Staurosporine Inhibits Agrin-induced Acetylcholine Receptor Phosphorylation and Aggregation

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Abstract. Agrin, a protein that mediates nerve-induced acetylcholine receptor (AChR) aggregation at developing neuromuscular junctions, has been shown to cause an increase in phosphorylation of the β, γ, and δ subunits of AChRs in cultured myotubes. As a step toward understanding the mechanism of agrin-induced AChR aggregation, we examined the effects of inhibitors of protein kinases on AChR aggregation and phosphorylation in chick myotubes in culture. Staurosporine, an antagonist of both protein serine and tyrosine kinases, blocked agrin-induced AChR aggregation in a dose-dependent manner; 50% inhibition occurred at ~2 nM. The extent of inhibition was independent of agrin concentration, suggesting an effect downstream of the interaction of agrin with its receptor. Staurosporine blocked agrin-induced phosphorylation of the AChR β subunit, which occurs at least in part on tyrosine residues, but did not reduce phosphorylation of the γ and δ subunits, which occurs on serine/threonine residues. Staurosporine also prevented the agrin-induced decrease in the rate at which AChRs are extracted from intact myotubes by mild detergents. H-7, an antagonist of protein serine kinases, inhibited agrin-induced phosphorylation of the γ and δ subunits but did not block agrin-induced phosphorylation of the AChR β subunit, AChR aggregation, or the decrease in AChR extractability. The results provide support for the hypothesis that tyrosine phosphorylation of the β subunit plays a role in agrin-induced AChR aggregation.

THROUGHOUT the nervous system certain transmitter receptors are clustered in postsynaptic membranes. The high density of receptors is crucial for normal synaptic transmission. As a step toward understanding how such aggregates of receptors are formed during development and maintained at adult synapses, we have been studying the mechanism by which agrin induces the formation of clusters of acetylcholine receptors on chick myotubes in culture. Agrin, a protein released by nerve terminals at developing neuromuscular junctions, triggers the formation of specializations that contain several components of the postsynaptic apparatus, including aggregates of acetylcholine receptors (AChRs)[1] (McMahan and Wallace, 1989; McMahan, 1990; Cohen and Godfrey, 1992; Reist et al., 1992). Agrin also has been shown to induce an increase in phosphorylation of the β, γ, and δ AChR subunits in myotubes in culture (Wallace et al., 1991). Agrin-induced phosphorylation of the γ and δ subunits can be blocked by the protein serine kinase inhibitor H-7 without preventing AChR aggregation, indicating that phosphorylation of these subunits is probably occurring primarily on serine or threonine residues and is not necessary for aggregation. On the other hand, agrin-induced phosphorylation of the β AChR subunit is not blocked by H-7, and occurs, at least in part, on tyrosine residues. Agrin-induced tyrosine phosphorylation largely precedes receptor aggregation and several treatments known to inhibit agrin-induced AChR aggregation also inhibit tyrosine phosphorylation of the β subunit. These and other findings have led to the hypothesis that agrin-induced tyrosine phosphorylation of the β subunit plays a role in AChR aggregation (Wallace et al., 1991; Wallace, 1992).

The experiments reported here were undertaken to test this hypothesis by examining the effects of protein tyrosine kinase inhibitors on agrin-induced AChR aggregation and phosphorylation. Several of those studied, including herbimycin A, genistein, lavendustin A, geldanamycin, methyl 2,5-dihydroxycinnamate, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid, and tyrphostin, are generally considered relatively selective inhibitors of protein tyrosine kinases (Uehara et al., 1986, 1988; Akiyama et al., 1987; Gazit et al., 1989; Onoda et al., 1989; Umezawa et al., 1990). One other, staurosporine, is a potent but nonselective antagonist of protein kinases (Ruegg and Burgess, 1989). Staurosporine inhibits both protein serine/threonine kinases, such as protein kinase C and cyclic nucleotide-dependent kinases (Tamaoki et al., 1986; Ruegg and Burgess, 1989), as well as protein tyrosine kinases, such as the trk protein tyrosine kinase activated by NGF in PC-12 cells (Berg et al.,

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

1992; Ney et al., 1992; Tapley et al., 1992). Of these kinase antagonists, staurosporine was the only one that inhibited any of agrin's effects, and it blocked both agrin-induced phosphorylation of the AChR β subunit and receptor aggregation.

