

Regulation of the Interaction of Nicotinic Acetylcholine Receptors with the Cytoskeleton by Agrin-activated Protein Tyrosine Kinase

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Abstract. Agrin induces the accumulation of nicotinic acetylcholine receptors (AChRs) in the myofiber membrane at synaptic sites in vertebrate skeletal muscle and causes an increase in tyrosine phosphorylation of the AChR β subunit. To examine further the mechanism of agrin-induced AChR phosphorylation and the relationship between changes in protein phosphorylation and AChR aggregation, the effect of the protein tyrosine phosphatase inhibitor sodium pervanadate was tested on chick myotubes in culture. Pervanadate caused an increase in the phosphotyrosine content of a variety of proteins, including the AChR. Pervanadate also prevented agrin-induced AChR aggregation and slowed the rate at which AChRs were extracted from

intact myotubes by mild detergent treatment. The rate at which phosphorylation of the AChR β subunit and receptor detergent extractability changed following pervanadate-induced phosphatase inhibition was increased by agrin, indicating that agrin activates a protein tyrosine kinase rather than inhibiting a protein tyrosine phosphatase. The present results, taken together with previous findings on the inhibition of agrin-induced AChR aggregation by protein kinase inhibitors, demonstrate that protein tyrosine phosphorylation regulates the formation and stability of AChR aggregates, apparently by strengthening the interaction between AChRs and the cytoskeleton.

A variety of studies have implicated protein tyrosine phosphorylation as an important mechanism for the regulation of cell proliferation and differentiation (Fantl et al., 1993; Walton and Dixon, 1993). Results of recent experiments suggest that the accumulation of nicotinic acetylcholine receptors (AChRs)¹ in the postsynaptic membrane at the neuromuscular junction may be regulated by protein tyrosine phosphorylation, perhaps of the AChR itself. The formation of AChR aggregates at developing synapses appears to be triggered by specific isoforms of the protein agrin (Tsim et al., 1992; Ruegg et al., 1992; Ferns et al., 1992, 1993). When added to chick myotubes in culture, such isoforms of agrin induce the formation of specializations that contain high concentrations of many components of the postsynaptic apparatus, including AChRs, a 43-kD receptor-associated protein, and several cytoskeletal and extracellular matrix proteins (Wallace, 1986, 1989; Shadiak and Nitkin, 1991). Agrin also induces an increase in phosphorylation of the AChR β , γ , and δ subunits (Wallace et al., 1991). Agrin-induced phosphorylation of the γ and δ subunits is not required for AChR aggregation (Wallace et al., 1991; Wallace, 1994). Agrin-induced phosphorylation of the β subunit occurs, at least in part, on tyrosine residues, and

protein tyrosine kinase antagonists that inhibit agrin-induced tyrosine phosphorylation of the β subunit also block agrin-induced receptor aggregation (Wallace, 1994). Thus, agrin-induced AChR aggregation appears to require increased protein tyrosine phosphorylation.

The agrin-induced increase in protein tyrosine phosphorylation could arise from an increase in protein tyrosine kinase activity and/or a decrease in protein tyrosine phosphatase activity. Protein tyrosine kinases and phosphatases for which AChRs are substrates have recently been identified in *Torpedo* electric organ (Mei and Haganir, 1991; Swope and Haganir, 1993). Accordingly, we undertook to study the effects of inhibitors of protein kinases and phosphatases on AChR aggregation and phosphorylation in chick myotube cultures. Previously, we reported that staurosporine, a protein kinase antagonist, inhibits selectively agrin-induced tyrosine phosphorylation of the AChR β subunit and prevents receptor aggregation (Wallace, 1994). In the experiments reported here, the effects of the protein tyrosine phosphatase inhibitor sodium pervanadate (Pumiglia et al., 1992) were investigated. Pervanadate was found to increase tyrosine phosphorylation of AChRs and other myotube proteins, prevent agrin-induced AChR aggregation, and slow the rate at which AChRs were extracted from intact myotubes by mild detergent treatment. The results indicate that agrin stimulates protein tyrosine kinase activity and that protein tyrosine phosphorylation regulates the attachment of AChRs to the cytoskeleton.

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1. *Abbreviation used in this paper:* AChR, acetylcholine receptor.

Materials and Methods

Chick Myotube Cultures

Myotube cultures were prepared from hindlimb muscles of 11- to 12-d-old White Leghorn chick embryos by the method of Fischbach (1972) with minor modifications (Wallace, 1989). Experiments were made on 5–6-d-old myotube cultures.

Agrin

Experiments were made with partially purified preparations of agrin (Cibacron pool) prepared from electric organ of *Torpedo californica* as previously described (Nitkin et al., 1987).

Isolation of AChRs

AChRs on the myotube surface were labeled with biotinylated α -bungarotoxin (Molecular Probes, Eugene, OR), the toxin-receptor complexes solubilized in buffer containing 1% Triton X-100, and the complexes isolated on streptavidin-conjugated Sepharose beads (Molecular Probes) as previously described (Wallace et al., 1991).

Measurement of AChR Phosphorylation

Cultures were rinsed with minimum essential medium without sodium phosphate (GIBCO BRL, Gaithersburg, MD) supplemented with 1 mg/ml bovine serum albumin (RIA grade; Sigma Chemical Co., St. Louis, MO), 20 μ g/ml conalbumin (type II; Sigma Chemical Co.), 100 U/ml penicillin, and 100 μ g/ml streptomycin, and then incubated in the same medium containing 0.5 mCi/ml [32 P]orthophosphate ([32 P]H₃PO₄ in H₂O; ICN Biomedicals, Irvine, CA) in the presence or absence of agrin and/or pervanadate. In some experiments cultures were preincubated with [32 P]orthophosphate for 3–14 h to label the ATP pool and phosphoproteins to steady state levels, then incubated with agrin and pervanadate. Both protocols gave similar results and the data have been combined. At the end of the incubation period, AChRs were isolated as described above, eluted into SDS sample buffer, and the eluate was boiled and electrophoresed on 7.5% SDS-polyacrylamide gels according to Laemmli (1970). The positions of the AChR β , γ , and δ subunits were determined from the positions of pre-stained molecular weight markers (Sigma Chemical Co.), based on previous results using subunit-specific antibodies (Wallace et al., 1991). The gels were fixed, dried under reduced pressure, and exposed to preflashed autoradiography film (Hyperfilm-MP; Amersham Corp., Arlington Heights, IL). The resulting autoradiograms were analyzed by densitometry as previously described (Wallace, 1994).

