Regulation of nicotinic acetylcholine receptor phosphorylation in rat myotubes by forskolin and cAMP
(protein kinases/ion channel/desensitization)

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Contributed by Paul Greengard, May 28, 1987

ABSTRACT The nicotinic acetylcholine receptor (AcChoR) from rat myotubes prelabeled in culture with [32P]orthophosphate was isolated by acetylcholine affinity chromatography followed by immunoaffinity chromatography. Under basal conditions, the nicotinic AcChoR was shown to be phosphorylated in situ on the β and δ subunits. Regulation of AcChoR phosphorylation by cAMP-dependent protein kinase was explored by the addition of forskolin or cAMP analogues to prelabeled cell cultures. Forskolin, an activator of adenylyl cyclase, stimulated the phosphorylation of the δ subunit 20-fold over basal phosphorylation and induced phosphorylation of the α subunit. The effect of forskolin was dose dependent with a half-maximal response at 8 μM in the presence of 35 μM Ro 20-1724, a phosphodiesterase inhibitor. Stimulation of δ subunit phosphorylation was almost maximal within 5 min, whereas stimulation of α subunit phosphorylation was not maximal until 45 min after forskolin treatment. Stimulation of AcChoR phosphorylation by 8-benzylthiodenosine 3',5'-cyclic monophosphate was identical to that obtained by forskolin. Two-dimensional thermolytic phosphopeptide maps of the δ subunit revealed a single major phosphopeptide. These results correlate closely with the observed effects of forskolin on AcChoR desensitization in muscle and suggest that cAMP-dependent phosphorylation of the δ subunit increases the rate of AcChoR desensitization in rat myotubes.

Signal transduction at the postsynaptic membrane of the neuromuscular junction is mediated through the nicotinic acetylcholine receptor (AcChoR). This pentameric complex, composed of four types of protein subunits (αβγδ), is both a neurotransmitter receptor and a membrane ion channel. The neurotransmitter acetylcholine binds to the AcChoR opening the ion channel, and the resulting influx of cations depolarizes the muscle cell (1). The neuromuscular junction represents an excellent model system for investigating the molecular mechanisms involved in modulating intercellular signal transduction processes.

Biochemical characterization indicates that the Torpedo electric organ AcChoR is a phosphoprotein (2, 3). The regulation of AcChoR phosphorylation in vitro has been shown to involve at least three different protein kinases present in the postsynaptic membrane of the Torpedo electric organ. The cAMP-dependent protein kinase phosphorylates the γ and δ subunits (4), protein kinase C phosphorylates the α and δ subunits (5), and an endogenous protein tyrosine kinase phosphorylates the β, γ, and δ subunits (6). Protein phosphorylation is a major posttranslational mechanism involved in regulating the function of proteins including ion channels and receptors (7–9). The effect of phosphorylation on AcChoR function was directly examined in vitro by analyzing the ion-transport properties of purified AcChoR that was reconstituted into phospholipid vesicles. Phosphorylation of the purified AcChoR by cAMP-dependent protein kinase in vitro was shown to accelerate desensitization, the process by which the receptor is progressively inactivated in the presence of acetylcholine (10). In addition, electrophysiological studies have suggested that agents that increase the intracellular levels of cAMP increase the rate of AcChoR desensitization at the neuromuscular junction (11, 12). Exposure of rat soleus muscle to forskolin, a potent activator of adenylyl cyclase, was shown to increase the rate of AcChoR desensitization. This effect of forskolin on the rate of desensitization occurred over the same concentration range as that for activation of adenylyl cyclase and was potentiated by a phosphodiesterase inhibitor. The rate of desensitization was also increased by cAMP analogues (13). However, it is not known whether the AcChoR is phosphorylated in muscle and whether changes in the state of AcChoR phosphorylation could be responsible for accelerating AcChoR desensitization.

To establish that the AcChoR in rat muscle is a phosphoprotein, we isolated the AcChoR following phosphate-labeling of rat myotubes in culture. In this paper we demonstrate that the AcChoR is phosphorylated in situ in rat myotubes and that the state of phosphorylation of the α and δ subunits of the AcChoR increases after treatment of the myotubes with forskolin and cAMP analogues. These findings suggest that the AcChoR is phosphorylated by the cAMP-dependent protein kinase in situ and that this phosphorylation increases the rate of AcChoR desensitization at the neuromuscular junction.