Materials and Methods

Staurosporine and prestained high molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO), herbimycin A from Calbiochem-Novabiochem Corp. (La Jolla, CA), and genistein, lavendustin A, geldanamycin, methyl 2,5-dihydroxycinnamate, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid, and tyrphostin from GIBCO BRL (Gaithersburg, MD).

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 routinely made on 5- to 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 isolated as previously described (Wallace et al., 1991). Briefly, cultures were labeled with biotinylated α-bungarotoxin (Molecular Probes, Inc., Eugene, OR) and the toxin-receptor complexes were solubilized in buffer containing 1% Triton X-100 and isolated on streptavidin-conjugated Sepharose beads (Molecular Probes, Inc.).

Measurement of AChR Phosphorylation

To measure changes in AChR phosphorylation, cultures were rinsed in 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, 100 μg/ml streptomycin, and incubated in the same medium containing 1 mCi/ml [^{32}P]orthophosphate ([^{32}P]H_3PO_4 in H_2O; ICN Biomedicals Inc., Costa Mesa, CA). In some experiments cultures were incubated for 3 h in medium containing [^{32}P]H_3PO_4 to label the ATP pool and phosphoproteins to steady state levels and then incubated an additional 3 h in the presence or absence of agrin and/or inhibitors. In other experiments the cultures were first incubated for 1 h with or without inhibitors and then incubated for 3 h in medium containing [^{32}P]H_3PO_4 in the presence or absence of agrin and/or inhibitors. Both protocols gave similar results and the data have been combined. After the 3-h incubation, AChRs were isolated as described above, the beads eluted into SDS sample buffer, and the eluate electrophoresed on 7.5% SDS-polyacrylamide gels according to Laemmli (1970). The gels were fixed, dried under reduced pressure, and exposed to pre-flashed autoradiography film (Hyperfilm-MP; Amersham Corp., Arlington Heights, IL). The resulting autoradiograms were imaged with a Star 1 chilled CCD camera (Photometrics Ltd., Tuscon, AZ) and the images were analyzed using WHIP Virtual Image Processing Software (G. W. Hannaway and Associates, Boulder, CO) to produce densitometric scans of the autoradiograms. The radioactivity associated with each subunit was determined using Sigma Plot Software (Jandel Sci., Corte Madera, CA) by obtaining the best fit to the scan assuming the density profile was the sum of three normal distributions.

Detergent Extraction of AChRs

Intact myotubes were labeled with 2×10^{-8} M [^{125}I]α-bungarotoxin (Amersham Corp.) and toxin-receptor complexes were solubilized by mild detergent treatment (0.05% Triton X-100 in 0.286 M sucrose, 50 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) as previously described (Wallace, 1992). Nonspecific binding was determined by adding 2×10^{-6} M α-bungarotoxin together with the radiolabeled toxin.

Quantitation of AChR Aggregation

Myotube cultures were labeled with 2×10^{-8} M rhodamine-conjugated α-bungarotoxin (Molecular Probes, Inc.), rinsed, and fixed as previously described (Wallace, 1989). Myotube segments were viewed with a Nikon Optiphot microscope equipped for phase contrast and epifluorescence. AChR aggregation was quantitated in two ways: (*a*) AChR aggregates were counted by eye and the data presented as number of aggregates/myotube segment; or (*b*) myotubes were imaged with a Star 1 chilled CCD camera and analyzed using WHIP Virtual Image Processing Software to determine the total fluorescence intensity from aggregated AChRs (Wallace, 1992), in which case the data are presented as AChR aggregation. In the experiments described here both methods yielded similar results.

