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Role of Protein Phosphorylation in the Regulation of Neuronal Sensitivity

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The project reported here focuses on the regulation of neuronal sensitivity by a novel class of protein kinase: an ecto-protein kinase which phosphorylates proteins at the cell surface by extracellular ATP. The progress we have made in this project includes the development of novel experimental paradigms for the determination of ecto-protein kinase and its substrates in cultured neuronal cells. We used these paradigms in the conclusive identification of the surface phosphoproteins in primary neurons cultured from embryonic brain and in PC 12 cloned neuronal cells induced to differentiate by nerve growth factor (NGF). We have determined which of these surface phosphoproteins are regulated by NGF. We have also identified a specific phosphorylation system at the surface of CNS neurons involved in neurogenesis. These specific phosphoproteins are at the focus of continued studies on the role of ecto kinase in synaptic plasticity. A pilot investigation conducted as part of this project revealed that the naturally occurring ether-phospholipit Platelet Activating Factor (PAF) induces Long-Term Potentiation (LTP) in hippocampal slices, and may have an important modulatory role in the process of memory formation.

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Concise Summary

The goal of our research is to determine the role of surface protein phosphorylation by ecto-protein kinase activity in neuronal development and synaptic plasticity. In this research we use two model systems. The first is a homogeneous population of cloned cells of the line PC12 and the induction of neuronal differentiation in these cells by nerve growth factor (NGF). A major aim of this project has been to complete the detailed characterization of surface protein phosphorylation in PC12 cells and to determine the mode of regulation of this activity by NGF. This goal has been accomplished. A manuscript entitled: "Ecto-Protein Kinase and Surface Protein Phosphorylation in PC12 Cells: Interactions With Nerve Growth Factor" has been accepted for publication in the Journal of Neurochemistry. The manuscript acknowledges the support by AFOSR grant #88-0290. Reprints (6) will be provided when they are sent to us by the Journal.

In parallel to the study of PC12 cells, we have continued our investigation of the developmentally regulated surface phosphorylation of proteins in primary cultures of neurons from embryonic chick brain. We completed to collect the evidence that these neurons posses ecto-protein kinase and to identify it specific substrates. We have carried out detailed characterization of this activity, identified specific surface phosphoproteins implicated in neuritogenesis and currently we continue the experiments designed to purify these surface phosphoproteins.

In addition to the above studies, a pilot investigation was conducted as part of this project and revealed that the naturally occurring ether-phospholipid Platelet Activating Factor (PAF) induces Long-term Potentiation (LTP) in hippocampal slices. PAF may thus have an important modulatory role in mechanisms underlying synaptic plasticity and the process of memory formation.

Research Objectives

The Specific Aims of this project have been:

(1) Biochemical characterization of the extracellular protein phosphorylation systems operating in PC12 cells and in primary CNS neurons differentiated in culture. This objective includes experiments demonstrating conclusively the ecto-enzymatic nature of the protein-kinases under investigation.

(2) Identification and characterization of the surface neuronal proteins whose phosphorylation by extracellular ATP alters during neuronal development and synaptogenesis.

(3) Identification and characterization of the surface neuronal proteins whose phosphorylation by extracellular ATP in PC12 cells is regulated by Nerve Growth Factor.

(4) Isolation and purification of a neuronal ecto-protein kinase and of specific surface proteins that serve as its substrates, and the preparation of antibodies against these components.

The tools and probes prepared in this project will be used in detailed investigation of the involvement of ecto-protein kinase in the regulation of neuronal function by extracellular ATP, with emphasis on studies of neurogenesis and synaptic plasticity.

