Induction of Phosphorylation and Cell Surface Redistribution of Acetylcholine Receptors by Phorbol Ester and Carbamylcholine in Cultured Chick Muscle Cells

Anthony Ross,* Mary Rapuano,‡ and Joav Prives*‡

*Department of Anatomical Sciences, and ‡Cellular and Developmental Biology Program, State University of New York at Stony Brook, Stony Brook, New York 11794

Abstract. We have investigated the mechanisms regulating the clustering of nicotinic acetylcholine receptor (AChR) on the surface of cultured embryonic chick muscle cells. Treatment of these cells with the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA), a potent activator of protein kinase C, was found to cause a rapid dispersal of AChR clusters, as monitored by fluorescence microscopy of cells labeled with tetramethylrhodamine-conjugated α-bungarotoxin. The loss of AChR clusters was not accompanied by an appreciable change in the amount of AChR on the surface of these cells, as measured by the specific binding of [^{125}I]Bgt. Analysis of the phosphorylation pattern of immunoprecipitable AChR subunits showed that the γ- and δ-subunits are phosphorylated by endogenous protein kinase activity in the intact muscle cells, and that the δ-subunit displays increased phosphorylation in response to TPA. Structural analogues of TPA which do not stimulate protein kinase C have no effect on AChR surface topography or phosphorylation. Exposure of chick myotubes to the cholinergic agonist carbamylcholine was found to cause a dispersal of AChR clusters with a time course similar to that of TPA. Like TPA, carbamylcholine enhances the phosphorylation of the δ-subunit of AChR. The carbamylcholine-induced redistribution and phosphorylation of AChR is blocked by the nicotinic AChR antagonist d-tubocurarine. TPA and carbamylcholine have no effect on cell morphology during the time-course of these experiments. These findings indicate that cell surface topography of AChR may be regulated by phosphorylation of its subunits and suggest a mechanism for dispersal of AChR clusters by agonist activation.

It is known that surface membranes in many cell types contain localized patches with a macromolecular composition and functional characteristics that are clearly distinguishable from the cell surface in surrounding regions (for examples see references 26 and 27). A well-studied example of a regional cell surface specialization is the motor end plate of skeletal muscle cells (for review see references 20 and 53). A major component of the postsynaptic muscle membrane is the nicotinic acetylcholine receptor (AChR), an integral membrane protein that binds the neurotransmitter acetylcholine and transduces this binding into a change in membrane permeability. Before innervation, AChR is distributed homogeneously on the surface of embryonic muscle cells. After innervation, the entire AChR population becomes concentrated into a small patch of membrane adjacent to the nerve ending (23). Although comprising a focus of current research, the structural mechanisms responsible for the formation and maintenance of high density AChR aggregates are not adequately understood. Recent studies have shown that AChR clusters are attached to the detergent resistant cytoskeleton (48, 49, 57), immobilized in the plane of the membrane (4, 57), and spatially associated with cytoskeletal structures enriched in actin, vinculin, α-actinin, and other proteins (8–10, 44).

Since AChR cell surface topography is under dynamic control in cultured muscle cells, this system has proven advantageous for studying the regulation of AChR surface distribution. As in the intact system, innervation of muscle cells in culture triggers the redistribution of existing surface AChR into high density clusters subjacent to nerve endings (3, 20). Even in aneurally cultured embryonic muscle cells, surface AChR spontaneously aggregates to form high density clusters (3, 24, 54, 60). Rapid AChR clustering can be induced by extracts of neuronal cells (17, 32, 40, 41, 45, 46, 48, 54). In addition, AChR clustering is reversible: rapid dispersal of clusters has been observed upon depletion of extracellular Ca^{2+} (6, 15), addition of the metabolic inhibitor sodium azide (5), or exposure to the AChR activator carbamylcholine (7, 11).