Results

Inhibition of Agrin-induced AChR Aggregation by Protein Tyrosine Kinase Antagonists

We screened several inhibitors of protein kinases for their

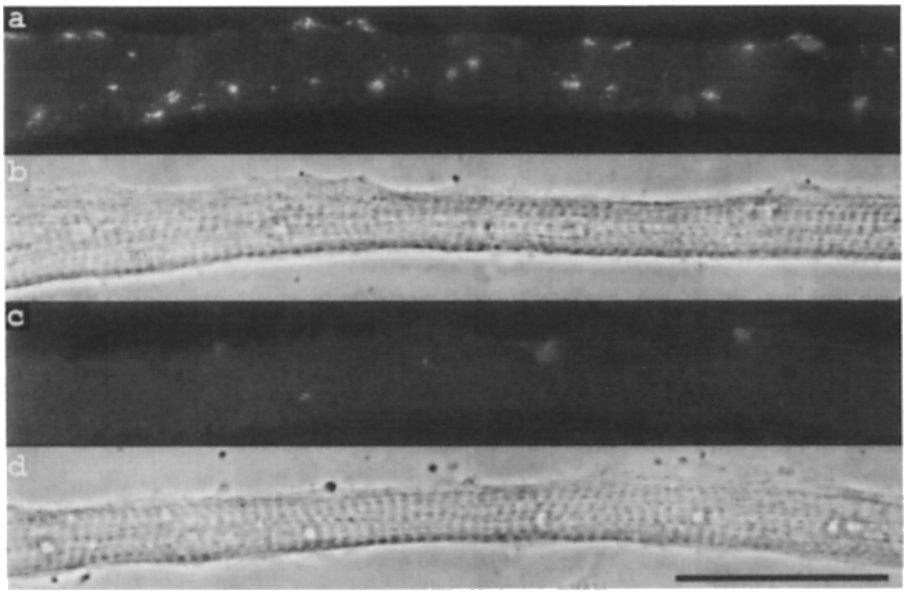

Figure 1. Staurosporine inhibits agrin-induced AChR aggregation. Phase contrast (*b* and *d*) and fluorescence (*a* and *c*) micrographs of myotubes incubated for 6 h with agrin in normal medium (*a* and *b*) or in the presence of 20 nM staurosporine (*c* and *d*), then labeled with rhodamine α-bungarotoxin to reveal the distribution of AChRs. Bar, 50 μm.

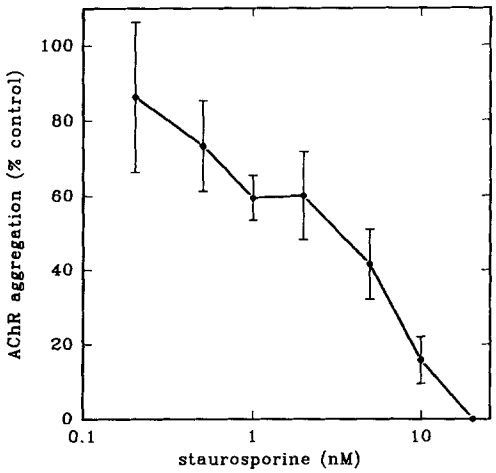

Figure 2. Dose dependence of the inhibition of agrin-induced AChR aggregation by staurosporine. Myotubes were incubated for 6 h with agrin and the indicated concentration of staurosporine. AChRs were labeled with rhodamine α-bungarotoxin and the extent of AChR aggregation compared to that seen in cultures incubated in medium without staurosporine. Data is expressed as mean ± SEM, $n = 6$ ($n = 3$ for 0.2 nM staurosporine).

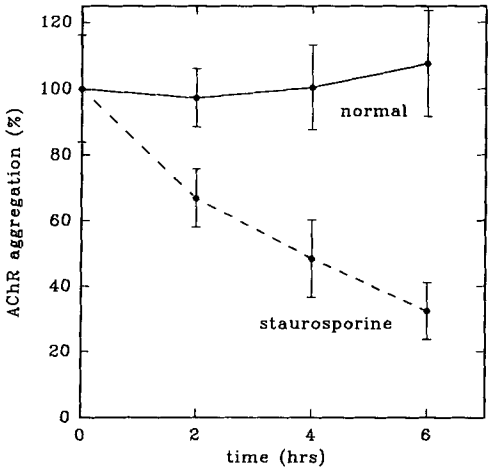

Figure 4. Staurosporine accelerates AChR aggregate disappearance. Myotube cultures were treated overnight with agrin, rinsed, and incubated for the designated time in normal medium or in medium containing 20 nM staurosporine. AChRs were visualized with rhodamine α-bungarotoxin and the extent of aggregation compared to that observed at the time agrin was removed. Data is expressed as the mean ± SEM, $n = 6$.

