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Phosphorylation of membrane proteins at a cholinergic synapse

(cholinergic ligands/protein kinase/acetylcholine receptor/postsynaptic membrane/nicotinic cholinergic synapses)

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ABSTRACT **Endogenous membrane protein kinase activity and protein kinase substrates have been found in membrane fractions enriched in the acetylcholine receptor that were prepared from the electric organ of *Torpedo californica*. Phosphorylation of four polypeptides is stimulated 9-fold by K^+. The specific cholinergic ligand, carbachol, inhibited phosphorylation of these four polypeptides by 72% in the presence of 1 mM Na^+ and 100 mM K^+. The 65,000-dalton component of the acetylcholine receptor in the membrane fraction appears to be phosphorylated by the endogenous protein kinase. These results suggest that protein phosphorylation may play an important role in synaptic events at nicotinic cholinergic synapses.**

In the nervous system, neurotransmitters react with their receptors at synapses to produce striking changes in Na^+, K^+, or Cl^- permeability across the postsynaptic membrane. The molecular events underlying such specific increases in ionic permeability are unclear; in addition, little is known about the biochemistry of other postsynaptic phenomena, such as desensitization. In analogous systems in which polypeptide hormones react with receptor sites on the cell membrane, the biologic response in the target cell appears to be mediated by changes in the phosphorylation of a regulatory protein (1). Since neurotransmitters ultimately affect ionic permeability, it has been postulated that changes in the level of phosphorylation of specific proteins at the synapse might regulate changes in ionic permeability at the postsynaptic membrane (2). This has been investigated by Greengard and others, who have found protein kinase, protein kinase substrates, and phosphoprotein phosphatase in synaptic membrane fractions from brain (3). However, polypeptide substrates of the same molecular weight as the substrates of the brain protein kinase have been found in many other innervated and noninnervated tissues (4–11), and the relationship between synaptic membrane phosphorylation and specific ionic permeability is still unclear. If phosphorylation and dephosphorylation are involved in postsynaptic events, then these reactions should be regulated by neurotransmitters. However, although neurotransmitters affect adenylate cyclase activity (12–17), no neurotransmitter-dependent changes in protein phosphorylation in membranes prepared from innervated tissues have been reported (6).

Membrane proteins that play a role in postsynaptic events at cholinergic synapses should be associated with the acetylcholine receptor (AChR). These proteins could be identified in synaptic membrane fragments enriched in AChR. The electric organ of *Torpedo californica* is a rich source of homogeneous nicotinic cholinergic synapses. We have used membrane fractions from this tissue to investigate the relationship between neurotransmitters and membrane protein phosphorylation. In this report we demonstrate that (*a*) protein kinase activity and protein kinase substrates appear to be associated with the AChR in cholinergic synaptic membrane fragments, (*b*) cholinergic ligands inhibit the phosphorylation of these membrane polypeptide substrates, and (*c*) the 65,000-dalton component of the AChR appears to be phosphorylated by an endogenous membrane protein kinase. These findings suggest that protein phosphorylation may play an important role in postsynaptic events at nicotinic cholinergic synapses.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; AChR, acetylcholine receptor.

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MATERIALS AND METHODS

Preparation of Membrane Fractions. Membrane fractions were prepared from the electric organ of *Torpedo californica* (Pacific Bio-Marine Lab., Venice, Calif.). The method used was a modification of that of Duguid and Raftery (18) for preparation of receptor-rich membranes banding at sucrose density 1.14. The electric organ (120 g) was homogenized at top speed in a Waring blendor for 3 min in 120 ml of 0.02 M sodium phosphate buffer, pH 7.4, containing 0.4 M NaCl. The homogenate was filtered through four layers of cheesecloth to remove large particles, and centrifuged at 27,000 × *g* for 90 min. The pellets were resuspended in the same buffer with the Waring blendor, washed twice at 39,000 × *g* for 90 min, and resuspended to 0.3 ml/g in 62.5 mM Tris·HCl, pH 6.8. The homogenate was layered on a discontinuous gradient, and membranes banding between sucrose of densities 1.13 and 1.15 were collected after centrifugation at 63,000 × *g* in an SW 25.1 rotor for 6 hr. The membrane fraction was recovered with a Buchler Densiflo apparatus, diluted to 50 ml with 62.5 mM Tris·HCl, pH 6.8, and centrifuged at 48,000 × *g* for 1 hr to remove Na^+ that entered the gradient. The fraction was washed three times and resuspended before use. Protein content was determined by the method of Lowry *et al.* (19).

