Role of Protein Phosphorylation in the Regulation of Neuronal Sensitivity

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Final

FROM 11/1/87 TO 6/30/88

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Neuronal phosphoproteins; Ectoprotein Kinase; Receptor sensitivity; Primary CNS neuron culture

The research carried out during the short period of this report represents a continuation of our studies on a previous project supported by the AFOSR (no. 84-0331). This grant (AFOSR 88-0004) was terminated at our request due to transfer of the Principal Investigator to another institution, where this work now continues. In the main study carried out during the period of this report we have found that primary neurons, cultured from the neostriata of embryonic mouse brain, store ATP within a synaptic vesicle and release it by exocytosis upon stimulation. It was also found that these cells possess ecto-protein kinase, and that specific phosphoprotein substrates for this activity are present at the cell surface. These findings open for investigation the role of extracellular protein phosphorylation in the regulation and adaptation of neurons in the Central Nervous System.
Principal Investigator: Yigal H. Ehrlich, Ph.D.

1) concise summary

The main project carried out in our laboratory during the period covered by this report has been focused on the finding that neural cells possess an ecto-protein kinase activity, which phosphorylates proteins localized at the outer surface of the plasma membrane. We have discovered this activity while carrying a previous project supported by the Air Force Office of Scientific Research (AFOSR 84-0331). The report submitted here summarizes the continuation of this research during the period Nov. 1987 – June 1988. The main new findings reported here are that primary CNS neurons, cultured from the neostriatum of embryonic mouse brain, have an ecto-protein kinase and surface phosphoprotein substrates for its activity. These cells were also found to store ATP within synaptic vesicles and secrete it in a calcium-dependent manner upon stimulation. These results open for investigation the role of extracellular protein phosphorylation in the regulation and adaptation of CNS neurons.

Two additional projects were pursued in our laboratory in parallel to the studies summarized above. We have continued our investigation on the role of extracellular protein phosphorylation in the regulation of the uptake of norepinephrine (NE). In these studies we found that neural cells of the clone PC12 have ecto-protein kinase activity, and that addition of ATP-gamma-S to the medium significantly stimulates high affinity NE-uptake by these cells. During the same period we have completed the first phase of our investigation on the interaction of the alkyl-ether phospholipid platelet-activating-factor (PAF) with neuronal cells. We have reported that PAF stimulates Ca++-uptake in the neural cells of the lines NG108-15 and PC12. This effect appears to be mediated by the activity of receptor-operated calcium channels. Since in previous studies we found in NG108-15 and N1E-115 similar stimulation of Ca++-uptake by extracellular ATP, future studies should investigate the interactions between these two systems.
2) Research Objectives.

The research proposal which led to award no. AFOSR 88-004 included studies designed for a three (3) years period. Six specific aims were listed in the original application for this 3 years period, as follows:

1. Biochemical characterization (Kinetic properties, ionic requirements, regulating factors) of the extracellular protein phosphorylation systems operating in primary CNS neurons grown and differentiated in-culture.

2. Determination of the effects of cell stimulation by neurotransmitters, depolarizing agents, neurohormones and growth factors on the activity of the phosphorylation systems defined in specific aim no. 1.

3. Isolation and purification to apparent homogeneity of specific components (ecto-protein kinase, certain protein substrates and phosphoprotein phosphatase) of the neuronal extracellular protein-phosphorylation systems.

4. Development of poly- and mono-clonal antibodies to specific components isolated under specific aim no. 3.

5. Identification of the extracellular protein phosphorylation systems associated with the function of mature synapses, by determining the developmental pattern of these systems during synaptogenesis in-culture (1-18 DIV).

6. Investigation on the role of the phosphorylation of N-CAM by ecto-protein kinases in neuronal function.

(3) Research Accomplishments.

During the period covered by this report we have made progress in one major project of our research and in two ancillary projects related to this research. The major project was studies of ATP secretion and extracellular protein phosphorylation by CNS neurons in primary culture. The two ancillary projects were: (a) Regulation of norepinephrine uptake by extracellular ATP, and (b) Regulation of calcium uptake in cultured neural cells. The main results obtained in these studies are summarized below.