Tumor-promoting phorbol esters such as 12-0-tetradecanoylphorbol-13-acetate (TPA) have been shown to exert marked effects on plasma membrane organization and cytoskeletal properties (12, 34, 55). These agents are thought to act by binding to a specific intracellular receptor, Ca^{2+}-phospho-

lipid-dependent protein kinase (protein kinase C) and stimulating the phosphorylation of its protein targets (16, 38). There is evidence that protein kinase C can modulate functional properties of several types of surface receptors including AChR (1, 22, 38).

In the present study, we have examined the effects of phorbol esters and the AChR activator carbamylcholine on the surface distribution and the phosphorylation state of AChR. We have observed that both treatments trigger the redistribution of surface AChR from cluster domains, and that in both cases the rapid dispersal of clustered AChR is associated with an increase in AChR phosphorylation. These findings indicate that AChR topology is influenced by protein phosphorylation and suggest a mechanism for the regulation of AChR topology.

Materials and Methods

Reagents

$^{32}P_i$ was purchased from ICN Radiochemicals (Irvine, CA). [^{125}I]α-bungarotoxin (Bgt)[1] and ^{125}I-protein A were purchased from New England Nuclear (Boston, MA). Protein A-Sepharose was purchased from Pharmacia Biotechnology, Inc. (Piscataway, NJ). Tetramethylrhodamine was purchased from Kramer Labs (New York, NY). Chemicals for PAGE and Western blotting were obtained from Bio-Rad Laboratories (Richmond, CA). α-bungarotoxin was obtained from Boehringer-Mannheim (Indianapolis, IN). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures

Myogenic cells were isolated from breast muscle tissue of 12-d chick embryos (50). Cells were plated on collagen-coated culture dishes at an initial density of 1.8×10^6 cells/60-mm culture dish. Cultures were grown in DME, supplemented with 10% horse serum and 2% chick embryo extract at 37°C in an atmosphere of 92% air/8% CO_2.

Embryonic chick brain extract (BE) prepared from brains of 12-d chick embryos as described (32), was added to cultures to stimulate the formation of surface clusters of AChR. This extract which contained ~5 mg/ml protein was stored at −70°C until use, and added to cultures at a dilution of 10% at 1-d and 2-d postplating. Both the amount of surface AChR and the number of clusters were enhanced 2–3 fold in BE-treated cultures relative to untreated cells. TPA was prepared as a 100 μg/ml stock in DMSO, and added directly to cultures at a final concentration of 100 nM. The equivalent concentration of DMSO was added to control cultures and had no effect on AChR cluster stability or $^{32}P_i$ incorporation.

Surface-labeling of AChR

AChR on the surface of intact cultured muscle cells was monitored by the binding of α-Bgt, as described previously (47, 50). For localization of AChR clusters by fluorescence microscopy, Bgt conjugated to tetramethylrhodamine (TMR-Bgt, 51) was used. [^{125}I]Bgt was used for quantification of surface AChR. Cultures were washed once in DME and incubated with TMR-Bgt or [^{125}I]Bgt (10^{-8} M) in DME containing BSA (1 mg/ml) for 1 h at 37°C. The labeling period was terminated by five washes with 3 ml vol of DME to remove unbound toxin. Nonspecific binding was established by measuring [^{125}I]Bgt binding in the presence of the competitive inhibitor decamethonium (20 μM) as described (50), and did not exceed 5% of total labeling. The degradation rate of AChR was measured with [^{125}I]Bgt-labeled cultures by monitoring the release of ^{125}I into the medium (21). Radioactivity was measured by dissolving the cells in 1% Triton X-100 in 1 N NaOH and gamma counting.

Fluorescence Microscopy

Cell surface distribution of AChR was visualized by labeling cultured muscle cells with TMR-Bgt and inspecting the fixed cultures with a Zeiss photomicroscope equipped with epi-illumination and the appropriate excitation and emission filters. After TMR-Bgt labeling was carried out as described above, cells were fixed with 3.7% formaldehyde in PBS for 20 min on ice, rinsed with water, and mounted with aquamount. Nonspecific staining in the presence of decamethonium (10^{-5} M) yielded very low background fluorescence. As described previously by ourselves (49) and others (3–8, 42, 53), AChR clusters are easily distinguished in TMR-Bgt-labeled cultures as discrete patches of intense fluorescence. These can be present on either the upper or the lower surface of myotubes, and are consistent in appearance and dimensions, displaying a circular to elliptical shape with an average area of ~35–55 μm^2 and a diameter of 15–20 μm. For each determination, clusters on myotubes in 50 randomly-selected fields were viewed by fluorescence microscopy and counted.

