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Cyclic AMP-regulated AChR assembly is independent of AChR subunit phosphorylation by PKA

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SUMMARY

Forskolin treatment of cells expressing *Torpedo* acetylcholine receptors leads to enhanced assembly efficiency of subunits, which correlates with increased phosphorylation of the γ subunit. To determine the role of the two potential protein kinase A sites of the γ subunit in receptor assembly, cell lines expressing different mutant receptors were established. Mouse fibroblast cell lines stably expressing wild-type *Torpedo* acetylcholine receptor α, β, δ subunits plus one of three γ subunit mutations (S353A, S354A, or S353,354A) were established to identify the protein kinase A phosphorylation sites of γ in vivo, and to determine if increased phosphorylation of the γ subunit leads to enhanced expression of receptors. We found that both serines (353, 354) in γ are phosphorylated in vivo by protein kinase A, however, phosphorylation of either or both of these sites does not lead to increased assembly efficiency. We established a cell line expressing α, β, and γ(S353,354A) subunits only (no δ), and found that the presence of δ (or its phosphorylation) is also not necessary for the observed stimulation by forskolin. αβγ, αγ, and βγ associations were stimulated by forskolin but αβ and αδ interactions were not. These data imply that the presence of γ is necessary for forskolin action. We postulate that forskolin may stimulate acetylcholine receptor expression through a cellular protein that is involved in the folding and/or assembly of protein complexes, and that forskolin may regulate the action of such a protein through phosphorylation.

Key words: phosphorylation, assembly, acetylcholine receptor

INTRODUCTION

The muscle-like nicotinic acetylcholine receptor (AChR) is the best characterized member of the ligand-gated family of ion channels, which also includes neuronal AChRs, $GABA_A$ and glycine receptors (Claudio, 1989). The AChR is an intrinsic membrane glycoprotein composed of four different subunits with the stoichiometry $\alpha_2\beta\gamma\delta$ (for reviews see Claudio, 1989; Galzi et al., 1991; Karlin, 1991). Evidence exists for the regulation of AChR expression at the level of mRNA transcription by neural factors (Harris et al., 1988; Horovitz et al., 1989; Falls et al., 1993), by muscle activity (Klarsfeld and Changeux, 1985; Goldman et al., 1988; Chahine et al., 1992; Dutton et al., 1993), by denervation (Goldman et al., 1985; Shieh et al., 1987; Goldman and Tamai, 1989; Tsay and Schmidt, 1989; Bambrick and Gordon, 1992; Brenner et al., 1992; Neville et al., 1992; Salmon and Changeux, 1992), and by the neuropeptide calcitonin gene-related peptide (CGRP) (Fontaine et al., 1987), which induces increased cAMP levels. However, in none of these studies does an increase in mRNA levels correlate tightly with changes in AChR cell surface expression, suggesting that there are additional sites of regulation.

Protein expression can be regulated at the level of DNA, RNA, protein synthesis, polypeptide folding, post-translational modifications, transport, and degradation. For a heterologous multisubunit protein, regulation can also occur during assembly, with both the incoming subunits and the assembly intermediates being potential sites of regulation. One mechanism of regulating a wide variety of cellular functions is protein phosphorylation (for reviews see Edelman et al., 1987; Sibley et al., 1987; Greengard, 1988; De Camilli et al., 1990; Greengard et al., 1993). There is increasing evidence for the regulation of function, expression and cellular distribution of cell surface receptors and ion channels by phosphorylation mechanisms. Such regulatory effects have been shown to be involved in exocytosis, endocytosis, and/or recycling (e.g. receptors for transferrin, asialoglycoprotein (ASGP), mannose 6-phosphate, epidermal growth factor (EGF), insulin; Klausner et al., 1984; May et al., 1984; Fallon and Schwartz, 1986; Lin et al., 1986; Braulke et al., 1990; Davidson et al., 1992; Meresse and Hoflack, 1993), in functional modulation and desensitization (e.g. AChR, $GABA_A$, cystic fibrosis transmembrane conductance regulator (CFTR); Huganir et al., 1986; Moss et al., 1992; Chang et al., 1993), and in assembly (e.g. assembly of AChR subunits in cultured chick muscle cells, connexin 43 in the assembly of gap junctional plaques; Ross et al., 1987; Musil and Goodenough, 1991). In this paper,

we will investigate the role of phosphorylation in regulating the assembly of AChR subunits.

We have shown previously that agents that increase intracellular levels of cAMP cause an increase in the number of cell surface AChRs expressed either in muscle cells or in fibroblasts expressing *Torpedo* AChRs (All-11 cells) (Green et al., 1991a). Similar enhancement in cell surface expression has been observed for $GABA_A$ receptors expressed in Cα12 cells, cells that produce ~ five times higher levels of the catalytic subunit of protein kinase A (PKA) than wild-type cells (L929) (Angelotti et al., 1994). The effect of forskolin on AChR expression was shown to be post-translational and it correlated with an increased stability of the partially assembled subunits through heterologous subunit-subunit interactions (Green et al., 1991a). Further, it was shown that forskolin increased subunit assembly efficiencies two-fold with a corresponding increase in cell surface AChRs (Ross et al., 1991). ^{32}P labeling experiments of All-11 cells indicated a parallel stimulation of the γ subunit phosphorylation by forskolin. The time-course of forskolin-stimulated phosphorylation preceded that of subunit assembly and surface expression. The same stimulation was observed in fibroblast cell lines transduced with *Torpedo* αβγ and αγ subunit cDNAs. The results together indicated a strong correlation between forskolin-enhanced AChR expression and γ subunit phosphorylation (Green et al., 1991b).

Torpedo AChR can be phosphorylated in vitro or in vivo by PKA (γ and δ), PKC (γ and δ) and tyrosine kinases (β, γ and δ) (Yee and Huganir, 1987; Safran et al., 1990; Qu et al., 1990; Schroeder et al., 1991; Wagner et al., 1991). Yee and Huganir (1987) reported that γ subunit phosphorylated in vitro by PKA was phosphorylated on a single consensus serine (353) site. However, there are two consensus PKA sites (Ser-353, Ser-354) in the sequence of the γ subunit. One question we will address in this paper is can both sites be phosphorylated in vivo. A second question is can increased phosphorylation of the γ subunit lead to increased AChR assembly. For both questions, three stable fibroblast cell lines were established: wild-type *Torpedo* αβδ cDNAs plus γ cDNA point-mutated to remove consensus phosphorylation sites Ser-353, Ser-354, or both Ser-353 and Ser-354. Our results demonstrate that both serines in γ are phosphorylated in vivo, however, phosphorylation of these sites does not lead to increased assembly efficiency of AChR subunits. Although the δ subunit is also phosphorylated by PKA, we show that it does not play a role in stimulating the assembly of mutant receptors. Our results strongly indicate that the presence of the γ subunit is necessary for increased AChR assembly efficiency, although its phosphorylation is not. We suggest that the forskolin effect may be mediated through a cellular protein that interacts with γ subunits.

