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cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor

(membrane channel/calmodulin/calcium)

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ABSTRACT **Postsynaptic membranes, rich in the nicotinic acetylcholine receptor, were isolated from the electric organ of *Torpedo californica* and shown to contain a cAMP-dependent protein kinase and a calcium/calmodulin-dependent protein kinase. The cAMP-dependent protein kinase phosphorylated the γ and δ subunits of the acetylcholine receptor. The phosphorylated subunits were identified after purification of the acetylcholine receptor by affinity chromatography on a choline carboxymethyl affinity gel. In contrast, the calcium/calmodulin-dependent protein kinase phosphorylated proteins that were separated from the acetylcholine receptor by affinity chromatography. Protein kinase inhibitor, a specific inhibitor of the catalytic subunit of cAMP-dependent protein kinase, abolished the basal endogenous phosphorylation of the γ and δ subunits of the receptor. cAMP activation of the endogenous phosphorylation of the γ and δ subunits was dose dependent with a half-maximal response at 25 nM. Studies were also carried out with acetylcholine receptor purified from *T. californica* and catalytic subunit of cAMP-dependent protein kinase purified from bovine heart. The purified acetylcholine receptor was rapidly and specifically phosphorylated on the γ and δ subunits by the purified catalytic subunit of cAMP-dependent protein kinase to a stoichiometry of 1.0 and 0.89 mol of ^{32}P per mol of receptor, respectively. The initial rates of phosphorylation of the γ and δ subunits of the receptor were comparable to those of histone f2B and synapsin I (protein I), two of the most effective substrates for the catalytic subunit. Under the conditions used, the γ and δ subunits had K_m values of 4.0 and 3.3 μM and V_{max} values of 2.7 and 2.1 μmol/min per mg, respectively. The results are consistent with the idea that the acetylcholine receptor is phosphorylated *in vivo* by a cAMP-dependent protein kinase.**

Protein phosphorylation, one of the principal mechanisms of regulation of cellular metabolism, has also been suggested to play a major role in the regulation of synaptic function (1, 2). However, investigation of the physiological significance of protein phosphorylation in the regulation of synaptic transmission has been limited by a lack of knowledge of the function of most phosphoproteins in the brain. In contrast, the nicotinic acetylcholine receptor, which mediates synaptic transmission at the postsynaptic membrane of the neuromuscular junction, is well characterized both structurally and functionally (3, 4). It has also been demonstrated to be a phosphoprotein *in vivo* (5). Postsynaptic membranes, rich in the acetylcholine receptor, have been isolated in large amounts (6, 7) and the receptor has been solubilized, purified (8, 9), and reconstituted into membrane vesicles with full retention of biological activity (10, 11). The purified acetylcholine receptor is a 250-kilodalton (kDal) membrane protein (12) that consists of four subunits, α (40 kDal), β (50 kDal), γ (60 kDal), and δ (65 kDal), in the stoichiometry $\alpha_2\beta\gamma\delta$ (12). The isolated acetylcholine receptor contains 9 phosphoserines, distributed 1, 1, 2, and 5 among the α, β, γ, and δ subunits, respectively (5).

Postsynaptic membranes rich in the acetylcholine receptor contain endogenous protein kinase (13, 14) and protein phosphatase activities (14, 15). The endogenous protein kinase has been demonstrated to phosphorylate the γ and δ subunits of the receptor *in vitro* (16, 17). It has been suggested that the β (18, 19) and α (19, 20) subunits are also phosphorylated *in vitro*. However, it has been difficult to identify the factor(s) responsible for the regulation of this endogenous phosphorylation. The phosphorylation of the receptor has been reported to be independent of cAMP (13, 18, 19), cGMP (13, 18), calcium (18), and calcium/calmodulin (20). Recently, it was reported that phosphorylation of the β, γ, and δ subunits and possibly of the α subunit of the receptor is regulated by calcium/calmodulin (19).

In view of the potential physiological significance of phosphorylation of the acetylcholine receptor, we have carried out a detailed investigation of the regulation of this phosphorylation reaction. For this purpose, we have studied endogenous phosphorylation in membrane preparations rich in the acetylcholine receptor, as well as phosphorylation of purified acetylcholine receptor by purified protein kinases.

MATERIALS AND METHODS

All procedures were performed at 0–4°C unless otherwise indicated.