Preparation of Antisera

Rabbit antiserum was prepared against the α- and δ-subunits of AChR purified by affinity chromatography from denervated leg muscle of adult chicken as detailed elsewhere (52). AChR subunits were resolved by SDS-PAGE (33) and identified by immunoblotting as described elsewhere (52) and shown in Fig. 1. The specificity of this antiserum was established by immunoblotting to extracts of cultured muscle cells fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose. Under these conditions the anti-δ-antiserum recognized only the δ-subunit and did not react with the other AChR subunits or with other proteins (52).

Labeling, Immunoprecipitation, and Immunoblotting

Cultures were labeled at 37°C with [^{32}P]orthophosphate (2 mCi/ml) in phosphate-free medium for 4 h. Labeling was terminated by two washes with a solution consisting of 150 mM NaCl, 10 mM Tris, pH 7.4, 2 mM EGTA, and 2 mM EDTA, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, 10 μg/ml leupeptin, 10 mM *n*-ethylmaleimide, 50 mM sodium fluoride, 40 mM sodium pyrophosphate, 20 mM potassium phosphate, 10 mM sodium molybdate, and 1 mM sodium orthovanadate (Buffer A). These washes and subsequent steps were carried out at 4°C. Cells were scraped with a rubber policeman, pelleted by centrifugation, and extracted for 30 min in Buffer A containing 0.5% Triton X-100 (a procedure that solubilizes total cellular AChR as monitored in [^{125}I]Bgt-labeled cultures). The extracts were clarified by centrifugation at 100,000 *g* for 30 min. The supernatants were incubated with anti-δ antisera for 3 h, then with Protein A-Sepharose beads for 1 h. The precipitates were washed five times with Buffer A and resuspended in 50 μl of SDS sample buffer. After incubation for 10 min at room temperature the beads were centrifuged and the supernatants were fractionated on 10% SDS-polyacrylamide gels according to Laemmli (33). The gels were stained with Coomassie Brilliant Blue, destained and exposed to Kodak XAR-5 X-ray film for 24–48 h. For immunoblotting experiments, AChR was purified by batchwise affinity chromatography on cobra toxin-sepharose. Samples were fractionated by SDS-PAGE and the proteins were transferred to nitrocellulose paper and visualized by labeling with the specified antisera and ^{125}I-protein A.

Results

Cultured embryonic chick muscle cells that were treated with BE displayed prominent surface clusters of AChR at 5-d postplating, as visualized by the fluorescence staining of intact cells with TMR-Bgt (Figs. 1 *A* and 2 *A*). Shown in Figs. 1 *B* and 2 *B* are similar fields after incubation for 10 h in growth medium containing 100 nM TPA. As shown, this TPA treatment resulted in a marked decrease in the number of AChR clusters. This effect was not accompanied by a significant change in cell morphology within the time course of these experiments (Figs. 1 and 2). Surface levels of AChR, determined by the specific binding of [^{125}I]Bgt, did not decrease significantly during TPA incubation periods sufficient to cause marked cluster loss (Table I). During an 8-h exposure to TPA, AChR levels decreased by less than 15% (Table I), whereas the number of AChR clusters decreased by greater than 70% (Fig. 3 *A*). This suggests that TPA-induced cluster loss reflects a lateral redistribution of cell surface

1. *Abbreviations used in this paper*: BE, chicken embryo brain extracts; Bgt, α-bungarotoxin; TMR, monotetramethylrhodamine.