Assays for Phosphorylation

To measure overall protein phosphorylation, aliquots of the AChR-containing extracts of cultures labeled with [32 P]orthophosphate were treated with trichloroacetic acid and the radioactivity associated with the precipitates determined by Cerenkov counting. In addition, the precipitates were resuspended in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as described above.

Protein tyrosine phosphorylation was measured by Western 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 the extracts were electrophoresed on 7.5% SDS-polyacrylamide gels as described above. The proteins were transferred to nitrocellulose membranes and probed with anti-phosphotyrosine antibodies as previously described (Wallace et al., 1991), except that bound anti-phosphotyrosine antibodies were detected with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, Inc., West Grove, PA) and the ECL Western blotting detection system (Amersham Corp.).

Detergent Extraction of AChRs

Myotubes were labeled with [125 I] α -bungarotoxin (Amersham Corp.) and the rate of extraction of toxin-receptor complexes into a solution containing 0.05% Triton X-100 was measured, as described previously (Wallace, 1992).

Quantitation of AChR Aggregation

Myotube cultures were labeled with 2×10^{-8} M rhodamine-conjugated α -bungarotoxin (Molecular Probes), rinsed, and fixed as previously described (Wallace, 1989). Myotube segments were viewed with a Nikon Optiphot microscope equipped for phase contrast and epifluorescence. To quantify AChR aggregation, either the number of AChR aggregates per myotube segment was counted by eye or myotubes were imaged with a Star I chilled CCD camera (Photometrics, Ltd., Tucson, AZ) and analyzed using WHIP Virtual Image Processing Software (G. W. Hannaway and Associates, Boulder, CO) to determine the total fluorescence derived from aggregated AChRs, as previously described (Wallace, 1992). Both methods gave similar results.

Preparation of Pervanadate

Sodium pervanadate was prepared by adding 1 part 500 mM H₂O₂ to 50 parts 10 mM sodium orthovanadate (Sigma Chemical Co.) in modified Tyrodes solution, incubating the mixture for 10 min at room temperature, and diluting it into culture medium immediately before use (Pumiglia et al., 1992).

Results

Pervanadate Selectively Inhibits Protein Tyrosine Phosphatases in Cultured Chick Myotubes

First we sought to assess the specificity of phosphatase inhibition by pervanadate. Since phosphoserine and phosphothreonine typically account for >99% of phosphoamino acids in proteins in cell extracts (Cooper et al., 1983), changes in incorporation of radiolabeled phosphate into phosphoproteins can provide a measure of protein serine/threonine phosphatase activity. Pervanadate had little or no effect on either incorporation of radiolabeled phosphate into trichloroacetic acid precipitates of cell extracts (30 min pervanadate treated = $107 \pm 5\%$ of controls [mean \pm SEM, $N = 6$]) or in the pattern of phosphoproteins seen on autoradiograms of SDS-polyacrylamide gels of cell extracts (Fig. 1). Thus, pervanadate did not appear to inhibit protein serine/threonine phosphatase activity in chick myotubes.

To assess the effect of pervanadate on protein tyrosine phosphatase activity, extracts of control and pervanadate-treated cells were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with anti-phosphotyrosine antibodies. As shown in Fig. 1, pervanadate caused a large increase in the phosphotyrosine content of a variety of proteins in the cell extracts. Thus, pervanadate was a selective inhibitor of protein tyrosine phosphatases in chick myotubes.

Sodium pervanadate did not adversely affect cell viability; myotubes appeared normal at the end of a 6-h incubation in 100 μ M pervanadate and had a normal complement of AChRs ($104 \pm 3\%$ of controls [mean \pm SEM, $N = 6$]). Moreover, the absence of any detectable effect of pervanadate on the incorporation of [32 P]orthophosphate into phosphoproteins indicated that phosphate uptake and ATP synthesis were also relatively normal in pervanadate-treated cells. Even a day after a 6-h exposure to pervanadate, myotubes appeared healthy by phase microscopy. Thus, pervanadate had no obvious cytotoxic effects.

Inhibition of Agrin-induced AChR Aggregation by Pervanadate

Results of previous experiments have suggested that protein tyrosine phosphorylation plays a role in agrin-induced AChR