The significant role of secreted ATP in the regulation of neuronal function and the activity of ecto-protein kinases which utilize extracellular ATP to phosphorylate proteins localized at the cell surface have been previously studied in peripheral neurons and in cloned neural cell lines. During the research period reported here we have utilized neostriatal neurons differentiated in primary culture to demonstrate vesicular secretion of ATP and phosphorylation of proteins by extracellular ATP in neurons derived from the central nervous system (CNS). Neostriatal neurons from embryonic mice were maintained in a chemically defined medium for 15-18 days. Functional differentiation was determined by measuring evoked GABA-release. ATP secretion was measured by luciferin-luciferase assays, and protein phosphorylation by adding 7-32P-ATP to the
extracellular medium. Depolarization by 50mM KCl induced a Ca**-dependent ATP release, and stimulation by 100μM veratridine resulted in secretion of ATP that could be blocked by tetrodotoxin. Phosphorylation of specific protein components with apparent molecular weights of 110K, 80K, 55K, and 30K was detected in striatal neurons incubated for 15 mins with γ-32P-ATP added to the medium, but not by labeling intracellular ATP pools with equivalent amounts of radioactivity presented as inorganic 32Pi. These results open for investigation the role of extracellular protein phosphorylation systems in processes underlying the responsiveness of CNS neurons to secreted ATP. A full account of this study has been published, and 6 copies of the paper are enclosed with this report.

In another study carried out during this period, the potential modulation of norepinephrine (NE) uptake by extracellular ATP and divalent cations was examined in PC12 cells. An analog of ATP, ATPγS, was chosen for these studies since ATPγS can be utilized by protein kinases and the resulting thiophosphate bonds are resistant to phosphatase activity. Previous work in our laboratory has shown that both ATP and ATPγS (0.1 μM) can stimulate NE uptake. In this study, cells were incubated for 5 min at 37°C, in a Krebs-Ringer buffer containing 1.87 mM Ca2+ and no added Mg2+, with and without 1 μM ATPγS. The cells were then washed and NE uptake assayed 30 min later. ATPγS produced a persistent stimulation of uptake and this effect was dependent on the presence of Ca2+ in the pretreatment medium. Preincubation with ATP produced no increase in uptake, suggesting that there was an inactivation by dephosphorylation. Pretreatment with ATPγS and an excess of ATP resulted in the inhibition of the ATPγS-induced stimulation. The ATPγS pretreatment protocol was used to examine kinetic changes and the results showed an increase in the Vmax, with no change in the apparent Km for NE. Experiments are currently in progress to determine ecto-protein kinase activity in PC12 cells, to identify the surface proteins phosphorylated by ATPγ35S, and examine their role in action of the NE-transporter system and its regulation by extracellular ATP.

We have reported previously that extracellular ATP can stimulate calcium uptake into neural cells and cause an increase in the levels of intracellular free Ca**-ions. It was suggested that the mechanism involves regulation of receptor-operated calcium channel. A potent regulator of such mechanism in diverse biological systems is the naturally occurring alkyl-ether phospholipid called platelet activating factor (PAF). However, whether this critical extracellular mediator plays a role in neuronal function has not been known. In the present study we have investigated the interactions of PAF with cloned neural cells. We have found that PAF increased the intracellular levels of free calcium ions in cells of the clones NG108-15 and PC12. The increase was dependent on extracellular calcium and was inhibited by the antagonistic PAF analog CV-3988 and by the calcium-influx blockers prenylamine and diltiazem. A functional consequence of this interaction was revealed by measuring a PAF-elicted, Ca**-dependent secretion of adenosine triphosphate from PC12 cells. Exposure of NG108-15 cells for 3 to 4 days to low concentrations of PAF induced neuronal differentiation; higher concentrations were neurotoxic. Thus, by influencing Ca** fluxes, PAF may play a physiological role in neuronal development and a pathophysiological role in the degeneration that occurs when neurons are exposed to circulatory factors as a result of trauma, stroke, or spinal cord injury. These effects of PAF appear to be mediated by the activity of receptor-operated calcium channels. Continued
Studies examine the role of extracellular ATP secreted by neurons in the regulation of this receptor-coupled activity in neurons.

(4) Written Publications.


ATP Secretion and Extracellular Protein Phosphorylation by CNS Neurons in Primary Culture

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A large body of evidence documents the significant role of protein phosphorylation systems in the regulation and adaptation of multiple neuronal functions (6, 8, 11, 17, 18, 22). In this process the enzyme protein kinase transfers the gamma phosphate of ATP to specific sites in various intracellular proteins (21, 24). In addition, conditions necessary for phosphorylation of extracellular proteins also exist in the nervous system (11). It is well known that ATP is stored within synaptic vesicles of adrenergic, cholinergic and purinergic feedback regulation of synaptic functions by ATP secreted system. Phosphorylation by CNS Neurons

METHOD

Primary Neuronal Cultures

Neostriatal neurons were dissected from the brain of 14-day mouse embryos, mechanically dissociated, plated in 6-well cluster plates (0.8-1.0 x 10^6 cells/ml, 2 ml/well), and maintained in a

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Eventually as described above for GABA release, except that aliquots (0.45 ml) of the blocked by TTX. The data presented in Table collected extracellular medium and in washed cells extracted after replacement of the medium with KRB containing 50 min incubation in KRB. ['HIGABA in aliquots of the collected extracellular medium was found to be about 0.5 nM (see bar marked C in Fig. 2). In the releasate collected during 5 min incubation by replacing the buffer with KRB supplemented with 50 mM KCI can occur in a Ca 0-free KRB medium. 

