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MEMBRANE-BOUND PROTEIN KINASE ACTIVITY IN ACETYLCHOLINE RECEPTOR-ENRICHED MEMBRANES

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Summary

Membrane protein phosphorylation may be a general regulatory mechanism mediating the response of cells to exogenous metabolic and physical signals. We have determined that the membrane-bound acetylcholine receptor is the major substrate phosphorylated in situ by a nearby membrane protein kinase. Moreover, these same membranes also contain phosphoprotein phosphatase activity which dephosphorylates the membrane-bound receptor. These findings suggest that reversible phosphorylation of the actylcholine receptor may be critical for receptor function at the synapse. Therefore, it is necessary to define the properties of the enzymes which mediate this phosphorylation-dephosphorylation mechanism. In this report we describe the properties of the first component of this system, the membrane-bound protein kinase in receptor-enriched membranes from the electric organ of *Torpedo californica*. Only ATP is effective as a phosphate donor for this cyclic AMP-independent membrane kinase; GTP does not support phosphorylation of the receptor. Both casein and histone can also be phosphorylated by the membrane protein kinase, but casein is a better substrate. Although phosphorylation of the receptor appears to be regulated by cholinergic ligands and K^+, casein phosphorylation is not specifically affected by these agents. Moreover, while phosphorylation of the acetylcholine receptor is maximal in receptor-enriched membranes, casein phosphorylation is similar in all membrane fractions prepared from the electric organ. Taken together, these findings suggest that the membrane protein kinase activity in receptor-enriched membranes is similar to most other membrane kinases. Therefore, the

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

unique characteristics of membrane-bound acetylcholine receptor phosphorylation appear to be determined by the receptor and its availability as a substrate for the membrane kinase.

Introduction

Acetylcholine released at the synapse reacts with the acetylcholine receptor to produce changes in the properties of the receptor and the post-synaptic membrane. The molecular mechanisms underlying these changes are not known. We have reported that the membrane-bound acetylcholine receptor of *Torpedo californica* is phosphorylated and dephosphorylated in situ by a membrane protein kinase and phosphatase [1,2]. Phosphorylation in acetylcholine receptor-enriched membranes is inhibited by cholinergic ligands and stimulated by K^+ [3], agents which are known to react specifically with the acetylcholine receptor [4—6]. Teichberg et al. have reported that the acetylcholine receptor in *Electrophorus electricus* can be phosphorylated by a protein kinase after solubilization of receptor-enriched membranes [7]. They also found that phosphorylation in membranes from *Torpedo marmorata* electric organ is stimulated by K^+ and inhibited by Na^+ [8]. These findings all suggest that changes in the level of acetylcholine receptor phosphorylation could be related to changes in the physiological properties of the acetylcholine receptor at the post-synaptic membrane.

There is increasing evidence that other neurotransmitters [9], light [10], polypeptide hormones [11] and growth factors [12] may act by regulating the level of phosphorylation of membrane proteins. These observations suggest that membrane protein phosphorylation may be a general regulatory mechanism affecting the response of cells to exogenous metabolic and physical signals. Acetylcholine receptor-enriched membranes from *T. californica* constitute an ideal model system in which to characterize the role of phosphorylation in the regulation of receptor function. The acetylcholine receptor is the major constituent of these membranes and, to date, is the only membrane-bound substrate for a membrane-bound kinase which has been identified. This report characterizes some of the properties of the membrane-bound kinase in acetylcholine receptor-enriched membranes. These findings should be of general interest since they may help to elucidate the mechanism of receptor function and regulation in many membrane systems.

Materials and Methods

Membrane fractions were prepared from the electric organ of *T. californica* (Pacific Bio-Marine, Venice, CA) as described [3]. Histone (Type III lysine-rich), α-casein (C-3883) and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP (30 Ci/mmol) was purchased from New England Nuclear.

Acetylcholine receptor assay. Acetylcholine receptor was measured using *Naja naja siamensis* toxin as reported [3].

