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Specificity and localization of the acetylcholine receptor kinase

(membrane protein phosphorylation/hypotonic lysis/detergents/thiophosphorylation)

C. GEOFFREY DAVIS, ADRIENNE S. GORDON, AND IVAN DIAMOND

Departments of Neurology, Pediatrics, and Pharmacology, University of California, School of Medicine, San Francisco, California 94143

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ABSTRACT **Acetylcholine receptor phosphorylation has been compared in sealed and lysed right-side-out membrane vesicles prepared from *Torpedo californica* electric organ. Phosphorylation was increased 5- to 12-fold in hypotonically lysed vesicles as compared with untreated vesicles. Control experiments confirm that this enhancement is a result of increased permeability of the membrane to ATP. These data suggest that the acetylcholine receptor kinase is located on the cytoplasmic side of the plasma membrane. Results with detergent lysis support this conclusion. Although the acetylcholine receptor constitutes less than 10% of the total protein in these membranes, the kinase was found to be highly specific for polypeptides corresponding in molecular weight to acetylcholine receptor subunits.**

Protein phosphorylation is one of the most important biochemical control mechanisms for regulating the function of cytoplasmic proteins. However, little is known about the significance of membrane protein phosphorylation. This is because the identity and function of the phosphorylated membrane proteins are usually unknown and analysis of the phosphorylation reaction is often hampered by the presence of multiple kinases and phosphatases on both sides of the plasma membrane.

We have been studying phosphorylation in postsynaptic membranes from *Torpedo* electric organ in order to understand the role of phosphorylation in synaptic function. This is an ideal model system to examine the significance of membrane protein phosphorylation because these preparations contain high concentrations of the acetylcholine receptor (AcChoR). The AcChoR can be identified unequivocally with specific antibodies, and the function of the AcChoR can be studied by physiologic, pharmacologic, and anatomic techniques. We have demonstrated that at least two of the four subunits of the AcChoR are phosphorylated *in situ* by an endogenous membrane protein kinase (1).

It has been reported that the AcChoR spans the membrane (2–4). Because both external and internal protein kinases have been described and ATP is released with acetylcholine at the synapse, the receptor kinase could be located on either side of the postsynaptic membrane. Therefore, we have investigated the localization and specificity of the receptor kinase. These questions have important implications for the role of phosphorylation in receptor function.

METHODS

Sealed Right-Side-Out Vesicles. Sealed right-side-out membrane vesicles (S-R vesicles) were prepared from *Torpedo californica* electric organ (stored at −70°C) by the method of Hartig and Raftery (5). The critical and final step of their procedure is centrifugation of the homogenized membranes on a 4–20% sucrose gradient. Intact vesicles, which are impermeable to sucrose, float at the top while damaged vesicles and membrane sheets equilibrate at the membrane density. As additional evidence of membrane integrity, we also have established that vesicles prepared in 400 mM KCl retain 95% of the potassium for at least 1 hr after dilution into 400 mM NaCl (unpublished data). Using the α-bungarotoxin binding assay for the AcChoR developed by Hartig and Raftery (5), we also confirmed that >95% of the vesicles are oriented right-side-out.

AcChoR-Enriched Membranes. Receptor-enriched membranes (R-E membranes) were prepared by a modification (6) of the method of Duguid and Raftery (7). The preparation of these membranes selects for AcChoR enrichment without regard for membrane integrity.

