Berlin, 23rd – 27th September 1990

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The Cholinergic Synapse

Invited Speakers:
E. X. Albuquerque, Baltimore
E. Barnard, Cambridge
H. Betz, Heidelberg
H. Breer, Stuttgart
U. Brodbeck, Bern
J.-P. Changeux, Paris
J. Dani, Houston
J. O. Dolly, London
M. E. Eldefrawi, Baltimore
G. C. Fishbach, St. Louis
S. Fuchs, Rehovot
J. Gershoni, Bethesda
E. Gundelfinger, Hamburg
S. F. Heinemann, San Diego
F. Hucho, Berlin
R. Huganir, Baltimore
M. Israel, Gif sur Yvette
R. Jahn, Martinsried
A. Karlin, New York
R. Kelly, San Francisco
J. Koenig, Bordeaux
P. Layer, Tübingen
J. Lindstrom, San Diego
R. Llinas, New York
A. Maelicke, Mainz
L. G. Magazanik, Leningrad
J. Massoulié, Paris
J. McMahan, Stanford
E. Neumann, Bielefeld
J. Patrick, San Diego
R. Rahamimoff, Jerusalem
H. Rehm, Zurich
B. Sakmann, Heidelberg
D. Sattelle, Cambridge
R. Scheller, Stanford
I. Silman, Rehovot
H. Soreq, Jerusalem
R. Stroud, San Francisco
P. Taylor, La Jolla
V. Tsetlin, Moscow
A. Vincent, Oxford
V. Witzemann, Heidelberg
H. Zimmermann, Frankfurt

Honorary Chairman: Sir Bernard Katz

Organizing Committee:
J. Gershoni, F. Hucho, I. Silman

International Symposium
THE CHOLINERGIC SYNAPSE
Berlin, September 23rd - 27th,1990

Honorary chairman: Sir Bernard Katz
Organizers: J.Gershoni, Washington,D.C., F.Hucho, Berlin, I.Silman, Rehovot

Local organizers:
F.Hucho, K.Buchner, A.Gülzow, S.Hertling-Jaweed, H.Otto, C.Weise

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GENERAL INFORMATION

LOCATION

Grand Hotel Esplanade, Lützowufer 15, 1000 Berlin 30
The lectures will be given in the "WEISSER SAAL A"

REGISTRATION DESK

The registration desk will be open on Sunday, September 23rd, from 7 to 9 p.m. and mornings during the meeting. Its phone number is 262 88 70.

BADGES

Members of the local organizing committee are identified by a yellow tape on their badge. They will be pleased to assist you.

SLIDES

Speakers are invited to preview and load their slides at a table next to the information desk. **Lectures are planned for twenty minutes plus ten minutes for discussion.**

POSTERS

All poster presentations will take place in the Wintergarten. Poster presenters are invited to put up their posters on the board bearing their poster number. This number corresponds to the page number of the abstract in the program book. Tape will be available.

Posters 44 through 64 will be on display on Monday and Tuesday. They should be removed on Tuesday evening.

Posters 66 through 91 will be on display from Wednesday morning until the end of the meeting. They must be removed by 2 p.m. Thursday afternoon.

Poster discussion for numbers 44-64 will be Monday evening from 6.30 to 8 p.m. and for nos. 65-91 Wednesday evening from 6 to 8 p.m.

Poster presenters are requested to leave a note at their poster indicating when they will be present at the poster for additional discussion, e.g. during the coffee breaks.

LUNCH TICKETS

Registered participants have received three meal tickets for the lunches on Monday, Wednesday and Thursday at the hotel. Additional lunch tickets may be purchased for DM 40/lunch at the information desk.

EXCURSION

On Tuesday afternoon all participants are invited for a boat trip through Berlin including lunch and a reception by the Senate Department of Science and Research of the Berlin city government, represented by Staatssekretär Dr. Hans Kremendahl. - Departure will be at 2 p.m. from the dock directly in front of the Grand Hotel Esplanade.

SCIENTIFIC PROGRAM

Monday, Sept. 24th, 1990

8:45 Opening Remarks

TRANSMITTER RELEASE

Chair: Sir Bernard Katz/F. Hucho

9:00 **R. Llinás** - Recent studies on depolarization release coupling in the squid giant synapse

9:30 **H. Betz** - The synaptopore - Fact or Fiction?

10:00 **R. Jahn** - Synaptic vesicles as tools for understanding of neurotransmitter release.

10:30 **R.Kelly** - Generation of synaptic vesicles in non-neuronal cells.

11:00 Coffee Break/Poster session

Chair: R. Llinas/H. Betz

11:30 **J. O. Dolly** - Transmitter release probed with botulinum neurotoxins.

12:00 **H. Rehm** - The relationship between the dendrotoxin binding protein and the mammalian *Shaker*-Type K^+ channels.

12:30 **R. Rahamimoff** - Channels and views of the cholinergic nerve terminal.

13:00 - 14:30 Lunch

Monday, Sept. 24th, 1990

Chair: J.-P. Changeux/B. Sakmann

14:30 **H. Zimmermann** - Molecular control of synaptic vesicle exocytosis and synaptic vesicle membrane recycling.

15:00 **R. Scheller** - Molecular studies of synaptic function and development.

15:30 **M. Israël** - Characterization of a nerve terminal membrane protein that translocates acetylcholine upon calcium action.

16:00 **H. Breer** - *High* affinity transporter for choline.

16:30 Coffee Break/Poster session

SYNAPTOGENESIS AND GENE EXPRESSION (SESSION ONE)

Chair: S. Fuchs/P. Layer

17:00 **G. Fischbach** - Further characterization of an ACh receptor inducing activity (ARIA) that may promote the accumulation of receptors at developing synapses.

17:30 **U. J. McMahan** - Molecules that mediate the neuron-induced formation of postsynaptic apparatus in skeletal muscle fibers.

18:00 **J.-P. Changeux** - The Acetylcholine receptor : Functional architecture and regulation.

18:30 - 20:00 **POSTERS** - Numbers 44 through 64.

Tuesday, Sept. 25th, 1990

TRANSMITTER RECEPTION (SESSION ONE)

Chair: J. Gershoni/E. Neumann

9:00	**E. Gundelfinger** - Molecular analysis of the nicotinic cholinergic receptor system of *Drosophila melanogaster*.
9:30	**E. X. Albuquerque** - Characteristics of neuronal nicotinic acetylcholine receptor ion channels of rat hippocampal neurons.
10:00	**V. Tsetlin** - α-Neurotoxin interactions with acetylcholine receptor and other proteins.
10:30	**L. G. Magazanik** - Physiological significance of desensitization in neuromuscular junction.
11:00	Coffee Break/Poster session
11:30	**J. Lindstrom** - Structures of nicotinic receptors from muscles and nerves and of neuronal α-bungarotoxin-binding proteins.
12:00	**I. Silman** - Structural and functional studies on the phosphatidylinositol-anchored dimer of acetylcholinesterase purified from electric organ tissue of *Torpedo californica*.
12:30	**J. Massoulié** - Molecular polymorphism of cholinesterases: various modes of anchoring.
13:00	**U. Brodbeck** - Studies on mammalian acetylcholinesterases: Subunit assembly, membrane anchoring and state of glycosylation.
13:30 - 14:00	Pause
14:00	**Excursion** (boat trip with lunch and reception by the city government - departure from the dock directly in front of the hotel)

Wednesday, Sept. 26th, 1990

TRANSMITTER RECEPTION (SESSION TWO)

Chair: I. Silman/R. Jahn

9:00	**E. Neumann** - Single conductivity events and lipid-kinase activity of the *Torpedo californica* nicotinic acetylcholine receptor.
9:30	**M. Eldefrawi** - Nicotinic receptor optical sensor as a diagnostic device.
10:00	**J. Patrick** - Functional diversity of neuronal nicotinic acetylcholine receptors.
10:30	**R. Huganir** - Regulation of the nicotinic acetylcholine receptor by serine and tyrosine protein kinases
11:00	Coffee Break/Poster session
11:30	**D. Sattelle** - Insect nicotinic receptors: Functional properties of native receptors and of a cloned subunit cDNA expressed in Xenopus oocytes.
12:00	**B. Sakmann** - Identification of structural determinants of ion flow and selection in membrane channels by site-directed mutagenesis.
12:30	**J. Dani** - Calcium permeability and MK-801 block of the acetylcholine receptor channel.

13:00 - 14:30 Lunch

Wednesday, Sept. 26th, 1990

Chair: V. Tsetlin/H. Breer

14:30 **J. Gershoni** - The cholinergic binding site : structure and application.

15:00 **F. Kucho** - Functional domains of the acetylcholine receptor - A novel phosphorylation site in position δS362.

15:30 Coffee Break/Poster session

16:00 **A. Karlin** - Structure of the acetylcholine receptor.

16:30 **A. Maelicke** - α-bungarotoxin and the competing antibody WF6 recognize different patterns of amino acids within the same cholinergic subsite.

17:00 **R. Stroud** - Three-dimensional structure of the nicotinic acetylcholine receptor - What the structure of the acetylcholine receptor at 22 A resolution by low dose electron microscopy and x-ray diffraction to 12.5 A implies for function, location of the major associated 43 kD cytoskeletal protein, and structure change.

17:30 **V. Witzemann** - The nicotinic acetylcholine receptor at the rat neuromuscular junction: structure, function, regulation.

18:00 - 20:00 **POSTERS** - Numbers 65 through 91.

20:00 - 22:00 Evening session - program to be announced

Thursday, Sept. 27th, 1990

SYNAPTOGENESIS AND GENE EXPRESSION (SESSION TWO)

Chair: R. Rahamimoff/U. Brodbeck

9:00	**H. Soreq** - Expression and in vivo amplification of human acetylcholinesterase and butyrylcholinesterase genes.
9:30	**P. Layer** - Cholinesterases and cell surface glycoconjugates in the chicken neural tube: a new look at presynaptogenetic neuronal specificity.
10:00	**P. Taylor** - Research directions emerging from the structure of acetylcholinesterase and its gene.
10:30	**S. Fuchs** - Acetylcholine receptor gene expression in experimental autoimmune *myasthenia gravis* .
11:00	Coffee Break/Poster session
11:30	**A. Vincent** - Molecular biology of the human acetylcholine receptor.
12:00	**M. Ballivet** - Electrophysiological properties of neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes.
12:30	**S. Heinemann** - The structure, function and expression of the brain nicotinic and glutamate receptor families
13:00	**J. Koenig** - Sequential events during neuromuscular synaptogenesis.
13:30	Lunch, End of the symposium.

LECTURES

Recent studies on depolarization release coupling in the squid giant synapse

LLINAS, R

No abstract received.

The Synaptopore - Fact or Fiction?

H. Betz, L. Thomas, P. Knaus and B. Marquèze-Poucy, ZMBH, Universität Heidelberg, and Max-Planck-Institut für Hirnforschung, Frankfurt, FRG.

From morphological and electrophysiological data, preformed proteinaceous channels have been implicated in the release of neurotransmitters from their vesicular stores, synaptic vesicles (1,2). We have characterized different synaptic proteins which qualify as potential components of such hypothesized "synaptopore" or "fusion pore" structures:

1. Synaptophysin is a major integral membrane protein of synaptic vesicles that shares its transmembrane topology and structural organization with connexins, the subunits of gap junction proteins. By crosslinking and reconstitution techniques we have demonstrated that native synaptophysin contains six identical subunits and forms channels upon reconstitution in planar lipid bilayers (3). This protein therefore may correspond to the vesicular portion of a putative fusion pore structure.
2. Synaptoporin is another member of the synaptophysin/connexin channel protein family that is localized in small synaptic vesicles (4). In situ hybridization shows that this protein is selectively expressed in a subset of central neurons. (5)
3. Using binding assays with intact vesicles we have identified a synaptophysin-binding protein of 36 Kd that fractionates with synaptic plasma membranes. This protein, named physophilin, behaves as a larger oligomer and is postulated to play a role in the docking of synaptic vesicles to pasma membranes.

   We conclude that, in accord with previous speculations, vesicular transmembrane channels and presynaptic plasma membrane proteins may play an important role in vesicle-plasma membrane interactions during transmitter release.

References

1. Pfenninger, K., Akert, K., Moor, H. and Sandri, C. (1972) J. Neurocyt. 1, 129-149.

2. Almers, W. and Tse, F.W. (1990) Neuron 4, 813-818.

3. Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W.W. and Betz, H. (1988) 242, 1050-1053.

4. Knaus, P., Marquèze-Pouey, B., Scherer, H. and Betz, H. (1990) Neuron, in press.

5. Thomas, L. and Betz, H. (1990) J. Cell Biol., in press.

Synaptic vesicles as tools for understanding of neurotransmitter release

R. Jahn, Department of Neurochemistry, Max-Planck-Institute for Psychiatry, D-8033 Martinsried, Fed. Rep. Germany

Synaptic vesicles are highly specialized organelles involved in neurotransmission. Several classes of synaptic vesicles are distinguished based on their morphological appearance. Small synaptic vesicles which occur in high numbers in every nerve terminal are the most abundant class and are supposed to contain non-peptide transmitters.
My laboratory started recently a systematic study of the structural and functional properties of small synaptic vesicles from mammalian brain. Small synaptic vesicles contain a group of unique membrane proteins which are highly specific for this organelle and occur on all vesicles irrespective or their neurotransmitter content. This includes the proteins synaptophysin, synaptobrevin, synaptotagmin (p65), p29 and the small GTP-binding protein rab3A. In contrast to their abundance on small synaptic vesicles, most of these proteins are absent from the membrane of peptide-containing large dense core vesicles demonstrating that these two organelles have a different membrane composition. In addition, these proteins are also present in an endomembrane system of endocrine cells which is different from the hormone-containing secretory vesicles. These proteins exhibit some unique structural features. Their function is still unknown. However, recent data suggest that at least some of them are involved in directing the membrane traffic in the nerve terminal. Furthermore, specific probes developed for these proteins proved to be versatile tools for studying the properties of synaptic vesicles and the related endomembrane system in endocrine cells. For example, a novel type of immunobeads was developed using monoclonal antibodies directed against various vesicle proteins. With these beads vesicles were isolated in one step at high purity allowing the analysis of their transmitter content.

Generation of synaptic vesicles in non-neuronal cells

KELLY, R.

No abstract received

TRANSMITTER RELEASE PROBED WITH BOTULINUM NEUROTOXINS

J. Oliver Dolly
Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, U.K.

Botulinum neurotoxin (BoNT) is a *Clostridial* di-chain protein that selectively inactivates an intracellular, ubiquitous component concerned with the release of several transmitters. Preferential inhibition of acetylcholine release (ACh) by BoNT arises from its targetting to ecto-acceptors present only on cholinergic nerve terminals in the periphery, and subsequent internalization. In attempts to decipher the molecular basis of the intoxication, we examined the ability of agents with known actions to perturb the inhibition by BoNT of transmitter release from synaptosomes. Destruction of actin-based cytoskeleton in cerebrocortical synaptosomes with cytochalasin D failed to alter the blockade of K^+-stimulated Ca^{2+}-dependent release of noradrenaline by BoNT A or B. In contrast, pre-treatment of the synaptosomes with colchicine, nocodazole or griseofulvin, under conditions that disrupt microtubules, antagonized the inhibition of K^+-evoked transmitter release by BoNT B but not type A. Stabilisation of the microtubules with taxol counteracted this effect of colchicine. On the other hand, effects of neither toxin were influenced by altering protein kinase C activity or cGMP levels. As colchicine did not affect binding of ^{125}I-labelled BoNT to intact synaptosomes and, apparently, could not prevent toxin internalization, it can be deduced that drug-induced reduction in the microtubule content interferes with the intra-neuronal action of type B BoNT. The inhibitory effect of BoNT A on noradrenaline release could be reversed much more readily by the Ca^{2+} ionophore A23187 than that of type B. These differences support the postulated interaction of BoNT A and B with distinct components of the release process, whilst the involvement of tubulin-based cytoskeleton in the action of type B accords with its ability to reveal asynchronous transmitter release.

The relationship between the dendrotoxin binding protein and the mammalian *Shaker*-type K^+ channels.

Hubert Rehm, Institut für Pharmakologie, Gloriastr. 32, CH-8006 Zürich

In the last two years considerable progress was made in the biochemistry of neuronal K^+ channels. K^+ channel specific toxins like the dendrotoxins from *Dendroaspis* venoms, ß-bungarotoxin from the venom of *B. multicinctus* or MCD peptide from bee venom led to the isolation of toxin binding proteins from brain membranes. After reconstitution into phospholipid bilayers, the toxin binding proteins displayed the characteristics of K^+ channels. The channels were activated by cAMP-dependent protein kinase and a novel endogenous kinase.

In paralell investigations, several laboratories showed that the *Shaker* locus of Drosophila expressed a family of A-type (transient) K^+ channels. Clones from the *Shaker* locus were used as probes to isolate a family of homologeous clones from mammalian brain libraries. In frog oocytes, the cRNAs of the mammalian *Shaker*-type clones induced K^+ currents of the delayed rectifier type, some of which were blocked by the dendrotoxins and MCD peptide.

Site-directed antibodies against a mammalian *Shaker*-type K^+ channel specifically recognized the dendrotoxin binding protein from mammalian brain. In addition, partial amino acid sequences from the dendrotoxin binding protein were nearly identical with sequences of one the mammalian *Shaker*-type K^+ channels. It is concluded that the dendrotoxin binding proteins of the mammalian brain constitute a subset of the mammalian *Shaker*-type K^+ channels.

CHANNELS AND VIEWS OF THE CHOLINERGIC NERVE TERMINAL

by R. Rahamimoff*#, M. Abdul-Chani*, S. A. DeRiemer#\,
J. Edry-Schiller*, S. Ginsburg*&, N. Melamed*, B. Sakmann∫#,
H. Stadler#, and N. Yakir*

* Dept. of Physiology, Hebrew University - Hadassah Medical School, Jerusalem, Israel;
\ Dept. of Biological Sciences, Columbia University, New York, NY, USA;
& Open University, Tel Aviv, Israel;
∫ Max-Planck-Institut für Medical Research, Heidelberg, FRG;
Max-Planck-Institut for Biophysical Chemistry, Göttingen, FRG.

Release of transmitter is governed by ionic currents throught the presynaptic nerve terminal membrane. The channels responsible for these currents are largely unknown. The cholinergic presynaptic nerve terminals and their subcellular components are too small for conventional single channel recording. Therefore, we fused, with the aid of DMSO and PEG-1500, synaptic vesicles and synaptosomes from Torpedo electric organ to produce structures amenable to the patch technique.

In the fused synaptosome membrane, two out of a large number of channels have been characterized. The most common one found in over 80% of the patches is a bursting K^+ channel with a large selectivity of K over Na. The open channel i-V relation is linear and has a slope conductance (γ) of 23.7 pS ± 1.33 (390 mM K^+ in the bath and 350 - 390 mM Na in the pipette). The probability of opening increases with depolarization, and the channel shows an outward rectification. The other frequent channel in the synaptosomal membrane is a Cl^- channel with γ of about 10 pS.