DETAILED REPORT

Role for Ecto-Protein Kinase in Synaptic Plasticity

In a previous project supported by the ASFOSR (no. 84-0331) we have discovered a neuronal ecto-protein kinase and identified its endogenous substrates at the surface of cloned cells of the neural cell line NG108-15 (Ehrlich et al., Nature, 320: 67-70, 1986). Detailed characterization of the ecto-protein kinase in NG108-15 cells revealed that in addition to activities with Km values for ATP in the micromolar range, there was another activity which saturated only at high ATP concentration. Detailed analysis of ecto-protein kinase activity at extracellular ATP concentrations between 100μM and 2mM and double -reciprocal plots of the data revealed the presence of an activity with a Km value for ATP of 525μM, with significant and detectable phosphorylation of specific proteins at 100-200μM $AT^{32}P$ (Ehrlich et al, J. Neurochem., 50: 245-301, 1988). This ecto-protein kinase may be uniquely activated during the brief accumulation of ATP in the synaptic cleft after repetitive stimulation, and thus provide a trigger for the chain of events leading to long-term changes in synaptic function, such as seen in long-term potentiation (LTP). The production of LTP was shown to involve increase in intracellular free Ca^{++}, alterations in intracellular second-messenger generating systems, and excessive neurotransmitter release. Therefore, we examined whether extracellular ATP in the concentration range of 100-1000μM could induce these changes in neural cells. Extracellular ATP at the concentration range of 100-1000μM was found to induce 7-fold increase in $^{45}Ca^{++}$-uptake by NG108-15 cells, and this increase was additive with K^+-induced depolarization. Use of the calcium probe quin 2 revealed that the ATP-induced increase in Ca^{++} -uptake was associated with significant elevation in the concentration of intracellular free Ca^{++} in N1E-115 NB cells. Similar results were obtained with NG-cells loaded with the Ca^{++}-probe aequorin. We then labeled intact neural cells with ^3H-adenine and ^3H-guanosine, to measure the effects of extracellular ATP on intracellular adenylate and guanylate cyclase, respectively, in intact neural cells. We found that extracellular ATP inhibits, in a concentration dependent manner, PGE_1-stimulated cyclic AMP formation, and stimulates net cyclic GMP formation, and that this increase is reversible. Another second-messenger system sensitive

to changes in [Ca^{++}]i is phosphoinositide metabolism. We found that 1mM extracellular ATP produced about a 15 fold increase in ^{3}H-IP accumulation in intact neural cells, and that this effect can be almost completely prevented by an inorganic blocker of Ca^{++} influx. Pooled data from all the experiments described above showed that the K0.5 value for extracellular ATP in exerting the effects on various signal transduction systems in neural cells is in the range of 510-540μM. Interestingly, the Km of ecto -protein kinase for ATP in this range is 525μM. These results have provided the basis for a systematic series of studies on the role of extracellular ATP, secreted by stimulated neurons, in the feed-back regulation of second messenger generating systems involved in neuronal adaptation and synaptic plasticity.

Ecto-protein kinase in the regulation of high-affinity NE-uptake

In another study of our previous project (AFOSR 84-0331) we have examined the hypothesis that ATP, released together with norepinephrine (NE) from brain noradrenergic nerve terminals, serves as a co-substrate for an extracellular protein phosphorylation system that regulates the uptake of the transmitter NE. The high affinity uptake (uptake 1) of ^{3}H-NE in rat cerebral cortical synaptosomes was examined with respect to regulation by divalent cations and ATP, both of which are involved in protein phosphorylation activity. A marked inhibition of uptake 1 was observed when App(NH)p, a non-hydrolyzable, competitive antagonist of ATP, was added during uptake assays. Similar inhibition of ^{3}H-NE uptake in synaptosomes was observed when calcium and magnesium were omitted from the medium. Calcium and magnesium exerted differential actions on the regulation of NE uptake. Magnesium-dependent uptake was not affected by App(NH)p, whereas calcium-dependent uptake-1 was inhibited. In parallel experiments, the patterns of protein phosphorylation in crude and purified preparations of synaptosomes were examined under conditions similar to those used in the uptake assays. We found a striking correlation between the inhibition of uptake 1 by App(NH)p or Ca^{++} omission, and inhibition of the phosphorylation of one specific protein component in synaptosomes. This synaptosomal protein, with 39-42Kd approximate molecular weight, did not exhibit the known properties of a mitochondrial protein of similar electrophoretic mobility, the alpha subunit of pyruvate dehydrogenase. These findings are consistent with the

possibility that ecto-protein kinase(s) utilize extracellular ATP and calcium in phosphorylating a protein component associated with the regulation of NE uptake (published in J. Neurochem., 50: 263-273, 1988).