Materials. Live *Torpedo californica* were obtained from Pacific Biomarine Laboratories (Venice, CA). Nonidet P-40 (NP-40), cAMP, and cGMP were obtained from Sigma. Histone f2B was obtained from Worthington. ^{125}I-Labeled α-bungarotoxin was obtained from New England Nuclear. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (21). cGMP-dependent protein kinase was purified from bovine lung by a modification of the procedure of Walter *et al.* (22). Calcium/calmodulin-dependent protein kinase was partially purified from rat brain by a modification of the procedure of Kennedy *et al.* (23). Calmodulin was purified from rabbit brain according to Grand *et al.* (24). Synapsin I (protein I) was purified from bovine brain by a modification of the procedure of Ueda and Greengard (25). Protein kinase inhibitor was purified from rabbit skeletal muscle by a modification of the procedure of McPherson *et al.* (26).

Preparation of Postsynaptic Membranes. Postsynaptic membranes rich in the acetylcholine receptor were prepared from freshly dissected electric organ of *T. californica* by a modification (11) of the method of Sobel *et al.* (27), except that the minced organs were homogenized for 30 sec in a Waring blender

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Abbreviations: kDal, kilodalton; NP-40, Nonidet P-40; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

in 20 mM Tris·HCl, pH 7.4/5 mM EDTA/5 mM EGTA/1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$)/10 mM 2-mercaptoethanol, containing pepstatin at 10 μg/ml, Trasylol at 20 units/ml, leupeptin at 10 μg/ml, and antipain at 10 μg/ml. The material at the interface between 35% and 37.5% sucrose of the discontinuous sucrose gradient was used for all experiments and had an α-bungarotoxin-binding specific activity (28) of 1.0–1.5 nmol/mg of protein.

Solubilization of the Postsynaptic Membranes. The postsynaptic membrane preparation was isolated as sealed right-side-out vesicles (29, 30) which are impermeable to hydrophilic molecules. The membranes therefore were solubilized with NP-40 in order to allow free access of added [^{32}P]ATP, cAMP, protein kinase inhibitor, and calmodulin to the protein kinases on the cytoplasmic side of the membrane (20). The membranes were solubilized by incubation with 1% NP-40 in 20 mM Tris·HCl, pH 7.4/0.1 mM EGTA/0.1 mM EDTA/10 mM 2-mercaptoethanol at a protein concentration of 2.5 mg/ml. After 30 min the extract was centrifuged at 150,000 × *g* for 20 min, the pellet was discarded, and the supernatant was used for all experiments.

Purification of the Acetylcholine Receptor. The acetylcholine receptor used for the experiments with exogenous protein kinase was purified from a Na cholate extract of the postsynaptic membranes by affinity chromatography on a choline carboxymethyl affinity gel as described by Huganir and Racker (11). The purified acetylcholine receptor preparations had an α-bungarotoxin-binding activity of 8–10 nmol/mg of protein.

Phosphorylation Assays. Endogenous phosphorylation was measured by incubating 20–30 μg of solubilized membrane protein in 20 mM Tris·HCl, pH 7.4/20 mM $MgCl_2$/1 mM ouabain/0.1 mM EGTA/10 mM 2-mercaptoethanol/0.5% NP-40/2 μM [γ-^{32}P]ATP (≈ 10^4 cpm/pmol) in a final volume of 50 μl. The reaction mixture was preincubated for 5 min at room temperature and the reaction was initiated by addition of the ATP. The assay was stopped at 30 sec by the addition of 60 μl of $NaDodSO_4$ sample buffer [5% $NaDodSO_4$/156 mM Tris·HCl, pH 6.8/25% (vol/vol) glycerol/12.5% (vol/vol) 2-mercaptoethanol/0.05% bromophenol blue]. The samples were then subjected to electrophoresis on $NaDodSO_4$/polyacrylamide gels (10%) as described by Laemmli (31). The gels were stained with Coomassie blue, dried, and autoradiographed with Kodak X-Omat film. The incorporation of ^{32}P was quantitated by cutting out the appropriate gel pieces and assaying them directly in Liquiscint.