Veratridine. A 5 min releasate was collected, followed by 2×5 medium were rinsed twice with KRB and then covered with buffer containing 10 μCi of γ-32P-ATP (ICN) and a final ATP concentration of 1 μM. Following incubation for 15 min at 37°C, the reaction was terminated by adding "SDS-stop solution" (7) and the proteins separated by slab-gel electrophoresis using 7–14% exponential polyacrylamide gel gradients, as described previously (9). Autoradiograms were used to quantify [32P]-incorporation into specific protein bands by scanning with a digitizing laser microdensitometer (LKB Ultrascan XL). Molecular weights (MW) of phosphoproteins were estimated by determining relative mobility compared to protein standards (BioRad) separated on the same gel and detected by staining with Coomassie-blue (7–9).

RESULTS

Striatal neurons were maintained in primary culture in a chemically defined medium for 15–18 days in vitro, as detailed in the Method section. Previous studies using these culturing conditions have demonstrated morphological differentiation and synaptogenesis in these cells during 10–14 DIV (29). To demonstrate a functional aspect of neuronal maturation in these cultures, we measured depolarization-induced neurotransmitter release in attached cells preloaded with [3H]GABA. Figure 1 demonstrates that under nonstimulating conditions, a stable basal release is exhibited by the cells, which can be maintained for at least 40 min. When the cells were stimulated with 50 mM KCl, about 10-fold increase in [3H]GABA release over basal conditions was evident. An even more pronounced release of GABA was evoked by stimulating the cells with 100 μM veratridine, and the veratridine-induced GABA release was completely blocked by 1 μM tetrodotoxin (TTX) (see Fig. 1).

The experimental protocol used to measure GABA release as shown in Fig. 1 was followed for measuring ATP-release by a luciferin-luciferase assay carried out with aliquots of medium collected at 5 min intervals from attached neostriatal neurons incubated in Krebs-Ringer buffer. Under nonstimulating conditions, ATP concentration in the medium was found to be about 0.5 nM (see bar marked C in Fig. 2). In the releasate collected during 5 min incubation after replacement of the medium with KRB containing 50 mM KCl (in place of NaCl), the ATP concentration was 17.3±4.3 nM. Figure 2 also shows that veratridine induced about a 50-fold increase in ATP release (23.7±1.1 nM ATP), and over 80% of the veratridine-evoked ATP release was blocked by TTX. The data presented in Table 1 demonstrate that 47% of the [3H GABA released by cells depolarized with 50 mM KCl can occur in a Ca 0-free KRB medium.

![Graph](image-url)
EXTRANECULAR PHOSPHORYLATION IN CNS NEURONS

FIG. 2. Secretion of ATP from striatal neurons in primary culture. Cells at 14-15 DIV were assayed following the protocol shown in Fig. 1 and detailed in its legend. ATP concentration in aliquots of the collected medium was determined by luciferin-luciferase reactions as described in the Method section. Bars show the mean data obtained under basal (C) and stimulated conditions. Arrows indicate the time point of stimulation by 50 mM KCl, 100 µM veratridine (VT) and VT + TTX (1 µM), corresponding to the arrow depicted in Fig. 1. Data shown are means from 4 experiments; SEMs are provided in the text and Table 1.

TABLE 1

DEPOLARIZATION-INDUCED SECRETION OF ATP AND GABA FROM STRIATAL NEURONS: DEPENDENCE ON Ca++ IN THE EXTRACELLULAR MEDIUM

<table>
<thead>
<tr>
<th>Incubation Conditions*</th>
<th>Incubation in KRB</th>
<th>Ca++-free KRB</th>
<th>Ca++ dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-Secretion (in nM; mean ± SEM)</td>
<td>17.3 ± 4.3 (n=4)</td>
<td>4.4 ± 1.5 (n=3)</td>
<td>75%</td>
</tr>
<tr>
<td>[3H]GABA-Release (in % of total uptake)</td>
<td>10.52 ± 0.86 (n=2)</td>
<td>4.95 ± 0.15 (n=4)</td>
<td>53%</td>
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</table>

*Neostriatal cells at 14-15 DIV were stimulated with 50 mM KCl in normal KRB or in KRB prepared without CaCl2, and the medium collected after 5 min incubation at 37°C as described in the legend to Fig. 1. ATP and [3H]GABA release were measured as detailed in the Method section. n = the number of experiments.

whereas 75% of the depolarization-induced ATP release is Ca++-dependent.

