Biochimica et Biophysica Acta, 770 (1984) 225–229
Elsevier

BBA Report

BBA 70148

cAMP, NOT Ca^{2+}/CALMODULIN, REGULATES THE PHOSPHORYLATION OF ACETYLCHOLINE RECEPTOR IN *TORPEDO CALIFORNICA* ELECTROPLAX

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(Received December 12th, 1983)

Key words: Phosphorylation; Calcium/calmodulin; cyclic AMP dependence; Protein kinase; Acetylcholine receptor; (T. californica electroplax)

The regulation of the phosphorylation of the acetylcholine receptor in electroplax membranes from *Torpedo californica* and of purified acetylcholine receptor was investigated. The phosphorylation of the membrane-bound acetylcholine receptor was not stimulated by Ca^{2+}/calmodulin, nor was it inhibited by EGTA, but it was stimulated by the catalytic subunit of cAMP-dependent protein kinase, and was blocked by the protein inhibitor of cAMP-dependent protein kinase. Purified acetylcholine receptor was not phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase activity in electroplax membranes, nor by partially purified Ca^{2+}/calmodulin-dependent protein kinases from soluble or particulate fractions from the electroplax. Of the four acetylcholine receptor subunits, termed α, β, γ and δ, only the γ- and δ-subunits were phosphorylated by the cAMP-dependent protein kinase (+cAMP), or by its purified catalytic subunits.

Acetylcholine receptor from the electroplax of *Torpedo californica* is a phosphoprotein [1] composed of four polypeptide subunits termed α, β, γ and δ ($M_r = 40000$, 50000, 60000 and 65000, respectively) that are present in the stoichiometric ratio of $\alpha_2\beta_1\gamma_1\delta_1$ [2]. We previously reported that the γ- and δ-subunits, and perhaps the β-subunit of the acetylcholine receptor was phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase activity present in *T. californica* electroplax particulate fractions [3]. This conclusion was based in part on the identical migration of several ^{32}P-labelled polypeptides with polypeptides of purified acetylcholine receptor on SDS-polyacrylamide gel electrophoresis. Subsequent attempts on our part to further characterize the system failed to verify the original finding that acetylcholine receptor phosphorylation was dependent upon a Ca^{2+}/calmodulin-requiring enzyme. Instead, we found that acetylcholine receptor phosphorylation is due to cAMP-dependent protein kinase and that only the γ- and δ-subunits are phosphorylated. While this work was in progress Huganir and Greengard [4] showed that the γ- and δ-subunits of acetylcholine receptor were phosphorylated by a cAMP-dependent protein kinase, and not by a Ca^{2+}/calmodulin-dependent protein kinase. Our present results are in complete agreement with their findings.

This work was reported in part at the annual meeting of the American Society of Biological Chemists (June 5–9, 1983) in San Francisco, CA; (1983) Fed. Proc. 42, 2024.

Four lines of evidence suggested that we re-evaluate both the role of cAMP in the electroplax,

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

and our hypothesis that acetylcholine receptor phosphorylation is regulated by Ca^{2+}/calmodulin.

(1) Fluoride was present in many of our assays because it stimulates endogenous protein phosphorylation of particulate fractions ** [3]. We found that this effect of F^- was primarily due to inhibition of ATPase activity that otherwise would have rapidly destroyed ATP required for the phosphorylation reaction. However, F^- also stimulates adenylate cyclase. Adenylate cyclase activity in two different electroplax particulate fractions, measured by the method of Salomon [6], was stimulated by fluoride (20 mM) 6- and 21-fold; i.e., from 12.9 ($\pm$2.3 S.D.) to 77.6 ($\pm$1.1) and from 8.1 ($\pm$3.9) to 167.0 ($\pm$3.8) pmol cAMP/mg protein. The presence of Ca^{2+} (total concentration = 0.8 mM) and calmodulin (1 μM) had no effect. The presence of adenylate cyclase suggested a role for cAMP-dependent phosphorylation in the particulate fractions. The concentration of cAMP attained was estimated to be 15 and 35 nM, sufficient to stimulate cAMP-dependent protein phosphorylation in electroplax membranes [4].