(Na^+ + K^+)-ATPase. Membrane protein (75 μg) was incubated for 10 min at

37°C in a final volume of 0.2 ml containing 1 mM $MgCl_2$, 5 mM KCl, 60 mM NaCl, 0.1 mM EDTA, 100 mM Tris-HCl (pH 7.4) and 2.5 mM ATP. The reaction was stopped with the addition of 1.0 ml of 10% trichloroacetic acid and centrifuged at 12 000 × *g* for 10 min. 1.0 ml of the supernatant was mixed with 1.0 ml of freshly prepared color reagent (40.0 mg $FeSO_4$ in 10 ml 1% ammonium molybdate and 1.15 N H_2SO_4). The resulting blue color was quantitatively determined by measuring the absorbance at 700 nm [13]. Inhibition by 1 mM ouabain was virtually 100%. A standard curve was constructed using inorganic phosphate (0.05—5 μg).

Acetylcholinesterase. Membrane protein (5 μg) was incubated at room temperature in a 1 cm cuvette in a final volume of 3 ml containing 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM acetylthiocholine and 0.5 mM Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)). The reaction was monitored by measuring the absorbance change at 412 nm [14]. Enzyme activity was proportional to the slope of the linear portion of the absorbance curve as a function of time ($\Delta A_{412}/\Delta t$).

Electrophoresis. Sodium dodecyl sulfate acrylamide slab gel electrophoresis was carried out as described [3].

Preparation of casein and histone. The proteins were dissolved at 25 mg/ml in 1 mM Hepes (pH 8) containing 1 mM EDTA, heated for 20 min at 70°C, dialyzed overnight at 4°C against 1 mM Hepes (pH 8) containing 0.1 mM EDTA and filtered through 0.45 μm Millipore filters into sterile tubes for storage at −20°C.

Protein phosphorylation

Endogenous substrate. The standard reaction mixture contained 50 μg of membrane protein, 0.25 mM EGTA, 20 mM magnesium acetate, 62.5 mM Tris-HCl (pH 6.8), 0.1 M KCl 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 4°C. The reaction was stopped by addition of 0.02 ml of 20% sodium dodecyl sulfate and after 0.5 min the samples were placed in a boiling water bath for 1 min. Bromophenol blue and sucrose were then added as previously described [3]. Under these conditions ATP degradation was less than 2%.

Exogenous substrate. Exogenous protein phosphorylation was assayed according to the method of Avruch et al. [15]. All assays were performed in duplicate. 25-μl of membranes (15 μg protein) were incubated in an equal volume of 0.2% saponin at 4°C for 30 min. This was diluted to a final volume of 100 μl containing 15 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 0.25 mM EGTA and 0.05% saponin. Casein or histone was present at a final concentration of 1 mg/ml. The mixture was pre-incubated for 3 min at 37°C and 50 μM [γ-^{32}P]ATP (2 μCi/tube) was added to start the reaction. Phosphorylation of substrate was allowed to occur for 20 min at 37°C (we have found that exogenous phosphorylation is linear for at least 30 min). The reaction was terminated by the addition of 4 ml of cold 0.25 M perchloric acid containing ATP, inorganic pyrophosphate and inorganic phosphate, each at 1 mM. The tubes were held on ice while 0.5 mg of carrier casein was added to each tube. The precipitates were collected by vacuum filtration on 24 mm Whatman GF/c

glass fiber filters, washed five times with 4 ml of the stop solution and washed once with 5—10 ml of cold 5% trichloroacetic acid. Filters were added to 10 ml of Instagel (New England Nuclear) and counted in a liquid scintillation counter. Membranes were omitted from control tubes. Phosphorylation of membrane protein may in part be due to incorporation of [^{32}P]phosphate into the ATPase intermediate [16]. Therefore, all reported values for exogenous phosphorylation were also corrected for endogenous membrane phosphorylation determined in the absence of histone or casein. Low membrane concentration and high exogenous substrate concentrations were chosen to minimize endogenous membrane protein phosphorylation.

Protein was determined by using the method of Lowry et al. [23].

Results

We have proposed that acetylcholine receptor phosphorylation is involved in synaptic function. In order to investigate the regulation of post-synaptic phosphorylation mechanisms it is necessary to study membranes of synaptic origin which are enriched in the acetylcholine receptor. We present the details of our membrane purification and characterization in order to establish that these membranes are of post-synaptic origin.