The predominant channel in the giant vesicle membrane is the P channel, which is cationic in nature, shows a marked rectification and is activated by calcium ions.

A number of speculations regarding the possible role of these channels will be brought forward.

In addition, preliminary results will be presented regarding confocal images of the cholinergic nerve terminals at the neuromuscular junction of the frog and the lizard.

Supported in part by CIF, BSF, Lower Saxony, CTR, Cowen and the Israeli Academy of Sciences.

MOLECULAR CONTROL OF SYNAPTIC VESICLE EXOCYTOSIS AND SYNAPTIC VESICLE MEMBRANE RECYCLING

H. Zimmermann, A. Henkel, F. Bonzelius, AK Neurochemie, Zoologisches Institut der J.W. Goethe-Universität, Siesmayerstr. 70, D-6000 Frankfurt am Main

A major unknown in the life cycle of the synaptic vesicle is the molecular cascade involved in synaptic vesicle exocytosis and retrieval. A presumably major step in exocytosis is the controlled binding of the synaptic vesicle membrane to a specific release site at the presynaptic membrane. We show that binding of iodinated synaptic vesicles from bovine brain to isolated presynaptic membranes (SPM) is enhanced by 400% if the SPM have previously been incubated with ATP and TPA, an activator of protein kinase C. Incubation of vesicles and membranes in the presence of Ca^{++} enhances vesicle binding by 200%. An SPM-protein of 92 kD is likely to be responsible for synaptic vesicle binding. It binds to synaptic vesicle membranes only when it has previously been phosphorylated or in the presence of micromolar concentrations of Ca^{++} (Zimmermann et al., Cell Biol Int 13: 993-1006, 1990).

Using perfused blocks of the Torpedo electromotor system we show that the synaptic vesicle membrane compartment is recycled directly on exo- and endocytosis and does not mix with the presynaptic membrane. The recycled synaptic vesicle retains an aliquot of the extracellular medium and this does not impair synaptic vesicle reloading with neurotransmitter.

Molecular Studies of Synaptic Function and Development

R. Scheller

Dept. of Biol. Sciences, Stanford University, Stanford CA 94305

Regulated release of chemical transmitters at the synapse is the cellular basis of neuronal communication. In spite of the central importance of the synapse in brain function, only a vague picture of the membrane flow which governs transmitter packaging and release is available, and little is known about the specific mechanisms of synapse information. To better understand transmitter metabolism at the synapse, several labs are characterizing proteins which are components of synaptic vesicles. Homogeneous cholinergic vesicles isolated from the electric organs of marine rays contain at least 20 vesicle enriched or specific proteins. We have characterized several of these proteins, including VAMP, VAT, synaptophysin, low MW GTP-binding proteins and components of the proton ATPase. Gene transfer and immunological reagents are being used to investigate the function of these molecules. In collaboration with the laboratory of U.J. McMahan, a ray cDNA clone for a component of the synaptic basal lamina has been isolated. In rat, a homologous gene is abundantly expressed in embryonic motor neurons. The protein is comprised of at least 14 EGF repeats and has 8 regions which are homologous to protease inhibitors. The potential role of this molecule in synapse development is being investigated.

CHARACTERIZATION OF A NERVE TERMINAL MEMBRANE PROTEIN THAT TRANSLOCATES ACETYLCHOLINE UPON CALCIUM ACTION.

M. Israël
Laboratoire de Neurobiologie Cellulaire et Moléculaire.
Departement de Neurochimie. C.N.R.S. 91198 GIF S/YVETTE
FRANCE.

The description of choline oxidase assay of acetylcholine(ACh) (Israël and Lesbats 1981) and the continuous monitoring of transmitter release obtained by coupling the choline oxidase reaction with a chemiluminescent detection of H_2O_2 has proven to be useful tool to study the mechanism of ACh release (Israël and Lesbats 1981, 1982, 1987). This method rendered possible the purification of the nerve terminal membrane protein that translocates ACh upon calcium action. This protein, the mediatophore seems made of 15 kD subunits forming a large structure of some 200 kD apparent molecular weight. It looks pentameric with a hollow center after electron microscopic examination. The reconstitution of mediatophore into artificial membrane showed that it shared with the release mechanism some common properties :

- calcium dependency of ACh release blocked by magnesium.
- inhibition of release by cetiedil:
- Desensitization of release by successive calcium pulses.

The mediatophore or a similar protein was also found in rat brain synaptosomes particularly in cholinergic areas. Antibodies raised against Torpedo electric organ mediatophore did not cross react with the rat motor end-plates but stained Torpedo muscles end-plates.

M. Israël and B. Lesbats. Neurochem. Int. (1981) 3, 81-90.
M. Israël, N. Morel, B. Lesbats, S. Birman and R. Manaranche Proc. Natl. Acad. Sci. USA (1986) 83, 9226-9230.
M. Israël and B. Lesbats. in Neurochemistry. A practical approach. IRL press. ed.by Turner and Bachelard. (1987)

HIGH AFFINITY TRANSPORTER FOR CHOLINE

H. Breer, M. Knipper, C. Kahle
University of Stuttgart-Hohenheim
Institute of Zoophysiology
7000 STUTTGART 70, FRG

There is considerable evidence indicating that the accumulation of choline via high affinity transporter is the rate-limiting step for the synthesis of acetylcholine in cholinergic nerve terminals. Thus, this transport system for choline, which is apparently unique for cholinergic nerve endings, plays a key role in cholinergic synapses and synaptic transmission. Although considerable progress in exploring the energetics of choline transport has recently been made in studies using synaptosomal ghosts, the mechanism of choline uptake mediated by high-affinity carrier is still poorly understood. It is one of the intriging properties of this transport system that its capacity appears to be coupled to the neuronal activity. Some recent experiments suggest that this coupling may be brought about via intracellular messengers. Rapid kinetic experiments revealed that stimulation of isolated nerve terminals either by depolarization or via activation of presynaptic receptors changes the intrasynaptosomal concentration of second messengers (cAMP, IP_3) in the subsecond time range. Modifying the concentration of cyclic nucleotides in synaptosomes, significantly increased the rate of choline accumulation; a similar effect was induced by phorbol esters, which activate protein kinase C. The observed changes in the rate of choline accumulation could either result from alterations in the velocity of the transport process or via changes in the actual number of functional carriers. In experiments using tritiated hemicholinium-3 as a specific probe for choline transporter, we have found that the number of binding sites significantly increased upon kinase activation. These results suggest that occult choline transporter may be recruited via kinase-dependent mechanisms and thus increasing the rate of choline accumulation. Towards an identification of the carrier protein, monoclonal antibodies were generated which specifically block the high affinity transport of choline. These antibodies were found to recognize a single polypeptide band of about 90,000 on Western blots and to label specific areas in nervous tissue sections. Using FPLC-ion-exchange and immunoaffinity-chromatography, the immunoreactive polypeptides have been purified to homogeneity and were subsequently reconstituted in liposomes; in proteoliposomes the purified protein catalyzed the accumulation of exogenous choline.

FURTHER CHARACTERIZATION OF AN ACH RECEPTOR INDUCING ACTIVITY (ARIA) THAT MAY PROMOTE THE ACCUMULATION OF RECEPTORS AT DEVELOPING SYNAPSES.

G.D. Fischbach, D.L. Falls, D.A. Harris, Department of Anatomy and Neurobiology, Washington University Medical School, St. Louis, MO 63110

A glycoprotein purified from chick brain that stimulates the synthesis of ACh receptors in cultured chick myotubes appears as a broad band centered at Mr 42,000 following SDS-PAGE. Acetylcholine receptor inducing activity (ARIA) can be eluted from gel slices that contain the band, and both the protein and the activity migrate more rapidly (ca. Mr 31,000) following digestion with N-glycanase. Although activity survives denaturation in the presence of SDS, activity was destroyed following exposure to reducing agents. The sequence of 24 of the first 27 amino acids was determined by automated Edman degradation. An antibody raised against a synthetic peptide corresponding to the 11 N-terminal amino acids labels motor neurons in the anterior horn of the spinal cord. R

The PCR was used with degenerate oligonucleotide primers to amplify a 34 nucleotide segment from chick brain poly A^+ mRNA that encodes 11 amino acids of the chemically defined sequence. This oligonucleotide was used to isolate a 2.2kb cDNA from an embryonic chick brain library that contained an open reading frame containing 267 amino acides including a putative signal sequence. The sequence includes four potential N-glycosylation sites, a 48 residue proline and glycine containing hexapeptide repeat near the N-terminal, two hydrophobic regions: one near the middle of the molecule and the other near the C-terminal end, and two cysteine residues that we assume are connected in a disulfide bond. Northern blots show a prominent 2.9kb message in the spinal cord as early as embryonic day 6, the time when motor axons first extend into the developing muscle mass in the limb. In situ hybridization shows that the mRNA is concentrated in the anterior horn. The mRNA is present in several other embryonic tissues, but it is more restricted to the CNS in adult animals.

The predicted amino acid sequence is 43% identical to the mouse prion protein allowing for one gap in the chick protein and five in the prion sequence. More definitive evidence that the chick sequence corresponds to the receptor inducing activity depends on immunoprecipitation and expression experiemnts now underway. Nevertheless, the possibility can be considered that the neurodegenerative diseases associated with an altered form of the prion protein are in fact due to loss of neurotrophic support.

MOLECULES THAT MEDIATE THE NEURON-INDUCED FORMATION OF POSTSYNAPTIC APPARATUS IN SKELETAL MUSCLE FIBERS.

U.J. McMahan. Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305.

The basal lamina in the synaptic cleft of the neuromuscular junction contains molecules that direct the formation of cell surface specializations on regenerating axon terminals and muscle fibers. Some of these specializations are directly involved in synaptic transmission. For example, the synaptic basal lamina induces regenerating axon terminals to form active zones, which are involved in the release of the transmitter, acetylcholine, and it directs regenerating muscle fibers to form aggregates of acetylcholine receptors and acetylcholinesterase. Over the last several years my colleagues and I have conducted experiments aimed at identifying and characterizing the basal lamina molecules that induce the aggregation of AChRs and AChE. Our results have led to the following hypotheses. 1) A single basal lamina molecule causes the aggregation of both AChRs and AChE as well as other components of the postsynaptic apparatus, 2) the active molecule is synthesized by motor neurons and released by their axon terminals to be incorporated into the basal lamina, and 3) the basal lamina molecule that directs formation of the postsynaptic apparatus on regenerating muscle fibers is the same molecule that mediates the nerve-induced formation of postsynaptic specializations on developing muscle fibers in the embryo and it helps maintain those specializations on mature muscle fibers in the adult. Our studies indicate further that the active molecule is identical, or very similar, to agrin, a protein we have purified from the electric organ of Torpedo californica. I will discuss evidence that supports these hypotheses and present results of studies that bear upon the steps and mechanisms involved in the formation of the AChR aggregates.

THE ACETYLCHOLINE RECEPTOR : FUNCTIONAL ARCHITECTURE AND REGULATION

J.P. Changeux, P. Benoit, A. Bessis, J. Cartaud*, A. Devillers-Thiéry, A. Duclert, J.L. Galzi, B. Jasmin*, A. Klarsfeld, R. Laufer, C. Mulle, H.O. Nghiêm, J. Piette and F. Revah.
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In the adult motor endplate, the AChR is strictly localized under the nerve ending while in the non innervated myotube it is distributed all over the surface of the cell. In situ hybridization with α-subunit probes, containing or not exonic sequences disclose high levels of unspliced and mature mRNA in mononucleated myotomal cells and differentiating myotubes. After the entry of the exploratory motor axons, the clusters of grains located outside the endplate decrease in number. In 15-day old chicks, AChR α-subunit mRNAs become restricted to the subneural "fundamental" nuclei. Denervation causes a reappearance of unspliced and mature mRNA in extrajunctional areas.

Chronic paralysis of the embryo by flaxedil interferes with the disappearance of extrajunctional AChR which, thus, represents an electrical activity- dependent repression of AChR genes. This process has been analysed with a chicken α-subunit genomic probe in primary cultures of chick myotubes. Blocking their spontaneous electrical activity by tetrodotoxin (TTX) causes increases of both precursor and mature α-subunit mRNA levels while α-actin mRNA levels do not change. The entry of Ca^{++} ions and possibly the activation of protein kinase C contribute to the repression of α-subunit gene transcription.

The maintenance and late increase in AChR number at the endplate level requires the intervention of an anterograde signal from neural origin. Calcitonin gene- related peptide (CGRP), a peptide shown to coexist with acetylcholine in chick spinal cord motoneurons, increases surface AChR and α-subunit unspliced and mature mRNA respectively by 1.5 and 3 fold. CGRP stimulates membrane-bound adenylate cyclase in the range of concentration where it enhances AChR α-subunit gene expression.

The data are interpreted in terms of a model which assumes that : 1) in the adult muscle fiber, nuclei may exist in different stages of gene expression in subneural and extrajunctional areas, 2) different second messengers elicited by neural factors or electrical activity regulate the state of transcription of these nuclei via trans-acting allosteric proteins binding to cis-acting DNA regulatory elements.

To look for such components, the 5'-end and part of the upstream flanking region of the α-subunit gene was isolated and sequenced in the chick. This α-subunit promoter, including 850 bp of the 5' flanking sequence, was inserted into a plasmid vector in front of a chloramphenicol acetyltransferase (CAT) gene. This construct directed high CAT expression in transfected mouse C2.7 myotubes but not in unfused C2.7 myoblasts or non myogenic mouse 3T6 cells. DNAse I foot-printing and gel retardation assays show that nuclear factors bind to three distinct domain AR I, II and III located within the most proximal 110 nt domains. Levels of several of these factors change during fusion of myoblasts into myotubes (AR IIb and III) and as a consequence of denervation (AR IIb and III).

Finally, multiple post-transcriptional processes involving, in particular the Golgi apparatus, proteins from the basal lamina and from the cytoskeleton (the 43 KD protein among others) contribute to the clustering, and stabilisation of the AChR in the post-synaptic membrane.

MOLECULAR ANALYSIS OF THE NICOTINIC CHOLINERGIC RECEPTOR SYSTEM OF *DROSOPHILA MELANOGASTER*

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Acetylcholine is a prevalent excitatory neurotransmitter in the insect nervous system which acts predominantly by the activation of nicotinic type receptors. Recent studies by our laboratories as well as others showed that, as in vertebrates, protein subunits of nicotinic acetylcholine receptors (nAChRs) are encoded by a family of related genes. Three members of this gene family expressed in the *Drosophila* central nervous system have been characterized so far: the **ARD** protein representing a potential structural or non-α nAChR subunit (Hermans-Borgmeyer et al., 1986, EMBO J. 5, 1889) and two putative ligand-binding α-subunits **ALS** (Bossy et al., 1988, EMBO J. 7, 611) and **Dα2** (Baumann et al., 1990, Nucl. Acids Res. 18, 3640; Sawruk et al., in press). A fourth gene whose ligand-binding region has not yet been defined is currently under investigation. The *ARD* gene maps at chromosomal region 64B. The *ALS* and *Dα2* genes both are located at region 96A suggesting that they form (part of) a nAChR gene cluster.

Antisera against bacterially expressed fusion proteins were raised to further characterize the nAChR proteins. Immunoprecipitation experiments revealed that ALS and ARD protein are integral components of the same receptor complex which binds ^{125}I-α-bungarotoxin with high affinity. Furthermore, an ALS-specific antiserum was employed to immunohistochemically localize ALS protein-containing receptors in fly heads. Essentially, structures within the protocerebrum, the α-, ß- and γ-lobes of the mushroom bodies and parts of the central body were stained by anti-ALS antibodies. In addition, distinct neurons of the lamina of the visual system are expressing the ALS protein.

Currently, reverse genetics methods are being applied to investigate function and expression of the various nAChR subunits. Results of these studies will be discussed.

Characteristics of neuronal nicotinic acetylcholine receptor ion channels of rat hippocampal neurons. E.X. Albuquerque and M. Alkondon. Department of Pharmacology & Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD 21201.

The pharmacological and developmental profile of neuronal nicotinic acetylcholine receptor ion channels (nAChR) in the hippocampus was studied using tissue cultured fetal (17 days gestation) rat hippocampal neurons. Ionic currents were measured by whole-cell patch-clamp technique, and agonists and antagonists were applied to the neurons through a 'U'-tube rapid delivery system. Acetylcholine (ACh) and (+)anatoxin (AnTX), two nAChR agonists, elicited inward currents in neurons which were clamped near their resting membrane potentials. AnTX was found to be nearly 10-20 times more potent than ACh in inducing these currents. Linear I-V plots for both agonists were obtained from -100 mV to -10 mV and the currents reversed near 0 mV. At positive membrane potentials, smaller outward currents were recorded when compared to that seen at equivalent negative potentials. The currents induced by both agonists showed a desensitization pattern during prolonged agonist application and a complete recovery from this process occurred in about 90 sec to 180 sec. The nAChR currents were either undetectable (<10 pA peak) or very small (<50 pA peak) up to 5-7 days in culture, and larger currents (200-2000 pA peak) were recorded in neurons grown in culture for 14 or more days. The currents produced by AnTX were reversibly blocked by d-tubocurarine, dihydro-β-erythroidine and (±)mecamylamine. However, they were unaffected by $MgCl_2$ and APV at concentrations which blocked NMDA-induced currents. The quisqualate antagonist DNQX had no effect whereas the $GABA_A$ receptor antagonist bicuculline had a marginal inhibitory effect on AnTX-currents. Atropine, the muscarinic receptor antagonist and tetrodotoxin, the sodium channel blocker did not block the responses to AnTX. VX, an irreversible acetylcholinesterase inhibitor, potentiated the responses to ACh but not AnTX. These results confirm the existence of a class of functional neuronal nAChR in the rat hippocampal neurons predominantly located at the axodendritic region. Support: US Army Med. Res. & Devel. Comm. Contr. DAMD17-88-C-8119 & NIH Grant NS25296.

α-NEUROTOXIN INTERACTIONS WITH ACETYLCHOLINE RECEPTOR AND OTHER PROTEINS

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Snake venom α neurotoxins are classical blockers of nicotinic acetylcholine receptors (AChR) of muscles and *Torpedo* electrocytes. Neurotoxin-AChR binding is of udoubt interest for understanding general principles of ligand-receptor interactions. Using various series of α neurotoxin derivatives, the regions of the toxin involved in AChR binding have been characterized in our laboratory. Although several groups have demonstrated that in isolated state only α-subunit binds α-neurotoxins, our data showed that in the intact AChR complex the toxins contact all subunits.

