA (10^{-7}M) or diC$_8$ ($5x10^{-5}$M) or Gn-RH (10^{-7}M) or a "superagonist"-RS 94991, followed by SDS-Page and autoradiography, induced phosphorylation of a 42-kDa, a 37-kDa, 11-kDa and 10-kDa proteins. Preincubation with potent Gn-RH antagonists (RS 29226 or RS 68439) for one hour followed by Gn-RH (10 min, 10^{-8}M) prevented the Gn-RH effect in a dose dependent manner, while leaving the effect of PMA or diC$_8$ intact. "Down-regulation" of PKC or preincubation with H-7, a PKC inhibitor, prevented the effect of both groups of agents (Gn-RH or PMA), suggesting that the phosphorylation induced by either agent is mediated via PKC either directly (PMA or diC$_8$) or through endogenous production of diacylglycerols (Gn-RH and RS 94991). By assessing the ability of various Gn-RH analogs to promote the translocation of PKC from the cytosol to the particulate fraction of the pituitary cells, the following rank of potency was observed: RS 94991 > Gn-RH > [Gly^2] LH-RH > [His^2] LH-RH = control. Taken together, these data suggest a direct correlation between the extent of translocation of PKC to the particulate form, phosphorylation of cellular substrates and ultimately LH/FSH secretion.

192 DIRECT STIMULATION OF PROTEIN KINASE C BY ARACHIDONATE IS ASSOCIATED WITH PLATELET ACTIVATION. Masakatsu Nishikawa., *Hiroyoshi Hidaka., **Robert S. Adelstein and Shigeru Shirakawa Dept. of Internal Med.II and *Dept. of Pharmacol.,Mie University School of Med. 2-174 Edobashi Tsu Mie 514 JAPAN & **NHLBI,NIH Bethesda MD 20892

Arachidonate (AA) and other unsaturated fatty acids stimulated protein kinase C in a dose-dependent manner(5-50 ug/ml), when protein C was assayed with either histone H-1 or the 20,000-dalton myosin light chain. Arachidic acid and methyl arachidonate did not stimulate protein kinase C. AA also stimulated the endogenous phosphorylation of 40,000-dalton and 20,000-dalton proteins in the cytosol (100,000g supernatant) of human platelets. The addition of H-7, an inhibitor of protein kinase C, inhibited AA-induced phosphorylation of these proteins. When human platelets were activated by AA, a biphasic pattern of platelet aggregation and secretion was observed corresponding to the dose of AA and these responses were closely associated with the phosphorylation of the 40,000 and 20,000-dalton proteins. Platelet activation induced by the low levels of AA(0.1-5ug/ml) was inhibited by aspirin, whereas that induced by high levels of AA(10-50ug/ml) was not inhibited. Other unsaturated fatty acids at the same concentration also induced platelet aggregation which was not inhibited by aspirin. Arachidic acid and methyl arachidonate did not induce platelet responses. These results suggest that the mechanism of platelet activation by high levels of AA is different from that induced by low levels of AA and that the ability of AA to directly activate protein kinase C may account for the activation of platelets by high levels of AA.

193 EXISTENCE OF THREE ISOZYMES OF PROTEIN KINASE C WITH CLOSELY RELATED PRIMARY STRUCTURES.

Shigeo OHNO, Masaki INAGAKI*, Hiroshi KAWASAKI, Shinobu IMAJOH, Kyoko KUBO, Hisayuki YOKOKURA*, Takashi SAKOH*, Hiroyoshi HIDAKA* and Koichi SUZUKI.
Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113 and *Department of Pharmacology, Mie University School of Medicine, Edobashi, Tsu, Mie 514, Japan

To clarify the roles of protein kinase C in the signal transduction and cell proliferation, we determined its primary structure by protein sequencing and cDNA cloning.

Protein kinase C was purified to homogeneity as reported by Inagaki et al. (J. Biol. Chem., 260, 2292, 1985). Peptides produced by digestion with lysylendopeptidase were separated and sequenced. cDNA clones were isolated from rabbit cDNA libraries with oligonucleotide probes synthesized on the basis of the peptide sequences. The isolated cDNA clones were classified into three types, each of which encodes a protein with closely related primary structures. The amino acid sequences of peptides obtained from the "purified" enzyme can be explained by assuming that the preparation is a mixture of these isozymes.

Existence of multiple protein kinase C species raises the possibility that these isozymes share different functional roles in signal transduction and cell proliferation.

194 DIFFERENT Ca^{2+} REQUIREMENT FOR THE PHOSPHORYLATION OF H_1 HISTONE AND ENDOGENOUS PROTEINS BY THYROID PROTEIN KINASE C.

B. Omri, M.F. Breton and M. Pavlovic-Hournac. Unité 96 INSERM - 94275 Le Kremlin-Bicêtre Cedex (F).

Thyroid protein kinase C (PKc) from cytosols of porcine and rat glands was characterized by using histone H_1 or endogenous proteins as substrates. Enzyme from both species had similar kinetic and chromatographic properties and phosphorylated the same substrates (18 KDa, 22-25 KDa and 32-36 KDa proteins). However, it was found that Ca^{2+} requirement for the activation of the PKc greatly differed, depending whether H_1 or endogenous proteins served as substrates. While for the phosphorylation of H_1 calcium was absolutely necessary ; its addition was not indispensable when endogenous proteins served as substrates. In the latter case, almost the same rate of phosphorylation was obtained with and without 0.2 mM Ca^{2+} in the incubation medium. However, the one dimensional PAGE revealed, that patterns of labelled proteins were different in essays with and without calcium. In the presence of Ca^{2+}, modifications were observed in the zone of 32-36 KDa proteins. However, calcium alone without phospholipids, did not have any effect on the phosphorylation pattern of these proteins.

When proteins, phosphorylated only when Ca^{2+} was present, were resolved by the two dimensional PAGE, at least 5 additional spots different from those labelled in the absence of calcium, were noted in the region of 32-36 KDa ; their Pi range was from 5-7.5.

In conclusion, a very low concentration of Ca^{2+}, if any, was sufficient for the activation of the PKc and phosphorylation of a large number of endogenous substrates. Higher Ca^{2+} concentrations induced the phosphorylation of additional proteins. It could be that modulations of Ca^{2+} concentration play a role in the selection of protein substrates that are phosphorylated by the PKc.

195 RAPID ACTIVATION OF CALMODULIN-KINASE III IN MITOGEN-STIMULATED HUMAN FIBROBLASTS.

H.C. Palfrey, A.C. Nairn*, J. Kuchibhotla, L.L. Muldoon and M. Villereal. Dept. of Pharmacol. & Physiol. Sci., Univ. of Chicago, Chicago, IL 60637 and *Rockefeller Univ., N.Y., N.Y. 10029.

We recently characterized (Nairn et al., PNAS, 82, 7939, 1985) a novel calmodulin-dependent protein kinase (CaM-PK III) in mammalian tissues that preferentially phosphorylates an abundant M_r 100,000 cytosolic protein (100K). Polyclonal antibodies have now been used to precipitate 100K from prelabelled quiescent human fibroblasts stimulated with a variety of growth factors and other agents. Human lung (WI38) or foreskin (HSWP) fibroblasts were serum-deprived for 4-36h prior to labelling for 1h in P_i-free medium with 0.2 mCi/ml $^{32}P_i$. Mitogens were then added for various times, cells solubilized with detergent and 100K immunoprecipitated. ^{32}P incorporation into 100K was quantitated by SDS-PAGE and counting of gel slices. Serum (10%) led to a 5-10 fold increase in 100K labelling within 1 min but by 5 min ^{32}P incorporation had returned to baseline. Similar effects were obtained with lys-bradykinin or vasopressin (100nM). Extracellular Ca was not required for these responses, consistent with previous findings that peptide mitogens activate the PI cycle to produce IP_3 and release of Ca from intracellular stores. The time course of 100K phosphorylation induced by peptide mitogens agreed well with the transient rise in free intracellular Ca (determined by fura-2 fluorescence) caused by the same agents. The Ca and CaM antagonists TFP (50uM), TMB-8 (50uM) and W-13 (100uM) all reduced growth factor effects on 100K phosphorylation. A23187 (10uM) led to a slower increase in 100K phosphorylation that was dependent on extracellular Ca. These results show that mitogenic stimulation of quiescent fibroblasts leads to a rapid activation of CaM-PK III and phosphorylation of its major substrate. (Supported by AM-20595 (HCP and MLV) and GM-28359 (MLV).

196 GOLD COMPOUNDS MAY ALTER TRANSDUCTION OF INFLAMMATORY SIGNALS AT THE LEVEL OF PROTEIN KINASE C.

J.E. Parente*, M.D. Tyers¶, C.B. Harley¶, P. Davis* and K. Wong*

Gold compounds such as auranofin (AUR) and gold sodium thiomalate (GST) are used therapeutically to treat rheumatoid arthritis however the mode of action of these drugs in the remission of disease symptoms remains unknown. Previous in vitro studies have shown that human neutrophil responses elicited by phorbol esters and chemotactic tripeptides are inhibited by AUR and less potently by GST. Since activation of protein kinase C (PKC) occurs in response to both agonists, the effects of AUR and GST on PKC were investigated. Cell-free incubations showed that AUR did not influence the activity of PKC isolated from the cytosol of resting cells. When intact neutrophils were incubated with 7.6 - 20.3 uM AUR, cytosolic PKC activity decreased in a time- and dose-dependent manner so that by 45 minutes PKC activity had dropped by 10 - 75 %. Membrane-associated PKC activity from drug treated cells was concomitantly elevated although the amount recovered could not account for that lost from the cytosol. GST (5.0 uM - 0.5 mM) displayed similar effects on PKC as AUR but to a lesser extent. Pretreatment of neutrophils with the gold compounds decreased the phorbol ester-induced accumulation of PKC activity in the membrane fraction to a level intermediate between that for cells stimulated with phorbol ester and either AUR or GST alone. Antiarthritic gold compounds appear to alter signal transduction at the level of PKC, possibly by increasing the affinity of the kinase for the plasma membrane without causing cellular activation.

Supported by the Alberta Heritage Foundation for Medical Research, the Canadian Arthritis Society and the Medical Research Council of Canada. *Department of Medicine, University of Alberta, Edmonton, Alberta, ¶Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada.

197 MULTI-SITE PHORBOL ESTER REGULATION OF GH_3 CELL ADENYLATE CYCLASE

Lawrence A. Quilliam, Pauline R.M. Dobson and B.L. Brown, Department of Human Metabolism & Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, UK.

We have recently demonstrated that a phorbol ester (PDBu) stimulates cyclic AMP production in GH_3 pituitary tumour cells, even after pretreatment with cholera or pertussis toxins (1). We now report that this effect persists in GH_3 cell membrane preparations and is enhanced by a cytosolic factor which can be translocated to the membrane by preincubation of cells with PDBu.

In addition to this stimulatory effect, PDBu attentuated VIP-stimulated cyclic AMP production in intact cells (1). We now show that in addition to decreasing the maximal VIP response, PDBu causes a small right shift in the VIP dose response curve. These observations cannot be repeated in broken cell preparations and preliminary studies suggest that maximum VIP-receptor binding is not affected by PDBu.

After preincubation of cells with oxotremorine (oxo) which inhibits adenylate cyclase via R_i receptors, PDBu caused a larger fold increase in cAMP production (4.8 $\pm$ 0.9 fold) than in the absence of oxo (2.2 $\pm$ 0.5 fold). Also, in contrast to the previously reported attenuation (1), PDBu was synergistic with VIP in stimulating cyclic AMP production after oxo treatment. This suggests that as well as stimulating basal and attenuating VIP stimulated adenylate cyclase in a pertussis toxin resistant fashion, PDBu can also resist inhibition of the enzyme through N_i. C-kinase mediated phosphorylation of N_s could explain all 3 observations, however, these effects may be regulated individually.

(1) LA Quilliam et al (1985) BBRC 129 898-903.

198 PHOSPHORYLATION-DEPHOSPHORYLATION OF A SYNAPTOSOMAL PROTEIN OF M_R 96,000 (P96).

P.J. Robinson and W. Lovenberg. Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, OH 45215.

When intact synaptosomes from rat brain are incubated with $^{32}P_i$, a protein of molecular mass 96,000 (termed P96) becomes labeled. Upon depolarization with 41 mM K^+ P96 is rapidly and extensively dephosphorylated. By contrast, depolarization stimulates the phosphorylation of synapsin I by activation of calmodulin-dependent protein kinase, and of an "87kDa" synaptosomal protein by activation of protein kinase C (PKC). Dephosphorylation of P96 was fully reversible when the increased K^+ was returned to 4.7 mM, and a total of 5 cycles of phosphorylation-dephosphorylation were achieved. Dephosphorylation of P96 was dependent on extracellular calcium, but when intracellular calcium was raised, without activating the calcium channel, dephosphorylation did not occur. Thus P96 dephosphorylation was not dependent simply on a rise in intracellular calcium, but also on the mechanism of calcium entry. The phosphorylation of P96 was regulated by cyclic nucleotides and calcium. In intact synaptosomes dibutyryl cAMP increased P96 phosphorylation. The increase was not mediated by calcium-dependent protein kinases since stimulation also occurred in the presence of fluphenazine, which inhibits these enzymes. The primary protein kinase that phosphorylates P96 appears to be PKC. In lysed synaptosomes labeled with [γ-^{32}P]ATP a 96,000 dalton protein was phosphorylated only in the presence of calcium and phosphatidylserine. Phosphopeptide mapping of this protein and of P96 from intact synaptosomes demonstrated that the two proteins are likely to be the same. P96 may play an important role in nerve terminal function.

199 A NOVEL INHIBITOR OF MLCK, ML-9, PRODUCES VASCULAR RELAXATION

M. Saitoh and H. Hidaka, Dept. of Pharmacology, Mie University School of Medicine, Mie 514, Japan.

Two types of Ca^{2+}-dependent protein phosphorylation are established major general mechanisms by which intracellular events are controlled by external stimuli. One is catalyzed by Ca^{2+}-calmodulin dependent protein kinase, myosin light chain kinase (MLCK). The other species of Ca^{2+}-dependent protein kinase is Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C). Recently we synthesized a potent and selective inhibitor of protein kinase C, referred to as H-7, and investigated the role of protein kinase C by using H-7 in many tissues. The phosphorylation of the regulatory light chain of myosin catalyzed by MLCK is obligatory for the stimulation of myosin ATPase in both smooth muscle and nonmuscle tissues, a prerequisite for tension development. "Calmodulin antagonists" have been used as pharmacological tools to elucidate the function of Ca^{2+}-calmodulin dependent MLCK in vitro and in vivo by inactivating calmodulin. However, no compound is available, which directly inhibit the catalytic activity of the enzyme not via calmodulin. 1-(5-chloro-naphthalenesulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), which is a newly synthesized compound, was found to exhibit the same Ki value for both Ca^{2+}-calmodulin dependent and independent activities of MLCK, and inhibit the other protein kinases such as protein kinase C with the Ki values of at least an order of magnitude higher than for MLCK. Kinetic analysis revealed that ML-9 was a competitive inhibitor vs. ATP and noncompetitive inhibitor vs. MLC in the catalytic reaction. These results suggest that ML-9 bind at the active center of MLCK resulting in inhibition of the catalytic activity of MLCK. Potent vaso-relaxing effect of ML-9 on rabbit vascular strips as well as saponin treated skinned vascular fibre was diacussed in the relation to the in vivo inhibition of MLCK by ML-9.

200 MENADIONE ALTERS FUNCTIONAL RESPONSE AND PROTEIN PHOSPHORYLATION EVENTS IN RESTING AND STIMULATED HUMAN NEUTROPHILS. M. P. Shakarjian and R. A. Carchman. Department of Pharmacology and Toxicology, Medical College of Virginia/VCU, Richmond, VA 23298.

Menadione, a derivative of the natural vitamins K, has antineoplastic and antiinflammatory properties. Its profound biochemical effects upon resting and stimulated neutrophils make these cells a likely target for several of its purported therapeutic actions. We found that superoxide dismutase-inhibitable O_2^- production, a major contributor to leukocyte-mediated inflammation, was enhanced in resting neutrophils by menadione, as a function of cell number, time and drug concentration. Conversely, in phorbol myristate acetate (PMA) stimulated neutrophils, menadione significantly reduced the rate and magnitude of O_2^- generation. The release of O_2^- induced by formylated peptide (fmlp) and calcium ionophore were also inhibited in concentration dependent manner by menadione. As protein phosphorylation may represent a common event preceeding the functional response produced by these stimuli, the effect of menadione on this parameter was also examined. In experiments with whole cell suspensions of human neutrophils, menadione produced a low level but concentration related increase in ^{32}P-phosphate incorporation into endogenous protein substrates with apparent molecular weights of 58K and 62K. Moreover, menadione enhanced protein phosphorylation in neutrophils stimulated by PMA, fmlp, or ionophore. These results further implicate the role of protein phosphorylation as possible loci of the interaction of menadione with the signal transduction pathways in the neutrophil. (Supported by NIEHS grant Nos. ES03572 and T 32 ES07087.)

201 S6 KINASE ACTIVITY OF ASTROCYTES STIMULATED BY INSULIN, IGF1, AND PHORBOL ESTER.

D. Toru-Delbauffe, M. Pierre, J.M. Gavaret, M. Pomerance, & C. Jacquemin. INSERM U.96 - 78, rue du Général Leclerc. 94275 KREMLIN BICETRE Cedex (France).

Treatment of cultured astrocytes from two day old rat cerebral hemispheres, with insulin or IGF1 leads to a rapid activation of a cytosolic protein kinase activity which phosphorylates the ribosomal protein S6. This activation is observed as early as five minutes after insulin addition to the culture medium and it is maximal by 60 minutes. Concentration-response curve shows an activation increasing from 10^{-9} M to a maximal value at 10^{-7} M of insulin. In cytosolic extracts from cells treated with insulin, the cyclic AMP dependent protein kinase activity, protein kinase C activity and protein kinase activities tested on histones H2b or phosvitin are not modified. The S6 kinase activity promoted by insulin treatment is separated from main cytosolic protein kinases by ion exchange chromatography.

Exposure of astrocytes to tumour promoter phorbol ester (TPA 10^{-7} M) also produces a rapid increase of the S6 kinase activity in the cytosolic fraction. Simultaneously protein kinase C activity disappears from the cytosol in which cyclic AMP dependent protein kinase and phosvitin kinase activities are not affected.

These effects of insulin, IGF1 and TPA are also observed in the presence of cycloheximide. These data indicate that S6 kinase activity in astrocytes is promoted from a preexisting molecule, probably via the tyrosine kinase-insulin receptor and/or protein kinase C.

202 THREE DISTINCT FORMS OF TYPE 2A PROTEIN PHOSPHATASE IN HUMAN ERYTHROCYTE CYTOSOL.

Hirofumi Usui, Michinori Imazu, Kouji Maeta, Haruhisa Tsukamoto, Kazuyoshi Azuma, Masao Takeda, Department of Biochemistry, Hiroshima University School of Medicine, Kasumi, Minami-ku, Hiroshima 734, Japan.

Three distinct forms (I, III, IV) of type 2A protein phosphatase in human erythrocytes were purified to near homogeneity, as judged by polyacrylamide gel electrophoresis (PAGE). The apparent molecular weights of I, III and IV were estimated from Stokes radii and $s_{20,w}$ values to be 180,000, 177,000 and 104,000 respectively. From the molecular weights and intensities of the Coomassie blue-stained bands which were separated by sodium dodesyl sulfate-PAGE (Laemmli), subunit structures were determined to be $\alpha_1\beta_1\delta_1$ for I, $\alpha_1\beta_1\gamma_1$ for III and $\alpha_1\beta_1$ for IV. α, β, γ and δ migrated as 34kDa, 63kDa, 53kDa and 74kDa bands respectively. The treatment with 80% ethanol converted I, III and IV into M_r = 31,000 forms (34kDa bands) which had essentially the same physical and catalytic properties, and heparin-Sepharose column chromatography converted more than 90% of I and 20~30% of III to lower molecular weight forms which were indistinguishable from IV ($\alpha_1\beta_1$) in physical and catalytic properties, indicating that I, III and IV have a common subunit structure of $\alpha_1\beta_1$. Specific activity (units/mg) of $\alpha_1\beta_1\delta_1$, $\alpha_1\beta_1\gamma_1$, $\alpha_1\beta_1$ and α were 400, 5000, 1500 and 6500 respectively, with P-H2B histone as substrate, and were 50, 50, 300 and 1000 respectively, with phosphorylase *a* as substrate. From these values, it was suggested that β suppressed these phosphatase activities of α, and γ stimulated P-H2B histone phosphatase activity of $\alpha_1\beta_1$ but inhibited the phosphorylase phosphatase activity. δ was assumed to suppress strongly these phosphatase activities of $\alpha_1\beta_1$. The molar ratio of I, III and IV in human erythrocyte cytosol was estimated to be 7:1:13.

203 WhAT IS THE FUNCTION OF N-DOMAIN THE STRUCTURE OF WHICH IS IDENTICAL AMONG METAZOAN CALMODULINS? Koichi Yagi Department of Chemistry, Hokkaido Universiy, Sapporo, JAPAN

Calmodulins have been isolated from various vertebrates, invertebrates, fungi and plants. The primary structures from N-terminal to glycine at 98th position are identical among the metazoan calmodulins. According to the ^{1}H-NMR study of calmodulin, N-domain and C-domain after separation by trypsin maintained an identical conformation as those in the mother protein, respectively. It indicates that no interaction between the two domains exists in calmodulin. Ca^{2+} exchange rates were estimated to be about 300-500 sec^{-1} in the N-domain and 3-10 sec^{-1} in the C-domain. Ca^{2+} binding were measured using flow-dialysis method. The two low affinity sites are located in N-domain and the two high affinity sites in C-domain. The two mol of Ca^{2+} binding to each domain were positively cooperative, but no interaction was observed between the Ca^{2+} binding of two domains. Therefore, the two domains behave independently. Because of their different Ca^{2+} binding properties, the two domains are expected to take different parts in receiving Ca^{2+} signals. Our results suggest that Ca^{2+} first binds to the C-domain and the Ca^{2+}-saturated C-domain anchors it to the target proteins. N-domain then quickly respond to a small change in the Ca^{2+} concentration, working directly in the Ca^{2+} regulation. Although it is not yet clear why the primary structures of N-domain are maintained strictly to be the same, it must be needed to fulfill the transfer of Ca^{2+}-carrying information precisely in Metazoa. It can also be speculated that a common structure to accept N-domain is present in many target proteins.