AChR Assay. AChR was assayed according to a modification of the method of Meunier *et al.* (20). Ten microliters containing 1.25 μg of membrane protein in 1% Triton-eel Ringer's solution were added to 50 μl of 0.25 μM ^{125}I-labeled *Naja naja* toxin (1.2×10^4 cpm/pmol) prepared by the method of Morrison (21). This solution was diluted to 200 μl to achieve a final concentration of 0.75% Triton in eel Ringer's solution. After the mixture was incubated for 1 hr at room temperature, 20 ml of eel Ringer's solution was added and 10 min was allowed for the receptor to aggregate. The assay mixture was then filtered on Millipore filters (type HA, 0.45 μm) that had been soaked in eel Ringer's solution. The filters were then washed with 10 ml of eel Ringer's solution and radioactivity was determined in a Packard gamma counter.

Sodium Dodecyl Sulfate ($NaDodSO_4$)-Acrylamide Slab Gel Electrophoresis of Membrane Fractions. Membranes (0.1 ml) containing 50 μg of protein and 5% 2-mercaptoethanol were solubilized by the addition of 0.02 ml of 20% $NaDodSO_4$. The mixture was placed in a boiling-water bath for 1.5 min, 5 μl of 60% sucrose and 5 μl of 0.05% bromphenol blue were added, and the sample was layered on $NaDodSO_4$-acrylamide slab gels, 1.5 mm thick. These were prepared according to the method

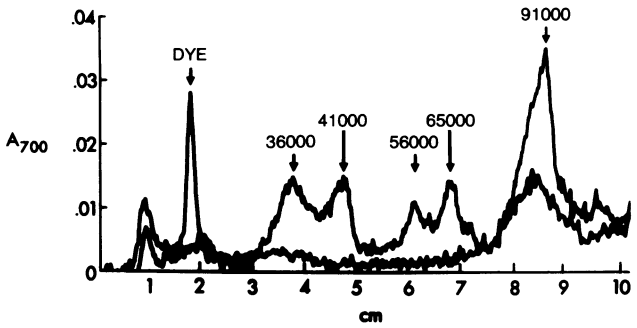

FIG. 1. Effect of 100 mM K^+ on phosphorylation of membrane polypeptides by endogenous protein kinase activity. Scan of autoradiogram of dried $NaDodSO_4$ gel. Ordinate represents absorbance at 700 nm. Upper curve, 100 mM KCl; lower curve, no additions.

of Laemmli (22), with 8% acrylamide in the running gel and 3% in the stacking gel. The slabs were run overnight at 15 mA and then simultaneously fixed and stained for protein for 2 hr in 25% isopropranol, 10% acetic acid, and 0.2% Coomassie blue. They were destained overnight in the same solution without dye. When protein kinase substrates (see below) were to be determined, the stained gels were subsequently dried at 80° under reduced pressure on filter paper and subjected to autoradiography. The films were cut into strips and scanned at 700 nm with a linear transport attachment on a Gilford spectrophotometer.

Protein Kinase Activity of Membrane Fractions. The reaction mixture contained 50 μg of membrane protein, 0.25 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 10 mM $Mg(OAc)_2$, 62.5 mM Tris·HCl (pH 6.8), cations where indicated, and 0.005% Triton X-100 in a final volume of 0.1 ml. The reaction was initiated by addition of [γ-^{32}P]ATP (1–2 μCi per tube) to a final concentration of 5 μM and allowed to proceed for 0.5 min at 0°. When carbachol was present, experimental and control tubes were first incubated for 5 min at 0° and then for 1 min at 20° before addition of [γ-^{32}P]ATP. The reaction was stopped by addition of 0.02 ml of 20% $NaDodSO_4$, and the samples were treated as above for electrophoresis.

