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Reversible Phosphorylation of the Membrane-Bound Acetylcholine Receptor

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We have found that the acetylcholine receptor (AChR) of Torpedo californica is phosphorylated and dephosphorylated in situ by a membrane-bound protein kinase and phosphatase [1]. There is increasing evidence that other neurotransmitters [2], light [3–6], polypeptide hormones [7], and growth factors [8–12] also 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.

To understand the role of membrane protein phosphorylation, we have chosen to study acetylcholine receptor-enriched membranes purified from the electric organ of T californica. This organ is an ideal model system for such studies since it is a rich source of the acetylcholine receptor. Membranes can be purified from the electric organ that are enriched in the AChR [13] and that show cholinergic agonist-dependent changes in cation flux [14]. Moreover, the AChR from T californica has been purified [15], biochemically characterized [16], and used to generate specific antibodies [17]. Receptor-enriched membranes contain only a few other proteins that are closely associated with the receptor in the postsynaptic membrane. Such associated proteins may play a critical role in regulating the function of the AChR in the postsynaptic membrane. We have taken advantage of these conditions to study phosphorylation of the membrane-bound AChR in this well-defined, homogeneous system.

CHARACTERIZATION OF AChR-ENRICHED MEMBRANES

When the electric organ of T californica is homogenized and the resulting membranes purified by ultracentrifugation on a discontinuous suscrose gradient, several membrane fractions are recovered (Fig. 1). We determined which membrane fraction was enriched in the AChR by using Naja naja siamensis toxin, a specific nicotinic cholinergic antagonist. The distribution of toxin binding in the various membrane fractions compared with other membrane markers is shown in Figure 2. The AChR was enriched in fractions C and D (Fig. 2a). Compared with the total membrane preparation (H), fraction D had nearly a 15-fold increase in receptor activity, while fraction C was 5-fold enriched. In contrast to

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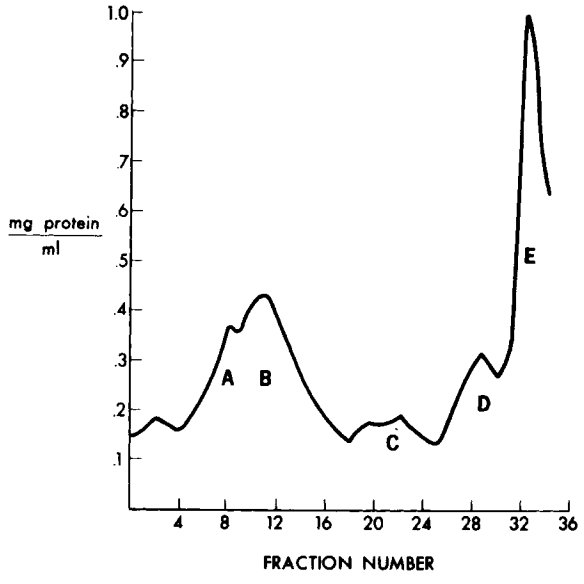

Fig. 1. Discontinuous sucrose gradient centrifugation of a total membrane homogenate from Torpedo californica.

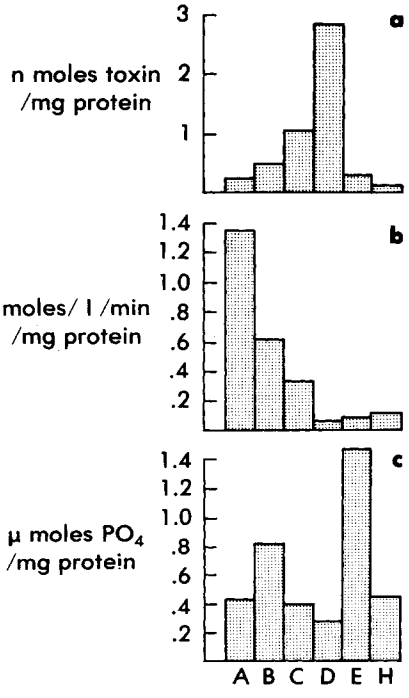

Fig. 2. Marker enzyme distribution in membranes purified by sucrose gradient centrifugation: (a) I^{125}-Naja naja siamensis toxin binding activity; (b) Acetylcholinesterase activity; (c) Na-K-ATPase activity. Fractions A–E are recovered from the sucrose gradient. Fraction H is the total membrane homogenate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

