Immobilization of Nicotinic Acetylcholine Receptors in Mouse C2 Myotubes by Agrin-induced Protein Tyrosine Phosphorylation

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Abstract. Agrin induces the formation of highly localized specializations on myotubes at which nicotinic acetylcholine receptors (AChRs) and many other components of the postsynaptic apparatus at the vertebrate skeletal neuromuscular junction accumulate. Agrin also induces AChR tyrosine phosphorylation. Treatments that inhibit tyrosine phosphorylation prevent AChR aggregation. To examine further the relationship between tyrosine phosphorylation and receptor aggregation, we have used the technique of fluorescence recovery after photobleaching to assess the lateral mobility of AChRs and other surface proteins in mouse C2 myotubes treated with agrin or with pervanadate, a protein tyrosine phosphatase inhibitor. Agrin induced the formation of patches in C2 myotubes that stained intensely with anti-phosphotyrosine antibodies and within which AChRs were relatively immobile. Pervanadate, on the other hand, increased protein tyrosine phosphorylation throughout the myotube and caused a reduction in the mobility of diffusely distributed AChRs, without affecting the mobility of other membrane proteins. Pervanadate, like agrin, caused an increase in AChR tyrosine phosphorylation and a decrease in the rate at which AChRs could be extracted from intact myotubes by mild detergent treatment, suggesting that immobilized receptors were phosphorylated and therefore less extractable. Indeed, phosphorylated receptors were extracted from agrin-treated myotubes more slowly than nonphosphorylated receptors. AChR aggregates at developing neuromuscular junctions in embryonic rat muscles also labeled with anti-phosphotyrosine antibodies, suggesting that tyrosine phosphorylation could mediate AChR aggregation in vivo as well. Thus, agrin appears to induce AChR aggregation by creating circumscribed domains of increased protein tyrosine phosphorylation within which receptors become phosphorylated and immobilized.

ORMATION of the vertebrate skeletal neuromuscular junction involves the localized accumulation of a variety of cytoplasmic, membrane, and extracellular matrix–associated proteins in the postsynaptic muscle cell. The accumulation of several such components, including the nicotinic acetylcholine receptor (AChR)[1], is induced by the protein agrin (McMahan, 1990; Reist et al., 1992), apparently by binding to a receptor on the muscle cell surface (Nitkin et al., 1987). Recent experiments on chick myotubes in culture demonstrate that agrin activates a protein tyrosine kinase, causing a rapid increase in tyrosine phosphorylation of the AChR β subunit and a decrease in the rate at which AChRs are extracted from intact myotubes by mild detergent treatment (Wallace et al., 1991; Wallace, 1994, 1995). Inhibitors of agrin-induced tyrosine phosphorylation prevent the agrin-induced de-

crease in AChR detergent extractability and block AChR aggregation. These and other findings suggest that agrin-induced protein tyrosine phosphorylation strengthens the interaction of AChRs with the cytoskeleton and that this process plays a role in the formation of AChR aggregates (Wallace, 1995).

A characteristic feature of agrin-induced AChR aggregation is that it is highly localized. AChR aggregates form selectively at sites where myotubes come into contact with cells or surfaces coated with agrin (Campanelli et al., 1991; Tsim et al., 1992; Ferns et al., 1992, 1993), or with agrin on axon terminals or in basal lamina (McMahan and Slater, 1984; Cohen and Godfrey, 1992; Reist et al., 1992; Cohen et al., 1995). This suggests that if increases in protein tyrosine phosphorylation induced by the interaction of agrin with its receptor play a role in AChR aggregation, then such increases are likely to be confined to the vicinity of the activated agrin receptor.

The aim of the experiments reported here was to understand how agrin-induced changes in tyrosine phosphorylation and AChR detergent extractability could lead to AChR aggregation. A simple explanation for the formation of AChR aggregates would be a modification of the

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1. *Abbreviations used in this paper:* AChR, nicotinic acetylcholine receptor; sConA, succinyl-Con A; TCA, trichloroacetic acid.

diffusion-trap model originally proposed by Edwards and Frisch (1976). According to this scheme, if agrin-induced increases in protein tyrosine phosphorylation and decreases in AChR detergent extractability resulted in immobilization of AChRs and if such changes were restricted to certain regions of the myotube, then AChRs, which are normally free to diffuse laterally in the plane of the membrane, would gradually accumulate within such regions, creating receptor aggregates. Myotubes appear to have a relatively small number of receptors for agrin scattered over their surface (Nastuk et al., 1991). At developing neuromuscular junctions, contact with agrin is restricted to the synaptic site, and so AChR aggregation would occur there. When agrin is added to the medium bathing myotubes in culture, as in the experiments reported here, each activated agrin receptor might stimulate protein tyrosine kinases in its immediate vicinity, thereby creating small patches scattered over the myotube surface within which AChRs would accumulate (Wallace, 1995).

To test the hypothesis that agrin creates regions of increased tyrosine phosphorylation which act as traps for AChRs, we have examined changes in AChR tyrosine phosphorylation, lateral mobility, and detergent extractability. The mobility of AChRs and other proteins on the surface of cultured mouse C2 myotubes was measured using the technique of fluorescence recovery after photobleaching (Axelrod et al., 1976; Dubinsky et al., 1989). This technique has been used previously to demonstrate that most randomly distributed receptors are relatively free to diffuse laterally in the lipid bilayer, while within aggregates most AChRs are relatively immobile. We found that in C2 myotubes agrin induced the formation of patches of increased tyrosine phosphorylation within which AChRs were relatively immobile. On the other hand, pervanadate, an inhibitor of protein tyrosine phosphatases (Pumiglia et al., 1992), caused an increase in tyrosine phosphorylation and a decrease in AChR mobility throughout the myotube. Both agrin and pervanadate caused a decrease in the rate at which AChRs were extracted from intact C2 myotubes by mild detergent treatment, as previously reported for chick myotubes (Wallace, 1995). In agrin-treated C2 myotubes phosphorylated receptors were extracted more slowly than nonphosphorylated receptors. These observations are consistent with the hypothesis that agrin induces the formation of domains of increased tyrosine phosphorylation within which AChRs become phosphorylated and immobilized as a consequence of interacting more strongly with the cytoskeleton. Preliminary accounts of some of these results have been published previously (Perez, G. M., T. Meier, and B. G. Wallace. 1994. *Soc. Neurosci. Abstr.* 20:867).

Materials and Methods

C2C12 Mouse Cell Line

C2C12 cells (Yaffe and Saxel, 1977; Blau et al., 1983) were grown in DME (GIBCO BRL, Gaithersburg, MD) containing 20% FBS (GIBCO BRL), 0.5% chick embryo extract, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gemini Products, Calabasas, CA) on 35-mm tissue-culture dishes (Gordon and Hall, 1989). As the cells approached confluence, the cultures were changed to fusion medium, consisting of DME-high glucose (Sigma Chemical Co., St. Louis, MO) supplemented with 5%

horse serum (GIBCO BRL), glutamine and antibiotics, to induce differentiation. Cultures were used after 4–6 d in fusion medium, by which time cells had fused to form myotubes. For detergent extraction experiments cells were grown on collagen-coated dishes. During incubations with agrin or pervanadate, the medium was changed to DME-F12 (Sigma Chemical Co.) supplemented with 0.8 mM CaCl₂, 1 mg/ml bovine serum albumin (RIA Grade, Sigma Chemical Co.), 20 μg/ml conalbumin (type II; Sigma Chemical Co.), glutamine and antibiotics (Wallace, 1989).