Sucrose gradient centrifugation of homogenized *Torpedo* electric organ membranes resulted in the separation of several membrane fractions (A—E) which could be recovered from the gradient (Fig. 1). The distribution of enzymatic activities in the membrane fractions obtained from the discontinuous sucrose gradient is shown in Fig. 2. Compared to the total membrane preparation (H), the acetylcholine receptor was enriched in fractions C and D. Frac-

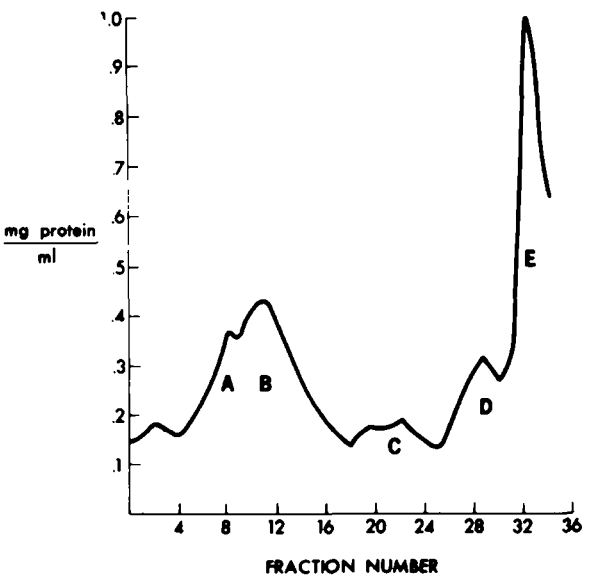


Fig. 1. Discontinuous sucrose gradient centrifugation of a total membrane homogenate from *Torpedo californica*.

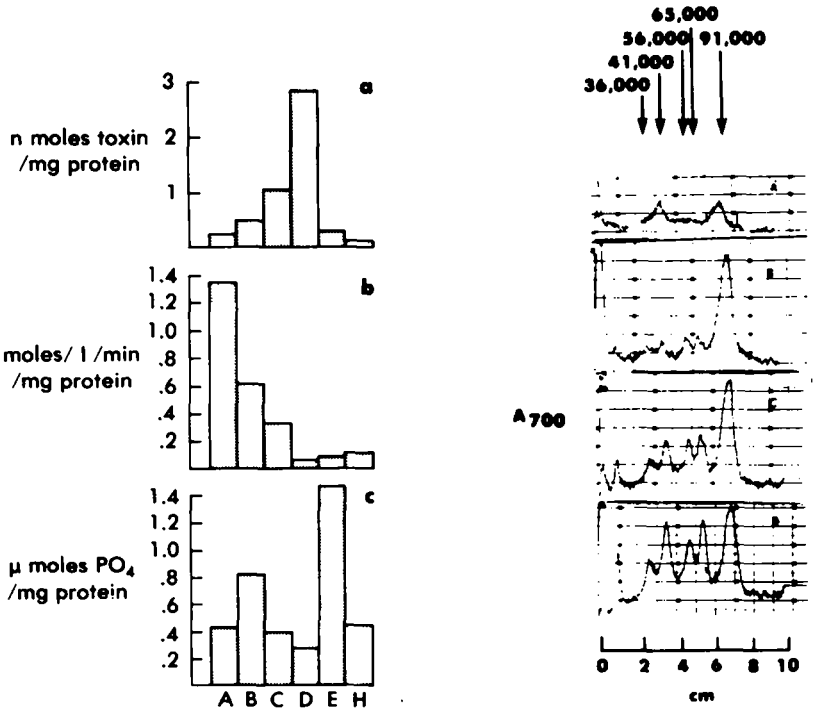


Fig. 2. Marker enzyme distribution in membranes purified by sucrose gradient centrifugation: (a) ^{125}I-labeled *Naja naja siamensis* toxin binding activity; (b) acetylcholinesterase activity; (c) $(Na^+ + K^+)$-ATPase activity. Fractions A—E are recovered from the sucrose gradient. Fraction H is the total membrane homogenate applied to the sucrose gradient.