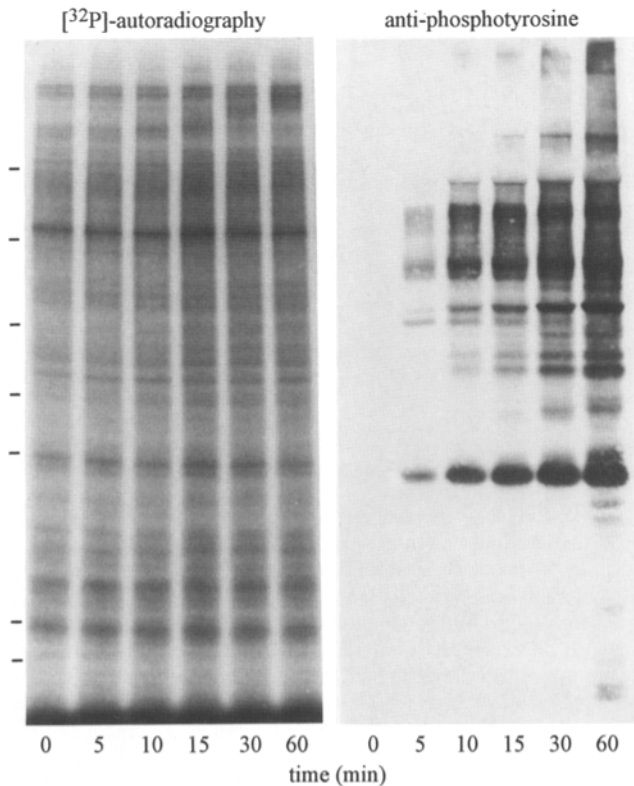


Figure 1. Inhibition of protein tyrosine phosphatases by pervanadate. (Left) Autoradiogram visualizing predominately serine/threonine phosphoproteins. Myotube cultures were equilibrated with [32 P]orthophosphate and then incubated with $100 \mu\text{M}$ sodium pervanadate for the indicated times, rinsed, and extracted. Proteins in the extracts were precipitated with trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. (Right) Western blot demonstrating tyrosine phosphoproteins. Extracts of unlabeled myotubes treated with $100 \mu\text{M}$ pervanadate for the indicated times were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with anti-phosphotyrosine antibodies. Molecular weight markers (top to bottom): 198, 116, 86, 66, 56, 40.5, 36 kD. In both experiments, equal amounts of protein were added to each lane.

aggregation (Wallace et al., 1991; Wallace, 1994). Accordingly, we examined the effects of pervanadate on the formation of AChR aggregates. When pervanadate was added together with agrin to cultured chick myotubes AChR aggregation was blocked; 50% inhibition occurred at a pervanadate concentration of $10 \mu\text{M}$ (Fig. 2).

As a step toward determining how pervanadate prevented AChR aggregation, the effect of pervanadate on the dose dependence of agrin-induced AChR aggregation was analyzed. As illustrated in Fig. 3, $30 \mu\text{M}$ pervanadate did not shift the dose dependence of aggregation to higher levels of agrin, but rather decreased the response at all agrin concentrations. Thus, pervanadate is not a competitive antagonist of the binding of agrin to its receptor on the myotube surface.

To determine if myotubes recovered the ability to respond to agrin after pervanadate treatment, cultures were exposed to $100 \mu\text{M}$ pervanadate for 1 h, rinsed, returned to the incubator for 6–24 h, and then assayed for agrin-induced AChR aggregation. As illustrated in Fig. 4, the myotubes recovered

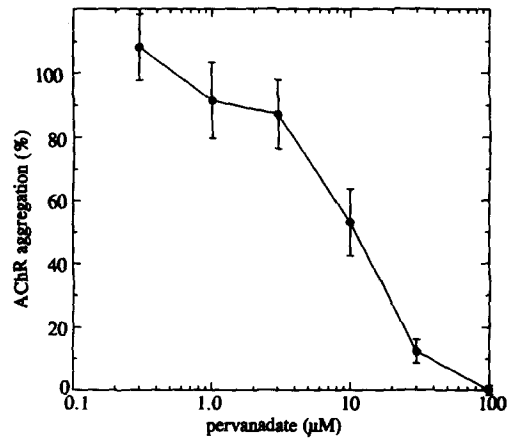


Figure 2. Inhibition of agrin-induced AChR aggregation by pervanadate. Myotube cultures were incubated for 3–4 h with 8 U agrin and the indicated concentration of sodium pervanadate. AChRs were labeled with rhodamine-conjugated α -bungarotoxin and the extent of AChR aggregation was compared to that in cultures incubated with agrin in normal medium. Results are expressed as mean \pm SEM, $N = 8$ ($N = 6$ for $0.3 \mu\text{M}$).

their ability to respond to agrin; the response reached 60% of that seen in untreated cultures 24 h after pervanadate treatment. Thus, inhibition of agrin-induced AChR aggregation was reversible. The slow time course of recovery may reflect the rate of reactivation of protein tyrosine phosphatase by cellular reducing agents (Pumiglia et al., 1992; Hecht and Zick, 1992) or the synthesis of new enzyme.

Pervanadate-induced AChR Phosphorylation

To determine the effects of pervanadate on the extent of AChR phosphorylation, myotubes were incubated for 4 h in radiolabeled orthophosphate with or without pervanadate,

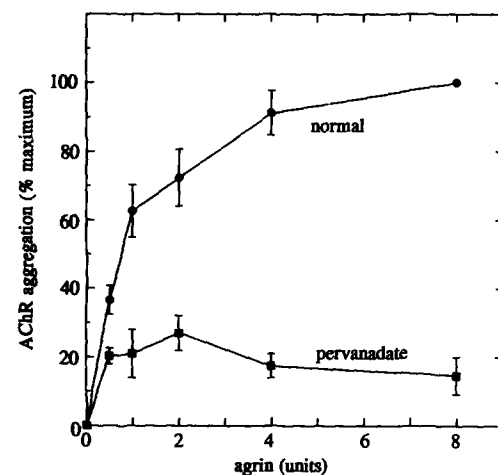


Figure 3. Effect of pervanadate on the dose-dependence of agrin-induced AChR aggregation. Myotubes were incubated for 6 h with the indicated concentration of agrin in normal medium (\bullet) or medium containing $30 \mu\text{M}$ sodium pervanadate (\blacksquare). AChRs were visualized with rhodamine-conjugated α -bungarotoxin and the extent of aggregation was compared to the maximal response (cultures incubated in normal medium with 8 U of agrin). Data is expressed as the mean \pm SEM; normal, $N = 7$; pervanadate, $N = 4$.