204 IDENTIFICATION OF THE ATP.Mg-DEPENDENT PROTEIN PHOSPHATASE ACTIVATOR (F_A) AS A MYELIN BASIC PROTEIN KINASE IN THE BRAIN. Shiaw-Der Yang, National Tsing Hua University, Hsinchu, Taiwan, Republic of China.

The activating factor F_A of the ATP.Mg-dependent protein phosphatase F_CM was purified to near homogeneity from pig brain by a procedure involving chromatography on phosphocellulose, phosvitin-Sepharose 4B and Blue Sepharose CL 6B. A specific myelin basic protein (MBP) kinase was found to co-purify with F_A in constant ratio throughout purification. It also proved impossible to separate the two activities on non-denaturing gel electrophoresis and 5-20% sucrose density gradient ultracentrifugation. Kinetic study indicated that MBP, presumably a substrate for F_A, could compete with F_CM for F_A and thereby prevent the F_A-mediated activation of the F_CM activity. All the results taken together demonstrate that MBP kinase and F_A are localized on the same protein. This, together with the data that F_A, by activating the ATP.Mg-dependent phosphatase, promotes the dephosphorylation of MBP phosphorylated by F_A itself, suggests the evidence for a protein bearing two opposing activities involved in the regulation of brain functions. Moreover, since F_A was tightly associated with the purified brain myelin membrane, the results further support the notion that F_A may well be an endogenous protein kinase responsible for the cyclic phosphorylation-dephosphorylation of the central nervous system myelin.

204a IDENTIFICATION OF THE ATP.Mg-DEPENDENT PROTEIN PHOSPHATASE ACTIVATOR F_A AS A MYELIN BASIC PROTEIN KINASE IN THE BRAIN

Shiaw-Der Yang
Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan Republic of China

The activating factor F_A of the ATP.Mg-dependent protein phosphatase F_CM was purified to near homogeneity from pig brain. A specific myelin basic protein kinase was found to co-purify with F_A in constant ratio throughout purification. It also proved impossible to separate the two activities on native disc gel electrophoresis and on 5-20 % sucrose gradient ultracentrifugation. Kinetic study indicated that myelin basic protein, presumably a substrate for F_A, could compete with F_CM for F_A and thereby prevent the F_A-mediated activation of the F_CM activity. All the results taken together demonstrate that MBP kinase and F_A are localized on the same protein. This, together with the data that F_A, by activating the F_CM activity, promotes the dephosphorylation of MBP phosphorylated by F_A itself, suggests evidence for a protein bearing two opposing activities involved in the regulation of brain functions. Since F_A was tightly associated with the purified intact brain myelin membrane, the results further support the notion that F_A is an endogenous protein kinase responsible for the cyclic phosphorylation-dephosphorylation of the central nervous system myelin.

ACKNOWLEDGEMENTS - This work was supported in part by National Science Council in Taiwan, Republic of China.

205 PHOSPHORYLATION OF BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEX IN ISOLATED ADIPOCYTES
by S.J. Yeaman & S.M.A. Jones (Department of Biochemistry, University of Newcastle upon Tyne, U.K.)

Branched-chain 2-oxoacid dehydrogenase complex (BCOADC) is regulated by reversible phosphorylation. Phosphorylation, which causes inactivation of the complex, is catalysed by a specific kinase intrinsic to the complex. The kinase is inhibited in vitro by the 2-oxoacid substrates of the complex. We have found that the phosphorylation state of the complex in isolated rat adipocytes is also regulated by the concentration of branched-chain 2-oxoacids in the medium. Purified complex has also been demonstrated to oxidise 2-oxobutyrate and 4-methylthio-2-oxobutyrate with kinetic constants close to those for oxidation of the branched-chain 2-oxoacids. This implicates BCOADC in the catabolism of five essential amino acids, namely leucine, isoleucine, valine, threonine and methionine. Furthermore these additional substrates inhibit the kinase in the isolated adipocyte system, indicating that they may play a role in regulation of the activity of the complex.

206 ADP-RIBOSYLATION OF NON-MUSCLE ACTIN BY BOTULINUM C2 TOXIN:
K. Aktories, M. Bärmann, M. Laux, P. Presek, K.H. Reuner and B. Schering. Rudolf-Buchheim-Institut für Pharmakologie, D-6300 Giessen, F.R.G.

Botulinum C2 toxin (BtoxC2), which causes hypotonic effects, increase in vascular permeability and gut secretion, mono-ADP-ribosylates a 43 kDa protein in various cell types including human platelets, S49 lymphoma cells, neuroblastoma x glioma hybrid cells and chick embryo cells (CEC). We identified the toxin substrate protein as actin by means of peptide mapping, immunoprecipitation and immunoblotting. Half-maximal and maximal ADP-ribosylation of platelet actin was found at 0.1 and 3 µg/ml BtoxC2. The K_m value of the ADP ribosylation reaction for NAD was about 1 µM. The ADP-ribosylated actin bound to DNase sepharose matrix and was eluted by guanidine-HCl (3 M) or formamide (40 %). Purified platelet and liver G-actin and much less platelet and liver F-actin, but not skeletal muscle F- or G-actin were ADP-ribosylated by BtoxC2. Phalloidin blocked the toxin induced ADP-ribosylation. Toxin-catalyzed ADP-ribosylation of purified actin decreased the viscosity of polymerized actin and prevented the formation of the typical microfilament network. BtoxC2 pretreatment of intact cells largely reduced the ADP-ribosylation of the protein substrate in lysed cell preparations. Furthermore, in ^{32}P-loaded CEC, BtoxC2 induced the labelling of actin. Treatment of CEC with BtoxC2 caused cell roundings in a time and concentration dependent manner, which correlated with the toxin-catalyzed ADP-ribosylation of actin. The findings indicate that actin is the pathophysiological substrate of BtoxC2 and suggest a fundamental role of actin in the pathogenetical mechanism of BtoxC2. BtoxC2 appears to be an important tool to study physiological processes which involve non-muscle actin.

Supported by the Deutsche Forschungsgemeinschaft.

207 ENERGY UTILIZATION IN SIGNAL TRANSDUCTION. R. Dean Astumian and P.B. Chock, Laboratory of Biochemistry, NHLBI, NIH, Bethesda, MD 20892.

Inter- and intracellular communication require the expenditure of energy by a cell. For example, signal amplification basically means an increase in the amount of information available to, and hence reflects a decrease in the entropy of, the system. This can be accomplished only by the utilization of stored free energy such as that in ATP.

Here we analyze a number of proposed schemes for intracellular signal transduction in terms of the efficiency and effectiveness of energy usage. Criteria used were sensitivity, responsiveness, and gain. We find that cyclic cascade mechanisms present a significant advantage over simple allosteric control in a number of respects. Also, some schemes for allosteric regulation (e.g., "stamping" mechanism) are shown to contradict simple thermodynamic principles.

The key enzymes responsible for directly mediating communication between the cell and its environment (intercellular signal transduction) are transmembrane proteins. We discuss ways in which these enzymes can directly use energy contained in the membrane potential and in chemical gradients of various substances to effect transmembrane information transfer and signal amplification.

208 INTERRELATIONSHIPS OF CYCLIC AMP(cA) AND CALCIUM(Ca2+) IN SECRETAGOGUE INDUCED STIMULATION OF ACID SECRETION IN RAT PARIETAL CELLS ENRICHED BY CENTRIFUGAL ELUTRIATION. E.B. Black, S.J. Strada and N.J. Thompson, Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL 36688

Secretagogue stimulation of acid secretion and the possible interrelationships of Ca2+ and cA in the regulation of stimulus-secretion coupling has been studied in enriched parietal cell (PC) populations using a newly developed centrifugal elutriation procedure for rat gastric mucosal cell fractionation. The PC fraction is 60-80% pure as judged by light microscopy and FACS analysis and greater than 95% viabile as determined by trypan blue dye exclusion. Comparative studies of the rates of cA accumulation in PC using forskolin (F), isoproterenol (Iso) and histamine (H) and hydrogen ion production measured indirectly with 14C-aminopyrine accumulation (AP) show similar maximal secretory rates despite widely variant cA accumulation rates. The calmoldulin antagonists, fenoctimine (Fn) and trifluoperazine (TFP), inhibit AP induced by all three agonists. Both agents also inhibited carbachol (C) induced AP accumulation (Ki = 60 nM). Further studies utilizing the calcium sensitive, fluorescent indicators Quin 2 or Fura 2 show that C increases intracellular Ca++ from 930 nM to 2295 nM with a transient duration of 1.5 to 2 min. Studies of H action with Fura 2 indicate lesser involvement of Ca2+ than those of C whereas neither F nor Iso appear to modify Ca2+ levels in rat parietal cells. These results suggest a possible interrelationship of both Ca++ and cA in H,F and Iso and C induced acid secretion. This research is supported by USPHS grant 33538.

209 ADENYLATE CYCLASE AS A PROBE OF THE RELATIONSHIP BETWEEN THE EFFECTS OF ETHANOL ON THE PHYSICAL AND FUNCTIONAL PROPERTIES OF THE MEMBRANE. D.C. Bode and P.B. Molinoff, University of Pennsylvania, Philadelphia, PA 19104

The effects of ethanol on the physical and functional properties of the plasma membrane were investigated using membranes prepared from wild-type S49 lymphoma cells. The effects of ethanol on the physical properties of the membrane were assessed by measuring the fluorescence anisotropy of diphenylhexatriene (DPH). The β-adrenergic receptor-coupled adenylate cyclase system was used as a probe of the effects of ethanol on the functional properties of the membrane. The effects of ethanol in vitro included a decrease in fluorescence anisotropy (equivalent to an increase in membrane fluidity), an increase in adenylate cyclase activity, and a rightward shift in the dose-response curve for stimulation of adenylate cyclase activity by isoproterenol. The last of these effects was accounted for by a decrease in the affinity of the receptor for isoproterenol in the presence of ethanol. Following chronic exposure of cells to 50 mM ethanol for 4 days, adenylate cyclase activity was decreased. There were no differences, however, between membranes prepared from control and ethanol-treated cells in terms of the effects of ethanol in vitro. Anisotropy was lower in membranes prepared from ethanol-treated cells than in control membranes. There were no differences in the cholesterol:phospholipid ratio or in the fatty acid composition of the plasma membrane between control and ethanol-treated cells. There were small differences in the phospholipid composition, but these could not account for the difference in anisotropy between control and ethanol-treated cells. (Supported by USPHS AA 06215)

210 STIMULUS-SECRETION-SYNTHESIS COUPLING IN LACTOTROPHS AND CORTICOTROPHS OF RAT ANTERIOR PITUITARY: ROLE OF CALCIUM AND CYCLIC AMP. J.R. Dave, L.E. Eiden, D. Lozovsky, J.A. Waschek and R.L. Eskay. LCS, NIAAA, DICBR and LCB, NIMH, Bethesda, MD-20892.

Stimulus-secretion-synthesis are continuous events occurring in most secretory cells; however, the nature of coupling between these events in a number of anterior pituitary hormone secreting cells is not clear. The objective of this study was to determine the role of calcium and cyclic AMP in stimulus-mediated secretion and synthesis events of POMC-derived peptides and prolactin (PRL) in cultured rat anterior pituitary (AP) cells. Treatment of AP cells with a calcium channel blocker (D600, 10 uM) had no effect on beta-endorphin (BE) release and POMC mRNA levels. However, this treatment produced a 70% and 25% decrease in PRL release and mRNA levels, respectively. Incubation of AP cells in calcium-free media did not alter BE release, but decreased PRL release and POMC and PRL mRNA levels by approximately 50%. Treatment of AP cells with either D600 or calcium-free media partially blocked CRF- or forskolin (FN)-stimulated BE release and completely blocked FN-stimulated PRL release and PRL mRNA levels. The calcium agonist barium (1 mM) produced a two-fold increase in BE and PRL release, which was blocked by D600 and enhanced in the absence of calcium. Treatment of cells with barium had no effect on POMC mRNA levels but increased PRL mRNA levels. Our results document that i) in the absence of calcium, basal or stimulus-mediated PRL secretion and PRL mRNA levels are dramatically reduced, whereas, stimulated but not basal BE secretion is partially blocked, and ii) the effect of calcium-free media on PRL mRNA, but not on POMC mRNA levels, can be reversed by the addition of barium. These findings support previous observations and suggest that both calcium and cAMP may act as intracellular second messengers in lactotrophs, whereas of the two, only cAMP functions as an intracellular second messenger coupling stimulus-secretion-synthesis events in corticotrophs of the anterior pituitary gland.

211 CLONED cDNA FOR SPECIES OF RNA THAT ARE REGULATED BY CYCLIC AMP IN NG108-15 NEUROBLASTOMA-GLIOMA HYBRID CELLS.

M. Y. Giovanni, B. Raj, B.S. Schrier, and M. Nirenberg. NIH, Bethesda, MD.

Prolonged elevation of cAMP levels of NG108-15 cells shifts relatively undifferentiated cells to a differentiated state with respect to some neuronal properties and increases the ability of the cells to form synapses in vitro with muscle cells. A cDNA library, prepared from poly A^+ RNA obtained from differentiated NG108-15 cells treated with 1 mM dibutyryl cAMP for 5-7 days, was screened by differential colony hybridization using cDNA probes prepared from cytoplasmic poly A^+ RNA isolated from undifferentiated (D^-) logarithmically dividing cells or from differentiated (D^+) cells that had been treated with 1 mM dibutyryl cAMP for 5-7 days. Colonies with recombinant plasmids were found which hybridize to more D^+ ^{32}P-DNA probe than D^- ^{32}P-DNA. These colonies comprise < 1% of the colonies screened. Some colonies also were found with more D^- ^{32}P-DNA hybridized than D^+ ^{32}P-DNA. Cloned DNA in most colonies hybridized with approximately the same amounts of D^+ and D^- ^{32}P-DNA. Different concentrations of cytoplasmic poly A^+ RNA from D^+ or D^- cells were applied to filters and hybridized with cloned ^{32}P-cDNA probes from different recombinant plasmids. Approximately 20 clones were found with cDNA inserts that hybridize to species of poly A^+ RNA that increased in abundance 10-90 fold when NG108-15 cells were treated for prolonged periods with dibutyryl cAMP. These results show that some species of NG108-15 poly A^+ RNA increase in abundance when cells are treated with dibutyryl cAMP and that other species decrease in abundance.

212 STRUCTURE-FUNCTION ANALYSIS of THREE cAMP-INDEPENDENT FORMS of the cAMP RECEPTOR PROTEIN. James G. Harman, Keith McKenney and Alan Peterkofsky. NIH, Bethesda, MD.

cAMP receptor protein (CRP)-dependent operon expression in Escherichia coli requires the CRP:cAMP form of wild-type CRP. One class of crp mutants (crp*) activates CRP-dependent promoters in strains incapable of endogenous cAMP synthesis (cya). Of fundamental interest is the difference in regulatory properties exhibited by crp* mutant strains, some of which respond to glucose-mediated repression of β-galactosidase synthesis, some of which do not. To gain a better understanding of the mechanisms of cAMP-independent promoter activation and repression we have: 1) determined through cloning and DNA sequence analysis the primary structure of three CRP* forms of CRP; 2) purified the mutant proteins; 3) characterized the effect of mutations on the secondary structure of these forms of CRP and 4) studied CRP* activated lac promoter regulation in a purified in vitro transcription system. The results of this study show that crp alleles containing mutations that substitute a) threonine for alanine at position 144, b) isoleucine for threonine at position 127 and lysine for glutamine at position 170, or c) isoleucine for threonine at position 127, lysine for glutamine at position 170 and arginine for leucine at 195 encode proteins which, in the absence of cAMP, have conformations that are similar to the wild-type CRP:cAMP complex conformation and that promote cAMP-independent activation of a CRP-dependent promoter in vitro. In addition, a CRP allele-specific inhibition of CRP* activity was observed in vitro in reaction mixtures containing spermidine that parallels crp* strain-specific sensitivity to glucose-mediated repression of β-galactosidase synthesis in vivo. This suggests that the catabolite repression observed in crp* strains that lack cAMP may be mediated through a mechanism that results in an inhibition of CRP* activity.

213 DIFFERENTIAL REGULATION BY CALMODULIN OF BASAL, GTP- AND DOPAMINE-STIMULATED ADENYLATE CYCLASE ACTIVITIES IN BOVINE STRIATUM. Jeffrey Harrison, C.K. Mickevicius and M.E. Gnegy. Dept. Pharmacology, Univ. of Michigan Med. Sch., Ann Arbor, MI 48109

We have examined the concentration requirements for calmodulin (CaM) in altering basal, GTP- and dopamine (DA)-stimulated adenylate cyclase activities in an EGTA-washed particulate fraction from bovine striatum. CaM stimulated basal adenylate cyclase activity 3.5-fold with an EC_{50} of 100 nM. GTP stimulated basal activity 2-fold with an EC_{50} of 300 nM. In the presence of CaM, GTP was able to further activate adenylate cyclase. With increasing concentrations of CaM, however, the EC_{50} for GTP was decreased 5-fold. In the presence of only 90 nM CaM, the EC_{50} for GTP was maximally reduced from 300 nM to 60 nM. CaM could also increase the maximal activation of adenylate cyclase by the neurotransmitter, DA. Similarly, the concentration of CaM required to maximally increase the DA-stimulated activity was significantly less than the concentration required to maximally activate basal adenylate cyclase activity. The EC_{50} for CaM in increasing the GTP sensitivity and maximal response of the adenylate cyclase to DA is 13 nM as compared to an EC_{50} of 100 nM in activation of basal adenylate cyclase activity. These data support the concept of a dual regulatory role for CaM in the activation of adenylate cyclase activity. Supported by NIMH 36044.

214 Adenylate cyclase modulators regulate glucose transport in rat adipose cells by changing glucose transporter intrinsic activity H.G.Joost, T.M.Weber, S.W.Cushman, and I.A.Simpson, Experimental Diabetes, Metabolism and Nutrition Section, National Institutes of Health, Bethesda.

In isolated rat adipose cells, isoproterenol reduces insulin-stimulated glucose transport activity by 60%. Plasma membranes isolated from these cells after sequential treatment with 10 nM insulin (25 min), 0.5 uM isoproterenol plus 2.5 ug/ml adenosine deaminase (15 min), and 2 mM KCN (2 min) contain an unchanged concentration of glucose transporters (31 ± 7, mean ± SEM, vs. 31 ± 4 pmol/mg protein in controls), but transport glucose at a reduced rate (19 ± 6 vs. 48 ± 9 pmol/mg protein/sec). This finding contrasts with the effect of insulin which comprises parallel increases in plasma membrane glucose transporter concentration and transport activity. Kinetic studies of isoproterenol-inhibited glucose transport reveal a 60% decrease in V_{max} (2900 ± 350 vs. 7200 ± 1000 pmol/mg protein/sec) and a small increase in K_m (15.1 ± 1 vs. 13.0 ± 0.6 mM). Incubation of intact cells in the presence of the adenylate cyclase inhibitor adenosine stimulates plasma membrane glucose transport activity compared to that in the absence of adenosine (44 ± 6 vs. 36 ± 6 pmol/mg protein/sec). These data indicate that modifications of glucose transport activity produced by lipolytic and antilipolytic agents in intact adipose cells can be fully retained in plasma membranes isolated under appropriate conditions. Furthermore, the effects of these agents occur through a modification of the glucose transporter intrinsic activity.

215 INHIBITION OF VASCULAR TYPE IV PDE: PROBLEMS IN EVALUATING POTENCY OF INHIBITORS. T. KARIYA and R.C. DAGE, MERRELL DOW RESEARCH INSTITUTE, CINCINNATI, OH 45215, U.S.A.

During evaluation of newly synthesized compounds for cardiotonic activity, we have noted that some compounds produced relatively greater lowering of blood pressure for a given cardiotonic effect. These compounds inhibited dog cardiac Type IV cAMP phosphodiesterase (PDE III). It was of interest to us to determine if differences in sensitivity between cardiac and vascular Type IV PDE could explain our observations. Dog heart and femoral arteries were homogenized with a Polytron and the PDEs were fractionated into Types I, II and IV PDE by DEAE-cellulose chromatography. Inhibition studies were done on Type IV PDE using an inhibitor that is specific for this isoenzyme (enoximone) and a non-specific, competitive inhibitor of PDEs (isobutylmethylxanthine, IBMX). For dog cardiac Type IV PDE, the IC50 of both enoximone and IBMX was 4 μM at a substrate concentration of 0.5 μM cAMP. At the same substrate concentration, the IC50 of enoximone for vascular Type IV PDE varied greatly (5 μM - >100 μM) from preparation to preparation, whereas inhibition by IBMX was relatively uniform (4 μM - 7 μM). It was shown that differences in homogenization of the samples of vascular tissue resulted in the observed variability. Prolonged homogenization with the Polytron yielded preparations of Type IV PDE that were relatively insensitive to inhibition by enoximone.

216 CYCLIC AMP ACCUMULATION IN RABBIT AORTA SMOOTH MUSCLE CELLS ALTERED IN THE PRESENCE OF HYPERLIPIDEMIC SERUM

Yukio Kishi, Fujie Numano, Keiji Nishiyama, Michiyoshi Yajima and Fujio Numano

Third Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo, Japan

We investigated the effect of hyperlipidemic serum on cAMP accumulation in cultured smooth muscle cells from the rabbit aorta. The cells were grown to confluence, then cultured for 24 h in hyperlipidemic medium (total cholesterol: 2.2 mmol/l). CAMP accumulation was enhanced in response to isoproterenol 10^{-6} M, as compared to control cells, and this enhancement was still detectable in the presence of IBMX 10^{-3} M, a potent inhibitor of phosphodiesterase. Application of propranolol 10^{-4} M at 5 min after ispoproterenol showed a similar time course for cAMP disappearance. The phosphodiesterase activity in the 40,000 g supernatant of the Triton X-100 solubilized homogenates of the cells in hyperlipidemic medium remained unchanged. β-receptor assays showed an increased B_{max} with a similar Kd, and such may contribute, at least in part, to the increased adenylate cyclase activity. An extended incubation in the presence of hyperlipidemic medium attenuated the cAMP accumulation, possibly due to excessive increases in the total cholesterol content.