Fig. 3. Endogenous phosphorylation of membrane fractions A—D. Densitometric scan at 700 nm of autoradiograms of dried SDS polyacrylamide gels.

tion D had nearly a 15-fold increase in receptor activity while fraction C was enriched 5-fold. In contrast to the distribution of acetylcholine receptor, acetylcholinesterase was enriched in fraction A and $(Na^+ + K^+)$-ATPase activity was enriched in fractions B and E. Fraction D, the fraction most enriched in acetylcholine receptor, contained the least amount of $(Na^+ + K^+)$-ATPase and acetylcholinesterase activity. This distribution of enzyme activities is similar to that found by Duguid and Raftery [17]. Minor differences probably are produced by changes in the construction of the gradient. Electron microscopy demonstrated that the acetylcholine receptor-enriched membrane fraction contained many septilaminar vesicular profiles which were not present in the other membrane fractions (Basbaum, C., unpublished data). Thus, homogenization and sucrose gradient centrifugation appear to have produced a functional dissection of membrane components.

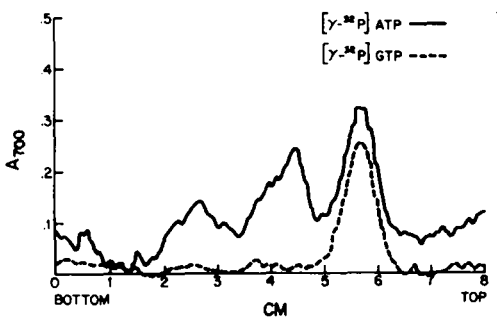
Protein phosphorylation

Endogenous substrates. If phosphorylation regulates receptor function in the post-synaptic membrane, then receptor-enriched membranes would be expected to show relatively high levels of endogenous acetylcholine receptor phosphorylation. When fractions A—D were examined for endogenous mem-

brane protein phosphorylation by incubating the membranes with [γ-^{32}P]ATP in the presence of Mg^{2+}, fraction D exhibited maximal phosphorylation (Fig. 3). The phosphorylated polypeptides have molecular weights of 36 000, 41 000, 56 000, 65 000 and 91 000. We have identified the 56 000 and 65 000 dalton phosphorylated polypeptides as components of the acetylcholine receptor (Ref. 1 and unpublished data). Significant phosphorylation of these polypeptides was also demonstrable in fraction C but markedly reduced in fraction B and virtually absent in fraction A. Thus, protein phosphorylation in membrane fractions from *Torpedo* electric organ correlated directly with the presence of the acetylcholine receptor as indicated by neurotoxin binding activity and polypeptides corresponding to the molecular weights of subunits of the acetylcholine receptor.

Specificity of the phosphate donor. We have reported that the protein kinase present in acetylcholine receptor-enriched membranes is a cyclic AMP-independent enzyme [3]. Most cyclic AMP-independent kinases can use GTP as well as ATP as a phosphate donor in the phosphorylation reaction [24]. We examined the specificity of the phosphorylation reaction in acetylcholine receptor-enriched membranes by comparing the effectiveness of [γ-^{32}P]ATP and [γ-^{32}P]-GTP as phosphate donors. Fig. 4 shows that in contrast to [γ-^{32}P]ATP, [γ-^{32}P]-GTP at the same concentration and specific activity supports only the phosphorylation of the 91 000 dalton polypeptide; the other polypeptides which are phosphorylated in the presence of [γ-^{32}P]ATP are unlabeled. This finding suggests that ATP appears to be the specific phosphate donor for endogenous phosphorylation of the membrane-bound acetylcholine receptor.