Immunoelectrophoresis. Immunoelectrophoresis was performed by the method of Converse and Papermaster (23). Membrane protein (14 μg) was subjected to electrophoresis as described above. Slices were then cut from the slab and subjected to electrophoresis at right angles into agarose containing 4% goat antiserum against AChR at a constant voltage of 50 V for 2 hr. The agarose gels were then incubated overnight in 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl to remove unreacted serum, dried, and then stained and destained as above. Control agarose gels run with pre-immune serum showed no reaction.

Antibody Production. Antibody to AChR was produced in two goats using AChR from *Torpedo californica* purified as described (24). Each goat was injected subcutaneously in multiple sites with a total of 1.5 ml of an emulsion containing 300 μg of AChR in 0.75 ml of buffer and 0.75 ml of complete Freund's adjuvant. After 6 weeks each goat received a second inoculation with a total of 0.6 ml of an identical emulsion. Each goat developed a severe myasthenia gravis-like disease on the seventh day after the second inoculation, and at this time 500 ml of immune serum was obtained by terminal bleeding. One precipitin band was seen on Ouchterlony double immunodiffusion of serum or purified IgG against purified AChR.

Reagents. Reagents for the preparation of membranes and enzyme assays were analytical reagent grade. ATP, carbachol, and EGTA were purchased from Sigma Chemical (St. Louis, Mo.). [γ-^{32}P]ATP, 32 Ci/mmol, was purchased from New England Nuclear Corp. (Boston, Mass.). *Naja naja siamensis* toxin was from the Miami Serpentarium (Miami, Fla.) and purified in our laboratory by the method of Karlsson *et al.* (25). All reagents for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, Calif.).

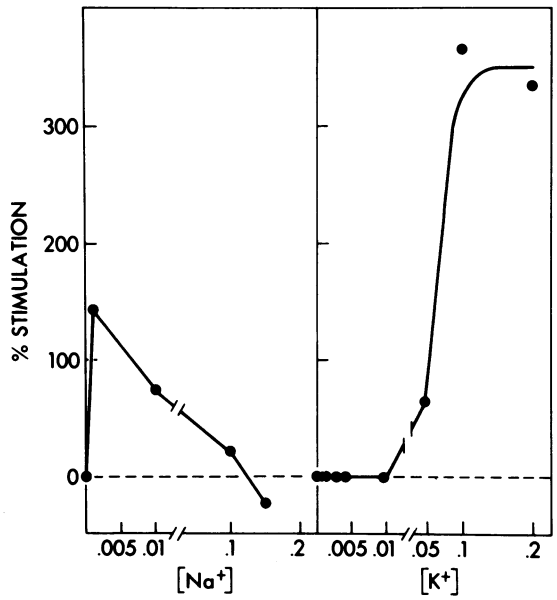

FIG. 2. Phosphorylation of 65,000-dalton polypeptide as a function of Na^+ and K^+ concentrations. Assay is as described in *Materials and Methods* for each cation concentration. Percent stimulation is calculated after measurement of absorbance at 700 nm for a given salt concentration for the peak corresponding to the 65,000-dalton polypeptide.

RESULTS

Membrane fractions banding at sucrose density 1.14 have been shown to be enriched in AChR (18). For example, the binding capacity for ^{125}I-labeled *Naja naja* toxin in the membrane fraction we have used is 1.1 nmol/mg of protein, compared to only 0.19 nmol/mg of protein in the resuspended homogenate. This membrane fraction was examined for protein kinase activity by incubation with [γ-^{32}P]ATP and subsequent $NaDodSO_4$ gel electrophoresis of the phosphorylated membranes. In the absence of K^+, only one major band, containing material with a molecular weight of 91,000, is seen (Fig. 1). This could be the phosphorylated intermediate of the Na-K-ATPase, since Jean *et al.* (26) have shown that the phosphorylated subunit of purified ATPase from *Electrophorus* electric organ has a similar molecular weight. Upon addition of 100 mM K^+, however, several other phosphorylated bands appear (Fig. 1), one traveling with the dye and four others of molecular weights 65,000, 56,000, 41,000, and 36,000. Stimulation by K^+ was not due to changes in the level of ATP. Under the conditions of our experiment ATP hydrolysis was only 2% whether or not K^+ was present. 3′:5′-cyclic AMP and 3′:5′-cyclic GMP (10^{-5}–10^{-9} M) were without effect on phosphorylation under various conditions (manuscript in preparation).