217 ROLE OF PROTEIN KINASE C (PKC) IN CELLULAR FUNCTIONS: INHIBITION BY SPHINGOID LONG-CHAIN BASES, J. D. Lambeth, E. Wilson, J. Kinkade, and A. H. Merrill, Jr.; Emory Univ. Med. Sch.; Atlanta, GA 30322. PKC has been implicated in growth and differentiation (eg. PMA-induced differentiation of HL-60 cells), secretion [eg. PMA-induced secretion of lactoferrin and vitamin B12-binding protein from neutrophils (PMN)], and PMA activation of the PMN oxidative burst. Long-chain bases (sphinganine and sphingosine) were recently found to be potent inhibitors of PKC in a micelle-reconstituted system [Hannun et al. (1986) J. Biol. Chem. in press]. We have tested long-chain bases as inhibitors of the above cellular functions and documented their utility in testing PKC-dependent vs. -independent processes. The following effects are described: (1) Long-chain bases block PMA-initiated differentiation of HL-60 into macrophage-like cells, but do not affect retinoate-induced differentiation. (2) Long-chain bases block activation of the oxidative burst not only by PMA, but also by other agonists (diacylglycerol, FMLP, A23187, arachidonate, and opsonized zymosan); inhibition occurs at the same concentration for all agonists, implying the same site of inhibition; sphinganine competition for [^{3}H]-phorbol dibutyrate binding to PMN strongly implicates PKC as the site of inhibition. (3) Neutrophil specific granule secretion appears to be regulated by PKC, while azurophilic granule secretion is not. Long-chain bases block secretion, of specific- but not azurophilic-granule markers. (4) Agonist-induced phosphorylations of various PMN and HL-60 proteins are blocked by sphinganine. In addition, these inhibitors appear to be specific; agonist-induced calcium transients, calmodulin-dependent processes, and cAMP-dependent processes were not affected. Thus, long-chain bases are naturally occurring inhibitors of PK-C which may function as physiologic negative regulators of this enzyme. [Supported by NIH grants AM27373, CA22294, and GM 33369].

218 ORGANELLE SYNESIS HYPOTHESIS OF SIGNAL TRANSDUCTION
A.N. Malviya, P. Anglard and A. Masmoudi

A protein phosphorylating activity, independent of calcium, has been separated from protein kinase-C (FEBS Lett. 199, 213-216, 1986). This activity has been termed as protein kinase-L and is manifested by phorbol ester (TPA) or diacylglycerol (DAG) in the presence of phospholipid. Protein kinase-L activity is reported here to be localized in the nuclei isolated from rat liver, calf thymus, and cultured chicken neurons. In all the three cell types studied protein kinase-L activity was abundantly present as contrasted from a negligible protein kinase-C activity. Protein kinase-L activity observed in rat liver nuclei seems distinct from the activity seen in nuclei isolated from calf thymus or cultured chicken neurons in its response towards TPA or DAG examined with or without phosphatidylserine. A strong prima-facie case is advanced for organelle synesis in signal transduction coupled to tumor promotion. In this hypothesis, for the trigger of signal transduction, catalytic potential of protein kinase-C, protein kinase-L, and IP_3 receptor seems best manifested at the site of plasma membrane, nucleus, and endoplasmic reticulum, respectively.

219 UBIQUITIN-ANTIBODIES BLOCK HIGH AFFINITY CHOLINE UPTAKE AT EXTRACELLULAR SITES IN RAT BRAIN SYNAPTOSOMES. E. M. Meyer, C. West and V. Chau, Depts. of Pharmacology, Biochemistry, Anatomy and Cell Biology, Univ. Florida School of Medicine, Gainesville, FL 32610

Sodium-dependent [^{3}H]-choline uptake and coupled [^{3}H]-acetylcholine synthesis were inhibited in rat cerebral cortical synaptosomes in a dose-(1-10 ug/ml) and time-dependent manner by affinity-purified antibodies directed against ubiquitin (anti-Ub). Neither sodium-independent [^{3}H]-choline uptake nor [^{3}H]-acetylcholine release was affected by up to 10 ug/ml anti-Ub, indicating that the cholinergic terminals were not depolarized by the anti-Ub. Binding of anti-Ub to the synaptosome preparation, as measured with [^{125}I]-protein A, was saturable and occurred over the same concentration range (1-10 ug/ml) at which uptake-inhibition was observed. Although preimmune IgG bound to the synaptosome preparation to a greater extent and was apparently not readily saturable, this fortuitous binding was without effect on high affinity choline uptake and conversion to acetylcholine. The results suggest that a ubiquitin-protein conjugate is closely associated, on the synaptosomal surface, with the sodium-dependent choline transport system.

220 CYTIDYLATE CYCLASE: EVIDENCE OF THE BIOSYNTHESIS OF CYTIDINE 3',5'-CYCLIC MONOPHOSPHATE AND OF FOUR NOVEL CYTIDINE CYCLIC PHOSPHATE DERIVATIVES

*R.P. Newton, +E.E. Kingston, *N.A. Hakeem and +J.H. Beynon
*Department of Biochemistry, School of Biological Sciences, and +Royal Society Research Unit, University College of Swansea, Swansea SA2 8PP, U.K.

Although the existence of phosphodiesterases capable of cyclic CMP hydrolysis is now established, controversy has existed over reports of endogenous cyclic CMP and of putative cytidylate cyclase activity. The natural occurrence of cyclic CMP, together with cyclic UMP, IMP and dTMP, has recently been unequivocally demonstrated by means of f.a.b. mass spectrometry with m.i.k.e.s. scanning. This technique, together with the use of dual labelled [^{14}C]- and [^{32}P]-CTP and h.p.l.c., t.l.c. and RIA, has enabled the identification of the eight labelled cytidine nucleotides resulting from the activity of cyclase preparations from brain, liver and kidney. The major component was the unused substrate, CTP, but the second major was 3',5'-cyclic CMP. In addition, CMP, CDP, cytidine 2'-aspartyl-3',5'-cyclic monophosphate, cytidine 2'-glutamyl-3',5'-cyclic monophosphate, cytidine 2'-monophosphate-3',5'-cyclic monophosphate and cytidine 3',5'-cyclic pyrophosphate were identified as minor components.

The presence of these four novel cytidine cyclic phosphate compounds may well be the cause of the conflicting reports concerning cytidylate cyclase assay and cyclic CMP-RIA; their identification has enabled the development of a cytidylate cyclase assay from which they are eliminated and a subsequent investigation of the properties of this enzyme.

221 UNAMBIGUOUS ASSAY OF CYTIDINE 3',5'-CYCLIC MONOPHOSPHATE AND CYTIDYLATE CYCLASE ACTIVITY

Russell P. Newton and Nabil A. Hakeem
Department of Biochemistry, School of Biological Sciences, University College of Swansea, Swansea SA2 8PP, U.K.

The structures of four novel cytidine cyclic phosphates have been elucidated. These labelled compounds are produced from [^{32}P]- or [^{14}C]-CTP during conventional assays for cytidine cyclase and are also immunoreactive with antisera raised against cyclic CMP. Knowledge of their structures has permitted development of (a) a single column composed of two separate tiers of ion-exchanger which separates cyclic CMP from these contaminants to form the basis of an assay for cytidylate cyclase, and (b), a single column composed of three tiers of ion-exchanger which separates cyclic CMP from these four novel compounds, from other cytidine nucleotides and from cyclic AMP and cyclic GMP, and is used as a separation stage prior to radioimmunoassay.

Cytidylate cyclase activity, distinct from adenylate and guanylate cyclase activity, was found in each tissue examined, the highest activity being in liver and kidney and the major subcellular site a 25000 x g fraction containing lysosomes, mitochondria and microsomes. This activity was elevated in regenerating tissue stimulated by testosterone and calmodulin and was inhibited by UTP and ITP. Cyclic CMP was found also in each mammalian tissue examined, usually within a concentration range of 0.1 to 40 pmol/g tissue. The highest concentrations were in the uterus and kidney and the lowest in brain, testis, ovary and plasma. These concentrations decreased with age but increased with increased cellular growth rate.

222 NEUROPEPTIDE BIOSYNTHESIS IN CHROMAFFIN AND NEUROBLASTOMA CELLS: REGULATION BY CYCLIC AMP, PROTEIN KINASE C AND CALCIUM. Rebecca M. Pruss, Lee E. Eiden and James A. Waschek. Laboratory of Cell Biology, NIMH, Bethesda, MD, 20892.

Stimulation of nicotinic receptors on the surface of chromaffin cells causes the calcium-dependent secretion of enkephalin peptides and vasoactive intestinal polypeptide (VIP) and is accompanied by a calcium-dependent increase in de novo biosynthesis of VIP and enkephalin, and proenkephalin mRNA. Peptide biosynthesis is also stimulated by agents which cause cell depolarization, such as elevated potassium and veratridine, and by the calcium agonist barium, indicating that calcium plays a second messenger, rather that merely a permissive role, in secretagogue stimulation of neuropeptide biosynthesis. Elevation of intracellular cyclic AMP also causes a stimulation of enkephalin and VIP synthesis in chromaffin cells, which is independent of extracellular calcium. Phorbol ester stimulates the biosynthesis of VIP, but not enkephalin, indicating a selective regulation of neuropeptide expression by two second messenger systems in chromaffin cells.

In human neuroblastoma cells in culture, synthesis of VIP and its messenger RNA is synergistically regulated by both cyclic AMP and phorbol ester. These cells do not exhibit a calcium-dependent release of VIP upon depolarization with elevated extracellular potassium, nor are they biosynthetically responsive to elevated potassium or barium.

These results indicate that the expression of the genes encoding proenkephalin A and VIP are differentially responsive to two protein kinase-stimulated pathways, and that biosynthesis is regulated by calcium influx in cells that secrete these peptides in a calcium-dependent way.

223

VANADATE POTENTIATES VASOPRESSIN-INDUCED INOSITOL PHOSPHATE TURNOVER.

N. Aiyar, P. Nambi, G. Gessner and S.T. Crooke, Mol. Pharm., SK&F Labs., Phila., Pa. 19101

Rat aortic smooth muscle cells (A-10) contain vasopressin receptors of V1 subtype. Incubation of these cells with vasopressin (AVP) resulted in an (a) inhibition of isoproterenol-stimulated cAMP accumulation and (b) stimulation of phosphatidylinositol turnover. In an attempt to delineate the mechanism of AVP action in these cells, we have used tumor promoting phorbol ester (activator of protein kinase C) to test its effect on AVP action. Pretreatment of the cells with active phorbol ester resulted in an inhibition of the AVP-mediated responses. Further binding studies with ^{3}H SK&F 101926, a novel vasopressin antagonist, suggested that these receptors are coupled to a guanine nucleotide binding protein and that pretreatment with phorbol ester resulted in the uncoupling of the receptor from the guanine nucleotide binding protein. Here we have used vanadate, an inhibitor of phosphatase to test its effects on AVP-mediated responses. Pretreatment of ^{3}H inositol-labeled cells with vanadate resulted in an increase of AVP-stimulated inositol di- and tris phosphates as much as twofold without significantly changing inositol monophosphate levels. Vanadate potentiated AVP response at all concentrations of hormone tested without affecting the half-maximal concentration of the hormone necessary for inositol phosphate accumulation. While in control cells, AVP-induced inositol phosphate accumulation was observed only in the presence of LiCl, in vanadate-treated cells significant increase in inositol di- and tris-phosphates was observed even in the absence of LiCl. Vanadate pretreatment had little effect on ^{3}H AVP binding to intact cells or on the AVP-induced inhibition of isoproterenol-stimulated cAMP accumulation. These data suggest that vanadate may be exerting its effect by inhibiting inositol di and tris phosphatases.

224 LIGAND-STIMULATED PRODUCTION, METABOLISM, AND RECEPTOR BINDING OF INOSITOL TRISPHOSPHATE DURING THE STEROIDOGENIC ACTION OF ANGIOTENSIN II. Tamas Balla, Gaetan Guillemette, Albert J. Baukal and Kevin J. Catt, NICHD, NIH, Bethesda, MD 20892

Angiotensin II (AII) exerts its steroidogenic effects through calcium-mobilizing receptors that promote the generation and actions of inositol trisphosphate (ins-P_3) via phospholipase-C-catalyzed breakdown of polyphosphoinositides. In rat adrenal glomerulosa cells labeled with [^{3}H]inositol, AII caused rapid (< 3 sec) breakdown of both PIP and PIP_2 with concomitant formation of inositol phosphates. In addition to rapid and prominent formation of ins-P_2, two isomers of both ins-P_3 and ins-P were detected by HPLC. Ins-1,4,5-P_3 showed only a slight and transient rise with a peak at about 5 sec, whereas ins-1,3,4-P_3 increased steadily over 60 sec. Significant increases in ins-P_2 and ins-4-P at 2.5 sec, with no change in ins-1-P during the first 60 sec, revealed that ins-1,4,5-P_3 is rapidly metabolized through ins-1,4-P_2 with accumulation of ins-4-P, and that PI breakdown is not an early response to AII. The progressive formation over 20 min of ins-1,3,4-P_3, ins-1,4-P_2, and ins-4-P, all enhanced in the presence of lithium, was indicative of continued breakdown of polyphosphoinositides. Specific and high affinity (Kd ∿ 1 nM) binding sites with low capacity (∿ 100 fmol/mg prot) for ins-1,4,5-P_3 were demonstrated in bovine adrenal cortex and shown to be distinct from the degradative enzyme ins-P_3-5'-phosphatase. The rapid kinetics of association and dissociation of ins-P_3 at these sites are consistent with its early and transient elevation during AII stimulation, and with its binding to receptors through which calcium is mobilized from intracellular storage pools. These results demonstrate that ins-1,4,5-P_3 formation and metabolism are early intermediate steps in the adrenal response to AII, and that ins-4-P production provides an index of sustained polyphosphoinositide hydrolysis during AII action in the glomerulosa cell.

225

INHIBITION OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C BY MANOALIDE.

C. Frank Bennett, Seymour Mong, Hsiao-Ling W. Wu and Stanley T. Crooke, Dept. of Mol. Pharm., Smith Kline and French Laboratories, Philadelphia, Pa. 19101.

Manoalide is a nonsteroidal sesterterpenoid isolated from sponge. Manoalide exhibits analgesic and anti-inflammatory activity in mouse models (Jacobs et al.,Tetrahedron 41:981). Investigations on the mechanism of action for manoalide revealed that it was a potent inhibitor of venom phospholipase A_2 (PLA_2), exhibiting IC_{50} values of 0.12, 0.7 and 1.9 μM for bee, rattlesnake and cobra venom PLA_2,respectively. However, intracellular PLA_2's assayed in cytosolic and membrane fractions from several mammalian tissues were less sensitive to inactivation by manoalide, exhibiting IC_{50} of 30 μM or greater. Cytosolic proteins, BSA, and poly-L-lysine protected purified bee venom PLA_2 from inactivation by manoalide, while free amino acids did not protect purified PLA_2. Investigations on other intracellular targets for manoalide revealed that PI-specific phospholipase C (PLC) was more sensitive to inactivation by manoalide than PLA_2(IC_{50}= 1.5 and 30 μM, respectively). A PI-specific PLC purified from guinea pig uterus (Mr = 64,000) also exhibited an IC_{50} of 1.5 μM. Manoalide inhibited, in a concentration dependent manner, the synthesis of inositol-1-phosphate from DDT_1MF-2 cells stimulated with norepinephrine (IC_{50} = 2.0 μM). These results suggest that the anti-inflammatory activity of manoalide may be due, in part, to inhibition of PI-specific PLC. These results also demonstrate, for the first time, the potent inhibitory activity of manoalide on phospholipase C isolated from guinea pig uterus.

226 Ca^{2+}-DEPENDENT REGULATION OF INOSITOL 1,4,5-TRISPHOSPHATE METABOLISM IN INSULIN SECRETING CELLS. T.J. Biden & C.B. Wollheim. Institut de Biochimie Clinique, Univ. of Geneva, Switzerland.

Inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) acts as a second messenger to mobilize intracellular Ca^{2+} stores during the muscarinic stimulation of insulin release. It is degraded by phosphomonoesterases which sequentially remove the phosphate groups. An alternative route has been proposed whereby Ins 1,4,5-P_3 is phosphorylated by a specific kinase to form inositol 1,3,4,5-tetrakisphosphate (Ins-P_4) which is in turn converted to Ins 1,3,4-P_3. Analysis of these metabolites by HPLC in insulin secreting RINm5F cells prelabelled with ^{3}H-inositol now shows that cholinergic stimulation in Ca^{2+}-depleted medium markedly increased the ratio of Ins 1,4,5-P_3 to Ins 1,3,4-P_3 + Ins-P_4, relative to cells stimulated in Ca^{2+} containing medium. Ins 1,4,5-P_3 kinase activity assayed in a cytosolic fraction was stimulated 2½ fold by raising Ca^{2+} from 10^{-7}-10^{-5}M (EC_{50} approx. 800nM). In contrast, 1,4,5-P_3-phosphomonoesterase activity was not Ca^{2+}-dependent. Moreover, the kinase displayed a K_m for Ins 1,4,5-P_3 of 1.5μM, rather than 30μM for the phosphomonoesterase. These results suggest that the rise in Ca^{2+} brought about by Ins 1,4,5-P_3 generation would in turn enhance its catabolism via the inositol tris/tetrakisphosphate pathway. In pancreatic islets Ca^{2+} also plays a role in the generation of Ins 1,4,5-P_3. Thus K^+ depolarization induced a Ca^{2+}-dependent, transient production of Ins 1,4,5-P_3 which was not secondary to neurotransmitter release. This Ca^{2+}-mediated effect played only a minor role following muscarinic stimulation of the islets, but was quantitatively important for the generation of Ins 1,4,5-P_3 by the nutrient stimulus glucose.

227 IDENTIFICATION OF A NUCLEOTIDE-SENSITIVE PHOSPHOLIPASE C IN LIVER PLASMA MEMBRANES. D. Bojanic, M.A. Wallace, R.J.H. Wojcikiewicz, L.S. Bradham and J.N. Fain. Dept. of Biochemistry, University of Tennessee, Memphis, TN 38163.

The hydrolysis of exogenous [^{3}H]phosphatidylinositol 4,5-bisphosphate ([^{3}H]PIP_2) to inositol phosphates by rat hepatic plasma membranes was investigated. In the absence of deoxycholate (DOC), phospholipase C activity was not readily detected. DOC produced a dose dependant increase in activity up to 2mM after which the rate of hydrolysis decreased. GppNHp (100μM) stimulated the DOC activated enzyme by 30 - 50% above basal. This stimulation was not specific for guanine nucleotides since AppNHp also stimulated the enzyme. GppNHp stimulation was only apparent with [^{3}H]PIP_2 as substrate; little or no effects were observed on [^{3}H]phosphatidylinositol 4-phosphate or [^{3}H]phosphatidylinositol hydrolysis. Phospholipase C had a pH optimum of 6.5-7.0 for both basal and GppNHp-stimulated activities. Time courses of [^{3}H]PIP_2 breakdown were linear up to 10 min of incubation and hydrolysis was temperature dependent with optimal nucleotide stimulation baing observed at 37°C. The main product of [^{3}H]PIP_2 hydrolysis was identified as inositol 1,4,5-trisphosphate. No added calcium was required for activition of phospholipase C but activity was blocked by inclusion of 1mM EGTA in the incubation. Magnesium partially inhibited inositol phosphate formation whereas all monovalent cations tested were stimulatory. These results provide further evidence for nucleotide regulation of phospholipase C. However, the lack of nucleotide specificity suggests that this enzyme may not be regulated solely by guanine nucleotides.

228 STIMULATION OF G1-- S TRANSITION OF Ca^{2+}-DEPRIVED SERUM-STIMULATED RAT LIVER EPITHELIAL CELLS BY INOSITOL 1,4,5-TRISPHOSPHATE AND PROTEIN KINASE C. Alton L. Boynton and Jean Zwiller. Cancer Research Center of Hawaii, 1236 Lauhala St., Honolulu, HI 96813.

Hydrolysis of phosphatidyl inositol 4,5-bisphosphate with the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) appears to be a major signal transduction pathway involved in several biological responses including cell proliferation. The putative function of DAG is to stimulate protein kinase C activity and of IP_3 to mobilize intracellular Ca^{2+} perhpas via an IP_3-dependent protein kinase recently discovered in our laboratory. We have identified two extracellular Ca^{2+}-dependent stages of the G1 phase of the cell cycle of T51B rat liver epithelial cells. Both protein kinase C and IP_3-dependent (via IP_3 dependent protein kinase) events are required for these cell cycle transitions because both protein kinase C activators (TPA, OAG, DiC_8) and the IP_3-dependent protein kinase activator (IP_3) must be added to serum-stimulated, Ca^{2+}-deprived T51B cells for successful G0-- G1 and G1-- S transition. An alteration of this membrane based signal transduction mechanism is indicated by the fact that neoplastic cells no longer require extracellular Ca^{2+} for G0-- G1 and G1-- S transition and also are able to maintain elevated levels of protein kinase C in Ca^{2+}-deficient medium while their non-neoplastic counterparts cannot. Supported by NIH/NCI grant no. CA39745, CA42942.

229 STIMULATION OF A PHOSPHOTRANSFERASE ACTIVITY BY INOSITOL 1,4,5-TRISPHOSPHATE. J. Zwiller, T. Rayson, A.L. Boynton, Cancer Research Center of Hawaii, Honolulu, HI 96813.

The hydrolysis of phosphatidylinositol 4,5-bisphosphate by a GTP-dependent phospholipase C results in two second messengers, diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). Recent results from our laboratory suggest that the function of both these second messengers is required for G0-- G1 and G1-- S transition of serum-stimulated T51B rat liver cells. DG is known to stimulate protein kinase C activity in conjunction with Ca^{2+} and phosphatidyl-serine. IP_3 has been demonstrated to stimulate the increase of intracellular free Ca^{2+} by releasing the ion from presumably the endoplasmic reticulum. We describe here an IP_3-dependent phosphotransferase activity which we have putatively termed protein kinase I. Protein kinase I is extracted from T51B rat liver cells or from rat brain. Small quantities of protein kinase I are present in the 100,000xg supernatant of EGTA extracted cells and larger quantities are present in the detergent-extracted 100,000xg pellet. At present we can only speculate as to the function of protein kinase I. One obvious role may be in the mechanism of intracellular Ca^{2+} release from endoplasmic reticulum. Like other protein kinases, protein kinase I probably has numerous phosphoprotein substrates. Like several other protein kinases such as protein kinase A, C and G, protein kinase I may play a central role in such cellular responses such as neurotransmitter release, secretion, muscle contraction, differentiation and cell proliferation. The characterization, purification and function of protein kinase I are being investigated. Supported by NIH/NCI grant no. CA39745.