Agrin and Pervanadate

Experiments were carried out with a saturating dose (4U) of partially purified agrin (Cibacron pool) prepared from electric organ of *Torpedo californica* as previously described (Nitkin et al., 1987). In most experiments cultures were treated with agrin for 4 h, by which time agrin-induced changes in AChR aggregation, phosphorylation, and detergent extractability had reached a maximum. Sodium pervanadate was prepared by adding 1 part of 500 mM H₂O₂ to 50 parts of 10 mM sodium orthovanadate (Sigma Chemical Co.) in modified Tyrodes solution, incubating the mixture for 10 min at room temperature, and diluting it into defined medium immediately before use (Pumiglia et al., 1992; Wallace, 1995). Cultures were routinely treated with 30 μM pervanadate for 1 h, which produced a maximal response.

Quantitation of AChR Aggregation

C2 myotubes were labeled with 2×10^{-8} M rhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR), rinsed, and fixed as described for chick myotubes (Wallace 1989, 1992). For each culture, AChR aggregates on 10 myotubes were counted by eye with a 40× objective on a Nikon Optiphot microscope equipped for phase and fluorescence microscopy.

Immunohistochemistry

C2 myotubes were labeled with rhodamine-conjugated α-bungarotoxin as described above, rinsed with PBS, fixed, and permeabilized for 5 min with –20°C 95% ethanol, rinsed with Tris buffered saline (TBS), incubated 1 h with 1:1,000 anti-phosphotyrosine mAb PY20 (ICN ImmunoBiologicals, Costa Mesa, CA) in TBS containing 0.05% Triton X-100 (TBS-T), rinsed in TBS, incubated 1 h with 1:200 fluorescein (DTAF)-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA) in TBS-T containing 10% normal goat serum, rinsed, dehydrated, and mounted in AF1 (Citifluor, Ltd., London). No staining was observed with anti-phosphotyrosine antibodies unless myotubes were first permeabilized, indicating that the phosphotyrosine-containing proteins were predominately intracellular.

Diaphragms were removed from E18 rat embryos, rinsed in PBS, fixed in 1% paraformaldehyde dissolved in 90 mM sodium phosphate, pH 7.2, containing 160 mM sucrose, rinsed with TBS containing 0.5% Triton X-100 (TBS-TT) and incubated 1 h in anti-phosphotyrosine monoclonal antibody PY20 or mAb 4G10 (Upstate Biotechnology Incorporated, Lake Placid, NY) diluted 1:2,000 in TBS-TT. The tissue was washed 3×10 min in TBS-TT, incubated 1 h in 1:300 fluorescein-conjugated goat anti–mouse IgG in TBS-TT containing 3% BSA, 10% normal goat serum and 2×10^{-6} M rhodamine-conjugated α-bungarotoxin, rinsed 4×15 min TBS-TT, and mounted in Citifluor AF1.

Measurement of AChR Phosphorylation

C2 cultures were rinsed with minimum essential medium without sodium phosphate (GIBCO BRL) supplemented with 1 mg/ml bovine serum albumin (RIA grade, Sigma Chemical Co.), 20 μg/ml conalbumin (type II, Sigma Chemical Co.), 100 U/ml penicillin, and 100 μg/ml streptomycin, followed by a 4- or 16-h incubation in the same medium containing 0.5 mCi/ml [³²P]orthophosphate ([³²P]H₃PO₄ in H₂O, ICN Biomedicals Inc.). A 4-h incubation was sufficient to label phosphoproteins to steady state levels. Biotinylated α-bungarotoxin (4×10^{-8} M, Molecular Probes, Inc.) was added for the final hour of incubation to label AChRs (Wallace et al., 1991). After washing the cultures in ice-cold phosphate buffered saline containing 1 mM phenylmethylsulfonyl fluoride, toxin-AChR complexes were solubilized by incubating each culture for 10 min on ice in extraction buffer (20 mM sodium phosphate buffer, pH 7.4, supplemented with 5 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 40 mM sodium pyrophosphate, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and

1% [wt/vol] Triton X-100). The extracts were centrifuged 10 min at 13,000 g and the supernatants incubated 2 h at 4°C with 2.5 μl streptavidin-conjugated agarose beads (Molecular Probes, Inc.). The beads were washed with 2 × 1 ml extraction buffer, 2 × 1 ml extraction buffer supplemented with 1 M sodium chloride, and 1 × 1 ml buffer containing 50 mM sodium phosphate, 50 mM sodium chloride, 1 mM sodium orthovanadate, pH 7.4. After washing the beads, AChRs were eluted into SDS sample buffer at room temperature and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels (Laemmli, 1970). Gels were fixed, dried, and exposed to preflashed autoradiography film (Hyperfilm-MP, Amersham, Arlington Heights, IL). Nonspecific binding was determined by adding 2×10^{-6} M α-bungarotoxin during incubation with biotin-conjugated toxin.

AChR β, γ, and δ subunits were tentatively identified by their positions relative to prestained molecular weight markers (Sigma Chemical Co.). The apparent molecular weights of the AChR subunits (β ~50 kD, γ ~58 kD, δ ~63 kD) were similar to those reported for AChRs isolated from C2 myotubes by Gu et al. (1989), although doublets of the γ and δ subunits, arising from partial proteolysis, were not observed. We confirmed the identity of the AChR β subunit on Western blots using mAb 148, which is specific for the AChR β subunit (Tzartos et al., 1993). The apparent lack of partially degraded subunits in our ^{32}P-labeled extracts may result from isolating surface AChRs selectively or from other differences in the isolation protocol. (We cannot rule out the possibility that the peptide we designate as the γ subunit is actually a partially degraded δ subunit, and that the γ subunit is missing entirely. The δ and particularly the γ subunits are known to be susceptible to proteolysis and have often been difficult to detect in muscle cells [Merlie and Sebbane, 1981; Gu et al., 1989; Forsayeth et al., 1990]. However, we found that our putative γ and δ subunits had different phosphopeptide maps, consistent with their being two distinct subunits.) Autoradiograms were analyzed by densitometry as previously described (Wallace, 1994). ·

Assays for Protein Serine/Threonine and Tyrosine Phosphorylation

To measure protein serine/threonine phosphorylation, cultures were labeled with 10 μCi/ml [^{32}P]orthophosphate, rinsed, and extracted as described above for AChR isolation. Soluble proteins were precipitated with 10% trichloroacetic acid (TCA) and the radioactivity associated with the acetone-washed precipitates determined by Cerenkov counting. Since phosphoserine and phosphothreonine typically account for >99% of phosphoamino acids in proteins in cell extracts (Cooper et al., 1983), changes in the incorporation of radiolabeled phosphate into TCA precipitates provides a measure of protein serine/threonine phosphorylation. Protein tyrosine phosphorylation was measured by dot blot analysis using affinity-purified rabbit polyclonal anti-phosphotyrosine antibodies from rabbits immunized with phosphotyrosine-conjugated bovine serum albumin (Ohtsuka et al., 1984). Cultures were extracted as described for isolation of AChRs and aliquots of the extracts were applied to nitrocellulose. The blots were probed with anti-phosphotyrosine antibodies and the ECL detection system (Amersham Corp.) as previously described (Wallace et al., 1991; Wallace, 1995), and analyzed by densitometry.

Phosphoamino Acid Analysis

Regions containing individual ^{32}P-labeled AChR subunits were excised from dried gels after autoradiography, the gel slices rehydrated, and proteins extracted and hydrolyzed in 5.8 N HCl at 110°C for 90 min according to standard protocols (Boyle et al., 1991; van der Geer and Hunter, 1994). Phosphoamino acids were dissolved in pH 1.9 buffer and separated in one dimension by thin layer chromatography (TLC) on Merck 0.1-mm cellulose plates (EM Separations, Gibbstown, NJ) in isobutyric acid:n-butanol:acetic acid:pyridine:water (65:2:3:5:29) (Boyle et al., 1991). The positions of phosphoamino acid standards were determined by spraying with 0.25% ninhydrin dissolved in acetone. TLC plates were exposed to preflashed autoradiography film followed by densitometric analysis as above. Alternatively, TLC plates were analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and densitometric measurements for each phosphoamino acid achieved using ImageQuant-software (Molecular Dynamics). Both methods gave similar results and the data were pooled for further analysis.