Phosphorylation of Membranes. Membranes were incubated for 1 min at room temperature in 100 μl of 400 mM NaCl/10 mM Tris·HCl, pH 7.4/1 mM EDTA/20 mM $MnCl_2$/1.6 mM ouabain/5 μm ATP with 2 μCi of [γ-^{32}P]ATP (1 Ci = 3.7×10^{10} becquerels). Triton or digitonin was added to the incubation where indicated. In some experiments, vesicles were hypotonically lysed by adding concentrated vesicles in 400 mM NaCl/10 mM Tris·HCl, pH 7.4/1 mM EDTA to an appropriate volume of 10 mM $MnCl_2$/1.6 mM ouabain/10 mM Tris·HCl, pH 7.4/5 μM ATP with 2 μCi [γ-^{32}P]ATP and a concentration of NaCl calculated to yield the desired osmolarity. The total volume was 92 μl. NaCl, in 20 mM Tris·HCl (pH 7.4) was added immediately to restore the incubation medium to 400 mM NaCl. In some experiments, ATP was added after a 10-min delay to allow vesicles to reseal before initiating the phosphorylation reaction. The reaction was stopped by adding 50 μl of 80 mM EDTA/6% $NaDodSO_4$/3% 2-mercaptoethanol/0.005% bromphenol blue (stop solution). The samples were then electrophoresed on a $NaDodSO_4$/polyacrylamide gel by the method of Laemmli (8). The dried gels were autoradiographed on Kodak XRMat AR film.

RESULTS

If the kinase which phosphorylates the AcChoR is located on the external side of the postsynaptic membrane, the receptor should become phosphorylated with [^{32}P]phosphate after addition of [γ-^{32}P]ATP to intact vesicles. However, if the kinase is on the cytoplasmic side of the membrane, receptor labeling should occur only after the vesicles are made permeable and [γ-^{32}P]ATP becomes available to the inside kinase.

Intact Vesicles. Incubation of intact S-R vesicles with [γ-^{32}P]ATP for 1 min resulted in only trace labeling of polypeptides in the M_r range of AcChoR subunits (Fig. 1, lane B). Significant labeling of higher M_r bands was frequently observed (see Fig. 4). Changing the incubation period to 0.1 min or 20

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Abbreviations: AcChoR, acetylcholine receptor; S-R vesicles, sealed right-side-out vesicles; R-E membranes, receptor-enriched membranes.

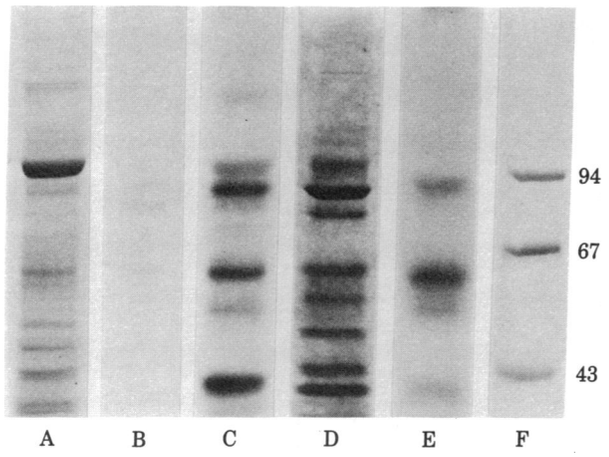

FIG. 1. Phosphorylation in S-R vesicles and R-E membranes: 50 μg of membranes were incubated with [γ-^{32}P]ATP for 1 min, at which time the reaction was stopped. The phosphorylated membranes were then applied to $NaDodSO_4$/polyacrylamide gels and electrophoresed. Lanes: A, Coomassie blue staining pattern of S-R vesicles; B, autoradiogram of untreated S-R vesicles; C, autoradiogram of S-R vesicles hypoosmotically shocked by dilution to 200 mM NaCl; D, Coomassie blue staining pattern of R-E membranes; E, autoradiogram of R-E membranes; F, molecular weight markers, shown $\times 10^{-3}$.

min did not affect the phosphorylation pattern. To test the possibility that AcChoR subunits had been rapidly phosphorylated and dephosphorylated, we used a common inhibitor of protein phosphatase activity, sodium fluoride, which we have shown inhibits AcChoR dephosphorylation (9). If rapid turnover of phosphate were occurring on the external surface of sealed vesicles, the addition of sodium fluoride to the reaction should result in greatly increased labeling. However, we found that 100 mM NaF had virtually no effect on the level of phosphorylation; it increased phosphorylation of low M_r bands by <5%. Therefore, we conclude that the low levels of phosphorylation in sealed vesicles is not due to rapid dephosphorylation.