230

A MASS MEASUREMENT OF INOSITOL(1,4,5)TRISPHOSPHATE IN CHEMOATTRACTANT-STIMULATED RABBIT NEUTROPHILS. Peter G. Bradford and Ronald P. Rubin, Medical College of VA, Richmond, VA 23298.

Previous studies from our laboratory using ^{32}P-Ins(1,4,5)P_3 demonstrated the presence of a specific intracellular receptor for this second messenger. In the present study, Ins(1,4,5)P_3 content of fMet-Leu-Phe stimulated rabbit neutrophils was determined. Two independent assays of Ins(1,4,5)P_3 mass were performed using ether-washed TCA-extracts derived from ^{3}H-inositol-labeled, fMet-Leu-Phe-stimulated cells. Ins(1,4,5)P_3 content of the extracts was assayed both by the ability of inositol phosphates in the extracts to displace ^{32}P-Ins(1,4,5)P_3 from its intracellular receptor and to release sequestered $^{45}Ca^{2+}$ from nonmitochondrial stores of saponin-permeabilized cells. The relative proportions of ^{3}H-Ins(1,3,4)P_3, ^{3}H-Ins(1,4,5)P_3 and ^{3}H-Ins(1,3,4,5)P_4 in a series of extracts derived from cells stimulated for various times (5s - 5 min) were determined by HPLC. The relative capacity of each fraction to compete with ^{32}P-Ins(1,4,5)P_3 at its receptor and to release sequestered $^{45}Ca^{2+}$ from permeablilized cells coincided perfectly with the content of radioactive ^{3}H-Ins(1,4,5)P_3 in the fraction and not with the content of either ^{3}H-Ins(1,3,4)P_3 or ^{3}H-Ins(1,3,4,5)P_4. Since these assays are selective for Ins(1,4,5)P_3 and are measures of mass, the amount of Ins(1,4,5)P_3 formed in cells can be determined. Thus, the Ins(1,4,5)P_3 content in neutrophils was found to rise from a basal value of 0.02 pmol/10^6 cells to a peak value of 0.40 pmol/10^6 cells after 10 sec stimulation and to return to baseline by 2 min. This study confirms the physiological relevance of the ^{32}P-Ins(1,4,5)P_3 binding site and for the first time measures the mass of Ins(1,4,5)P_3 formed in stimulated cells.

231 STUDIES ON THE INTERACTIONS BETWEEN THE CALCIUM MOBILIZATION AND CYCLIC AMP PATHWAYS IN GUINEA-PIG HEPATOCYTES.

G.M. Burgess., R.K. Dooley., J.S. McKinney., E. Nånberg* and J.W. Putney, Jr. Division of Cellular Pharmacology, Medical College of Virginia, VA 23298 and *Wenner Gren Institute, Biologihus F3, University of Stockholm, S-106 91, Sweden.

The adenylate cyclase activating hormone isoproterenol (50nM) which, when given alone had no effect on ^{86}Rb or ^{45}Ca efflux, potentiated the effects of angiotensin (1-50nM) on ^{86}Rb efflux and ^{45}Ca efflux from guinea-pig hepatocytes. This effect occurred in the presence or absence of extracellular Ca^{2+}, and required the simultaneous presence of both isoproterenol and angiotensin. Neither the Ca-ionophore A23187, nor 4-β phorbol dibutyrate, separately or together, could substitute for angiotensin (cAMP can substitute for isoproterenol). The effects of isoproterenol were greatest with submaximal concentrations of angiotensin, while maximal concentrations of angiotensin were affected little. Isoproterenol did not substantially increase the formation of [^{3}H]inositol trisphosphate, or alter the ratio of isomers, [^{3}H]inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,3,4-trisphosphate, formed in response to angiotensin, suggesting that isoproterenol acts by increasing the sensitivity of the endoplasmic reticulum to the Ca^{2+}-releasing action of inositol 1,4,5-trisphosphate. Isoproterenol also enhanced the phase of Ca^{2+} mobilisation involving Ca^{2+} entry which is consistent with the previously proposed functional linkage between receptor-regulated Ca^{2+} release and Ca^{2+} entry. (Supported in part by N.I.H. grant #AM-32823).

232 ALTERATION IN VASCULAR PHOSPHOINOSITIDE METABOLISM AND HEPATIC PHOSPHOLIPASE C COUPLED RECEPTORS IN A RAT MODEL OF SEPSIS. J. A. Carcillo*+, E. A. Suba*, R. Z. Litten†, and B. L. Roth*, *Naval Medical Research Institute, Bethesda, MD 20814-5055; †Armed Forces Radiobiological Research Institute, Bethesda, MD; and +Childrens Hospital, National Medical Center, Washington, DC.

We previously predicted that alterations in phosphoinositide (PI) metabolism and phospholipase C coupled receptors may cause the altered glucose metabolism and diminished vascular tone seen in septic shock (Chernow and Roth, Circ Shock 18:141-155, 1986). To test this hypothesis, we subjected rats to cecal ligation and puncture (CLP) or a sham operation and measured PI metabolism and receptors 18-24 hours later. The PI metabolism in rat aorta was measured as previously detailed (Roth et al., *Neuropharmacol* 23:1223-1225, 1984). We observed a 50-60% reduction in basal phosphoinositide metabolism (N=16; $p < 0.05$) as well as a 50% reduction in α_1-adrenergic and $PGF_2\alpha$-induced PI hydrolysis in aortas from CLP rats. We also discovered 40-60% ($p < 0.05$) reductions in hepatic α_1-adrenergic and [Arg^8]-vasopressin receptors. Silver-stained two-dimensional gels of liver plasma membranes disclosed no evidence of gross proteolysis. Finally, we found no changes in cardiac β-adrenergic or dihydropyridine receptors or in brain opiatergic or serotonergic receptors. These findings suggest that hepatic and vascular PI metablism and PI-coupled receptors are selectively attenuated in sepsis and that these alterations contribute to the pathogenesis of septic shock. This work was supported by Naval Medical Research and Development Command Work Unit No. M0095.01.1032.

233 THE DETECTION OF CALCIUM BINDING PROTEINS IN RAT CORTEX AND IN AMMONIUM SULFATE FRACTIONS OF WHOLE RAT BRAIN. G.J. Creed, D.M. Santer, W.E. Heydorn, T. Fukuda and D.M. Jacobowitz, Laboratory of Clinical Science, NIMH, Bethesda, MD 20892 & FDA, Rockville, MD 20857.

The detection of calcium binding proteins (CBP) can give insights into the character and possible identity of particular proteins. Protein samples of rat cortex and ammonium sulfate fractions of whole rat brain were separated by two-dimensional gel electrophoresis (2DE). The proteins were then electroeluted onto nitrocellulose paper and the membranes incubated in $^{45}Ca^{+2}$. CBP were detected by autoradiography. Five proteins were consistently detected in rat cortex. Two of these, calmodulin (CaM) and calcineurin (Cn), were identified using purified protein and specific antisera. The others have M_r and pI ranges of 13,500-16,000 and 4.8-5.3 respectively. These same 5 proteins were again seen in the various ammonium sulfate fractions but 4 previously undetected CBP were also seen. The M_r and pI ranges of these newly detected CBPs were 12,500-27,500 and 5.0-5.3 respectively. Two proteins were visualized in the 20% fraction and one each in the 80%, 100% and S-100 fractions. The protein (M_r 27,500, pI 5.2) found in the 80% fraction shows an intense binding of calcium similar to the β subunit of Cn. Its intensity may indicate high binding affinity, as the other proteins show lower intensities even though they are also enriched. These results demonstrate that using 2DE, CBP are detectable. The abundant CBP, CaM and Cn, were identified by this method, and with enrichment, as in ammonium sulfate fractionation, previously undetected CBP are detectable.

234 ALLOSTERIC REGULATION OF INOSITOL 1,4,5-TRISPHOSPHATE-3-KINASE BY SODIUM. A.B. Cubitt, P.R.M. Dobson and B.L. Brown. Department of Human Metabolism & Clinical Biochemistry, University of Sheffield, Sheffield, U.K.

The importance of inositol 1,4,5-trisphosphate Ins (1,4,5) P_3 as a second messenger mediating the actions of many hormones and neurotransmitters is now established. The recent discovery (1) of a Ins (1,4,5) P_3-3-kinase has, however, complicated previous ideas of the metabolism of this important molecule and lead to a search for a role of this pathway. When [^{3}H]-Ins (1,4,5) P_3 (~ μM; 1 Ci/mM Amersham) was added to a neuroblastoma hybrid (NCB-20) supernatant in a buffer approximating to the intracellular ionic composition (KCl 110 mM, NaCl 10 mM, $MgCl_2$ 200 μM, ATP 200 μM, EGTA 1 mM, HEPES 25 mM pH 7.6 at 37°C). Some Ins (1,4,5) P_3 was phosphorylated to Ins (1,3,4,5) P_3 (5%) (as determined by HPLC), while the bulk of Ins (1,4,5) P_3 was dephosphorylated to IP_2, IP_1 and Ins. Raising the concentration of sodium (compensated for by potassium) $^+$ increased the amounts of IP_4 formed dose dependently. The effect was maximal at 50 mM Na^+ while changing the concentration of sodium from 2 mM to 20 mM enhanced the activity of the kinase 3 fold. Previous studies (1) have demonstrated that the IP_3-3-kinase may be activated by the alkalinisation induced by growth factors. It is proposed here that in vivo the kinase may also undergo allosteric activation as a consequence of the influx of sodium ions resulting from activation of the sodium-hydrogen antiport system. Estimates (2) of the resting sodium concentration suggest a value of 8 mM, whilst upon alkalinisation this may rise as high as 26 mM. This is consistent with the view that changes in sodium ion concentration may be important in regulating the activity of the IP_3 kinase and raises the possibility that sodium may be important in regulating the activities of other enzymes 1. R.F. Irvine et al Nature 320,631-634 (1986) 2. M.L. Villereal J.Cell Phys.111:163-170 (1982).

235 SEPARATION OF MULTIPLE ISOMERS OF INOSITOL PHOSPHATES FORMED IN GH_3 CELLS, Nicholas M. Dean and James D. Moyer, Laboratory of Biological Chemistry, DCT, DTP, NCI, Bethesda, MD 20892

High performance liquid chromatography (HPLC) separations have been developed to resolve inositol phosphates. One set of conditions separates isomers of inositol monophosphate (IP), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3), another IP_4, IP_5 and IP_6. The separations were used to investigate the thyrotropin releasing hormone (TRH) stimulated production of inositol phosphates in a rat pituitary tumor (GH_3) cell line. In cells labeled with [^{3}H]inositol and treated with lithium, radiolabeled components identified as I(1)P, I(2)P, I(4)P, I(1,4)P_2, I(1,3,4)P_3, I(1,4,5)P_3, IP_4, IP_5 and IP_6 are present as are multiple unidentified IP_2 peaks. Low levels of glycerophosphoinositol are also found, however no glycerophosphoinositol phosphate, glycerophosphoinositol bisphosphate or inositol(1,2)cyclic.monophosphate are present. Subsequent to stimulation with TRH both I(1)P and I(4)P increase, the increase in I(4)P preceding that of I(1)P; I(1,4)P_2 and an unknown IP_2 increase; and both I(1,3,4)P_3 and I(1,4,5)P_3 increase, the increase in I(1,4,5)P_3 being rapid and transient while the increase in I(1,3,4)P_3 is slower and more sustained. The most rapidly appearing inositol phosphates produced subsequent to TRH stimulation are I(1,4)P_2 and I(1,4,5)P_3. No I(1)P is formed at early time points indicating that a phospholipase C mediated breakdown of phosphatidylinositol is not an early event in these cells.

236 PRODUCTION OF INOSITOL TRISPHOSPHATE ISOMERS IN HUMAN NEUTROPHILS. Susan B. Dillon, John J. Murray, and Ralph Snyderman. Howard Hughes Medical Institute, Duke Univ. Med. Ctr., Durham, N.C. 27710.

Stimulation of PMN chemoattractant (CMT) receptors for N-formyl peptides (f-met-leu-phe), platelet activating factor (PAF) and leukotriene B4 (LTB4) utilize a common transduction pathway that results in the rapid elevation of cytoplasmic Ca^{2+} levels, and in production of inositol trisphosphate (IP_3). Since the 1,4,5, and 1,3,4 isomers of IP_3 have disparate biological activities it is important to determine the relative amounts and kinetics of their production following PMN activation. Inositol phosphates were TCA extracted from ^{3}H-myo-inositol labelled PMN and separated by H.P.L.C. by anion exchange. In unstimulated cells, c.p.m. in the 1,4,5 IP_3 peak were low (38±14; n=8) and 1,3,4 IP_3 was not detected. The initial IP_3 response to 0.1 - 1.0 uM f-met-leu-phe, PAF, or LTB4 (measured at 5 seconds) consisted entirely of the 1,4,5 IP_3 isomer. Peak levels of 1,4,5 IP_3 were comparable for optimal doses of all 3 CMT tested, reaching between 330-500% of resting levels by 5 seconds. For each CMT, the 1,3,4 isomer first appeared at 15 seconds after stimulation, constituting 25±4% (n=5) of total IP_3. By 60 seconds, 50% or more of the total IP_3 consisted of the 1,3,4 isomer, however by this time the total IP_3 in PAF or LTB4 stimulated cells had declined to 10-20% of maximum. Elevated IP_3 levels in f-met-leu-phe stimulated cells did not subside until 5 minutes after stimulation. The proposed mechanism for formation of 1,3,4 IP_3 is via a 1,3,4,5 inositol tetrakisphosphate (IP_4) intermediate (Irvine et.al. Nature 320; 1986). In studies using plasma membranes prepared from ^{3}H-myo-inositol labelled PMNs, only the 1,4,5 isomer was produced in response to Ca^{2+} or GTP mediated activation. This suggests that a 1,4,5 IP_3 kinase activity may be present in cytoplasmic fractions of PMN.

237 CALCIUM RELEASE FROM INOSITOL 1,4,5 TRISPHOSPHATE-SENSITIVE ISLET CELL MEMBRANES CO-LOCALIZED WITH PLASMA MEMBRANE. Marjorie Dunlop and Richard G. Larkins. University of Melbourne, Department of Medicine, Parkville. Vic. Australia. 3050.

Inositol 1,4,5 trisphosphate (IP_3), arising from hydrolysis of phosphatidylinositol 4,5 bisphosphate [PtdIns (4,5)P_2], is proposed as the link between membrane receptor activation and mobilization of Ca^{2+} from intracellular sites in hormone secreting cells. The location of IP_3-sensitive membranes was investigated in neonatal β cells. Membranes were obtained after lysis of cultured cells attached to positively charged Sephadex. Scanning electron microscopy indicated a plasma membrane attachment face of 5-10μm diameter with attached membrane sheets. Enzyme markers: 5'-nucleotidase (5'NT), 4.14; glucose-6-phosphatase (G-6-Pase), 1.31; UDP-galactosyl transferase (UDP-GT), 0.21; succinate dehydrogenase (SD), 0.28μmol/h/mg protein. Following sonication vesicles (0.17 ± 0.04μm diameter) remained at the attachment face (5'NT, 5.54; G-6-Pase, 0.09μmol/h/mg protein, UDP-GT and SD undetected). The endoplasmic reticulum marker, G-6-Pase represents 5.2% of total cell activity. ATP-dependent $^{45}Ca^{2+}$ accumulation was shown in this preparation (410 ± 24 (6) pmol/mg protein at 150nM free Ca^{2+}) and was inhibited by vanadate (100μM). Half maximal Ca^{2+} release occurred with 0.5μM IP_3, maximal release with 5μM IP_3, $t_{½}$ 12 seconds. With concurrent use of [γ^{32}P] ATP to label phosphoinositides, Gpp(NH)p effected PtdIns(4,5)P_2 hydrolysis and release of Ca^{2+} (half maximal Ca^{2+} release, 50μM, $t_{½}$ 20 seconds). GTP was without effect on PtdIns(4,5)P_2 hydrolysis but effected release of Ca^{2+} (half maximal Ca^{2+} release, 5μM, $t_{½}$ 75 seconds) indicative of a separate release mechanism. A close association between plasma membrane and elements of the endoplasmic reticulum is indicated in this model, providing a possible mechanism for local alterations in free Ca^{2+} in the sub-plasma membrane region.

238 ACTIVATION OF CALCIUM CHANNELS IN ENDOPLASMIC RETICULUM (ER) BY GUANINE NUCLEOTIDES AND INOSITOL 1,4,5-TRISPHOSPHATE (IP_3). Donald L. Gill, Shesu-Hwei Chueh, and Mark W. Noel, Dept. Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201.
A highly sensitive and specific guanine nucleotide regulatory mechanism has been observed to directly control Ca^{2+} efflux from ER within cultured neuronal and smooth muscle cell lines. Using saponin permeabilized cells and microsomal membrane vesicles from N1E-115 neuroblastoma cells, studies have revealed release of loaded Ca^{2+} in response to both IP_3 and guanine nucleotides. Thus, GTP activates a rapid and substantial release of Ca^{2+} with an EC_{50} of 0.8 μM; in contrast, mM levels of ITP, CTP, ATP, UTP, GMP, 2'3'-cGMP, 3'5'-cGMP, and guanosine are ineffective. Neither GTPγS nor GppNHp effect release, however, GTPγS (but not GppNHp) blocks the action of GTP. GDP effects full but delayed Ca^{2+} release, but only after its conversion to GTP via nucleoside diphosphokinase activity; thus the effects of GDP are prevented by addition of ADP. In fact GDP is a potent competitive inhibitor of GTP-mediated Ca^{2+} release with K_I=3 μM. The results suggest that a specific GTPase controls opening of a Ca^{2+} channel in ER, activation of which is dependent on the cytosolic GTP:GDP ratio. GTP has no effects on release of Ca^{2+} accumulated within mitochondria or plasma membrane vesicles. GTP appears to induce Ca^{2+} release from the same specific subcompartment of ER targeted by IP_3. Distinctions in the action of the two agents with respect to temperature-dependence and GDP-reversiblity suggest they initially activate separate mechanisms. However, the permissive effects of PEG on both GTP and IP_3-mediated Ca^{2+} release from microsomal vesicles and the common nature of the Ca^{2+} pool involved suggest coupling between the actions of IP_3 and GTP. (Supported by NIH grant NS19304 and NSF grant DCB-8510225).

239 MECHANISMS OF AGONIST-STIMULATED INOSITOL(1,3,4,5)P_3 FORMATION IN LIVER. C. A. Hansen, R. Johanson, and J. R. Williamson, University of Pennsylvania, Dept. of Biochemistry & Biophysics, Philadelphia, PA 19104 USA

Ins(1,4,5)P_3 can either be dephosphorylated to Ins(1,4)P_2 by a 5-phosphatase or phosphorylated to Ins(1,3,4,5)P_4 by a soluble ATP-dependent Ins(1,4,5)P_3 kinase (Irvine *et al.* Nature 320:631, 1986, Hansen *et al.* J. Biol. Chem. 261:8100, 1986). The ability of a variety of Ca^{2+}-mobilizing agonists to stimulate Ins(1,3,4,5)P_4 formation was examined using [^{3}H]-*myo*inositol prelabeled hepatocytes with the [^{3}H]-inositol phosphates being analyzed by HPLC. Vasopressin (20 nM) resulted in the greatest increase in both Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3. The relationship of Ins(1,3,4,5)P_4 accumulation to that of Ins(1,4,5)P_3 suggested that the IP3 kinase was saturated with Ins(1,4,5)P_3. Accumulation of inositol phosphates with maximal phenylephrine (10μM) was 20% of that with VP. However, the Ins(1,3,4,5)P_4 levels were linearly related to the Ins(1,4,5)P_3 levels, indicating the IP_3 kinase was not saturated. EGF (100 nM) and glucagon (10 nM) both caused small but statistically significant accumulations of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3. There were no indications with any of these agonists for a specific stimulation of IP_3 kinase relative to the other agonists. Phenylephrine, glucagon, and EGF-stimulated accumulations of all inositol phosphates were abolished by pretreatment of hepatocytes with PMA. This suggested that these agents were acting via a common mechanism, i.e. by modulation of phospholipase C activity (e.g. via a G-protein). Alternately, however, the small increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 with glucagon and EGF could result from inhibition of the 5'phosphatase, thus diverting basal flux through phospholipase C predominantly to Ins(1,3,4,5)P_4. Current efforts are focussed on the potential modulation of 5'phosphatase activity by cAMP-dependent protein kinase and protein kinase C. Supp: NIH grant AM 15120

240 ATP-INDUCED CALCIUM MOBILIZATION AND INOSITOL 1,4,5-TRISPHOSPHATE FORMATION IN H-35 HEPATOMA CELLS. D.A. Horstman, K.A. Tennes and J.W. Putney, Jr., Division of Cellular Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298.

Addition of ATP (but not epinephrine, angiotensin II, vasopressin, or platelet-activating factor) to H-35 hepatoma cells whose cellular lipids have been pre-labelled with [^{3}H]inositol, causes a rapid increase in [^{3}H]inositol trisphosphate. The formation of inositol trisphosphate isomers was examined. Control cells contained [^{3}H](1,4,5)IP_3 but no detectable [^{3}H](1,3,4)IP_3. On stimulation with ATP (300μM) [^{3}H](1,4,5)IP_3 levels were increased more than three-fold and a roughly equivalent amount of [^{3}H](1,3,4)IP_3 was formed. In H-35 cells pre-incubated in the presence of $^{45}Ca^{2+}$, ATP causes a similarly rapid release of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ efflux was transient, maximal within 30 sec and back to basal levels 3 min after the initial application of ATP. The transience of this response presumably reflects the finite size of the ATP-sensitive Ca^{2+} pool. The concentration-effect relationships for inositol trisphosphate formation and Ca^{2+} efflux are similar to those reported previously for differentiated hepatocytes. These results demonstrate that at least one of the Ca^{2+}-mobilizing receptors normally found on hepatocytes is functionally retained in the H-35 hepatoma cell line and thus could provide a useful model for the study of these receptor mechanisms in liver. (Supported by NIH Grant #AM-32823).