the distribution of AChR, acetylcholinesterase was enriched in fraction A (Fig. 2B) and Na-K-ATPase activity was enriched in fractions B and E (Fig. 2C). Fraction D, the fraction most enriched in AChR, contained very low levels of Na-K-ATPase and acetylcholinesterase activity. This suggested that fraction D was enriched in membranes of postsynaptic origin.

If phosphorylation regulates receptor function in the postsynaptic membrane, then receptor-enriched membranes should contain endogenous protein kinase activity. When fractions A–D were examined for endogenous membrane protein phosphorylation by incubating the membranes with γ-^{32}P-ATP in the presence of Mg^{+2}, fraction D exhibited maximal phosphorylation (Fig. 3). Several of the phosphorylated polypeptides have molecular weights corresponding to some of the subunits of the purified AChR. Significant phosphorylation of the same polypeptides was also demonstrable in fraction C but was markedly reduced in fraction B and virtually absent in A. Thus, fraction D, a specialized membrane preparation enriched in the AChR, also showed maximal endogenous membrane protein phosphorylation.

In the experiments described above, we found phosphorylation of several major polypeptides that had the same molecular weight as subunits of the AChR. In addition, phosphorylation of these polypeptides appeared to be regulated by K^+ and cholinergic ligands [18]. These results suggested that the membrane-bound AChR itself might be a substrate for the endogenous protein kinase. To investigate this possibility directly, we used anti-

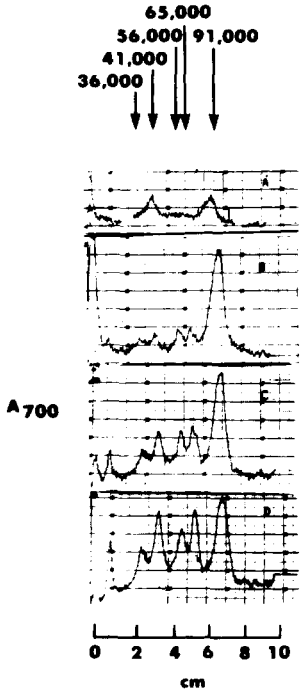

Fig. 3. Endogenous phosphorylation of membrane fractions A–D. Densitometric scan at 700 nm of autoradiograms of dried SDS-polyacrylamide gels. Membranes were incubated for 0.5 min at 4°C with γ-^{32}P-ATP (5 μM, 10 μCi/ml), and Mg^{2+}. The reaction was stopped by the addition of SDS, and the phosphorylated polepeptides were separated by SDS-polyacrylamide gel electrophoresis [18].

bodies prepared against the purified AChR to prove that several of the phosphorylated polypeptides are components of the AChR [19]. The technique we employed was two-dimensional immunoelectrophoresis as described by Converse and Papermaster [20]. Acetylcholine receptor-enriched membranes were incubated with γ-^{32}P-ATP and Mg^{2+}, and the reaction was terminated by the addition of SDS. The solubilized membranes were then electrophoresed in duplicate in SDS-polyacrylamide gels (Fig. 4a). The gels were cut into longitudinal strips along the sample wells; one gel was stained for protein, another was subjected to immunoelectrophoresis at right angles into two layers of agarose. The first layer contained 1.5% Lubrol PX to remove excess SDS, and the second contained goat anti-AChR antiserum.

Polypeptides derived from the AChR that are present in optimal concentrations form immunoprecipitates with the anti-AChR antiserum and are recognized as "rockets." Figure 4b shows a major Coomassie blue-stained rocket corresponding to the 65,000-dalton polypeptide of the AChR. Control gels run against preimmune serum show no rockets. Autoradiography of the dried gel (Fig. 4c) demonstrates that the same polypeptide precipitated by anti-AChR antiserum contains ^{32}P. The stoichiometry of the phosphorylation reaction is low, varying between 0.1 and 1.0 moles of phosphate per 100 moles of AChR. However, these values appear to grossly underestimate the actual stoichiometry of the reaction since, as we present in this paper, there is considerable phosphoprotein phosphatase activity present that dephosphorylates the AChR.