Hypotonically Lysed Vesicles. When S-R vesicles were hypotonically lysed in the presence of 5 μM [γ-^{32}P]ATP, phosphorylation of five polypeptides of M_rs 40,000, 60,000, 65,000, 92,000, and 94,000 was greatly increased (Fig. 1, lane C). Similar results were obtained with 0.25 mM ATP. In many experiments, increased phosphorylation of a M_r 36,000 polypeptide was also observed. The specificity of the reaction in S-R vesicles was strikingly similar to the phosphorylation pattern of receptor-enriched membranes, even though the S-R preparation had many more proteins than did the R-E preparation. Indeed, with the exception of the M_r 94,000 band, the phosphorylation patterns of the two membrane preparations were identical, despite the fact that the Coomassie blue staining patterns were very different. Whereas the S-R membranes showed more than 30 polypeptides (Fig. 1, lane A), R-E membranes have only 6–8 major polypeptides (Fig. 1, lane D), and four are subunits of the AcChoR.

West and Huang (10) have reported that membrane vesicles from *Torpedo* electroplax can be made permeable to large molecules such as sucrose and inulin by osmotic shock. The degree of induced permeability is determined by the strength of the osmotic shock—i.e., the magnitude of the change in osmolarity. Consistent with their observation, we found that when S-R vesicles were hypoosmotically shocked in the presence of [γ-^{32}P]ATP, the absolute increase in phosphorylation of the four bands correlated directly with the strength of the hypoosmotic shock (Fig. 2). Vesicles in 400 mM NaCl, which were lysed by a 1:20 dilution to 20 mM NaCl, showed increases in phosphorylation of the M_r 36,000, 40,000, 60,000, and 65,000 bands of 12-, 8.6-, 10.2-, and 5.5-fold, respectively.