Fluorescence Recovery after Photobleaching

Lateral mobility of fluorescently labeled AChRs was measured by the technique of fluorescence recovery after photobleaching, as first described by Axelrod et al. (1976). C2 myotube cultures were labeled with rhodamine-conjugated α-bungarotoxin as described and rinsed several times with Puck's saline G (Puck et al., 1958) supplemented with 1.1 g/l glucose. To estimate changes in overall mobility of surface proteins, myotubes were labeled with 10 μg/ml rhodamine-conjugated succinyl-Con A (sConA; Vector Labs, Burlingame, CA) for 15 min at room temperature; conditions were chosen to maximize labeling while minimizing sConA-induced patching of surface proteins. Experiments were carried out at room temperature within 2 h of the last wash using a MRC 600 laser confocal microscope (Bio-Rad Laboratories, Hercules, CA) equipped with a krypton-argon laser light source and a 40x water immersion objective. A circular spot with a diameter of ~2 μm was bleached by attenuating the beam to 10% of its nominal energy by means of neutral density filters and focusing on a preselected location for three seconds. For experiments on nonaggregated AChRs and sConA-labeled surface proteins, images were obtained by scanning the field with the beam attenuated to the same extent. For experiments on aggregated AChRs, the beam was attenuated to 3% while images were acquired. Images were obtained immediately before bleaching, just after bleaching, after a 3-min recovery period, and, for sConA-labeled proteins, after 6 min. The recovery periods were chosen to provide sufficient time for the more rapidly diffusing fraction of receptors and sConA-labeled proteins to equilibrate (Axelrod et al., 1976; Koppel et al., 1976; Dubinsky et al., 1989). The images were digitized, aligned, and analyzed using WHIP Virtual Image Processing Software (G. W. Hannaway and Associates, Boulder, CO). The average pixel intensity within the area bleached was determined for each frame, and the values corrected for background pixel intensity. Bleaching resulted, on average, in a 56% decrease in fluorescence. Recovery is expressed as a percent of the difference in average pixel intensity before and immediately after the bleach. This simplified protocol did not allow determination of apparent diffusion constants, but did provide a measure of the proportion of AChRs free to diffuse laterally in the plane of the membrane.

Detergent Extraction of AChRs

Cultures were incubated for 1 h with 2×10^{-8} M [^{125}I]α-bungarotoxin (Amersham) to label surface AChRs, and then rinsed twice with DME-F12 medium at room temperature, twice with ice cold Prives buffer A (Prives et al., 1982), and extracted with ice cold Prives buffer A supplemented with 0.05% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride and 100 μM sodium pervanadate (to inhibit tyrosine phosphatases during the prolonged extraction). The dishes were gently agitated on a cold block and detergent-containing buffer was replaced at 5-min intervals. Residual AChRs were extracted into 1 N NaOH at the end of each experiment. Fractions were analyzed by gamma counting and the percent of AChRs remaining associated with the myotube cytoskeletons at each time point calculated, after correcting for nonspecific binding as determined from cultures labeled in the presence of a 50-fold excess of noniodinated α-bungarotoxin.

To examine the relationship between AChR phosphorylation and detergent extractability, sister cultures were labeled with either [^{125}I]α-bungarotoxin or with biotin-α-bungarotoxin and [^{32}P]orthophosphate as described above. Cultures were rinsed and extracted for 30 min on ice with Prives buffer A containing 0.05% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride, and 100 μM sodium pervanadate. Residual AChRs were solubilized by 10 min incubation with extraction buffer containing 1% Triton X-100, see above. These conditions were chosen so that each fraction (extracted and residual) contained approximately the same number of AChRs, as determined from cultures labeled with [^{125}I]α-bungarotoxin. AChRs in fractions from ^{32}P-labeled cultures were isolated on streptavidin-conjugated agarose beads and analyzed by SDS-PAGE and autoradiography. Densitometric scans of the autoradiograms were quantified for each AChR subunit.

Results

Agrin Induces Domains of Tyrosine Phosphorylation

To determine the effect of agrin on the distribution of phosphotyrosine-containing proteins in mouse C2 myotubes, myotube cultures were treated overnight with agrin and labeled with anti-phosphotyrosine antibodies. Control myotubes had spontaneously occurring accumulations of
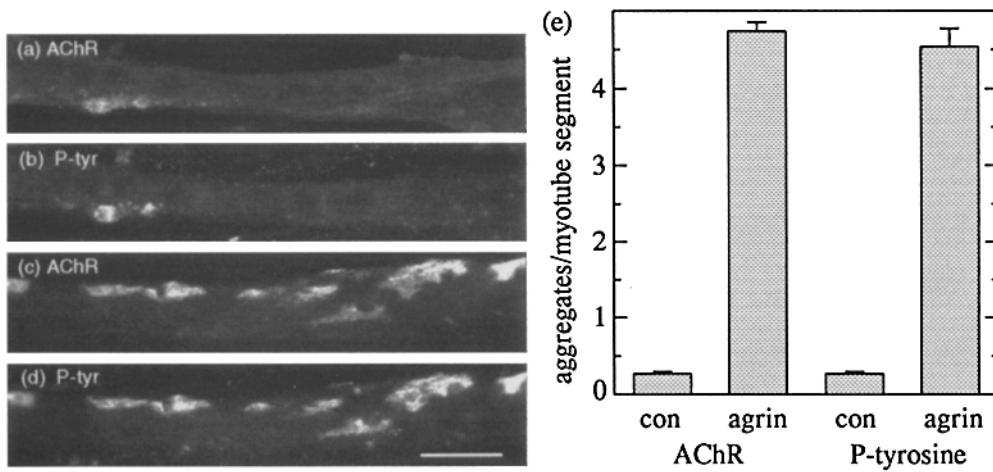
Figure 1. Agrin induces domains of increased protein tyrosine phosphorylation. Fluorescence micrographs of control (*a* and *b*) and agrin-treated myotubes (*c* and *d*) labeled with rhodamine-conjugated α-bungarotoxin (*AChR*) and anti-phosphotyrosine monoclonal antibody PY20 and fluorescein-conjugated secondary antibody (*P-tyr*). Control myotubes have many small patches that label for phosphotyrosine but not for AChRs and a few larger domains composed of coextensive aggregates of phosphotyrosine and AChRs. Agrin induces the formation of additional large domains that stain intensely for phosphotyrosine and AChRs. (*e*) The number of AChR or phosphotyrosine-containing (*P-tyrosine*) aggregates per myotube segment was determined for control (*con*) and agrin-treated (*agrin*) myotubes. Data is expressed as mean ± SEM, *N* = 3. Bar: (*a–d*), 15 μm.

phosphotyrosine-containing proteins (Fig. 1). Many such spontaneous patches were small (<2 μm²) and were not sites of AChR accumulation, as indicated by a low level of labeling with rhodamine-α-bungarotoxin. Control myotubes also had a few larger patches at which both phosphotyrosine-containing proteins and AChRs were concentrated. Treating cultures with agrin resulted in an increase in the number of relatively large patches labeling with anti-phosphotyrosine antibodies (Fig. 1). Such patches also labeled intensely with rhodamine-conjugated α-bungarotoxin. Indeed, all AChR aggregates, both in control and agrin-treated cultures, stained for phosphotyrosine. Thus, agrin induced the formation of patches on C2 myotubes at which phosphotyrosine-containing proteins and AChRs accumulated.