241 PHOSPHORYLATION OF THE 47,000 DALTON PROTEIN AND PHOSPHOINOSITIDES TURNOVER ARE INVOLVED IN ABNORMAL PLATELET ACTIVATION IN SPONTANEOUSLY HYPERTENSIVE RATS. Huzoor-Akbar and Khursheed Anwer. Program in Physiology and Pharmacology, The Ohio University, Athens, OH 45701, USA.

In this study platelets from spontaneously hypertensive (SHR) and normotensive (Wistar Kyoto, WKY) rats were used to investigate the factors responsible for abnormal platelet aggregation (PA) in hypertension. Thrombin (0.04 - 0.05 U/ml) induced two- to three-fold increased PA in SHR than in WKY. Incubation of (^{32}P)-PO_4 labeled platelets with thrombin for 10, 15, 30 and 60 seconds caused significantly increased phosphorylation of 47,000 Dalton protein (P47) in SHR platelets (58%, 70%, 105% and 110%) than in WKY platelets (15%, 29%, 46% and 58%). The effect of thrombin on incorporation of (^{32}P)-PO_4 into platelet phosphatidic acid was examined as an index of phosphoinositides (PIns) turnover. Thrombin (0.05 U/ml) caused a four-fold increase in phosphatidic acid production in SHR platelets (85%) than in WKY platelets (21%) in 15 seconds. The extent of serotonin secretion (SS) and thromboxane A_2 (TXA_2) synthesis induced by 0.04 - 0.05 U/ml of thrombin was similar in WKY and SHR platelets. Aspirin (500 uM) inhibited thrombin (0.05 U/ml) induced TXA_2 synthesis by >75% but did not affect PA or SS in WKY or SHR platelets. Thrombin (0.1 - 0.3 U/ml) produced significantly larger amounts of TXA_2 in SHR than in WKY platelets. However, no differences in PA or SS were seen at these higher concentrations of thrombin. In other experiments up to 1000 uM arachidonic acid or up to 20 uM U46619, a stable analog of prostaglandin H_2, did not induce PA in WKY or SHR platelets. These findings lead us to suggest that increased turnover of PIns and increased phosphorylation of P47 and not the increased TXA_2 synthesis is responsible for abnormal platelet activation in SHR. (Supported in part by the COHC #84-1, #86-01-A and the Ohio University College of Osteopathic Medicine.)

242 CALCIUM MODULATION OF INOSITOL 1,4,5-TRISPHOSPHATE- BUT NOT OF GTP-INDUCED CALCIUM RELEASE FROM NG108-15 MICROSOMES. Thierry JEAN and Claude B. KLEE

The mechanism of calcium release induced by inositol 1,4,5-trisphosphate IP_3 or by GTP was studied in subcellular fractions of neuroblastoma x glioma (NG108-15) hybrid cells. A post-mitochondrial fraction, enriched in endoplasmic reticulum and plasma membranes was the most active in both IP_3 and GTP-dependent calcium release. However, the effects of GTP and IP_3 were strikingly different: i) the release of calcium induced by GTP was slow ($t_{\frac{1}{2}}$=90 seconds) as compared to that produced by IP_3 ($t_{\frac{1}{2}}$<10 seconds). ii) GTP released calcium only in the presence of polyethyleneglycol whereas the fusogene had no effect on IP_3-induced calcium release. iii) The presence of GTP (or IP_3) did not affect the efflux of calcium mediated by the other agent. iv) Increasing the free calcium concentration in the incubation medium stimulated the GTP-induced calcium release but inhibited the effect of IP_3. IP_3-induced calcium release was maximum at 10^{-6}M free calcium and was undedectable at $2x10^{-5}$M free calcium. These results suggest that IP_3 and GTP act by distinct mechanisms on different calcium stores. The physiological significance of a GTP-induced calcium release remains to be demonstrated. On the other hand, the feedback inhibition of the IP_3-induced calcium release by cytoplasmic calcium constitutes an important self-regulatory mechanism and may permit the uncoupling of the IP_3 and diacylycerol signals following hormonal stimulation of the membrane-bound phospholipase C.

243

TOXICITY OF LIPOSOMES CONTAINING PLANT PHOSPHATIDYLINOSITOL TO TUMOR CELLS: EFFECTS OF PHOSPHOLIPASE A_2 INHIBITORS. Marti Jett and Carl R. Alving, Div. Biochem., Walter Reed Army Inst. Res., Washington, D.C. 20307-5100.

Plant phosphatidylinositol (PI) has been shown by us to have a direct cytotoxic effect on cultured tumor cells but not on normal cells. Synthetic PI containing ^{14}C-linoleic acid in the sn-2 position, also showed the same pattern of selective cytotoxicity. When the metabolic fate of synthetic PI was examined with tumor cells, the radioactivity which no longer occurred as PI, was found as either products of phospholipase A_2 (93%, free fatty acids and phosphatidylcholine) or phospholipase C (7%, diglycerides). Uptake of liposomal PI was directly correlated with cytotoxicity. We tested a variety of inhibitors to see the effect on uptake and/or cytotoxicity of plant PI. General metabolic inhibitors such as metrizamide or sodium azide reduced cellular uptake of the plant PI liposomes to 70%. Inhibitors of lipoxygenase formation, such as indomethacin, did not alter the uptake or cytotoxicity induced by plant PI. Quinacrine, an inhibitor of phospholipase A_2, decreased the uptake of the PI containing liposomes to 50% of that seen in the presence or absence of any other inhibitor. Although quinacrine is itself toxic to cells, at low concentrations of quinacrine, plant PI did not show the same degree of cytotoxicity as in the absence of quinacrine. Procaine and lidocaine, also inhibitors of phospholipase A_2, reverse the toxicity of plant PI to tumor cells. These data are compatible with our hypothesis that plant PI exerts cytotoxicity by serving as a substrate for phospholipase A_2.

244 THE ROLE OF GTP AND INOSITOL TRISPHOSPHATE IN MEDIATING Ca^{2+} RELEASE FROM LIVER MICROSOMES. S. K. Joseph, C. V. Nicchitta, and J. R. Williamson, Dept. of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6089 USA.

Inositol trisphosphate (IP_3) can release Ca^{2+} from liver microsomes only when GTP and polyethyleneglycol (PEG) are present in the incubation medium [*c.f.* Dawson, A. P. (1985) FEBS Lett. 185: 147]. Under some conditions GTP itself can release Ca^{2+}. However, the Ca^{2+} releasing effect of GTP can be selectively removed by decreasing the amount of Ca^{2+} sequestered into the microsomal vesicles without affecting the ability of the nucleotide to render the microsomes sensitive to IP_3. The ability of GTP to release Ca^{2+} could also be inhibited by the addition of a soluble fraction prepared from a high speed centrifugation of liver homogenate. This fraction retained inhibitory activity after boiling and desalting on Sephadex-G25. Non-hydrolysable analogues (e.g. GTPγS) had no effects when added alone but did suppress the response to GTP. The binding of ^{35}S-GTPγS to microsomal membranes was unaffected by IP_3 or PEG. Similarly, GTP and PEG did not significantly affect ^{32}P-IP_3 binding. These studies tend to exlude models involving GTP-binding proteins analogous to those coupled to cell surface receptors. No evidence was obtained for direct GTP-dependent phosphorylation of microsomal proteins. The data suggest that IP_3 can open a divalent cation channel only after the channel is first 'primed' in some unknown manner by GTP hydrolysis. In subcellular fractions this process is promoted by PEG and regulated by the intravesicular Ca^{2+} content and cytosolic factors. Supported by JDF Career Development Award (SKJ) and NIH grants AM-34804 (SKJ) and AM-15120 (JRW).

245 CHOLECYSTOKININ OCTAPEPTIDE ACTIVATES INOSITOL PHOSPHATE ACCUMULATION IN A HUMAN EMBRYONIC PITUITARY CELL LINE. William W.Y. Lo*+, Colin R. Clark+ and John Hughes+, +Parke-Davis Research Unit, Addenbrooke's Hospital Site, Hills Road, Cambridge, U.K. *Downing College, Cambridge University, Cambridge, England.

The effect of CCK-8(S) on polyphosphoinositide (PPI) turnover was investigated in a human embryonic pituitary cell line (i.e. Flow 9000). CCK-8(S) stimulated inositol phosphate accumulation in a dose- (EC_{50}=0.79 ± 0.12nM) and time-dependent manner. Time course studies showed that significant amounts of [^{3}H]InsP_3 were formed as early as 15 sec indicating that an initial CCK-8(S)-mediated breakdown of phosphatidylinositol-4,5-bisphosphate is the principal mechanism operating during receptor activation. External calcium is not required for the CCK-8(S)-PPI effect. Benzotript, a specific CCK receptor antagonist, competitively inhibited CCK-8(S)-induced [^{3}H]InsP formation with a K_i value of 12.75 ± 1.55 μM. These results suggest that stimulated PPI hydrolysis is the biochemical mechanism which CCK receptor utilizes following its activation in Flow 9000 cells. Moreover, the human embryonic pituitary cell line Flow 9000 appears to be a useful model system for the examination of CCK-mediated pituitary cell function.

William W.Y. Lo is a Commonwealth Scholar.

INHIBITION OF HISTAMINE. INDUCED Ca^{++} EFFLUX BY AN ANTIHYPERTENSIVE DRUG (CICLETANINE) IN CULTURED VASCULAR SMOOTH MUSCLE CELLS.

246 M. Lonchampt, M. Cabanié, A. Esanu, C. Demerlé and P. Braquet
IHB Research Labs, 17 avenue Descartes, F-92350 Le Plessis Robinson (France)

Histamine (Hist) -induced Ca^{++} efflux was studied in smooth muscle cells (S M C) cultured from guinea-pig up to 4th passage. The cells were labeled with $^{45}Ca^{++}$ (2 μ Ci/ml) to a stable specific activity. The effect of cicletanine (CIC) a new dihydrofuropyridine antihypertensive drug was studied on this model.

During the efflux, 2 phases could be distinguished. The Ca^{++} efflux during the initial phases was rapid (50% in the first 2 minutes) and Hist stimulated this efflux by 80%. A H_1 agonist (2 -pyridylethylamine dihydrochloride, 10^{-4} M) stimulated Ca^{++} efflux (50% compared to Hist) whereas H_2 agonist (dimaprit 10^{-4} M) had no effect. Anti H_1 drugs (mepyramine 10^{-5} M and terfenadine 10^{-5} M) blocked the Hist stimulated efflux.

Whatever the doses, (S) CIC did not modify the Ca^{++} efflux. In the same manner (R) CIC and Rac CIC did not change the efflux. When cells were preincubated with these products (10^{-5} M) and then stimulated by Hist (10^{-4} M), the effect of Hist was completely inhibited by S (CIC) and Rac (CIC) but not by R (CIC).

these data suggest that:

1- Hist activates H_1 receptors to mediate a release of Ca^{++} from internal stores.
2- CIC acts as a H_1 antagonist. Its enantiomers have opposite effects.

247 PROTEIN KINASE C STIMULATES INOSITOL TRISPHOSPHATE DEPHOSPHORYLATION. Luis M. Molina y Vedia and Eduardo G. Lapetina. Molecular Biology Department, Burroughs Wellcome Co.,Research Triangle Park, NC 27709

Inositol trisphosphate (IP3) is formed in response to specific agonists that cause activation of phospholipase C and degradation of phosphatidylinositol bisphosphate. IP3 is a second messenger that releases Ca^{2+} from the dense tubular system to the cytosol in stimulated platelets. Our present information indicates that [^{3}H]IP3 is dephosphorylated to [^{3}H]inositol bisphosphate (IP2) and [^{3}H]inositol monophosphate (IP) by human platelets treated with 0.05% Triton X-100. This detergent allows IP3 to access cytosolic IP3-phosphatase and to activate the dephosphorylation of IP2 to IP by IP2-phosphatase. Pretreatment of intact platelets with phorbol dibutyrate (PDBu) and 1-oleyl,2-acetyl,diacylglycerol (OAG) for 30 s, at concentrations that maximally activate protein kinase C, stimulates the conversion of IP3 to IP2 and IP. This suggests a role for protein kinase C in the regulation of IP3 degradation.

248 MEASUREMENT OF INTRACELLULAR Ca^{2+} IN SINGLE SMOOTH MUSCLE CELLS. J. R. Monck, E. E. Reynolds and J. R. Williamson, Univ. of Pennsylvania School of Medicine, Dept. of Biochemistry and Biophysics, Philadelphia, PA 19104 USA.

The newly developed Ca^{2+} indicator Fura2 has been employed to study hormone-induced Ca^{2+} changes in single cells using videomicroscopy and image analysis. A10 Smooth muscle cells, derived from rat thoracic aorta, responded to maximal concentrations of vasopressin with a single, large increase in free Ca^{2+} which decayed over 5-10 min. However, at low vasopressin concentrations (0.2, 0.5 nM) there were two distinct peaks of Ca^{2+}. The initial peak rose rapidly and had predominantly decayed before onset of the second peak which reached a maximum after 3 min and decayed more slowly. No sign of the biphasic response was seen when the Ca^{2+} transient was recorded with a population of cells in a fluorometer, using a monolayer of cells grown on ACLAR. Furthermore, the rise time for the transient in the monolayer was considerably slower than for the single cells. This suggests that the different transient observed in the cell population is due to individual cells responding asyncronously. When the response of a single cell to low concentrations of vasopressin was measured in the absence of extracellular Ca^{2+}, the second phase was abolished, but the first phase was unchanged. Extracellular Co^{2+} (2 mM) also greatly reduced the second phase. These data indicate that the initial peak is due to mobilization of intracellular Ca^{2+} and the second phase to influx of extracellular Ca^{2+}. The observation that the second phase does not become apparent until the initial phase has largely decayed raises the interesting possibility that influx of extracellular Ca^{2+} is mediated by a Ca^{2+} and IP_3-independent mechanism, perhaps through a secondary interaction of the vasopressin receptor with a guanine nucleotide binding protein as has been suggested for K^+ channels coupled to muscarinic receptors. Supp: HL-14461

249 INVOLVEMENT OF INOSITOL PHOSPHATES, DIGLYCERIDE AND ARACHIDONATE IN GnRH ACTION ON GONADOTROPH LH SECRETION. R.O. Morgan, J.P. Chang and K.J. Catt. ERRB/NICHD, Bethesda, MD 20892.

The GnRH-induced elevation of intracellular [Ca^{2+}] required for LH secretion results from the mobilization of calcium from intracellular pools as well as by external Ca^{2+} entry via voltage-sensitive calcium channels (J. Biol. Chem. 261, 9105). Certain products of phosphoinositide metabolism, viz. inositol-1,4,5-trisphosphate (IP_3) and arachidonic acid, have been proposed to mediate changes in cytoplasmic calcium levels. HPLC characterization of gonadotroph phosphoinositides and inositol polyphosphates (IPs) has provided unequivocal evidence that phospholipase C-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to IP3 and diglyceride (DG) is a selective, primary event in GnRH action. Two novel findings were that gonadotrophs contain "higher" IPs, identified by dual-label analysis as IP_4, IP_5 and IP_6, which increase in response to GnRH, and that inositol-4-monophosphate (I-4-P) rather than the expected I-1-P is the chief intermediate of IP catabolism in gonadotrophs.

The DG resulting from GnRH-stimulated PIP_2 hydrolysis is a known activator of protein kinase C (PKC) and a source of free arachidonic acid (AA) in rat gonadotrophs. Results from pituitary cell column perifusion studies using AA, DG, phorbol ester, nordihydroguaiaretic acid and retinal suggest that AA (or its metabolites) may participate mainly in the early secretory response to GnRH, while PKC may regulate later events of GnRH action. The demonstrated changes in IP_3 and higher IPs, and the effects of DG and AA on LH secretion substantiate the hypothesis that these intermediates represent intracellular signals in the calcium- and protein kinase-dependent secretory mechanisms of GnRH action.

250 CHARACTERIZATION OF A POLYETHYLENE GLYCOL STIMULATED HIGH AFFINITY MICROSOMAL GTPase.

C. V. Nicchitta, S. K. Joseph, and J. R. Williamson, University of Pennsylvania, Dept. of Biochemistry and Biophysics, Philadelphia, PA 19104 USA.

Recent studies of the Ca^{2+} transport properties of the microsomal fraction of rat liver have shown that GTP, in the presence of polyethylene glycol (PEG), will stimulate the release of sequestered Ca^{2+} (Dawson et al., 1986, Biochem. J. 234:311). In this system PEG (3%w:v) is necessary to allow Ca^{2+} release in response to GTP as well as to inositol 1,4,5-trisphosphate (IP_3). In order to investigate possible mechanisms responsible for the PEG requirement, the effects of PEG on microsomal GTPase activity were determined. When assayed in the presence of 0.25 μM GTP, ATP, AppNHp, and an ATP regenerating system PEG stimulated the rate of GTP hydrolysis by 40 to 70%. This effect was dependent on the PEG concentration; maximal effects were observed at 3%(w:v) PEG. GTP hydrolysis was inhibited at PEG concentrations above 5%(w:v). The stimulation by PEG of microsomal GTP hydrolysis was only observed at GTP concentrations of 0.01 to 5uM. At GTP concentrations above 5μM the rate of GTP hydrolysis was identical in the presence and absence of PEG. PEG-stimulated GTP hydrolysis was inhibited by the nonhydrolyzable GTP analogs GTPγS and GppNHp. Complete inhibition was observed with equimolar concentrations of GTPγS. GppNHp was approximately one order of magnitude less effective than GTPγS. A similar sensitivity to GTP analogs was observed for the GTP-sensitive Ca^{2+} release system. Inhibition of basal GTPase activity required far greater concentrations of the GTP analogs (10^{-4}M). Neither IP_3 nor IP_4 significantly altered the rate of GTP hydrolysis in the presence or absence of PEG. Our current studies are devoted to further characterization of this GTPase activity as well as its regulation by cytosolic components.

251

USE OF TRITIATED MYO-INOSITOL IN STUDIES OF PHOSPHATIDYLINOSITOL METABOLISM: FACTORS AFFECTING SENSITIVITY AND THE SELECTION OF OPTIMUM CONDITIONS FOR EXPERIMENTS.

Kenneth G Oldham, Amersham International, Cardiff, Wales.

Receptor-stimulated phospholipase C-mediated hydrolysis of PtdIns 4,5-P_2 results in the formation of two second messengers: diacylglycerol, which exerts its effect by activating protein kinase C; and Ins(1,4,5)P_3 which acts by mobilising calcium from cellular stores. Tritiated myo-inositol is increasingly being used in a highly sensitive new assay for receptor activation which is based on the ability of Li^+ to inhibit the enzymatic dephosphorylation of Ins$_3$I-P. This inhibition prevents recycling of the inositol and results in the accumulation of [^{3}H]inositol phosphates which can easily be separated from the neutral inositol by ion-exchange chromatography. Only very low conversions (0.1% or less) to inositol phosphates are commonplace and there is much interest in methods of increasing both the sensitivity and the amount of activity incorporated in such experiments. These attempts have often involved the use of high specific activity [^{3}H]inositol and special inositol-depleted culture media. It will be shown that in this application high sensitivity does not automatically result from the use of high specific activity labelled substrates but is best obtained by careful experimental design particularly in relation to the use of procedures aimed at minimising blanks, and to the use of high radioactive concentrations.

252 LPS INDUCES FORMATION OF MYOINOSITOL TRISPHOSPHATE (IP_3) IN MURINE PERITONEAL MACROPHAGES.

V. Prpic+, J.E. Weiel+, S.D. Somers+, B. Herman*, and D.O. Adams+x. Depts. of +Pathology and xMicrobiology-Immunology, DUMC, Durham, N.C. and *Dept. of Anatomy, UNC, Chapel Hill, N.C.

LPS is a potent signal for macrophages but the mechanisms of signal transduction by which it acts are not known. Treatment of murine peritoneal macrophages with LPS (10μg/ml) produced a rapid and substantial (85%) increase in IP_3 at 10 sec. The increased level peaked at 1 min and was sustained for 5 min. The increase in IP_3 was dose-dependent and observable with doses of LPS as low as 1 ng/ml. Lipid A, the active moiety of LPS, mimicked the effect of LPS on IP_3 generation with similar dose-dependency, but with a more transient time course. Lipid A (10μg/ml) mobilized intracellular Ca^{++} in Quin 2-loaded macrophages. This effect peaked at 30-45 sec, rising from approximately 200 nM basal to approximately 400 nM stimulated level. Macrophages loaded with Fura 2 showed a larger magnitude of increase in cytosolic calcium when stimulated with Lipid A (10μg/ml) rising from 90 nM basal to 336 nM at 30 sec. Stimulation of macrophages by these agonists shows, in addition to I-1,3,4-P_3, the formation of another isomer of IP_3 whose phosphate position is unknown, as a degradative pathway of I-1,4,5-P_3. Either LPS or Lipid A, when added to macrophages prelabeled to a high specific activity with $H_3{}^{32}PO_4$, can stimulate the enhanced phosphorylation of protein species at 103, 67, 33, and 28 kD minimally detectable at 15 min. Previous observations have shown that PMA can also induce the enhanced phosphorylation of the macrophage proteins at 67, 33, and 28 kD, suggesting a role for protein kinase C in this process. Taken together, the data indicate one mechanism, by which LPS stimulates macrophages, is the breakdown of polyphosphoinositides.