Since striatal neurons secrete ATP, the cosubstrate for protein kinase activity, they may also possess extracellular protein phosphorylation systems. To measure the phosphorylation of proteins by extracellular ATP we added γ-32P-ATP to the medium covering intact striatal neurons while still attached to their substratum. In control wells of the same cluster plate, the intracellular ATP pools of the cells were labeled by adding an equivalent amount (in µCi) of radioactive, inorganic 32Pi and incubating for the same time period (15 min). Figure 3 depicts an autoradiogram of 32P-containing protein bands labeled by these two procedures. It can be seen that under these incubation conditions proteins...
FIG. 3. Autoradiogram of phosphorylated proteins in neostriatal cells incubated for 15 min at 37°C with 60 μCi per well of γ-32P-ATP (ATP) or inorganic 32P (P) in KRB. Reactions were carried out as described in the Methods section and terminated by adding SDS-stop solution. Solubilized, reduced proteins [2% beta-mercaptoethanol; 5 min 95°C] were resolved in 7-14% exponential gradient of polyacrylamide. The gel was autoradiographed with Kodak X-Omat film for 6 days. Each sample is shown in duplicate. Representative of 4 experiments with neostriatal cells assayed at 15-18 DIV. Mobility of standard MW markers is shown on the left.

with apparent molecular mass of 110,000 kilodaltons (Kd), 80 Kd, 55 Kd and approximately 30 Kd and 20 Kd incorporated 32P from γ-32P-ATP added to the extracellular medium, but not by labeling the neurons for the same period with equivalent amount of radioactivity provided as α32P. Densitometric scans of the autoradiograms of separated proteins from neostriatal cells incubated for 15 min with γ-32P-ATP added in normal KRB, in high K-KRB, and in KRB supplemented with 100 μM veratridine are shown in Fig. 4A, 4B and 4C, respectively. The results of these experiments (n=8) were quantitated by measuring peak areas (see Table 2). This analysis revealed that compared to basal incubation conditions, depolarization of the cells by 50 mM K- caused a significant decrease in the phosphorylation of a protein component with apparent MW of 80 K. In contrast, incorporation from extracellular γ-32P-ATP into a 55 K protein increased significantly in striatal neurons stimulated with veratridine. Functional studies of ecto-protein kinase activity in differentiated CNS neurons can now focus on these specific phosphoprotein components.

It is important to point out that in striatal neurons that were assayed at 15-18 DIV (namely, after differentiation and synaptogenesis), only minimal phosphorylation by extracellular ATP was detected in proteins with molecular mass greater than 110 Kd (see Fig. 4), which comigrate with neuronal cell adhesion molecules (N-CAMs). On the other hand, pronounced phosphorylation of N-CAMs by ecto-protein kinase activity was found during the phase of rapid neurite extension in cloned neural cells differentiating in culture (9). Similar experiments, to be reported in detail elsewhere, have found phosphorylation by extracellular ATP of proteins recognized by anti-D-CAM antibodies (9) in CNS neurons maintained in primary culture for 4-6 DIV, namely, during neuronal differentiation. These results suggest that in the nervous system phosphorylation of different proteins by ecto-protein kinase activity is developmentally regulated.

Discussion

Procedures for obtaining pure neuronal populations by culturing cells from central nervous tissue in chemically defined, serum-free media have enabled detailed investigation of various molecular aspects of the development, differ-
EXTRACELLULAR PHOSPHORYLATION IN CNS NEURONS

TABLE 2

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>**P-Incorporation Into Specific Protein Components</th>
<th>Significant Change in %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20 K</td>
<td>30 K</td>
</tr>
<tr>
<td>Basal (KRB) (n=8)</td>
<td>4.99±0.51</td>
<td>5.95±0.44</td>
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<tr>
<td>50 mM KCl (n=8)</td>
<td>5.48±0.25</td>
<td>6.83±0.14</td>
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<tr>
<td>Veratridine (n=8)</td>
<td>5.96±0.43</td>
<td>6.90±0.22</td>
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*Values of **P-incorporation are means ± SEM for the number of experiments shown (n), expressed as percentage of relative area of specific peaks obtained by digitizing densitometric analysis of autoradiograms, as described in the Method section.

ACKNOWLEDGEMENTS

We thank Dr. S. Weiss and Ms. D. Kemp for advice and help in culturing neostriatal cells, and are grateful to Mr. I. Galbraith for expert technical assistance, and to Ms. M. Philbrick for typing the manuscript. These studies were supported by USAFOSR grants 84-0331 and 88-0004, and in part by NSF grant BNS82-09265.
REFERENCES