MATERIALS AND METHODS

Materials. Pregnant rats (14–18 days of gestation) were obtained from Taconic Farms (Germantown, NY). Tissue culture media and supplies were obtained from Gibco, except for phosphate- and methionine-free media, which were purchased from Flow Laboratories. Radioisotopes and EnHance were obtained from New England Nuclear. The affinity resin was synthesized by incubating bromoacetylcholine with reduced Affi-Gel 401 (Bio-Rad) (14). Hybridoma cells secreting monoclonal antibody 88-B, a monoclonal antibody directed against the Torpedo AcChoR, were a generous gift from Stanley Froehner (Dartmouth University, Hanover, NH) and rabbit anti-mouse IgG was purchased from DAKO (Santa Barbara, CA). Reverse-phase HPLC was conducted using a Waters μBondapac C18 column. Forskolin and 1,9-dideoxyforskolin were obtained from Calbiochem and 8-benzylthiodenosine 3',5'-cyclic monophosphate (8-benzylthio-cAMP) was obtained from ICN. Protein A-Sepharose was purchased from Pharmacia. ortho-Na2VO4 was purchased from Fisher. Trasylol was obtained from FBA.

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Abbreviation: AcChoR, acetylcholine receptor.
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Pharmaceuticals. Leupeptin, pepstatin, and antipain were purchased from Chemicon (El Segundo, CA). Ro 20-1724 was a gift from Hoffmann-La Roche. All other chemicals were obtained from Sigma.

**Muscle Cell Culture and Metabolic Labeling.** Primary cultures were established from the hind limb muscle of 20- to 21-day rat embryos. Myoblasts were enzymatically dissociated with 0.05% trypsin and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) horse serum, 2% (vol/vol) chicken embryo extract, and 33 mM glucose. Experiments were performed on 7- to 12-day-old myotube cultures. For metabolic labeling with phosphate or methionine, cultures were washed either with phosphate-free or methionine-free Eagle’s minimal essential medium. Cells were then incubated either with [32P]orthophosphate (0.5 mCi; 1 Ci = 37 GBq) in 1 ml of phosphate-free medium per 35-mm dish for 3.5 hr or [35S]methionine (0.5 mCi) in 1 ml of methionine-free medium per 35-mm dish for 3.5 hr or overnight as indicated.

**Forskolin Treatment of Cultured Myotubes.** Cultured cells were treated with forskolin for the indicated times and concentrations along with 35 μM of the phosphodiesterase inhibitor Ro 20-1724. Both compounds were dissolved in 95% (vol/vol) ethanol and were added directly to the culture medium prior to the radioactive label such that the cells were not exposed to an ethanol concentration exceeding 1%. Ethanol alone (1%) was found to have no effect on phosphorylation.

**Acetylcholine Receptor Purification.** Cultured myotubes were rinsed with isotonic phosphate-buffered saline, and the AcChoR was isolated from each culture dish by solubilization in 500 μl of a “lysis solution” containing 1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM N-ethylmaleimide, 100 μM NaVO₃, 100 mM NaF, 10 mM sodium pyrophosphate, leupeptin (10 μg/ml), pepstatin (10 μg/ml), antipain (10 μg/ml), and Trasylol (10 units/ml). Insoluble material from each culture extract was removed by centrifugation for 10 min at 10,000 x g, and the supernatants containing AcChoR were passed through individual 200-μl acetylcholine affinity columns. Each flow through was reapplied to affinity resin, and the columns were rinsed twice with 500 μl of the lysis solution. The specificity of AcChoR binding to the columns was demonstrated by preincubating parallel culture extracts with 25 mM carbamylcholine. The AcChoR complex was eluted from the affinity resin with 500 μl of lysis solution containing 25 mM carbamylcholine. The eluates from each acetylcholine affinity column were then immediately passed through individual 100-μl immunoaffinity columns consisting of monoclonal antibody 88-B linked to protein A-Sepharose by a rabbit anti-mouse IgG. The columns were washed with 500 μl of lysis solution, and the AcChoR subunits were eluted from the immunoaffinity columns with sample buffer containing 125 mM Tris (pH 6.8), 2% (wt/vol) NaDodSO₄, 10% (vol/vol) glycerol, and 5% (vol/vol) β-mercaptoethanol. The yield of AcChoR from parallel cultures was consistently the same. The eluates from immunoaffinity columns were subjected to electrophoresis on 9% NaDodSO₄/polyacrylamide gels as described by Laemmli (15). Gels were dried and autoradiographed with the exception that those containing 35S were treated with Enhance prior to drying.

**Two-Dimensional Phosphopeptide Maps.** Two-dimensional thermolytic phosphopeptide mapping of proteins contained in gel pieces was performed as described (6).