253 MUSCARINE-STIMULATED INOSITOL TRISPHOSPHATE FORMATION AND CALCIUM MOBILIZATION IN PC12 CELLS. C.S. Rabe, *E. DeLorme and F.F. Weight, NIAAA, Rockville, MD 20852 and *Dept. Pharmacol., Georgetown U., Washington, DC 20007

The neurosecretory cell line, PC12, was used to examine the effect of muscarine on inositol trisphosphate (IP_3) and intracellular Ca^{2+}, $[Ca^{2+}]_i$, levels. IP_3 was assayed as described by Berridge et al. (Biochem J. 212:473, 1983) and $[Ca^{2+}]_i$ was measured using Quin 2 as described by Tsien et al. (Nature 295:68 1982). Exposure of PC12 cells to muscarine produced rapid increases in both cellular IP_3 and $[Ca^{2+}]_i$. Maximal increases in both phenomena were observed within 30 sec and levels remained elevated for several min. Threshold stimulation of IP_3 and $[Ca^{2+}]_i$ occurred between 1 and 10 uM muscarine and both events increased with concentration through 3 mM. Both the increase in IP_3 and $[Ca^{2+}]_i$ obtained at 300 uM muscarine were totally inhibited by atropine (100 nM) but were unaffected by curare (10 uM). Neither the increase in IP_3 or $[Ca^{2+}]_i$ was affected by nifedipine (1 uM), an inhibitor of voltage-dependent Ca^{2+} influx, suggesting that elevation of $[Ca^{2+}]_i$ was not due to Ca^{2+} influx through voltage-activated Ca^{2+} channels. In addition, elevation of extracellular K^+ to a concentration which produced a comparable increase in $[Ca^{2+}]_i$ (measured with Quin 2) as that seen with 300 uM muscarine caused a tripling of $^{45}Ca^{2+}$ uptake into the PC12 cells, whereas 300 uM muscarine produced no detectable increase in $^{45}Ca^{2+}$ uptake. These observations suggest that the increased $[Ca^{2+}]_i$ observed upon stimulation of the cells with muscarine may be due to mobilization of Ca^{2+} from an intracellular site. However, the ability of muscarine to elevate IP_3 and $[Ca^{2+}]_i$ were partially dependent on the presence of extracellular Ca^{2+}. Incubation of the cells in Ca^{2+}-free medium reduced both the basal and muscarine-stimulated levels of IP_3 and $[Ca^{2+}]_i$. Thus, extracellular Ca^{2+} may modulate the level of $[Ca^{2+}]_i$ and in turn modulate the ability of muscarine to elevate IP_3 and $[Ca^{2+}]_i$.

254 TWO FORMS OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C FROM BOVINE BRAIN: PROTEIN KINASE C PHOSPHORYLATES THE 145KDa POLYPEPTIDE.

Sue Goo Rhee*, Key Seung Cho*, Kee-Young Lee*, Sung Ho Ryu* and Kuo-Ping Huang**
*Laboratory of Biochemistry, NHLBI, **Endocrinology and Reproduction Branch, NICHHD

Phosphatidylinositol-specific phospholipase C (PLC) plays a crucial role in initiating the surface receptor mediated signal transduction by generating two second messenger molecules, diacylglycerol and inositol 1,4,5-triphosphate.

We resolved two forms of bovine brain enzymes, PLC-I and PLC-II on a HPLC-DEAE column and purified PLC-II near homogeneity. Upon analysis of PLC-II on SDS-PAGE, a single band of M_r=145,000 was observed. When the same sample was subjected to native gradient polyacrylamide gel, four bands, one major band of M_r=200,000 and three minor bands with M_r corresponding to different oligomeric states of the 200KDa protein, were visible. Western blot experiments using anti-PLC-II antibody indicated that PLC-I might be derived from PLC-II by proteolytic cleavage. Multiple forms of brain PLC enzymes described in literature might be therefore, attributed to the oligomerization and proteolysis of PLC-II. PLC-I and PLC-II hydrolyzed both phosphatidylinositol (PI) and phosphatidylinositol 4,5-diphosphate (PIP_2). Both activities were stimulated by Ca^{++}. However, the presence of Ca^{++} was not an absolute requirement for the hydrolysis of PIP_2 while PI hydrolysis at neutral pH required Ca^{++}.

Protein kinase C phosphorylates PLC-II in a Ca^{++} and phospholipid dependent manner. The physiological meaning of this phosphorylation is not known yet.

255 INCREASED POST-RECEPTOR RESPONSE AS A MECHANISM FOR INCREASING ADRENERGIC SENSITIVITY: THE EFFECT OF ESTROGEN ON ALPHA-1 MEDIATED INOSITOL TRIPHOSPHATE PRODUCTION IN UTERINE SMOOTH MUSCLE R. Kirk Riemer, Alan Goldfien, and James M. Roberts, Reproductive Endocrinology Center, University of California, San Francisco, CA 94143

Estrogen increases alpha-1 adrenergic sensitivity for rabbit uterine contraction independent of effects on alpha-1 receptor concentration. Muscarinic cholinergic sensitivity and receptor concentration is unaffected by estrogen treatment. We asked whether the post-receptor response to alpha-1 adrenergic stimulation, inositol triphosphate (IP3) production, was increased by estrogen. Since muscarinic stimulation is also coupled to IP3 production in rabbit uterus, we compared the effects of estrogen on both adrenergic and cholinergic mediated IP3 production. Tritiated myoinositol was used to label the phosphatidylinositol pool in minced uterine tissue. We found that estrogen increased maximal IP3 production in response to 100 uM norepinephrine from 138±11 to 264±19 percent of control, $P<0.05$ ($n = 6, 11$, ovariectomized control and estrogen treated, respectively). Cholinergic response to 1 mM carbachol was not changed by estrogen treatment: 195±17 to 147±11 percent of control, $P>0.05$ ($n = 4, 9$). No affect of estrogen on the uterine sensitivity for this response was observed.. These results demonstrate that increased IP3 production accompanies the estrogen-induced increase in adrenergic sensitivity. Thus, enhanced post-receptor response correlates with increased sensitivity for physiologic response. The lack of a similar effect on cholinergic uterine IP3 and contractile response demonstrates that the IP3 response mediated by different receptors can be independently modified by a nuclear active hormone.

256 VASCULAR PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C'S: MODULATION OF ACTIVITY BY GUANINE NUCLEOTIDES, CALCIUM AND MAGNESIUM. B. L. Roth and E. A. Suba. Naval Medical Research Institute, Bethesda, MD 20814-5055.

We previously demonstrated that rat aortic $5HT_2$, α_1-adrenergic, and $PGF_{2\alpha}$ receptors activate phosphoinositide (PI) hydrolysis in rat aorta. We also showed that a phospholipase C inhibitor (NCDC) and phorbol esters attenuate both receptor-stimulated PI hydrolysis and rat arotic contraction, and suggested a coupling between receptors that mediate vasoconstriction and phospholipase C. Here we characterized aortic phospholipase C activities. Homogenates and membranes were prepared in Tris-maleate buffer (pH=6.80) and incubated with exogenous [^{3}H]-phosphatidylinositol (PL) and [^{3}H]-phosphatidylinositol-4,5-biphosphate (PIP_2). Activity for both substrates was increased by $CaCl_2$ and abolished by EGTA (1 mM), although the PL-specific phospholipase C was activated to a greater extent by μM Ca++. Both activities were inhibited by mM Mg++. The PIP_2-specific activity was increased by Gpp(NH)p and GTPγS, and to a lesser extent by GTP. Guanine nucleotide-stimulated activity showed an absolute requirement for Ca++. The PL-specific activity was not altered by guanine nucleotides. These results suggest that two PI-specific phospholipase Cs exist in vascular tissue. Both may be activated by Ca++ and inhibited by Mg++. Only the PIP_2-specific enzyme is coupled to a guanine nucleotide regulatory protein as previously predicted (Chernow and Roth, Circ Shock 18:141-155, 1986). This work was supported by Naval Medical Research and Development Command Work Unit No. M0095.01.1032 and MR04120.05.

257 INHIBITION BY PHORBOL ESTERS OF ANTI-IMMUNOGLOBULIN INDUCED CALCIUM SIGNALLING AND B CELL ACTIVATION. TL Rothstein, DL Kolber, ER Simons and TR Baeker. Departments of Medicine, Microbiology and Biochemistry, Boston University Medical Center, Boston, MA 02118.

Phorbol esters inhibit B cell proliferation induced through interaction with surface immunoglobulin, although phorbol esters co-stimulate B cells with calcium ionophore. The inhibitory effect of phorbol dibutyrate (PDB) on B cell stimulation was evaluated using a model in which activation is induced by modest doses of anti-immunoglobulin antibody (anti-Ig) and progression to DNA synthesis is induced by cytochalasin (J Immunol 135:106; 136:813). PDB preferentially inhibited anti-Ig induced activation and did so during brief (2 hour) preincubation with anti-Ig. Activation was inhibited whether PDB was added before, or shortly after, anti-Ig. Since activation for cytochalasin responsiveness appears to be mediated by Ca++ (J Immunol 136:749), the effect of PDB on the anti-Ig induced rise in intracellular Ca++ was evaluated (using the Ca++ sensitive fluorescent dye, Indo-1AM). PDB (and other phorbol esters that activate protein kinase C) inhibited the rise in Ca++ normally associated with anti-Ig treatment; moreover, PDB reversed an established anti-Ig induced Ca++ response. These data suggest that phorbol esters inhibit B cell activation by interferring with the elevated levels of intracellular Ca++ produced by cross-linking of surface immunoglobulin by anti-Ig. This could represent a "feedback inhibition" type of response but it remains to be seen if this occurs under physiological conditions of protein kinase C activation.

258

INVOLVEMENT OF Ca^{2+} AND CALMODULIN IN MORPHOGENESIS OF Candida albicans

Bipasha Gupta Roy and Asis Datta, Jawaharlal Nehru University, New Delhi-110067, INDIA.

Candida albicans, an opportunistic human pathogen, exists in yeast and mycelial forms through an intermediate germ tube. Investigations carried out in our laboratory demonstrate that though N-acetylglucosamine can induce germ tube as well as N-acetylglucosamine catabolic enzymes, these are mutually exclusive events. The germ tube formation can be stopped by inhibitors of calmodulin, e.g. trifuoperazine (TFP) and compound 48/80. Furthermore, ionophore which opens up ion channels in biological membrane also inhibits the germ tube formation and this can be reversed by Ca^{2+}. This result suggests the involvement of the calcium binding protein, calmodulin, in morphogenesis of Candida albicans. Further investigations into the molecular mechanisms involved in germination induced by N-acetylglucosamine revealed that there is a progressive increase in the rate of protein phosphorylation. However, TFP brought back the rate almost to yeast phase level by also inhibiting the rate of protein phosphorylation. An increase in protein phosphorylation which was inhibited by TFP was also observed during germination with the other inducers. Our observations indicate that the action of calmodulin in germination is possibly exerted via calmodulin modulated protein phosphorylation.

259 IMPAIRED PHOSPHOINOSITIDE-MEDIATED SIGNALING IN HEPATOCYTES OF CONTINUOUSLY ENDOTOXEMIC RATS: PARTIAL SIMULATION OF THE EFFECT BY A PHORBOL ESTER. J.A. Spitzer and E.R. deTurco Louisiana State Univ. Med. Ctr., New Orleans, LA.

Rats were infused i.v. with a non-lethal dose (0.1 mg/100g body wt./day) of E. coli endotoxin (ET) or saline via implanted osmotic minipumps (Fish, R.E. & Spitzer, J.A. Circ. Shock 12, 135-149, 1984). After 30h of continuous infusion hepatocytes were isolated, labelled in vitro with ^{32}P and the status of basal and vasopressin (VP)-stimulated phosphoinositide turnover assessed. In cells of ET-infused rats, the early response to VP stimulation (within 30 sec) in terms of phosphatidyl inositol 4,5-bisphosphate (PIP_2) and phosphatidyl inositol-4phosphate (PIP) breakdown was inhibited by 40% as compared to saline-infused controls. Subsequent resynthesis of PIP_2 and PIP was also impaired. The ability of VP to stimulate ^{32}P incorporation into phosphatidyl inositol (PI) was markedly depressed from 160 to 130%. This endotoxemia-induced perturbation in phosphoinositide metabolism elicited by the activation of VP receptors is associated with concomitant impairment in cytosolic free [Ca^{2+}] and glycogen phosphorylase a activation (Deaciuc, I.V. and Spitzer, J.A., Am. J. Physiol. In press). Infusion i.v. of the phorbol ester TPA via the implanted pump (240 nmoles into a 360g rat over 30h) resulted in similar impairment of the VP-associated PIP_2 and PIP degradation and resynthesis as observed due to ET-infusion. The results support the concept that some of the pathophysiologic effects observed in endotoxemia may be brought about in part through activation of protein kinase C. (Supported by NIH grants GM32654 and GM30312).

260 THE INOSITOL TRIS/TETRAKIS PATHWAY IN GUINEA-PIG HEPATOCYTES. K.A. Tennes and J.W. Putney, Jr., Division of Cellular Pharmacology, Medical College of Virginia, Virginia Commonwealth University Richmond, Virginia 23298.

Inositol(1,4,5)trisphosphate-3-kinase activity was identified in permeabilized guinea-pig hepatocytes. The conversion of [^3H](1,4,5)IP_3 to [^3H](1,3,4,5)IP_4 occurred rapidly and was detected as early as 5 sec, whereas a significant increase in [^3H](1,3,4)IP_3 occurred only after a definite lag period. Incubation of permeabilized hepatocytes with [^{32}P](1,3,4,5)IP_4 resulted in the formation of [^{32}P](1,3,4)IP_3, no [^{32}P](1,4,5)IP_3 was produced. The half-time of hydrolysis of [^{32}P](1,3,4,5)IP_4 of approximately 1 min was increased to 3 min by 2,3 bisphosphoglyceric acid. Similarly, the rate of production of [^{32}P](1,3,4)IP_3 from the [^{32}P]tetrakisphosphate was reduced by 2,3 bisphosphoglyceric acid. In the absence of ATP there was no conversion of [^3H](1,4,5)IP_3 to [^3H](1,3,4,5)IP_4 or [^3H](1,3,4)IP_3, which eliminates the possibility that the [^3H](1,3,4)isomer occurs as a result of isomerization of [^3H](1,4,5)IP_3. The results of this study demonstrate that the origin of the (1,3,4)isomer of inositol trisphosphate in isolated hepatocytes is IP_4 and that (1,4,5)IP_3 is rapidly converted to this tetrakisphosphate. The ability of 2,3 bisphosphoglyceric acid, an inhibitor of 5'-phosphomonoesterase, to inhibit the breakdown of (1,3,4,5)IP_4 suggests that the enzyme which removes the 5'-phosphate from (1,4,5)IP_3 also acts to convert the tetrakisphosphate to (1,3,4)IP_3. It is not known if the role of the (1,4,5)IP_3-kinase is solely to remove (1,4,5)IP_3 or whether it also produces second messengers. (Supported by NIH grant #AM-32823).

261 GTP-DEPENDENT ACTIVATION OF THE INS(1,4,5)P_3-SENSITIVE CALCIUM RELEASE SYSTEM IN PERMEABILIZED HEPATOCYTES. Andrew P. Thomas, Department of Pathology, Thomas Jefferson University, Philadelphia, Pa 19107.

Hepatocytes were permeabilized using digitonin in a medium containing Quin2 to simultaneously buffer and monitor the free Ca^{2+} concentration. After a steady-state level of Ca^{2+} uptake had been achieved in the presence of ATP, the addition of 1.5 uM Ins(1,4,5)P_3 caused a maximal release of about 30% of the sequestered Ca^{2+}. The remaining Ca^{2+} could all be released by ionomycin. Pretreatment of the permeabilized cells with GTP (50 uM) for 3 min resulted in a 2-fold increase in the amount of Ca^{2+} released by Ins(1,4,5)P_3 with an equivalent decrease in the residual ionomycin-releasable Ca^{2+} pool. GTP alone did not cause any Ca^{2+} release and did not alter steady-state Ca^{2+} uptake. The potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release by GTP was half-maximal at 5uM and maximal at 50uM GTP and did not involve a shift in the sensitivity to Ins(1,4,5)P_3. Non-hydrolyzable GTP analogues such as GTP-γ-S had no effect on Ca^{2+} release by Ins(1,4,5)P_3 but were potent in blocking the effects of GTP. Experiments using vanadate to inhibit the ATP-driven Ca^{2+} uptake pump indicated that GTP acts directly on the Ca^{2+} release system. Although GTP did not cause Ca^{2+} release in the absence of Ins(1,4,5)P_3 under normal conditions, it was found that GTP alone could cause substantial Ca^{2+} release in permeabilized hepatocytes which had been washed to remove cytosolic and loosely associated membrane components. The data presented here demonstrate the existence of a multicomponent system capable of modulating the Ca^{2+} mobilization response to the second messenger Ins(1,4,5)P_3.

262 CALCIUM REGULATION IN BOVINE SPERMATOZOA. Srinivasan Vijayaraghavan, David Triebwasser and Dale D. Hoskins. Oregon Regional Primate Research Center, Beaverton, OR 97006.

Mammalian sperm acquire the capacity for motility during their passage through the epididymis. While intracellular levels of cyclic AMP are elevated during epididymal maturation of bovine sperm, the ability of sperm to accumulate calcium declined during maturation. The present study was undertaken to examine aspects of regulation of intracellular calcium in bovine sperm. First, using the intracellular calcium indicator Quin 2 we measured cytoplasmic calcium levels in bovine caudal sperm. When sperm were loaded with Quin 2 in the absence or presence of external calcium the levels were 62.5 $\pm$ 4.3 nM (n=25) and 188.8 $\pm$ 18.8 nM (n=16), respectively. Also, when sperm were loaded with Quin 2 in the presence of calcium and the mitochondrial uncoupler CCCP the levels of calcium were 55.5 $\pm$ 2.6 nM (n=13). These results, along with those using $^{45}Ca^{2+}$, suggested that calcium uptake in bovine sperm occurred only at the midpiece of the cell. Secondly, we examined the effects of bovine seminal plasma (BSP) and caudal epididymal fluid (CF) on calcium ($^{45}Ca^{2+}$) uptake in immature caput spermatozoa. BSP is known to contain a protein, caltrin, that inhibits calcium uptake in mature caudal sperm. Immature caput sperm, however, showed a biphasic response to BSP - a stimulation at 1 min and a subsequent inhibition of calcium uptake. Pre-incubation with BSP or CF eliminated the biphasic response and CF by itself stimulated calcium uptake in caput sperm, suggesting the presence of a factor(s) in these fluids that affects calcium uptake in immature caput sperm. Studies on the nature of the factor(s) would be useful in confirming the hypothesis that bovine sperm possess a unique mid-piece calcium regulatory mechanism. Supported by NIH grant HD18737.

263 CHARACTERIZATION OF cAMP-REGULATED, CALCIUM/CALMODULIN-REGULATED AND CALCIUM PHOSPHOLIPID-REGULATED PROTEIN PHOSPHORYLATION SYSTEMS IN RAT SARCOLEMMA. Walaas, S.I. (1,2) and R.S. Horn (1), (1) Institute of Medical Biochemistry, University of Oslo, 0317 Oslo, Norway, and (2) Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021.

Relatively little is known about the properties of protein phosphorylation systems in skeletal muscle sarcolemma. We have therefore characterized possible endogenous phosphoproteins in rat skeletal muscle sarcolemma. Purified sarcolemma has been phosphorylated in vitro with either cAMP-, cGMP-, calcium/calmodulin- or calcium/-phospholipid-dependent protein kinase, and the resulting phosphoproteins have been analyzed by various techniques. A minimum of 30 distinct phosphoproteins could be seen in these preparations, more than 20 of which could be phosphorylated by cAMP-dependent protein kinase, and 4 of which also could be phosphorylated by calcium/phospholipid-dependent protein kinase. In contrast, cGMP and calcium/calmodulin-dependent protein kinases catalyzed phosphorylation of only a few proteins. The majority of sarcolemma proteins phosphorylated by cAMP- or calcium/phospholipid-dependent protein kinase appeared to represent integral membrane proteins, being soluble in nonionic detergents. In contrast, only one 15 kilo dalton phosphoprotein behaved as a proteolipid, being soluble in acidified chloroform: methanol. This 15 kDa proteolipid may be a target for multiple receptor-regulated second messenger systems in the sarcolemma (see abstract by Walaas, O. et al., this meeting). We thank drs. K.A. Albert and A.C. Nairn, The Rockefeller University for the gift of purified protein kinases.

264 MULTISITE PHOSPHORYLATION OF A 15 KILODALTON SARCOLEMMA PROTEOLIPID, CATALYZED BY cAMP-DEPENDENT AND CALCIUM/PHOSPHOLIPID-DEPENDENT PROTEIN KINASE. Walaas, O. (1,2), Horn, R.S. (1) and Walaas, S.I. (1,3), (1) Institute of Medical Biochemistry, University of Oslo, 0317 Oslo, Norway, (2) California Metabolic Research Foundation, La Jolla, California 92038, and (3) Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021.

Rat sarcolemma contains a major 15 kDa proteolipid which can be phosphorylated in vitro both by cAMP- and calcium/phospholipid-dependent protein kinases (see abstract by S.I. Walaas and R.S. Horn, this meeting). The present study has characterized the phosphorylation of this proteolipid in more detail. Rat skeletal muscle sarcolemma has been phosphorylated with cAMP- and calcium/phospholipid-dependent protein kinases, aliquots have been lyophilized and extracted with acidic chloroform: methanol, and the phosphoproteins have been analyzed with a variety of techniques. Our results show that both the sarcolemma-localized and the extracted 15 kDa phosphoprotein could be electrofocused in urea-containing gels, with an apparent pI of 5.9. Thermolytic digests showed a minimum of two phosphorylation sites in the protein, which were independently phosphorylated on serine residues by cAMP- and calcium/phospholipid-dependent protein kinases, respectively. This sarcolemma proteolipid may thus be a target for distinct protein phosphorylation systems in intact skeletal muscle.

We thank drs. K.A. Albert and A.C. Nairn, The Rockefeller University, for the gift of purified protein kinases.

265 GUANYL-NUCLEOTIDE INDUCED LEYDIG CELL MEMBRANE PHOSPHORYLATION A CALCIUM DEPENDENT EVENT: IMPLICATIONS FOR HORMONE ACTIVATED ADENYLATE CYCLASE Christine Winters and Maria L. Dufau, Mol. Endo. Section, ERRB, NICHD, NIH, Bethesda, MD 20892

Rapid membrane events in the gonadotropin activated Leydig cell include GTP-induced phosphorylation of a 44,500 Mr protein. This phosphorylation is Ca^{++} modulated, being increased at low (nM) but inhibited at high (μM) concentration of free Ca^{++}. Adenylate cyclase activity is also effected by Ca^{++} in a similar biphasic manner. At submaximal levels of GTP (10^{-6}M), LH increases membrane phosphorylation by 30% over control levels. This event is cAMP independent, and is also induced by the phospholipids, phosphatidylserine (PS) and phosphatidylinositol (PI) and is enhanced by Ca^{++} but not the addition of diacylglycerols or phorbol esters. Phosphoaminoacid analysis demonstrated an increase in ^{32}P incorporation in phosphoserine. The Ca^{++} effects on adenylate cyclase activity are associated with changes in the number of enzyme units activated by the hormone with no changes in the apparent affinity, as reflected in the ED_{50} and IC_{50} of activation and inactivation by the hormone. These results indicate that a Ca^{++} dependent kinase system exists in the purified Leydig cell membrane. The molecular weight of the substrate, the nucleotide requirement, and the similar modulation by Ca^{++} of cyclase and phosphorylation, suggest that these events could influence the hormonal dependent G_s subunit-mediated interactions in the plasma membrane.