The immunoelectrophoresis experiment provided unambiguous evidence that the 65,000-dalton component of the AChR was phosphorylated in situ by an endogenous membrane protein kinase present in AChR-enriched membranes. This was the first component of a membrane receptor protein to be identified as a substrate for an endogenous membrane protein kinase. Unlike work with other membrane preparations, the phosphorylated substrate in our studies is a subunit of a postsynaptic membrane protein whose function is known and that has been biochemically characterized. This is an important first step in correlating phosphorylation of membrane proteins with membrane function.

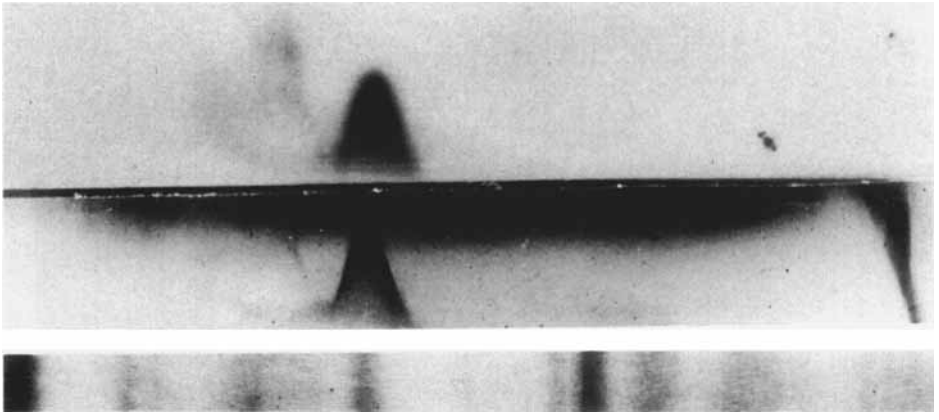

Fig. 4. Two-dimensional immunoelectrophoresis of AChR-enriched membranes phosphorylated in situ [19]: (a) Coomassie-blue stained SDS-gel; (b) Coomassie-blue stained agarose gel after immunoelectrophoresis of the SDS strip into goat anti-AChR-containing agarose; (c) autoradiography of (b).

We have also investigated some of the factors that regulate protein kinase activity in the postsynaptic membrane. ATP appears to be released with ACh from presynaptic nerve terminals at the neuromuscular junction. We wondered whether ATP might have a specific effect at the synapse to support the phosphorylation of the postsynaptic AChR. Figure 5 shows that ATP is the specific phosphate donor for phosphorylation of the membrane bound AChR. With GTP as phosphate donor, only the 100,000-dalton band was phosphorylated; no receptor phosphorylation is seen. We also used histone and casein to study phosphorylation of exogenous substrates by the membrane-bound protein kinase present in AChR-enriched membranes. Figure 6 shows that both histone and casein can be phosphorylated by the membrane-bound protein kinase present in AChR-enriched membranes, and the amount of $^{32}P\text{-}PO_4$ incorporated into either protein was proportional to the amount of membrane protein. Histone appears to be phosphorylated optimally by cAMP-dependent protein kinases [21], whereas casein is ordinarily the optimal substrate for cAMP- independent kinases. We have previously shown that endogenous phosphorylation in these membranes is cAMP-independent [18] and cAMP did not stimulate phosphorylation of casein (not shown) or histone (Fig. 6). These results suggest that the protein kinase that phosphorylates the AChR in the postsynaptic membrane is not a cAMP-dependent enzyme. However, we have not excluded the possibility that the regulatory subunit of a cAMP-dependent kinase has been lost during the purification of the AChR-enriched membranes.