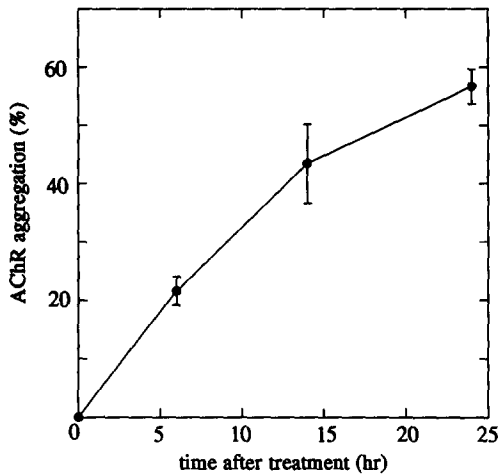


Figure 4. Recovery from pervanadate treatment. Myotubes were treated with 100 μ M sodium pervanadate for 1 h, rinsed, and returned to the incubator in fresh medium. After the indicated time had elapsed, agrin was added and the cultures were incubated an additional 3 h. AChRs were labeled with rhodamine-conjugated α -bungarotoxin and the extent of aggregation was compared to that seen after adding agrin to cultures not exposed to pervanadate. Results are expressed as mean \pm SEM, $N = 3$.

surface AChRs were isolated, and the receptors were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Table I, pervanadate caused an increase in phosphorylation of the AChR β , γ , and δ subunits; agrin caused no further increase in AChR phosphorylation in pervanadate-treated cells.

Effects of Pervanadate on Detergent Extractability

Previous experiments have demonstrated that in agrin-treated cultures the rate at which AChRs are extracted from intact myotubes by mild detergent treatment is reduced (Wallace, 1992). Such a decrease in detergent extractability has been interpreted as evidence that AChRs are attached to the cytoskeleton (Prives et al., 1982; Podleski and Salpeter, 1988; Phillips et al., 1993). Several lines of evidence suggest that agrin-induced decreases in AChR detergent extractability might be regulated by protein tyrosine phosphorylation (Wallace, 1992, 1994). To determine if increased protein tyrosine phosphorylation induced by phosphatase inhibition

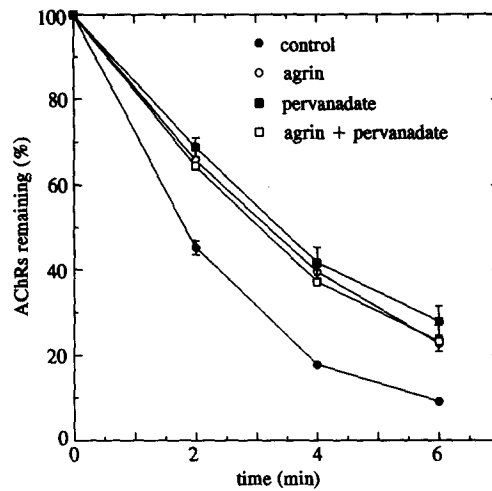


Figure 5. Effects of pervanadate on the rate of detergent extraction of AChRs. Myotube cultures were incubated for 4 h with or without agrin in the presence or absence of 100 μ M sodium pervanadate. Myotube cultures were labeled with [125 I] α -bungarotoxin, rinsed, and incubated in buffer containing 0.05% Triton X-100. At 2-min intervals the detergent-containing buffer was replaced and the amount of 125 I in the detergent extracts was used to calculate the percentage of AChRs remaining associated with the myotube cytoskeletons. Pervanadate decreased the rate of detergent extraction to the same extent as seen with agrin treatment. Results, expressed as mean \pm SEM, $N = 3$, are from a single experiment with triplicate samples. Data from three such experiments are summarized in Table I.

could also cause a change in the attachment of AChRs to the cytoskeleton, the rate of detergent extraction of AChRs was measured in cultures treated with pervanadate. As illustrated in Fig. 5 and summarized in Table I, pervanadate reduced the rate at which AChRs were extracted from myotubes to the same extent as was seen with agrin treatment. In the presence of pervanadate, agrin caused no further change in detergent extractability. Thus, pervanadate, like agrin, appeared to strengthen the association of AChRs with the cytoskeleton.

Pervanadate Stabilizes AChR Aggregates

If pervanadate causes AChRs to become linked to the cytoskeleton, then one might expect that pervanadate would

Table I. Effects of Pervanadate on AChR Phosphorylation and Detergent Extractability

	No inhibitor		Pervanadate	
	Control	Agrin	Control	Agrin
Phosphorylation*				
β	12.8 \pm 1.9	32.6 \pm 3.0 \S	89.1 \pm 8.4 \S	78.8 \pm 7.7
γ	100 \pm 2.5	127.2 \pm 5.0 \S	237.3 \pm 21.2 \S	220.9 \pm 20.2
δ	67.35 \pm 3.2	78.4 \pm 4.0 \S	123.2 \pm 16.4 \S	104.4 \pm 14.1
		($N = 10$)		
Detergent extraction \ddagger	55.7 \pm 2.8	67.1 \pm 1.5 \parallel	65.3 \pm 1.6 \parallel	68.3 \pm 1.7
		($N = 9$)		

Data expressed as mean \pm SEM.

* Autoradiogram densities normalized to γ subunit of control myotubes in normal medium (=100).

\ddagger Percent AChRs remaining after 2 min detergent extraction.

\S Differs from control cultures with no inhibitor, $P < 0.02$, Bonferroni's t -test, paired comparisons.

\parallel Differs from control cultures with no inhibitor, two way ANOVA, Student-Newman-Keuls test, $P < 0.05$.

stabilize pre-existing aggregates. However, in cultures pretreated overnight with agrin, rinsed, and then incubated in medium without agrin, pervanadate had no significant effect on the number of agrin-induced aggregates remaining after 6 h ($109 \pm 22\%$ of controls [mean \pm SEM, $N = 4$]). Thus, pervanadate had little effect on the rate of disappearance of aggregates in agrin-pretreated myotubes.