Agrin Reduces the Lateral Mobility of AChRs

Several lines of evidence indicate that in muscle cells non-aggregated AChRs are relatively free to diffuse laterally in the plane of the membrane while receptors within aggregates are immobile (Froehner, 1993). To assess the lateral mobility of AChRs in mouse C2 myotubes, we used the technique of fluorescence recovery after photobleaching (Axelrod et al., 1976; Dubinsky et al., 1989). AChRs on the myotube surface were labeled with rhodamine-conjugated α-bungarotoxin, a small spot was illuminated with intense light to bleach the rhodamine bound to receptors in that region, and the rate at which receptors bearing unbleached rhodamine-α-bungarotoxin molecules diffused into the bleached spot was estimated from the recovery of
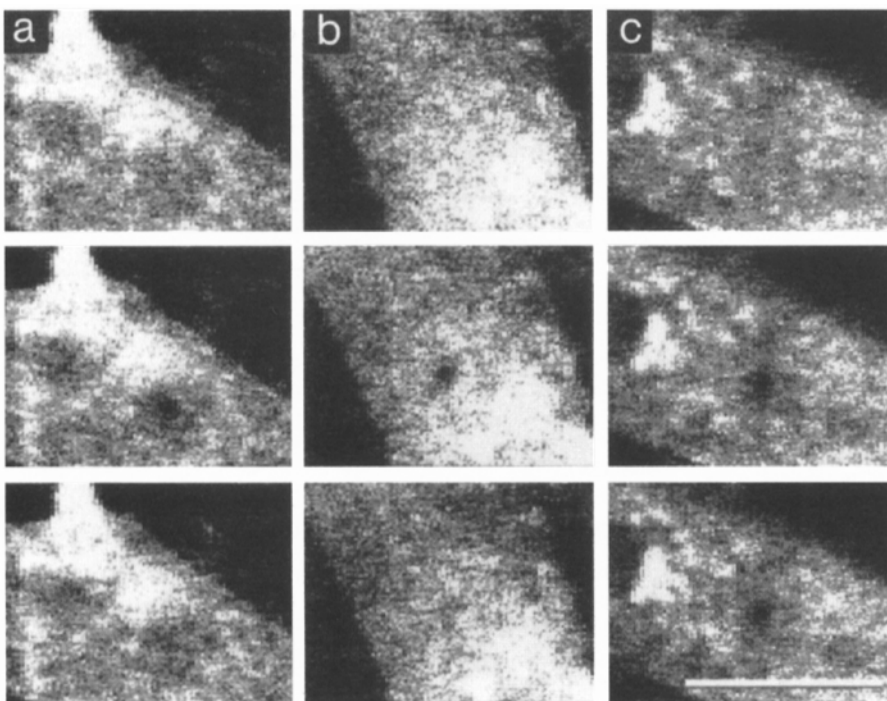

Figure 2. Fluorescence recovery after photobleaching measurements of AChR lateral mobility. Fluorescence micrographs of agrin-treated (*a*), control (*b*), and pervanadate-treated (*c*) myotubes before (*top row*), immediately after (*middle row*), and 3 min after (*bottom row*) a 2.5-μm diam spot was photobleached in an area devoid of AChR aggregates. Recovery of fluorescence within the bleached area in control and agrin-treated myotubes indicates that many of the AChRs are free to diffuse laterally in the membrane; the lack of recovery in the pervanadate-treated myotube demonstrates that AChRs are less mobile. Bar, 25 μm.
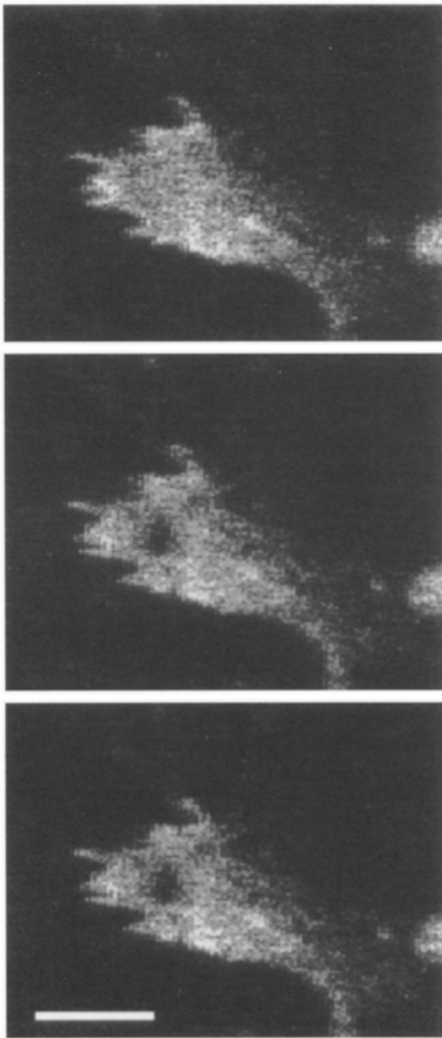
Figure 3. Aggregated AChRs are immobile. Fluorescence micrographs of a C2 myotube showing an AChR aggregate before (top), immediately after (middle), and 3 min after (bottom) a 2.5-μm diam spot was photobleached. The lack of recovery of fluorescence within the bleached spot demonstrates that aggregated AChRs are immobile. Bar, 10 μm.

fluorescence. As expected, when measurements were made on areas of the myotube surface where there were no recognizable receptor aggregates, there was an obvious recovery of fluorescence in both control myotubes and myotubes treated overnight with agrin, indicating that diffusely distributed receptors were mobile in both cases (Fig. 2, *a* and *b*; Fig. 4). There was little recovery of fluorescence, however, when spots within spontaneous or agrin-induced aggregates were bleached, indicating that aggregated AChRs were relatively immobile (Figs. 3 and 4). Thus agrin treatment created domains in cultured myotubes where tyrosine phosphorylation was increased and AChR mobility was reduced.

Pervanadate Selectively Inhibits Protein Tyrosine Phosphatases

If AChRs become immobilized in agrin-induced specializations because of the localized increase in tyrosine phos-
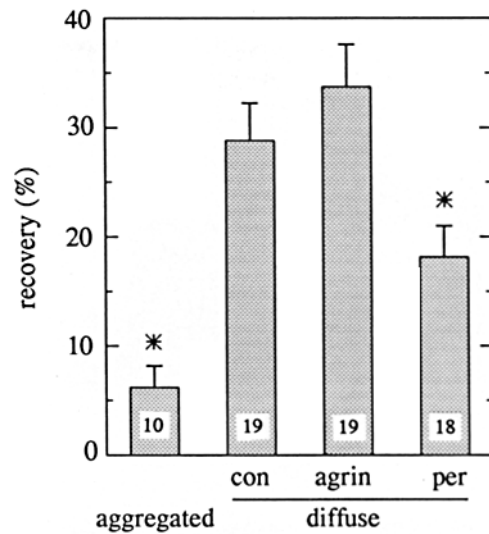

Figure 4. AChR lateral mobility. Analysis of the extent of fluorescence recovery 3 min after photobleaching for AChRs within aggregates (*aggregated*) and for diffusely distributed AChRs (*diffuse*) in control (*con*), agrin-, and pervanadate-treated (*per*) myotubes. For aggregated AChRs, data were pooled from spontaneously occurring and agrin-induced aggregates. Data is mean ± SEM, the number of observations is given within the columns. *Differs significantly from control, $p < 0.05$ (one-way ANOVA, Tukey-Kramer HSD test).