In view of colocalization of receptors of classical neurotransmitters and neuropeptides, we examined possible effects of α-neurotoxins, capable of multiple interactions, on the neuropeptide receptors. It was found that α-bungarotoxin (Bgt) and toxin 3 *N.naja siamensis*, but not the short-chain neurotoxins inhibit with K_I 10^{-7}-10^{-8}M binding of iodinated substance P (SP) to the rat brain NK-1 tachykinin receptors. Less potently the toxins competed for the NK-2 and NK-3 receptors. At 10^{-4}-10^{-5} M, inhibition was manifested by d-tubocurarine, phencyclidine, and triphenylmethylphosphonium. On the other hand, carbamoylcholine, nicotine and decamethonium were without effect even at higher concentrations. The results obtained, in view of preservation of the inhibitory activity of toxins towards solubilized membranes, indicate that some of the Bgt-binding polypeptides of mammalian brain are tightly associated with tachykinin receptors.These polypeptides are distinct from those having a high affinity for thymopoietin.

Physiological significance of desensitization in neuromuscular junction

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Desensitization, a fall of sensitivity to transmitter due to its long-lasting action, was commonly considered as negative feedback mechanism protecting postsynaptic membrane against transmitter excess. Evidently, a transition of cholinoreceptors into desensitized form which saves the ability to interact with ACh but failed to operate the ionic channels plays a leading role in the process of desensitization in cholinergic neuromuscular junction. Desensitized receptors have also much higher affinity to ACh in comparison with normal resting ones. These disabled but ready for catch receptors may effectively compete for free ACh with resting ("ready-for-use") ones decreasing thereby effective concentration of ACh. The latter sequence of desensitization may be involved in the mechanism of some phenomena observed in the experiments which were done on isolated frog neuromuscular preparations treated with anticholinesterase drugs.

The 15-30 min treatment with either prostigmine (6 μM) or organophosphorus inhibitor, armine (1 μM), induced an increase of miniature endplate currents (MEPC) amplitude and decay time constant (τ) by factors of 1.3 and 4.4, respectively. However, this prominent prolongation of MEPC decay was followed by the slow and continuous fall of τ without pronounced changes of MEPC amplitude. We suppose that after treatment with anticholinesterase drugs the number of desensitized receptors increases. These receptors can serve as traps for free ACh attenuating thereby the repetitive ACh binding and activation of channels.

The hypothesis was supported by the fact that any procedures affecting the desensitization are able to modify the rate of MEPC shortening. Thus the decrease of temperature to 11°C prevented MEPC shortening completely, and maximal values of τ and amplitude remained stable during several hours. On other hand, desensitization can be promoted by the application of exogenous ACh. In the experiments on anticholinesterase-treated preparations after relatively short-term (10-15 min) bath application and following washout of 2 μM ACh amplitude and τ dropped to 74 ± 3 % and 49 ± 3 %, respectively (n=6). During further washout in contrast to rapid recovery of amplitude, τ restored very slowly. Prominent shortening of MEPC without decrease of amplitude was observed in prostigmine-treated muscle during the action of desensitization-promoting drug proadifen. After pretreatment with non-hydrolysable agonist, carbacholine (10 μM, 15 min), followed by 15 min washing, MEPC were changed negligibly, but subsequent AChE inhibition by prostigmine led to much smaller increase in τ than in control.

Hence, the increase of the number of desensitized receptors (traps) may compensate in some extent the lack of ACh hydrolysis in anticholinesterase-treated muscles.

Structures of Nicotinic Receptors from Muscles and Nerves and of Neuronal α-Bungarotoxin-Binding Proteins

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Three gene families within the ligand-gated ion channel gene family encode subunits of proteins with cholinergic ligand-binding properties. Of these, muscle nicotinic acetylcholine receptors (AChRs) are the best characterized. The various subtypes of neuronal nicotinic AChRs are also ACh-gated cation channels, but they differ from muscle AChRs in pharmacology, subunit composition, and (in some cases) in functional roles. Neuronal α-bungarotoxin-binding proteins (αBgtBPs) comprise the third of these gene families Their endogenous ligand and functions are unknown, but cDNAs and mAbs for some subunits of two subtypes are now available.

Muscle AChRs are composed of two α1 subunits, one β1 subunit, one γ or ε subunit, and one δ subunit, organized in a pentagonal array around a central cation channel. Our studies have concentrated on muscle-type AChRs from *Torpedo* electric organ and human TE671 cells. Assembly of subunits expressed in various combinations in *Xenopus* oocytes has been studied. Epitopes on α subunits have been mapped using synthetic peptides and using *in vitro* mutagenesis and expression in *Xenopus* oocytes.

Neuronal AChRs are composed of only two kinds of subunits. The primary subtype in mammalian brain is composed of α4 ACh-binding subunits and β2 structural subunits. These subunits are found in an $(\alpha4)_2(\beta2)_3$ stoichiometry which resembles the pentagonal subunit arrangement of muscle AChRs. In chicken retina AChRs with α3 and β2 subunits are a major subtype. cDNAs have been cloned for human α3 and β2 subunits.

Neuronal αBgtBPs of two subtypes have been identified in chicken brain. One subtype comprising ≥75% of the total includes αBgtBP α1 subunits and an unknown complement of structural subunits Another subtype comprising ~15% of the total includes both αBgtBP α1 subunits and αBgtBP α2 subunits as well as an unknown complement of structural subunits. These two subunits exhibit 82% sequence identity with each other, and 44-49% sequence identity with α subunits of AChRs of either muscles or nerves. The presence of cysteines homologous to α192-193 of muscle and neuronal AChRs suggests that these are ligand-binding subunits. Sequence identities in putative transmembrane domains suggest that they could be components of ligand-gated cation channels.

STRUCTURAL AND FUNCTIONAL STUDIES ON THE PHOSPHATIDYL-INOSITOL-ANCHORED DIMER OF ACETYLCHOLINESTERASE PURIFIED FROM ELECTRIC ORGAN TISSUE OF *TORPEDO CALIFORNICA*

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In electric organ tissue of the electric fish, *Torpedo*, a substantial amount of the acetylcholinesterase (AChE) occurs in the form of a membrane-bound hydrophobic dimer. In this molecular species of AChE the membrane-anchoring domain is provided by the diacylglycerol moiety of a single phosphatidylinositol (PI) residue which is covalently attached, via an intervening oligoglycan, to the COOH-terminus of each of the two catalytic subunit polypeptides of the dimer. This form of AChE can be selectively solubilized from electric organ tissue by a PI-specific phospholipase C of bacterial origin, and subsequently purified by affinity chromatography. The highly purified water-soluble AChE so obtained provides a convenient preparation for structural studies, including chemical modification and identification of residues involved in catalytic activity, physicochemical studies of conjugates of the enzyme with organophosphorus poisons, and characterization of the membrane-anchoring domain. Furthermore, it has provided us with an ideal source for preparation of high quality crystals of AChE which have proved suitable for use in the determination of its three-dimensional structure by X-ray crystallography. Recent results in these various areas will be presented and discussed.

MOLECULAR POLYMORPHISM OF CHOLINESTERASES : VARIOUS MODES OF ANCHORING

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In *Torpedo*, a single gene produces several transcripts for acetylcholinesterase (AChE), through alternative splicing and probably the use of several transcription origins and polyadenylation sites.

Three types of coding sequences have been identified in cDNA clones. The more abundant ones, in the mRNA population, encode the precursors of the catalytic subunits of the collagen-tailed, asymmetric forms (A), and of the amphiphilic glycolipid-anchored dimers (Ga)[1-2]. These precursors consist of a signal peptide, a major common catalytic domain, and divergent C-terminal peptides. A third type of cDNA, diverging at the same position in the coding sequence, seems to exist in a small minority of mRNAs. However, the divergent sequence corresponds to the intron following the last common exon, and the existence of the predicted protein has not yet been demonstrated.

Torpedo also possesses a butyrylcholinesterase (BuChE), presenting both asymmetric and globular forms. A detailed study of amphiphilic forms of AChE and BuChE led to the distinction of two classes of amphiphilic dimers[3]. Class I corresponds to glycolipid-anchored dimers : they are sensitive to PI-PLC and PI-PLD and aggregate in the absence of detergent. Class II dimers do not present these characteristics ; they are readily soluble without detergent, although they clearly bind detergent micelles. In the case of BuChE, only class II dimers were observed. Amphiphilic dimers and monomers of AChE from chicken and rabbit can be considered as class II, by extension[4]. The nature of the hydrophobic domain at class II cholinesterases is not known.

Glycosylation introduces variations in the mature AChE forms, producing distinct electrophoretic variants, or electromorphs, in different tissues. In *Torpedo* and *Electrophorus* electric organs, some forms of AChE carry the Elec-39/HNK-1 glycosidic epitope, which is characteristic of adhesion molecules, suggesting that electric organ AChE may be involved in a structural function, in addition to its catalytic activity[5].

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Studies on Mammalian Acetylcholinesterases:
Subunit Assembly, Membrane Anchoring and State of Glycosylation

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Mammalian acetylcholinesterase (AChE) exists in brain as tetrameric globular enzyme (G_4 form) of which approximately 80% are amphiphilic, membrane bound through a structural subunit linked by disulfide bridges to one pair of the four catalytic subunits. Our results show that pairs of dimers assemble to the G_4 form by strong non-covalent hydrophobic interactions near their C-terminus. On the other hand, G_2-AChE from mammalian erythrocytes and a number of other sources consists of two disulfide linked catalytic subunits which are membrane bound through a glycosylphosphatidylinositol (GPI) moiety covalently attached to the C-terminus of each subunit. While the complete chemical structure of the GPI-membrane anchor of dimeric AChE from human eythrocytes has been worked out, relatively little is known about the anchor of the brain enzyme. By labelling with the photoactivatable reagent 3-trifluoromethyl-3-(*m*[^{125}I]-iodophenyl) diazirine ([^{125}I]TID), a non-catalytic structural subunit of about 20 kD molecular mass was identified in human and bovine brain AChE which is sensitive to proteolytic cleavage. In non-denaturing conditions the reduced and alkylated enzyme remained tetrameric and the anchor remained bound to the catalytic subunit. This indicated that the anchor not only interacted hydrophobically with the lipid bilayer, but also with the catalytic subunits. The [^{125}I]TID-label could in part be removed by treatment with hydroxylamine, indicating that the [^{125}I]TID-labelled structural subunit is composed of hydrophobic amino acids as well as other hydrophobic constituents.

Acetylcholinesterases have varying amounts of carbohydrates attached to the core protein. Sequence analysis of the known primary structures gives evidence for several asparagine linked carbohydrates. From the differences in molecular mass determined on SDS-gels before and after deglycosylation with N-glycosidase F, it is seen that bovine AChE (both dimeric and tetrameric forms) are more heavily glycosylated than the corresponding human enzymes. Further, dimeric AChE from red cell membranes is more heavily glycosylated than the tetrameric brain enzyme. N-acetylgalactosamine could neither be detected in bovine brain nor in bovine erythrocyte AChE indicating that both enzyme forms contain no O-linked carbohydrates.

Monoclonal antibodies (MAB) raised against AChE from electric organs of *Torpedo nacline timilei* showed interesting properties towards mammalian AChE. Three of them crossreacted with AChE from bovine and human brain but not with AChE from erythrocytes. Treatment of the brain enzyme with N-glycosidase F abolished binding of MAB suggesting that the epitope, or part of it, consists of N-linked carbohydrates. The remarkable difference in reactivity of MAB towards AChE from brain and erythrocytes as well as the differences observed after treatment with N-glycosidase F suggest different glycosylation patterns in dimeric erythrocyte and in tetrameric brain AChE.

Single conductivity events and lipid kinase activity of the Torpedo californica nicotinic acetylcholine receptor

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The dimer ($M_r \approx 580{,}000$) and the monomer ($M_r \approx 290{,}000$) of the detergent-solubilized, affinity chromatographically purified nicotinic acetylcholine receptor (nAcChR) of Torpedo californica electrocytes exhibit different channel conductances and different enzyme properties.

Patch clamp data of the dimer, reconstituted in large lipid vesicles, show that the dimer double channel behaves as a 'single channel', reflecting simultaneous, concerted switching of the two constituing monomeric parts. The vesicle-reconstituted monomer, if prevented from aggregation, exhibits a channel conductance which is only half of that of the dimer. The conductance of the reconstituted dimer is the same, and shows the same Ca^{2+}-dependence, as the Torpedo receptor expressed in Xenopus oocytes (Imoto et al., 1986). The comparison implies that it is the dimer which is the physiological switching unit of the nAcChR. Both the isolated highly purified monomer and dimer are associated with AcCh-dependent lipid kinase activity. The dimer catalyzes the formation of phosphatidylinositol phosphates (PIP) in a much stronger manner than the monomer and, in addition, shows an AcCh-dependent hydrolase activity for PIP. The lipid phosphorylation data analysis in terms of an extended Michaelis-Menten-'Ansatz'yield $k_{cat} = 0.2\ s^{-1}$ and $K_M = 10^{-4}$ M. The AcCh-dependence is characterized by the half saturation constant $Q_A = 7(\pm 1) \times 10^{-8}$ M (30°C, pH 7.5), comparable to $Q_A = 3(\pm 1) \times 10^{-8}$ M characterizing the lower branch of the AcCh-binding hysteresis. Thus the enzymatic activities are associated with the desensitized receptor forms, present to 2% ($K_O = 2.5 \times 10^{-2}$) in the absence of AcCh. In conclusion, the desensitized receptor state, sometimes considered as pathological because of channel inactivity, is biochemically active. The endogeneous lipid kinase activity suggests that the desensitized nAcChR can modulate the signal cascade of the phosphoinositide second messengers. The hysteretic AcCh-binding endows the signal transduction of the synaptic transmission with memory and learning properties.

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NICOTINIC RECEPTOR OPTICAL SENSOR AS A DIAGNOSTIC DEVICE

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Nicotinic acetylcholine receptor protein isolated from the electric organ of *Torpedo*, and immobilized on a quartz fiber is a sensitive biosensor of α-neurotoxins of snake venoms and antireceptor antibodies. The density of immobilized receptor sites, determined by specific binding of ^{125}I or fluorescein isothiocyonate (FITC)-labeled α-bungarotoxin (α-BGT) was dependent on reaction time, pH, and concentration of the receptor protein in the coupling medium. Each fiber carried an average of 4 pmoles of receptor (i.e. 20 fmol/mm^2). Detection limits of FITC labeled α-BGT or α-cobratoxin were in the pM range. The optical signal was generated by the fluorescent toxins when they bound to the receptor protein, that was immobilized on the optic fiber, and excited by the evanescent wave.

The pharmacological specificity of the acetylcholine receptor optical biosensor was investigated using FITC-labeled-α-BGT, -α-*Naja* toxin (α-NTX) or -α-conotoxin GI. Binding and dissociation time courses for the FITC-labeled toxins were markedly different, with association constants (k_{+1}) of 8.4×10^6 M^{-1} min^{-1} for FITC-α-BGT, 6.0×10^6 M^{-1} min^{-1} for FITC-α-NTX and 1.4×10^6 M^{-1} min^{-1} for FITC-α-conotoxin GI. The dissociation constants (k_{-1}) were 7.9×10^{-3} min^{-1}, 4.8×10^{-2} min^{-1} and 8.0×10^{-1} min^{-1} for FITC-α-BGT, FITC-α-NTX and FITC-α-conotoxin GI, respect-ively. K_i values for d-tubocurarine using the optical sensor were similar to those reported for radioisotope binding to the membrane-bound receptor. However, K_i values for carbamylcholine were 1 to 2 orders of magnitude higher for the optical sensor. (Supported in part by DOD Grant No. DAAA-15-89-C-0007).

Functional Diversity of Neuronal Nicotinic Acetylcholine Receptors

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A family of genes encodes subunits of the neuronal nicotinic acetylcholine receptors and homologous proteins. These genes are expressed in unique but overlapping brain regions and encode similar but non-identical proteins. Functional nicotinic receptors can be generated from the pairwise combination of either the beta2 or the beta4 subunit with either the alpha2, alpha3, or alpha4 subunits. We have examined the pharmacological properties of the six receptors thus generated and find that each has a spectrum of sensitivities to both agonists and antagonists that serve to distinguish them one from the other. We find that both the alpha and beta subunits contribute to agonist and antagonist specificities and that a ligand that functions as an agonist on receptors containing a beta4 subunit actually blocks function on receptors composed of beta2 subunits. We propose that this ligand acts as a channel blocker on beta2 containing receptors but not on beta4 containing subunits. We also report the sequence of a new member of the gene family that we have called alpha6. The protein encoded by this gene has the contiguous cysteines at positions 192 and 193 that are hallmarks of the alpha subunits. The alpha6 gene is expressed in a limited number of regions in the adult rat brain including the habenula and the substantia nigra pars compacta.

REGULATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR BY SERINE AND TYROSINE PROTEIN KINASES

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The nicotinic acetylcholine receptor (AChR) has served as an excellent model for studying the structure, function, and regulation of neurotransmitter receptors. The AChR is a pentameric complex of four types of subunits: α (40 KD), β (50 KD), γ (60 KD), and δ (65 KD) in the stoichiometry $\alpha_2\beta\gamma\delta$. Postsynaptic membranes from Torpedo californica are enriched in AChR and contain at least three different protein kinases which phosphorylate the AChR. cAMP-dependent protein kinase phosphorylates the γ and δ subunits, protein kinase C phosphorylates the δ subunit, while a protein tyrosine kinase phosphorylates the β, γ, and δ subunits of the AChR. The proposed phosphorylation sites are clustered together on a homologous domain of each subunit. The major functional effect of phosphorylation of the AChR by all three protein kinases appears to be the regulation of the desensitization of the receptor.

The AChR is phosphorylated in rat myotubes by the same protein kinases found to phosphorylate the AChR in postsynaptic membranes of Torpedo californica. The phosphorylation of the AChR in muscle cells by protein kinase C is regulated by acetylcholine itself, while the phosphorylation of the AChR by cAMP-dependent protein kinase is regulated by CGRP (calcitonin gene-related peptide), a neuropeptide which is a co-transmitter with acetylcholine at the neuromuscular junction. In addition, phosphorylation of the AChR on tyrosine residues is regulated by innervation of the muscle cells. These results suggest that phosphorylation of neurotransmitter receptors such as the AChR by various protein kinases is an important mechanism in mediating the effects of several regulatory pathways on synaptic transmission.