Because processes other than protein tyrosine phosphorylation appeared to regulate the normally very slow disappearance of AChR aggregates, we sought to determine if pervanadate would antagonize the increased rate of aggregate disappearance caused by the protein kinase inhibitor staurosporine. As previously reported, staurosporine selectively inhibits agrin-induced phosphorylation of the AChR β subunit, increases the rate at which AChRs are extracted from intact myotubes by detergent, and increases the rate at which pre-existing aggregates disappear (Wallace, 1994). To test the effect of pervanadate on staurosporine-stabilized aggregates, myotubes were treated overnight with agrin to induce aggregate formation, then rinsed and incubated without agrin for an additional 6 h in normal medium or medium containing pervanadate and/or staurosporine. At the end of the 6-h incubation the number of AChR aggregates and the rate of detergent extraction were assayed. Pervanadate prevented the increase in rate of detergent extraction that would otherwise have occurred in staurosporine-treated myotubes (Fig. 6 *a*) and slowed the rate at which AChR aggregates disappeared (Fig. 6 *b*). Thus, under conditions where the rate of aggregate disappearance was increased by treatment with staurosporine, pervanadate had a stabilizing effect.

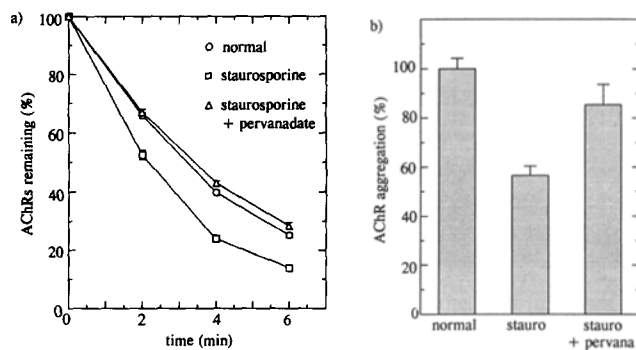


Figure 6. Pervanadate antagonism of the effects of staurosporine on AChR detergent extractability and aggregate stability. Myotube cultures were incubated overnight with agrin to induce AChR aggregation and reduce the rate of detergent extraction of AChRs. Agrin was removed and the myotubes were incubated for an additional 6 h in normal medium, 40 nM staurosporine, or 40 nM staurosporine and 100 μ M sodium pervanadate. (a) Myotubes were labeled with [125 I] α -bungarotoxin and the rate of extraction of AChRs into detergent solution was determined. Data is expressed as mean \pm SEM, $N = 9$ (b) Myotubes were labeled with rhodamine-conjugated α -bungarotoxin and the number of AChR aggregates per myotube segment was determined. Data is expressed as mean \pm SEM, $N = 3$. Myotubes incubated in staurosporine (*stauro*) had significantly fewer aggregates remaining than myotubes incubated in normal medium (*normal*) or myotubes incubated in staurosporine plus pervanadate (*stauro + pervana*) (one way ANOVA, Student-Newman-Keuls test, $P < 0.05$). The number of AChR aggregates in myotubes treated with staurosporine plus pervanadate did not differ significantly from normal.

Time Course of Pervanadate-induced Changes in Protein Phosphorylation and Detergent Extractability

When myotubes are treated with agrin, the rate of detergent extraction changes in parallel with the increase in tyrosine phosphorylation of the AChR β subunit, each reaching a half-maximum response in approximately 1.5 h (Wallace, 1992). As shown in Fig. 7, pervanadate-induced phosphorylation of the AChR β subunit reached half-maximum in less than 10 min, suggesting a rapid turnover of phosphate residues in control myotubes. Detergent extractability changed with the same rapid time course. Thus, although the time course of agrin- and pervanadate-induced effects differed by approximately an order of magnitude, in each case detergent extractability and phosphorylation of the AChR β subunit changed in parallel. Moreover, in pervanadate-treated cultures inhibition of agrin-induced AChR aggregation developed with the same time course as receptor phosphorylation and decreased detergent extractability (Fig. 7).

Staurosporine Antagonizes the Effects of Pervanadate

The decrease in AChR detergent extractability induced by agrin is prevented by the protein kinase antagonist staurosporine (Wallace, 1994). To determine if changes induced by pervanadate and agrin involve similar kinases, the ability of staurosporine to block the effects of pervanadate was tested. As shown in Fig. 8, staurosporine inhibited the pervanadate-induced decrease in AChR detergent extractability. Thus, those substrates of pervanadate-sensitive phosphatases that mediate changes in AChR detergent extractability are phosphorylated by staurosporine-sensitive kinases, consistent

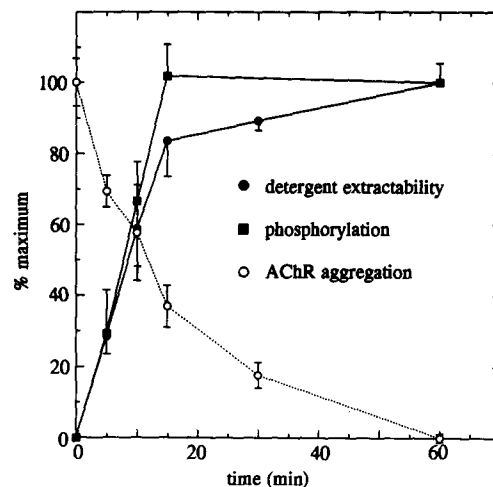


Figure 7. Time course of pervanadate-induced changes in detergent extractability, phosphorylation of the AChR β subunit, and AChR aggregation. Myotube cultures were labeled with either [32 P]orthophosphate or [125 I] α -bungarotoxin, incubated for the indicated time in 100 μ M pervanadate, and the extent of phosphorylation of the AChR β subunit or the rate of detergent extraction of AChRs was assayed. Data, expressed as mean \pm SEM ($N = 4$), are calculated as the percent of the maximal change induced by 60-min treatment with pervanadate. Other cultures were incubated for the indicated time in pervanadate, rinsed, incubated with agrin for 6 h, and assayed for AChR aggregation. Data, expressed as mean \pm SEM ($N = 6$), are calculated as a percent of the increase in AChR aggregation observed in cultures not treated with pervanadate.