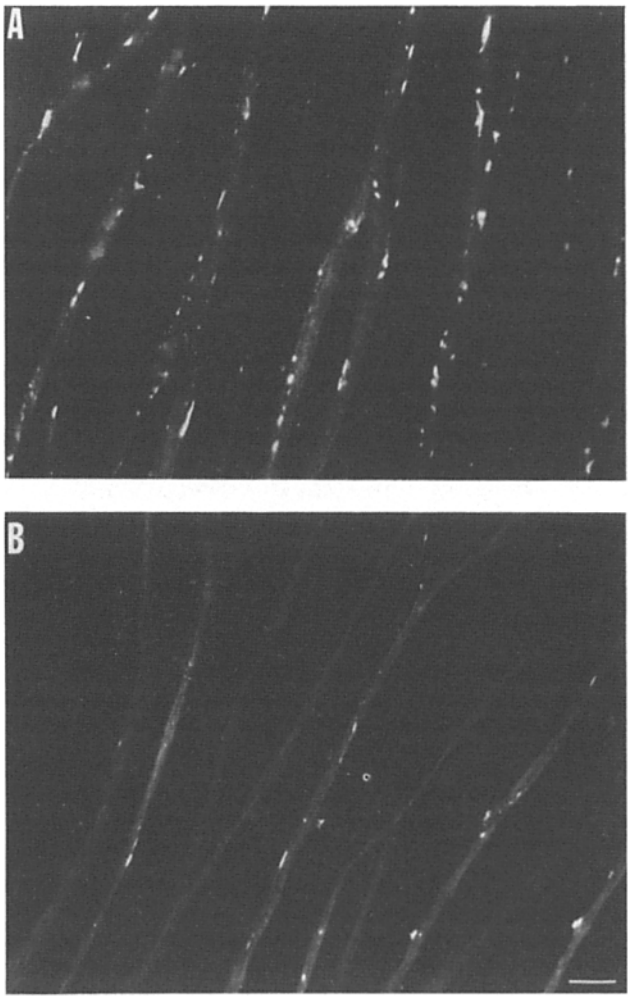

Figure 1. Effect of TPA on distribution of AChR on the surface of cultured muscle cells. Cells were stained with TMR-Bgt and fixed as described in Materials and Methods. (*A*) Fluorescent image of untreated cultures on which numerous AChR clusters are visible. (*B*) Cultures after a 10-h exposure to TPA (100 nM). Note the decrease in number of AChR clusters. Bar, 50 μm.

AChR. Supporting this possibility is our observation that the dispersal of AChR clusters in TPA-treated cultures is frequently accompanied by an increase in diffuse TMR-Bgt labeling.

The loss of AChR clusters caused by exposure of myotubes to 100 nM TPA was quantified by counting the number of clusters per myotube at increasing times after TPA addition.

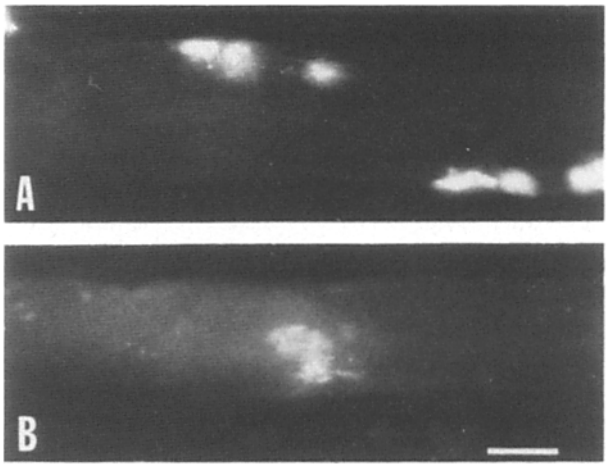

Figure 2. AChR cluster dispersal induced by TPA. (*A*) Fluorescent image of TMR-Bgt-labeled untreated myotube. (*B*) Myotube from a replicate culture after a 6-h exposure to TPA (100 nM). Bar, 10 μm.