MATERIALS AND METHODS

Mutagenesis and cloning

A 318 bp fragment containing Ser-353 and -354 of the γ subunit cDNA in pSV2 (pSV2γ) was subcloned into Bluescript KS^+ vector (Stratagene, La Jolla, CA), through unique *Sal*I and *Cla*I sites; 24-30 bp oligonucleotides were made with mutations Ser353Ala(-1), Ser354Ala(-2), and Ser353,354Ala(-3). In vitro mutagenesis (Taylor et al., 1985) was carried out using an Amersham mutagenesis kit (Amersham, IL). The mutants were analyzed and confirmed by DNA sequencing. The mutant DNA fragments (318 bp) were cloned back into pSV2(γ –318 bp) vectors and used for transfections. Cloning and all manipulations were carried out using standard techniques (Sambrook et al., 1989).

Cell lines

Cell lines were grown at 37°C in the presence of 5% CO_2 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (DMEM+CS). All-11 cells (mouse fibroblast L cells cotransfected with *Torpedo californica* AChR α, β, γ, δ subunit cDNAs) have been described previously (Claudio et al., 1987). Cell lines expressing wild-type *Torpedo* α, β, δ + γ mutations -1, -2, -3 and a cell line expressing α, β + γ(-3) mutation were established by lipofection (Felgner et al., 1987; Claudio, 1992). Each subunit cDNA was engineered behind a SV40 promoter (pSV2 vectors) and cotransfected with thymidine kinase cDNA (ptk) into Ltk^- cells. For lipofection, 50%-confluent 10 cm dishes of Ltk^- cells were rinsed three times with DMEM, and incubated at 37°C in 3 ml of DMEM containing 100 μl of DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; BRL, MD) plus 100 ng ptk and 2.5 μg each of α, β, δ, and mutant γ cDNA. After 5 hours, 10 ml of DMEM+CS was added and cells were grown until confluent. Cells were split into DMEM+CS containing 15 μg/ml hypoxanthine, 1 μg/ml aminopterin, and 5 μg/ml thymidine (HAT). Subsequent steps followed the same protocol as in Claudio et al., 1987. All-11, βγδ, -1, -2, -3 and αβ(γ -3) cell lines are mouse fibroblast L cells stably expressing wild-type *Torpedo* αβγδ, βγδ, αβδ + γ(S353A), αβδ + γ(S354A), αβδ + γ(S353,354A) and αβ + γ(S353,354A), respectively. NIH3T3 cells expressing *Torpedo* αβ, αγ, αδ and αβγ have been cotransfected with the neomycin resistance gene (pSV2-neo) and αβ, αγ, and αβγ subunit cDNAs engineered into pSV2 vectors. To induce expression in each of these cell lines, cells were grown at 37°C until confluent, 20 mM sodium butyrate (NB) was added for 24-48 hours, and then the cells were placed in an incubator maintained at 20°C for the indicated time period.

^{125}I-αBungarotoxin (BuTx) binding

To measure cell surface AChRs, cells were rinsed twice with PBS + 0.6 mM $CaCl_2$ and incubated at room temperature in PBS containing 5 nM ^{125}I-BuTx (a peptide neurotoxin that binds specifically to AChRs) (sp. act. 140-170 cpm/fmol; NEN Radiochemicals, MA), 0.6 mM $CaCl_2$ and 0.1% BSA. Cultures were labeled for 2 hours on a shaker table, washed three times with PBS, solubilized in 1% Triton X-100, and radioactivity quantitated using a γ-counter. Nonspecific binding, determined by measuring ^{125}I-BuTx binding in the presence of the competitive inhibitor carbamylcholine (10 mM), did not exceed 5% of total labeling. Total cellular levels of AChR or partial complexes were determined by incubating cell lysates in 10 nM ^{125}I-BuTx from 6 hours to overnight at 4°C on a Labquake shaker, followed by immunoprecipitation and quantitation with a γ-counter.

Labeling and immunoprecipitations

For ^{35}S labeling, confluent 10 cm dishes of cells were treated with 20 mM NB containing medium for 24-48 hours at 37°C, washed twice with PBS, incubated for 15 minutes at the labeling temperature (37 or 20°C) in methionine-free DMEM (JR Scientific, CA) and then incubated in 2 ml methionine-free DMEM containing 300 μCi [^{35}S]methionine (Amersham Radiochemicals, IL) for the given time and temperature. After labeling, cells were immediately lysed or washed twice with DMEM and chased in 4 ml of DMEM+NB with or without cycloheximide (10 μg/ml) at 20°C for the indicated time. Before lysis, cells were washed twice with PBS, scraped, and the cells pelleted by centrifugation at 5,000 ***g*** for 3 minutes. The remaining steps were carried out at 4°C. Pellets were resuspended in 400-700 μl of lysis buffer (LB) (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 1% Triton X-100, 0.02% NaN_3) containing fresh 2 mM phenyl-

methylsulfonyl fluoride (PMSF) and 2 mM *N*-ethylmaleimide (NEM). In some experiments, 1% Triton X-100 was replaced with 1% LPC (1% Lubrol Px (Calbiochem, CA) + 183 μl (per 10 ml LB) of phosphatidyl choline (Sigma)), freshly made once a week. In ^{32}P labeling experiments, phosphatase inhibitors NaF (50 mM), pyrophosphate (5 mM) and vanadate (100 μM) were included in the lysis buffer, along with the protease inhibitors. Cells were lysed overnight, and the extracts clarified by centrifugation at 10,000 ***g*** for 10 minutes. Polyclonal or monoclonal antibodies (α-specific mAb 35 (American Type Culture Collection, Rockville, MD), α-specific mAb 14, β-specific mAb 148, or γ-specific mAb 168 (Tzartos and Lindstrom, 1980; Gullick and Lindstrom, 1983; Wan and Lindstrom, 1985)) were added to the supernatants and the samples were rocked overnight. Then 50 μl of Protein G-Sepharose (Pharmacia) or Protein A-Sepharose (Sigma) (diluted 2× in PBS) was added, the samples were rocked a minimum of 3 hours, and the resin was washed five times with lysis buffer. After the final wash, 35 μl of gel-loading buffer (4% SDS, 20% glycerol, 0.125 M Tris, pH 6.8, 0.01% Bromophenol Blue) containing fresh 10 mM DTT was added to the pellets and incubated at room temperature for 20 minutes. The resins were spun down and the supernatants were loaded onto a 7.5% discontinuous SDS-polyacrylamide gel (Laemmli, 1970). Gels were fixed and stained for 30 minutes in 25% methanol and 10% acetic acid containing 0.3% Coomassie Brilliant Blue, destained, and then soaked in Amplify (Amersham, IL) for 30 minutes, dried on a gel dryer, and exposed to film (Kodak X-Omat RP XRP-1, Parker Xray, CT) at −70°C with an intensifying screen. Autoradiographs were analyzed and quantified by scanning densitometry using a Bioimage/Visage system (Millipore).