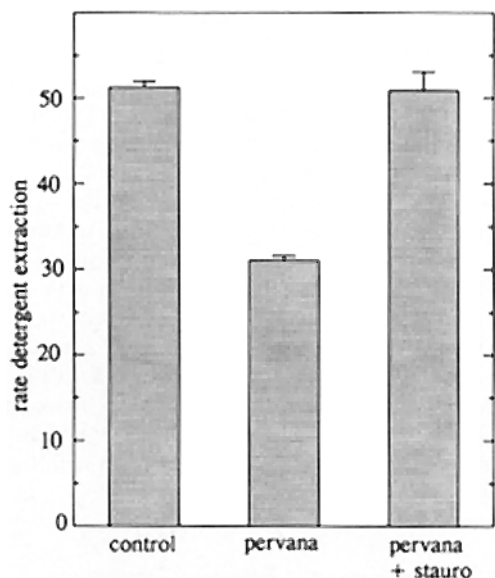


Figure 8. Effect of staurosporine on pervanadate-induced changes in detergent extractability. Myotubes were labeled with [¹²⁵I]α-bungarotoxin, incubated with (*pervana*) or without (*control*) 100 μM pervanadate for 30 min, and the rate of detergent extraction of AChRs determined. In other cultures 40 nM staurosporine was added 10 min prior to adding pervanadate (*pervana + stauro*). Staurosporine completely blocked the decrease in detergent extractability caused by pervanadate. Results, expressed as the fraction of receptors solubilized during the first 2 min of detergent treatment, are mean ± SEM, *N* = 3. (*Pervana* differs significantly from *control* and from *pervana + stauro*; *pervana + stauro* is not significantly different from *control* [one way ANOVA, Student-Newman-Keuls test, *P* < 0.05]).

with the idea that agrin and pervanadate affect the same pathway.

Agrin Activates a Protein Tyrosine Kinase

Agrin-induced increases in tyrosine phosphorylation of the AChR β subunit could result from kinase activation and/or phosphatase inhibition. To determine if agrin inhibits a phosphatase, kinase activity was blocked with staurosporine and the effect of agrin on the rate at which detergent extractability increased was measured. We chose to monitor changes in detergent extractability rather than incorporation of radiolabeled phosphate as an initial test because this assay is simpler and requires much lower levels of radioactivity. Cultures were first treated overnight with agrin to induce AChR phosphorylation and reduce detergent extractability. The myotubes were rinsed, incubated for 4 h with agrin, staurosporine, or staurosporine and agrin, and then detergent extractability was assayed. During the 4-h incubation the rate of detergent extractability gradually increased in staurosporine-treated cultures, presumably due to spontaneously active phosphatases. If agrin normally stimulated a kinase, then under these conditions agrin would have no effect on how rapidly detergent extractability changed because kinase activity was inhibited by staurosporine. On the other hand, if agrin inhibited a spontaneously active phosphatase, it would slow the rate at which detergent extractability changed. As shown in Fig. 9, agrin had no effect on the increase in rate of detergent extraction in staurosporine-treated

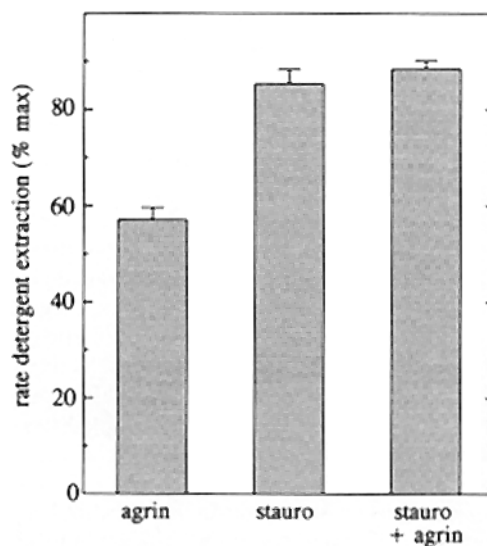


Figure 9. Effect of agrin on staurosporine-induced changes in detergent extractability. Myotube cultures were incubated overnight with agrin, rinsed, and incubated an additional 4 h with agrin (*agrin*), 40 nM staurosporine (*stauro*), or 40 nM staurosporine and agrin (*stauro + agrin*). Cultures were labeled with [¹²⁵I]α-bungarotoxin and the rate at which AChRs were extracted into detergent solution was determined. The rate was normalized to the maximum rate of extraction, measured after prolonged treatment with staurosporine. Overnight treatment with agrin reduced the rate of detergent extraction by 43%. Exposure to staurosporine for 4 h partially reversed the effect of agrin; the extent of the reversal was the same in cultures treated with both staurosporine and agrin. Data are expressed as mean ± SEM, *N* = 9. (*Stauro* and *stauro + agrin* differ significantly from *agrin* but not from each other; one way ANOVA, Student-Newman-Keuls test, *P* < 0.05).

cultures. Thus, there is no evidence that agrin inhibits a protein tyrosine phosphatase.

To determine if agrin activates a protein tyrosine kinase, the rate at which detergent extractability decreased in cultures treated with pervanadate was compared to that in cultures treated with both pervanadate and agrin. The decrease in rate of detergent extraction in pervanadate-treated myotubes presumably reflects increased phosphorylation catalyzed by spontaneously active kinases. Pervanadate appears to inhibit any protein tyrosine phosphatase involved in agrin-induced changes in AChR extractability, because agrin or pervanadate alone produced the same maximum effect as agrin and pervanadate together. Therefore, in the presence of pervanadate, agrin would enhance the rate at which detergent extractability changed only if it increased protein tyrosine kinase activity. Indeed, the decrease in detergent extractability was found to occur more rapidly in cultures treated with agrin and pervanadate than in cultures treated with pervanadate alone (Fig. 10), suggesting that agrin activates a protein tyrosine kinase.