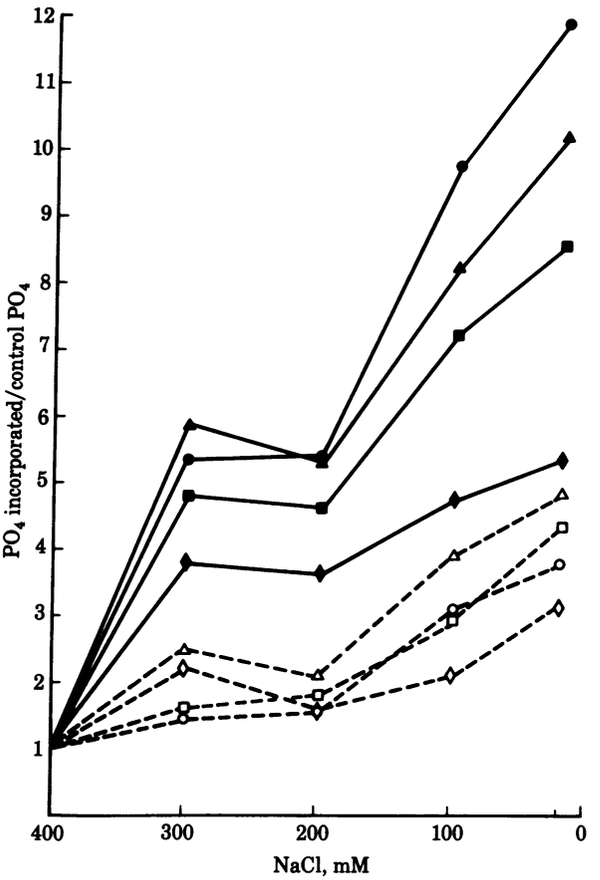

FIG. 2. Phosphorylation of S-R vesicles after hypotonic lysis and after resealing. S-R vesicles were lysed in the presence of [γ-^{32}P]ATP by dilution from 400 mM NaCl to the concentration of NaCl indicated on the abscissa and incubated for 1 min (——). The incorporation of ^{32}P into each band was measured by densitometric scanning of autoradiograms and is reported as fold enhancement over untreated vesicles. Vesicles were lysed in the absence of ATP and then resealed for 10 min; [γ-^{32}P]ATP was then added for 1 min (----). ●, M_r 36,000 band; ■, M_r 40,000 band; ▲, M_r 60,000 band; ◆, M_r 65,000 band. Each point is the mean of four experiments.

Many of the vesicles lysed in the absence of [γ-^{32}P]ATP can be resealed by incubation in 400 mM NaCl for 10 min. As expected, ATP added after resealing did not support phosphorylation to the same extent as did ATP added to the vesicles when they were lysed (Fig. 2). Moreover, the level of phosphorylation measured in resealed vesicles also appeared to correlate with the harshness of the initial shock.

It was possible that during hypotonic lysis, the kinase or substrates were released from the membrane and that solubilization was responsible for the appearance of phosphorylation. To investigate this possibility, vesicles were lysed in the presence of [γ-^{32}P]ATP and centrifuged in a Beckman Airfuge for 2 min at 100,000 $\times$ g (Fig. 3) to pellet the membranes. The supernatant and pellet were then subjected to $NaDodSO_4$ gel electrophoresis and autoradiography. There was no detectable release of protein by the hypoosmotic shock procedure (Fig. 3, lane B). Analysis of the autoradiograms revealed that >95% of the total phosphorylation remained in the pellet (Fig. 3, lane C). Phosphorylation in the supernatant was negligible and similar to that in unlysed control preparations (Fig. 3, lanes D and E). These results indicate that the substrates for the kinase remained in the membrane. However, in order to confirm further that the