effects on agrin-induced AChR aggregation. Inhibitors were added alone or together with agrin to chick myotubes in culture. Herbimycin A, genistein, lavendustin A, geldanamycin, methyl 2,5-dihydroxycinnamate, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid, and tyrphostin had little or no consistent effect on AChR aggregation at concentrations that were not obviously toxic to the cells. Staurosporine, on the other hand, consistently reduced agrin-induced receptor aggregation (Fig. 1).

Staurosporine inhibited AChR aggregation in a dose-dependent manner; 50% inhibition occurred at a concentration of ~2 nM (Fig. 2). To characterize further the inhibition of AChR aggregation by staurosporine, we examined the dose dependence of agrin-induced AChR aggregation in the presence and absence of staurosporine. As illustrated in Fig. 3, 5 nM staurosporine inhibited aggregation by ~70% regardless of the agrin concentration. Thus, staurosporine did not appear to compete with agrin for its receptor on the myotube surface, but rather to inhibit some subsequent step in receptor aggregation.

Cells appeared healthy after a 6-h incubation in ≤20 nM staurosporine (Fig. 1). This suggests that the effect of staurosporine on agrin-induced AChR aggregation was selective and not due to general cytotoxity. Consistent with this idea is the finding that treatment with staurosporine did not reduce the number of AChRs on the myotube surface, as determined by specific binding of ^{125}I-α-bungarotoxin (binding to staurosporine-treated cultures was 97 ± 3% of controls [mean ± SEM, $n = 22$]). High concentrations of staurosporine (≥50 nM) appeared toxic, causing many of the cells to detach from the substrate during a 6-h incubation.

Although myotubes appeared healthy at the end of a brief exposure (6 h) to concentrations of staurosporine ≤20 nM, many of the myotubes subsequently detached from the substrate, even if staurosporine was removed from the medium. However, cells that did survive regained to some extent their ability to respond to agrin. Thus, although brief exposure to staurosporine was detrimental to myotube survival, inhibition of agrin-induced receptor aggregation was, at least to a limited extent, reversible.

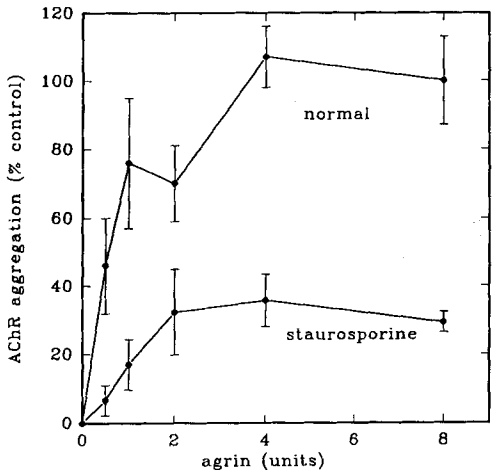

Figure 3. Dose dependence of agrin-induced AChR aggregation in the presence or absence of staurosporine. Myotubes were incubated for 6 h in the indicated concentration of agrin in the presence or absence of 5 nM staurosporine. AChRs were visualized with rhodamine α-bungarotoxin and the extent of aggregation compared to that seen in cultures incubated in normal medium with 8 U of agrin. Data is expressed as mean ± SEM, $n = 5$.