As a further test of this hypothesis, the experiment was repeated and the rate of incorporation of radiolabeled phosphate into the AChR β subunit was measured. Agrin increased the rate of phosphorylation of the AChR β subunit in pervanadate-treated cultures (Fig. 10), confirming that agrin activates a protein tyrosine kinase. As was the case during exposure to pervanadate alone, during treatment with

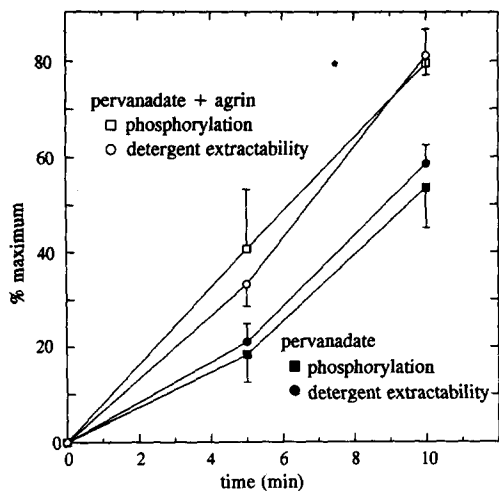


Figure 10. Effect of agrin on pervanadate-induced changes in detergent extractability and AChR phosphorylation. Myotube cultures were labeled with [32 P]orthophosphate or [125 I] α -bungarotoxin, treated for 5 or 10 min with pervanadate (*closed symbols*) or pervanadate and agrin (*open symbols*), and assayed for detergent extractability (\bullet , \circ) or phosphorylation of the AChR β subunit (\square , \blacksquare). The results were normalized to the maximum change in detergent extractability or phosphate incorporation, measured after 1-h treatment with pervanadate. Data are expressed as mean \pm SEM, $N = 16$ for detergent extraction, $N = 4$ for phosphorylation. Agrin accelerates the changes observed when protein tyrosine phosphatases are inhibited by pervanadate, indicating that agrin increases protein tyrosine kinase activity. (*Pervanadate + agrin* differs significantly from *pervanadate* [t -test, paired comparisons, $P < 0.05$], except for phosphorylation at 5 min $P \approx 0.16$).

pervanadate and agrin the change in phosphorylation of the AChR β subunit paralleled the change in detergent extractability (Fig. 10).

Discussion

The results reported here demonstrate that sodium pervanadate, an inhibitor of protein tyrosine phosphatases, blocks agrin-induced AChR aggregation, causes an increase in phosphorylation of a variety of proteins, including the AChR β , γ , and δ subunits, and reduces the rate at which AChRs are extracted from intact myotubes by detergent. Results of experiments comparing the effects of pervanadate, staurosporine, and agrin indicate that agrin-induced changes in AChR phosphorylation and detergent extractability are due to kinase activation rather than phosphatase inhibition. We conclude that agrin stimulates a protein tyrosine kinase whose activity results in a strengthening of the association of AChRs with the cytoskeleton. The effects of pervanadate indicate that there are spontaneously active protein tyrosine kinases that produce similar cytoskeletal attachment of AChRs.

AChR Phosphorylation and Attachment to the Cytoskeleton

Results of three different experiments indicate that the rate at which AChRs are extracted from intact myotubes by mild detergent treatment is reduced by increased protein tyrosine phosphorylation. First, agrin causes an increase in phos-

phorylation of the AChR β subunit and a decrease in the rate of detergent extraction (Wallace et al., 1991; Wallace, 1992). Second, as reported here, the protein tyrosine phosphatase inhibitor pervanadate causes an increase in protein tyrosine phosphorylation and a decrease in AChR detergent extractability. Third, staurosporine, at concentrations shown to inhibit protein tyrosine kinases but not protein serine/threonine kinases, blocks both the agrin- and pervanadate-induced changes in detergent extractability (Wallace, 1994; Fig. 8). Thus, we conclude that protein tyrosine phosphorylation regulates the strength of the association of AChRs with the cytoskeleton, and that staurosporine-sensitive protein tyrosine kinases mediate the reduction in detergent extractability induced by both agrin and pervanadate.

Pervanadate, and perhaps agrin as well, causes tyrosine phosphorylation of many different proteins. Not all such proteins are phosphorylated at the same rate, however (see Fig. 1). Therefore, the fact that the pervanadate-induced increase in AChR phosphorylation and decrease in detergent extractability occurred with the same time course (Figs. 7 and 10) raises the possibility that these two changes are causally related. Likewise, agrin-induced AChR phosphorylation and reduced detergent extractability also occurred contemporaneously, but at rates 10-fold slower than pervanadate-induced changes. The striking temporal correlation between changes in AChR phosphorylation and detergent extractability suggests that cytoskeletal attachment may be regulated by tyrosine phosphorylation of the AChR β subunit itself. One test of this hypothesis would be to transfect into myotubes AChR β subunits lacking the tyrosine phosphorylation site and determine if agrin and pervanadate induce changes in the detergent extractability of AChRs bearing such mutant β subunits.