The phosphorylation of substrates by exogenous catalytic subunit was measured by incubating the various protein substrates in 20 mM Tris·HCl, pH 7.4/20 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/10 mM 2-mercaptoethanol/0.1% NP-40/100 μM [γ-^{32}P]ATP (10^2 cpm/pmol) with 5 nM purified catalytic subunit of cAMP-dependent protein kinase in a final volume of 50 μl. The final concentrations of the purified acetylcholine receptor, histone f2B, and Synapsin I (Ia plus Ib) were 0.4 μM. The assays were initiated and stopped and the phosphoproteins were analyzed as described above.

Protein Determinations. Protein concentrations were measured by a modification (32) of the procedure of Lowry *et al.* (33) with bovine serum albumin as standard.

RESULTS

Endogenous cAMP-Dependent and Calcium/Calmodulin-Dependent Protein Phosphorylation. The postsynaptic membrane preparation from the electric organ of *T. californica* contained seven major polypeptide bands, 40, 43, 50, 55, 60, 65, and 95 kDal (Fig. 1). Four of these polypeptide bands comigrated on $NaDodSO_4$/polyacrylamide gels with the α (40 kDal),

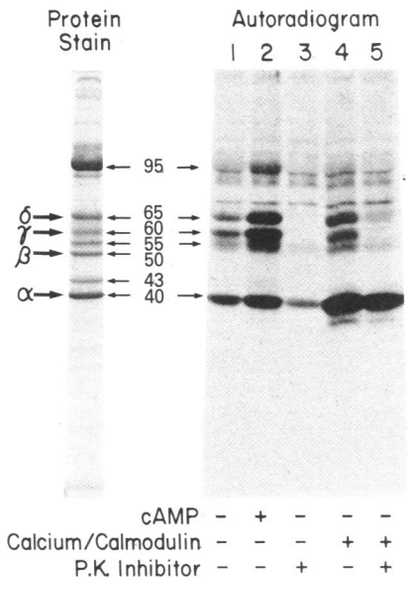

FIG. 1. Endogenous cAMP-dependent and calcium/calmodulin-dependent protein phosphorylation of solubilized postsynaptic membranes rich in the acetylcholine receptor. Endogenous protein phosphorylation was carried out in the absence or presence of 1 μM cAMP, 1 μM protein kinase (P.K.) inhibitor, and 0.5 mM $CaCl_2$/1.8 μM calmodulin, as indicated. Size markers are in kDal.

β (50 kDal), γ (60 kDal), and δ (65 kDal) subunits of the purified acetylcholine receptor. When the postsynaptic membrane preparation was incubated in the presence of [γ-^{32}P]ATP and $MgCl_2$ under basal conditions, five major phosphoprotein bands, 40, 55, 60, 65, and 95 kDal, were observed (Fig. 1, lane 1). Three of these phosphoprotein bands comigrated with the α, γ, and δ subunits of the purified acetylcholine receptor.

When 1 μM cAMP was included in the reaction mixture, a consistent 2- to 3-fold stimulation of the incorporation of ^{32}P into all five major phosphoprotein bands was seen (Fig. 1, lane 2). When protein kinase inhibitor, a specific inhibitor of the catalytic subunit of cAMP-dependent protein kinase (26), was added to the reaction mixture, the phosphorylation of the 60- and 65-kDal proteins was completely inhibited and the phosphorylation of the 40-, 55-, and 95-kDal proteins was partially inhibited (Fig. 1, lane 3).

The addition of calcium/calmodulin caused a small stimulation of the phosphorylation of the 40-, 55-, 60-, and 65-kDal bands, both in the absence (Fig. 1, lane 4) and presence (Fig. 1, lane 5) of protein kinase inhibitor. In other experiments using cruder fractions of membranes from the discontinuous sucrose gradient, the calcium/calmodulin stimulation of these bands was more dramatic. The calcium/calmodulin stimulation of the phosphorylation of these proteins was most dramatic (>10-fold) in the cytosol fraction (H. C. Palfrey and J. E. Rothlein, personal communication; data not shown).

The following information concerning the components of the membrane preparation rich in the acetylcholine receptor can be deduced from the results shown in Fig. 1. The stimulation of phosphorylation by cAMP indicates the presence of the holoenzyme of cAMP-dependent protein kinase, and the inhibition of the basal phosphorylation by the protein kinase inhibitor indicates the presence of free catalytic subunit of the cAMP-dependent protein kinase. The stimulation of phosphorylation by calcium/calmodulin indicates the presence of a calcium/calmodulin-dependent protein kinase.