Since K^+ appeared to have a striking effect on protein phosphorylation, the effects of Na^+ and K^+ were investigated in detail. Fig. 2 shows the phosphorylation of one of the polypeptides of molecular weight 65,000 as a function of both Na^+ and K^+ concentrations. Phosphorylation is maximally stimulated at 1 mM Na^+, but higher concentrations of Na^+ are less effective or inhibitory. In contrast, phosphorylation is insensitive

Table 1. Effect of K^+ and carbachol on protein phosphorylation

Polypeptide molecular weight	K^+ (0.1M), % stimulation	Carbachol (10^{-4} M), % decrease
36,000	600	68
41,000	1300	69
56,000	1000	76
65,000	833	75
91,000	135	53

to K^+ at low concentrations and exhibits maximal activity at about 100 mM K^+. Thus, optimal phosphorylation of the 65,000-dalton polypeptide occurs at nearly physiologic intracellular concentrations of Na^+ and K^+ for *Torpedo* (27). Three polypeptides of molecular weight 56,000, 41,000, and 36,000 also show the same type of Na^+,K^+-dependent phosphorylation. In contrast, phosphorylation of the 91,000-dalton ATPase intermediate is not as sensitive to these ions (Table 1).

Since membrane fragments enriched in AChR contained substrates for a protein kinase, we investigated the effects of carbachol and other cholinergic ligands on the phosphorylation of these polypeptides. As shown in Fig. 3, carbachol at 10^{-4} M produces a 75% decrease in the level of phosphorylation of the same four K^+-dependent bands. The 91,000-dalton band corresponding to the phosphorylated intermediate of ATPase shows a similar but less striking change (Table 1). Carbachol inhibition of protein phosphorylation has an absolute requirement for K^+, with an optimal concentration of 100 mM K^+. Na^+ is not required, but no carbachol effect is seen at high Na^+ concentrations (data not shown). Qualitatively similar results were obtained with carbachol at concentrations as low as 10^{-6} M, tubocurare at 10^{-6} M, and decamethonium at 10^{-5} M. Hexamethonium at 5×10^{-4} M had no effect. Addition of these compounds did not produce changes in the intensity of Coomassie blue staining. Since several agonists and antagonists known to react specifically with the nicotinic acetylcholine receptor produced a similar response, it is possible that receptor occupancy is related to a decrease in protein phosphorylation

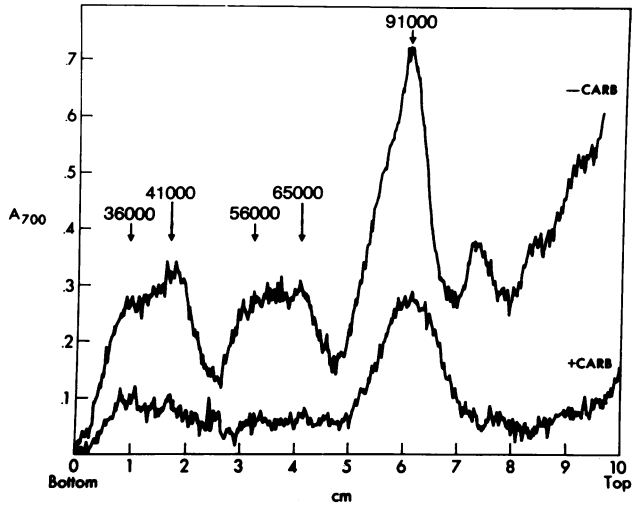

FIG. 3. Effect of 10^{-4} M carbachol on phosphorylation of membrane polypeptides by endogenous protein kinase activity. Scan of autoradiogram of dried $NaDodSO_4$ gel. Ordinate represents absorbance at 700 nm. Upper curve, no additions; lower curve, 10^{-4} M carbachol. Assay is in *Materials and Methods*, with salt concentrations of 1 mM NaCl and 100 mM KCl.