How the change in detergent extractability relates to interactions between AChRs and the underlying cytoskeleton remains to be determined. Studies of AChR mobility in postsynaptic membranes from *Torpedo* electric organ and, more recently, of AChR distribution in transfected cells suggest that AChR localization and attachment to the cytoskeleton can be regulated by the 43-kD receptor-associated protein (Froehner, 1993). It is not clear which subunits of the receptor normally mediate the interaction of AChRs with the 43-kD protein and the cytoskeleton. Crosslinking studies suggest that in *Torpedo* postsynaptic membranes the 43-kD protein is bound to the AChR β subunit (Burden et al., 1983). In transfected fibroblasts, however, the 43-kD protein can bind to each of the AChR α , β , γ , and δ subunits (Phillips et al., 1993). In transfected COS cells, deletion experiments indicate that binding of the 43-kD protein to the receptor does not require the cytoplasmic domains of either the ϵ or δ subunits, suggesting that binding to the 43-kD protein is mediated by the α and/or β subunits (Yu and Hall, 1994). Unfortunately, in these experiments interactions between the 43-kD protein and the α and β subunits could not be assessed directly due to effects of deletions on receptor assembly. Yu and Hall (1994) also reported that binding of the 43-kD protein to AChRs does not require the consensus tyrosine phosphorylation site on the β subunit. The present results suggest that protein tyrosine phosphorylation, perhaps of the AChR β subunit itself, strengthens the interaction of AChRs with the underlying cytoskeleton. This effect may be mediated through alteration of the interaction of the AChR with the 43-

kD protein, or may involve interactions of AChRs with other components of the cytoskeleton, such as β -spectrin (Bloch and Morrow, 1989) or components of the dystrophin-associated protein complex (Tinsley et al., 1994).

Localized Changes in Protein Kinase and Phosphatase Activity During AChR Aggregation

A characteristic feature of agrin-induced AChR aggregation is that the effect of agrin is localized. For example, when myotubes in culture come in contact with cells or surfaces coated with agrin, AChR aggregates form selectively at the sites of contact (Campanelli et al., 1991; Tsim et al., 1992; Ferns et al., 1992, 1993). This result is not unexpected; in normal developing and adult skeletal muscle agrin, either released from nerve terminals or bound in the basal lamina, induces formation of AChR aggregates precisely at sites of neuromuscular contact, not scattered over the myotube surface (McMahan and Slater, 1984; Cohen and Godfrey, 1992; Reist et al., 1992). This suggests receptor aggregation involves localized activation of kinases and/or inhibition of phosphatases.

The results reported here suggest that spread throughout chick myotubes there are spontaneously active protein tyrosine kinases and phosphatases that catalyze a relatively rapid turnover of phosphotyrosine residues on AChRs and other proteins. We find that a widespread increase in protein tyrosine phosphorylation, produced by inhibiting such protein tyrosine phosphatases with pervanadate, results in diffusely distributed AChRs becoming associated more strongly with the cytoskeleton. If one assumes that pervanadate-induced attachment to the cytoskeleton reduces AChR mobility, then application of pervanadate would be expected to inhibit AChR aggregation, since AChRs normally accumulate in aggregates by lateral migration (Godfrey et al., 1984; Wallace, 1988). The results reported here demonstrate that pervanadate inhibits agrin-induced AChR aggregation, as predicted by such a hypothesis. We have no direct evidence regarding the identity of the tyrosine phosphoproteins responsible for inhibiting AChR aggregation. However, pervanadate-induced inhibition of aggregation developed with the same time course as phosphorylation of the AChR β subunit (Fig. 7), consistent with the idea that phosphorylation of the β subunit links AChRs to the cytoskeleton, thereby immobilizing them and preventing their redistribution into aggregates.

The ability of agrin to induce formation of AChR aggregates when added to the culture medium may reflect localized stimulation of protein tyrosine kinase activity determined by the number and distribution of agrin receptors. Myotubes in culture appear to have a relatively small number of receptors for agrin scattered over their surface (Nastuk et al., 1991). When agrin is added to the culture medium, each activated agrin receptor may stimulate kinases in its immediate vicinity, creating the patchwork of small AChR aggregates seen after 1–2 h agrin treatment (Wallace, 1988). Whether the agrin receptor is itself a protein tyrosine kinase or stimulates distinct protein kinases remains to be determined. One component of the dystrophin-associated protein complex, α -dystroglycan, appears to comprise at least part of a receptor that binds agrin to the muscle surface and, therefore, may play a role in AChR aggregation (Bowe et al., 1994; Gee et al., 1994; Campanelli et al., 1994; Sugiyama et al., 1994). Whether this or any component of the dys-

trophin-associated protein complex has agrin-inducible protein tyrosine kinase activity remains to be determined. On the other hand, at least two soluble protein tyrosine kinases appear to be associated with AChRs in *Torpedo* postsynaptic membranes (Swope and Haganir, 1993). Each of these kinases can phosphorylate AChRs, but there is as yet no evidence as to whether or not these kinases play a role in receptor aggregation.

Localized activation of protein tyrosine kinases by agrin, leading to localized tyrosine phosphorylation and immobilization of AChRs, may be sufficient to account for the formation of AChR aggregates. However, the variety of components that accumulate in agrin-induced specializations (Wallace, 1986, 1989; Shadiak and Nitkin, 1991) and the observation that accumulation of at least some of these appears to be independent of AChR aggregation (Nitkin and Rothschild, 1990) makes it seem likely that kinases (and perhaps phosphatases) regulated directly or indirectly by agrin will affect phosphorylation not only of AChRs but also of additional proteins as well. The resulting agrin-induced changes in protein tyrosine and serine/threonine phosphorylation may play a role in AChR aggregation or may regulate the accumulation of other components of the postsynaptic apparatus.

I thank Drs. T. Meier, J. Caldwell, and G. Perez for their constructive criticism of the manuscript, Donna Kautzman-Eades for her excellent technical assistance, Drs. H. Gates and T. Meier for preparation of anti-phosphotyrosine antibodies, and Kathy Fernandez for her unfailing secretarial support.

These studies were funded by National Institutes of Health Grant NS29673.

Received for publication 22 September 1994 and in revised form 14 December 1994.

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