Insect nicotinic receptors: Functional properties of native receptors and of a cloned subunit cDNA expressed in *Xenopus* oocytes

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Molecular cloning was performed of a locust (*Schistocerca gregaria*) central nervous system nicotinic acetylcholine receptor (nAChR) cDNA encoding an α-type subunit with homology to vertebrate neuronal nAChR subunits and possessing the Cys-Cys α-subunit motif at a position homologous to 191. Expression of the transcribed locust mRNA in *Xenopus* oocytes results in a depolarizing response on exposure to nicotine (10^{-7} M and above). In voltage-clamp studies, the response to nicotine was antagonized by both α-bungarotoxin and kappa-bungarotoxin; reversible block of nicotine-induced currents by d-tubocurarine, tetraethylammonium, bicuculline and strychnine has also been observed. This unusual pharmacology is characteristic of some insect neuronal nAChRs. The results show that an insect α-subunit alone is sufficient to form a functional nAChR with a homo-oligomeric structure, which shares some pharmacological properties with a native insect nAChR and with some non-cholinergic members of the same ligand-operated superfamily. This insect nAChR may retain some properties of an ancestral receptor of the vertebrate neuronal and peripheral nAChRs.

Voltage-clamp studies on cockroach ganglion neurones show that the α-bungarotoxin-sensitive insect nAChR there is also blocked by dihydro-β-erythroidine, lophotoxin, lophotoxin analog-1 and neosurugatoxin. Patch-clamp experiments on dissociated insect neuronal cell bodies yield three conductances (60-80 pS; 30-40 pS and 20 pS) of which the 30-40 pS conductance is the most abundant. Open times are best fitted by 2 exponential components. Closed time distributions show greater variation and require 2-4 exponential fits. The molecular basis of these distinct conductance states remains to be resolved, but the insect nicotinic receptor formed by a single subunit may offer direct experimental approaches to such questions.

IDENTIFICATION OF STRUCTURAL DETERMINANTS OF ION FLOW AND SELECTION IN MEMBRANE CHANNELS BY SITE-DIRECTED MUTAGENESIS

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By combined use of complementary DNA manipulation and single channel current analysis for *Torpedo californica* nicotinic acetylcholine receptor (AChR), we have identifed three clusters of negatively charged and glutamine residues neighbouring the hydrophobic segment M2, presumably forming three anionic rings, as major determinants of the AChR channel conductance for monovalent cations and as sites of interactions with divalent cations. To gain a better insight for the molecular basis of the weak, but distinct ion selectivity of this cation channel, we have measured the conductances and the permeability ratios for alkali cations of the mutant AChR channels expressed in *Xenopus laevis* oocytes. The results show that mutations in these rings can alter the conductance and permeablity sequences consistent with the view that permeant ions come into a close contact with the amino acid residues in the anionic rings and suggest that the ion selectivity arises from a series of interactions between permeant ions and the channel wall.

Calcium Permeability and MK-801 Block of the Acetylcholine Receptor Channel. John A. Dani, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX, USA, 77030

For the muscle acetylcholine receptor there is a general picture that relates structure and function. Our research has focused on how the open channel structure governs ion permeation, and an ion permeation model was developed to describe permeation data based explicitly on the general structure of the channel. The model helps to explain, in structural terms, the calcium permeability and the MK-801 inhibition of nAChR channels.

MK-801 is a potent inhibitor of the NMDA subtype of glutamate receptors. Our single-channel and macroscopic currents indicate that MK-801 also inhibits nicotinic acetylcholine receptors. Although there is a slight inhibition of the closed nAChR, the main action of MK-801 is to enter and block the open channel with a $K_d(0)$ of about 7 μM, which is about 40 times higher than the $K_d(0)$ for MK-801 binding to the NMDA receptor. The relative potency of tricyclic compounds like MK-801 for various neurotransmitter systems points out that the pharmacologic action of these drugs could involve complicated interactions *in vivo*.

The calcium permeability of the nAChR was determined using patch-clamp techniques. The results indicate that the nAChR channel carries calcium surprisingly well. With pure 110 mM calcium as the external permeant cation, the slope conductance of the nAChR is 12 pS, which is similar to the conductance of the NMDA receptor channel under these conditions. Because nAChRs are densely packed at synapses, the calcium influx at an active synapse could produce a locally high calcium environment.

THE CHOLINERGIC BINDING SITE: STRUCTURE AND APPLICATION

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The binding site to α-neuro-toxins such as α-bungarotoxin has been shown to contain the amino acid sequence α-180-200. This short peptide (synthetic or recombinant) can bind toxins with affinities similar to those measured for the isolated α-subunit ($Kd=10^{-7}$ M) however, this affinity is about 100 fold less than that of the intact receptor. A number of questions have be addressed: does this sequence also partake in the recognition of the cholinergic agonists? what residues of the region are critical for the binding activity? how flexible is the site or, how do amino acid changes effect the binding constants? what is the minimal sequence that can be identified that continues to bind the ligands with reasonable affinity? are there similarities between other known sites that recognize common ligands? Our approach has employed recombinant proteins that are expressed in *E. coli* as fusion proteins between the truncated form of *trpE* and a carboxy terminal extension that contains the sequence of interest. The fusion proteins are then subjected to biochemical analyses and to NMR spectroscopy. As a result of our studies the 13 amino acid sequence, α-186-198, has been found sufficient for toxin binding ($Kd=5x10^{-7}$ M) and the general requirements of the binding site are more clearly understood. Through selective T1 relaxation, it is demonstrated that the toxin binding site, binds acetylcholine and nicotine as well.

Although the mechanism of ligand recognition is still not fully understood a different question can none the less be tested, namely; can the recombinant binding site act as a receptor mimic and provide a therapeutic value? The prospects of the use of such mimics as molecular decoys will be discussed.

FUNCTIONAL DOMAINS OF THE ACETYLCHOLINE RECEPTOR
A novel phosphorylation site in position δS362

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The acetylcholine receptor is composed of three main functional components, (i) the transmitter (and competitive antagonist) binding domain,(ii) the ion channel, and (iii) regulatory domains. To detect these domains within the quaternary and primary structures of the transmembrane protein among others immunological methods, site directed mutagenesis in combination with patch clamp electrophysiology and protein chemical methods are applied. We investigated among the regulatory sites of the receptor the phosphorylation sites. Rapid and transient phosphorylation of the receptor was implicated in regulatory phenomena like rapid desensitization (1). We obtained evidence that the phosphorylation sites under discussion are stably phosphorylated and are therefore not available for short-term regulatory events.

The δ-subunit of the nicotinic acetylcholine receptor isolated in absence of protein phosphatase inhibitors contains a total of four phosphate groups, three of which are ester-bonded to serine groups, one to tyrosine. We localized these groups within the primary structure: We found phosphoserine in positions δS361 and δS377, the predicted sites phosphorylated by PKA and PKC, respectively. In addition we found that δS362 is also phosphorylated, a residue not shown to be phosphorylated before. Phosphorylation experiments with the synthetic peptide δL357-δK368 showed that phosphorylation of this novel site can be catalyzed by PKA and PKC. Phosphorylation of this sequence with PKC without identifying the exact amino acid residue had been accomplished before (2). δY372, the predicted substrate site for a tyrosine kinase (3), was also found to be loaded with phosphate.
These localization experiments were performed with classical Edman technologies (4) and for the first time with ion spray mass spectrometry (5).

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Structure of the acetylcholine receptor

KARLIN, A.

No abstract received

α-BUNGAROTOXIN AND THE COMPETING ANTIBODY WF6 RECOGNIZE DIFFERENT PATTERNS OF AMINO ACIDS WITHIN THE SAME CHOLINERGIC SUBSITE

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Studying the binding of α-bungarotoxin and three different monoclonal antibodies able to compete with α-neurotoxins and cholinergic ligands, to a panel of synthetic peptides as representative structural elements of the nicotinic acetylcholine receptor (nAChR) from Torpedo, we recently identified the sequence segments α181-200 and α55-74 as contributing to form the cholinergic site (Conti-Tronconi et al., 1990). As a first attempt to elucidate the structural requirements for ligand binding to the subsite formed by the sequence α181-200, we have now studied the binding of α-bungarotoxin and of antibody WF6 to the synthetic peptide α181-200, and to a panel of peptide analogues differing from the parental sequence α181-200 by substitution of a single amino acid residue. CD-spectral analysis of the synthetic peptide analogues indicated that they all have comparable structures in solution, and they can therefore be used to analyze the influence of single amino acid residues on ligand binding.

Distinct clusters of amino acid residues, discontinuously positioned along the sequence α181-200, seem to serve as attachment points for the two ligands studied, and the residues necessary for binding of α-bungarotoxin are different from those crucial for binding of antibody WF6. In particular, residues at positions α188-190 (VYY) and α192-194 (CCP) were necessary for binding of α-bungarotoxin, while residues W_{187}, T_{191}, Y_{198} and the three residues at positions α193-195 (CPD) were necessary for binding of WF6. Comparison of the CD-spectra of the toxin/peptide complexes, and those obtained for the same peptides and α-bungarotoxin in solution, indicates that structural changes of the ligand(s) occur upon binding, with a net increase of the β-structure component.

The cholinergic binding site is therefore a complex surface area, formed by discontinuous clusters of amino acid residues from different sequence regions. Within this relatively large structure, cholinergic ligands bind with multiple points of attachment, and ligand-specific patterns of the attachement points exist. This may be the molecular basis of the wide spectra of binding affinities, kinetic parameters and pharmacologic properties observed for the different cholinergic ligands.

THREE-DIMENSIONAL STRUCTURE OF THE NICOTINIC ACETYLCHOLINE RECEPTOR: WHAT THE STRUCTURE OF THE ACETYLCHOLINE RECEPTOR AT 22Å RESOLUTION BY LOW DOSE ELECTRON MICROSCOPY AND X-RAY DIFFRACTION TO 12.5Å IMPLIES FOR FUNCTION, LOCATION OF THE MAJOR ASSOCIATED 43kD CYTOSKELETAL PROTEIN, AND STRUCTURE CHANGE

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ABSTRACT

The three-dimensional structure of the nicotinic acetylcholine receptor (AChR) from *Torpedo californica*, crystallized both before and after removal of associated proteins, most notably the main 43 kD cytoskeletal protein that interacts both with AChR and actin, is determined to a resolution of 22 Å. This is the first structural analysis where the 43 kD protein has been removed from the sample prior to crystallization. Thus it provides the most reliable assessment of what constitutes the structure of the minimal five subunit AChR complex, and by comparison with the native membrane, of the location of the 43 kD cytoskeletal protein. Image reconstruction from electron images of up to ± 52^0 tilted specimens of latticed AChR and hybrid density maps that include X-ray diffraction perpendicular to the membrane to 12.5 Å resolution were used.

The actin-binding 43 kD component is closely associated with the lipid bilayer as well as with the cytoplasmic domain of the AChR. It binds beside the AChR. Acetylcholine receptors aggregate in the absence of any cytoskeletal proteins, suggesting that the AChR alone is sufficient to encode and stabilize clustering, and perhaps to do so during synaptogenesis. The main 43 kD component may play a role in location and rate of association of AChR. The disulfide bond that crosslinks δ–δ chains of adjacent pentamers in about 80% of AChR, is not required to stabilize the lattice of AChR. Latticed tube structures are stable indefinitely. The lattices described here have 20% less volume of lipid than those we originally obtained and characterized Kistler,J.and R.M.Stroud. 1981. *Proc. Natl. Acad. Sci. USA*. 78: 3678-3682., or those subsequently characterized by Unwin et al.

Changes in structure that accompany opening and interaction with local anaesthetics are discussed in relation to the mechanism of action of AchR.

The nicotinic acetylcholine receptor at the rat neuromuscular junction: Structure, function, regulation

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Two acetylcholine receptor (AChR) subtypes exist in mammalian muscle which differ in their structure, function and subcellular distribution. We have cloned all five AChR subunit specific cDNAs.Their functional expression in *Xenopus*-oocytes supports the notion that channels comprised of α-,β-,γ-and δ-subunits correspond to the receptors predominating during early development and in denervated muscle while channels containing α-,β-,ε-, and δ-subunits represent the synaptically localized AChR of the adult , innervated muscle.During development the AChR subunit specific mRNA levels change in a characteristic manner indicating that all subunits are regulated differentially. Both myogenic factors as well as signals linked to the electrical activity of the muscle regulate the expression of the two AChR subtypes. Experiments were conducted to differentiate between the signals of 'neural'or 'muscular'origin: The effects of denervation or muscle inactivation using neurotoxins which block neuromuscular signal transmission at different synaptic sites reveal that extrasynaptic AChR mRNAs are strongly regulated by muscle activity. The accumulation and maintenance of AChR mRNA at the synapse , however, is mediated by neural signals.Genomic DNA fragments of the γ- and ε- subunit genes were isolated to analyze mechanisms regulating AChR expression at the level of the genes. The experiments suggest that myogenic factors such as MyoD1 control the γ- and ε- subunit genes in a differential manner.

Expression and in vivo amplification of human acetylcholinesterase and butyrylcholinesterase genes

Soreq, H.

No abstract received

Cholinesterases and cell surface glycoconjugates in the chicken neural tube: a new look at presynaptogenetic neuronal specificity.

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The role of acetylcholinesterase (AChE) in neurotransmission is well known. But long before synapses are formed in vertebrates, AChE is expressed in young postmitotic neuroblasts that are about to extend the first long tracts. AChE histochemistry can thus be used to map primary steps of brain differentiation. Preceding and possibly inducing AChE in avian brains, the closely related butyrylcholinesterase (BChE) spatially foreshadows AChE-positive cell areas and the course of their axons. In particular, before in the trunk spinal motor axons grow out, their corresponding rostral sclerotomes and myotomes express BChE, and both their neuronal source and myotomal target cells express AChE (ref. 1). Similarly in the head, cholinesterases precede the outgrowth of cranial nerves from segmented rhombomeres.

Whereas BChE demarcates areas of positive neurite growth, the peanut lectin (PNA) binding specifically to sugar moieties of cell surface glycoconjugates, labels matrices that are void of neurites. This will be demonstrated for the caudal halves of the sclerotomes, for the mesenchymal matrix that embeds cranial nerves, and for the radial neuroepithelial borders that separate individual rhombomeres from each other (ref. 2). The possible functions of cholinesterases and of PNA-binding glycoconjugates in attracting or retracting growing neurites, respectively, will be briefly discussed.

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1. Layer, P.G., *Bioessays* 12(9), September 1990.
2. Layer, P.G. & R. Alber (1990) *Development* 109, 613-624.

Research Directions Emerging from the Structure of Acetylcholinesterase and its Gene. Palmer Taylor, Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093

The cloning of the genes encoding the various cholinesterases, initially from Torpedo and now in several other species, has provided a structural framework for examining residues and domains in the enzyme critical for catalysis, inhibitor susceptibility and localization within the cell. Acetylcholinesterase (AChE) gene structure has also shown that alternative mRNA processing is responsible for the biosynthetic divergence of the major molecular species (asymmetric or A) and the glycophospholipid containing (amphiphilic G) species in Torpedo. These conclusions are now buttressed by studies of expression of the cDNA's encoding individual enzyme species. Both of these classes of molecular forms of the enzyme exist in neurons and muscle of fish and amphibians, but the glycophospholipid form appears largely confined to hemopoetic cells in mouse.

Current research in our laboratory has employed transient transfection of cDNA's encoding the glycophospholipid-containing form of Torpedo AChE, the predominant brain species of murine AChE, and murine butyrylcholinesterase. This approach has defined residues critical to proton transfer within the active center, nucleophilicity of the serine and substrate inhibition. By deletion and exchange of the third exon in the open reading frame, it has been possible to alter the cellular disposition of the enzyme when expressed in COS cells. Expression in the Spodoptera-baculovirus system allows for production of large quantities of AChE and its mutant forms.

Analysis of the genomic sequence has also provided several leads into mechanisms of regulation of AChE expression and it is likely that conserved base sequences will prove important to detailing critical nucleic acid domains. Although certain differences in gene organization have been detected between Torpedo and mammals, the general features of the exon-intron junctions and alternative mRNA processing are preserved. The Torpedo AChE gene contains AP-1, AP-2 and a partial myo D cis elements which are subject to regulation when inserted into synthetic constructs. The gene also contains inverted repeat sequences in the 3' untranslated regions of the alternative exons encoding the two major species of enzyme. While the influence of such sequences in the control of AChE expression during development and tissue differentiation remains to be determined, transfection studies show a potential role for both transcriptional and translational regulation of gene expression.

Acetylcholine receptor gene expression in experimental autoimmune myasthenia gravis

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The nicotinic acetylcholine receptor (AChR) has been shown to be a major autoantigen in myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG). Humoral and cellular anti-AChR immune responses are observed in myasthenia, and are accompanied by receptor loss. In an attempt to elucidate the molecular mechanisms underlying the induction of EAMG and subsequent decrease in AChR, we have addressed the question whether AChR gene expression is altered in myasthenic animals. Rabbits, mice and rats were injected with purified Torpedo AChR in order to induce EAMG. Development of antibody and EAMG were followed, and AChR loss as well as AChR gene expression were analyzed. An increase in AChR transcripts was demonstrated to be exclusively associated with myasthenic symptoms and with a severe loss in membrane AChR. An increase of α-, β-, ε-, and δ- subunit specific mRNAs (5.2, 1.6, 3.2 and 3.7 fold, respectively), which code for the adult type of AChR ($\alpha_2\beta\varepsilon\delta$) was observed in EAMG in rats. The γ-subunit transcript was not detectable in myasthenic or healthy rats. It appears that the regulatory control of AChR gene expression in EAMG is different from that observed upon denervation.

MOLECULAR BIOLOGY OF THE HUMAN ACETYLCHOLINE RECEPTOR

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The acetylcholine receptor at the neuromuscular junction is the target for autoantibodies in myasthenia gravis (MG), and may be involved in some forms of congenital or inherited neuromuscular disorders.

We have isolated cDNA clones for the human α, β, γ and δ subunits, and part of the ϵ subunit. The genes coding for the α and δ subunits have been localised on chromosome 2 and the β subunit on chromosome 17.

The human AChR α subunit exists as two isoforms, one of which includes a 25 amino acid insert encoded by a previously unreported exon located between exons P3 and P4, which we have termed P3A.

The cDNAs are being used to detect polymorphisms within the human genes, in healthy individuals and in those with inherited forms of myasthenia, and to generate recombinant polypeptides in *E. coli* to help in our studies of the autoimmune response.

MG and experimental (mouse) anti-AChR antibodies are of high affinity, and bind to several regions on the extracellular surface of the molecule. In contrast, mice immunised against recombinant α subunit make antibodies that are mostly directed towards determinants within the cytoplasmic domain. Recombinant polypeptides have been used to stimulate and clone T lymphocytes from MG patients and from control individuals. The T cell epitopes involved are being mapped using smaller recombinants and synthetic peptides.

Electrophysiological properties of five different avian neuronal nAChRs expressed in Xenopus oocytes.

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Department of Biochemistry and *Physiology (Medical School), University of Geneva, 1211 Geneva 4, Switzerland.

cDNAs and genes encoding six neuronal nAChR alpha subunits (α2 to α7) and three non-alpha subunits (nα1 to nα3) have been cloned from chicken CNS and PNS cDNA libraries and from a chicken genomic library.