(2) Synapsin 1 is a protein from brain that is known to be phosphorylated at two distinct sites by Ca^{2+}/calmodulin- and cAMP-dependent protein kinases, respectively. These sites can be resolved by partial proteolysis with *Staphylococcus aureus* V8 protease followed by SDS-polyacrylamide gel electrophoresis [7]. A 30 kDa polypeptide fragment is phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase, whereas a 10 kDa polypeptide fragment is phosphorylated by a cAMP-dependent and by a Ca^{2+}-dependent protein kinase [7]. We purified synapsin 1 from calf brain [8,9] and used it as a test substrate for protein kinases in electroplax particulate fractions. Both polypeptide fragments were phosphorylated. The phosphorylation of the 30 kDa fragment was stimulated by Ca^{2+}/calmodulin and inhibited by EGTA. The phosphorylation of the 10 kDa fragment was blocked by the cAMP-dependent protein kinase inhibitor, and unaffected by trifluoperazine or EGTA. These experiments showed that both Ca^{2+}/calmodulin- and cAMP-dependent protein kinases were present in the particulate fraction.

(3) Acetylcholine receptor that had been purified by affinity chromatography [10] was added to particulate fractions with [γ-^{32}P]ATP. This exogenous, solubilized acetylcholine receptor added to the assay was subsequently separated from most of the particulate fraction proteins by centrifugation and then analyzed by SDS-polyacrylamide gel electrophoresis. Phosphorylated γ- and δ-subunits of acetylcholine receptor were recovered in the supernatants. Corresponding polypeptides were not found in the supernatants of particulate fractions incubated with Triton X-100 but without purified acetylcholine receptor. There was no

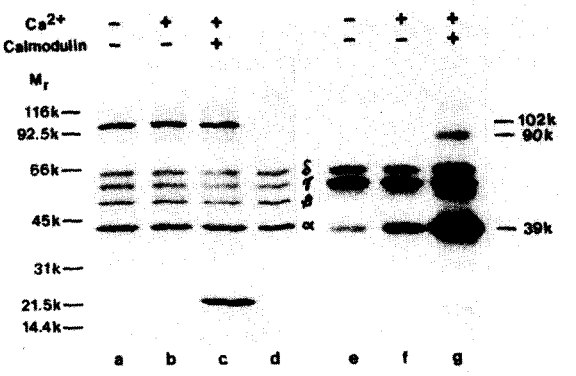


Fig. 1. Phosphorylation of exogenous, purified acetylcholine receptor by protein kinase activity present in the particulate fraction of *T. californica*. Particulate fractions (26 μg total protein) containing approx. 2.5% acetylcholine receptor were incubated for 2 h with [γ-^{32}P]ATP (50 μM, 0.2 μCi/nmol), 20 mM K_2 Pipes, pH 7.0, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ouabain, 100 mM vanadate, 20 mM KF, 1 mM EGTA and purified acetylcholine receptor (22 pmol) solubilized in 0.2% Triton X-100. The final concentration of Triton X-100 was 0.05% which did not solubilized membrane-bound acetylcholine receptor. The samples were centrifuged in a Beckman Airfuge (5 min at 30 lb/inch^2) and the supernatants and pellets analyzed by SDS-polyacrylamide gel electrophoresis (5–15% acrylamide gradient). The gels were stained with Coomassie blue, dried and autoradiographed. Only the supernatants are shown here. Additions of Ca^{2+} (free Ca^{2+} = 163 μM) and calmodulin (1.5 μM) are as indicated. Lanes a–d are Coomassie blue stains, lanes e–g are autoradiographs of lanes a–c. Lane d is the purified acetylcholine receptor standard. The M_r = 102 000 protein in lanes a–c and the phosphorylated M_r = 90000 and 39000 polypeptides in lanes e–g are solubilized from the particulate fraction by 0.05% Triton X-100.

** Particulate fractions used in this work were prepared either by the method we previously described (1.25–5% of the total protein is acetylcholine receptor) [3], or by the method of Elliott et al. [5], which fractions contained more acetylcholine receptor (15–25% of total protein).

stimulation of phosphorylation of the acetylcholine receptor subunits by added Ca^{2+} or Ca^{2+}/calmodulin (Fig. 1). This experiment strongly suggested that phosphorylation of acetylcholine receptor subunits did not involve a Ca^{2+}/calmodulin-dependent protein kinase.

(4) Ca^{2+}/calmodulin dependent protein kinase activity was partially purified from both the particulate and soluble fractions of electroplax homogenates. Samples of particulate fractions were adjusted to pH 11.0 with KOH and centrifuged at 30000 rpm for 1 h in a type 65 Beckman rotor. The supernatant was adjusted to pH 7.5 with HCl, frozen at −80°C, then thawed and recentrifuged as above to remove precipitated material and finally applied to a calmodulin-Sepharose column. The kinase activity that was eluted contained three major polypeptides (M_r = 62000, 53000 and 39000–40000) which were all phosphorylated in the presence of ATP and Ca^{2+}/calmodulin. When this kinase-containing fraction was incubated with purified acetylcholine receptor only the polypeptides (analyzed by SDS-polyacrylamide gel electrophoresis, data not shown) from the enzyme fraction were phosphorylated. In contrast, this enzyme activity could readily phosphorylate synapsin 1.