When these data were plotted on a semilogarithmic scale (Fig. 3 *A*) and analyzed as described (56), the points were well fit by a simple linear regression. In addition, an analysis of residual autocorrelation (13) shows that a linear model of these data is appropriate. The loss of AChR clusters from the surface of myotubes exposed to TPA occurs with a half-time of ~4.5 h, as obtained from the slope of the linear plot (Fig. 3 *A*). In contrast, no time-dependent loss of AChR clusters was observed in replicate cultures that were treated with 100 nM phorbol-12,13-diacetate, a structural analogue of TPA that is biologically inactive (12, 16). A line drawn with the aid of a computer to fit the points representing the number of clusters per myotube at different times of exposure to phorbol-12,13-diacetate (Fig. 3 *A*, open circles) gave a slope not significantly different from zero ($P > 0.6$). Moreover, we observed that dioctanylglycerol (0.1 mM), a cell-permeable diacylglycerol that is structurally different than TPA yet is an efficient activator of protein kinase C in cultured cells (19), mimics TPA in causing the time-dependent dispersal of AChR clusters (not shown). These data support a role for protein kinase C in AChR cluster dispersal.

Exposure to the AChR activator carbamylcholine was reported to cause dispersal of AChR clusters from membranes of cultured rat myotubes (7) as well as at developing rat neuromuscular junctions (11). We have found that carbamylcholine also causes the declustering of cell surface AChR in chick muscle cultures, and have compared this action of carbamylcholine with the effects of TPA. BE-treated

Table I. Effects of TPA and Carbachol on AChR Levels in Cultured Muscle Cells

	[^{125}I]Bgt binding	Percentage of control	
Incubation time	*0.5 h*	*3.0 h*	*8.0 h*
Experimental condition			
TPA	102 ± 3.46 (6)	88 ± 1.0 (3)	88 ± 2.74 (6)
Carbachol	93 ± 5.66 (6)	87 ± 3.32 (5)	77 ± 8.67 (6)

Cultures were incubated at 37°C in growth medium with or without TPA (100 nM) or carbamylcholine (1 mM) for the time specified. The cultures were then washed extensively and labeled with [^{125}I]Bgt (10^{-8} M) for 1 h at 37°C as described in Materials and Methods. Values represent mean ± SD, followed by the number of determinations, in parentheses.

5d myotubes were exposed to 1 mM carbamylcholine for increasing intervals, then washed five times with fresh medium to remove carbamylcholine and labeled with TMR-Bgt. The time-dependence of AChR cluster dispersal induced by carbamylcholine is shown in Fig. 3 *B*. As in the case of TPA-induced cluster dispersal (Fig. 3 *A*), the data were adequately fit by a simple linear regression determined by computer analysis. Upon comparison of the slopes of the linear plots of AChR cluster dispersal induced by TPA and carbamylcholine, an analysis of covariants indicates that the 2 slopes are not significantly different from each other (the common slope is 0.131; $P > 0.58$). Since the slope corresponds to the rate constant, these results indicate that TPA and carbamylcholine induce AChR cluster dispersal at similar rates, with similar half times of 4–5 h.

The marked loss of AChR clusters induced by carbamylcholine was found not to be associated with significant morphological changes showing that the loss of clusters is not a secondary consequence of altered cell shape, in agreement with previous findings (7). The carbamylcholine-induced dispersal of AChR clusters is accompanied by a small decrease in surface labeling by [^{125}I]Bgt (Table I). After 30 min in carbamylcholine, [^{125}I]Bgt levels are reduced by 5–10%, possibly due to persistent presence of carbamylcholine acting as a competitive inhibitor of Bgt binding. Over the next 7.5 h in carbamylcholine surface [^{125}I]Bgt labeling decreases an additional 10–15%. A carbamylcholine-induced decline of AChR has previously been shown to occur in mouse (39) and chick (25) muscle cells. Carbamylcholine-induced cluster loss, then, may result from either a lateral redistribution of AChR from clustered to diffuse areas, as happens with TPA, or by an internalization of AChR which results in a loss of surface AChR clusters. The first possibility is supported by our observation that the time course of cluster loss is similar in TPA and carbamylcholine-treated cultures (Fig. 3). To test the possibility that cluster dispersal reflects the internalization of cell surface AChR, cultures were labeled for 1 h with TMR-Bgt, and subsequently incubated at 37°C for 8 h before cell fixation and visualization of AChR by fluorescence microscopy. During this interval labeled cell surface AChR was diminished ~30% due to normal internalization and degradation. Although under these conditions the decrease in labeled cell surface AChR exceeds the decrease observed after an 8-h incubation in carbamylcholine, the number of clusters was not decreased (not shown). The loss of clusters induced by carbamylcholine or TPA was not associated with a change in metabolic degradation rates of AChR (not shown). Although it is possible that clustered AChR is selectively internalized in carbamylcholine-treated cultures, we have no evidence that this occurs.