Using an improved and simplified DNA injection procedure, we examine and compare the responses to ACh of the four pairwise combinations α3/nα1, α3/nα3, α4/nα1, and α4/nα3 in voltage clamped oocytes. We find that receptors assembled from α3 are much less sensitive to ACh and desensitize more rapidly than those formed with α4. In addition, we find that nα3 confers a lower ACh sensitivity to both α3- and α4-containing receptors. We argue that "ganglionic" receptors are likely to assemble from α3 and nα3 because α3 and nα3 transcripts are readily detected in neurons of the PNS and because the reconstituted α3/nα3 channel has typical ganglionic ACh sensitivity and desensitization properties.

α7, the most recently isolated neuronal nAChR subunit, has fascinating properties. In oocytes, it assembles into a functional homo-oligomeric nAChR which is i) blocked by αBTX (IC_{50} 0.7 nM), ii) more sensitive to nicotine (EC_{50} 11 μM) than to ACh (EC_{50} 110 μM) and, iii) desensitizes very rapidly at elevated ACh concentrations. As α7 transcripts in the developing avian optic tectum accumulate in accord with αBTX binding activity and as an α7 bacterial fusion protein binds αBTX efficiently, we conclude that some and perhaps all of the saturable, high-affinity αBTX binding activity in the vertebrate nervous system consists of heretofore unrecognized nAChRs.

The structure, function and expression of the brain nicotinic and glutamate receptor families

HEINEMANN, S.

No abstract received

SEQUENTIAL EVENTS DURING NEUROMUSCULAR SYNAPTOGENESIS.

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During embryogenesis neurons, Schwann cells and muscle cells are tighly juxtaposed and interact for synapse formation and synapse maturation.

Cocultures of spinal cord neurons and muscle cells taken from rat embryos were used for in vitro reproduction of embryonic synapses. This system did not display the synaptic maturation characteristics of post natal development: decreased multiple innervation and the presence of developed subneural apparatus. Studies on cultures consisting of 3 cell types (muscle cells, nerve cells, Schwann cells), or on cocultures (muscle cells, nerve cells), in the presen- or absence of a monoclonal antibody (6.17) directed against an antigen from Schwann cells, have shown that the Schwann cells play a role in synaptic maturation. (The synapses were visualised for optical microscopy by co-localisation of acetylcholinesterase(AChE) spots and acetylcholine receptor (AChR) clusters.)

The results indicate that: (1) the synaptic population decreased when Schwann cells were added to muscle/neuron cocultures, and increased when antibody 6.17 was present in the cocultures. (2) In two-cell cocultures (muscle/neuron) we could observe several synapses on a myotube, and in 3-cell cocultures (muscle/neuron/Schwann cell) we saw only one synapse for a similar length of myotube. (3)Synaptic maturation as well as the colocalisation of AChR and antibody 6.17 were only noted when the 3 cell types were present.

This suggests that Schwann cells express one or more signals necessary for the deseappearance of multiple innervation and for synapse maturation.

Antigen 6.17 has been purified and characterized.

POSTERS

PART 1

Monday and Tuesday

THE CALCIUM-DEPENDENT ACETYLCHOLINE RELEASER MEDIATOPHORE MAY BELONG TO A NEW FAMILY OF CATION-TRANSLOCATING PROTEINS.

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The mediatophore is an oligomeric transmembrane protein isolated from Torpedo electric organ synaptosomes. Several lines of data argue for a direct role of this protein in the mechanism of acetylcholine release (see abstract by M. Israël). Upon SDS PAGE of purified mediatophore, a doublet at 15 kDa was identified. Amino-acid sequencing after CNBr cleavage gave a 17-residue long peptide, allowing synthesis of a 51-mer oligodeoxynucleotide. A library of Torpedo electric lobe cDNAs was constructed in λZap II and screened with this probe. A positive recombinant clone was isolated and found to encode the complete sequence of a 15.5 kDa protein, and this was confirmed by in vitro translation of sense RNA transcripts of the clone. The peptide presents sequence homology with the bovine chromaffin granule or yeast vacuolar ATPase proteolipid, a 23 kDa yeast protein of unknown function, and a 16 kDa protein co-isolating with bovine brain gap junctions. In Torpedo, high levels of expression of the 1.3 kb mRNA encoding the mediatophore subunit were only found in nervous tissues.

Pharmacological distinction of cholinergic processes at nerve terminal using cetiedil analogs.

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In cholinergic nerve terminals,two essential physiological functions are dependent on acetylcholine(ACh) fluxes: 1-translocation of ACh to the presynaptic cleft,as the last event of presynaptic transmission 2-translocation of cytoplasmic ACh into the synaptic vesicles where the transmitter is concentrated.Another related process is the presynaptic choline transport.

The present study aimed to compare the pharmacological properties of these three cholinergic functions,studied on synaptosomes and synaptic vesicles isolated from the electric organ of Torpedo.Among the drugs tested,two were highly specific for one function : vesamicol for vesicular ACh transport , hemicholinium-3 for high affinity choline uptake. Other compounds such as cetiedil have been shown to be less specific since they affect all three functions.In fact,cetiedil resembles ACh with a cumbersome end and on kinetic studies of vesicular ACh transport it was shown to act as a competitive inhibitor.A series of cetiedil analogs was then synthezised and a pharmocological pattern of choline uptake,evoked ACh release and vesicular ACh transport has been established.Some drugs that block vesicular ACh transport were shown either to be ineffective on evoked ACh release or even to potentiate it. Choline uptake has still a different pattern.

Tritiated vesamicol and cetiedil are currently being used as high affinity ligands on presynaptic and vesicular membranes.

CAPACITANCE CHANGES DURING EXO- AND ENDOCYTOSIS

IN SINGLE VERTEBRATE NERVE TERMINALS

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[2] Centre de Neurochimie, CNRS, 5 rue Blaise Pascal F-67048 Strasbourg, France

We have monitored capacitance changes associated with exocytosis and endocytosis in single isolated nerve terminals using time-resolved patch-clamp capacitance measurements in the 'whole-terminal configuration. These experiments directly demonstrate fusion of large numbers of granules in response to depolarisation under voltage clamp. The fine structure of the capacitance changes suggest the fusion of individual granules with the plasma membrane as well as reversible fusion events of large vacuoles.

In cells which were heavily stimulated by prolonged depolarisation large ON and OFF steps were observed which apparently represent exo- and endocytosis of large vacuoles. During fusion of such a large vacuole a short lived state with a narrow fusion pore of ~ 1 nS conductance could be observed preceding the long lived fused state with a wide fusion pore.

When terminals are loaded with fura-2 in the 'whole-terminal' patch-clamp configuration, the intracellular calcium concentration increases with a half time of 500 - 800 ms in response to depolarisation and may stay elevated for minutes. The capacitance measurements demonstrate that exocytotic granule fusion may continue for many seconds during prolonged depolarisation. Elevation of intracellular calcium in the absence of depolarisation also induces granule fusion indicating that the membrane potential change is not essential in stimulating exocytosis. These experiments directly demonstrate the correlations between depolarisation, intracellular calcium and exocytosis in nerve terminals. However, the details in the time course of exocytosis and intracellular calcium suggest that the calcium at the plasma membrane, rather than the bulk intracellular calcium appears to be the essential regulator of exocytotic granule fusion.

VAT-1, A Membrane Protein of Synaptic Vesicles from Marine Rays - A Structural Analysis

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VAT-1, a synaptic vesicle membrane protein, was isolated from *Torpedo* electric lobe cDNA library. The RNA is specifically expressed in the electric lobe of marine rays and to a much lesser extent in other parts of the brain. No expression is detected in non-neural tissues. The product is an abundant 41 kDa protein of the cholinergic synapse, specifically located within the membrane of synaptic vesicles. A small amount (15-20%) of VAT protein is located to the plasma membrane (Linial et al., 1989, Neuron 2, 1265-1273).

The topology of VAT-1 within the membrane was studied by applying a combination of proteases digestion on intact purified synaptic vesicles ,and probing the digested products by specific antibodies. This analysis revealed an in/out topology within the vesicular membrane. The protein carboxyterminal tail faces the lumen while the aminoterminal portion faces the cytoplasm. This aminoterminal of the protein is very hydrophilic and shares a significant similarity (67% similarity over 32 amino acids) to Ca^{++} binding domain of Calmodulin.

The native form of VAT-1 was analysed and its hydrodynamic properties were determined. As revealed by glycerol gradient and by gel filtration on FPLC, VAT-1 in its native form is part of a large protein complex of about 180 kDa, which is composed of repeated units of the 41 kDa protein. By cross-linking solubilized vesicles membranes by gluteraldehyde, the native form of VAT protein in the complex was confirmed, each complex is composed of 3-4 subunits of 41 kDa protein. An immunoprecipitation of iodinated synaptic vesicles membranes resulted in co-precipitation of VAT-1 and an additional 34 kDa protein which represents an abundant specific protein in *Torpedo* synaptic vesicles.

The protein complex of VAT-1 is copurified with an ATPase activity. This activity shares some properties of the vesicular ATPase P type. Partial depletion of this ATPase activity from the solubilized synaptic vesicles membranes was obtained by antibodies against VAT-1. We are further exploring the function of VAT protein and its interplay with other components of the vesicular membrane.

Characterization of a novel protein of synaptic vesicles

MARQUEZE-POUEY, Béatrice

No abstract received

Synaptoporin: A novel putative channel protein of synaptic vesicles

KNAUS, Petra

No abstract received

DIFFERENT EFFECTS OF $GABA_A$-BENZODIAZEPINE RECEPTOR LIGANDS ON THE IN VITRO ACETYLCHOLINE RELEASE IN CORTICAL SLICES AND SYNAPTOSOMES

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Abstract

Alzheimer's disease is a progressive disorder involving deterioration of intellect, memory and personality. There is considerable evidence implicating cortically- and hippocampally-projecting cholinergic systems located in basal forebrain in learning and memory processes, although their roles have not been clearly defined. The functional integrity of these systems declines with age and they are severely affected in Alzheimer's disease. Therapeutic approaches involving direct manipulation of cholinergic neurotransmission have not been successful. An alternative approach may be to manipulate cholinergic transmission indirectly, possibly by reducing the efficacy of gabaergic inhibition. This may occur at the soma level in basal forebrain, or at the terminal level, by influencing presynaptically located receptors. Some studies have reported interactions between cholinergic and gabaergic systems in the basal forebrain complex. In particular, benzodiazepine receptor agonists have been shown to reduce the efficacy of cholinergic transmission. Compounds acting as antagonists or inverse agonists at the BDZ-receptor may therefore have facilitating effects on cholinergic transmission. To study the effects of such compounds, the *in vitro* [^{3}H]-ACh release from frontal cortex slices and cortical synaptosomes was investigated and compared.

$GABA_A$ and BDZ-receptor ligands influenced the *in vitro* [^{3}H]-ACh release from cortical synaptosomes and slices. In contrast, in our hands, these compounds did not effect the [^{3}H]-ACh release from hippocampal and striatal slices. The results obtained with cortical preparations showed that the $GABA_A$ receptor agonist, muscimol, dose-dependently inhibited the [K^+]-elicited release of [^{3}H]-ACh. This small but consistent reduction (to approx. 80% of control level) is postulated to be due to a chloride shunting mechanism following $GABA_A$ receptor activation. In keeping with this hypothesis, BDZ-receptor agonists (Diazepam, ZK 93423) enhanced and BDZ-receptor inverse agonists (DMCM) antagonized muscimol effects in cortical synaptosomes and slices. BDZ-receptor antagonists (ZK 93426, Ro 15-1788) and partial inverse agonists (FG 7142; CGS 8216; Ro 15-4513) also influenced the [^{3}H]-ACh release. They antagonized the muscimol induced reduction in [K^+]-stimulated [^{3}H]-ACh release from cortical slices, while having no (ZK 93426; Ro 15-1788) or the same effect (Ro 15-4513) on [^{3}H]-ACh release from cortical synaptosomes. It is possible that these differing effects of BDZ-receptor antagonists and partial inverse agonists on the [^{3}H]-ACh release in cortical slices and synaptosomes are mediated by $GABA_A$-BDZ-receptor subtypes. This points to a local, presynaptic modulation of ACh release mediated by gabaergic interneurons in cortex. These presynaptically located BDZ-receptors could constitute targets for cognition or vigilance enhancing antagonists or partial inverse agonists.

Neurotransmitter distribution and content analysis in the rat brain

BURGER, Peter

No abstract received

Vesicular amino acid uptake: characterization and reconstitution

MAYCOX, Peter

No abstract received

EXPRESSION OF HIGH AFFINITY SODIUM DEPENDENT CHOLINE TRANSPORT IN *XENOPUS* OOCYTES INJECTED WITH *TORPEDO* ELECTRIC LOBE mRNA

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During culture, *Xenopus laevis* oocytes developed the capacity to accumulate choline by an apparently high affinity mechanism. Endogenous choline uptake was not changed upon addition of 10^{-5} M hemicholinium-3 or when Li^{+} replaced Na^{+} in the medium, indicating that this transport mechanism is independent of sodium.

Oocytes injected with electric lobe poly $(A)^{+}$ mRNA from *Torpedo marmorata* showed an additional component of choline uptake that was sensitive to a low concentration of hemicholinium-3, and whose presence could only be revealed in the presence of a high concentration of sodium, thus resembling choline transport by the cholinergic transporter from electric organ nerve terminals.

These results suggest that high affinity choline uptake may occur by two different mechanisms and are consistent with the hypothesis that the neuronal choline transporter uses the sodium gradient to translocate choline.

THE STIMULATION-DEPENDENT EFFECTS OF (-)-VESAMICOL ON MINIATURE END-PLATE CURRENT AMPLITUDES IN THE CUT RAT HEMI-DIAPHRAGM

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( )-Vesamicol inhibits the uptake of acetylcholine (ACh) into isolated synaptic vesicles from the *Torpedo* electric organ. Biochemical studies have shown, in a wide variety of preparations, that vesamicol inhibits the release of newly synthesised ACh with little or no effect on the release of previously loaded ACh (for review see Marshall & Parsons, 1987). (-)-Vesamicol therefore represents a potentially powerful agent for investigating the recycling and release of quanta at the neuromuscular junction. We have previously reported that stimulation at 10 Hz for 5 min in (-)-vesamicol results in the production of a bi-modal distribution of miniature end-plate current (MEPC) amplitudes consisting of both a pre-stimulation amplitude population of MEPCs and a population of smaller amplitude MEPCs whose amplitude is dependent on the concentration of (-)-vesamicol (Marshall, Prior & Searl, 1990). We have now investigated the stimulation-dependence of these two populations. End-plates were voltage-clamped at -55 mV. A two minute recording was made of the pre-stimulation MEPCs. Following this, the nerve was stimulated for 1 min periods at 10 Hz. Between each successive period of stimulation, a two min recording was made of the post-stimulation MEPCs. This protocol was repeated until the preparation had received a total of 10 blocks of stimulation. In control experiments, stimulation had no effect on MEPC amplitudes. However, in the presence of 100 nM ( )-vesamicol, stimulation caused a progressive reduction in the mean MEPC amplitude. In addition to a reduction in the mean amplitude of MEPCs, the distribution of MEPC amplitudes also changed with stimulation. Following 3 - 4 blocks of stimulation, MEPC amplitudes had distinct bi-modal distributions, consisting of both pre-stimulation amplitude MEPCs and a population of smaller amplitude MEPCs. With continued stimulation, the numbers within the small-mode MEPC population increased, whilst the numbers of pre-stimulation amplitude MEPCs became reduced. Following 8 - 9 blocks of stimulation, the reduction in MEPC amplitude approached a plateau, only a small number of pre-stimulation amplitude MEPCs remained and MEPC amplitudes were well fitted by single Gaussian distributions. These results are consistent with our previous suggestion (Marshall, Prior & Searl, 1990), that the small-mode MEPCs seen following stimulation in the presence of 100 nM (-)-vesamicol, represent recycled quanta whose filling has been inhibited by the presence of vesamicol, while the normal-mode MEPCs represent pre-formed quanta. These observations represent further evidence in support of the vesicular hypothesis of neuromuscular transmission.

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THE PANCREATIC ACINAR CELL LINE AR42J IS AMPHICRINE: COMBINED EXPRESSION OF EXOCRINE AND NEUROENDOCRINE PROTEINS

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The permanent cell line AR 42J, derived from a rat pancreatic acinar carcinoma, is widely used and considered to be a useful in vitro system for exocrine cell function. While detailed data on the cytoskeletal and secretory membrane components are still lacking, it is assumed that these zymogen granule containing cells are of epithelial differentiation. We now present evidence that AR42J cells are clearly epithelial cells and express polypeptides characteristic of the exocrine as well as the neuroendocrine phenotype. As determined by immunohistochemistry and biochemical analysis, these cells contain an extensive meshwork of bundles of cytokeratins, equivalent to human cytokeratin polypeptide Nos. 8 and 18. Other intermediate filament proteins were not found. In addition, immunofluorescence microscopy, Western and Northern blot analyses with monospecific antibodies and cDNA probes for SV2 and synaptophysin showed an expression pattern of these neuroendocrine markers that is characteristic of the neuroendocrine phenotype. Biophysical characterization of subcellular fractions immunoreactive for synaptophysin revealed particles similar in size and density to presynaptic vesicles known to contain synaptophysin as a major membrane component. Glucocorticoids known to increase the synthesis of zymogen granules did not affect neuroendocrine vesicle expression as determined by quantitative dot immunoasssay and Northern blot analysis for synaptophysin. In summary, the pancreatic AR42J cell represents the first permanent cell line with combined features of exocrine and neuroendocrine phenotypes, i.e. coexpression of zymogen granules and small neuroendocrine vesicles. These findings support the hypothesis of free interconversion of exocrine and endocrine cells.

The Monoclonal Antibody 2G8 is Carbohydrate Specific and Distinguishes between Different Forms of Vertebrate Cholinesterases

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An inhibitory monoclonal antibody (mAb) 2G8 (subclass IgG2a), raised against acetylcholinesterase (AChE, EC 3.1.1.7) from electric organ of *Torpedo nacline timilei*, crossreacted with AChE from *Torpedo marmorata*, electric eel, flounder (*Platichthys flesus*) body muscle, rat brain, bovine brain and human brain, suggesting that the epitope to which mAb 2G8 bound, had been highly conserved during evolution. No crossreaction was found with AChE from human and bovine erythrocytes, nor with butyrylcholinesterase (BtChE, EC 3.1.1.8) from human serum. Binding of mAb 2G8 to the globular G2-form of AChE from *Torpedo marmorata* strongly decreased enzyme activity, while no significant inhibition was found with either collagen-tailed, asymmetric forms, or with the enzymes from flounder body muscle and from mammalian sources. The possibility that mAb 2G8 bound to anionic sites of AChE could be excluded since neither edrophonium chloride nor decamethonium bromide influenced the binding of 2G8 to the enzymes. Enzyme-linked immunosorbent assay and western blot showed that heat denatured, DFP-treated, CNBr and trypsin digested AChE from *Torpedo marmorata* still reacted with mAb 2G8, indicating that the epitope to which 2G8 bound at least partially belonged to a continuous determinant. Treatment of cholinesterases with N-glycosidase F abolished crossreaction with 2G8 showing that an essential part of the epitope consisted of N-linked carbohydrates.