Direct examination of the involvement of ecto-protein kinase in norepinephrine uptake required studies of a homogenous population of intact cells. Therefore, we initiated in the project reported here studies on the effects of extracellular ATP on NE uptake using cells of the cloned line PC12 as a model of adrenergic neurons. In this study, we examined the high-affinity uptake of NE in PC12 cells in the presence of varying concentrations of extracellular ATP. In the presence of Ca^{2+}, low concentrations of ATP (0.1μM) increased uptake 1 by approximately 36%. This increase could be mimicked by adenosine-5'-0-(3-thiotriphosphate) tetralithium salt (ATPγS), an analogue of ATP which can be utilized by protein kinases, but not by 5'-adenylylimidodiphosphate tetralithium salt, a nonhydrolyzable analogue of ATP. Adenosine, GTP, and ADP also had no effect on uptake 1. Preincubation of the cells with NE and ATPγS, followed by washing and assaying NE uptake 30 min later, resulted in a persistent increase in uptake 1 as expected from a mechanism involving irreversible protein-thiophosphorylation. Similar pretreatment with ATP did not show this increase, however, simultaneous pretreatment with ATP and ATPγS blocked the activation produced by ATPγS alone. Kinetic analysis revealed that ATPγS pretreatment produces an increase in the Vmax of uptake 1 without altering the apparent K_m for NE. These results support the hypothesis that extracellular ATP can regulate NE uptake via an ecto-protein kinase. Calcium-dependent phosphorylation of a 39K surface protein by extracellular ATP was recently detected in PC12 cells (see below) and provided the basis for focusing the investigation of the role of ecto-protein kinase inNE-uptake on this specific surface phosphoprotein (published in J. Neurochem., 53: 1512-1518, 1989).

Surface Protein Phosphorylation in PC12 Cell: Interations With NGF.

To identify proteins which serve as specific substrates for ecto-protein kinase in PC12 cells, we followed the criteria and experimental guidelines published by Ehrlich et al., 1990 (Ann N.Y. Acad. Sci., 603: 401-416). Accordingly, intact cells were grown in 48 well plates and incubated with 15μCi of [γ-^{32}P]ATP or ^{32}Pi. We found that phosphorylation by ATP is blocked when the ATP hydrolyzing enzyme,

apyrase, is added to the medium. Incubation of the cells with extracellular [γ-^{32}P]ATP for 1 min results in labeling of 5 proteins migrating with molecular weight of 20K, 39K, 97K, 105K and 116K. Proteins with MW 20K, 39K and 105K are not phosphorylated by Pi even after 1 hr incubation time. The time course for the phosphorylation of the ecto-protein kinase substrates of 20K, 39K, and 105K, during the period of 10 sec to 60 min incubation with [γ-^{32}P]ATP showed that incorporation of ^{32}P into the 20K and 39K proteins increases with reaction time for up to 10 min. The phosphorylation of the 105K protein continuously increases during a 60 min. incubation of intact cells with [γ-^{32}P]ATP.

The criteria outlined above established that these three phospho-proteins are the specific substrates of ecto-protein kinase in PC12 cells. We then initiated studies on the involvement of this enzymatic system in neuronal development by examining its interaction with Nerve Growth Factor (NGF). To determine the effect of NGF on phosphorylation of extracellular proteins, PC12 cells were incubated for 5 min with 50 ng/ml of NGF before initiating the reaction. In other experiments, PC12 cells were treated with the same concentration of NGF for 3-4 days. We found that during a short incubation with NGF there is an increase in phosphate incorporation into these proteins. Three day incubation of the cells with 50 ng/ml of NGF leads to a further significant increase ($p < 0.05$) in phosphorylation of the 20K protein. Treatment of PC12 cells with NGF for 3 days induced phosphorylation of an additional specific band at MW 53K, which was minimally phosphorylated without NGF treatment.

In conjunction with our previous studies, we have tested the effects of divalent cations on phosphorylation of the specific protein substrates of ectokinase in PC12 cells. This effect was examined by comparing regular KRB, modified KRB containing Mg^{++} only, or Ca^{++} only . This phosphorylation of most protein bands did not occur in the absence of Mg^{++}. However, as seen previously in synaptosomes with NE uptake system, we detected also in PC12 cells surface phosphorylation of a 39K protein that did not require Mg^{++}, and was observed to be phosphorylated in the presence of Ca^{++} only. The surface phosphorylation of this protein is now a specific target in studies on the role of ecto-protein kinase in the regulation of Ca^{++}-dependent NE-uptake by extracellular ATP.