PROPERTIES OF THE DEPHOSPHORYLATION REACTION

If phosphorylation of the AChR is an important regulatory event at the synapse, then dephosphorylation of the AChR must also occur in situ. Therefore, the AChR-enriched membranes should have endogenous membrane phosphoprotein phosphatase activity that can dephosphorylate the AChR. We first studied phosphoprotein phosphatase activity using $^{32}P\text{-}PO_4$-labeled casein as exogenous substrate [22]. Figure 7 shows the time course for release of ^{32}P from phosphorylated casein after incubation with AChR-enriched membranes. Boiled membrane controls or phosphorylated casein without membranes did

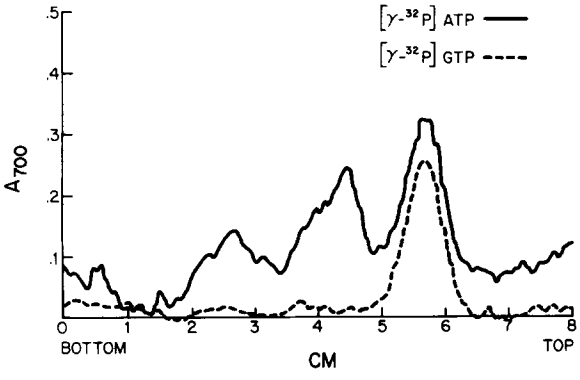

Fig. 5. Comparison of γ-^{32}P-ATP and γ-^{32}P-GTP at equal concentration and specific activities as phosphate donors for the phosphorylation of the acetylcholine receptor.

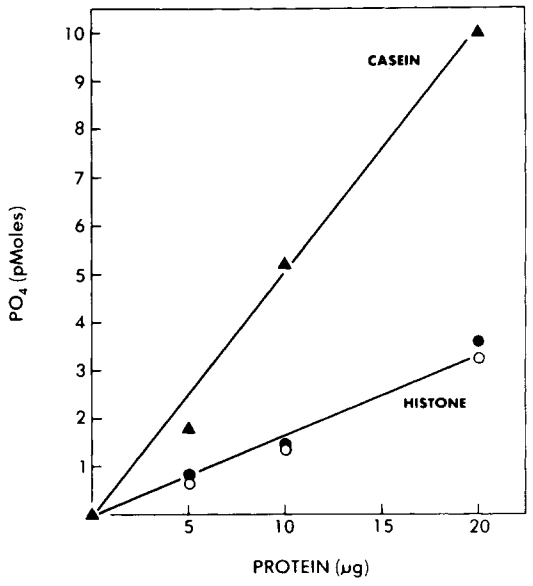

Fig. 6. Phosphorylation of exogenous substrates by membrane protein kinase present in AChR-enriched membranes as a function of membrane protein concentration [28]. Reported values are corrected for endogenous membrane protein kinase activity in the absence of exogenous substrate. ▲, casein; ○, histone; ●; histone and 10 μm, 3′,5′-cAMP.

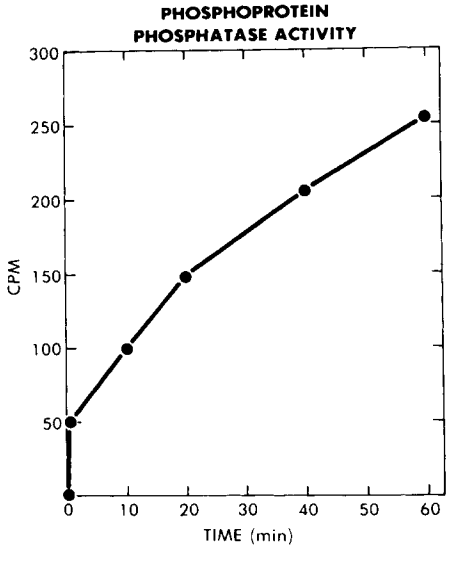

Fig. 7. Time course of phosphoprotein phosphatase activity in AChR-enriched membranes using ^{32}P-PO_4-casein as substrate. Reaction carried out as described in Table I.

not show a release of radioactivity over this same time interval. Release of ^{32}P from casein was proportional to the concentration of membrane protein (Fig. 8). ^{32}P released from casein after incubation with membranes could be due to proteolytic breakdown of casein into labeled amino acids. We investigated this possibility and found that ^{32}P-PO_4 casein migrated as one band on SDS-acrylamide gel electrophoresis visualized by both Coomassie blue staining and autoradiography and that the patterns were unchanged after incubation of the casein with membranes. In addition, all of the ^{32}P released from casein was extractable into isobutanol in the presence of ammonium molybdate and sulfuric acid. This confirmed that the label was inorganic phosphate. The effect of activators and inhibitors on protein phosphatase activity in AChR-enriched membranes is shown in Table I. The data indicate that this membrane protein phosphatase is similar to other cAMP independent protein phosphatases.