EXPRESSION OF ACTIVE *TORPEDO CALIFORNICA* ACETYL-CHOLINESTERASE IN INSECT CELLS

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The enzyme acetylcholinesterase (acetylcholinhydrolase, AChE) plays an important role in the cholinergic mechanism by hydrolysing acetylcholin. To get insight, at a molecular level, of the structure/function of the enzyme a molecular approach would be of interest. Complementary DNA (cDNA) clones from a *Torpedo californica* λgt10 cDNA library (1) encoding AChE have been identified by hybridisation with oligonucleotides complementary to a previously cloned AChE gene (2). Several cDNA clones were isolated and sequenced. To a clone encoding the mature form of AChE oligonucleotides were fused to form hybrid constructs encoding secreted and mature forms of the enzyme. A hybrid AChE construct, in which the mature form of AChE is preceded by the signal peptide of *Torpedo marmorata* AChE, was cloned into the Baculovirus cloning vehicle pAc373 (3). This plasmid was cotransfected with wild-type Baculovirus DNA into *Spodoptera frugiperda* cells and recombinant virus carrying the AChE gene were identified by filter-hybridisation and isolated. Purified recombinant virus clones were multiplied and used for infection of *S. frugiperda* cells. Infected cells were harvested 2-4 days following infection and cell extracts were analysed by enzyme activity measurements and immunoblotting. The localisation of AChE was analysed by electronmicroscopy and immunofluorescens labeling.

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Exclusion of the Asp70-->Gly substitution in human BCHE from correlation with tumorogenicity by PCR-RFLP autoradiography

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Abstract

Substitution of Asp70 by Gly in human butyrylcholinesterase (BCHE) has been found in DNA from individuals with the "atypical" BCHE phenotype (McGuire et al., 1989) and from glioblastoma and neuroblastoma tumors (Gnatt et al, 1990), raising the question whether this particular variation in the BCHE gene is correlated with tumorogenicity. To answer this question, the appearance of the mutation causing the Asp70 --> Gly substitution was pursued in DNA samples from various tumors and blood cell disorders where abnormalities in the BCHE gene were previously found as compared with samples from individuals with no record of tumors or BCHE abnormalities. For this purpose, the fragment of the BCHE gene including this site of substitution was amplified in vitro by the polymerase chain reaction (PCR) and the resultant DNA was subjected to enzymatic restriction with SauIIIA, which digests the "usual" BCHE gene but not the "atypical" one. End labeling of the SauIIIA restriction fragments with [^{32}P], followed by polyacrylamide gel electrophoresis, enabled their detection by autoradiography with high resolution and sensitivity, creating an informative pattern of restriction fragment length polymorphism (RFLP) characteristic of this substitution.

Using this PCR-RFLP autoradiography method, a heterozygous A-->G alteration disrupting this informative SauIIIA site was detected in PCR-amplified BCHE DNA from four different sources, out of 32 samples which were examined (14%). These included the previously characterized glioblastoma and neuroblastoma tumors, a benign ovarian cyst and peripheral blood cells from an individual with amplified BCHE gene who was exposed to parathion. In contrast, this mutation was <u>not</u> observed in 5 different samples of glioblastoma multiform, 6 ovarian carcinomas, 10 peripheral blood cell samples from hemopoietic disorders and 7 samples from individuals with occupational exposure to organophosphorous (OP) poisons, history of post-anasthetic apnea in the family or no clinical records. Altogether, 13 DNA samples with amplified BCHE genes did not display this variation.

Recombinant variant serum BCHE produced in microinjected oocytes and serum BCHE in individuals which were thus found to carry the mutation responsible for the Asp70-->Gly substitution was found to be equally resistant to inhibition by succinylcholine and the naturally occuring alkaloid solanidine, while "usual" BCHE was sensitive to both these ihibitors <u>in vivo</u> and <u>in ovo</u>. These findings confirm the validity of the PCR-RFLP autoradiography approach to detect the Asp70-->Gly substitution, demonstrate the phenotypic effect of this sequence alteration and exclude the Asp70-->Gly substitution in human BCHE from correlation with tumors and/or BCHE gene amplification; further analyzes will be required to determine whether this is also the case for the other 9 variations which were so far found in the human ACHE gene or the alterations in the related ACHE gene encoding acetylcholinesterase.

Molecular cloning and analysis of the human acetylcholinesterase coding sequence.

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To study the primary structure of human acetylcholinesterase (ACHE EC 3.1.1.7) and its gene expression and amplification, cDNA libraries from human tissues expressing oocyte-translatable ACHE mRNA were constructed and screened with labeled oligodeoxynucleotide probes. Several cDNA clones were isolated which encode a polypeptide with ≥50% identically aligned amino acids to Torpedo ACHE and human butyrylcholinesterase (BCHE; EC 3.1.1.8). However, these cDNA clones were all truncated within a 300 nucleotides long G,C-rich region with a predicted pattern of secondary structure having a high free energy (-117 kcal/mole) downstream from the expected 5'-end of the coding region. Screening of a genomic DNA library revealed the missing 5'-domain. When ligated to the cDNA and constructed into a transcription vector, this sequence encoded a synthetic mRNA translated in microinjected oocytes into catalytically active ACHE with marked preference for acetylthiocholine over butyrylthiocholine as a substrate, susceptibility to inhibition by the ACHE inhibitor BW284C51 and resistance to the BCHE inhibitor iso-OMPA. The ACHE coding sequence was found to be particularly rich in G,C residues (64%),, in contrast with the A,T-rich composition (63%) of BCHE coding sequence. However, the complete ACHE and BCHE polypeptides display a considerable level (52%) of identically aligned amino acid residues, homology which is accounted for by differential codon usage. Furthermore, clear differences were observed by comparative surface probability analysis of the primary amino acid sequences. Thus, both the specific control of gene expression and the distinct immunological and catalytic activities of ACHE and BCHE appear to be inherent to their nucleotide and amino acid sequences. Blot hybridization of genomic DNA from different individuals carrying amplified ACHE genes revealed variable intensities and restriction patterns with probes from the regions upstream and downstream from the predicted G,C-rich structure. Thus, the human ACHE gene includes a putative G,C-rich attenuator domain and is subject to structural alterations in cases of ACHE gene amplification.

CHOLINESTERASE (ChE) AND DIFFERENT CILIARY STRUCTURES IN TWO INVERTEBRATES.

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ChE activity is localized by histochemical methods in adult Philodina roseola and Rotaria rotatoria (Rotifera Bdelloidea) and larvae (trochophore, veliger) of the mussel Mytilus galloprovincialis (Mollusca Pelecypoda) in order to investigate ciliary structures. Different substrates and inhibitors are employed to distinguish acetylcholinesterase (AChE) and pseudocholinesterase (BuChE) activities.

Both the rotifers and the mussel larvae have ciliary sensory organs intimately connected with the main nervous ganglion which stain intensely for AChE. In the rotifers ciliated sensory neurons wrapped by epithelial cells are localized at the tip of the dorsal tentacle and connected by AChE-active nerve fibres to the brain. On each side of the latter a "cerebral eye" is found, also consisting of a ciliated sensory neuron associated with an epithelial cell. A comparable, bilaterally symmetrical structure develops in the mussel larvae during the pre-trochophore and trochophore stages together with the rudiment of the viscero-parietal ganglion. This organ becomes more complex in the veliger larva, where is connected with a thick nerve bundle stained intensely for AChE and can either protrude or invaginate.

On the other hand, all the cilia-bearing cells of the rotatory apparatus of the rotifers and the velum of the veliger show a moderate histochemical staining for AChE and BuChE activities, localized around the nucleus, at the cell membrane and most evident at the base of the cilia. A few longer ciliary tufts stain all along their lenght. AChE-active nerve endings seem to contact sporadically ciliated epithelial cells. ChE is also localized in other ciliary organs, the protonephridial flame cells devoid of innervation.

The results suggest that ciliary sensory organs of these invertebrates are characterized by a strong ChE activity and a direct relationship with the nervous system involving possibly cholinergic synapses (Villeneuve & Clément, 1971). The enzyme activity of other ciliary structures may have a role in the mechanism of the ciliary beat and its coordination from one cell to another independent from innervation. The nerve endings which seem to reach a few ciliated cells of the locomotory apparatus may modulate the rate of the ciliary beat (Raineri, 1984).

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PARTIAL SEQUENCE OF ACETYLCHOLINESTERASE FROM THE VENOM OF THE COBRA NAJA NAJA

AChE and lysophospholipase have similar sequences

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The venom of a variety of elapid snakes has been known as a rich source of acetylcholinesterase (AChE). The enzyme has been purified from several snake venoms including the venom of the cobra Naja Naja oxiana (Raba et al., 1979). It is isolated from the venom as a monomer and consists of a single polypeptide chain with a molecular weight of 67 kDa (Raba et al., 1982).

About 30 % of the primary structure of the acetylcholinesterase (AChE) from Cobra venom were determined by protein chemical methods. The sequence of the tryptic peptide around the active site serine labeled by diisopropyl-fluorophosphate (DFP) was found to be **TVTLFGESAGAASVGM** which is similar to other cholinesterases. The part of the structure determined shows 76 % identity with AChE from Torpedo and 42 % identity with the enzyme from Drosophila (Weise et al., 1990). - A surprisingly large sequence identity (42 % in the sequence determined) was found with lysophospholipase from rat (E.C. 3.1.1.5.) as determined by Han et al., 1987. The sequence immediately flanking the active site serine is invariably FGESAG in both enzymes.

This prompted us to investigate if AChE exhibits hydrolytic activity towards lysophospholipid substrates. AChE from Torpedo as well as from Cobra venom has no detectable lysophospholipase activity towards lysophosphatidylcholine (lyso-PC) and lysophosphatidylethanolamine (lyso-PE) under standard test conditions (pH 7.2, with and without addition of 10 μM Ca^{2+}). From this lack of overlapping enzymatic activity we conclude that even the very pronounced sequence similarity around the active serine residue is not sufficient to confer substrate specifity upon the enzymes.

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ANIONIC SUBSITES OF THE ACETYLCHOLINESTERASE FROM TORPEDO CALIFORNICA

Affinity labelling with the cationic reagent N,N-dimethyl-2-phenyl-aziridinium

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The neurotransmitter hydrolyzing enzyme AChE (E.C. 3.1.1.7.) is thought to contain several anionic binding sites, one of which is part of the catalytic center where it is responsible for the binding of the positively charged choline moiety of the physiological substrate (Nachmansohn and Wilson, 1951). One or several additional anionic sites have been postulated to be located peripherally (Changeux, 1966). - N,N-dimethyl-2-phenyl-aziridinium (DPA) was designed as an affinity reagent for the anionic subsite of the ACh binding site, combining the features of a true quarternary ion with high electrophilic reactivity (Belleau and Tani, 1966). The inhibition of the enzyme by the alkylation reaction can be prevented by alkylammonium salts (Palumaa et al, 1984); this supports the idea that DPA blocks the anionic site within the catalytic center.

Several peptides of acetylcholinesterase of *Torpedo californica* labelled with ^{3}H-DPA were localized within the primary structure. One peptide had the sequence **KPQELIDVE** (position 270-278); the incorporation of DPA into this peptide could be specifically suppressed by propidium, which suggests that it is part of the peripheral anionic site. The incorporation of DPA into two other peptides was insensitive to propidium but could be prevented by edrophonium; the sequence of one of the peptides assumed to be part of the anionic site in the catalytic center was found to be **DLFR** (positions 217-220). Decamethonium efficiently blocked alkylation by DPA in all three investigated peptides.

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Cloning and expression of the 58k β subunit of the inhibitory glycine receptor

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The inhibitory glycine receptor (GlyR) mediates postsynaptic inhibition in spinal cord and other regions of the CNS. Purified mammalian GlyR contains two membrane-spanning subunits of 48 kd (α) and 58 kd (β) plus a 93 kd receptor-associated cytoplasmic protein. Here the primary structure of the β subunit was deduced from cDNAs isolated from rat spinal cord and brain cDNA libraries. The predicted amino acid sequence exhibits 47 % identity to the previously characterised rat α1 polypeptide. Northern blot analysis revealed [illegible] levels of β subunit transcripts in postnatal spinal cor[illegible]ellum and cortex. Nuclear injection into Xenopus oocytes of a β subunit cDNA engineered for efficient expression generated weak glycine-activated chloride currents that were insensitive to the classic GlyR antagonist, strychnine. Our data indicate a differential expression of GlyR α and β subunits in the rat nervous system and support a structural role of the β polypeptide in the native receptor complex.

Site-directed mutagenesis of ligand-binding sites of the inhibitory glycine receptor

KUHSE, Jochen

No abstract received

POSTERS

PART 2

Wednesday and Thursday

Side-dependent effect of Mg^{2+} on single-channel conductance of nicotinic receptor-channels in rat PC12 (pheochromocytoma) cells.

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We have investigated the effect of Mg^{2+} on the conductance of neuronal nicotinic acetylcholine receptors (nAChRs) in nerve growth factor (NGF) treated rat pheochromocytoma (PC12) cells (Greene & Tischler, 1976). The patch-clamp technique was used to record acetycholine-induced single-channel currents from cell-attached and excised, outside-out patches. Both sides of the membrane faced a Ca^{2+}-free isotonic CsCl solution to which various amounts of Mg^{2+} were added.

The maximal conductance of the channel measured in a divalent cation-free solution was approximately 60 pS for both outward and inward currents. Increasing the internal Mg^{2+} concentration reduced the outward conductance of the neuronal nAChR channel in a concentration-dependent manner with an IC_{50} of 0.7 mM. The inward conductance of the channel was much less sensitive to increases of the external Mg^{2+} concentration as indicated by an IC_{50} of 9 mM. It has been suggested that the effect of Mg^{2+} is due to screening of rings of negatively charged amino acids placed on either side of the ion channel (Imoto *et al.*, 1988). The asymmetry of the effect of Mg^{2+} on the conductance of neuronal nAChR channels in PC12 cells suggests that the density and/or the number of charges differ on either side of the ion pore. Furthermore, whereas the effect of Mg^{2+} on outward conductance could be overcome by strong depolarization, the effect of Mg^{2+} on inward conductance displayed no voltage-dependence. This implies that the amino acids screened by divalent cations sense electric fields only weakly and are presumably outside the lipid bilayer.

It has recently been postulated that the Mg^{2+}-induced inward rectification of single channel current-voltage (I-V) relationship underlies the known rectification of whole-cell I-V curves (Ifune & Steinbach, 1990). However, whole-cell ACh-induced currents recorded in the absence of internal Mg^{2+} (20 mM EDTA inside) displayed clear inward rectification whereas the single-channel I-V did not (Neuhaus & Cachelin, 1990). Thus our and other's (Mathie *et al.*, 1990) results indicate that the inward rectification of whole-cell ACh induced currents is an intrinsic, probably kinetic, property of neuronal nicotinic receptors.

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FUNCTIONAL ARCHITECTURE OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

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Agonists and competitive antagonists of the nicotinic receptor reversibly bind, in a mutually exclusive manner, to a class of primary sites carried, at least in part, by the α-subunits. The photoactivatable cation p-(N,N-dimethylamino)benzene diazonium DDF, which acts in the dark as a competitive antagonist, efficiently labels upon irradiation the AcCho-binding sites of the native membrane bound receptor. This labeling i) is inhibited by the agonist carbamylcholine, the competitive antagonist α-bungarotoxin and ii) is distributed among three cyanogen bromide peptides (I-III) of the α-subunit, at the level of Tyr 93 (III)[1], Trp 149 (I), Tyr 190, Cys 192 and Cys 193 (II)[2]. In addition, carbamylcholine sensitive DDF incorporation is also detected, to a lower extent, at the level of Trp 86, Tyr 151 and Tyr 190[1,2]. These amino acids are located in the large hydrophilic amino terminal part of the α-subunit which is thus exposed, at least in part, to the synaptic cleft. Their labeling by DDF provides evidence for a multiple loop site for cholinergic ligands.

The noncompetitive blocker chlorpromazine has been used to probe the structure of the ion channel. When bound to its high affinity site, ^{3}H-chlorpromazine covalently labels all the subunits upon UV irradiation, suggesting that this site is located on the axis of symetry of the receptor. The labeled amino acids (α-Ser 248, β-Ser 254, β-Leu 257, γ-Thr 253, γ-Ser 257, γ-Leu 260 and δ-Ser 262)[3,4,5,6] all belong to a putative transmembrane, highly conserved, hydrophobic sequence of each subunit referred to as MII. The distribution of the labeled residues of the β- and γ-subunits is consistent with an α-helical organization of this hydrophobic segment. These results, together with site directed mutagenesis experiments[7,8] support the notion that the MII segments are involved in the ion permeation.

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Lipid phosphorylation by the nicotinic acetylcholine receptor of Torpedo californica

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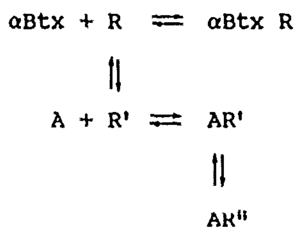
The detergent-solubilized nicotinic acetylcholine receptor (nAcChR) of Torpedo californica electrocytes shows lipid phosphorylation activities which are strongly dependent on the concentration of the neurotransmitter AcCh. In the absence of AcCh there are 2 % of the receptor which are catalytically active. In the presence of the inhibitor α-bungarotoxin (αBtx), this activity is reduced to 1 %.
Both the AcCh and αBtx effects can be summarized in a cyclic reaction scheme, involving the channel activatable conformation R, and one or more desensitized conformations R', R" which mediate lipid kinase activity.



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Determination of the Minimal α-Bungarotoxin Binding Site of the Nicotinic Acetylcholine Receptor.

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In this study, we have continued our analysis of the interaction between the area α184-200 of the nicotinic acetylcholine receptor of Torpedo californica and α-bungarotoxin.

Our approach was to construct a series of synthetic oligonucleotides corresponding to variations of the binding site sequence α 184-200 and to produce fusion proteins which could be biochemically tested for α-bungarotoxin binding.

Two types of modifications were tested: those with stepwise deletions of the NH_2-terminal residues from Trp-184 up to His-186 and those in which the COOH-terminal residues were removed sequentially from Asp-200 back to Thr-196.