For ^{32}P labeling, cells were treated with DMEM+NB at 37°C for 24-36 hours and then treated with DMEM+100 μM forskolin (Calbiochem, CA) at 20°C for 25-30 hours. Cells were washed and incubated in phosphate-free DMEM at 20°C for 15 minutes, and labeled with 4 ml of phosphate-free DMEM containing [^{32}P]orthophosphate (1-2 mCi/10 cm dish, NEN) plus forskolin (100 μM) for 15-18 hours. Cells were lysed and processed as above.

Phosphoamino acid analysis

All-11 cells were ^{32}P-labeled in the presence of forskolin (100 μM) at 20°C, lysed in 1% Triton X-100 LB, and the unassembled subunits were isolated by first running the cell lysate on a 5-20% linear sucrose gradient and then collecting the 6 S peak (Green et al., 1991b). The samples were run on an SDS gel, transferred to immobilon membranes (Millipore, MA), the γ subunit band was excised and subjected to partial acid hydrolysis at 110°C for 1 hour in 5.7 M HCl (Kamps and Sefton, 1989). Phosphoamino acids were resolved by two-dimensional electrophoresis on thin-layer cellulose plates (Stern et al., 1991). Electrophoresis in the first dimension was at pH 1.9, and at pH 3.5 in the second dimension. Non-labeled phosphoamino acid standards mixed in with the samples were located by ninhydrin staining.

In vitro phosphorylation by PKA

Ten confluent 10 cm dishes of All-11 cells were treated with or without forskolin (100 μM) for 2 hours during the last two hours of the 20°C incubation period. Cells were lysed in 225 μl of 1% Triton X-100 LB in the presence of protease and phosphatase inhibitors. The in vitro phosphorylation was performed by incubating 100 μl of cell lysate with 1 μM purified catalytic subunit of PKA (Promega,WI), 25 μM ATP, and 25 μCi of [γ-^{32}P]ATP (NEN) at 30°C for 3 hours in the presence of the protease inhibitors, 2 mM PMSF and 2 mM NEM (Yee and Huganir, 1987).

Phosphopeptide mapping

Cells labeled with [^{32}P]orthophosphate were lysed in 1% Triton X-100 LB, γ subunits were isolated by immunoprecipitation with mAb 168 (γ-specific mAb), the precipitates were run on a 7.5% gel, and the γ subunit band was cut from the gel. Sample preparation for phosphopeptide mapping used the method of Lai et al. (1987). Gel pieces were washed in 20 ml of 10% acetic acid/10% isopropanol for 12-24 hours, and then twice with 50% methanol for 12 hours. Gel pieces were washed in deionized distilled water and dried in a Speedvac. Gel pieces were then incubated in 500 μl of digestion buffer (50 mM NH_4CO_3, 0.5 mM DTT, Phenol Red, 50 μg/ml thermolysin) for 24 hours at 37°C. The supernatant was dried in a Speedvac, washed several times with water and dissolved in 5 μl of pH 3.5 running buffer (1% pyridine, 10% acetic acid) plus 1 μl of a 1 μg/ml solution of basic furisin in running buffer. The tubes were vortexed and spun in a microfuge, the supernatant was spotted (1 μl at a time) on a thin-layer nitrocellulose plate (Baker, NJ), and dried with a hair dryer after each application. The sample was subjected to electrophoresis at 350 V for 75 minutes in the first dimension. The plates were allowed to dry overnight and subjected to thin-layer ascending chromatography in the second dimension. The plates were then dried and analyzed by autoradiography.

RESULTS

The γ subunit is phosphorylated only on serine residues

In previous studies, we reported that forskolin and other cAMP-activators induced a two- to three-fold increase in cell surface AChRs for both *Torpedo* AChRs expressed in AChR-fibroblasts and endogenous rat AChRs expressed in rat L6 muscle cells. For both systems, the forskolin effect was shown to be mediated through a post-translational mechanism (Green et al., 1991a). Further analysis demonstrated that the stimulation occurs at the level of subunit assembly (Ross et al., 1991) and it correlated with an increase in phosphorylation of the γ subunit (Green et al., 1991b). These observations led to the hypothesis that AChR subunit assembly may be regulated by phosphorylation of the γ subunit. To further investigate this correlation, we first characterized the phosphorylation sites of the γ subunit.

In order to obtain maximum expression of AChRs in All-11 cells (*Torpedo* AChR-fibroblasts), a temperature-shift protocol was followed. Confluent dishes of cells were incubated in 20 mM NB medium at 37°C for 24-48 hours (to enhance subunit synthesis), and then shifted to 20°C for 48-72 hours (to allow assembly of AChR subunits (Claudio et al., 1987; Paulson and Claudio, 1990). Forskolin treatment was carried out during the 20°C incubation period. To identify the phosphoamino acid residues of the γ subunit, confluent dishes of All-11 cells were labeled with [^{32}P]orthophosphate in the presence of 100 μM forskolin. Unassembled subunits were isolated by running the cell lysate on a sucrose gradient and immunoprecipitating fractions with mAb 168, an antibody directed against the γ subunit. Immunoprecipitates were run on SDS-polyacrylamide gels and purified γ subunit was subjected to partial acid hydrolysis (see Materials and Methods). Phosphoamino acids were resolved by two-dimensional electrophoresis on thin-layer cellulose plates and analyzed by autoradiography (Fig. 1). The results indicate that γ is phosphorylated only on serine residues.

Forskolin pre-treatment of All-11 cells blocks in vitro phosphorylation by PKA

To determine if the in vivo phosphorylation of the γ subunit is mediated through PKA, confluent dishes of All-11 cells were treated with NB at 37°C and then shifted to 20°C in the absence

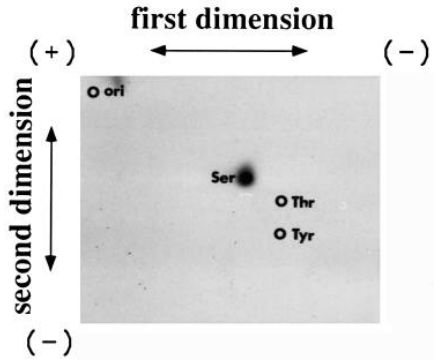

Fig. 1. Phosphoamino acid analysis of the γ subunit. Confluent 10 cm dishes of All-11 cells were incubated in growth medium containing 20 mM NB for 28 hours at 37°C. The cells were treated with forskolin (100 μM) at 20°C for 26 hours and labeled with [^{32}P]orthophosphate (2 mCi/10 cm dish) plus forskolin for 18 hours. Unassembled γ subunits were isolated as described in Materials and Methods and subjected to phosphoamino acid analysis. Electrophoresis in the first dimension was at pH 1.9 (anode at the left), and at pH 3.5 in the second dimension (anode at the top). Ser, Thr, and Tyr mark the positions of the serine, threonine and tyrosine standards, respectively, and ori marks the position of the origin.