If phosphatase activity is important in regulating the level of AChR phosphorylation, then the enzyme should dephosphorylate the AChR in situ. We used two different assay systems to study this question. In the first, we measured time-dependent release of ^{32}P-PO_4 from AChR-enriched membranes that had been phosphorylated in the presence of γ-^{32}P-ATP and Mg^{2+}. Table IIA shows that ^{32}P-labeled inorganic phosphate was released from the phosphorylated membranes after incubation at 37°C. This reaction was also inhibited by fluoride ion. Thus, the enzyme does dephosphorylate endogenous membrane protein.

In the second assay, we measured changes in the level of phosphorylation of the membrane-bound AChR directly after incubation at 37°C for 10 min. SDS-gel electrophoresis of the phosphorylated membranes shows that a major subunit of the AChR had been dephosphorylated since there was less covalently bound ^{32}P-PO_4 (Table IIB). As before, endogenous dephosphorylation of the AChR was also inhibited by 0.1 M NaF. Therefore the AChR-enriched membranes contain both endogenous protein kinase and phosphatase activities that regulate the level of phosphorylation of the AChR in situ.

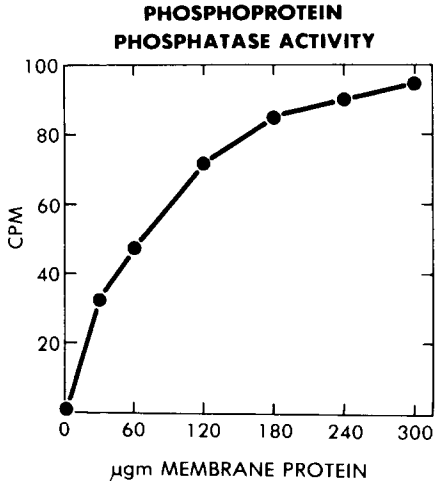

Fig. 8. Phosphoprotein phosphatase activity as a function of membrane protein concentration. Blank values in the absence of membranes have been subtracted.

TABLE I. Casein Phosphatase Activity*

Additions	mM	Relative activity (%)
None	–	100
NaF	10	50
NaF	100	16
NaCl	100	77
DTT	4	340[a]
Mg^{+2}	10	77
Mn^{+2}	10	58
cAMP	10^{-3}–10^{-6}	100
cGMP	10^{-3}–10^{-6}	100
ATP	1	11
GTP	1	15
P	10	46

*AChR-enriched membranes containing 0.1 mg of protein were incubated in duplicate with 50 μg of ^{32}P-PO_4 casein at 37°C for 20 min in a total volume of 100 μl, containing 4 mM dithiothreitol (DTT), 0.1% Triton, and 15 mM Tris-HCl, pH 6.8. The reaction was stopped with 3 ml of an ice-cold solution containing Norit A (40 mg/ml), 0.1 M HCl, 0.2 mg/ml bovine serum albumin, 1 mM NaP_i, and 1 mM NaP_i. Releasable ^{32}P not absorbed to the charcoal was determined by measurement of Cerenkov radiation in the filtrate.

[a]Relative to a control without DTT. All other samples contained 4 mM DTT.