Selective Inhibitors of Surface Protein Phosphorylation

The protein kinase inhibitors K-252a and K-252b (Ehrlich et al. 1990, see above) were tested for their effect on the intracellular protein phosphorylation in PC12 cells incubated with $^{32}P_i$ for 60 min. K-252a in a concentration dependent manner inhibited the phosphorylation of proteins in cells in which intracellular ATP was labeled with $^{32}P_i$. However, there were only minimal effects of K-252b on intracellular phosphorylation even when used at a concentration of 10 μM. This result supports the finding that K-252b does not penetrate through the cell membrane. We have, therefore, tested the possibility that K-252b is a selective inhibitor of ecto-protein kinase activity. PC12 cells were assayed for ectokinase activity by 5 min incubation with [γ-^{32}P]ATP at 37°C. Compared to control cells, 60-75% of kinase activity is inhibited after 10 min preincubation with 2 μM of K-252b. Almost complete inhibition was obtained with 10 μM of K-252b.

To determine whether extracellular protein phosphorylation is involved in cell differentiation in PC12 cells, the effect of 6-thioguanine (6-TG), a known inhibitor of neurite extension in these cells, was studied. At the concentration used (10μM), 6-TG produced 20% inhibition of the phosphorylation of 20K, 60% inhibition of the 39K protein, and approximately 40% inhibition of the phosphorylation of the 105K protein at the surface of PC12 cells. 100μM of 6-TG inhibited about 40% of the phosphorylation of the 20K protein, 80% of 39K and 75% of the 105K protein. The correlation of these results with the inhibition of neurite extension by 6-TG suggests that ecto-protein kinase activity may play an important role in the process of differentiation in PC12 cells.

Ecto-Protein kinase is a Threonine-kinase.

The presence of threonine protein kinase activity at the surface of PC12 cells was identified by analysis of phosphoamino acid composition of 20K, 39K and 105K proteins isolated by electroelution from SDS-gels. These phosphoproteins were labeled by incubation of intact cells for 5 min with extracellular [γ-^{32}P]ATP, isolated by electroelution, and then analyzed by thin-layer chromatography. In all three phosphoproteins, the ^{32}P-radioactivity detected by autoradiography appeared in the position of phosphothreonine. For the 20K, 39K and 105K proteins, 75.7%, 87.8% and 87.1%, respectively, of the total incorporated phosphate was detected in

phosphothreonine, with trace amounts in the phosphoserine position. In this regard ecto-protein kinase represents a unique kinase that has not been described before.

Ecto-protein kinase at the surface of CNS neurons.

Our studies with lines of cloned neural cells served to: 1) develope the experimental criteria necessary for the identification of ecto-protein kinase activity, 2) provide evidence for the existence of a neuronal ecto-protein kinase, and 3) identify specific proteins substrates for this enzyme at the surface of neural cells. Studies with the cloned neural cell lines NG108-15 and PC12 indicated a potential role for this activity in neuronal differentiation. In order to investigate the involvement of ecto-protein kinase in the regulation of physiological processes underlying neuronal development, we have undertaken the task of identification and characterization of the surface protein phosphorylation systems operating in primary cultured neurons obtained from embryonic brain. In these studies, primary neurons were prepared from the telencephalon of 7 day chick embryos and grown for 6 days in 48 well dishes coated with poly-L-lysine. These neurons were differentiated and exhibited many fasiculated neurites. Phosphorylation reactions were performed on 6 day cultured neurons that were maintained in a chemically-defined medium for 2 days prior to the reaction. In a typical reaction, primary neurons were incubated with [γ-^{32}P]ATP (15 μCi, 0.1 μM) for 5 min with or without apyrase. The proteins were then separated by polyacrylamide gel electrophoresis and analyzed by autoradiography and densitometric scanning. A protein duplex between 11.7K and a 13K was prominently phosphorylated. Protein bands of MW 11.7K, 13K, 30K, and 105K did not become labeled when apyrase was added, indicating their extracellular location. On the other hand, the phosphorylation of a protein band of 53K is completely unaltered by apyrase. Two protein bands (64K and 80K) have increased labeling when apyrase is added to the reaction. This increase in labeling can be the result of increased availability of ^{32}Pi that is produced by apyrase and then used to label intracellular proteins. Since no labeling of the 11.7K, 13K, 30K and 105K proteins occurs even after 40 mins in the presence of apyrase, it can be concluded that these protein bands represent phosphorylation that takes place EXCLUSIVELY in extracellular locations by ecto-protein kinase. Intracellular protein phosphorylation in primary neurons was investigated by labeling cells with ^{32}Pi for 40 mins. The extracellular phosphorylated bands of MW

11.7K, 13K, 30K and 105K are notably absent, indicating that these proteins are not labeled in an intracellular location. To confirm these finding, apyrase was added to the reaction, and indeed, it had no effect on the labeling of intracellular proteins with ^{32}Pi.