Effects of Staurosporine on Aggregate Stability

Staurosporine caused a slight, although not statistically significant, decrease in the extent of AChR aggregation in control cultures, suggesting that phosphorylation may be involved in the maintenance of spontaneously occurring AChR aggregates. To test the effect of staurosporine on the stability

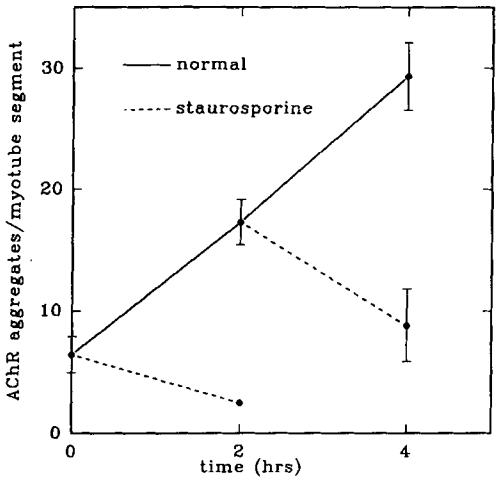

Figure 5. Staurosporine inhibits AChR aggregate formation. Cultures were incubated with agrin with or without 20 nM staurosporine for 2–4 h as indicated. AChRs were then labeled and the number of aggregates was determined. Data is expressed as mean ± SEM, $n = 3$.

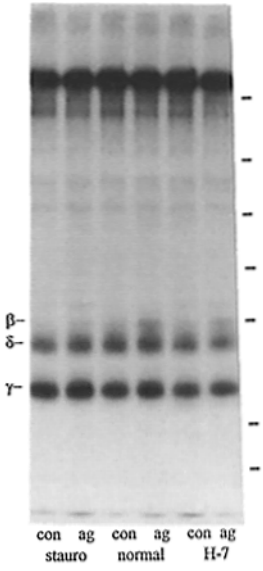

Figure 6. Effects of staurosporine and H-7 on AChR phosphorylation. Autoradiogram of AChRs from myotubes incubated for 4 h with 1 mCi/ml $[^{32}P]H_3PO_4$ with (*ag*) or without (*con*) agrin in normal medium (*normal*) or medium containing 20 nM staurosporine (*stauro*) or 0.2 mM H-7 (*H-7*). AChRs were isolated, the subunits separated by SDS–polyacrylamide gel electrophoresis, and the incorporation of radiolabeled phosphate determined by autoradiography. The positions of the β, γ, and δ subunits are indicated (determined with subunit-specific monoclonal antibodies [Wallace et al., 1991]), as are the positions of prestained molecular mass standards (top to bottom: 180, 116, 84, 58, 48.5, 36.5, and 26.6 kD).

of agrin-induced aggregates myotubes were treated overnight with agrin to induce aggregate formation, rinsed, and incubated in normal medium with or without staurosporine for 2–6 h. Receptors were then labeled and aggregation measured. In normal medium the extent of AChR aggregation did not change significantly during the 6-h incubation, as expected (Wallace, 1988), while in the presence of staurosporine receptor aggregation was reduced by 70% (Fig. 4). The acceleration in the rate of disappearance of aggregates caused by staurosporine suggests that ongoing phosphorylation may play a role in the maintenance of both spontaneously occurring and agrin-induced receptor aggregates.

Effects of Staurosporine on Aggregate Formation

The effect of staurosporine on aggregate stability appeared insufficient to account for the complete inhibition of aggregate formation observed during a 6-h incubation. To determine if staurosporine did indeed block the formation of receptor aggregates, the rate of appearance of AChR aggregates was measured in control and staurosporine-treated cultures. As illustrated in Fig. 5, during a 2-h incubation with agrin, staurosporine completely inhibited the formation of new aggregates, while causing the number of preexisting aggregates to decrease by ~50%. Thus, we conclude that staurosporine not only destabilizes existing aggregates but also inhibits the process by which aggregates form.