phorylation, then other treatments that cause an increase in protein tyrosine phosphorylation might also lead to AChR immobilization. One mechanism by which the level of protein tyrosine phosphorylation can be increased is to
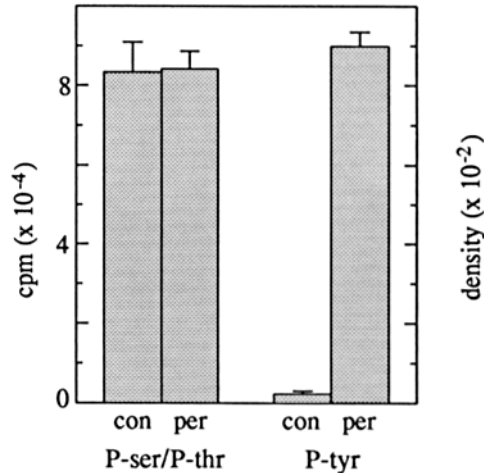

Figure 5. Pervanadate specifically inhibits protein tyrosine phosphatases. (*P-ser/P-thr*) Control (*con*) and pervanadate-treated (*per*) myotube cultures labeled with [^{32}P]orthophosphate were rinsed and extracted. The extracts were analyzed for radioactivity associated with total TCA-precipitable material. (*P-tyr*) Control and pervanadate-treated cultures were extracted, samples applied to nitrocellulose, and probed with anti-phosphotyrosine antibodies. Pervanadate (1 h, 30 μM) had no significant effect on TCA-precipitable counts, which measure levels of protein phosphoserine and phosphothreonine, but increased phosphotyrosine content nearly 40-fold. Left ordinate applies to P-ser/P-thr, right ordinate to P-tyr. Data is mean ± SEM; $N = 5$ for P-ser/P-thr; $N = 3$ for P-tyr.
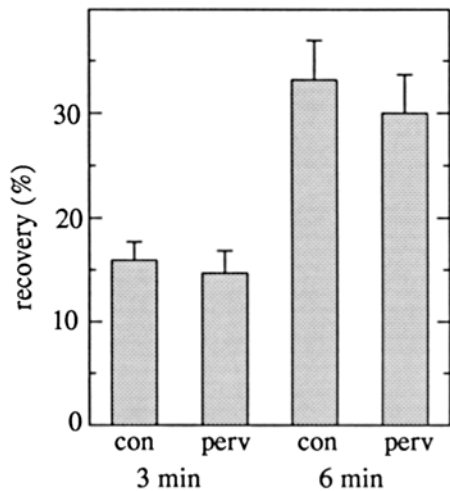
Figure 6. Pervanadate has little effect on the overall lateral mobility of surface proteins. Analysis of the extent of fluorescence recovery 3 and 6 min after photobleaching spots on control (con) and pervanadate-treated (perv) myotubes labeled with rhodamine-conjugated succinyl Concanavalin-A (sConA). The extent of recovery was similar in both control and pervanadate-treated myotubes, indicating that pervanadate did not have a significant effect on the average mobility of surface proteins that bind sConA. Data is expressed as mean ± SEM, N = 30–39.

Figure 8. Pervanadate inhibits agrin-induced AChR aggregation. C2 cultures were incubated in normal medium (normal), or medium containing 30 μM sodium pervanadate (pervanadate) and treated with (agrin) or without (con) agrin for 4 h. AChRs were visualized with rhodamine α-bungarotoxin and the number of AChR aggregates per myotube counted. Data is mean ±SEM, N = 3.

prevent ongoing dephosphorylation by inhibiting tyrosine phosphatases. To determine the effect of inhibiting phosphatases in C2 myotubes, cultures were treated with sodium pervanadate, a protein tyrosine phosphatase inhibitor (Pumiglia et al., 1992) and changes in protein phosphorylation
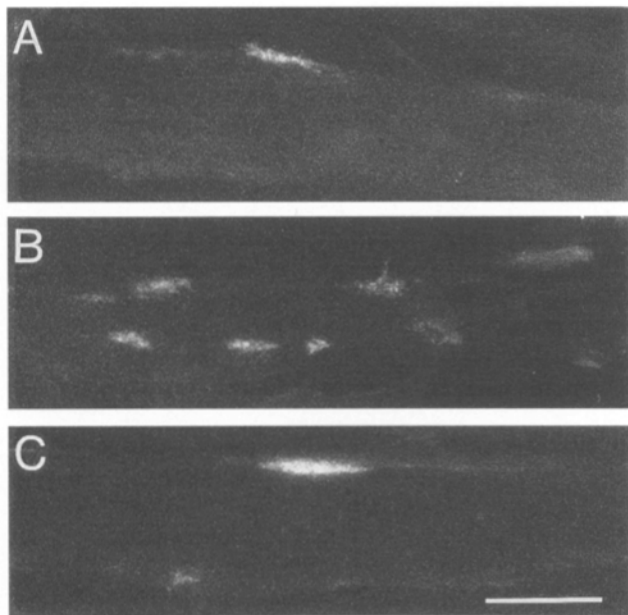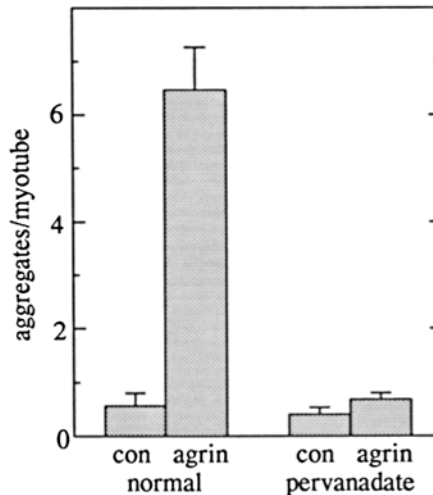

Figure 7. Effects of agrin and pervanadate on the distribution of surface AChRs. Fluorescence micrographs of C2 myotubes labeled with rhodamine-α-bungarotoxin after incubation for 4 h in control medium (A), with agrin (B), or with agrin and 30 μM sodium pervanadate (C). Pervanadate had little effect on spontaneously occurring AChR aggregates but prevented agrin-induced AChR aggregation. Bar, 20 μm.

were assayed. As illustrated in Fig. 5, treating myotubes with pervanadate (30 μM, 1 h) caused a large increase in their phosphotyrosine content, but no change in phosphoserine/phosphothreonine content. Thus, pervanadate selectively inhibited protein tyrosine phosphatases in C2 myotubes.

Pervanadate Induces Tyrosine Phosphorylation and AChR Immobilization throughout Myotubes

Pervanadate-treated myotubes stained more intensely with anti-phosphotyrosine antibodies, but the distribution of label was indistinguishable from that in control cultures. Thus, pervanadate increased tyrosine phosphorylation throughout the myotubes. To determine the effects of this widespread increase in tyrosine phosphorylation on the mobility of surface AChRs, receptors were labeled with rhodamine α-bungarotoxin and their lateral mobility measured by fluorescence recovery after photobleaching. As illustrated in Figs. 2 c and 4, in myotubes treated with 30 μM pervanadate for 1 h the mobility of diffusely distributed AChRs was significantly lower than in control myotubes. Thus, pervanadate caused a widespread increase in tyrosine phosphorylation and reduced the mobility of nonaggregated AChRs throughout the myotubes.