To determine if the induction of AChR cluster dispersal by carbamylcholine was related to the direct action on AChR of this agonist, these experiments were repeated in the presence of the competitive inhibitor d-tubocurarine (*open circles* in Fig. 3 *B*). As shown, the presence of d-tubocurarine prevented the dispersal by carbamylcholine of surface AChR clusters. Thus carbamylcholine must bind to AChR to cause cluster dispersal, in agreement with recent findings in rat myotubes (7), showing that carbamylcholine caused AChR cluster loss via the activation of AChR.

Recent evidence indicates that TPA exerts its effects by activation of protein kinase C at the surface membrane (38).

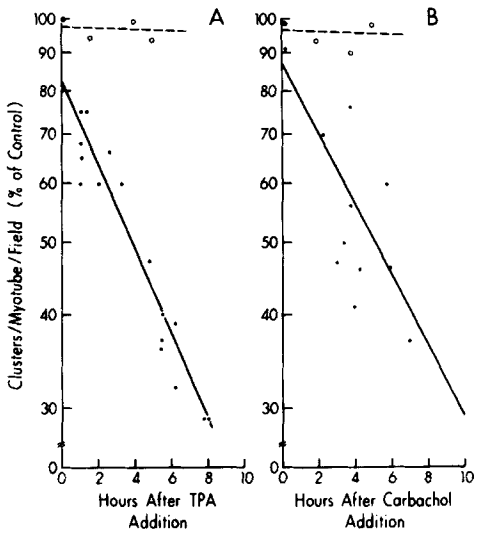

Figure 3. The time course of AChR cluster loss in the presence of TPA and carbamylcholine. (*A*) Cultures were incubated with 100 nM TPA (*closed circles*) or 100 nM phorbol-12,13-diacetate (*open circles*) for the indicated times. (*B*) Cultures were incubated for the indicated times with 1 mM carbamylcholine (*closed circles*) or with 1 mM carbamylcholine plus 1 mM d-tubocurarine after a 20-min preincubation with d-tubocurarine alone (*open circles*). Cultures were stained with TMR-Bgt and fixed, and clusters per myotube were counted as described in Materials and Methods. Lines were drawn with the aid of a computer as described in Materials and Methods.

In doing so, TPA substitutes for the endogenous activator of this enzyme, diacylglycerol, that is transiently produced through phospholipase C-catalyzed hydrolysis of phosphatidylinositol bisphosphate (38). This pathway is activated by a number of hormones and neurotransmitters and constitutes a mechanism for transmembrane signal transduction (38). Recent findings indicate that activation of nicotinic AChR in cultured muscle may stimulate this pathway and cause activation of protein kinase C (1, 22). AChR from *Torpedo* has been reported to be a substrate for protein kinase C (14, 30). These considerations led us to investigate the possibility that TPA and carbamylcholine induce AChR phosphorylation in intact muscle cells.