266 NUCLEOTIDE-REGULATED DEGRADATION OF EXOGENOUSLY ADDED PHOSPHOINOSITIDES BY MEMBRANES AND CYTOSOL R.J.H. Wojcikiewicz, M.A. Wallace, D. Bojanic, L.S. Bradham and J.N. Fain, Department of Biochemistry, University of Tennessee, Memphis; Memphis, TN 38163

We have examined the phospholipase C activities of membranes and cytosol from a variety of tissues using [^{3}H]-phosphatidylinositol 4,5-bisphosphate (^{3}H-PIP_2), [^{3}H]-phosphatidylinositol 4-phosphate (^{3}H-PIP) and [^{3}H]-phosphatidylinositol (^{3}H-PI). GH_3 cell membrane preparations degraded ^{3}H-PIP_2 and ^{3}H-PIP at similar rates. Degradation of ^{3}H-PI was much less rapid. The water soluble products of these reactions were inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate and inositol 1-phosphate respectively. Hydrolysis of ^{3}H-PIP_2 but not ^{3}H-PIP or ^{3}H-PI was stimulated by GTP, GTPγs and GppNHp (ED_{50} = 5-10μM). Other nucleotides, e.g. AppNHp, also stimulated ^{3}H-PIP_2 breakdown. AppNHp and GppNHp were equipotent and produced the same degree of maximal stimulation (160% of control). In contrast to membranes, GH_3 cell cytosol degraded ^{3}H-PIP_2, ^{3}H-PIP and ^{3}H-PI at similar rates. Again, though, only ^{3}H-PIP_2 degradation was nucleotide-sensitive. Nucleotide-stimulated ^{3}H-PIP_2 degradation was also seen in cytosol preparations from brain, adipose tissue, platelets and liver. NAD^+ (1mM) inhibited basal ^{3}H-PIP_2 degradation in membrane and cytosol preparations from GH_3 cells and adipose tissue by 60-90%. This inhibition was reversed by either AppNHp or GppNHp (100μM). These data show that degradation of exogenously added ^{3}H-PIP_2 by a phospholipase C is regulated by nucleotides. Whether the nucleotide effects on phospholipase C activity are direct or mediated by a discrete nucleotide-binding protein is currently under investigation.

267 SITE SELECTIVE cAMP ANALOGS IN THE REGULATION OF CELL PROLIFERATION AND TRANSFORMATION Y.S. Cho-Chung[1],T. Clair[1],P. Tagliaferri[1],S. Ally[1],D. Katsaros[1] and R.K. Robins[2];[1]National Cancer Institute, Bethesda, MD 20892, and [2]Nucleic Acid Res.Inst., ICN Pharm. Costa Mesa,CA 92626.

Sixteen site-selective cAMP analogs were used to study the mechanism of reverse transformation of the Ha-Mu-SV transformed NIH 3T3 clone 13-3B-4 cells. Treatment with combinations of analogs allowed the study of:(1)involvement of the cAMP receptor in growth control,(2) cooperativity of Site 1 and Site 2 binding,and (3) the use of lower analog concentrations with the combinations showing synergism of action. These cAMP analogs modified at either the C-8 position (Site-1 selective) or the C-6 position (Site-2 selective) were tested. The growth inhibitory potency for the individual analogs correlated better with their known K_a for protein kinase rather than with their known lipophilicity that measures the ability of the analog to penetrate the cell membrane. Both growth inhibition and morphological changes were enhanced synergistically by C-6 and C-8 analog combinations. The synergism of both processes by N^6 analogs when combined with 8-thio derivatives far exceeded that by N^6 analogs in combination with 8-amino derivatives suggesting a response of type II protein kinase. Further, within a few hours after cAMP analog treatment, the ratio of the R^{II}/R^I cAMP receptor proteins markedly increased, especially in the cell nucleus, and this increase in the R^{II}/R^I ratio was inversely related to a decrease in p21 protein expression.

It is concluded that the observed synergism of growth inhibition and phenotypic reversion is due to the binding of cAMP analogs to both intrachain sites of the cAMP receptor proteins and thus the cAMP receptor is most likely the cellular effector mediating cAMP regulation of the reverse transformation processes.

268 CYCLIC AMP AS A CO-INDUCER OF HORMONE DEPENDENT GENE EXPRESSION
Piotr Chomczynski, Elisabeth Bone and Leonard Kohn
Laboratory of Biochemistry and Metabolism NIDDK, NIH, Bethesda, MD.

The induction of thyroglobulin (Tg) gene expression by TSH is the first example for which cyclic AMP has been implicated as the physiological mediator of the transcriptional effects of a hormone. We have shown recently, however, that cyclic AMP does not faithfully mimic the action of TSH in rat thyroid FRTL-5 cells, indicating a more complex cooperation between cyclic AMP and TSH in the activation of the Tg gene. Evidence is presented here that a similar cooperation of cyclic AMP with TSH and insulin is observed in rat thyroid explant cultures. Explants were cultured for 6 days in TSH and insulin free medium followed by a 2 day culture period in the presence of appropriate hormones and agents. The effect of cyclic AMP, TSH and insulin on Tg gene expression was determined by Northern blot analysis of Tg mRNA accumulation. A small increase of Tg mRNA accumulation was observed when cyclic AMP or cholera toxin was added alone to the culture medium. However, cyclic AMP markedly potentiated stimulation of Tg mRNA by TSH or insulin. The highest level of Tg mRNA was observed in explants exposed to both TSH and insulin. Addition of cyclic AMP to these explants did not further increase accumulation of Tg mRNA. The addition of phenylisopropyladenosine (an inhibitor of hormonal activation of adenylate cyclase) diminished TSH-insulin induced accumulation of Tg mRNA by more than 50%. A marginal effect of cyclic AMP on Tg mRNA accumulation in explants cultured without TSH and insulin demonstrates clearly that cyclic AMP is not a sole mediator of TSH action in rat thyroid explants, thus confirming our previous results with thyroid FRTL-5 cells. Presented results indicate that cyclic AMP acts as a co-inducer of Tg gene expression by enhancing the transcriptional signals of TSH and insulin.

269 KINETICS OF EPIDERMAL GROWTH FACTOR BINDING AND PROCESSING IN ISOLATED INTACT RAT HEPATOCYTES. I.P. Gladhaug and T. Christoffersen. Department of Pharmacology, University of Oslo, P.O.Box 1057 Blindern, 0316 Oslo 3, Norway.

The kinetics of binding, internalization and degradation of epidermal growth factor (EGF) was studied in freshly isolated rat hepatocytes. After isolation the hepatocytes had a nonhomogeneous population of surface EGF receptors consisting of approximately 9,000 high affinity sites (K_d 21 pM) and 165,000 low affinity sites (K_d 0.62 nM) as determined by Scatchard analysis. Incubation at 37°C (45 min) increased the number of surface receptors per cell to about 260,000. This increase was cycloheximide-sensitive and selective for the low-affinity receptors. During 5 h incubation at 37°C the hepatocytes internalized 6-7 times more EGF molecules than the number of cell surface receptors, based on clearance measurements. The uptake was unaffected by cycloheximide. Concomitant estimation, using acid/salt elution, of surface-bound EGF and internalized EGF showed that the number of internalized EGF molecules exceeded the decrease in surface-binding 6 times. The ratio between internalized EGF and the decrease in surface binding was temperature-dependent, being reduced to a one-to-one stoichiometry at 10°C. After down-regulation (80%) induced by 5 nM unlabeled EGF the surface EGF receptors did not recover during subsequent incubation (2 h) at 37°C. However, the remaining surface receptors internalized EGF in 9-fold excess of their number. The large discrepancy between internalization capacity and cell surface binding capacity was also found in the presence of cycloheximide. The results suggest that internalized EGF receptors are partly replaced by externalization of preformed intracellular receptors during EGF uptake in isolated hepatocytes, involving recycling of a small population of EGF receptors and/or recruitment of unexposed, preexisting receptors.

270 TRANSFORMING GROWTH FACTOR α(TGFα) AND EPIDERMAL GROWTH FACTOR (EGF) INHIBIT PTH-RESPONSIVE ADENYLATE CYCLASE (AC) OF CLONAL OSTEOBLAST-LIKE CELLS. G.E. Gutierrez, G.R. Mundy, R. Derynck, E.L. Hewlett, and M.S. Katz. Univ. of Texas Health Science Center and Audie Murphy VA Hospital, San Antonio, TX; Genentech Inc., So. San Francisco, CA; and Univ. of Virginia, Charlottesville, VA, USA.

A number of solid tumors produce TGFα and factors which activate PTH-sensitive AC of osteoblasts. Since growth factors such as EGF influence hormone activation of AC in several systems, we evaluated the effects of TGFα and EGF on the PTH-responsive AC of clonal osteoblast-like osteosarcoma cells (UMR-106). AC of UMR-106 cells incubated for 48hr+TGFα or EGF was assayed in intact cells after incorporation of [^{3}H]-adenine by measuring production of [^{3}H] cAMP from [^{3}H] ATP. Recombinant TGFα and EGF each produced concentration-dependent inhibition of PTH ($4x10^{-7}$ M)-responsive AC, with maximal inhibition of 38-44% at 3.0ng/mL. Half-maximal inhibition occurred at 0.07ng/ml TGFα and 1.0ng/ml EGF. TGFα and EGF also inhibited beta adrenergic agonist (isoproterenol, Iso; 10^{-6} M)-stimulated AC of UMR-106 cells by 32%, but neither growth factor affected enzyme response to PGE_2 (10^{-5} M) or basal activity. Nonreceptor-mediated activation of AC by forskolin (10^{-4} M) and cholera toxin (1μg/ml) was inhibited 19% by TGFα and EGF. Pertussis toxin (PT; 100ng/ml) augmented PTH-stimulated AC, suggesting modulation of PTH response by a functional inhibitory guanine nucleotide-binding regulatory protein (N_i). However, PT had no effect on TGFα inhibition of PTH response. Treatment of UMR-106 cells with cycloheximide (1-5μg/ml), an inhibitor of protein synthesis, decreased the degree of TGFα inhibition of PTH- and Iso-stimulated AC to 10-18%. In conclusion, TGFα and EGF selectively impair PTH- and beta adrenergic-responsive AC of osteoblast-like cells (UMR-106) by actions which 1) may be exerted at the receptor for stimulatory agonist and also at nonreceptor components excluding N_i, and 2) may require protein synthesis. In addition, inhibition of PTH responsiveness of osteoblasts by growth factors conceivably explains why solid tumors secreting both PTH-like factors and growth factors may be associated with decreased bone formation.

271 IS THE ANTIAGGREGATORY EFFECT OF AD_6 CORRELATED WITH INHIBITION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES?

G. Hakim, D. Fiorentini, A. Falasca, M. Prosdocimi and C.A. Rossi
Institute of Biochemistry, University of Bologna, Bologna and Fidia Neurobiological Research Laboratories, Abano Terme, ITALY

Cyclic AMP is clearly implicated in platelet function, since an increase in cyclic AMP levels leads to the inhibition of platelet aggregation. On the other hand, the effect of cyclic GMP remains controversial. AD_6 (8-monochloro-3-beta-diethylaminoethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin) has been shown to be a powerful inhibitor of human platelet aggregation in vitro induced by a variety of different stimuli (Prosdocimi et al., Naunyn-Schmiedeberg Arch. Pharmacol. (1986) 332, 305). An investigation on the inhibitory effect of AD_6 upon phosphodiesterases (PDE) from human platelets is reported.
A cyclic GMP specific PDE and a cyclic AMP specific PDE have been prepared by ion-exchange chromatography of a platelet extract. Cyclic GMP PDE is selectively inhibited by AD_6 (Ki =0.2 mM); cyclic AMP PDE is quite less sensitive to AD_6 (IC_{50}> 1.2 mM at 1 µM cAMP). In fact AD_6, at a concentration (0.1 mM) which strongly inhibits platelet aggregation, has no effect on cAMP levels in platelets.
The effect of AD_6 has been also investigated on a calcium-calmodulin sensitive PDE partially purified from bovine brain; the drug has a poor inhibitory effect on the stimulated enzyme.
Our results suggest that the antiaggregatory effect of AD_6 is not related to cAMP PDE, while some role for cyclic GMP could be involved. Studies on the effects of AD_6 on cyclic GMP content in platelets are now in progress.
Supported by MPI grants.

272 EXPRESSION OF c-fos AND c-myc mRNA IN GROWTH STIMULATION OF PORCINE THYROID FOLLICLE CELLS IN CULTURE: N.-E. Heldin and B. Westermark, Dept. of Pathology, University Hospital, University of Uppsala, Sweden.

The proto-oncogenes c-fos and c-myc are believed to be involved in normal growth control. The growth regulation of the thyroid gland is rather complex. Thyrotropin (TSH) was until a few years ago generally accepted as the only regulator of thyroid growth. In porcine thyroid cells in culture epidermal growth factor (EGF) is the most potent mitogen, but also high concentrations of iodide stimulates the incorporation of ^{3}H-thymidine. TSH is not a growth promoting agent for these cells although it increases their functional capacity. The object of the present investigation was to study the expression of c-fos and c-myc mRNA in thyroid follicle cells after growth stimulation.

Addition of 10 ng/ml of EGF resulted in a transient increase in the c-fos mRNA (2.2 kb) level with a peak reached after 30 minutes. Enhanced c-myc mRNA (2.4 kb) expression was observed after 30 min., with a maximum after 2 hours and remained at a high level during the 24 h investigated.

TSH (10 mU/ml) had no stimulatory effect on the c-fos and c-myc mRNA expression.

Addition of 10^{-4} M of iodide (KI) enhanced the expression of c-myc mRNA but not c-fos mRNA. The increase was observed 6 h after stimulation, i.e. 4-5 h later than after an EGF-stimulation.

Our results show that an increased c-myc and c-fos (only with EGF) expression are early events in the prereplicative phase of porcine thyroid follicle cells, whether stimulated by a polypeptide growth factor (EGF) or an ion (iodide). The growth promoting activity of iodide has previously been shown to be coupled to a decrease in adenylate cyclase activity. In view of the fact that this effect may require several metabolic steps, including iodide organification, the finding of a lag in c-myc expression in iodide-treated cells compared to cells exposed to EGF, is not unexpected.

273 RELATIONSHIP BETWEEN THE $pp60^{v\text{-}src}$ PHOSPHORYLATION SITE AND THE INHIBITOR-2 BINDING SITE OF PROTEIN PHOSPHATASE 1 INVOLVED IN THE FORMATION OF THE MgATP-DEPENDENT PROTEIN PHOSPHATASE.

T.S. Ingebritsen and J.W. Johansen, Department of Pharmacology, University of Colorado Medical School, Denver, CO 80262.

Protein phosphatase 1 is regulated through the phosphorylation and dephosphorylation of three heat stable inhibitor proteins, inhibitor-1, inhibitor-2 and DARPP-32. Prolonged incubation of protein phosphatase 1 with inhibitor-2 leads to the inactivation of the protein phosphatase due to a change in the conformation of the enzyme. This inactivation can be reversed by phosphorylation of inhibitor-2 on a threonine by glycogen synthase kinase 3 (GSK-3). Protein phosphatase 1 is also regulated through the direct phosphorylation of the enzyme by $pp60^{v\text{-}src}$, the protein product of the src oncogene. Phosphorylation of the protein phosphatase results in a loss of activity due to a decrease in affinity for substrate proteins. The $pp60^{v\text{-}src}$ phosphorylation site has been localized to a small, protease-sensitive domain (Mr<4,000). Protein phosphatase 1 is not a substrate for $pp60^{v\text{-}src}$ when it is complexed with inhibitor-2 to form the MgATP-dependent protein phosphatase. This result is observed both before and after activation of the protein phosphatase by GSK-3. This indicates that the presence of bound inhibitor-2 limits the access of $pp60^{v\text{-}src}$ to the phosphorylation site presumably via steric hinderance and suggests that the $pp60^{v\text{-}src}$ phosphorylation site is either closely associated with or is part of the inhibitor-2 binding site involved in the formation of the MgATP-dependent protein phosphatase.

274 TRANSFORMING GROWTH FACTOR-β REGULATES FOLLICLE-STIMULATING HORMONE-INDUCED GRANULOSA CELL DEVELOPMENT THROUGH cAMP-DEPENDENT AND cAMP-INDEPENDENT MECHANISMS. Michael Knecht, Pei Feng, and Kevin Catt, ERRB/NICHD/NIH, Bethesda, MD 20892

Ovarian granulosa cells from diethylstilbestrol-implanted immature rats respond to follicle-stimulating hormone (FSH) with several hundred-fold increases in steroidogenesis and polypeptide hormone receptor expression. These stimulatory effects of FSH are dependent upon the activation of adenylate cyclase and are reproduced by cAMP-inducing ligands, while removal of cAMP results in the regression of granulosa cells. Transforming growth factor-β (TGF-β) had a bifunctional effect on FSH-induced cAMP production during a 72 h culture. The growth factor (16 pM) enhanced the effects of 2.5-10 ng FSH/ml by 2 to 3-fold, but suppressed responses to FSH levels over 25 ng/ml by 50%. The actions of TGF-β were concentration-dependent over the range of 0.8 to 80 pM, with maximal effects at 16 pM of the growth factor. TGF-β alone had no effect upon cAMP formation or cellular growth. In contrast to its bifunctional effects on FSH action, TGF-β did not alter choleragen- or forskolin-induced cAMP production. Thus, the growth factor may modify FSH receptor content or coupling of the FSH receptor:cyclase system. TGF-β also altered the actions of exogenous cAMP, since it enhanced the effects of 8-bromo-cAMP, choleragen, and forskolin on the expression of both luteinizing hormone and EGF receptors by 2-fold. These results indicate that TGF-β modifies gonadotropin-induced cAMP production and cAMP action in the developing granulosa cell and is involved in the maturation of an endocrine-responsive target cell.

275 BINDING OF 2-CHLOROADENOSINE TO BOVINE SPERMATOZOA. Ling, Xias-Yang, S. Vijayaraghavan, and D. D. Hoskins. Oregon Regional Primate Research Center, Beaverton, OR 97006.

Mammalian sperm are unique in that receptors for modulating signal transduction through binding to receptors is not known to occur. This study was undertaken to determine if receptors are involved in the mechanism by which adenosine and a number of analogs initiate motility in immotile, bovine caput epididymal sperm and stimulate motility in motile, inactive caudal sperm (Biol. Reprod. 34:468-477, 1986). More recently we have shown that addition of adenosine deaminase inhibitors and competitive substrates markedly stimulates motility in submotile caudal sperm. Specific binding of 2-chloroadenosine (2-Cl-Ad) to washed caudal sperm was determined as a function of time, temperature, and concentration. Highest specific binding (80%) occurs at 0° for 2.5 hrs, associated constants are: K_d, 10 μM and Bmax, 3.15×10^{-2} μM/10^8 sperm. Theophylline has no effect on binding and numerous adenosine analogs do not displace 2-Cl-Ad in a manner related to motility initiation. Nitrobenzylthioinosine (0.2 mM) completely blocks 2-Cl-Ad binding to intact sperm. These data suggest that 2-Cl-Ad may bind to an adenosine uptake site and that binding is unrelated to motility initiation. Binding to sperm plasma membranes requires the presence of protease inhibitors during membrane isolation. High specific binding (80%) to head plasma membranes has been found but activities have been low and variable. Supported by NIH grant HD18737.

276 CALMODULIN IN BLOOD AND SALIVA AND ITS ASSOCIATION WITH EPIDERMAL GROWTH FACTOR (EGF). S. Mac Neil, R. Dawson, C. H. Barton, L. Hanford, M. McGurk, R. Metcalfe and D. S. Munro Department of Medicine, Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU, U.K.

We have previously reported that calmodulin activity in human serum originates from the platelet which specifically releases biochemically-identifiable calmodulin in response to thrombin stimulation (Mac Neil et al. Bioscience Reports, 4, 643-650). As extracellular calmodulin can stimulate cell division in cultured cells (Mac Neil et al. J. Invest. Dermatol. 83, 15-19, 1984) we are investigating a possible extracellular role for calmodulin. Levels of serum calmodulin (20 volunteers) ranged from 27 to 1220 ng/ml ($\bar{x}$ of 297 ± 70 SEM) and plasma levels from 10 to 286 ng/ml (12 volunteers, $\bar{x}$ of 135 ± 30). Release of calmodulin from platelets (which contained up to 15 μg of calmodulin per mg of platelet protein) was associated with the release of other platelet products - EGF was released in all experiments where calmodulin was released and neither was released when platelet release was prevented. Investigating a possible association between calmodulin and EGF we next examined saliva and found EGF levels of 2-7 ng/ml and calmodulin activity of 0.2 to 4μg/ml. Oral surgery elevates EGF secretion in normal individuals. Both EGF and calmodulin secretion were similarly elevated (by 100% $p < 0.05$ for both) 24 hr post-surgery. The correlation between the rate of EGF secretion and the rate of calmodulin secretion was $r = 0.82$ (based on 26 salivary samples from 7 patients). Approximately 80 moles of calmodulin were secreted per mole of EGF. The calmodulin activity in saliva is recognised by calmodulin antibody and satisfies several other criteria for authentic calmodulin. We suggest that the high levels of calmodulin activity in blood and saliva associated with at least one other growth factor (EGF) merits further investigation of an extracellular role for calmodulin.