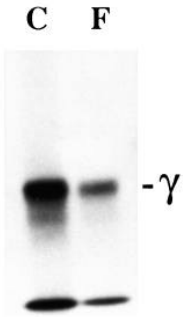

Fig. 2. In vitro phosphorylation of control and forskolin-treated cell lysates by PKA. Confluent 10 cm dishes of All-11 cells were incubated in 20 mM NB medium at 37°C for 24 hours and shifted to 20°C for 48 hours in the absence (C lane) or presence (F lane) of forskolin (100 μM). Equal amounts of cell lysates were phosphorylated in vitro by 1 μM catalytic subunit of PKA in the presence of [γ-^{32}P]ATP. Assembled subunits were immunodepleted from the lysate using mAb 35, then unassembled γ subunits were isolated by mAb 168 immunoprecipitation and run on a 7.5% SDS-polyacrylamide gel.

or presence of forskolin. Cells were lysed and equal amounts of control and forskolin-treated membrane extracts were phosphorylated in vitro in the presence of 1 μM catalytic subunit of PKA and 25 μM [γ-^{32}P]ATP at 30°C. Assembled subunits were immunodepleted from the lysate using mAb 35 (an antibody directed against the α subunit). Unassembled γ subunits could then be isolated with γ-specific mAb 168 and analyzed by SDS-PAGE autoradiography (Fig. 2). In vitro PKA phosphorylation was higher in control samples (lane C) compared with forskolin-treated (lane F) samples. The ^{32}P-labeled γ subunit bands were cut from the gel, dissolved, and counted in order to measure the number of moles of ^{32}P incorporated into subunits: 0.23 pmol of ^{32}P were incorporated under control conditions and 0.11 pmol were incorporated under forskolin-treated conditions. The amount of subunit present in the control and forskolin-treated cells was quantitated by parallel [^{35}S]methionine labeling experiments, and the total number of unassembled γ subunits was determined to be 0.40 pmol under control conditions and 0.70 pmol under forskolin-treated conditions. When ^{32}P incorporation was

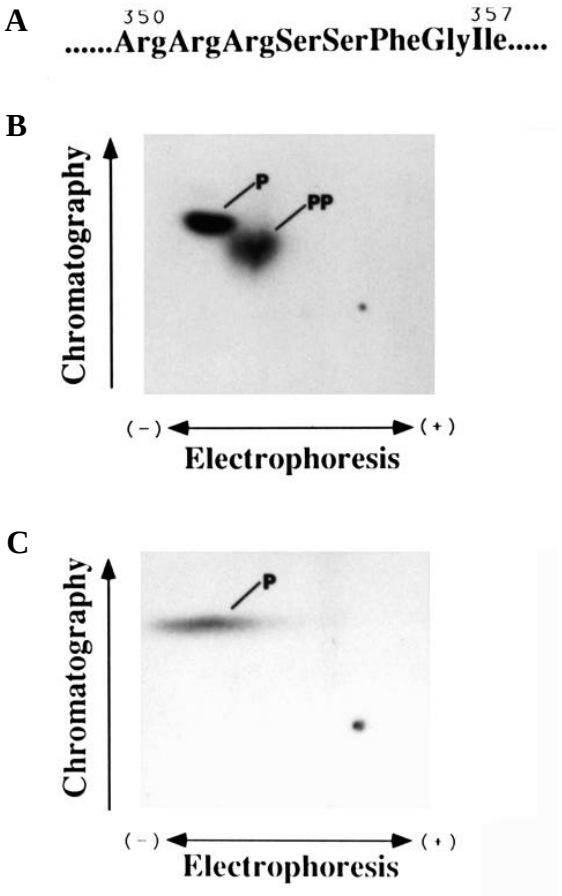

Fig. 3. Phosphopeptide mapping of γ subunit. Confluent 10 cm dishes of All-11 cells were incubated in 20 mM NB medium for 24 hours and shifted to 20°C for 26 hours in the absence or presence of forskolin (100 μM). Cells were then labeled with [^{32}P]orthophosphate (2 mCi/dish) plus or minus forskolin (100 μM) for 18 hours at 20°C. Cells were lysed in 1% Triton X-100 lysis buffer, assembled subunits were immunodepleted from the lysate using mAb 35, then unassembled γ subunits were isolated by mAb 168 immunoprecipitation and processed for phosphopeptide mapping as described in Materials and Methods. The peptides were subjected to electrophoresis at pH 3.5 in the first dimension (cathode at the left) and thin-layer chromatography in the second dimension (pH 13.5). (A) partial amino acid sequence of γ subunit (residues 350-357) (B) forskolin-treated (C) control-treated.

corrected for the amount of subunit present, we calculated that 0.57 pmol of ^{32}P per mol of subunit were incorporated under control conditions and 0.15 pmol of ^{32}P per mol of subunit were incorporated under forskolin-treated conditions. These results indicate that in vivo, forskolin-treated samples are phosphorylated at the same sites as those that are phosphorylated in vitro by PKA and therefore suggest that, upon forskolin treatment, PKA is the kinase responsible for phosphorylation of the γ subunit in vivo.

PKA phosphorylates γ on multiple sites

The γ subunit has two consensus PKA phosphorylation sites at Ser-353 and Ser-354 (Fig. 3A). Yee and Huganir (1987) reported that when membranes from *Torpedo* electric organs were phosphorylated in vitro by the catalytic subunit of PKA,

only Ser-353 was phosphorylated. In All-11 cells, forskolin and cAMP analogs stimulated the phosphorylation of the γ subunit. To identify the site(s) that is stimulated by forskolin, All-11 cells were labeled with [^{32}P]orthophosphate at 20°C in the presence or absence of forskolin, cells were lysed, membrane extracts were immunoprecipitated with mAb 168, and samples were subjected to SDS-PAGE autoradiography. The purified γ gel bands were processed for digestion with thermolysin and the resulting peptides were analyzed by phosphopeptide mapping, followed by autoradiography (Fig. 3B,C). Control samples (Fig. 3C) migrated predominantly as a single spot whereas the forskolin-treated samples (Fig. 3B) showed two major spots. The pattern shown in Fig. 3B is consistent with a multiply phosphorylated single peptide (Boyle et al., 1991), and thus suggested that the γ subunit was phosphorylated at more than one site.