**Determination of [32P]ATP Specific Activity.** Cell cultures labeled in situ with [32P]orthophosphate were washed in isotonic phosphate-buffered saline and then treated individually with 1 ml of 0.4 M perchloric acid. The insoluble material from each culture was removed by centrifugation for 10 min at 10,000 x g, and the resulting supernatants were neutralized with 0.1 ml of 4 M KOH. The KClO₄ precipitates that formed were removed by centrifugation for 10 min at 10,000 x g. Nucleotides, extracted into the resulting supernatants, were purified by reverse-phase HPLC employing isotonic elution with 1 mM potassium phosphate buffer (pH 6.0), pumped at 1 ml/min, and monitored by UV detection at 254 nm (16). ATP from 100-μl aliquots of each cell extract was identified by retention time and quantitated by peak height compared to known ATP standards. Fractions eluting from the column were measured for radioactivity in a liquid scintillation counter.

**RESULTS**

In Situ [35S]Methionine Labeling and Isolation of Myotube AcChoR. AcChoR was rapidly isolated, following solubilization of myotubes from cultures, by a two-column chromatography procedure consisting of acetylcholine affinity chromatography followed by immunoaffinity chromatography using monoclonal antibody 88-B. Monoclonal antibody 88-B, raised against Torpedo AcChoR, has been shown to recognize specifically the γ and δ subunits of the Torpedo AcChoR and to crossreact with cytoplasmic domains of mammalian AcChoR (17). With [35S]methionine labeling for 3.5 hr, three proteins of molecular masses 42, 50, and 62 kDa were isolated (Fig. 1, lane 1). Labeling cultures with [35S]methionine for 12 hr revealed an additional protein of 66 kDa (Fig. 1, lane 2). Confirmation that these proteins are subunits of the rat AcChoR was obtained by preincubating the cell homogenates with 25 mM carbamylcholine before affinity chromatography thus preventing the AcChoR from binding to the acetylcholine affinity column (Fig. 1, lane 3). Alternatively, pretreating the immunoaffinity column with an excess of purified Torpedo AcChoR also resulted in the specific exclusion of these proteins.
proteins (data not shown). The 42-, 50-, and 62-kDa proteins most likely correspond to the α, β, and δ subunits of the AcChoR. AcChoR isolated from a clonal mouse cell line (BC3H1) has been reported to have subunits of 42, 46, 48, and 60 kDa, which have been suggested to be the α, β, γ, and δ subunits, respectively (18). The absence of the γ subunit in our preparations may reflect its known sensitivity to proteolysis (18, 19). Prolonged incubation with [35S]methionine revealed trace labeling of a 66-kDa protein that appears to be due to a delayed posttranslational processing of the δ (62-kDa) subunit.

Phosphorylation of Myotube AcChoR in Situ. To measure the extent of AcChoR phosphorylation in situ, muscle cell cultures were incubated with [32P]orthophosphate. [32P] incorporation into total cell protein reached a steady state after 3.5 hr of metabolic labeling as measured by the 32P content of trichloroacetic acid precipitated proteins. Rapid isolation of the AcChoR following labeling of cell cultures with [32P]orthophosphate demonstrated that the AcChoR is a phosphoprotein in situ. Proteins of 50, 62, and 66 kDa were phosphorylated (Fig. 1, lane 4). Preincubation of cell homogenates from parallel cultures with 25 mM carbamylcholine prevented the isolation of these phosphorylated proteins (Fig. 1, lane 7). These 32P-labeled proteins comigrated with proteins isolated after [35S]methionine labeling and were specifically competed by carbamylcholine during affinity adsorption strongly suggesting that the 50- and 62-kDa proteins correspond to the β and δ subunits of the AcChoR. The specific incorporation of radioactive phosphate into AcChoR subunits was also found to plateau at 3 hr. To determine whether the 66-kDa phosphoprotein was related to the δ subunit, two-dimensional thermolytic phosphopeptide mapping was performed. Phosphopeptides generated by thermolysin digestion of the 62- and 66-kDa proteins have identical mobility by two-dimensional thin-layer chromatography suggesting that these proteins are variants of the same subunit (Fig. 2). The evidence that the 66-kDa protein is only apparent after prolonged [35S]methionine labeling suggests that the 66-kDa protein is related to the 62-kDa protein by posttranslational processing rather than proteolytic digestion. We have, therefore, designated the 66-kDa protein as the δ' subunit.