TABLE II. Dephosphorylation of the Membrane-Bound AChR*

Condition	A pmoles ^{32}P-PO_4 released	B ^{32}P-PO_4 in the 65,000 dalton subunit of the AChR A_{700}
Zero time control	0.57 ± 0.03	4.5
NaCl (0.1 M)	0.74 ± 0.02	2.8
NaF (0.1 M)	0.50 ± 0.03	4.2

*Membranes were initially phosphorylated as described [18]. The phosphorylated membranes were then incubated for 10 min at 37°C in either 200 mM NaCl or NaF. A: Reaction was stopped by the addition of trichloracetic acid. ^{32}P-PO_4 released was measured by extraction into isobutanol and liquid scintillation counting of the organic layer. B: Reaction was stopped by the addition of SDS and mercaptoethanol to final concentrations of 3.7% and 4%, respectively. The samples were then subjected to SDS-gel electrophoresis and autoradiography.

IDENTIFICATION OF ATP BINDING PROTEINS IN THE MEMBRANE

The purified AChR of T californica has four subunits, but only the 40,000 dalton polypeptide binds acetylcholine. The function of the other polypeptides is unknown. We reasoned that one of the other AChR subunits might have protein kinase or phosphatase activity. Therefore, we set out to determine whether subunits of the AChR or membrane polypeptides associated with the AChR are the enzymatic subunits of the protein kinase and phosphoprotein phosphatase. Since our data showed that both the kinase and phos-

phatase probably had ATP binding sites, we used arylazido-β-alanyl ATP as a photoaffinity ligand to identify these sites [23]. These experiments were done in collaboration with Drs. Ferdinand Hucho and Richard Guillory. Arylazido -β-alanyl ATP was synthesized as described by Jeng and Guillory [24], using either unlabeled ATP or α-^{32}P-ATP. The AChR-enriched membranes were incubated with the photoaffinity label and then irradiated so that the label remained covalently bound to the polypeptide containing the ATP binding sites. The polypeptides were then separated by SDS-polyacrylamide gel electrophoresis, and the dried gels autoradiographed to determine which polypeptides reacted with the ATP analog. Figure 9 shows that the AChR-enriched membranes used in this study contained about eight major polypeptides including four subunits of the AChR ($\alpha,\beta,\gamma,\delta$). After photoirradiation, the ATP photoaffinity analog, arylazido -β-alanyl ^{32}P-ATP, reacted with only three polypeptides and none of these were components of the AChR. In the absence of irradiation, there was no incorporated radioactivity (data not shown). Thus, comparison of the Coomassie blue stained gel (Fig. 9B) with the autoradiogram (Fig. 9A) of the gel shows that the affinity ligand-labeled bands migrate with molecular weights of 45,000, 55,000, and 100,000.

The specificity of binding of the photoaffinity label for ATP binding sites is shown in Figure 10. Unlabeled ATP at increasing concentrations correspondingly inhibited the

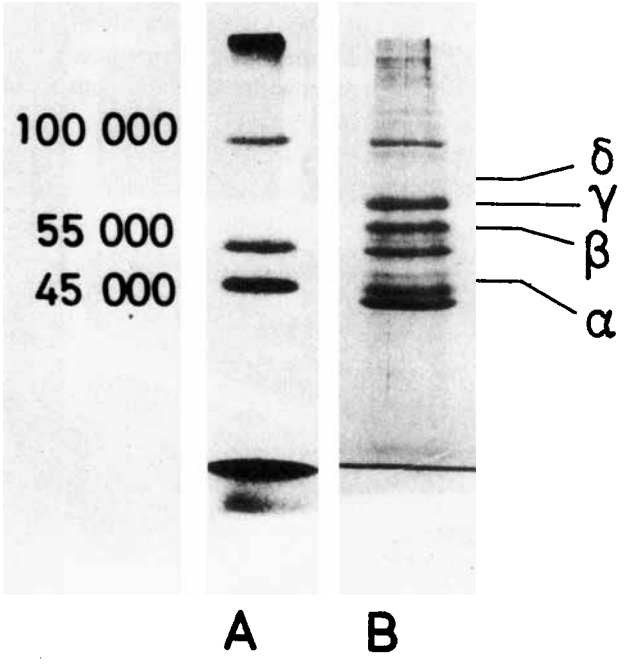

Fig. 9. Photoaffinity labeling of AChR-enriched membranes with arylazido-β-alanyl ATP. Protein (1 mg/ml) was suspended in 15 mM Tris-HCl, pH 7.5, containing 10 M ouabain and 0.225 mM arylazido-β-alanyl α-^{32}P-ATP. Irradiation was performed four times for 15 sec each at room temperature. The samples were chilled in ice following each 15 sec irradiation. SDS electrophoresis was carried out according to [18] in 7.5% acrylamide gels: (A) autoradiograph and (B) Coomassie blue-stained SDS-polyacrylamide gel.