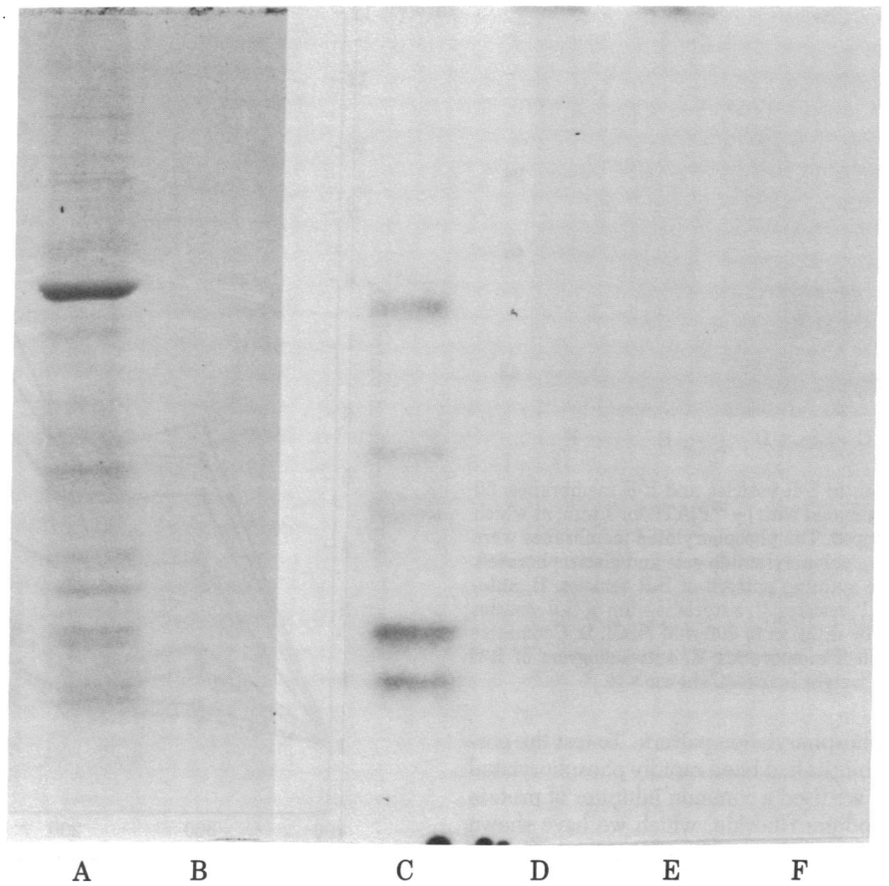

FIG. 3. Centrifugation of hypotonically lysed S-R vesicles. Lanes: A and B, Coomassie blue staining patterns of the pellet (lane A) and supernatant (lane B) from vesicles lysed by dilution into 200 mM NaCl and then centrifuged at 100,000 × *g* for 2 min; C and D, autoradiograms of the pellet (lane C) and supernatant (lane D) of vesicles lysed in the presence of [γ-^{32}P]ATP (the reaction was stopped by the addition of 20 mM EDTA before centrifugation); E, autoradiogram of the supernatant from vesicles lysed in the absence of ATP and then centrifuged ([γ-^{32}P]ATP was added to the isolated supernatant for 1 min); and F, autoradiogram of the supernatant from vesicles lysed in the absence of ATP, combined with intact vesicles, and incubated with [γ-^{32}P]ATP for 1 min.

kinase itself was not released, the supernatant from lysed vesicles was incubated with intact vesicles (Fig. 3, lane F). This procedure did not increase phosphorylation of intact vesicles.

Detergent Lysis. Because hypoosmotic shock appears to produce membrane lysis, other treatment that lyses sealed vesicles also should promote phosphorylation of the same bands. Sealed vesicles (20 μg of protein) were incubated with [γ-^{32}P]ATP and 100 mM NaF and treated with digitonin or Triton X-100 to lyse vesicles (Fig. 4 *Upper* and *Lower*, respectively). Phosphorylation of the M_r 60,000 and 65,000 bands increased with detergent concentration up to 0.02% digitonin and 0.1% Triton. Further increases in detergent concentration did not affect phosphorylation. Phosphorylation of the other four bands followed a very different pattern; the optimal concentration for phosphorylation was at 0.017% digitonin and 0.02% Triton. Further increases in detergent concentration produced a decrease in phosphorylation. These results were also observed if NaF was omitted from the reaction (data not shown). The effect of detergent on phosphorylation in AcChoR-containing vesicles was a function of the ratio of detergent to protein and not of the detergent concentration alone.

Thiophosphorylation. ATP[γ-S] can be used by protein kinases to thiophosphorylate substrate proteins (11). Thiophosphorylated proteins behave functionally as phosphorylated proteins, but the thiophosphoryl bond cannot be hydrolyzed by protein phosphatase activity (11). When ATP[γ-^{35}S] was substituted for [γ-^{32}P]ATP in the kinase reaction, no thiophosphorylation of AcChoR polypeptides was observed in intact S-R vesicles during 90 min (Fig. 5). However, if the vesicles were made permeable to ATP[γ-^{35}S] by the addition of 0.1% Triton X-100, phosphorylation of the M_r 65,000, 60,000, 40,000, and 36,000 bands continued to increase during the 90-min incubation. This result also confirms that sealed right-side-out vesicles must be lysed before ATP can gain access to the protein kinase to promote receptor phosphorylation.