^{125}I-labeled α-bungarotoxin overlays of protein blots and Scatchard analysis of toxin binding at equilibrium revealed that the stepwise removal of residues Asp-200 or Leu-199 caused only a marginal drop in affinity for α-bungarotoxin; However, the removal of residues Tyr-198, Pro-197 and Thr-196 had a marked effect on the toxin binding. The analysis of the NH_2-terminal variants demonstrated that deletion of His-186 caused greater than a 5-fold drop in affinity.

These data allowed the prediction of a minimal sequence of the binding site which should efficiently bind α-bungarotoxin. The 13 amino acid sequence, α186-198 was constructed and found to bind α-bungarotoxin with an affinity of $K_D = 4.7 \times 10^{-7}$ M.

NMR Studies of Specific Binding of Acetylcholine, Nicotine, Gallamine, and d-Tubocurarine to Recombinant Active Site Peptides of the nAChR.

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Localization of the binding site of the nicotinic acetylcholine receptor (nAChR) has been achieved by the use of snake venom neurotoxins which bind to the receptor with considerably higher affinity than acetylcholine (ACh). Binding studies to synthetic peptides as well as to recombinant peptides associated with the bacterial protein have indicated that the neurotoxins bind to the receptor in the area α170-200. Applying nuclear magnetic resonance (NMR) methods, in particular the selective T_1 relaxation measurements, have demonstrated this domain from Torpedo californica to contain the cholinergic binding site as well. Thus. ACh, nicotine, gallamine, and d-tubocurarine (TC) have been found to bind specifically to the α184-200 sequence (1).

We have extended our approach by checking additional recombinant peptides from various sources, all containing the α183-204 sequence. Respective K_D's of the above mentioned ligands with these sequences are given in the Table, together with the previously measured values for BTX (B. Ohana et al. in preparation). BTX displays a two order magnitude differences in its K_D's along the series, while K_D's of the other ligands do not differ by more than a factor of four, in line with the general rule that reduced reactivity is associated with reduced selectivity. Unlike the binding of BTX, the binding of the other ligands is particularly weak for the Drosophila sequence.

In order to assess the role of specific amino acids we turned to point mutations on the Torpedo californica sequence, attacking individual conserved and non-conserved amino acids. Thus the following mutations: ^{187}ser, ^{189}phe, ^{189}thr, ^{191}ser, have been made and checked for their ACh and TC binding. The role of the adjacent cysteins, ^{192}cys, and ^{193}cys was assessed by mutating them into ala or to ser. Again the trends are different than those found for BTX except for ^{193}ala which is weak for both.

K_D values (mM) for ligands binding to recombinant peptides.

Sequence	ACh	Nicotine	TC	GA	BTX
Torpedo	1.9	3.6	0.24	0.15	6.3×10^{-5}
Chick	1.9	3.8	0.17	0.11	1.5×10^{-4}
Xenopus	2.1	3.1	0.18	0.21	5.4×10^{-4}
Drosophila	3.9	12.0	0.46	0.39	1.7×10^{-3}
Mouse	3.3	3.25	0.34	0.21	3.2×10^{-3}
Cat	2.5	3.4	0.40	0.32	6.2×10^{-3}
Human	2.7	2.5	0.12	0.11	6.5×10^{-3}
^{187}ser	2.5	-	0.27	-	2.2×10^{-4}
^{189}phe	2.9	-	0.21	-	1.7×10^{-4}
^{189}thr	2.8	-	0.11	-	$>1\times10^{-2}$
^{191}ser	2.5	-	0.10	-	2.2×10^{-4}
^{193}ser	2.2	-	-	-	1.2×10^{-4}
^{192}ser	2.5	-	-	-	5.5×10^{-4}
^{193}ala	6.6	-	-	-	$>1\times10^{-2}$

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Comparison of Natural and Novel Toxin Binding Sites of the Nicotinic Acetylcholine Receptor

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In order to understand the mechanism of ligand binding the sequence α 183–204 of the nicotinic acetylcholine receptor has been produced as a recombinant fusion protein in bacteria and analyzed for α-bungarotoxin binding. The experimental system used consisted of DNA structure, corresponding to the binding domain, which were constructed in two fragments representing the NH_2 terminal and COOH terminal of the site (Cys 192 and Cys 193 serve as the midpoint). The binding sites of the cholinergic receptor derived from seven organisms (*Torpedo californica*, *Xenopus*, chick, mouse, calf, human and Drosophila). These were compared with respect to their toxin binding capacity. Scatchard analyses show that the K_D values of α-bungarotoxin binding to the above sites are 63, 536, 150, 3200, 6200, 6470, and 1700 nM respectively. These results reiterate the importance of α183-204 as a ligand binding site.

In order to learn which part of the native sequence is essential for efficient binding and to determine which amino acids are responsible for ligand binding we generated chimeric sites. From the analysis of such chimeras, some themes of the gross anatomy of the binding site can be learned. A positive sub-site followed by a hydrophobic patch preceding a nucleophilic domain appear to be required for efficient toxin binding.

On the basis of the preceding experiments, specific residues were point mutated and the effect of the change on toxin binding was tested.

A NEW CLASS OF CHANNEL ACTIVATION SITES AT THE NICOTINIC ACETYLCHOLINE RECEPTOR.

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Recent electrophysiological studies have suggested that reversible acetylcholine esterase inhibitors of the carbamate type, not only prevent hydrolysis of acetylcholine but apparently also directly interact with the nicotinic acetylcholine receptor (nAChR). Two types of interaction have been identified: blockade of the acetylcholine-activated channel and, in some cases, "agonistic" behavior. We have studied the agonistic behavior of the carbamate eserine by means of eserine-induced ion fluxes into nAChR-rich membrane vesicles from *Torpedo marmorata*. We find that eserine induces cation fluxes even in the presence of saturating concentrations of antagonists of acetylcholine or when the nAChR is "desensitized". Competition studies with monoclonal antibodies further establish that eserine exerts its agonistic properties from site(s) separate from the binding sites for the natural transmitter. Our results raise the possibility that there may exist a second pathway of activation of the nAChR-channel.

NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN DROSOPHILA MELANOGASTER

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Two genes - ard and als - are known to code for subunits of the neuronal nicotinic acetylcholine receptor (nAChR) in Drosophila. Genomic Southern blot analysis with probes encoding conserved regions of these genes, suggested the existence of additional nAChR genes in Drosophila. Upon screening a genomic library we have recently isolated several new crosshybridizing DNAs. An alignment of the amino acid sequence, deduced from partial nucleotide sequence analysis of one genomic clone, revealed significant homology to the fourth transmembrane region of ARD and ALS. Using this genomic DNA as probe, we have isolated additional hybridizing clones from an embryonic Drosophila cDNA library. The screening of the different libraries resulted in isolation of DNAs encoding three novel nAChR subunits. Here, we present data on two novel subunits, designated SAD (second alpha subunit of Drosophila) and SBD (second beta subunit of Drosophila). The expression of both genes is developmentally regulated and is restricted to the CNS during late embryogenesis. Cytogenetically, the genes encoding SAD and SBD map together with the als-gene on the 3R chromosome at position 96A. Our data indicate diversity of nAChR proteins in Drosophila.

OOCYTE EXPRESSION AND α-BTX BINDING STUDIES OF *DROSOPHILA* NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

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Nicotinic acetylcholine receptors (nAChRs) are thought to play an important role in the CNS of invertebrates. By cDNA cloning, we and others have identified four different nAChR subunits in *Drosophila*: the ALS, ARD, SAD [Dα2] and SBD proteins. Equilibrium binding and kinetic studies revealed two different high affinity binding sites for the classical nicotinic antagonist α-bungarotoxin (α-Btx) in head membranes of *Drosophila*. Their K_d values were ≈0.1 nM (site 1) and ≈4 nM (site 2). We now demonstrate that both the ALS and ARD proteins are components of the high affinity α-Btx binding site 1, and that a nicotinic cation-selective channel is generated in *Xenopus* oocytes upon coinjection of synthetic ARD and ALS RNAs. In addition we show that the ALS protein is the ligand binding subunit of this complex.
Expression of *in vitro* transcribed SAD RNA in *Xenopus* oocytes also forms a functional nAChR but its association with one of the defined toxin binding sites remains to be investigated.

Molecular genetics of Drosophila acetylcholine receptors

HESS, Norbert

No abstract received

Identification of phosphopeptides of the acetylcholine receptor by mass spectrometry

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It has been investigated whether ion spray mass spectrometry can be used for identifying phosphorylation sites in a protein. The method was tested with phosphokemptide and with a proteolytic digest of one subunit (δ-subunit) of the nicotinic acetylcholine receptor from *Torpedo* electric tissue. Both phospho- and dephosphokemptide could be clearly identified by their respective mass peaks. This shows that the phosphoester bond is stable under the conditions of the spectrometer. The mass difference of 80 dalton represents the $-H_2PO_3$ group.

δ-subunit digested with endoproteinase LysC was separated by HPLC which was connected directly to the mass spectrometer. Several phosphopeptides were identified: A mass peak corresponding to MW 1514 represents the di-protonated, di-phosphorylated peptide δ-LRRSSSVGYISK (position 357-368). The corresponding monophosphorylated peptide was identified as the mass peak (m/z) 1434. This confirms that two serine residues in this peptide are phosphorylated only one of which had been shown to be phosphorylated previously (Yee & Huganir, 1987). - Another mass peak with m/z 1o93 matches exactly the molecular weight of the phosphorylated peptide δ-AQEYFNIK (position δ-369-376) containing the site predicted to be phosphorylated by a tyrosine kinase. A further peptide identified by its m/z value of 12o8 (monoprotonated) could be the phosphorylated peptide position δ-377-387 (SRSELMFEK), predicted to be phosphorylated by protein kinase C. Unequivocal localization of phosphorylation sites in proteins is possible only by MS/MS tandem mass spectrometry or in combination with Edman sequencing.

Crystallization of a membrane protein from nAChR-rich solution

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Although the primary structure of the nicotinic acetylcholine receptor (nAChR) has long been known (1, 2, 3) there is still no information about the tertiary structure of this membrane protein from x-ray diffraction data. In order to, hopefully, avoid any change in conformation from the native state we have attempted to crystallize this protein under conditions that keep denaturation or desensitization at a minimum. The procedure for a "gentle" preparation and purification of the nAChR thus circumvents the use of a specific affinity column (4). Thus there is a possibility that with our present conditions a copurifying Na^+/K^+-ATPase*) actually crystallizes rather than the nAChR.

The crystals (shown to be protein by absorbance at 280nm in a photometer microscope) which were obtained from AChR-rich solutions so far could not directly be identified by electrophoresis or similar means as they are too small and hard to separate from the surrounding amorphous protein precipitate.

)The primary structure of this membrane protein is known as well (5, 6), but as of yet only 2D crystals have been described in literature (7).

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FINE LOCALIZATION OF THE MAJOR α-BUNGAROTOXIN BINDING SITE TO RESIDUES α189-195 OF THE TORPEDO ACETYLCHOLINE RECEPTOR. RESIDUES 189, 190 AND 195 ARE INDISPENSABLE FOR BINDING

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A major binding region for α-bungarotoxin on the acetylcholine receptor (AChR) has been recently identified within parts of the segment 170-204 of the α-subunit. We used the Pepscan systematic peptide synthesis system to determine the minimum _Torpedo_ AChR segment required for α-bungarotoxin binding, and to investigate the role of each residue within this segment. Continuously overlapping decapeptides within α179-203 and several decapeptides covering other α-subunit sequences showed that α188-197 and α189-198 exhibited the best ^{125}I-α-bungarotoxin binding activity (K_D= 7.3x10^{-8}M and 4.3x10^{-8}M respectively). Several continuously overlapping nona-, octa-, hepta-, hexa- and tetra-peptides showed that the heptapeptide α189-195 was the minimum sequence with high binding activity (K_D = 5.6x10^{-8}M). d-tubocurarine, but not carbamylcholine, blocked toxin binding. Twenty-six analogs of the α188-197, most having one residue substituted by Ala or Gly, showed that Tyr189, Tyr190 and especially Asp195 were indispensable for ^{125}I-α-bungarotoxin binding. Cys192 and Cys193 could be substituted by other amino acids proving that the disulfide bond between α192-193 was not required for α-bungarotoxin binding. The decreased α-bungarotoxin binding capacity of the equivalent human muscle AChR α188-197 peptide was the result of substitution of Tyr by Thr at α189.

FINE CHARACTERIZATION OF THE MAIN IMMUNOGENIC REGION OF THE ACETYLCHOLINE RECEPTOR BY THE SYSTEMATIC PEPSCAN PEPTIDE SYNTHESIS APPROACH. MINIMUM RESIDUE REQUIREMENTS FOR ANTIBODY BINDING.

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The Main Immunogenic Region (MIR) is an immunodominant area of the nicotinic acetylcholine receptor (AChR) both in human and in experimental autoimmune Myasthenia Gravis. Binding of anti-MIR monoclonal antibodies (mAbs) has been located between residues 67-76 (1) of the α-subunit of Torpedo electric organ and human muscle AChR. Because of its potentially significant pathogenic role, studies of MIR structure and antigenicity are of considerable importance.

A detailed study of the MIR was carried out, using synthetic peptides and anti-MIR mAbs. Groups of peptides were synthesized in parallel using the Pepscan systematic peptide synthesis technique (2) and tested directly by ELISA assays.

We synthesized and tested peptides of varying length and concluded that the shortest peptide capable of significant mAb binding is the pentapeptide 67-71. A systematic screening of peptide analogs, where each residue within the segments Torpedo α67-74 and both Torpedo and human α67-76 was substituted by 6-9 different aminoacids, was performed. Residues N68 and D71 were found indispensable for anti-MIR mAb binding. Peptides corresponding to α67-76 of AChR species which do not bind the anti-MIR mAbs, like Xenopus muscle and some mammalian neuronal AChRs, also did not bind these mAbs. Some of the studied analogs having single substitutions (like H76, R76) or selected combinations of two substitutions (such as V73A76, H73A76, V73R76) exhibited higher mAb binding activities than the original peptides. Further search for improved MIR analogs may find applications in studies of therapeutic models of Myasthenia Gravis.

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Tyrosine Kinases of the Torpedo Electric Organ

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Many protein tyrosine kinases are abundantly expressed in the nervous system where protein phosphorylation is thought to play a crucial role in the regulation of neuronal function. The peripheral nicotinic acetylcholine receptor (nAchR) has been shown to be phosphorylated and regulated by an unidentified protein tyrosine kinase endogenous to postsynaptic membranes of skeletal muscle and Torpedo electric organ. The receptor is endogenously phosphorylated on β, γ and δ subunits at a high stoichiometry. This phosphorylatation has been shown to increase the rate of desensitization of the receptor. Preliminary studies to identify the tyrosine kinase which phosphorylates the nAchR suggest that multiple tyrosine kinases are expressed in the Torpedo electric organ. Several of these kinases have been cloned from Torpedo cDNA libraries and include members of the *src*, *fps* and *yes* families of tyrosine kinases.

Nicotinic cholinergic receptors in porcine pituitary intermediate lobe cells

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The intermediate lobe (IL) of hypophysis contains an homogenous population of endocrine cells (melanotrophs) which secrete α-MSH and other pro-opiomelanocortine related peptides in a calcium-dependent fashion. Multiple innervations from hypothalamus form synaptic endings in IL cells. Previous studies have revealed the predominant inhibitory effects of GABA and dopamine on electrical activity and secretion of the melanotroph. Here we present recent results of nicotinic acetylcholine (ACh) responses in culture IL cells, suggesting a possible excitatory control in this structure.

Using patch-clamp techniques, we studied ACh induced responses in cells maintained in primary cell culture. Under current-clamp, the IL cells showed a resting potential of about -50 mV and spontaneous action potentials were generally present. ACh and nicotine, applied by fast microperfusion, depolarized the cells and provoked cell firing. In cells voltage-clamped at -60 mV, application of ACh or nicotine induced inward currents accompanied by a substantial increase in noise. Single channel data obtained in cell-attached configuration shows a conductance of 26 pS and a mean open time of 1.8 ms for the ACh receptor channels in IL cells. These channels were cation selective: they were permeant to Na^+ and Cs^+. The I-V curve of whole-cell responses of ACh displayed a strong inward rectification. This rectification stayed unchanged when either Cs^+ or Na^+ was in the recording pipette, and whether the intracellular Ca^{2+} and Mg^{2+} were present or not.

The ACh-induced responses in IL cells were blocked by d-tubocurarine, hexamethonium and mecamylamine, but were insensitive to a-bungarotoxin. An interesting finding of the present study is the inhibitory effect of bicuculline on ACh-evoked responses in IL cells. Surprisingly, the classical antagonist of GABA-ergic responses bicuculline methiodide, was shown to also block the effects of ACh in a nearly competitive fashion. Experiments in whole-cell recording configuration showed that the inhibition of bicuculline on ACh-induced currents was reversible and dose-dependent. The concentration of bicuculline producing half-maximum blockade (IC_{50}) was 44 μM.

Our studies demonstrate the presence of a neuronal-type nicotinic receptor in the IL cells of pituitary gland and suggest a cholinergic control on these endocrine cells. The antagonism of bicuculline on ACh-induced responses suggests a possible blocking effect of bicuculline on nicotinic ACh receptors in mammalian nervous system.

ACETYLCHOLINE RECEPTOR GENE EXPRESSION FOLLOWING α-BUNGAROTOXIN INJECTION

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Muscle Acetylcholine Receptor (AChR) is a heterologous pentamer which has an $\alpha_2\beta\gamma\delta$ composition during ontogenic development. Soon after birth a change is observed with an ε-subunit replacing the γ one. Muscle electrical activity, as well as nerve released factors, have been implicated in the dis-coordinated regulation of AChR genes. Experimental Autoimmune Myasthenia Gravis (EAMG) was recently reported to be accompanied by an increase in AChR-specific mRNAs which correspond to the adult type of receptor (Asher *et al.*, 1990, FEBS Lett. 264:231). This is different from denervation, which causes mainly an increase in the embryonic form. α-Bungarotoxin (α-BTX) was used in this study to investigate the effect of the blockade of acetylcholine-induced muscle activity on the AChR subunits gene expression. Rats were injected i.p. on 5 consecutive days with α-BTX (27 μg/kg body weight/day), residual muscle AChR content was measured by ^{125}I-α-BTX binding, and subunit-specific mRNA levels were analyzed using Northern blots of total and poly(A)+ RNA, hybridized with ^{32}P-labelled DNA probes. We observed a significant increase in the level of mRNAs specific for the α, β, ε and δ subunits of the AChR, reaching a peak between days 5 and 7 after the first injection, and followed later by a gradual decrease to normal levels. An increase in α-subunit mRNA levels was also observed in rats following a single injection with a sub-lethal dose of α-BTX. Reduction in functional AChR content following α-BTX treatment results in activation of AChR subunit gene expression, with a good correlation between residual membrane AChR and increase in receptor transcripts, as was previously observed in EAMG.