Regulation of Phosphorylation of Myotube AcChoR in Situ. The regulation of AcChoR phosphorylation by cAMP was explored by the addition of forskolin and the phosphodiesterase inhibitor Ro 20-1724 or by addition of cAMP analogues. Addition of the phosphodiesterase inhibitor Ro 20-1724 for up to 1 hr did not influence the basal phosphorylation level of the AcChoR (Fig. 1, lane 5). However, treating myotube cultures for 45 min with 20 μM forskolin in the presence of 35 μM Ro 20-1724 increased the phosphorylation of the α, δ, and δ' subunits (Fig. 1, lane 6). A comparison of phosphate and methionine labeling indicated that while phosphate incorporation into the AcChoR increased in the presence of forskolin, methionine labeling of AcChoR subunits did not increase, suggesting that forskolin increased the phosphorylation state of the AcChoR rather than having an effect on the AcChoR levels under these circumstances. In addition, this observation is consistent with the assumption that our purification procedure isolated both the phosphorylated and nonphosphorylated forms of the AcChoR. Two-dimensional thermolytic phosphopeptide mapping indicated that phosphorylation of the δ subunit occurred on a single major phosphopeptide (Fig. 2). Forskolin treatment during phosphate labeling did not induce additional phosphopeptides (data not shown); therefore, it is likely that this major phosphopeptide includes the phosphorylation site for the cAMP-dependent protein kinase. Addition of 20 μM 1,9-dideoxyforskolin, a derivative of forskolin that does not activate adenylate cyclase, in the presence of 35 μM Ro

Fig. 2. Two-dimensional thermolysin phosphopeptide maps of the 62-kDa (A) and 66-kDa (B) AcChoR subunits isolated from rat muscle cell cultures. Myotube cultures were incubated with 2 μCi of [32P]orthophosphate for 4 hr, and AcChoR was isolated as described in Fig. 1. Thermolysin phosphopeptide mapping of proteins contained in gel pieces was performed (circle designates origin).

20-1724 had no effect on AcChoR phosphorylation in myotube cultures (data not shown).

To investigate the possibility that forskolin caused an increase in the specific activity of ATP in cultured myotubes, we purified ATP from prelabeled cultures by HPLC to measure its specific activity. Neither the yield nor specific activity of ATP purified from forskolin-treated cultures differed significantly from control cultures (data not shown).

The dose dependence of AcChoR phosphorylation in response to forskolin was studied by treating myotube cultures for 1 hr with forskolin at concentrations ranging from 0 to 50 μM (Fig. 3). An increase in phosphorylation of the α, δ, and δ' subunits was detected that was dependent on the dose of forskolin and saturated at 20 μM forskolin. Quantitation of the autoradiogram optical density by scanning densitometry indicated that forskolin stimulated the phosphorylation of the δ subunit 20-fold with a half-maximal effect occurring at 8 μM forskolin. The stimulation of phosphorylation of the δ subunit by forskolin varied among experiments and ranged from 5- to 20-fold. Although the effect of forskolin

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Fig. 3. Dose dependence of the effect of forskolin treatment of rat myotubes on AcChoR phosphorylation. Myotube cultures were incubated for 3.5 hr with 0.5 mCi of $^{32}$Porthophosphate. Cells were then treated with 35 μM Ro 20-1724 and the indicated concentrations of forskolin for 1 hr. AcChoR was solubilized, isolated, and analyzed as described in Fig. 1.

on phosphorylation of the β subunit was highly variable, a decrease in phosphorylation was most consistently observed.

The time course of the increase in AcChoR phosphorylation using 20 μM forskolin showed that the rate of phosphorylation of the α subunit was slower than that of the δ and δ' subunits (Fig. 4A). Phosphorylation of the δ and δ' subunits reached half-maximal response within 5 min, whereas phosphorylation of the α subunit required 30 min to achieve half-maximal phosphorylation. The time course of δ plus δ' subunit phosphorylation after exposure to 20 μM forskolin was quantitated by scanning densitometry (Fig. 4B).

To test whether cAMP analogues had an effect similar to that of forskolin on AcChoR phosphorylation, cultured myotubes were treated with the cAMP analogue 8-benzylthio-cAMP (100 μM) for 60 min. 8-Benzylthio-cAMP stimulated the phosphorylation of the α, δ, and δ' subunits of the AcChoR, an effect similar to that observed with forskolin treatment (Fig. 5).

DISCUSSION

The results presented in this paper demonstrate that the nicotinic AcChoR is phosphorylated in intact muscle cells and that agents that increase intracellular cAMP levels stimulate phosphorylation of the AcChoR. Treatment of muscle cells with forskolin or 8-benzylthio-cAMP increased phosphorylation of the α, δ, and δ' subunits of the AcChoR.