To test if pervanadate was causing a nonselective decrease in the mobility of surface proteins, myotubes were labeled with rhodamine-conjugated succinyl Concanavalin A (sConA) and fluorescence recovery after photobleaching tested. sConA binds to terminal α-D-mannosyl and α-D-glucosyl residues, carbohydrates found on many surface proteins, and so can be used to measure the average mobility of a large subset of membrane proteins (Koppel et al., 1976). In control cultures, spots bleached in cells labeled with rhodamine-conjugated sConA took somewhat longer to recover than did spots bleached in myotubes la-
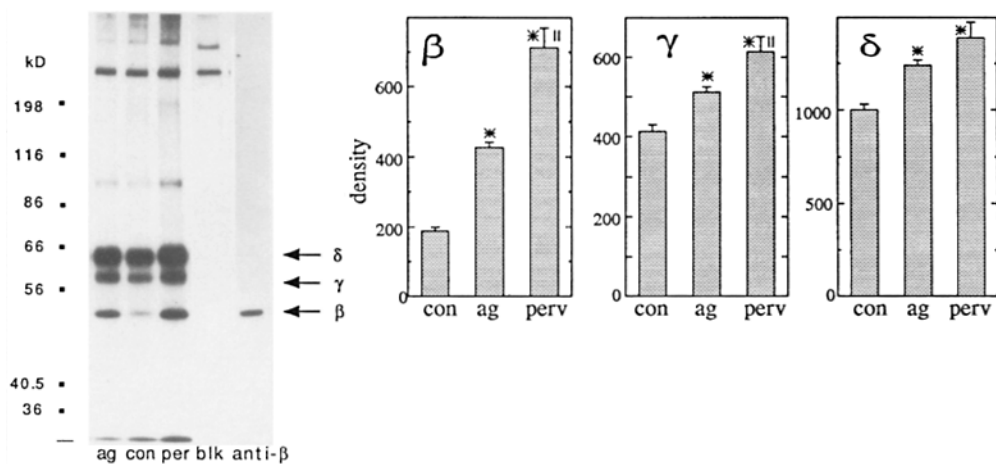
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density

con ag perv   con ag perv   con ag perv

Figure 9. Agrin and pervanadate induce phosphorylation of the AChR β, γ, and δ subunits. (*left panel*) C2 myotube cultures were labeled for 4 h with [^{32}P]orthophosphate and AChRs were isolated and analyzed by SDS-PAGE and autoradiography. Compared to control cultures (*con*), addition of agrin (*ag*) or 30 μM sodium pervanadate (*per*) during the 4-h incubation with [^{32}P]orthophosphate increased phosphorylation of the AChR β, γ, and δ subunits. Bands labeled as β, γ, and δ were identified as AChR subunits because they were absent from samples in which the isolation of AChRs was specifically blocked by including an excess of unconjugated α-bungarotoxin during incubation with biotin-conjugated toxin (*blk*). (*anti-β*) Western blot of C2 AChRs probed with anti-β subunit monoclonal antibody (mAb 148). The position of prestained molecular weight markers is shown on the left. (*right panels*) Quantitative analysis of agrin- and pervanadate-induced changes in AChR subunit phosphorylation. The level of incorporation of [^{32}P]orthophosphate into AChR subunits was measured by densitometric analysis of autoradiograms; data from different experiments were combined by normalizing the results to the δ subunit of control myotubes in normal medium (=1,000). Both agrin (*ag*) and pervanadate (*perv*) significantly increased phosphorylation of the β, γ, and δ subunits. Data is mean ± SEM, $N = 18$ for control, 27 for agrin, and 7 for pervanadate. * Differs significantly from control cultures, ‖differs significantly from agrin-treated cultures, $p < 0.05$ (one-way ANOVA, Tukey-Kramer HSD test).

beled with rhodamine-conjugated α-bungarotoxin, indicating that the lateral mobility of sConA-binding proteins was on average less than that of AChRs (Koppel et al., 1976). Pervanadate (30 μM, 1 h) had little or no effect on the lateral mobility of sConA-binding proteins (Fig. 6). Thus, pervanadate-induced AChR immobilization was selective and not the result of a general decrease in the mobility of surface proteins.

Pervanadate Prevents Agrin-induced AChR Aggregation

Agrin-induced AChR aggregation occurs by the lateral migration of AChRs already in the myotube membrane (Godfrey et al., 1984; Wallace, 1988). If pervanadate causes AChR immobilization, this would be expected to prevent agrin-induced AChR aggregation. As illustrated in Figs. 7 and 8, pervanadate completely inhibited agrin-induced AChR aggregation, consistent with the finding that pervanadate reduced the mobility of diffusely distributed AChRs.

Agrin and Pervanadate Induce AChR Tyrosine Phosphorylation

As a step toward determining how increased tyrosine phosphorylation might cause AChR immobilization, we examined the effects of agrin and pervanadate on AChR phosphorylation. Treating myotubes with agrin caused a significant increase in phosphorylation of the AChR β, γ, and δ subunits (Fig. 9). Similar increases were observed by phosphoamino acid analysis of isolated receptor subunits, although the only agrin-induced change that was statistically significant by this analysis was an increase in tyrosine phosphorylation of the AChR β subunit (Table I). Pervanadate also caused a significant increase in AChR phosphorylation (Fig. 9), including tyrosine phosphorylation of the AChR β subunit (Table I). In addition, pervanadate increased tyrosine phosphorylation of the AChR γ and δ subunits (Table I) and of a large number of other proteins (data not shown, see Wallace, 1995). Thus, both agrin and pervanadate caused an increase in AChR tyrosine phosphorylation.

Table I. Effects of Agrin and Pervanadate on AChR Phosphoamino Acid Content

Subunit	Control		Agrin[‡]		Pervanadate	
	P-ser*	P-tyr	P-ser	P-tyr	P-ser	P-tyr
β	140 ± 19	123 ± 19	175 ± 19	388 ± 44[§]	233 ± 29	755 ± 175[§‖]
γ	342 ± 30	172 ± 25	361 ± 41	215 ± 22	410 ± 72	309 ± 43[§]
δ	708 ± 26	292 ± 23	714 ± 77	390 ± 51	1070 ± 205[‖]	714 ± 75[§‖]
	(10)		(13)		(5)	

Data expressed as mean ±SEM, number of observations in parentheses.
*Total phosphoamino acid content determined from autoradiogram densities normalized such that phosphoserine (*P-ser*) + phosphotyrosine (*P-tyr*) content =1000 for δ subunit of control myotubes.
[‡]Experiments with 4- and 16-h agrin treatment pooled.
[§]Differs significantly from control (one-way ANOVA, Tukey-Kramer HSD test, $p < 0.05$).
[‖]Differs significantly from agrin (one-way ANOVA, Tukey-Kramer HSD test, $p < 0.05$).
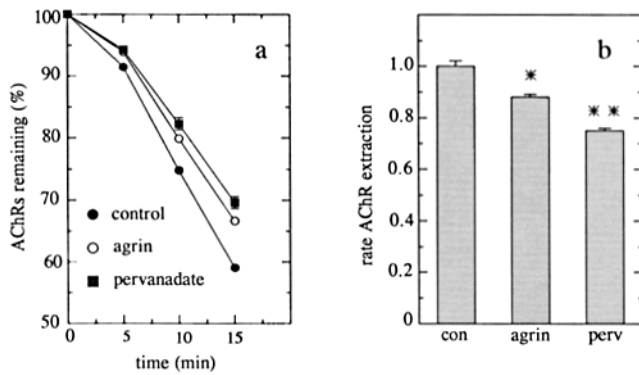
Figure 10. Agrin and pervanadate reduce the detergent extractability of surface AChRs. (*a*) Control, agrin-, and pervanadate-treated C2 cultures were incubated with [^{125}I]α-bungarotoxin to label surface AChRs, rinsed, and extracted with buffer containing 0.05% Triton X-100. The percent of receptors remaining associated with the myotube cytoskeletons is plotted as a function of time of extraction for triplicate cultures (Data is mean ± SEM, $N = 3$; error bars are shown where they exceed the size of the data point). (*b*) Summary of results from seven experiments on control (*con*), agrin- (*agrin*), and pervanadate-treated (*perv*) cultures. For each culture, the rate of AChR extraction was determined by least squares fit to the 5-, 10-, and 15-min time points, and then normalized to the slope observed under control conditions (= 1.0) in the same experiment. Data is expressed as mean ± SEM, $N = 21$. *Differs significantly from control, **differs significantly from control and from agrin, $p < 0.05$ (one-way ANOVA, Tukey-Kramer HSD test).