DEVELOPMENTAL CHANGES IN THE NICOTINIC ACETYLCHOLINE RECEPTOR OF TORPEDO.

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It has been shown in some mammalian species that during development, the embryonic γ-subunit of the nicotinic acetylcholine receptor (AChR) is replaced by an adult ε-subunit. It is of interest to find out whether Torpedo AChR also displays two alternative forms of the γ-subunit. We have prepared antibodies against a synthetic peptide corresponding to residues 346-359 of the Torpedo AChR γ-subunit, and containing a putative phosphorylation site that is present in all known ε-subunits, but is absent in their respective γ-subunits. These antibodies react strongly with adult and hardly with embryonic Torpedo γ-subunit. The differential reactivity of the anti-γ peptide antibodies does not reflect differences in the state of phosphorylation. Also, the γ-subunit of the adult Torpedo AChR is phosphorylated by exogenous cAMP-dependent protein kinase to a much higher extent than its embryonic counterpart. It thus appears that the known Torpedo γ-subunit represents an adult form of this subunit and is different, at least in the region of the phosphorylation site, from the crossreactive embryonic form. It should still be tested whether the embryonic and adult forms of the Torpedo AChR γ-subunit are coded by two different genes, as has been demonstrated in the bovine and murine muscle AChR, or whether other mechanisms are responsible for the observed differences.

EXPRESSION OF HUMAN MUSCLE AChR cDNAs

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Muscle acetylcholine receptor (AChR) is the autoantigen in myasthenia gravis. The cDNAs which encode the subunits of the AChR have been well characterised, but the low level of the AChR protein in human muscle (around 1 pmol/g of muscle) restricts the study of myasthenia. To overcome this difficulty we have isolated cDNAs containing the full coding sequence of the human α, β, γ and δ subunits, and have a partial clone for the ϵ subunit, and are using them in both prokaryotic and eukaryotic expression systems to generate AChR subunit polypeptides as a source of antigen.

Using a modification of the vector pKK 233-2 (Pharmacia), we have constructed plasmids for the expression of full-length α, β, γ and δ subunits. In particular, for the α subunit, which contains the 'Main Immunogenic Region', we have a library of expression constructs coding for overlapping polypeptides within the subunit. It has been possible to synthesise and purify milligram quantities of these polypeptides.

Using the SP6/T7 system, cRNAs have been synthesised for the α, β, γ and δ subunits, and have been expressed using rabbit reticulocyte lysates.

Metabolic stabilization of endplate AChR.s at developing neuromuscular junctions is mediated by Ca^{++} influx associated with muscle activity.

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The effects of muscle activity on the metabolic AChR stability and on the growth of synaptic acetylcholine receptor (AChR) accumulations were investigated in rat skeletal muscle. Ectopic endplates induced surgically in adult soleus muscle were denervated early during development when junctional AChR number and stability were still low and, subsequently, muscles were either left inactive or they were kept active by chronic exogenous stimulation. AChR numbers per ectopic AChR cluster and AChR stabilities were estimated from the radioactivity and its decay with time, respectively, of endplate sites whose AChR.s had been labelled with ^{125}I-α-bungarotoxin. The results show that the number of AChR.s in ectopic clusters and their metabolic stability is reversibly increased by muscle activity even when innervation is eliminated very early in development; one day of stimulation is sufficient to stabilize the AChR.s in ectopic AChR clusters. Muscle stimulation also produced an increase in the number of AChR.s at early denervated endplates. Activity-induced cluster growth occurs mainly by an increase in area rather than in AChR density, and for at least 10 days after denervation is comparable to that in normally developing ectopic endplates.

Activity dependent stabilization of endplate AChR.s is mediated by an influx of Ca^{++} through dihydropyridine sensitive membrane channels but it is insensitive to Ca^{++}-release from the sarcoplasmic reticulum. Metabolic stabilization of endplate AChR.s but not of extrajunctional AChR.s could be induced in chronically denervated fibres in the absence of muscle activity if muscles were treated with the Calcium ionophore A23187. AChR stabilization was also induced by culturing non-stimulated muscles in elevated K^+ and the Ca^{++} channel activator (+)-SDZ202-791. Conversely, the activity-dependent AChR stabilization was prevented in muscles stimulated in the presence of the Ca++-channel blockers (+)-PN200-110 or D-600. Treatment of muscles with ryanodine to induce Ca++-release from the sarcoplasmic reticulum in the absence of activity did not cause stabilization of junctional AChR.s.These data show that, during muscle activity, AChR stabilization is mediated by an influx of Ca++ ions through dihydropyridine-sensitive Ca^{++}-channels in the muscle membrane.

MYOGENIN AND MYOD REGULATE TRANSCRIPTION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR ALPHA SUBUNIT

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ABSTRACT

A family of myogenic factors that activate transcription of a battery of skeletal muscle genes has been recently identified. Transfection of non-muscle cells with cDNAs coding for myogenin and MyoD, two members of this family, leads to the transcriptional activation of skeletal muscle genes and commits the cells to become myoblasts. Both factors are nuclear proteins shown to bind the transcriptional enhancers of the creatine kinase and myosin light chain genes. Herewith I present evidence that myogenin and MyoD bind to two sites present in the enhancer of the chicken nicotinic acetylcholine receptor (nAChR) α subunit gene . Gel retardation assays and DNA footprinting were used to show that the site nearest to the CAP site (transcription initiation site) is bound preferentially by both factors. When 3T3 fibroblasts are cotransfected with a construct containing the nAChR α subunit enhancer linked to the reporter gene chloramphenicol acetyltransferase (CAT), plus a vector expressing either myogenin or MyoD, transcription of the CAT gene is activated. The introduction of two-base mutations to either binding site, however, dramatically reduces the expression of CAT in cotransfected 3T3 fibroblasts. In addition, mutations in the myogenin and MyoD binding sites also inactivate the expression of the CAT reporter gene in transfected C2C12 mouse myotubes; these muscle cells express both factors endogenously. In view of these results it is interesting to speculate, that myogenin and MyoD may regulate the expression of a repertoire of skeletal muscle genes that are transcriptionally activated during muscle differentiation, and whose expression is repressed by innervation.

In vitro neuromuscular synaptogenesis in the presence of Myasthenia Gravis patient serums

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Myasthenia Gravis (MG) is a neuromuscular disorder characterized by muscles'weakness and fatiguability. The basic defect is a decrease of the available acetylcholine receptor (AChR) at the neuromuscular junctions. MG is mediated by circulating antibodies directed against AChR, since anti AChR antibodies are detectable in nearly 80% of patient's serums. Several non-exclusive mechanisms were described to explain the role of these antibodies: (1) blockage of the acetylcholine (ACh) binding sites, (2) accelerated degradation of AChR requiring antibodies dependent cross-linking of AChR.

We analysed the structural and biochemical modifications (AChR and acetylcholinesterase (AChE) level and distribution) during synaptogenesis in the presence of MG patient's serums, in rat's nerve-muscle cocultures. The results were compared to the effect of an ACh antagonist, the α-bungarotoxin. In the case of antibodies directed against AChR in MG serums, receptors are internalised and degraded. In the case of the α-bungarotoxin, non-functional receptors are still present in the muscular membrane. Comparison between these different results gives the opportunity to better understand the mechanisms of AChE concentration during the synaptogenesis, in relation with the AChR concentration.

In the presence of MG serums, the formation of AChE spots, which normally occurs during the synaptogenesis, dramatically decreased, proportionally to the anti-AChR antibody's titer. The AChE total activity and the proportion of the different molecular forms are unchanged. In the presence of the α-bungarotoxin, the AChE activity and concentration are equivalent to the control. These results suggest that during the synaptogenesis, the presence and/or the concentration of AChR is a limitant factor for normal AChE concentration.

In order to analyse the mechanisms by which the AChE concentration is perturbed, we studied the presence of basal lamina's two components localised between the nerve-ending and muscle membranes, and in which AChE is anchored. By immunofluorescence methods, the two components studied, laminine and heparan sulfate proteoglycan (HSPG), are uniformaly distributed along the muscle membrane. In the presence of MG serums, the fluorescence intensity is increased, without distinguishing if there is a correlation with the anti-AChR antibody's titer of the serums. Two non-exclusive hypothesis can be suggested : (1) serums act directly on the synthesis and/or the secretion of basal lamina components, (2) serums have a mitogenic effect on the cells which synthesize these components.

MyoD1 binding sites regulate the acetylcholine receptor γ- and ε-subunit genes differentially

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In rat muscle the expression of two acetylcholine receptor (AChR) subtypes is under developmental control. At early developmental stages a low conductance channel comprised of α-, β-, γ- and δ-subunits predominates while higher conductance channels (α, β-, ε- and δ-subunits) are found in the adult, innervated muscle. The functional changes are correlated with defined structural changes in that the γ-subunits are replaced by the ε-subunits (1). To analyze the mechanism regulating the differential expression of the AChR-genes we isolated genomic DNA fragments encoding the 5'-flanking regions of the γ- and ε-subunit genes. DNA fragments of 350 bp length for the γ-subunit and 250 bp for the ε-subunit where shown to contain sequences which are necessary for muscle specific expression. Both fragments contain sequences exhibiting a high similarity to sequence motifs which bind myogenic regulatory factors such as MyoD1. While the γ-5'-flanking region has two MyoD1 binding sites reminiscent of the MyoD1 binding sites in the muscle creatine kinase (2) the ε-5'-flanking region has only one such site. To evaluate the role of the MyoD1 binding sites for the γ- and ε-subunit gene promoter activity we analyzed the corresponding DNA fragments, in primary cultures of rat Myotubes, CH310T 1/2 and HeLa cells. Transfection assays using the wild type DNA as well as mutated fragments or fragments where the MyoD1 binding sites had been deleted indicate that MyoD1 could regulate the γ- and ε-subunit genes in a differential manner.

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CARBOHYDRATE STRUCTURES OF THE PURE CHOLINERG... SYNAPSE OF TORPEDO ELECTRIC ORGAN. Subcellular distribution detected by cytochemical and immunocytochemical techniques

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It has been described a regional distribution and temporal regulation of expression of certain sugar structures by plants lectins or carbohydrate-specific monoclonal antibodies at the CNS and PNS (Margolis and Margolis, 1990). Although an abundant information is available about glycoproteins of the synapse (see Gurd, 1990 for a review), those studies has mainly been located at the CNS where a multiple synaptic types and neurotransmitters exist. The carbohydrate components of the typical cholinergic synapse of the PNS, the neuromuscular junction, has been involved in the establishment or stabilization of synaptic connections (Sanes and Cheney, 1982) and the carbohydrate pattern has also been studied (Scott et al., 1988). Due to electric organ of Torpedo marmorata is a homologous tissue to neuromuscular junction, we have studied the carbohydrate patterns present in this pure cholinergic synapse by morphological and colloidal gold techniques using lectins and monoclonal antibodies (MAbs) raised against carbohydrate epitopes. Small tissue pieces were fixed in diverse combinations of fixatives and embedded in Lowicryl K4M. Ultrathin sections were procesed by the post-embedding method. We have used lectins and MAbs described as follow: Lectins: Limax flavus agglutinin (LFA), NeuAc; Ricinus communis lectin I (RCL I), βGal; Datura stramonium agglutinin (DSA), Galβ1,4GlcNAcβ1; Concanavalin A (Con A), αMan; Wheat germ agglutinin (WGA), GlcNAc and NeuAc; Ulex europaeus agglutinin (UEA I), L-Fuc; Soybean agglutinin (SBA), Dolichos biflorus agglutinin (DBA) and Helix pomatia agglutinin (HPA), αGalNAc; Peanut agglutinin (PNA), Galβ1,3GalNAcα (epitope T) and αGalNAc. Monoclonal antibodies (MAbs): B72.3, NeuAcα2,6 GalNAcα-0-Ser/Thr (epitope sialyl-Tn), Cu-1, GalNAcα-0-Ser/Thr (epitope Tn). Furthermore we have use glycohydrolases (sialidase, N-glycanase). The main structures we have focus on has been the presynaptic membrane, synaptic vesicles, postsynaptic membrane, Schwannn cell, myelin and basal membranes. The conclusions we have obtained from the subcellular distribution of glycoconjugates with respect to the restricted synaptic structures are the following: 1.Presynaptic membrane: (1) We do not observe high mannose oligossacharides or glycoproteins (Con A-); (2) There is no terminal αGalNAc (HPA, DBA, SBA -); (3) There is no L-Fucose (UEAI-); (4) Carbohydrate epitopes T, Tn and sialyl-Tn are not present (respectively PNA, Cu-1 and B72.3-); (5) We detect the O-linked sequence NeuAcα2,3Galβ1,3GalNAcα (sialidase-PNA +); (6) Galβ1,4GlcNAcβ is the dissacharide most abundant among the N-linked glycoproteins (DSA+); (7) βGal is the most abundant monossacharide (RCL I) rather than NeuAc (LFA); (8) Probably there is NeuAc O-acetylated (LFA+ after sialidase treatment). 2. Synaptic vesicles: (1) There are terminal NeuAc (LFA+) and βGal (RCL I+) facin into the synaptic lumen; (2) Some of the NeuAc is resistant to sialidase. Probably it is O-acetylated; (3) Probably the NeuAc belongs to the oligossacharide sequence NeuAcα2,3Galβ1,3GalNAcα since some synaptic vesicles become positive to PNA after sialidase treatment; (4) There is Galβ1,4 GlcNAcβ (DSA+); (5) There is no T, Tn and sialyl-Tn epitopes (respectively, PNA, Cu-1, B72.3 -). 3. Postsynaptic membrane: (1) There is no L-fucose and αGalNAc terminal non-reduced; (2) there is high mannose oligossacharides (Con A+); (3) There is no T, Tn and sialyl-Tn epitopes (respectively, PNA, Cu-1, B72.3 -).

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Assembly of the muscle AChR in COS cells. Y. Gu, J.R. Forsayeth and Z.W. Hall, University of California, San Franciso.

COS cells were transiently transfected with cDNAs for α, β, γ and δ subunits of the mouse muscle nicotinic acetylcholine receptor (AChR). The properties of the AChRs expressed on the surface closely resembled those of the AChRs of C2 myotubes. When incomplete combinations of AChR subunits were expressed, no surface expression was detected except in the case of αβγ which gave 15% of that seen with all four subunits. Immunoprecipitation experiments showed that in cells that expressed pairs of subunits, αδ and αγ heterodimers were formed, but αβ were not. When three subunits were expressed, αδβ and αγβ complexes were formed, but αδγ were not. Variation of the ratios of the four cDNAs used in the transfection mixture showed that surface AChR expression was decreased by high concentrations of δ and γ cDNAs in a mutually competitive manner. These results are consistent with a scheme for assembly in which αδ and αγ heterodimers are formed first, followed by association with the β subunit and with each other to form the complete AChR. (Supported by NIH grants (NS28062 and NS13521) to JRF and ZWH and a grant from the Muscular Dystrophy Association.

Properties of embryonic and adult muscle acetylcholine receptors (AChRs) transiently expressed in COS cells. Y. Gu, J.R. Forsayeth, A. Franco, Jr., P. Gardner, J.B. Lansman and Z.W. Hall, University of California, San Francisco.

We have used transient transfection in COS cells to compare the properties of mouse muscle nicotinic AChRs containing α, β, δ and either γ or ε subunits. Following transfection with cDNAs of either the γ- or ε-AChR, toxin-binding activity corresponding to fully assembled receptor was expressed on the surface of COS cells at a level comparable to that seen in cultured muscle cells. Toxin-binding to γ-, but not ε-, AChR was partially blocked by a myasthenic serum reported to be specific for the embryonic form of muscle AChR. The ε-AChR was also degraded more slowly in the membrane than the γ-AChR. Patch-clamp recordings showed that the γ-AChR had the channel characteristics of embryonic AChR and the ε-AChR had those of the adult AChR. These results demonstrate that some but not all of the differences between AChRs at the adult endplates and those in the extrasynaptic membrane can be explained by the difference in subunit composition of γ- and ε- AChRs. Transient expression of AChRs in COS cells is convenient, reproducible, yields high levels of surface AChR, and should be generally applicable to the study of other multi-subunit proteins. Supported by NIH grants (NS28062 and NS13521) to JRF and ZWH, and a grant from the Muscular Dystrophy Association.

Postnatal changes in neuromuscular transmission in rats.

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New born infants show a greater than normal sensitivity to the effects of non-depolarizing muscle relaxants. Meakin (1988) and Meretoja (1988) showed that neonates are 2.8 and 2.6 times more sensitive to atracurium and vecuronium respectively than adults. Such enhanced sensitivity could result from a reduced safety factor for transmission due to a low quantal content of the endplate response in immature junctions (Kelly and Roberts, 1977). We are studying the development of normal transmission at the neuromuscular junction in the absence of blocking drugs by using a cut fibre preparation (Barstad & Lilleheil, 1968) to prevent action potential generation and contraction. This initial report concerns an investigation of the rundown or fade of the endplate response recorded with intracellular microelectrodes from the neonate rat diaphragm during repetitive stimulation and of the effects of low concentrations of curare.

Cut fibre rat phrenic nerve/diaphragm preparations were made from rats aged from 3 to 5 weeks and bathed in Krebs solution at 20 ± 2°C. Two KCl-filled microelectrodes were inserted at the endplate region of a fibre. One electrode was used to pass current to polarize the membrane to -60mV, the other to record miniature endplate potentials (MEPPs) and endplate potentials (EPPs) evoked by stimulation of the cut phrenic nerve held in a suction electrode. Initially, the rundown of EPPs was determined at stimulation frequencies between 1 and 50 Hz. Subsequently, the effect of curare on EPP rundown was determined. Synaptic potentials were analysed by computer using Spike2 (Cambridge Electronic Design, UK).

MEPPs were relatively infrequent, occurring at 0.56±0.06 sec^{-1} (N=32). All train rates resulted in rundown of EPPs to a plateau within 10 stimuli. For example, stimuli at 1 Hz resulted in an EPP plateau after 10 seconds which was 85% of the size of the first evoked EPP (N=21). Increasing the rate to 10 Hz depressed the plateau EPP to 65% within 1 second (N=20). Bathing fibres in 10^{-7}M curare for 45 minutes reduced the plateau EPP even further. At 1 Hz the plateau was reduced to 72% of the first EPP (N=20) and at 10 Hz to 51% (N=21). Higher rates of stimuli resulted in an even lower plateau EPP.

Taken in conjunction with the observed low rate of MEPPs, the increased rundown of evoked EPPs in preparations from these young rats suggests that they may have limited reserves of releasable transmitter. Our finding that curare futher reduced the plateau EPP at all rates of stimuli indicates that the drug has a significant pre-junctional effect at this age. Together, these results could account for the increased sensitivity of newborn infants to curariform drugs.

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