Ser-353 and Ser-354 mutants assemble properly

In order to determine if Ser-353 and Ser-354 can both be phosphorylated by PKA, and if the phosphorylation of the γ subunit can regulate AChR assembly, these two serines were singly or doubly mutated to alanines by in vitro site-directed mutagenesis. Mouse L fibroblasts stably expressing *Torpedo* αβδ and mutant γ subunits were identified by screening cell lines with ^{125}I-BuTx. The cell lines containing Ser353Ala, Ser354Ala, and Ser353,354Ala are referred to as -1, -2, -3, respectively. Clonal isolate number 1 (for -1), 14 (for -2) and 16 (for -3) were selected for further characterization and they expressed ~8, 10 and 30 fmols of surface ^{125}I-BuTx sites per 60 mm dish, respectively (All-11 cells express ~55 fmols/60 mm dish).

Torpedo αβγ subunits will assemble and form a functional complex which is expressed on the surface of fibroblasts (Green et al., 1991b). To determine if each mutant cell line contained all four subunits and if the subunits assembled properly, All-11, -1, -2, and -3 cells were pulse-labeled at 37°C for 1 hour with [^{35}S]methionine, then chased at 20°C for 48 hours. Cells were lysed, membrane extracts were immunoprecipitated with mAb 14 (anti-α) or mAb 168 (anti-γ), and immunoprecipitates were analyzed by SDS-PAGE autoradiography (Fig. 4). As shown, all four subunits were expressed in each cell line and the subunits assembled into complexes that could be immunoprecipitated with anti-α or anti-γ specific antibodies.

Both serines can be phosphorylated by PKA

In order to determine whether serines 353 and 354 are both phosphorylated by PKA in the presence of forskolin, All-11 and mutant AChR cells were labeled with [^{32}P]orthophosphate in the presence of forskolin at 20°C. Cells were lysed, membrane extracts immunoprecipitated with mAb 168, and immunoprecipitates analyzed by SDS-PAGE autoradiography (Fig. 5). Both -1 and -2 AChR mutants were phosphorylated on γ but the -3 AChR mutant did not show any ^{32}P incorporation. The observation that -1 and -2 were both phosphorylated demonstrates that either Ser-353 or Ser-354 can be phosphorylated. The observation that -3 was not phosphorylated, demonstrates that Ser-353 and Ser-354 are the only sites in γ that can be phosphorylated by PKA.

The γ gel bands from -1 and -2 were subjected to phosphopeptide mapping. The peptide maps (Fig. 6) showed single

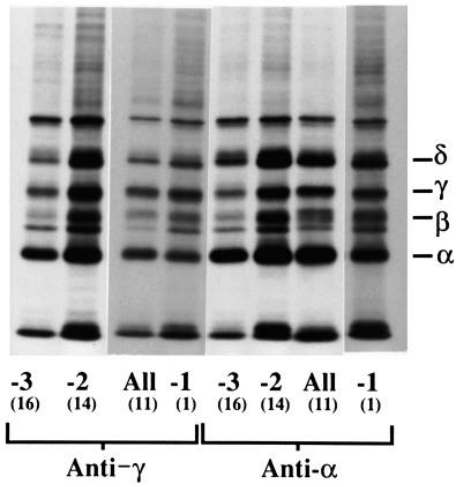

Fig. 4. Mutant AChRs assemble properly. Confluent 10 cm dishes of All-11 (1 dish/lane), -1 (S353A, 2 dishes/lane), -2 (S354A, 2 dishes/lane), -3 (S353,354A, 1 dish/lane) were incubated in 20 mM NB medium for 40 hours at 37°C. They were then labeled with [^{35}S]methionine (0.3 mCi/dish) at 37°C for 1 hour and chased in NB medium at 20°C for 24 hours. Cells were lysed in 1% Triton X-100 lysis buffer and assembled receptors were immunoprecipitated with mAb 14 or mAb 168. Immunoprecipitates were analyzed by SDS-PAGE autoradiography. The number in parentheses below each cell line is the clonal isolate number.

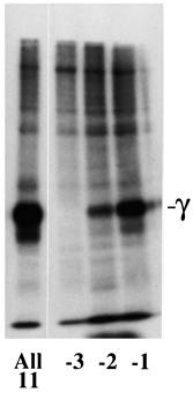

Fig. 5. Both serines in γ are phosphorylated. Confluent 10 cm dishes of All-11, -1(S353A), -2(S354A), -3(S353,354A) cells were incubated in 20 mM NB medium at 37°C for 24 hours. Cells were shifted to 20°C in the presence of forskolin (100 μM) for 31 hours and then they were labeled with [^{32}P]orthophosphate (1 mCi/dish) for 13 hours at 20°C. Cells were lysed in 1% Triton X-100 lysis buffer, subunits were denatured in 1% SDS, renatured in 5% Triton X-100, immunoprecipitated with mAb 168, and analyzed by SDS-PAGE autoradiography.

spots for both mutants. During ascending chromatography, the mutant phosphopeptides migrate with a similar R_f value, indicating that they are singly phosphorylated forms of the same peptide. As shown in Fig. 3B, two spots were observed on the phosphopeptide map of wild-type γ. Such a pattern of migration is indicative of similarly sized peptides being singly and doubly phosphorylated. During electrophoresis in the first dimension, both peptides migrate towards the cathode showing that they are basic. The doubly phosphorylated peptide (PP) migrates more slowly toward the cathode due to its one extra negative charge. During ascending chromatography in the second dimension, the singly phosphorylated peptide (P) migrates faster since it has 1 Ser + 1 phosphoserine compared to the doubly phosphorylated peptide which has 2 phosphoserines. This difference makes the doubly phosphorylated peptide less hydrophobic and therefore it migrates slower during chromatography. The results from Figs 3 and 6 demonstrate that the γ subunit can be phosphorylated on Ser-353, Ser-

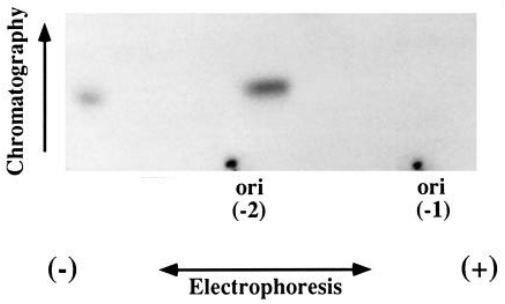

Fig. 6. Singly mutated γ subunits run as single phosphopeptides. Phosphorylated γ subunit bands (Fig. 5) of -1(S353A) and -2(S354A) cells were processed for phosphopeptide mapping as described in Materials and Methods. The peptides were subjected to electrophoresis at pH 3.5 in the first dimension (cathode at the left) and thin-layer chromatography in the second dimension (pH 13.5).