Samples of soluble fractions were loaded on a DEAE-cellulose column (Whatman DE 52) and eluted with a 0–400 mM KCl gradient in 20 mM Tris, pH 7.5, 2.5 mM dithiothreitol, 1 mM EGTA and 10 μM leupeptin and pepstatin. The kinase activity (detected between 125 and 275 mM KCl) was pooled, concentrated by ultracentrifugation and dialyzed against the above buffer. The concentrate was loaded on a Sephadex G-200 column and eluted with 10 mM K_2 Pipes, pH 7.0, and 5 mM dithiothreitol. The kinase activity eluted with the void volume. This protein kinase readily phosphorylated synapsin 1 (only in the M_r = 30000 polypeptide fragment) and myelin basic protein only in the presence of Ca^{2+}/calmodulin. Purified acetylcholine receptor and phosphorylase *b* were not phosphorylated under any conditions. The partially purified protein kinase described here is similar to a Ca^{2+}/calmodulin-dependent protein kinase purified from the soluble fraction of *T. californica* by Palfrey et al. [11].

In view of these findings we re-assessed the role

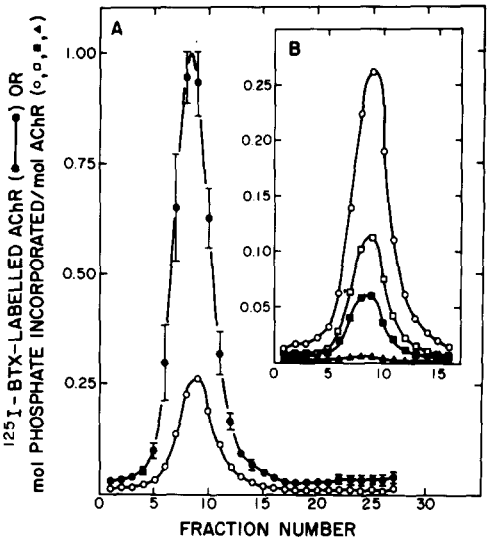
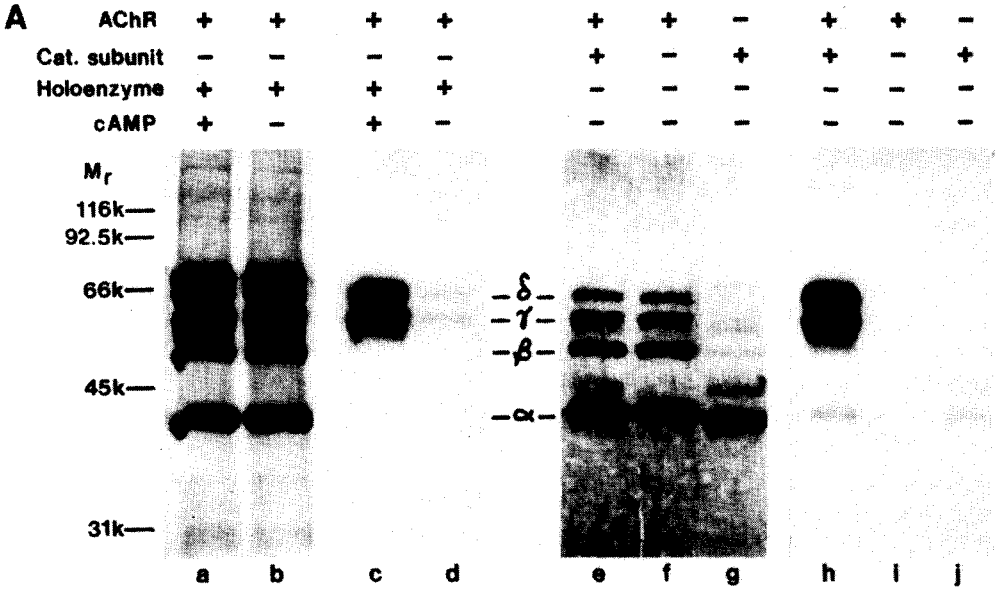