Separation of the Phosphorylated Acetylcholine Receptor from Nonreceptor Phosphoproteins. For further study of the

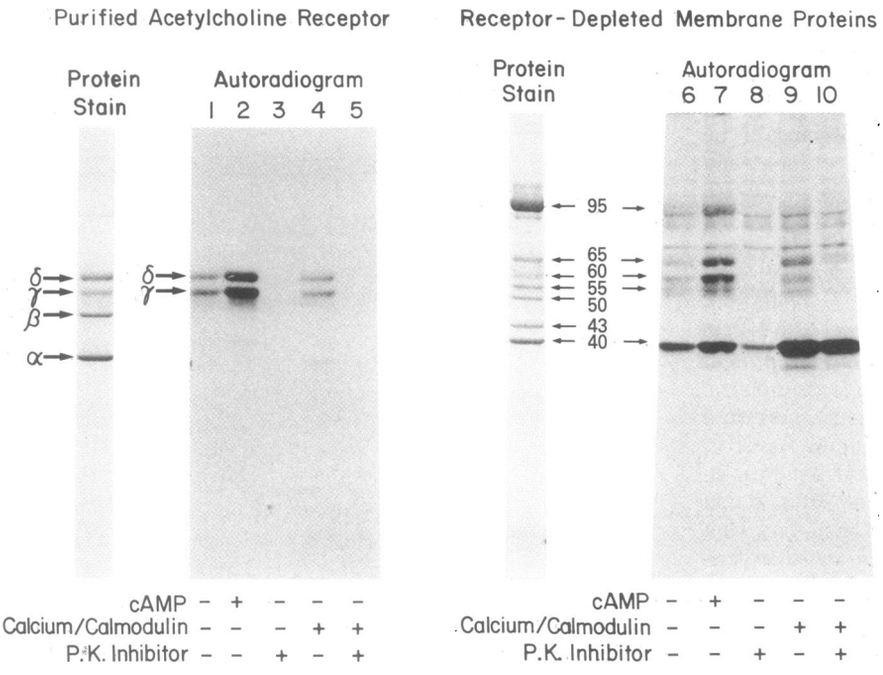

FIG. 2. Separation of the purified acetylcholine receptor phosphoprotein (*Left*) from nonreceptor phosphoprotein (*Right*) after endogenous cAMP-dependent and calcium/calmodulin-dependent phosphorylation of solubilized postsynaptic membranes. Endogenous phosphorylation was carried out in the absence or presence of 1 μM cAMP, 1 μM protein kinase (P.K.) inhibitor, or 0.5 mM $CaCl_2$/1.8 μM calmodulin, as indicated. The final volume was 200 μl and the reaction was stopped by the addition of 20 μl of 0.25 mM EDTA/10 mM ATP/0.5 M NaF. The phosphorylated proteins were then incubated for 3 hr with 0.1 ml of choline carboxymethyl affinity gel. The gel was centrifuged for 30 sec in a Beckman Microfuge. Aliquots of the supernatant (*Right*; receptor-depleted membrane proteins) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The affinity gel was washed twice with 1.0 ml of 20 mM Tris·HCl, pH 7.4/0.1 mM EGTA/0.1 mM EDTA/10 mM 2-mercaptoethanol/100 mM NaCl/0.1% NP-40 and eluted with $NaDodSO_4$ sample buffer. Aliquots of this eluate (*Left*; purified acetylcholine receptor) were analyzed by $NaDodSO_4$/polyacrylamide electrophoresis. Size markers are in kDal.