Divalent cations. Most protein kinases are Mg^{2+}-dependent enzymes. Fig. 5 shows the effects of divalent cations on phosphorylation of the 65 000 dalton component of the acetylcholine receptor. Mg^{2+} was the most effective enzyme



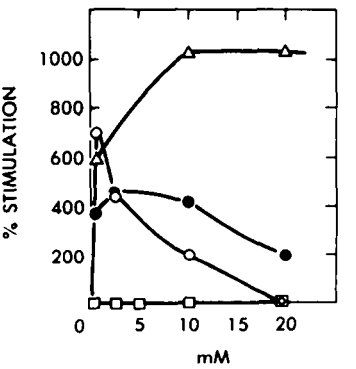


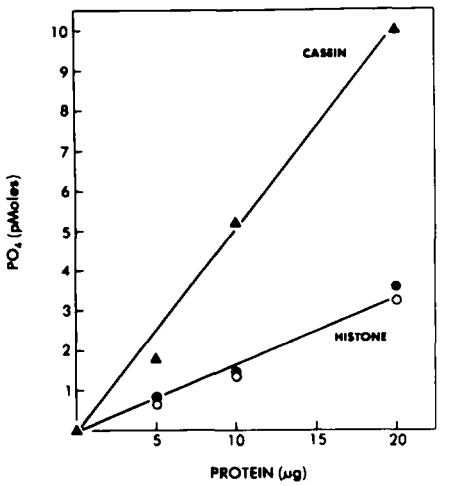
Fig. 4. Endogenous phosphorylation of acetylcholine receptor-enriched membranes. Densitometric scan at 700 nm of autoradiogram of dried SDS polyacrylamide gels. Membranes were incubated with either $5 \cdot 10^{-6}$ M ATP (———) or GTP (- - - - -) (2 μCi/tube). The reaction was stopped with SDS and the phosphorylated polypeptides were separated by SDS polyacrylamide gel electrophoresis.

Fig. 5. Effect of divalent cations on phosphorylation of the 65 000 dalton polypeptide in acetylcholine receptor-enriched membranes. Ordinate, absorbance at 700 nm of autoradiograms of membranes phosphorylated in the presence of a given divalent cation relative to the absorbance of membranes phosphorylated in the absence of the cation: △, Mg^{2+}; ○, Zn^{2+}; ●, Mn^{2+}; □, Ca^{2+}.

activator. Both Zn^{2+} and Mn^{2+} could be substituted for Mg^{2+} at low concentrations, but Ca^{2+} did not support receptor phosphorylation even after extensive dialysis of the membranes against EGTA.

Exogenous substrates. Since fraction D, which is enriched in acetylcholine receptor, exhibited maximal membrane protein phosphorylation, the ATP-dependent membrane kinase in fraction D might have special properties. This was investigated by studying phosphorylation of soluble exogenous substrates by the membrane-bound protein kinase in fraction D. In other systems histone and casein have both been used as convenient substrates to study membrane-bound protein kinase activity. Histone appears to be phosphorylated optimally by cyclic AMP-dependent protein kinases whilst casein appears to be phosphorylated optimally by cyclic AMP-independent protein kinases [18]. Fig. 6 shows that both histone and casein can be phosphorylated by the membrane-bound protein kinase present in acetylcholine receptor-enriched membranes. The amount of [^{32}P]phosphate incorporated into either protein was proportional to the amount of membrane protein (Fig. 6). We have previously shown that endogenous phosphorylation in these membranes is cyclic AMP-independent [3]. Therefore, as expected, casein was a better substrate than histone for the enzyme. Cyclic AMP did not stimulate the phosphorylation of casein (not shown) or histone (Fig. 6). Fig. 7 shows that phosphorylation of casein was linear to 30 min. These studies indicated that casein could be used effectively as a substrate for the membrane kinase.

We have reported that endogenous phosphorylation in acetylcholine receptor-enriched membranes is regulated by K^+ and cholinergic ligands [3]. To determine whether K^+ and cholinergic ligands alter membrane protein kinase



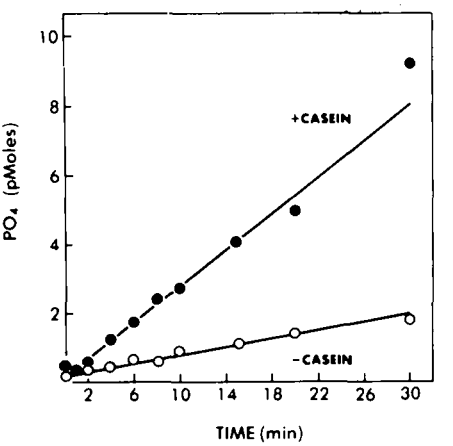


Fig. 6. Phosphorylation of exogenous substrates by membrane protein kinase present in acetylcholine receptor-enriched membranes. Reported values are corrected for endogenous membrane protein kinase activity in the absence of exogenous substrate. ▲, casein; ○, histone; ●, histone and 10 μM 3',5'-cyclic AMP.