DISCUSSION

Results from our laboratory (1) and other investigators (12) have indicated that the AcChoR is subject to reversible phosphorylation in the postsynaptic membrane. This suggests that phosphorylation may regulate the function of the AcChoR. If true, then the AcChoR should be the primary substrate for the phosphorylation reaction *in situ*. In our previous studies, receptor phosphorylation occurred in membranes in which the AcChoR constitutes 65% of the total membrane protein (1, 6). Therefore, selective AcChoR phosphorylation might occur because the AcChoR is the major protein component in these membranes. However, Fig. 1 shows that phosphorylation occurs in membrane vesicles in which the receptor constitutes only 10% of the membrane protein. Indeed, the AcChoR is barely detectable on Coomassie blue-stained gels. Despite the presence of many nonreceptor proteins available for phosphorylation, autoradiograms of these membranes show that the AcChoR remains the

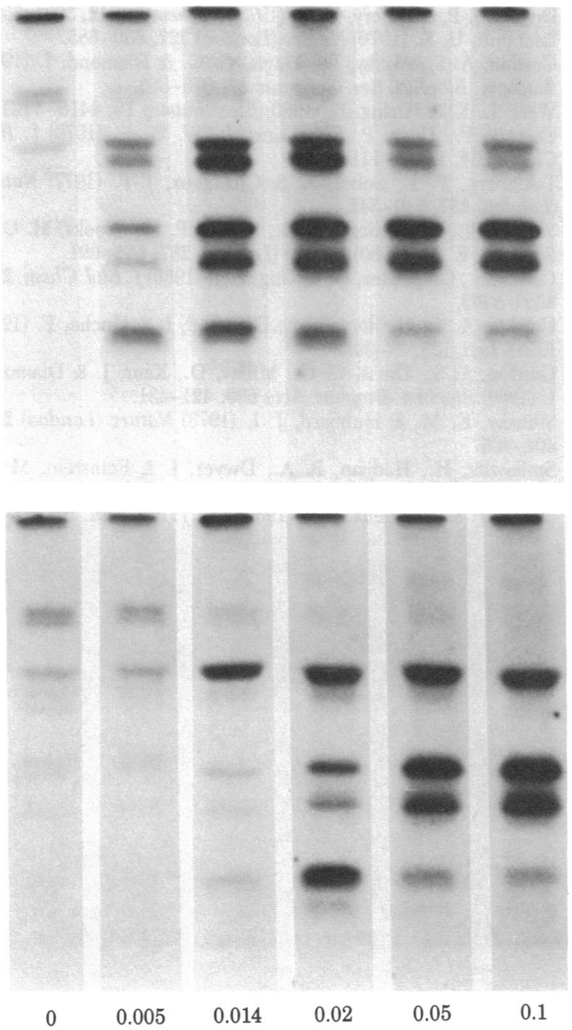

FIG. 4. Effect of detergent concentration on phosphorylation. (Upper) Membranes (20 μg) were incubated with [γ-^{32}P]ATP and various concentrations of digitonin for 1 min in the presence of 100 mM NaF. (Lower) Same as *Upper* except that Triton X-100 was used in place of digitonin.

primary substrate for the phosphorylation reaction *in situ* (Fig. 1). This indicates that the receptor kinase in postsynaptic membranes is highly specific for the membrane-bound AcChoR.

If the AcChoR itself were a protein kinase, autophosphorylation of the AcChoR could account for this high degree of specificity (13, 14). However, we already have shown that the membrane-bound AcChoR appears not to have ATP binding sites (15), and we have found that the purified AcChoR does not have protein kinase activity (unpublished observations). Therefore, although the receptor kinase is not a component of the AcChoR, it appears to be intimately associated with the AcChoR in the postsynaptic membrane.