phosphoproteins present in the solubilized postsynaptic membrane preparation, the phosphorylation reaction was stopped under nondenaturing conditions by the addition of EDTA, unlabeled ATP, and NaF. The preparation was then incubated with a choline carboxymethyl affinity gel (11) in order to separate the acetylcholine receptor from nonreceptor polypeptides. Under the experimental conditions used, 60–70% of the acetylcholine receptor was specifically adsorbed to the gel. The purified receptor and the receptor-depleted membrane proteins were then analyzed on $NaDodSO_4$/polyacrylamide gels for phosphoprotein content. Only the 60- and 65-kDal phosphoproteins copurified with the acetylcholine receptor; all other phosphoproteins eluted with the receptor-depleted membrane proteins, indicating that only the γ and δ subunits of the receptor were phosphorylated (Fig. 2). This experiment also demonstrated that the phosphorylation of the γ and δ subunits was stimulated by cAMP and was inhibited by protein kinase inhibitor. The cAMP-stimulated phosphorylation of the 60- and 65-kDal protein bands seen in the receptor-depleted membrane preparation is attributable to the γ and δ subunits of nonadsorbed acetylcholine receptor (data not shown). None of the substrates for the calcium/calmodulin-dependent protein kinase copurified with the receptor; instead, all of them eluted quantitatively (data not shown) with the receptor-depleted membrane proteins.

Regulation by cAMP of Endogenous Phosphorylation of the γ and δ Subunits of the Acetylcholine Receptor. cAMP increased both the initial rate and the final level of phosphorylation of the γ and δ subunits of the acetylcholine receptor (Fig. 3). At longer times, a slight decrease in phosphorylation of the γ and δ subunits was observed, suggesting the presence of protein phosphatase activity in the membrane preparation (14, 15). Protein kinase inhibitor, but not trifluoperazine, abolished the phosphorylation under control conditions (i.e., in the absence of cAMP and calcium), indicating that the background phosphorylation was due to the free catalytic subunit of cAMP-dependent protein kinase.

Stimulation of the endogenous phosphorylation of the γ and δ subunits as a function of cAMP concentration is shown in Fig. 4. cAMP produced up to a 3-fold stimulation over basal values with a half-maximal effect at 25 nM. cGMP was less effective than cAMP, stimulating phosphorylation only 2-fold with a half-maximal effect at 1 μM (not shown).

Phosphorylation of the Purified Acetylcholine Receptor by Exogenous Catalytic Subunit of cAMP-Dependent Protein Kinase. Studies with purified catalytic subunit of cAMP-dependent protein kinase from bovine heart and purified acetylcholine receptor from *T. californica* confirmed that only the γ and δ subunits of the receptor were phosphorylated by this en-

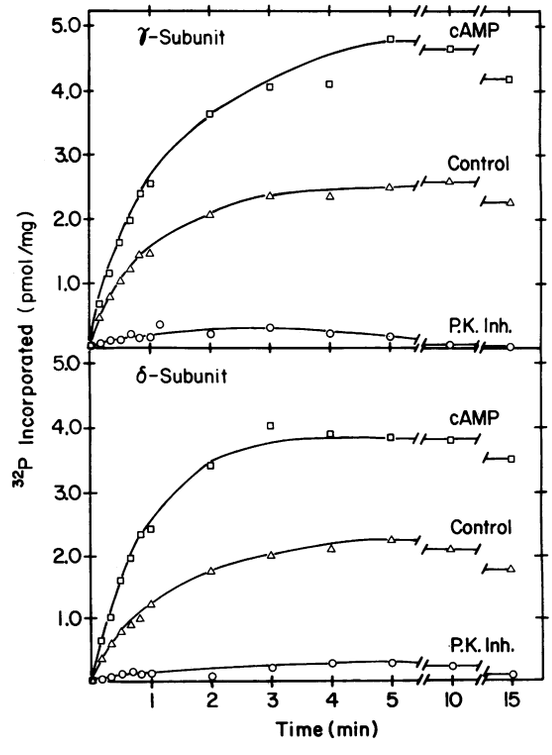

FIG. 3. Time course of endogenous phosphorylation of the γ and δ subunits of the acetylcholine receptor. Solubilized postsynaptic membranes rich in the acetylcholine receptor were subjected to endogenous phosphorylation with the assay time varied as indicated. Phosphorylation was carried out in the absence (control) or presence of 1 μM cAMP or 1 μM protein kinase inhibitor (P.K. Inh.), as indicated.