Inhibition of Agrin-induced AChR Phosphorylation by Staurosporine

To assess the effects of tyrosine kinase inhibitors on AChR phosphorylation, $[^{32}P]H_3PO_4$ was added to the culture medium to label phosphoproteins and the cultures were incubated 3 h in the presence or absence of agrin and/or the inhibitor. At the end of the 3-h incubation, AChRs were isolated, the subunits separated by SDS–polyacrylamide gel electrophoresis, and the extent of phosphorylation of each subunit determined by autoradiography. As illustrated in Fig. 6, in control cultures incubated in normal medium radiolabeled phosphate was incorporated into the γ and δ AChR subunits, and to a much lesser extent the β subunit. Most tyrosine kinase inhibitors tested, including genistein, lavendustin A, geldanamycin, methyl 2,5,-dihydroxycinnamate, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid, and tyrphostin had no significant effect on the level of spontaneous AChR phosphorylation. Staurosporine, on the other hand, caused an increase in phosphorylation of the γ subunit, no detectable change in the δ subunit, and a decrease in phosphorylation of the β subunit (Fig. 6; Table I). These results suggest that staurosporine inhibits selectively a spontaneously active kinase(s) that phosphorylates the AChR β subunit, or activates the corresponding phosphatase(s). Moreover, the modest effects of 20 nM staurosporine on spontaneous phosphorylation provide additional evidence that staurosporine is neither cytotoxic nor does it nonselectively disrupt protein phosphorylation during such brief (3–6 h) incubations.

As reported previously (Wallace et al., 1991), agrin increased the incorporation of phosphate into all three subunits, the increase in labeling of the β subunit was most conspicuous (Fig. 6; Table I). (In the experiments reported here the increase in phosphorylation of the δ subunit, although consistently observed, was not statistically significant.) Staurosporine (20 nM) inhibited the agrin-induced increase in AChR phosphorylation of the β subunit by 88%, but did not block agrin-induced phosphorylation of the γ or δ subunits (Figs. 6 and 7; Table I). Measurements of receptor aggregation made on sister cultures showed that staurosporine inhibited agrin-induced AChR aggregation by 92% (Fig. 7; Table I). Thus, staurosporine blocked selectively agrin-induced phosphorylation of the β subunit and agrin-induced AChR aggregation. We also confirmed that H-7 markedly reduced agrin-induced phosphorylation of the γ and δ subunits, while inhibiting only slightly agrin-induced AChR aggregation and phosphorylation of the β subunit (Figs. 6 and 7; Table I). None of the other tyrosine kinase inhibitors tested had any significant effect on agrin-induced changes in AChR phosphorylation (data not shown).

Table I. Effects of Inhibitors on AChR Phosphorylation, Aggregation, and Detergent Extractability

	Control			Agrin treated		
	No inhibitor	Staurosporine	H-7	No inhibitor	Staurosporine	H-7
Phosphorylation*						
β	13.5 ± 1.5	7.1 ± 0.7‖	10.7 ± 1.1	26.9 ± 3.4‖	8.7 ± 1.1¶	19.3 ± 2.5¶
γ	100	128.0 ± 10.1‖	96.6 ± 3.3	111.4 ± 4.2‖	138.2 ± 10.2¶	100.1 ± 3.8
δ	83.1 ± 2.4	84.4 ± 9.9	77.8 ± 4.9	90.4 ± 4.8	92.2 ± 8.7¶	77.1 ± 4.7
Aggregation‡	2.9 ± 0.8	1.4 ± 0.2	2.9 ± 0.9	16.1 ± 4.4‖	2.4 ± 0.8	15.4 ± 5.6¶
Detergent extraction§	50.5 ± 1.6	46.3 ± 4.6	48.9 ± 1.2	61.6 ± 1.2‖	46.8 ± 1.3	59.2 ± 1.4¶

Date expressed as mean ± SEM; $n = 5$ for phosphorylation, $n = 4$ for aggregation and detergent extraction.
* Autoradiogram densities normalized to γ subunit of control myotubes in normal medium (= 100).
‡ Number of aggregates/myotube segment.
§ Percent AChRs remaining after 2 min extraction.
‖ Differs from control cultures with no inhibitor, $P < 0.05$, Student's t test.
¶ Differs from control cultures with corresponding inhibitor, $P < 0.05$, Student's t test, paired comparisons.