Fig. 7. Time course of phosphorylation of casein by protein kinase in acetylcholine receptor-enriched membranes. Membrane protein concentration is 5 μg. ○, endogenous phosphorylation in the absence of casein; ●, exogenous phosphorylation.

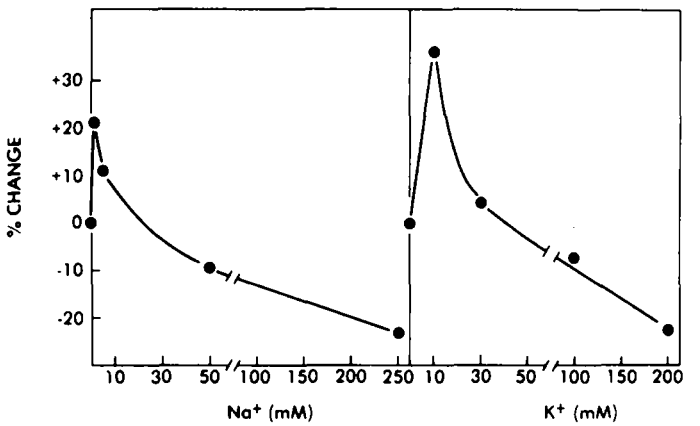


Fig. 8. Effects of Na^+ and K^+ on exogenous phosphorylation of casein by acetylcholine receptor-enriched membranes. Membrane protein concentration is 5 μg.

activity per se, we studied the effects of these agents on phosphorylation of casein by acetylcholine receptor-enriched membranes. As has been found for other membrane protein kinases, low concentrations of K^+ or Na^+ produced a small stimulation of casein phosphorylation but higher concentrations of either cation inhibited this reaction (Fig. 8). This is in contrast to studies of endogenous protein kinase activity in *Torpedo* electric organ where high concentrations of K^+ appeared to stimulate membrane protein phosphorylation [3,8]. Carbachol at $1 \cdot 10^{-4}$ M did not affect phosphorylation of casein by acetylcholine receptor-enriched membranes.

Membrane fractions enriched in the acetylcholine receptor exhibited the greatest amount of endogenous membrane phosphorylation when compared to other fractions (Fig. 3). Although this result appeared to be due to the fact that subunits of the acetylcholine receptor are the major substrates for the phosphorylation reaction [1], it was possible that protein kinase activity might be increased in the acetylcholine receptor-enriched membrane fraction. However, when casein was used with ATP as a substrate to compare kinase activities, the amount of phosphorylation per mg membrane protein was the same in all membrane fractions. This suggests that increased phosphorylation in acetylcholine receptor-enriched membranes is not due to increased kinase activity but probably to an increased amount of substrate (acetylcholine receptor) for the phosphorylation reaction.

Discussion

We have identified the acetylcholine receptor as a substrate for phosphorylation by a membrane-bound protein kinase in acetylcholine receptor-enriched membranes [1] and have proposed that reversible phosphorylation and dephosphorylation of the acetylcholine receptor may play a role in regulating the function of the acetylcholine receptor at the synapse [2]. Many laboratories have used membrane fractions from the electric organ of *T. californica* to investigate the properties of the membrane-bound acetylcholine receptor. We have

used similar preparative techniques to produce a functional dissection of the electric organ into specific membrane fractions (Fig. 2). This permitted us to study the properties of the membrane protein kinase which is associated with the acetylcholine receptor in the post-synaptic membrane. Using *Naja naja* toxin binding activity as a marker for the acetylcholine receptor we find in this study that acetylcholine receptor-enriched membranes show increased endogenous protein phosphorylation when compared to membrane fractions enriched in other enzyme activities (Fig. 3). Moreover, since subunits of the acetylcholine receptor are the major substrates for this reaction, endogenous membrane phosphorylation correlates directly with the presence of the acetylcholine receptor.