Agrin and Pervanadate Decrease AChR Detergent Extractability

If AChR phosphorylation caused the decrease in receptor mobility, then only immobilized receptors would be phosphorylated. Unfortunately, we could not determine if this was the case by the fluorescence recovery after photobleaching technique. However, results of previous experiments (Prives et al., 1982; Stya and Axelrod, 1983; Podleski and Salpeter, 1988) suggest that immobile receptors are relatively resistant to detergent extraction. Accordingly, we sought to compare the detergent extractability of phosphorylated and nonphosphorylated AChRs.

We first examined the effects of agrin and pervanadate on the overall detergent extractability of AChRs. Treating myotubes for 4 h with agrin reduced the rate of detergent extraction of AChRs by ~12%; treating myotubes with pervanadate for 1 h caused a 25% decrease (Fig. 10). Thus, both agrin and pervanadate decreased the rate at which AChRs were extracted from intact myotubes by mild detergent treatment.

To determine if phosphorylated AChRs were extracted more slowly than nonphosphorylated receptors, myotubes were incubated with agrin and [^{32}P]orthophosphate for 4 h, and then extracted for 30 min with buffer containing 0.05% Triton X-100, removing approximately half of the AChRs. Subsequently, residual AChRs were solubilized in buffer containing 1% Triton X-100. Extracted and residual AChRs were isolated and analyzed by SDS-PAGE and autoradiography. The number of AChRs in each fraction was determined by measuring the appearance of [^{125}I]
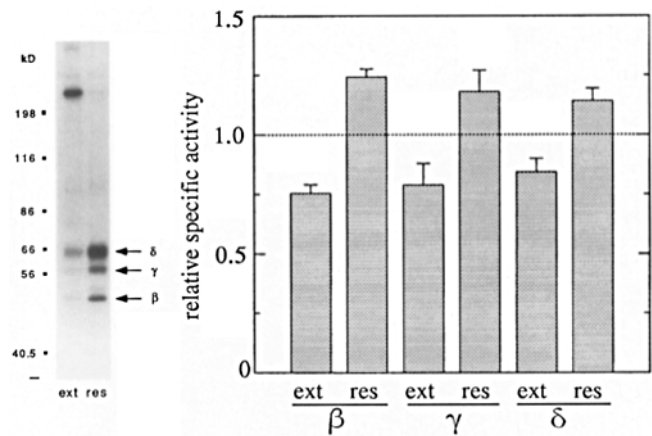

Figure 11. Phosphorylated AChRs are extracted from agrin-treated myotubes more slowly than nonphosphorylated AChRs. C2 myotube cultures were incubated with agrin and [^{32}P]orthophosphate for 4 h and extracted for 30 min with 0.05% Triton X-100. (*left panel*) Autoradiogram of AChRs extracted during detergent treatment (*ext*) and the residual AChRs remaining associated with the myotube cytoskeletons (*res*). The fraction of AChRs in the samples, determined from parallel extractions of [^{125}I]α-bungarotoxin–labeled cultures, was 39% extracted, 61% residual. (*right panel*) The relative specific activity of extracted (*ext*) and residual (*res*) AChR subunits was determined from densitometric analysis of AChRs in extracts of ^{32}P-labeled cultures and quantitation of AChRs in parallel extracts of [^{125}I]α-bungarotoxin–labeled cultures. If phosphorylated and nonphosphorylated AChRs had similar detergent extractabilities, the relative specific activity would be one for both extracted and residual receptors (*dotted line*). Data is expressed as mean ± SEM, $N = 10$. For each subunit, the specific activity of residual AChRs differs significantly from that of extracted AChRs, $p < 0.05$ (one-way ANOVA, Tukey-Kramer HSD test).

in corresponding extracts of sister cultures labeled with [^{125}I]α-bungarotoxin. As shown in Fig. 11, in agrin-treated cultures AChRs extracted during 30 min of incubation with 0.05% Triton were predominately nonphosphorylated; phosphorylated AChRs tended to remain associated with the myotube cytoskeleton. The results were similar for β, γ, and δ subunits. Thus, in agrin-treated myotubes phosphorylated AChRs were relatively resistant to detergent extraction compared to nonphosphorylated receptors.

Tyrosine Phosphorylation at Developing Neuromuscular Junctions In Vivo

There is considerable evidence that agrin mediates AChR aggregation at developing synapses (McMahan, 1990; Reist et al., 1992; Hall and Sanes, 1993). Accordingly, we sought to determine if AChR aggregates at developing synapses corresponded to sites of increased tyrosine phosphorylation. As illustrated in Fig. 12, anti-phosphotyrosine monoclonal antibody PY20 labeled developing neuromuscular junctions in rat diaphragm as early as embryonic day 18 (the earliest time yet examined). The labeling was blocked by 0.2 mM phosphotyrosine, but not by phosphoserine or phosphothreonine, indicating that the binding was specific. Similar results were obtained with a second anti-phosphotyrosine monoclonal antibody, 4G10. Thus, in rodent muscle cells both nerve- and agrin-induced in-
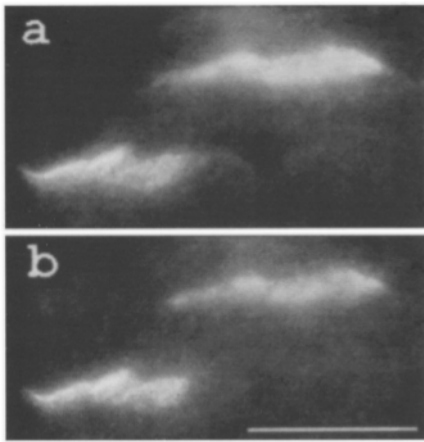
Figure 12. Developing neuromuscular junctions label with anti-phosphotyrosine antibody. Fluorescence micrographs of two end-plates in an E18 rat diaphragm in whole mount labeled with rhodamine-conjugated α-bungarotoxin to visualize the distribution of AChRs (a) and anti-phosphotyrosine monoclonal antibody PY20 and fluorescein-conjugated secondary antibody (b). Bar, 20 μm.

creases in tyrosine phosphorylation appear to occur early enough to mediate AChR aggregation.

Discussion

The results reported here demonstrate that agrin induces the formation of patches in cultured C2 myotubes at which phosphotyrosine-containing proteins and AChRs accumulate. Within these patches AChRs are relatively immobile, as judged by fluorescence recovery after photobleaching measurements. Treating myotubes with the tyrosine phosphatase inhibitor pervanadate caused an increase in tyrosine phosphorylation and a decrease in the mobility of AChRs throughout the myotube. Both agrin and pervanadate caused an increase in tyrosine phosphorylation of the AChR β subunit. Phosphorylated AChRs were less rapidly extracted from intact myotubes by mild detergent treatment than were nonphosphorylated receptors. Sites of AChR aggregation in developing rat muscles also labeled with anti-phosphotyrosine antibodies. We conclude that both in vitro and in vivo agrin induces the formation of domains of increased protein tyrosine phosphorylation in myotubes within which AChRs become phosphorylated and immobilized.