The effect of the addition of unlabeled ATP on the labeling of proteins for a 10 min reaction period was determined. As the concentration of unlabeled ATP was increased, the amount of labeling by [γ-^{32}P]ATP decreased significantly. The 11.7K band was affected to a great extent by the addition of unlabeled ATP. Following the addition of as little as 0.1μM to the standard reaction, the labeling of the 11.7K band was decreased by approximately 40%, and following the addition of 10 μM, there was total loss of protein labeling. We observed that the radiolabeling of three protein bands: 11.7K, 13K and 105K completely disappeared at an extracellular ATP concentration of 100μM, further confirming their identity as substrates for ecto-protein kinase.

To further characterize the extracellular nature of the neuronal ecto-protein kinase, the exogenous substrate casein was utilized. Casein was highly phosphorylated when added to a 10 min. phosphorylation reaction carried-out with intact cells, further indicating that the ecto-protein kinase is indeed extracellular. In this experiment we also found a significant difference in the extracellular phosphorylation activity between immature neurons grown in-culture for 2 days, and mature neurons grown in-culture for 6 days. Immature neurons were observed to have greater kinase activity than differentiated 6 day neurons. The phosphorylation of the endogenous substrates 11.7K and 13K proteins, as well as phosphorylation of the exogenous substrate casein, by extracellular [γ-^{32}P]ATP is much higher with 2 day neurons than in 6 day neurons, suggesting greater ecto-kinase activity in the earlier stages of development.

Primary neurons were incubated with a buffer containing Ca^{++} and Mg^{++}, Ca^{++} only or Mg^{++} only, and labeling of neurons with [γ-^{32}P]ATP for 10 min was assessed under these conditions. When Mg^{++} is removed (Ca^{++} only buffer), the protein bands of MW 11.7K, 13K, 64K and 105K are not labeled. Phosphorylation of the 17K band increases in Ca^{++} only buffer conditions. This data indicates that the ecto-protein kinase responsible

for the phosphorylation of the 11.7K and 13K proteins is a Mg^{++} dependent enzyme. We have determined that over 90% of the phosphate transfered by ecto-protein kinase activity from extracellular [γ-^{32}P]ATP to the 13K and 11.7K proteins is incorporated into phosphothreonine. Thus, as in PC12 cells, the ecto-protein kinase in primary CNS neurons in also a Mg^{++}-dependent threonine kinase.

Role for Ecto-Protein Kinase in Neuritogenesis and Synaptogenesis

The time course of the phosphorylation of the 11.7K and 13K protein substrates by ecto-protein kinase in immature 2 day was compared to mature 6 day cultured neurons. Two day primary neurons displayed greater ecto-protein kinase activity than did 6 day neurons over all time points studied for both the 11.7K and 13K proteins. The incorporation of phosphate increased in 6 day neurons for both the 11.7K and 13K proteins for up to about 20 min and then reached plateau. Initial incorporation of label into the 13K and 11.7K proteins of 2 day neurons increased at a faster rate and reached plateau for the 13K protein, but labeling of the 11.7K protein continued to increase for up to 60 min. These extracellular protein phosphorylation systems are thus developmentally regulated, with the measured kinetic parameters of enzymic activity showing enhancement in the earlier phase of development.

To assess the specificity in the activity of ecto-protein kinase, we examined the effects of inorganic phosphate. The addition of unlabeled Pi should specifically dilute the labeling by ^{32}Pi, but have no effect on labeling of extracellular proteins by [γ-^{32}P]ATP. We found that the addition of 1 mM unlabeled Pi to reactions carried-out with primary neurons eliminated all labeling by ^{32}Pi for both the 10 min and the 60 min reactions. Protein bands labeled specifically by [γ-^{32}P]ATP, expecially the 11.7K and 13K proteins, were unaffected when unlabeled Pi was added to either the 10 min or 60 min reaction. It is noteworthy that phosphorylation of the 11.7K and 13K proteins is greater in 2 day neurons than in 6 day neurons, especially during the initial time points of the reaction, both in the initial rate and extent of phosphorylation.