reaction of the labeled photoaffinity compound with the membranes. On the other hand, arylazido-β-alanine, ie, the photolabel without the ATP moiety, did not cause a significant decrease in reactivity of the radioactive affinity label (data not shown). These results strongly suggest that the analog is acting as a photoaffinity probe of adenine nucleotide binding sites in AChR-enriched membranes.

The 100,000-dalton band probably is the Na-K-ATPase that has the same molecular weight [25]. This band is not a component of the AChR but is present in variable amounts in receptor-enriched membrane preparations (see Fig. 2) and does have an ATP binding site. The 45,000- and 55,000-labeled bands probably are related to the protein kinase and protein phosphatase activities present in the receptor-enriched membranes. ATP is a substrate for the protein kinase reaction; therefore, this enzyme must have an ATP binding site. As we have shown in Table I, the phosphatase is inhibited by ATP and, therefore, probably also has a specific recognition site for the nucleotide.

If the photoaffinity ATP analog reacts with protein kinase in the membrane, it would be expected to inhibit protein kinase activity. Figure 11 shows that the photoaffinity label has a striking inhibitory effect on AChR phosphorylation in receptor-enriched membranes. The membrane-bound AChR was phosphorylated in situ with γ-^{32}P-ATP as described in the figure legend. Increasing concentrations of unlabeled photoaffinity ligand progressively inhibited receptor phosphorylation. Since we know that the ATP analog does not bind to the AChR (Fig. 9), the ATP photoaffinity label must be reacting with the membrane kinase to inhibit phosphorylation of the membrane-bound AChR.

We have determined that ATP inhibits phosphatase activity in the same membrane preparation. However, because of methodologic limitations we have not been able to study the interaction of the ATP photoaffinity label with this endogenous membrane phospho-

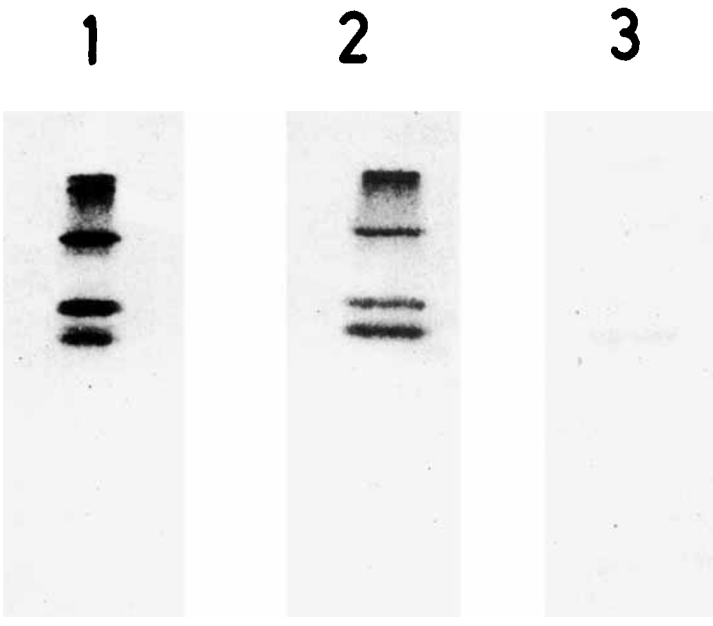

Fig. 10. Inhibition of photolabeling with ATP. Autoradiograms of SDS gels: (1) control as in Figure 9; (2) and (3) incubation mixture also contained 50 mM (2) and 100 mM ATP (3).

protein phosphatase. Therefore, we cannot assign a specific function to the 45,000- and 55,000-dalton bands labeled by the ATP photoaffinity probe. We can conclude, however, that at least one of these bands appears to be the kinase and that neither is a component of the purified AChR. We are now in the process of purifying the 45,000- and 55,000-dalton bands so that we can generate specific antibodies against these polypeptides. We anticipate that the antibodies will allow us to determine the function of each of these polypeptides. Once we establish that such antibodies inhibit specific enzyme activities, we will be able to identify the kinase and phosphatase and use the antibodies to manipulate the level of phosphorylation of the AChR in situ.