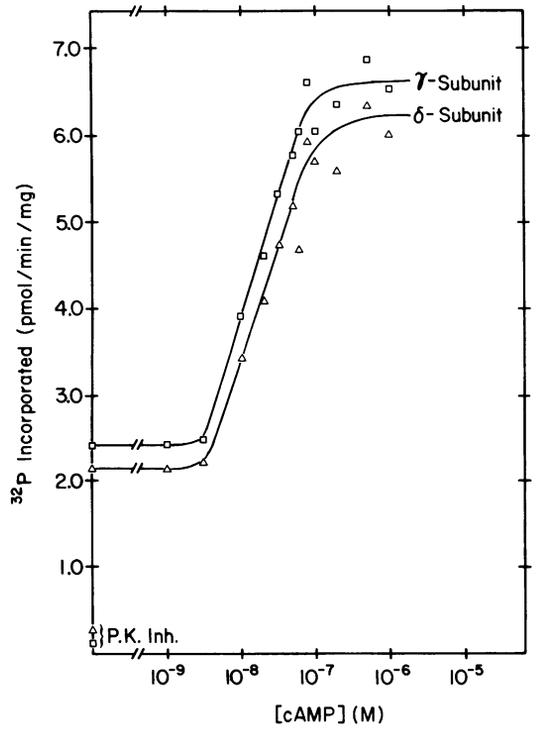

FIG. 4. Effect of various concentrations of cAMP on the endogenous phosphorylation of the γ and δ subunits of the acetylcholine receptor. Solubilized postsynaptic membranes were subjected to endogenous phosphorylation in the presence of the indicated concentrations of cAMP or 1 μM protein kinase inhibitor (P.K. Inh.).

zyme. Fig. 5 compares the rates of phosphorylation of the γ and δ subunits of the receptor to the rates of phosphorylation of histone f2B and synapsin I, two of the most effective substrates for the catalytic subunit. Under identical conditions the γ and δ subunits were phosphorylated at similar rates, which were slightly faster than the rate of phosphorylation of histone f2B and somewhat slower than the rate of phosphorylation of synapsin I. All of the substrates were phosphorylated to a high degree (≈0.6 mol of ^{32}P per mol of substrate). In other experiments, with a 10-fold higher concentration of the catalytic subunit, values of 1.0 and 0.89 mol of ^{32}P per mol of receptor were obtained for the γ and δ subunits, respectively.

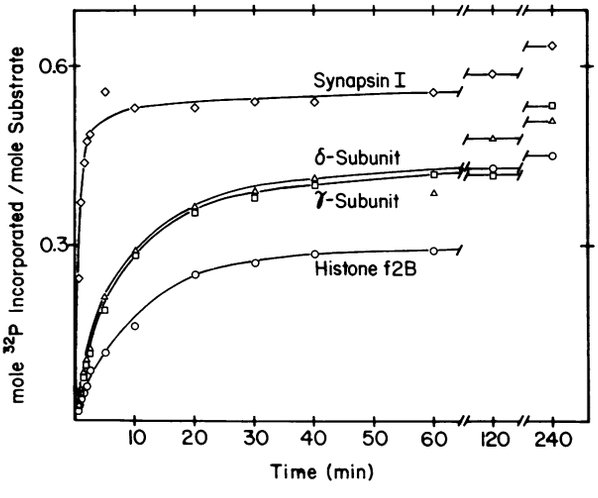

FIG. 5. Rates of phosphorylation of the γ and δ subunits of the purified acetylcholine receptor, of histone f2B, and of synapsin I by added catalytic subunit of cAMP-dependent protein kinase.

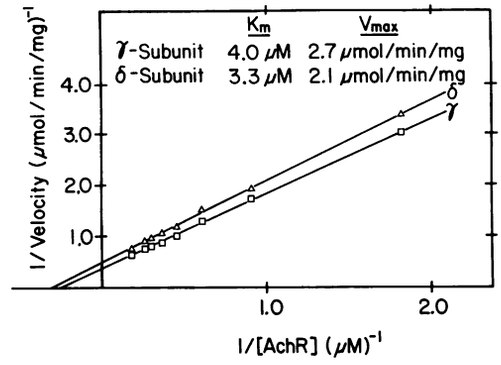

FIG. 6. Double-reciprocal plots of the effect of various concentrations of acetylcholine receptor on the initial rate of phosphorylation of the γ and δ subunits of the receptor. Concentration of receptor was varied as indicated, and the assay time was 1 min. Reactions were linear with respect to incubation time and to amount of enzyme.