354, or both, in vivo. They also show that the phosphorylation of one site does not prohibit the other site from being phosphorylated.

Forskolin stimulates mutant AChR assembly

We have shown previously that forskolin stimulated the assembly of wild-type AChRs in All-11 cells, and this stimulation correlated with an increase in γ subunit phosphorylation. To determine if phosphorylation of Ser-353, Ser-354, or both led to enhanced assembly efficiencies, All-11 and all three mutant AChR-expressing cells were treated with NB at 37°C, then shifted to 20°C for 48 hours in the presence or absence of forskolin. The expression of surface AChRs was measured by ^{125}I-BuTx binding and the ratios of forskolin-treated to control cells are given in Table 1. As shown, forskolin stimulated the assembly of all three mutants to the same extent as it stimulated the assembly of All-11 AChRs. These results clearly demonstrate that either or both PKA sites on γ can be phosphorylated, however, phosphorylation of the sites appears not to result in enhanced assembly efficiency of the subunits since the double mutant also showed enhanced expression. It is possible that when the PKA sites are removed from γ, phosphorylation of PKA sites on δ substitute for, and fulfill the role of, phosphorylated γ.

Does δ play a role in stimulating assembly?

Previously, we reported that γ and δ are both phosphorylated by PKA although the effect of forskolin on unassembled δ subunit phosphorylation was less pronounced than it was on γ (Green et al., 1991b). However, because δ is the only subunit other than γ that is phosphorylated by PKA, and because γ and δ are 49% identical and thereby possibly capable of substituting for one another, we wished to test the effect of forskolin on AChRs lacking a δ subunit in order to observe directly the effect of γ in regulating assembly. We showed previously that fibroblasts expressing wild-type *Torpedo* α, β, and γ subunits form αβγ complexes that are expressed on the cell surface, the complexes bind BuTx, and their expression is stimulated by forskolin treatment (Green et al., 1991b). Thus to test directly the role of γ phosphorylation, we established a cell line expressing α, β, and the γ double mutant, αβ(γ -3). The αβ(γ -3) cell line expressed all three subunits; the subunits assembled into complexes that were expressed on the cell surface, and they bound BuTx (~20 fmols per 60 mm dish). To test their response to forskolin, confluent dishes of cells were treated with NB at 37°C, shifted to 20°C in the presence or absence of forskolin, and cell surface expression was assayed by ^{125}I-BuTx binding. We found that expression of αβ(γ -3) complexes was also stimulated in the presence of forskolin (Table 1). These results strongly suggest that stimulation of AChR assembly by forskolin is not mediated by PKA phosphorylation of γ or δ subunits.

Table 1. Ratio of [^{125}I]BuTx counts (forskolin/control)

Cell line	Ratio (forskolin/control)*
All-11	1.9±0.1 (4)
-1	1.9±0.1 (4)
-2	2.2±0.1 (2)
-3	2.0±0.2 (2)
αβγ(-3)	1.7±0.1 (2)

*Numbers in parentheses represent the number of experiments from which the means ± s.d. were calculated. Each experiment was carried out in triplicate.

Is the forskolin stimulation γ-specific?

The above results demonstrate that forskolin stimulates the assembly of AChR subunits, and further, that the mechanism of action is not through direct PKA phosphorylation of subunits. The forskolin effect is thus indirect, but does it act specifically through one or more subunits? Previously, we reported that α-γ associations in αγ-expressing cells were stimulated by forskolin treatment whereas α-δ associations in αδ cells were not stimulated by this treatment (Green et al., 1991b). These and other results supported the observed correlation between an increase in γ subunit phosphorylation and an increase in assembly efficiency. In a more recent study (Green and Claudio, 1993) designed to delineate the order of subunit assembly, we have been able to isolate AChR assembly intermediates in cells expressing the full complement of subunits and found that an αβγ intermediate is formed very rapidly (<5 minutes after polypeptide synthesis). We demonstrate in that study that δ subunits do not add to these complexes until ~6 hours and that αδ associations do not occur in cells capable of expressing fully functional AChR complexes. Based on several lines of experimentation, we proposed that there is not a unique dimer precursor to αβγ trimers, but rather, αβ, αγ, and βγ all serve as precursors to trimers.

Thus, to address the questions of whether single or multiple subunits are the indirect targets of the forskolin effect, and to identify the subunit(s), we investigated the effect of forskolin on αβ, αγ, and βγ complexes using cells expressing αβ, αγ, and βγδ, respectively. Because the steady-state combination of α and δ subunits binds BuTx, the αδ cell line was also tested for modulation by forskolin. Two methods were used to detect interactions between subunits: (1) label α-containing cell lines with ^{125}I-BuTx, forskolin-treat cells, immunoprecipitate with an antibody directed against the non-α subunit, and quantitate the ^{125}I-BuTx co-precipitated by counting in a γ-counter; or (2) label cells with [^{35}S]methionine, forskolin-treat cells, immunoprecipitate, run the immunoprecipitates on SDS gels, treat for fluorography, and quantitate by scanning the band of the co-precipitated subunit. As shown in Table 2, both coprecipitation

Table 2. Forskolin treatment on partial subunit complexes

Assembly intermediates	[^{125}I]BuTx Counts* (forskolin/control)	[^{35}S]Methionine† (forskolin/control)
αβ	1.2±0.1 (2)	1.0
αγ	1.8±0.2 (2)	1.9
βγ	–	1.8
αδ	0.8±0.2 (2)	n.d.

*Numbers in parentheses indicate the number of experiments from which the means ± s.d. were calculated. Each experiment was carried out in triplicate.

Confluent 10 cm dishes of cells were incubated at 37°C in 20 mM NB media for 24 hours. Cells were shifted to 20°C for 48 hours, lysed in 1% LPC lysis buffer, labeled with [^{125}I]BuTx, immunoprecipitated with antibodies specific to the non-α subunit, and quantitated using a γ-counter.

†Values represent the average of duplicates.

Confluent 10 cm dishes of cells were incubated at 37°C in 20 mM NB media for 24 hours, labeled with [^{35}S]methionine, chased at 20°C for 48 hours, lysed in 1% LPC lysis buffer, immunoprecipitated and analyzed by SDS-PAGE autoradiography. For αβ, αγ, and βγ intermediates, [^{35}S]methionine ratios were obtained by scanning α, α, and β bands coimmunoprecipitated with anti -β, -γ, and -γ specific antibodies, respectively.

n.d. not determined.

of ^{125}I-BuTx-α subunits or coprecipitation of [^{35}S]methionine-labeled subunits, gave similar results. Using either or both methods, the data in Table 2 demonstrate that only complexes composed of γ subunits respond to forskolin treatment. Our conclusion from this series of studies is that the γ subunit is a specific, and indirect target of forskolin treatment.

Is the forskolin stimulation temperature-dependent?