277 MONOCLONAL ANTIBODIES AGAINST TYPE II RAT BRAIN PROTEIN KINASE C. Hiroki Nakabayashi and Kuo-Ping Huang, NICHD, NIH, Bethesda, MD 20892

Three isozymic forms of rat brain protein kinase C had been purified to near homogeneity. These enzymes were designated type I, II, and III protein kinase C based on their elution profile from hydroxylapatite column in an order of increasing phosphate buffer concentration. All three isozymes have the same molecular weight of 82,000, undergo autophosphorylation in the presence of Ca^{2+}, phosphatidylserine (PS), and diolein (DG), and bind [^{3}H]phorbol-12,13-dibutyrate (PDBu) dependent on the presence of Ca^{2+} and PS. Three monoclonal antibodies (8/1, 10/10, and 25/3) of the IgG_1 class had been prepared against the type II rat brain protein kinase C. All these antibodies immunoprecipitated the type II isozyme in a dose-dependent manner but did neither to type I nor type III isozyme. The immune complexes of type II isozyme and the antibodies retain all the kinase activity, which is dependent on Ca^{2+}/PS and further stimulated by DG. The concentrations of Ca^{2+} and PS required for half-maximal kinase activity were not influenced by the presence of antibodies. The initial rate and final extent of protein kinase C autophosphorylation, however, were reduced by the addition of the antibodies. The concentration of PS required for half-maximal PDBu binding and the Kd of PDBu were unchanged in the presence of the antibodies. The total binding of PDBu in the presence of one antibody (8/1) but not the other two, was reduced by 50%. Immunoblot analysis of the trypsin-degraded protein kinase C revealed that all three monoclonal antibodies recognized the 33-38-KDa fragment, the PS/PDBu-binding domain, but not the 45-48-KDa fragment, the kinase catalytic domain, of the enzyme. These results indicate that the PS/PDBu-binding domain of the type II protein kinase C contains the major immuno-reactive determinant for all these monoclonal antibodies; however, the effects of these antibodies on the binding of PDBu are different.

278 DIFFERENTIAL DISTRIBUTION AND DEVELOPMENTAL EXPRESSION OF THE ISOZYMIC FORMS OF PROTEIN KINASE C IN RAT BRAIN. Yasuyoshi Yoshida, Freesia Huang, Hiroki Nakabayashi, and Kuo-Ping Huang, NICHD, NIH, Bethesda, MD 20892.

Polyclonal antibodies against rat brain PKC were raised in goat. These antibodies can neutralize completely the purified rat brain PKC as well as the total PKC activity in the crude brain homogenate. The antibodies also recognize the same enzyme from other rat tissues. Neuronal tissues and lymphoid organs were found to be highly enriched in PKC, while lung, kidney, liver, heart, and skeletal muscle contained relatively low level of this kinase. We have also characterized three isozymic forms of PKC from rat brain. Monospecific antibodies against type I, II, and III isozymes were prepared by specific absorption of polyclonal antibodies to each type of isozyme previously electrophoretically transferred to nitrocellulose membrane. Each monospecific antibody preparation recognized preferentially its own antigen by immunoblotting. These antibodies were used to type the presence of isozymic forms of PKC in the various regions of rat brain and to determine the expression of these isozymes during brain development. The type I isozyme was found to be enriched in cerebellum, the type II isozyme in cerebral cortex, thalamus, and corpus callosum, and the type III isozyme in olfactory bulb. During brain development the type I enzyme was very low in the fetus and rats within one-week of age. At least a 3-5-fold increase in the type I enzyme was observed between one-and two-week of age and remained constant thereafter up to six-week of age. Both the type II and type III enzymes were progressively increased from three day before birth up to two-week of age and remained constant thereafter up to six-week of age. These results demonstrate that the various protein kinase C isozymes are enriched in distinct regions of rat brain and the expression of these isozymes is developmentally controlled.

279 PROTEOLYTIC DEGRADATION OF PROTEIN KINASE C IN THE PHORBOL ESTER-INDUCED INTERLEUKIN-2 SECRETING THYMOMA CELLS. Freesia Huang, Prince Arora, Edgar Hanna, and Kuo-Ping Huang, NICHD, NIH, Bethesda, MD 20892

Phorbol 12-myristate 13-acetate (PMA) induces the Interleukin-2 (Il-2) production in mouse thymoma cells of EL4, though the mechanism of action is not known. The effects of PMA on EL4 protein kinase C (PKC) were studied by using polyclonal antibodies raised against rat brain PKC. These antibodies inhibited PKC activity completely and detected an 82-KDa protein by immunoblot analysis in both crude homogenate and partially purified kinase preparation. PMA at 10ng/ml elicited a time-dependent loss of PKC (82 KDa) protein from the soluble fraction (earliest time tested was 2 min); an early rise (peak at 5 min) and subsequent loss of PKC from the particulate fraction was also observed. Consequently, PKC in the whole homogenate disappeared gradually (>50% loss in 30 min). These effects were dose-dependent and were not caused by the inactive analog, 4-α phorbol. With continuous presence of PMA, PKC remained very low. However, removal of PMA from the medium caused the reappearance of PKC after 24 hr, and the newly synthesized PKC was mainly located in the soluble fraction. These PMA effects were tested on a cloned line of EL4 (IEL4) which was known to be unresponsive to PMA-induced Il-2 production. At the same concentration (10ng/ml), PMA also induced a fast and time-dependent translocation of PKC from the soluble to the particulate fraction of IEL4. However, the degradation of membrane associated PKC was greatly retarded; up to 30 min the 82-KDa protein remained high and there was no change of PKC in the whole homogenate. These results suggested that translocation and degradation of PKC were early events of the PMA-induced Il-2 production; the retardation of PKC degradation resulted in the failure of Il-2 production in IEL4.

280 cAMP REGULATES THE TRASCRIPTIONAL ACTIVITY OF M-MuLV LTR

N. Majam[1], H. Fan[2], and Y.S. Cho-Chung [1]; [1]National Cancer Institute, NIH, Bethesda, MD 20892; [2]Dept. of Molecular Biology and Biochemistry, University of California, Irvine, CA, 92717.

Site 1- and Site 2- selective analogs in combination synergistically produced growth inhibition and phenotypic reversion of Ha-MuSV-transformed NIH/3T3 clone 13-3B-4 cells (P.Tagliaferri, et.al. Biochem. Biophys. Res. Commun. 130, 1193-1200, 1985). In this reverse transformation process, the increase of the ratio of the R^{II}/R^{I} cAMP receptor proteins , especially at the cell nucleus was found to be a primary event and this increase in the R^{II}/R^{I} ratio was inversely related to the suppression of p21 ras protein expression. In this work we investigated whether the cAMP analog effect on the p21 ras protein suppression in 13-3B-4 cells could be at the level of LTR expression.The effect of cAMP on the trascriptional activities of the wild type and deleted M-MuLV LTRs was assessed by fusion of the LTR to the bacterial CAT gene followed by transient expression assays in NIH/3T3 cells. Treatment of NIH/3T3 cells containing the wild Type LTR for 24 hr with the Site-2 analog, N^6-butyryl cAMP (25um)+ the Site-1 analog, 8-thiomethyl-cAMP (5μM) resulted in over 80% inhibition of the CAT expression ; the same inhibition was also obtained with each of these analogs or with other analogs singly but at 10-100 fold higher concentrations. A series of deletions of the tandemly repeated enhancer sequences (-194 to -150 bp) decreased the promoter activity and abolished the cAMP analog effect on the CAT expression. Deletions of the sequences below the tandem repeats between the Xba I site and CAAT box (-150 to -120), however, decreased the promoter activity only without affecting the cAMP inhibitory effect on the CAT expression.

These results suggest that cAMP in mammalian cells regulates gene expression at specific sequences.

281 CYCLIC AMP INCREASES THE mRNA LEVELS FOR THE REGULATORY SUBUNIT ($R\text{-}II_{51}$) OF TYPE II cAMP-DEPENDENT PROTEIN KINASE IN RAT SERTOLI CELLS.

O. Øyen[1,2], W. Eskild[2], H. Attramadal[1,2], I.P. Gladhaug[3], F.O. Levy[2], G. Knutsen[2], J.S. Richards[4], V. Hansson[2], and T. Jahnsen[1,2]. [1]Institute of Pathology, Rikshospitalet, Oslo, Norway; [2]Institute of Medical Biochemistry, University of Oslo, Norway; [3]Institute of Pharmacology, University of Oslo, Norway; [4]Department of Cell Biology, Baylor College of Medicine, Houston, Texas.

Testicular function is dependent on FSH/cAMP stimulation of Sertoli cells. All effects of cAMP are supposed to be mediated via cAMP-dependent protein kinases. The present study was undertaken in order to investigate FSH/cAMP regulation of $R\text{-}II_{51}$ in rat Sertoli cells. Total RNA was extracted from rat testes of different ages (5-100 days) and from rat Sertoli cell cultures after stimulation with FSH or dibutyryl-cAMP (db-cAMP). Messenger RNA for $R\text{-}II_{51}$ was quantitated by Northern analyses using 20 μg of total RNA and a nick-translated rat $R\text{-}II_{51}$ cDNA probe. Two mRNA species for $R\text{-}II_{51}$ (1.8 kb and 3.2 kb) were detected in rat testes. The mRNA levels for $R\text{-}II_{51}$ in rat testes increased from day 5 to day 15, showed high levels until day 25 after which it gradually decreased. Sertoli cell cultures from immature rat testes stimulated with db-cAMP (0.1-1.0 mM) and FSH (750 ng/ml) for 48hrs showed a 10-50 fold increase in $R\text{-}II_{51}$ mRNA. The elevated levels were due to an increase in the 3.2 kb mRNA for $R\text{-}II_{51}$. No increase in $R\text{-}II_{51}$ was obeserved when primary hepatocytes were incubated for 48hrs in the presence of 1 mM db-cAMP. These results show that FSH and cAMP in cultured Sertoli cells cause a cell-specific increase in mRNA for $R\text{-}II_{51}$ and that this may represent an important self-amplifying mechanism for FSH and cAMP action on Sertoli cells.

282 **INHIBITORY EFFECT OF A RAS ONCOGENE ON PLATELET-DERIVED GROWTH FACTOR (PDGF)-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN MURINE FIBROBLASTS.**

Gregory Parries, Robin Hoebel, and Efraim Racker. Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853.

We have investigated the influence of a *ras* oncogene (Land *et al.* (1983) *Nature* 304, 596) on PDGF-stimulated phosphatidylinositol (PI) metabolism in Rat-1 and NIH 3T3 fibroblasts. Incubation of [^{3}H]inositol-labeled Rat-1 cells with purified human PDGF (5 ng/ml) resulted in 2- to 3-fold increases in [^{3}H]IP_3 levels within 90 s and, in the presence of 25 mM Li^+, a 4-fold increase in the level of [^{3}H]IP_1 after 30 min. In contrast, incubation of EJ-*ras*-transfected Rat-1 cells (R1-EJ2 line) with PDGF resulted in little or no accumulation of either [^{3}H]IP_3 or [^{3}H]IP_1. This lack of response was not due to an absence of PDGF receptors since the number of specific [^{125}I]PDGF binding sites on R1-EJ2 cells was at least 80% of that on Rat-1 cells. In serum-free media the basal rates of PI turnover (i.e., the kinetics of [^{3}H]IP_1 accumulation in the presence of Li^+) in Rat-1 and R1-EJ2 cells were similar. Whereas PDGF stimulated PI turnover only in the parental Rat-1 cells, both the parental and EJ-*ras*-transfected cell lines were stimulated approximately 2-fold by thrombin (250 ng/ml) and 4- to 5-fold by 5% plasma-derived serum (PDS). In the presence of PDS, the rate of PI turnover in R1-EJ2 cells was similar to that in PDGF-stimulated Rat-1 cells. PDGF also induced a marked stimulation of PI metabolism in NIH 3T3 cells, but not in v-Ha-*ras*-transfected or Kirsten murine sarcoma virus-transformed 3T3 cell lines. On the other hand, NIH 3T3 cells that were transfected with v-*src* responded to PDGF to a similar extent as the parental cells. Using NIH 3T3 cells transfected with an expression vector containing a glucocorticoid-sensitive mouse mammary tumor virus LTR-linked v-Ha-*ras* gene, the induction of $p21^{v\text{-}Ha\text{-}ras}$ with 2 μM dexamethasone for 26 h was associated with a 91% reduction in PDGF-stimulated [^{3}H]inositol incorporation into PI; dexamethasone decreased the PDGF stimulation by about 40% in control 3T3 cells. These results suggest that a *ras* oncogene product, p21, may exert an inhibitory effect on the PDGF activation of phosphoinositide hydrolysis. (Supported by American Cancer Society grant PF-2683 and NIH grant CA-08964).

283

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE GENE. **Gregory Podgorski, Jakob Franke, Marie-Lise Lacombe, and Richard Kessin, Dept. of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, 630 W 168th St, New York, New York, 10032.**

We have cloned a cyclic nucleotide phosphodiesterase gene which codes for a developmentally regulated glycoprotein secreted by Dictyostelium discoideum. The organism uses cAMP as a chemoattractant during aggregation and the enzyme is part of the system which controls the levels of extracellular cAMP. The complete sequence of the phosphodiesterase has been determined from overlapping cDNA clones; the cDNA codes for a protein of 51,000 daltons. The genomic sequence has also been determined and except for a small 5' intron is identical to the cDNA sequence. The gene exists as a single copy in the genome and has no homology with other known cyclic nucleotide binding proteins. During the growth phase of the Dictyostelium life cycle, the mRNA of the phosphodiesterase is present at low levels as a 1.8 kb transcript. Early in the developmental phase a 2.3 kb phosphodiesterase transcript is induced. The level of mRNA is proportional to the amount of enzyme secreted. Mutants which lack phosphodiesterase activity, but which do not appear to be defective in other functions, have greatly reduced levels of transcript.

284

PROTEINS RELATED TO THE PROTO-ONCOGENE PROTEIN KINASES fes, abl, src and erb-B IN THE RAT STRIATUM AND HIPPOCAMPUS: A DEVELOPMENTAL PROFILE. R. Simantov, W.M. Cowan and H. Niman*. The Salk Institute for Biological Studies and The Research Institute of Scripps Clinic*, La Jolla, CA 92138.

It is now widely accepted that protein kinases play a pivotal role in the function of mammalian cells in general, and oncogenesis in particular. The abundance of several proto-oncogenes in the nervous system prompted us to investigate the appearance of proteins related to fes, abl, src and erb-B protein kinases during the development of two well defined brain structures, the striatum and the hippocampus. Sequence directed monoclonal antibodies recognizing a specific portion of the above mentioned proto-oncogenes were used to identify the proteins in embryonic, newborn, adult and aged rats. The immunoreactive proteins were analyzed on Western blots and visualized with ^{125}I-protein A. It was found that proteins related to each of the four oncogenes undergo characteristic developmental changes; e.g., a clear decline was observed in the level of the 18K, 60K and 130K proteins recognized by the antibodies to fes, abl and erb-B, respectively. The antibody against fes, however, recognizes a 150K protein in the adult but not embryonic striatum. Similarly, an antibody to the src kinase detects much higher levels of a 60K protein in the adult tissues as compared to the embryos. In conjunction with recent findings concerning the tyrosine-protein kinase activity of the c-src in cultured striatal cells and during development (Simantov et al., Neuroscience Abstracts, 1986), these experiments indicate that several proto-oncogene related protein kinases are developmentally regulated in the brain.

285 GANGLIOSIDES AS BIMODAL REGULATORS OF CELL GROWTH by Sarah Spiegel, Section on Membrane Biochemistry, Developmental and Metabolic Neurological Branch, NINCDS, Bethesda, MD

Gangliosides, sialic acid containing glycosphingolipids have been implicated in the regulation of cell growth, based on the dramatic changes in ganglioside composition during oncogenic transformation, cell cycle, and density-dependent growth inhibition. To directly examine potential functions for endogenous gangliosides, we have recently developed a new approach (1). The B subunit of cholera toxin (B), which binds exclusively to several molecules of gangliosides GM1 on the cell surface, was used as a ganglioside-specific probe to induce cell proliferation. B stimulated DNA synthesis and cell division in quiescent, non-transformed mouse 3T3 cells (NIH, Balb/C, and Swiss) in a dose-dependent fashion. Previously, we had shown that B was mitogenic for resting lymphocytes (1). Thus, the ability of B to stimulate resting cells to divide appears to be a general phenomenon. In addition, B remarkably potentiated the effects of other mitogens, such as serum, EGF, PDGF, and insulin. This synergistic effect indicates that B regulates the biological activity of these growth factors through a different, not yet identified pathway. In distinct contrast to its effects on quiescent cells, B inhibited the growth of transformed 3T3 cells as well as rapidly dividing normal 3T3 cells. Thus, we were able to demonstrate a bimodal response to B by the same cells just by varying their state of growth. Transformed 3T3 cells have less GM1 than normal 3T3 cells and it has been reported that levels of surface gangliosides increase as normal 3T3 cells reach confluency. Thus, the bimodel response to B may be related to the amount of surface GM1 being occupied. Our findings suggest that endogenous gangliosides may play a role as membrane transducers of both positive and negative signals that regulate cell growth. (1) Spiegel, S et al (1985) Science 230, 1285

286 **COMPARATIVE CHARACTERIZATION OF RECEPTOR AND NON-RECEPTOR ASSOCIATED PROTEIN TYROSINE KINASES.** Ashok K. Srivastava, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, (Quebec), H2W 1R7, CANADA

By using poly (glu: Tyr; 4:1) as an exogenous substrate, the characteristics of insulin receptor associated protein tyrosine kinase (PTK) from rabbit skeletal muscle has been compared with a growth factor-independent, non-receptor-PTK partially purified from rat lung. The two PTK's phosphorylated poly (glu: Tyr; 4:1) very effectively with apparent Km values of 0.3 mg/ml for insulin receptor-PTK and 0.8 mg/ml for non-receptor-PTK. ATP was the preferred phosphoryl donor for both PTK's (Km = 150 μM), however, in the case of non-receptor PTK, GTP was able to partially replace ATP. ATP analogues, AMP-PNP and ATP-Y-S inhibited the activities of both enzymes. Receptor PTK showed more activity in presence of Mg^{2+} whereas non-receptor PTK required either Mg^{2+} or Mn^{2+} for activity although higher concentrations of Mg^{2+} were needed as compared to Mn^{2+}. Para hydroxy-mercurobenzoate (PHMB), a SH-group blocking agent, inhibited the activities of both PTK's suggesting the requirement of SH groups for enzymatic activities. Both enzymes were inhibited by fluorosulfonylbenzoyl 5' adenosine (FSBA) and bioflavonoid quercetin. NaCl also inhibited both kinases, although, non-receptor PTK was more sensitive to inhibition by NaCl. Autophosphorylation resulted in the activation of both the kinases. These data indicate that there are only few but significant differences in the characteristics of receptor and non-receptor associated PTK's. (Supported by Medical Research Council of Canada).

287 **SITE SELECTIVE cAMP ANALOGS ALTER THE cAMP RECEPTOR RATIO (R^{II}/R^{I}) AND PRODUCE GROWTH INHIBITION AND MORPHOLOGICAL CHANGE IN SEVERAL HUMAN BREAST AND COLON CANCER CELL LINES.** P. Tagliaferri[1], D. Katsaros[1], R.K. Robins[2], T. Clair[1] and Y.S. Cho-Chung[1]; [1] National Cancer Institute, Bethesda, MD 20892, and [2]Nucleic Acids Res. Instit., ICN Pharm. Corp. Costa Mesa, CA 92626.

The growth regulatory effect of site selective cAMP analogs, which have been shown to be many fold more active in protein kinase activation than previously studied derivatives, was investigated in seven breast and three colon human cancer cell lines. The regulatory subunit of the cAMP dependent protein kinase contains two intrachain binding sites, Site 1 and Site 2. cAMP analogs which bind to one of the two sites are known as Site 1-selective (C-2 and C-8 substituted analogs) and Site 2-selective (C-6 substituted analogs). Thirty five analogs, C-2, C-6, and C-8 monosubstituted, or C-2 + C-8 disubstituted, or C-6 + C-8 disubstituted, were tested for their growth regulatory effect on the breast cancer lines MCF-7, MCF-7ras, T-47D, MDA-MB-231, ZR-75-1 , BT-20 and HBL 100, and the colon cancer lines LS-174T, WIDr, and HT-29. At uM concentrations most of the analogs caused appreciable growth inhibition of all ten cell lines. The greatest inhibition was observed when using analogs containing a chlorine atom at the C-8 position: 8-Cl-cAMP and N^6-phenyl-8-thioClphenyl-cAMP, at 50uM, caused 70-80% growth inhibition of all ten cell lines. The C-6 analog, N^6-benzyl-cAMP, was the next most potent inhibitor, causing 50-60% growth inhibition at 50 μM. Growth inhibition was accompanied by a change in cell morphology and an increase in the cAMP receptor ratio (R^{II}/R^{I}), as measured by photoaffinity incorporation of [^{32}P]-8N_3cAMP into the cell lysates , followed by SDS-PAGE and autoradiography. The site selective cAMP analogs are potent inhibitors of the growth of human cancer cells and can serve to elucidate the mechanisms of cell growth regulation.

288 FUNCTION OF THE CYR1 AND RAS2 GENES IN YEAST

Isao Uno, Takehiro Oshima*, Hiroshi Mitsuzawa and Tatsuo Ishikawa; Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; *Suntory Institute for Biomedical Research, Shimamoto-cho, Osaka 618, Japan

Regulatory roles of cAMP in yeast, Saccharomyces cerevisiae, have been studied by isolation of mutants requiring cAMP and their suppressors. The cyr1 mutants possessed no detectable levels of adenylate cyclase activity and cAMP, and the growth of cells carrying the cyr1 mutation was arrested at the G1 phase of cell cycle in the absence of cAMP. On the other hand, S. cerevisiae contains two closely related, but distinct genes, RAS1 and RAS2. The ras2 mutants produced a low level of cAMP, and growth of these mutants were suppressed by the bcy1 mutation which suppressed the cyr1 mutation. The relationship between the cyr1 and ras2 mutations of yeast in production of cAMP was studied. Cloned CYR1 and RAS2 genes were expressed in S. cerevisiae and Escherichia coli cells. The products of the CYR1 and RAS2 genes were reconstituted GTP-dependent adenylate cyclase in vivo and in vitro. To determine the catlytic domain of the CYR1 gene product and the domain interacting with RAS2 gene product, several kinds of truncated CYR1 gene were made. Further certain promoters and initiation codon were connected to the truncated genes adjusted to their frame. Genetical and biochemical analyses of transformants carrying these genes revealed that the catalytic domain of the CYR1 product was coded by the 3'-terminal 1.2 Kb of the gene, and the domain interact with the RAS2 product was located adjacent to the catalytic domain. The results suggest that yeast GTP-dependent adenylate cyclase is composed of catalytic and regulatory proteins encoded by the CYR1 and RAS2 genes, respectively.