Fig. 2. Isolation of phosphorylated membrane-bound acetylcholine receptor (AChR) by detergent solubilization and sucrose density gradient centrifugation. Particulate fractions containing 5% acetylcholine receptor (200 μg total protein) were incubated with 0.25% Triton X-100, ^{125}I-labelled α-bungarotoxin (BTX) (3.2 μM, 1.9 μCi/nmol), [γ-^{32}P]ATP (50 μM, 8 μCi/nmol), 20 mM K_2 Pipes, pH 7.0, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ouabain, 100 μM vanadate, 20 μM KF, 0.25% Triton X-100 and 2 mM EGTA (250 μl total volume) under four conditions: (1) no additions (■), (2) +60 μg porcine heart protein kinase inhibitor (▲), (3) +10 phosphorylating units of purified catalytic subunit of cAMP-dependent protein kinase (○) and (4) +protein-kinase inhibitor and catalytic subunit (□). After 1 h of incubation acetylcholine receptor was extracted with 3% Triton X-100, separated from unreacted ATP and unbound α-bungarotoxin on Sephadex G-50 (3 ml), and isolated by two cycles of sucrose density gradient centrifugation (5–20%, 55000 rpm for 1.75 h in a Beckman VTi65 rotor). After the first cycle, fractions containing acetylcholine receptor bound to ^{125}I-labelled α-bungarotoxin were pooled and layered on a second sucrose gradient and centrifuged as above to increase resolution and decrease background counts. Samples from the gradient fractions were assayed on a gamma counter, corrected for ^{32}P spillover (28%) and then on a liquid scintillation counter using a channel that omits all ^{125}I gamma counts. (A) Normalized distribution of acetylcholine receptor (●); the content of acetylcholine receptor in the peak fraction under each condition is set = 1.0 (bars = range of acetylcholine receptor content). Incorporation of phosphate into acetylcholine receptor was calculated and plotted as a ratio: mol phosphate per mol acetylcholine receptor, as shown in (A) (○), and on an expanded scale in (B) (○, □, ■, ▲). The sucrose gradient was calibrated with thyroglobulin (20.3 S), aldolase (8.3 S) and carbonic anhydrase (3.2 S), the peaks of which appeared in fractions 1, 11 and 21, respectively. The acetylcholine receptor monomer is 9.0 S.

of cAMP as a modulator of acetylcholine receptor phosphorylation. Particulate fractions were incubated with [γ-^{32}P]ATP and ^{125}I-labelled α-bungarotoxin under several conditions: (1) no additions, (2) + cAMP-dependent protein kinase inhibitor, (3) + catalytic subunit of cAMP-dependent protein kinase and (4) + catalytic subunit and protein kinase-inhibitor. Following incubation the acetylcholine receptor was extracted, isolated and the phosphate incorporation determined as described in the legend to Fig. 2. The basal level of acetylcholine receptor phosphorylation in particulate fractions was found to be almost completely prevented by the protein inhibitor of