Studies from this laboratory have provided the first documented evidence that a membrane-bound neurotransmitter receptor protein is subject to phosphorylation by an endogenous membrane protein kinase [1]. This reaction appears to be stimulated specifically by K^+, inhibited by cholinergic ligands, and unaffected by cyclic AMP [3]. Most cyclic AMP-independent protein kinases use GTP as well as ATP as phosphate donors [24]. However, since ATP but not GTP is known to be released at the synapse [25,26], ATP might be required specifically by the post-synaptic membrane kinase. This proved to be the case. When we compared GTP to ATP as a phosphate donor for the phosphorylation reaction in receptor-enriched membranes, GTP could not substitute for ATP in the phosphorylation of the acetylcholine receptor (Fig. 4).

The acetylcholine receptor-enriched membranes also contain an endogenous phosphoprotein phosphatase activity which dephosphorylates the acetylcholine receptor [2]. Therefore, phosphorylation of the acetylcholine receptor at the nicotine synapse appears to be a reversible biochemical reaction which has unique properties and which may relate to receptor function at the synapse. It would be useful to learn more about the regulation of this phosphorylation-dephosphorylation reaction. However, the kinase which phosphorylates the acetylcholine receptor is present in the same membranes as the acetylcholine receptor, its substrate. To overcome the difficulty of studying the regulation of membrane-bound kinases in the presence of their membrane substrates, may investigators have used soluble exogenous substrates such as casein. We investigated the possibility that the membrane kinase in acetylcholine receptor-enriched membranes had special properties which regulated protein phosphorylation. However, we found with soluble substrates that protein kinase activity in acetylcholine receptor-enriched membranes was similar to that described with other membrane preparations. Phosphorylation of casein was not specifically affected by ions (Fig. 8) or cholinergic agents. Also, exogenous kinase activity was similar in all membrane fractions whether or not they had an increased concentration of receptors. It should be pointed out that studies with casein may not accurately reflect the interaction of the kinase with the membrane-bound acetylcholine receptor. Despite this limitation, our results are consistent with the possibility that the kinase itself may not be an important site for regulating phosphorylation. While it would have seemed preferable to use purified and solubilized acetylcholine receptor as an exogenous substrate for these experiments, this approach was not feasible because it is prohibitively expensive to prepare sufficient quantities of solubilized receptor for adequate

study. In addition, studies with purified acetylcholine receptor would also have been limited because acetylcholine receptor solubilized in detergent is in a desensitized state [27]. Experiments under these conditions probably would not have physiological significance.

K^+ and cholinergic ligands are known to react specifically with the acetylcholine receptor to produce conformational changes in the receptor protein [4—6]. Since K^+ stimulates and cholinergic ligands inhibit acetylcholine receptor phosphorylation [3], it is possible that these agents interact directly with the membrane-bound acetylcholine receptor to alter its conformation and thereby its availability as a substrate for the membrane kinase. This kind of regulation would be analogous to the light-dependent regulation of rhodopsin phosphorylation which has been studied in great detail [19—22]. Rhodopsin is the only other purified membrane protein known to be involved in neural transduction. This protein undergoes light-stimulated phosphorylation while it is membrane-bound [19—21]. It has been shown that light-stimulated phosphorylation of rhodopsin is due to a light-dependent conformational change in rhodopsin itself and not due to an effect of light on protein kinase activity [22]. Perhaps after reaction with acetylcholine, changes in the conformational state of the membrane-bound acetylcholine receptor also determine its availability as a substrate for the endogenous phosphorylation reaction.

ATP has been demonstrated in synaptic vesicles [25] but the function of ATP at the synapse is not clear. It is also known that ATP is released at a nicotinic synapse [26] and is probably released at other synapses. Some investigators have proposed that ATP binds to neurotransmitter in synaptic vesicles [25] or serves as a neurotransmitter itself [28]. Our data suggest that after release into the synaptic cleft, ATP may function as a phosphate donor to support the phosphorylation of the acetylcholine receptor in the post-synaptic membrane. Therefore, it is possible that release of ATP from the nerve terminal may be part of a biochemical mechanism which regulates the activity of the post-synaptic acetylcholine receptor.

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