We have shown that assembly of *Torpedo* AChR subunits is temperature-sensitive and that although subunits are synthesized at 37°C, they do not assemble unless the temperature is lowered ~10°C (Claudio et al., 1987; Paulson and Claudio, 1990). To test if subunits fully synthesized at 37°C could assemble when the temperature was lowered to 20°C, we labeled All-11 cells with [^{35}S]methionine for 30 minutes at 37°C, rinsed at 37°C, and incubated them an additional 1 hour at 37°C before shifting to 20°C for 30 hours. This protocol allows sufficient time for labeled subunits to be fully synthesized before assembly is attempted. Using such a protocol, we found that subunits fully synthesized at 37°C then shifted to a permissive temperature, assemble with the same efficiency (20-35%) as those synthesized at 20°C (Jayawickreme, 1993).

The experiments described up to this point (and in previous papers on the forskolin effect from our laboratory) have all used a protocol in which the cells were labeled at 37°C and then shifted to 20°C in the presence or absence of forskolin. Thus the forskolin effect has been on subunits partially or fully synthesized at 37°C. Now that we have shown that the forskolin effect is not through direct phosphorylation of the γ subunit, could the secondary effect we are observing on γ subunits be due to a temperature artifact, such as a 37°C misfolded γ polypeptide being reshaped to a 20°C assembly-competent conformation? To test this idea, we asked if forskolin had the same stimulatory effect on subunits synthesized at 20°C as it did on subunits synthesized at 37°C.

Confluent dishes of αβγ cells were incubated at 37°C in NB for 24 hours, shifted to 20°C, then pulse labeled at 20°C for 1 hour with [^{35}S]methionine. Cells were washed twice with DMEM, one dish of cells was lysed immediately, and the remaining dishes were chased in DMEM containing (10 μg/ml) cycloheximide ± forskolin. After 1, 3, 6, 10 and 26 hours of chase at 20°C, control and forskolin-treated dishes of cells were lysed in 1% LPC buffer. Membrane extracts were incubated with 10 nM ^{125}I-BuTx and immunoprecipitated with mAb 168. Immunoprecipitates were analyzed by ^{125}I counting followed by SDS-PAGE autoradiography (Fig. 7A). Several conclusions can be drawn from this experiment. The assembly efficiency of subunits synthesized at 20°C is enhanced by forskolin treatment. Thus the secondary forskolin effect on γ subunits does not appear to be a mechanism by which the cell can salvage misfolded polypeptides at 37°C and shape them into assembly competent polypeptides. It still may be that the secondary effect is helping to shape the γ subunit, but it does not appear to be a temperature artifact. The forskolin effect is visible as early as 3 hours (Fig. 7A(lanes 4,5),B,C) and since AChR complexes are not formed before ~6 hours (Ross et al., 1991), these results further support our previous findings that forskolin stimulation occurs at the level of subunit assembly.

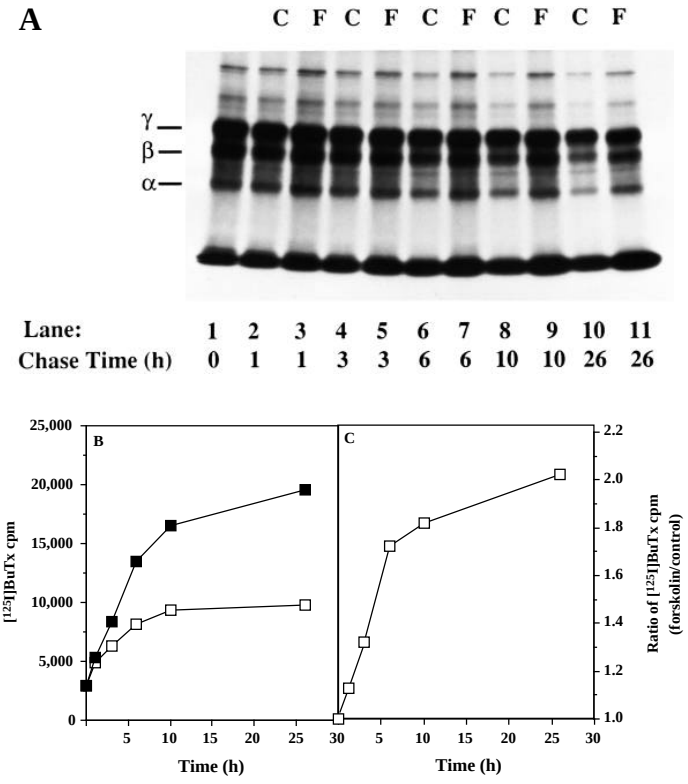

Fig. 7. Forskolin stimulates association of subunits synthesized at 20°C. Confluent 10 cm dishes of αβγ-expressing cells were incubated in 20 mM NB at 37°C for 24 hours. Cells were placed at 20°C and labeled with [^{35}S]methionine (0.3 mCi/dish) for 1 hour, then they were immediately lysed in 1% LPC lysis buffer or chased in 10 μg/ml cycloheximide containing 20 mM NB medium in the presence (lanes 3, 5, 7, 9, 11) or absence (1, 2, 4, 6, 8, 10) of forskolin (100 μM). Cells were lysed at the indicated times, lysates were labeled with 10 nM ^{125}I-BuTx, immunoprecipitated with mAb 168, counted in a γ-counter and analyzed by SDS-PAGE autoradiography. (A) SDS-PAGE analysis (B) ^{125}I-BuTx counts of control (□) and forskolin-treated (■) samples. (C) Ratio of ^{125}I-BuTx counts of forskolin and control-treated samples (□).

The increased efficiency of assembly observed indicates a decreased rate of degradation or an increased rate of association of AChR subunits during assembly.

DISCUSSION

In this study we show that upon forskolin treatment of All-11 cells γ subunits are phosphorylated only on serine residues. Forskolin pretreatment of All-11 cells blocked in vitro phosphorylation of the γ subunit by PKA. We also found that both serines (353, 354) in γ are phosphorylated in vivo. In earlier studies, membranes were isolated from *Torpedo* electric organ, and phosphorylated in vitro by the catalytic subunit of PKA (Yee and Huganir, 1987). Peptide maps of this material showed a single spot, and subtractive manual Edman degradation showed Ser-353 as the site of phosphorylation. However, due to the qualitative nature of their determination, Yee and Huganir could not rule out the possibility that Ser-354 was also phosphorylated. The reason they observed a single spot on phosphopeptide maps and we observe two spots, could be due to variations in the purity or the specific activity of the catalytic subunit of PKA. Another possibility is that the AChRs they isolated from postsynaptic membranes were already phosphorylated on a single site. Thus, during in vitro phosphorylation, only the second site could be phosphorylated, which would give doubly phosphorylated peptides which would run as a single spot.