The effect of various concentrations of the acetylcholine receptor on the initial rate of phosphorylation of the γ and δ subunits is shown in Fig. 6. When these data were plotted as double reciprocal plots, a linear relationship was observed between the initial rate of phosphorylation and the concentration of γ and δ subunits. The kinetic properties of the γ and δ subunits were similar, with K_m values of 4.0 and 3.3 μM and V_{max} values of 2.7 and 2.1 μmol/min per mg, respectively. Under the experimental conditions used (0.1% NP-40, 20°C, and pH 7.4), a V_{max} of only 1.5 μmol/min per mg was obtained with histone f2B compared to the value of 16.9 μmol/min per mg under optimal conditions (34). Thus, the V_{max} values for the γ and δ subunits can be estimated to be about 20–30 μmol/min per mg under optimal conditions. The similarity in the kinetic constants for the γ and δ subunits suggests that the regions surrounding the phosphorylation sites on the two subunits may be similar, an interpretation consistent with the known high degree of amino acid homology between the subunits of the receptor (35).

In support of the specificity of phosphorylation of the receptor by the catalytic subunit of cAMP-dependent protein kinase, purified cGMP-dependent protein kinase from bovine lung phosphorylated the γ and δ subunits of the receptor at a rate 1/100th that of the catalytic subunit. In addition, no phosphorylation of the receptor was detected in the presence of partially purified calcium/calmodulin-dependent protein kinase from either *Torpedo* (H. C. Palfrey and J. E. Rothlein, personal communication) or rat brain (data not shown).

DISCUSSION

It is demonstrated in this paper that the endogenous phosphorylation of the nicotinic acetylcholine receptor is mediated by cAMP-dependent protein kinase. The inability of previous investigators (13, 18, 19) to demonstrate an effect of cAMP on endogenous phosphorylation of the receptor may have been due to proteolysis of the regulatory subunit of cAMP-dependent protein kinase (36) by the large amounts of proteases present in *Torpedo* tissue (37, 38) or to the high level of background phosphorylation.

It is also demonstrated in this paper that the postsynaptic membrane preparation from *T. californica* contains a calcium/calmodulin-dependent protein kinase activity that phosphorylates endogenous substrate proteins. Our data indicate that these phosphoproteins are not the subunits of the acetylcholine receptor although they comigrate with the receptor subunits on $NaDodSO_4$/polyacrylamide gels. This conclusion supports the results of Palfrey and Rothlein (personal communication) and

Raphaeli and Parsons (39), who have recently demonstrated calcium/calmodulin-dependent phosphorylation of endogenous substrates in *Torpedo* electric organ. These substrates were distinct from the acetylcholine receptor but comigrated with the receptor subunits on $NaDodSO_4$/polyacrylamide gels. The occurrence of such substrates may explain the results of Smilowitz *et al.* (19), who reported that the phosphorylation of the β, γ, and δ subunits and possibly of the α subunit of the receptor is regulated by calcium/calmodulin.

In addition, the purified acetylcholine receptor is shown to be rapidly and specifically phosphorylated on the γ and δ subunits by purified catalytic subunit to final values of 1.0 and 0.89 mol of ^{32}P per mol, respectively. The K_m and V_{max} values for the phosphorylation of the γ and δ subunits are comparable to the kinetic constants of the most effective substrates for the catalytic subunit. These data, together with the demonstration that the endogenous phosphorylation *in vitro* is mediated by cAMP-dependent protein kinase, strongly suggest that the *in vivo* phosphorylation of the receptor is catalyzed by cAMP-dependent protein kinase.

Although it has been demonstrated that the nicotinic acetylcholine receptor is phosphorylated *in vitro* (refs. 16 and 17; this paper) and *in vivo* (5), the effects of this phosphorylation on the function of the acetylcholine receptor are not known. It is clear that the phosphorylation/dephosphorylation reaction does not mediate the opening and closing of the channel because the purified acetylcholine receptor is biologically functional (11) even though it has no protein kinase activity (data not shown). However, phosphorylation of the receptor may modulate some aspect of the channel activity of the receptor. For instance, the junctional form of the receptor has a mean channel-open time 3–5 times more rapid than the extrajunctional form (40, 41), and this property may be attributable to the state of phosphorylation of the receptor (42). Alternatively, desensitization of the channel may be modulated by phosphorylation. It will be interesting to compare the phosphorylated and dephosphorylated forms by using the patch-clamp technique to study the single-channel kinetics of the reconstituted receptor [unpublished results in association with D. Tank and W. Webb].

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