Effects of Staurosporine on Detergent Extractability of AChRs

Previous studies have shown that agrin causes a decrease in the rate at which AChRs are extracted from intact myotubes by mild detergent treatment (Wallace, 1992). This change in detergent extractability occurs with the same time course as AChR phosphorylation. As a step toward understanding the sequence of events triggered by agrin, we sought to determine whether staurosporine inhibited the change in detergent extractability. Myotubes were incubated for 4 h with agrin with or without staurosporine, labeled with [^{125}I]α-bungarotoxin, rinsed, and the rate at which AChRs were extracted into saline containing 0.05% Triton X-100 was measured (Fig. 8). Staurosporine caused a slight, but not statistically significant, increase in the rate at which receptors were extracted from control myotubes, and blocked completely the agrin-induced decrease in the rate of detergent extraction (Figs. 7 and 8 *a*; Table I). H-7, on the other hand, had little effect on the rate at which receptors were extracted from either control or agrin-treated myotubes (Fig. 7 and 8 *b*; Table I). This is consistent with the hypothesis that the agrin-induced decrease in detergent extractability is a consequence of phosphorylation of the AChR β subunit (Wallace, 1992).

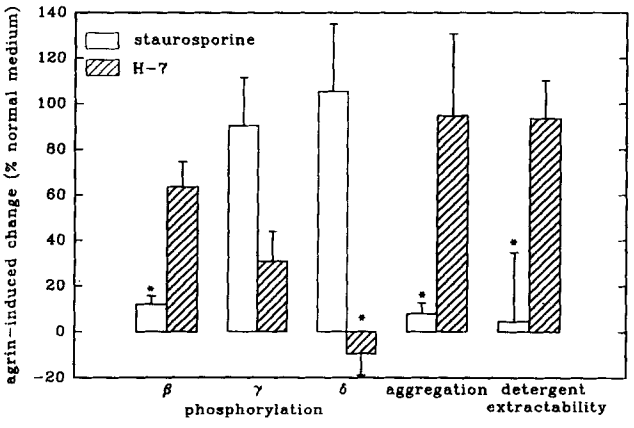

Figure 7. Effects of staurosporine and H-7 on the agrin-induced change in AChR phosphorylation, aggregation, and detergent extractability. Data from experiments summarized in Table I. * differs from normal medium, $p < 0.05$, Student's t test.

Discussion

Regulation of AChR Distribution by Protein Phosphorylation

The distribution of AChRs on the surface of muscle cells can be regulated, directly or indirectly, by the activity of several protein kinases and phosphatases. In some cases activation of a kinase prevents AChR aggregation. For example, treating chick myotubes with phorbol 12-myristate 13-acetate, an activator of protein kinase C, blocks the formation of agrin-induced AChR aggregates and causes preexisting aggregates to disperse (Ross et al., 1988; Wallace, 1988). Likewise, myotubes transformed with the Rous sarcoma virus do not have spontaneously occurring AChR aggregates, nor do they respond to aggregating factors such as agrin (Anthony et al., 1984). These effects of transformation are mediated by pp60$^{v\text{-}src}$, a protein tyrosine kinase, and arise, at least in part, from changes in the cytoskeleton induced by this kinase activity (Anthony et al., 1988; Marazzi et al., 1989).

On the other hand, activity of certain protein tyrosine kinases appears to be necessary for AChR aggregation. AChR aggregates can be induced to form in Xenopus myocytes by basic fibroblast growth factor attached to microbeads; the receptor for basic fibroblast growth factor is a protein tyrosine kinase and the formation of such aggregates of AChRs is prevented by tyrphostin RG-50864, an inhibitor of protein tyrosine kinases (Peng et al., 1991). The formation of AChR aggregates in Xenopus muscle cells in response to electric fields is also abolished by tyrphostin RG-50864 (Peng et al., 1993), implicating protein tyrosine kinase activity in this phenomenon as well. Here we report that while tyrphostin had little effect on AChR distribution in cultured chick myotubes, another inhibitor of protein tyrosine (and serine) kinases, staurosporine, effectively blocked agrin-induced AChR aggregation. The broad and diphasic nature of the relationship between staurosporine concentration and AChR aggregation (Fig. 2) suggests that staurosporine may be inhibiting