Cultures were labeled with [^{32}P]orthophosphate, extracted, and immunoprecipitated with antisera directed against the δ-subunit of chick muscle AChR as detailed under Materials and Methods. The immunoprecipitates were fractionated by SDS-PAGE and $^{32}P_i$-labeled polypeptides were visualized by autoradiography. The fractionation pattern of AChR subunits was established by immunoblotting as described elsewhere (52). As shown in Fig. 4 *A*, the α-subunit was identified in immunoblots as a 40-kD peptide by binding of ^{125}I[Bgt] (lane *1*) and anti-α-subunit antiserum (lane *2*). Similarly, the δ-subunit was identified by the binding of anti-δ-subunit antiserum (lane *3*). We have observed that two phosphorylated AChR peptides, the δ-subunit (M_r = 55 kD) and the γ-subunit (M_r = 50 kD), are immunoprecipitated by Bgt-anti Bgt (Fig. 4 *B*, lane *1*) and mAb 35 (Fig. 4 *B*, lane *3*), as well as by other anti-AChR antisera. The γ-subunit is coimmunoprecipitated under nondenaturing con-

ditions by several anti-AChR antisera, and its identification as γ- (rather than β-) is more tentative (52).

As can be seen in Fig. 5, a 30-min exposure of the intact myotubes to either 100 nM TPA (lane *2*) or 1 mM carbamylcholine (lane *3*) resulted in a dramatic increase in phosphorylation of the δ-subunit (*upper arrow*). This subunit, in contrast to the γ-subunit, contains a potential phosphorylation site for protein kinase C (30, 36). In addition, a slight stimulation of γ-subunit phosphorylation was seen in carbamylcholine treated cells (lane *3*). The increased phosphorylation of the δ-subunit was detected within 5–10 min of addition of carbamylcholine or TPA (not shown). The effect of TPA on AChR phosphorylation was reproduced by the cell-permeable diacylglycerol dioctanoylglycerol, while no change in phosphorylation state was observed when cells were treated with the inactive phorbol ester phorbol-12,13-diacetate (not shown). Moreover, the presence of d-tubocurarine was found to prevent the enhanced phosphorylation induced by carbamylcholine. Thus, AChR cluster dispersal by activators of either protein kinase C or AChR is accompanied by increased AChR subunit phosphorylation.

Discussion

Several lines of evidence suggest that the TPA-induced AChR cluster dispersal and the increased AChR phosphorylation observed in the present study both result from activation of protein kinase C. Cluster dispersal is not induced by phorbol diacetate (Fig. 3 *A*), a structural analogue of TPA that is ineffective as a tumor promoter and does not activate protein kinase C (16). Conversely, the cell permeable diacylglycerol dioctanylglycerol, a potent activator of protein kinase C (19) that is structurally distinct from phorbol esters, mimics the actions of TPA on both the surface distribution and phosphorylation of AChR. Moreover, the effects of TPA on both AChR phosphorylation and cell surface distribution are exerted rapidly. Increased phosphorylation can be detected within 5 min of TPA addition, and the decrease in the number of clusters per myotube is significant within 30 min. In addition, the dispersal of individual receptors may occur considerably earlier than the measurable loss of AChR clusters. Thus the time course of phosphorylation and cluster dispersal is consistent with a mechanism by which phosphorylation induces AChR redistribution.

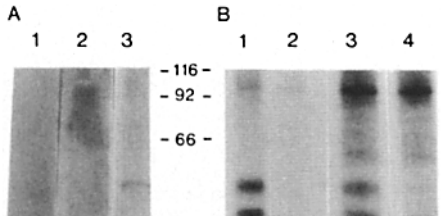

Figure 4. Identification and phosphorylation of AChR subunits in cultured chick muscle cells. (*A*) Overlay of blots of partially purified AChR from cultured chick muscle with [^{125}I]Bgt (lane *1*), anti-α-subunit antiserum (lane *2*), and anti-δ-subunit antiserum (lane *3*). Bound immunoglobulins in lanes *2* and *3* were visualized by ^{125}I-protein A autoradiography. (*B*) $^{32}P_i$-labeled cultures extracted and immunoprecipitated with Bgt anti-Bgt (lane *1*); Bgt anti-Bgt using anti-Bgt which was preabsorbed with free Bgt (lane *2*); mAb35 (lane *3*); mAb35 preabsorbed with purified *Torpedo* AChR (lane *4*). Note that the δ- and γ-subunits are visible as phosphoproteins of 55- and 50-kD, respectively.