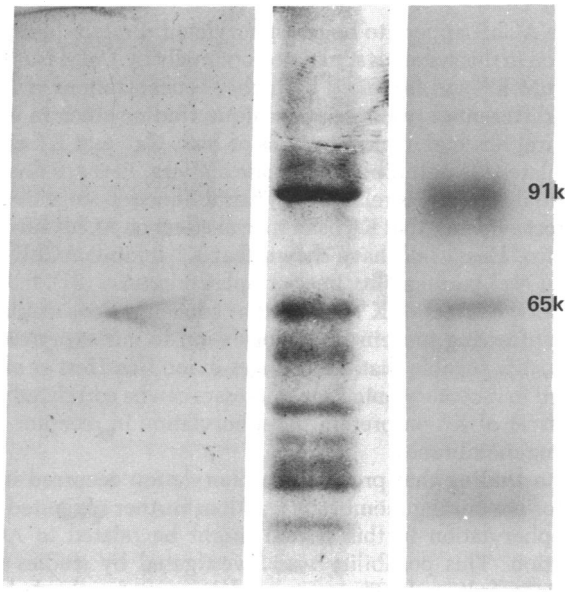

FIG. 4. Identification of a component of the 65,000-dalton band as a subunit of the AChR by two-dimensional immunoelectrophoresis. (a) Immunoelectrophoresis of a duplicate of the stained $NaDodSO_4$ gel pictured in (b). Membranes were phosphorylated by [γ-^{32}P]ATP in the presence of 0.1 M K^+, as described in *Materials and Methods*. (c) Autoradiogram of the $NaDodSO_4$ gel of (b); 91k, 91,000; 65k, 65,000.

and that there is an interaction between the AChR and protein phosphorylation. In fact, our data suggest that phosphorylation of a polypeptide component of the AChR may occur in these preparations.

The purified AChR of *Torpedo californica* has four polypeptide subunits of molecular weights 65,000, 58,000, 52,000, and 40,000 (unpublished observations; refs. 28 and 29). Three of the phosphorylated polypeptides in receptor-enriched membranes have virtually identical molecular weights (Fig. 3). We have used two-dimensional immunoelectrophoresis to investigate whether these phosphorylated polypeptides are subunits of the acetylcholine receptor. Fig. 4 shows a study combining $NaDodSO_4$ gel electrophoresis, two-dimensional immunoelectrophoresis, and autoradiography of the $NaDodSO_4$ gel. Fig. 4b shows the polypeptide distribution of membranes by Coomassie blue staining. Fig. 4c is an autoradiogram of this gel, indicating the polypeptides that are phosphorylated by the endogenous membrane protein kinase. Fig. 4a is the Coomassie blue stain of the agarose gel after immunoelectrophoresis of the $NaDodSO_4$ gel against antiserum against AChR. Fig. 4a demonstrates crossreactivity of antibodies raised against the AChR with the 65,000-dalton polypeptide. Under the conditions of this reaction, the antibody reacts only with this polypeptide. Autoradiography of the $NaDodSO_4$ gel (Fig. 4c) shows that the 65,000-dalton component is also phosphorylated. Although not completely conclusive, since we have yet to demonstrate phosphorylation of the immunoprecipitated "rocket" (Fig. 4a), these data provide strong suggestive evidence that a component of the AChR is phosphorylated by an endogenous membrane protein kinase.