Lateral Mobility of AChRs

In the experiments reported here, AChRs within aggregates were found to be relatively immobile, as expected. On the other hand, diffusely distributed AChRs in both control and agrin-treated C2 myotubes appeared equally mobile. This observation differs somewhat from results of experiments in which an extract of Torpedo electric organ was found to restrict partially the mobility of a portion of the diffusely distributed AChRs in chick myotubes (Dubinsky et al., 1989). The reason for this discrepancy is unclear. First, although the extract used in the earlier experiments probably contained agrin, there might have been additional factors present that affected AChR mobility. Second, the overall density of AChRs is considerably higher on mouse C2 cells than on chick myotubes, and a large number of freely diffusing receptors might obscure a small population of AChRs whose mobility is partially restricted. Alternatively, the discrepancy might reflect differences in when during agrin treatment and where with respect to emerging aggregates receptor mobility measurements were made. Clearly, more detailed information concerning the time course of agrin-induced changes in phosphorylation, AChR aggregation, and receptor mobility in mouse C2 myotubes is required.

Pervanadate caused a significant reduction in the average lateral mobility of diffusely distributed receptors. This effect may account for the ability of pervanadate to inhibit agrin-induced AChR aggregation, which normally occurs by lateral migration (Godfrey et al., 1984; Wallace, 1988). Pervanadate did not reduce the mobility of diffusely distributed AChRs to the same extent as seen for receptors within aggregates. This might reflect the proportion of receptors phosphorylated in each location. Alternatively, tyrosine phosphorylation of components in addition to AChRs might contribute to AChR immobilization within aggregates. This could account for the observation that at adult rat neuromuscular junctions denervation leads to a rapid reduction in tyrosine phosphorylation of the AChR β subunit but relatively little change in receptor aggregation (Qu et al., 1990). Indeed, we have found that both agrin and pervanadate induce increased phosphorylation of several components of the dystrophin/utrophin complex (Meier, T., and B. G. Wallace, manuscript in preparation). It has been suggested that components of the dystrophin complex might play a role in AChR aggregation (Kramarcy et al., 1994).

Distribution of Protein Tyrosine Kinases and Phosphatases Regulating AChR Phosphorylation and Mobility

The fact that pervanadate treatment caused phosphorylation and reduced the mobility of receptors throughout C2 myotubes suggests that there are diffusely distributed, spontaneously active protein tyrosine kinases and phosphatases catalyzing ongoing phosphorylation and dephosphorylation of AChRs. The lack of effect of agrin on the mobility of nonaggregated AChRs is consistent with the hypothesis that interaction of agrin with its receptor creates circumscribed domains of increased protein tyrosine phosphorylation that correspond to the dimensions of receptor aggregates. The identities of the agrin receptor and the protein tyrosine kinases and phosphatases that regulate AChR phosphorylation in muscle cells have yet to be determined. One of the components of the dystrophin-associated protein complex, α-dystroglycan, appears to comprise at least part of a receptor for agrin (Bowe et al., 1994; Gee et al., 1994; Campanelli et al., 1994; Sugiyama et al., 1994; Cohen et al., 1995), but it is not clear if binding of agrin to α-dystroglycan mediates AChR aggregation (Sugiyama et al., 1994). AChRs isolated from Torpedo electric organ postsynaptic membranes have associated with them two src-like protein tyrosine kinases capable of phosphorylating the AChR β subunit (Swope and Huganir, 1993). A protein tyrosine phosphatase that dephosphory-

lates AChRs has also been purified from *Torpedo* electric organ (Mei and Huganir, 1991). Whether these or similar enzymes play a role in AChR aggregation in muscle remains to be determined.

AChR Phosphorylation and Interaction with the Cytoskeleton

In previous experiments on chick myotubes changes in tyrosine phosphorylation of the AChR β subunit were correlated with changes in AChR detergent extractability (Wallace, 1992, 1994, 1995). Likewise, in mouse C2 myotubes both agrin and pervanadate increased tyrosine phosphorylation of the AChR β subunit and decreased detergent extractability of AChRs. Indeed, compared to agrin, pervanadate caused a 2–3-fold greater increase in β-subunit tyrosine phosphorylation and a twofold greater decrease in the rate of detergent extraction.

The decrease in the rate of detergent extractability was not large, although it was very reproducible. This might indicate that only a small fraction of all AChRs are phosphorylated, even in pervanadate-treated myotubes. Alternatively, the composition of the detergent treatment buffer might not be appropriate for maintaining interactions between the receptor and the underlying cytoskeleton.

If phosphorylation of AChRs caused the decrease in their detergent extractability, then phosphorylated receptors would be extracted more slowly than nonphosphorylated receptors. Analysis of the extent of phosphorylation of AChRs released during detergent treatment demonstrated that in agrin-treated C2 myotubes phosphorylated AChRs were indeed more resistant to detergent extraction than nonphosphorylated AChRs. However, if tyrosine phosphorylation of the AChR β subunit were the sole determinant of the rate of detergent extraction, as suggested by our previous results in chick myotubes, then one might expect that phosphorylated γ and δ subunits would be evenly distributed among more rapidly and more slowly extracted AChRs. This was not the case, AChRs bearing phosphorylated γ and δ subunits were also relatively resistant to detergent extraction. One explanation for this finding might be that phosphorylation of any subunit slows the rate of detergent extraction. This does not appear to be the case in chick myotubes, however, where in the presence of the protein tyrosine kinase inhibitor staurosporine agrin induces phosphorylation of the AChR γ and δ subunits, but does not reduce the rate of AChR detergent extraction (Wallace, 1994). A second possibility is that phosphorylation of the various subunits might not be mutually independent. For example, agrin might preferentially induce tyrosine phosphorylation of the β subunit of receptors that are already phosphorylated on the γ and δ subunits. Alternatively, agrin might create domains of increased tyrosine kinase activity within which AChRs become attached to the cytoskeleton and immobilized by a mechanism that does not rely on phosphorylation of AChRs but on phosphorylation of other membrane or cytoskeletal proteins, such as components of the dystrophin complex. Under such circumstances, AChRs more slowly extracted by detergent treatment might be preferentially phosphorylated simply as a consequence of their location within such a domain.

Tyrosine Phosphorylation at Developing Synapses

We report here that AChR aggregates at developing neuromuscular junctions in embryonic rat diaphragm label with anti-phosphotyrosine antibodies, as would be expected if tyrosine phosphorylation mediates AChR aggregation. No labeling was seen in the absence of primary antibody or when 0.2 mM phosphotyrosine (but not phosphoserine or phosphothreonine) was included during incubation with the primary antibody, indicating that the labeling was specific for phosphotyrosine. This result stands in contrast to a previous report that AChR aggregates at neuromuscular junctions in embryonic and neonatal rat diaphragm do not label with anti-phosphotyrosine antibodies (Qu et al., 1990). The reason for this discrepancy is not clear. Perhaps the methods used in the present study were more sensitive than those used in the earlier report. For example, Qu et al. (1990) incubated permeabilized whole mounts of neonatal diaphragm for only 30 min in primary antibody diluted in phosphate buffered saline, whereas we routinely incubated tissue for 1 h in primary antibody diluted in Tris buffered saline.

The data presented in this report demonstrate that agrin induces tyrosine phosphorylation and immobilization of AChRs in mouse C2 myotubes. We also demonstrate that sites of AChR aggregation in rodent myotubes in vivo and in vitro label with anti-phosphotyrosine antibodies. While it is tempting to conclude that such staining is due to phosphorylated receptors, it seems likely that other phosphotyrosine-containing proteins contribute. For example, in Xenopus myocytes sites at which AChRs will accumulate can be visualized with anti-phosphotyrosine antibodies before they can be detected with rhodamine-conjugated α-bungarotoxin (Baker and Peng, 1993). To understand the role of tyrosine phosphorylation in postsynaptic differentiation, it will be important to determine directly when during embryonic development in vivo AChRs and other postsynaptic proteins become phosphorylated and if such phosphorylated proteins are localized at neuromuscular junctions.

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