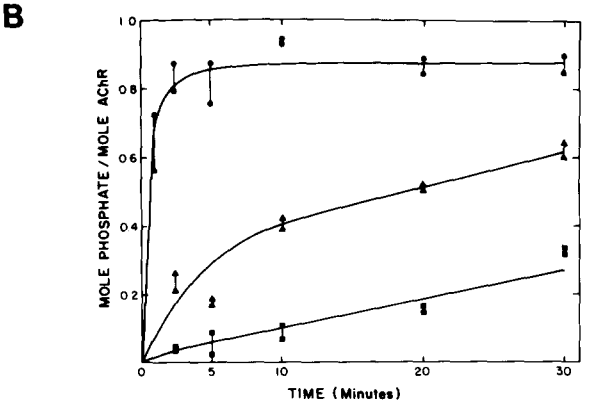


Fig. 3. Phosphorylation of purified acetylcholine receptor by cAMP plus cAMP-dependent protein kinase or by purified catalytic subunit. (A) Lanes a–d: Solubilized, purified acetylcholine receptor (20 pmol) was incubated with purified cAMP-dependent protein kinase (holoenzyme) from rabbit muscle (15 μg) for 30 min in the presence of 20 mM K_2 Pipes, pH 7.0, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ouabain, 100 μM vanadate, 20 mM KF, 1 mM EGTA and [γ-^{32}P]ATP (50 μM, 0.1 μCi/nmol). The samples were analyzed on SDS-polyacrylamide gel electrophoresis (10% acrylamide), silver stained, dried and autoradiographed. Lanes a, b are silver stain patterns of acetylcholine receptor (α-, β-, γ- and δ-subunits) and an unidentified polypeptide present in the protein kinase preparation that migrated to a point just above the δ-subunit. Lanes c,d are autoradiographs of lanes a, b, respectively. Lanes e–j: Solubilized, purified acetylcholine receptor (4 pmol) was incubated with purified catalytic subunit from cAMP-dependent protein kinase of beef heart (0.84 μg) (reconstituted in 300 mM dithiothreitol, final dithiothreitol concentration = 19 mM) for 5 min in the presence of 20 mM K_2 Pipes, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA and [γ-^{32}P]ATP (50 μM, 0.4 μCi/nmol). The samples were analyzed on SDS-polyacrylamide gel electrophoresis as above. Lanes e–g are silver stain patterns, lanes h–j are autoradiographs of lanes e–g, respectively. (B) Solubilized, purified acetylcholine receptor (4 pmol) was incubated with 20mM K_2 Pipes, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA and [γ-^{32}P]ATP (50 μM, 0.4 μCi/nmol) and 20 (●), 4 (▲) or 0.8 (■) pmol of the catalytic subunit of cAMP-dependent protein kinase (reconsituted in 300 mM dithiothreitol, final dithiothreitol concentration = 19 mM). At the indicated times 10% trichloroacetic acid was added to stop the reaction. The protein precipitate was retained on Nucleopore filters (0.4 μm pore size), washed and the radioactivity measured in a liquid scintillation counter. Non-specific phosphate binding and phosphate incorporation into catalytic subunit alone was subtracted.

cAMP-dependent protein kinase. The addition of purified catalytic subunit of cAMP-dependent protein kinase markedly stimulated acetylcholine receptor phosphorylation, this was partially inhibited by the protein kinase-inhibitor (Fig. 2). These results indicate that the particulate fractions contained active catalytic subunits of cAMP-dependent protein kinase which were able to phosphorylate acetylcholine receptor. Employing the sucrose-gradient method, or isolaton of acetylcholine receptor by affinity chromatography, we found that Ca^{2+}/calmodulin had no effect on the level of phosphorylation of acetylcholine receptor. In these experiments the particulate fractions were incubated in the absence of any detergent. We conclude that acetylcholine receptor is phosphorylated in situ by a cAMP-dependent protein kinase. To verify this result we incubated purified solubilized acetylcholine receptor with: (1) The catalytic subunit and [γ-^{32}P]ATP, or, (2) with cAMP-dependent protein kinase, cAMP and [γ-^{32}P]ATP. These incubations resulted in the selective phosphorylation of the γ- and δ-subunits of acetylcholine receptor as visualized on SDS-polyacrylamide gel electrophoresis (Fig. 3A). Phosphorylation ranged from 0.82 to 1.0 mol phosphate per mol acetylcholine receptor. When the holoenzyme was used added cAMP produced a 20-fold increase in phosphorylation above the basal level. Phosphorylation of the acetylcholine receptor by catalytic subunit was prevented by the protein kinase-inhibitor. The rate of acetylcholine receptor phosphorylation was dependent on the concentration of catalytic subunit (Fig. 3B).

Some previous reports have not identified the mechanism by which acetylcholine receptor is phosphorylated [3,12–14]. The data presented here demonstrate that acetylcholine receptor is phosphorylated in its γ- and δ-subunits by a cAMP-dependent protein kinase. We attribute our previous report [3] of a Ca^{2+}/calmodulin-dependent phosphorylation of acetylcholine receptor to the identical migration on SDS-polyacrylamide gel electrophoresis of non-acetylcholine polypeptides that are phosphorylated in this way. These polypeptides probably originate from two sources: (1) The polypeptides present in a membrane-associated Ca^{2+}/calmodulin-dependent protein kinase fraction that we partially purified (see above), and, (2) polypeptides from synaptosomes contaminating our particulate fractions that have been shown to be phosphorylated in a Ca^{2+}/calmodulin-dependent manner [15].

Our findings are in complete agreement with Huganir and Greengard [4] who studied the phosphorylation of acetylcholine receptor in membranes that were solubilized by the detergent Nonidet P-40. In our experiments phosphorylation of the acetylcholine receptor in situ by endogenous protein kinase was also detected in particulate fractions in the absence of any detergents, indicating that the enzyme(s) responsible maybe located in the same membrane as the acetylcholine receptor itself. The aim of this work was to differentiate between cAMP and Ca^{2+}/calmodulin-dependent phosphorylation of acetylcholine receptor, it does not rule out the possible involvement of other kinases.

This work was supported by a PHS Research Fellowship Award GM09248 to GBZ, NIH grants NS13860 and NS17119 to H.S., and GM17536 to M.B.F..

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