Our finding that the double mutant was not phosphorylated by forskolin treatment confirms that there are only two PKA sites on the γ subunit. All three mutant cell lines expressed receptors on the cell surface. The amount of AChR expression paralleled the total amount of polypeptide synthesized in the cells (data not shown). Furthermore, the mutant assembled receptors were shown to contain all four subunits. Together, these results indicate that the mutations did not affect AChR assembly or transport. However, despite the removal of phosphorylation sites of γ, AChR expression could still be stimulated by forskolin. To test the hypothesis that phosphorylation of the δ subunit can fulfill the role of γ in mutant AChRs, we established the cell line αβ(γ -3). The observation that forskolin could stimulate the assembly of αβ(γ -3) complexes strongly suggests that forskolin action on subunit assembly does not require PKA phosphorylation of any of the subunits. However, the observation that in cells expressing different combinations of subunits (αβ, αγ, αδ, βγ), only those cells expressing a γ subunit demonstrated increased associations in the presence of forskolin, strongly suggests that forskolin action requires the presence of the γ subunit.

We know that forskolin acts post-translationally (Green et al., 1991a; Ross et al., 1991; this study), and that it stimulates subunit assembly (which occurs in the ER; Ross et al., 1991). As shown in Fig. 7A, αβγ subunits are already associated with one another at 0 hours chase (1 hour label at 20°C) but the forskolin effect is not obvious until ~3-6 hours of chase. We showed in another study that αβγ associates within ~5 minutes of synthesis and that δ subunits do not add to the complex until ~6 hours (Green and Claudio, 1993). The major transformations subunits appear to undergo during this 5 minute to 6 hour period are folding and assembly. Because of the acute temperature sensitivity of *Torpedo* subunits, we investigated the forskolin effect at assembly-permissive and non-permissive temperatures. We showed that subunits labeled at 37°C and fully synthesized at this temperature will assemble when shifted to 20°C (Jayawickreme, 1993), that subunits labeled at 20°C will assemble at 20°C, and that both respond similarly to forskolin treatment. Thus the forskolin effect is not temperature-dependent and it appears not to be a mechanism for reshaping temperature-induced misfolded polypeptides.

A number of proteins appear to be regulated post-translationally by phosphorylation. It was found that phosphorylation of Thr-654 of the EGF receptor is necessary for ligand-independent internalization triggered by phorbol esters. However, site-specific mutagenesis of the phorbol ester-stimulated phosphorylation site in the EGF receptor (Thr-654) did not hinder constitutive or ligand-induced internalization (Lin et al., 1986). Similarly, phorbol ester-induced internalization of the T-cell surface antigen CD4 depends directly on the three serines that are phosphorylated by protein kinase C (Shin et al., 1990). However, in many other cases, such modulations appear not to be due to direct phosphorylation effects. Mutation of the unique phosphorylation site of the transferrin receptor, Ser-24, did not alter ligand uptake or receptor recycling, and further, its redistribution (up regulation of cell surface receptors) upon phorbol ester treatment was independent of phosphorylation at Ser-24 (Davis and Meisner, 1987; McGraw et al., 1988). It was suggested that the effect of phorbol esters may result from a more general perturbation of membrane trafficking. Similarly, phorbol esters induced hyperphosphorylation of human ASGP, and altered its intracellular redistribution (down-regulation of cell surface receptors). However, mutation of all five cytoplasmic serine residues in the ASGP receptor subunits did not affect the redistribution of ASGP receptors by phorbol esters. It was suggested that ASGP receptor redistribution was more likely an indirect effect on membrane traffic (Geffen et al., 1991; Geffen and Spiess, 1992). Similarly, mutation of two cytoplasmic serine residues to alanines of the human mannose 6-phosphate receptor did not alter its steady-state cellular distribution or its cycling and transport functions (Hemer et al., 1993).

Several PKA activators have been shown to regulate or modulate ion channel function and other properties. In the case of CFTR, the activity of the channel was proposed to be regulated by phosphorylation-dephosphorylation of a cluster of PKA sites present in the regulatory domain (Cheng et al., 1991; Rich et al., 1991; Tabcharani et al., 1991). However, when all ten consensus PKA sites were mutated to alanines, the channel activity was still regulated by PKA. It was proposed that phosphorylation on cryptic PKA sites may be responsible for the observed regulation of channel activity by PKA stimulation. However, there was no significant ^{32}P incorporation into the mutant CFTR protein upon in vivo labeling with [^{32}P]orthophosphate (Chang et al., 1993). Another example where channel modulation by PKA was observed is the slowly activating K^+ channel (minK protein). It is an interesting example since this channel, although it does not contain consensus PKA sites, is modulated by PKA (Blumenthal and Kaczmarek, 1992). It was proposed that the observed modulation by PKA is an indirect phosphorylation mechanism that might involve a protein intrinsic to the functional channel (Blumenthal, 1993). Supporting these observations is the evidence that the regulatory subunit of PKA has been localized to sub-

cellular compartments active in endocytosis and recycling of membrane receptors (Griffiths et al., 1990).

It appears that in several of the above examples discussed, the observed effects due to stimulation of kinases were still observed when the consensus phosphorylation sites were removed from the putative target proteins. A plausible explanation for these observations is the involvement of an ancilliary protein(s). The enhanced assembly of AChR caused by forskolin did not appear to require PKA phosphorylation of any of the subunits but rather, it appeared to act through an indirect phosphorylation mechanism. The observed forskolin effect was at the level of subunit assembly, and since AChR assembly occurs sequentially with multiple steps (Green and Claudio, 1993), the effector could be a protein assisting at the level of folding or assembly.

It is evident from a variety of studies that folding in the cell is assisted by at least two classes of proteins. The first class includes conventional enzymes that catalyze specific isomerization steps that may otherwise limit the rate of folding of some proteins. Examples for this class include protein disulfide isomerase (PDI) (Freedman, 1989) and peptidyl prolyl *cis-trans* isomerase (PPIase) (Lang et al., 1987) etc. The second class of proteins 'chaperones' stabilize unfolded or partially folded structures and prevent the formation of inappropriate intra- or interchain interactions. The endoplasmic reticulum (ER) of eucaryotic cells contain BiP (immunoglobulin heavy chain binding protein, Bole et al., 1986), grp94 (Lee et al., 1984) and other uncharacterized proteins (reviewed by Gething and Sambrook, 1992). We are currently investigating the associations of these and other unidentified proteins, with AChR subunits during assembly. Folding and assembly events in the ER have only begun to be understood; the cellular proteins involved are still being identified, the modes of interaction of these proteins are not known, and the functions of already identified proteins such as BiP are still not well defined. Identification of cellular proteins involved in the assembly of AChR subunits may contribute to our understanding of the components involved in the regulation and modulation of heterologous subunit folding and assembly.

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