These studies open a new avenue for investigating biochemical regulatory mechanisms at the synapse. In most studies of synaptic membrane phosphorylation, the identity of the phosphorylated proteins is unknown. In our studies, however, the AChR substrate for the phosphorylation-dephosphorylation reaction is an identified protein and the function of this receptor has been well-studied in many systems. Although the specific function of the 65,000-dalton polypeptide is not known, we can now begin to correlate the level of receptor phosphorylation with receptor function. The AChR in these membranes has been well-characterized with respect to its affinity for cholinergic agonists and antagonists [16].

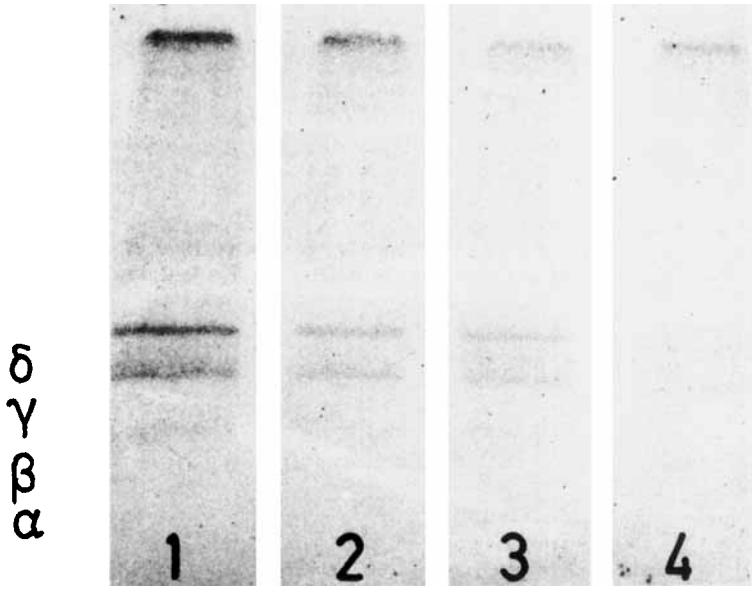

Fig. 11. Inhibition of endogenous protein kinase activity in AChR-enriched membranes by arylazido-β-alanyl ATP. Photoaffinity labeling of membranes carried out as in Figure 9 using unlabeled arylazido-β-alanyl ATP. Endogenous kinase activity was then determined [18]. Membranes containing 50 μg photoaffinity-labeled protein were incubated in 0.1 ml for 0.5 min at 0°C with 5 μm of γ-^{32}P-ATP (1–2 μCi per tube), 0.25 mM EGTA, 10 mM $MgAc_2$, 0.0625 M Tris-HCl, pH 6.8, and 100 mM KCl. The reaction was stopped by the addition of 15 μl of 20% SDS, and electrophoresis was carried out according to [18]. 1: Autoradiogram of a 7.5% SDS-acrylamide gel showing phosphorylation of the 62,000-dalton and 68,000-dalton bands of the AChR by an endogenous protein kinase. 2: Phosphorylation in the presence of 0.03 mM arylazido-β-alanyl ATP in the dark. 3: Same conditions as before with 4 × 15 sec irradiation. 4: Phosphorylation after photoaffinity labeling with 0.3 mM arylazido-β-alanyl ATP.

Changes in binding affinity (desensitization) also occur as a result of preincubation with an agonist [26]. In addition, these same preparations show agonist-induced Na^+ flux, and desensitization of this response has also been observed in vitro [27]. Using antibodies against the protein kinase and phosphatase, we plan to maintain the receptor in a given state of phosphorylation. Then, it will be possible to correlate the effects of phosphorylation and dephosphorylation of the AChR with receptor affinity for agonists as well as receptor-mediated ion fluxes.

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