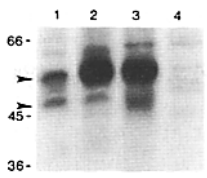

Figure 5. Effects of TPA and carbamylcholine on phosphorylation of AChR subunits. Cultures were labeled at 37°C with [^{32}P]-orthophosphate (2 mCi/ml) in phosphate-free medium for 4 h. Cultures were exposed to 100 nM TPA or 1 mM carbamylcholine for the final 30 min, then rinsed and extracted as described in Materials and Methods. The extracts were immunoprecipitated with antisera against δ-subunit, and immunoprecipitates were resolved by SDS–PAGE and radioautography. (Lane *1*) control; (lane *2*) TPA; (lane *3*) carbamylcholine; (lane *4*) preimmune serum. Upper arrow shows δ-subunit. Lower arrow indicates γ-subunit.

There is evidence that the regulation of AChR aggregation resides in interactions between surface AChR and proteinaceous structures adjacent to the lipid bilayer, including the cytoskeleton and the extracellular matrix. There is a good possibility that AChR surface topology is controlled via interactions with the cytoskeleton. AChR in clusters is selectively anchored to the detergent-insoluble cytoskeletal framework (48, 49, 57), laterally immobile (4, 58) and spatially associated with underlying cytoskeletal foci displaying elevated concentrations of actin (10), vinculin (8), α-actinin (9), and a postsynaptic 43-kD protein (44). Electron microscopic studies have shown that filamentous components of the cytoskeleton directly underlie AChR aggregates in cultured muscle (43), *Torpedo* electroplax (28), and neuromuscular junctions (29). TPA treatment of cultured kidney cells has been reported to cause the rapid redistribution of submembrane actin, α-actinin and vinculin (34, 55). Thus, it is possible that AChR cluster dispersal that we have observed in TPA-treated myotubes is a consequence of the reorganization of these submembranal cytoskeletal elements triggered by phorbol ester. The protein kinase C–catalyzed phosphorylation of myosin was shown to inhibit myosin–actin interaction (37) and consequently could induce reorganization of microfilament based cytoskeletal structures involved in anchorage of aggregated AChR.

An alternative possibility is that the dispersal of AChR clusters by TPA is a more direct consequence of the stimulated phosphorylation of AChR which we have observed to occur under these conditions (Fig. 5). Based on the location of potential phosphorylation sites for several protein kinases including protein kinase C on a putative cytoplasmic domain of AChR subunits, Browning et al. (14) have suggested that phosphorylation could regulate cytoskeletal associations and clustering of AChR. It is noteworthy that the δ-subunit of chick AChR possesses a potential phosphorylation site for protein kinase C (30, 36) and it is this subunit which has enhanced phosphorylation in response to TPA.

A major finding of this study is that carbamylcholine, an AChR activator, causes a marked elevation of AChR phosphorylation. This observation supports a role for phosphorylation in AChR autoregulatory mechanisms, as has been suggested by recent findings implicating phosphorylation in

modulating AChR desensitization by activators (2, 22, 31, 35). Our present findings indicate that AChR activators may influence AChR surface topography through phosphorylation–dephosphorylation mechanisms. The ability of the competitive inhibitor d-tubocurarine to block both AChR phosphorylation and cluster dispersal induced by carbamylcholine indicates that these effects are directly initiated by agonist action. Recent evidence documents the activation of second messengers by nicotinic AChR agonists. Nicotinic stimulation of AChR has been shown to rapidly activate protein kinase C in chromaffin cells (59). In addition, in cultured muscle cells carbamylcholine activation of AChR has been observed to stimulate phosphatidylinositol turnover (1), a physiological pathway that leads to protein kinase C activation (38).

The present study demonstrates that both TPA and the receptor agonist carbamylcholine cause elevation of AChR phosphorylation and cluster dispersal in cultured muscle. These findings indicate that cellular phosphorylation mechanisms can modulate AChR surface distribution.

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