DISCUSSION

In this paper we demonstrate that (*a*) an endogenous membrane protein kinase is present in membrane fractions enriched in the AChR, (*b*) phosphorylation of endogenous membrane polypeptides is responsive to cholinergic ligands, and (*c*) subunits

of the AChR appear to be phosphorylated. Protein phosphorylation in this system is stimulated optimally by 1 mM Na^+ and 100 mM K^+ and inhibited by higher concentrations of Na^+. This differential response is a unique finding since in other preparations high concentrations of both Na^+ and K^+ either stimulate (11) or inhibit (30) kinase activity. Perhaps this differential response is related to synaptic function since there is indirect evidence that K^+ has a special effect on AChR function *in vitro*. Hess *et al.* have shown that K^+ inhibits AChR-mediated Na^+ permeability in electroplax microsacs (31). In their studies the effect of K^+ occurred at concentrations similar to those affecting protein phosphorylation in our experiments. Thus, it is possible that the changes in ion flux Hess *et al.* reported in receptor-containing microsacs can be correlated with an effect of K^+ on protein phosphorylation in receptor-containing membranes.

The finding that protein phosphorylation occurred in receptor-containing membrane fractions further suggested that phosphorylation in this system might be related to AChR function. This possibility was investigated by studies with cholinergic ligands. The acetylcholine agonist carbachol produced a striking decrease in protein phosphorylation when added to membrane fractions containing AChR. This decrease represents an inhibition of protein phosphorylation since there was no measurable phosphatase activity under the conditions of study (manuscript in preparation). At the concentrations used, carbachol and the other cholinergic ligands are known to react specifically with the AChR (20). Using fluorescence techniques, Changeux *et al.* (32) have shown that both cholinergic agonists and antagonists produce similar conformational changes in the AChR. It is possible that in our system the conformational changes resulting from receptor occupancy by agonists and antagonists may be related to a decrease in phosphorylation. The reason other investigators have not found an effect of cholinergic ligands on protein phosphorylation may be because they have not used optimal concentrations of Na^+ and K^+. Thus, earlier studies have usually included 10 mM Na^+ and omitted K^+ (5, 33); under these conditions carbachol does not have an effect.

The effect of carbachol and the presence of protein kinase activity in membrane fragments containing the AChR suggested that a component of the receptor might be a substrate of an endogenous membrane protein kinase. The purified AChR from *Torpedo californica* has four polypeptide subunits of molecular weights 65,000, 58,000, 52,000, and 40,000 (unpublished observations; refs. 28 and 29). Three of the phosphorylated polypeptides in receptor-containing membranes have nearly identical molecular weights, but this observation does not confirm the identity of these polypeptides. We have used immunoelectrophoresis to determine which polypeptides crossreact with antibody raised against the purified *Torpedo* AChR. We found that the 65,000-dalton polypeptide is the only component that reacts with antibody under the conditions of the immunoelectrophoresis. This same polypeptide is phosphorylated by the endogenous membrane protein kinase. Because it is possible that the 65,000-dalton material is composed of more than one polypeptide, we are trying to demonstrate incorporation of ^{32}P into the immunoprecipitated "rocket." Unfortunately, these studies are hampered by the low specific activity of the phosphorylated protein, the limited amount of material that we can introduce into the gels, and the time required for exposure of the autoradiographic films. Nevertheless, these results provide strong suggestive evidence that a component of the AChR is phosphorylated by an endogenous membrane protein kinase.

As a result of the studies of Kasai and Changeux (34) and Michaelson and Raftery (35), it has been generally assumed that the AChR is the only component necessary to produce ionic permeability changes at nicotinic synapses. However, Karlin (36) has calculated that the average conductance increase per AChR site in these membrane preparations is 10^3–10^5 times less than observed *in vivo*. Furthermore, Chagas *et al.* have shown that at low concentrations, caffeine and theophylline modify depolarization in the isolated electroplax of *Electrophorus* (37). These observations suggest that at nicotinic synapses, other components in addition to the AChR may be required for optimal ion permeability. It is possible that protein phosphorylation may play a role in this process. However, it is also possible that phosphorylation is involved in the mechanism of other postsynaptic events at nicotinic synapses, such as desensitization. The exact relationship between the AChR, protein kinase activity, and the substrates of this enzyme is still not clear. If the AChR is a substrate for protein kinase, then receptor occupancy with cholinergic ligands may cause the receptor to undergo a conformational change such that it is no longer as accessible to the enzyme. Alternatively, the AChR could be directly or indirectly coupled to a protein kinase system so that receptor occupancy inhibits enzyme activity.

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