The concentrations of forskolin that were effective in stimulating phosphorylation of the AcChoR are in the dose-response range known to activate adenylate cyclase in other cell systems (20). These results suggest that the effect of forskolin on phosphorylation of the AcChoR is most likely mediated through activation of cAMP-dependent protein kinase. The rapid time course of phosphorylation of the δ and δ' subunits of the AcChoR following treatment with forskolin is consistent with direct phosphorylation of the AcChoR by cAMP-dependent protein kinase. In contrast, phosphorylation of the α subunit follows a much slower time course after a considerable lag time and may reflect an indirect effect of cAMP-dependent protein kinase. It is possible that another protein kinase whose activity or synthesis is regulated by cAMP-dependent protein kinase phosphorylates the α subunit of the AcChoR.

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the δ subunit of the Torpedo AcChoR (G. Yee and R.L.H., unpublished results). The phosphorylation site for cAMP-dependent protein kinase has been conserved in the δ subunit but not in the γ subunit of the mammalian AcChoR (22, 23). Interestingly, the ε subunit, which has been reported to substitute for the γ subunit in the mature form of the calf AcChoR, does contain the phosphorylation site for cAMP-dependent protein kinase (24). The experiments presented here confirm that the δ subunit of the mammalian AcChoR is phosphorylated but no conclusions may be drawn concerning phosphorylation of the γ subunit. It may be that the γ subunit present in primary muscle cell cultures is not phosphorylated by the cAMP-dependent protein kinase. Although the primary sequence for the δ subunit from rat is not available, it is interesting to note that the thermolysin phosphopeptide of the δ subunit from rat has a mobility very similar to that of the Torpedo AcChoR δ subunit phosphorylated by cAMP-dependent protein kinase. It is likely, therefore, that cAMP-dependent protein kinase regulates the phosphorylation of a site on the muscle AcChoR δ subunit homologous to the site determined on the Torpedo AcChoR δ subunit.

Forskolin has been shown to increase the rate of AcChoR desensitization in rat muscle cell cultures (13). Forskolin accelerated AcChoR desensitization with a half-maximal effect that occurred at 8 μM and was complete within 5 min after forskolin treatment. This corresponds most closely to the time course and dose dependency of δ and δ' subunit phosphorylation observed after stimulating muscle cell cultures with forskolin. In contrast, since the increase in phosphorylation of the α subunit undergoes a longer time course, the stimulation of α-subunit phosphorylation by forskolin appears not to be correlated directly to AcChoR desensitization rates. Physiological effects of forskolin on AcChoR receptor function that display a long time course have been observed. Miniature end-plate currents recorded extracellularly from rat soleus end plates exposed to forskolin for at least 30 min were found to decay faster than nontreated controls suggesting a shorter mean channel open time for the AcChoR in muscle treated with forskolin (11). However, it has also been reported that single-channel currents recorded from chicken myotubes in the presence of forskolin for 30 min showed a lengthening of mean channel open time of the AcChoR (25).

The endogenous activator(s) of cAMP-dependent protein kinase at the neuromuscular junction has not been identified. The neuropeptide calcitonin gene-related peptide has been shown to increase cAMP levels (26) and to stimulate AcChoR synthesis (27) in chicken myotubes. If calcitonin gene-related peptide is released at the neuromuscular junction, where it has been shown to be localized presynaptically in the mouse (28), it is an excellent candidate for modulating AcChoR function by stimulating cAMP-dependent phosphorylation of the AcChoR.

Protein kinase C may also be involved in regulating AcChoR function in cultured myotubes. Muscle cells treated with phorbol esters, activators of protein kinase C, displayed a decreased sensitivity to acetylcholine and an increased rate of AcChoR desensitization (29). An endogenous protein tyrosine kinase, regulated by an unknown mechanism, may also phosphorylate the AcChoR at the neuromuscular junction. In the Torpedo AcChoR the phosphorylation sites for cAMP-dependent protein kinase, protein kinase C, and a protein tyrosine kinase are contained in one cytoplasmic domain of the AcChoR subunits. This may reflect a convergence of various protein kinase systems toward a single functional modification, an increased rate of AcChoR desensitization. It will be interesting to discern the functional role as well as the activating signal for each protein kinase system and to understand how they interact to modify AcChoR function.

Note Added in Proof. Phosphorylation of the nicotinic acetylcholine receptor in mouse BC3H1 myocytes has been demonstrated (21).

We thank Angus Nairn and Robert Nichols for a critical reading of this manuscript. This work was supported by a grant from The Council for Tobacco Research USA, Inc. (R.L.H.) and a postdoctoral fellowship from the Muscular Dystrophy Association (K.M.).