It is now possible to investigate the molecular architecture of the receptor phosphorylation. We know that the kinase is not dependent on cAMP (6). We also know that ATP but not GTP (16) is required for the phosphorylation reaction. ATP is available in the synaptic cleft (17) and in the cytoplasm. In addition, it is known that the AcChoR spans the membrane (2–4). Therefore, the receptor kinase, which utilizes ATP, could be located on either side of the postsynaptic membrane. We investigated this question using the sealed right-side-out membrane vesicle preparation of Hartig and Raftery (5). We found that phosphorylation did not occur when sealed vesicles were incubated

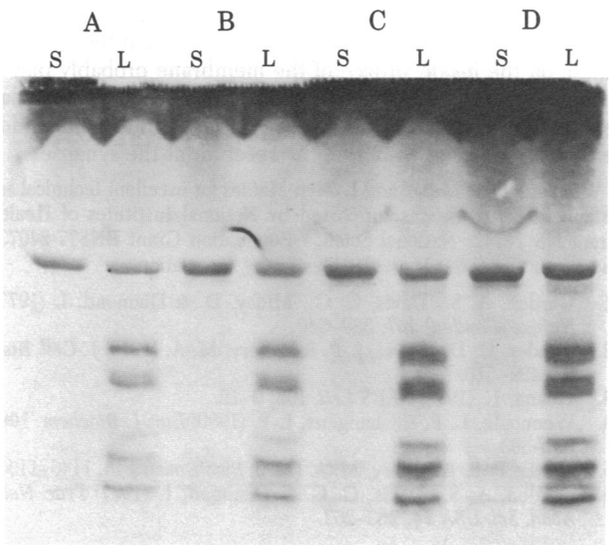

FIG. 5. Thiophosphorylation of S-R membranes. S-R membranes (50 g) were incubated at 4°C with 5 μCi of ATP[γ-^{35}S] for 15 min (lanes A), 30 min (lanes B), 60 min (lanes C), or 90 min (lanes D). S, lanes containing sealed vesicles; L, lanes containing vesicles lysed with 0.1% Triton X-100.

with ATP on the outside (Fig. 1). By contrast, when vesicles were lysed by hypoosmotic shock or exposure to low concentration of detergent, phosphorylation of the AcChoR was readily demonstrable (Figs. 1 and 4). Indeed, a variety of control experiments showed that phosphorylation of the membrane-bound AcChoR occurred only when ATP gained access to the cytoplasmic side of the membrane. These results provide strong evidence that the AcChoR kinase is restricted to the cytoplasmic surface of the postsynaptic membrane.

Other laboratories have confirmed our findings that several subunits of the AcChoR are phosphorylated (12, 18), but phosphorylation of the α subunit has not been demonstrated. Because this M_r 40,000 polypeptide contains the binding site for acetylcholine, it is important to determine whether it is phosphorylated *in situ*. When vesicles are made permeable to ATP, we observed striking phosphorylation of a M_r 40,000 polypeptide (Fig. 1). Phosphorylation of this polypeptide is extremely sensitive to experimental conditions and is much more affected by detergent concentration than are other substrates (Fig. 4). This sensitivity may explain why phosphorylation of the M_r 40,000 polypeptide is not observed consistently in different laboratories (18, 19).

Because the receptor kinase is located on the cytoplasmic side of the postsynaptic membrane, optimal conditions for studying the stoichiometry of AcChoR phosphorylation require unrestricted availability of ATP to the receptor kinase with minimal disruption of membrane integrity. However, hypotonically lysed vesicles reseal quickly, and low concentrations of detergent disrupt membrane integrity. We are now investigating the use of polypeptide ionophores to make the membranes permeable to ATP without solubilizing membrane proteins. Once optimal conditions for phosphorylation are established, it will be possible to determine whether the M_r 40,000 polypeptide is the α subunit of the AcChoR.

Covalent biochemical modification of the AcChoR by reversible phosphorylation probably regulates receptor function. A recent report by Smilowitz *et al.* suggests further that calmodulin might affect phosphorylation of the AcChoR (18). Because we have not found calmodulin stimulation in highly purified AcChoR-enriched membranes, it is possible that the

calmodulin-stimulated kinase is not the receptor kinase. In any event, it seems likely that intracellular factors and the receptor kinase on the inside surface of the membrane probably play a role in regulating the response of the AcChoR to extracellular signals. This may be related to such postsynaptic events as desensitization or localization of the receptor at the synapse.

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