To investigate further the developmental changes, the phosphorylation pattern of proteins was determined in 2, 3, 4, 5, 6 and 7

DIV neurons incubated with extracellular [γ-^{32}P]ATP or ^{32}Pi for 10 min. The labeling of the 13K and 11.7K protein bands increased up to day 3 followed by a distinct reduction in the labeling of these two proteins by extracellular ATP. A significant reduction in the phosphorylation of all proteins occured in neurons pretreated with either K252a or K252b for 10 min and then incubated with [γ-^{32}P]-ATP for an additional 10 min. Both K252a and K252b are protein kinase inhibitors. However, while K252a can cross the plasma membrane, it has been reported that K252b can not penetrate intact cells. Thus, only K252a, but not K252b, should inhibit intracellular protein phosphorylation. When primary neurons were treated with K252a we found that there was a very significant reduction in the intracellular labeling of all proteins. In contrast, K252b had virtually no effect on the phosphorylation of intracellular proteins. Our data therefore confirms that K252b will not cross the plasma membrane and when applied to intact cells, it is a selective inhibitor of ecto-protein kinase activity. In other studies, K252b was found to inhibit synaptogenesis in cultured brain neurons. The combined results suggest a significant role for surface protein phosphorylation in synapse formation.

The effects of various gangliosides on ecto-protein kinase activity were investigated. Primary neurons were pretreated with 5nM of the ganglioside GM3 for 1 hour and then incubated with [γ-^{32}P]-ATP for 10 min. The phosphorylation of the 11.7K and 13K proteins, the specific substrates of ecto-protein kinase, was significantly stimulated by GM3. The monosialo-gangliosides GM1 and GM2 had similar stimulatory effects on phosphorylation of these two proteins. GD1a, a disialoganglioside, also had stimulatory effects on the phosphorylation of the 11.7K and 13K proteins. The gangliosides tested in these experiments were shown in previous studies to stimulate neurite extension. Their selective stimulatory effects on the extracellular phosphorylation of the specific protein substrates of ecto-protein kinase further support our suggestion of a role for this enzymatic system in neuritogenesis, and indicates that detailed investigation of this system can reveal novel and important regulatory mechanisms operating during neuronal development.

REGULATION OF SYNAPTIC PLASTICITY

Molecular mechanisms as described above, that are involved in neuritogenesis and synaptogenesis, may play a role not only in the regulation of neuronal development but also be used in the mature brain in

processes underlyning synaptic plasticity. As part of the project reported here we have carried out a pilot study of the role of a novel extracellular regulator of neuronal function, in the regulation of synaptic plasticity. Platelet Activating Factor (PAF; PAF-acether) is a naturally occurring alkyl-ether phospholipid (12-0-alkyl, 2-acetyl-sn-glyceryl-3 phosphorylcholine) that acts as an extracellular mediator in a wide range of biological processes. Our finding that psychoactive drugs of the triazolobenzodiazepine class are potent antagonists of PAF action were the first that suggested a role for PAF in the nervous system (Kornecki & Ehrlich, SCIENCE, 240: 1792-1794, 1988). Subsequently, biosynthesis of PAF was detected in neuronal cells and brain tissue, neuroregulatory and neuropathological actions of PAF were demonstrated in cultured neural cells and in brain synaptosomes, and specific binding sites for PAF were found in brain membranes. However, a physiological role for PAF in the mature brain has not yet been established. In this project we have obtained direct evidence for the regulation of synaptic plasticity by PAF. We have found that PAF produces a stable, concentration-dependent (5-100 nM) increase in evoked potentials recorded from hippocampal slices. This PAF induced synaptic facilitation could be blocked by PAF receptor antagonists and by N-methyl-D-aspartate (NMDA) receptor antagonists. Once induced, however, PAF-triggered facilitation could not be reversed by PAF antagonists. These findings demonstrate that exposure of hippocampal tissue to PAF induces long-term potentiation (LTP). LTP is a type of synaptic plasticity associated with the formation of memory, and PAF may play an important modulatory role in this process. The mechanism responsible for LTP is known to include increase in intraneuronal levels of free Ca^{2+}-inos, and this effect can be induced in neurons by PAF (Kornecki and Ehrlich, 1988), and may involve ecto-protein kinase activity (Ehrlich et al, 1988). Thus, this project has opened a <u>new direction</u> in the investigation of molecular mechanisms underlying synaptic plasticity and by extension-the formation of memory.