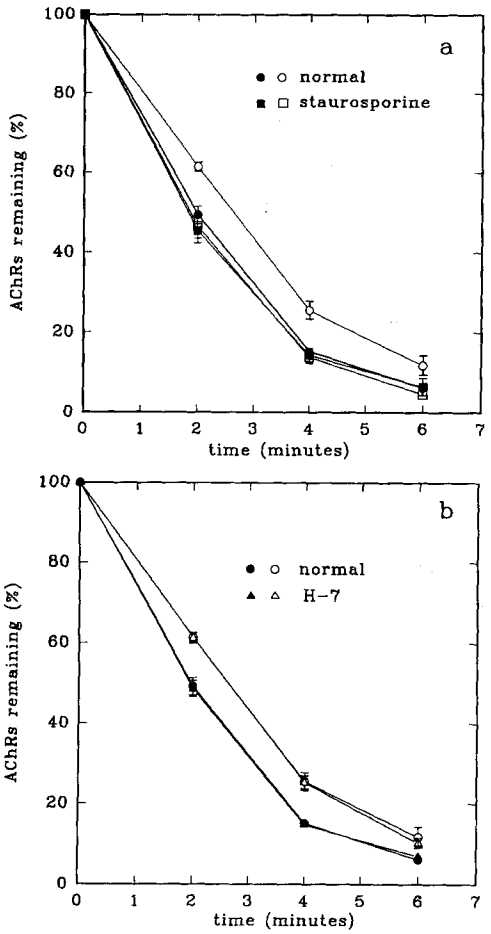

Figure 8. Effects of staurosporine and H-7 on the agrin-induced decrease in the rate of detergent extraction of AChRs. Cultures were incubated for 4 h with (*open symbols*) or without (*filled symbols*) agrin in normal medium (*a* and *b*, *circles*) or medium containing 20 nM staurosporine (*a*, *squares*) or 0.2 mM H-7 (*b*, *triangles*). Myotubes were labeled with [^{125}I]α-bungarotoxin, rinsed, and incubated with buffer containing 0.05% Triton X-100. The appearance of ^{125}I in the detergent extracts was used to calculate the percentage of receptors remaining associated with the myotubes. Agrin caused a decrease in the rate of detergent extraction. Staurosporine, but not H-7, prevented the agrin-induced change in detergent extractability. Data, from one of four such experiments, is expressed as the mean ± SEM of triplicate determinations.

more than one kinase involved in regulating receptor distribution.

Okadaic acid, an inhibitor of protein serine phosphatases, and sodium pervanadate, a tyrosine phosphatase inhibitor, also blocked agrin-induced AChR aggregation (unpublished observations), suggesting that the distribution of AChRs may be regulated by protein phosphatase activity as well.

Phosphorylation of AChRs and Other Proteins During Receptor Aggregation

In chick myotubes in culture, direct measurements of phosphotyrosine content and of radiolabeled phosphate incorporation demonstrate that agrin-induced AChR phosphorylation occurs during the first few hours of receptor aggregation, and immunohistochemical experiments show that agrin-induced receptor aggregates stain with antiphosphotyrosine antibodies as soon as they begin to form (Wallace et al., 1991). On the other hand, the intensity with which developing rat neuromuscular junctions stain with antiphosphotyrosine antibodies increases conspicuously weeks after AChR aggregates are first formed, suggesting that in the rat AChR tyrosine phosphorylation occurs well after receptors aggregate at synaptic sites (Qu et al., 1990). This could indicate that AChR aggregation is mediated by different mechanisms in these two species. However, in neither case is it known to what extent the intensity of immunohistochemical staining reflects antiphosphotyrosine antibodies binding to phosphorylated proteins other than AChRs. Indeed, in Xenopus myocytes it has been shown that phosphotyrosine-containing proteins other than AChRs accumulate at sites of receptor aggregation (Baker and Peng, 1993); we find the same to be true for chick myotubes in culture (Wallace, B. G., unpublished observations). Direct measurements of the extent of tyrosine phosphorylation of AChRs in developing rat or Xenopus muscle have yet to be made. Thus, the evidence suggests that AChR aggregation is accompanied by increased tyrosine phosphorylation of AChRs and of other proteins that accumulate at the same location as the AChRs. Since not all of these phosphotyrosine-containing proteins accumulate at the same rate (Baker and Peng, 1993), the time course of staining with antiphosphotyrosine antibodies cannot be assumed to reflect the time course of AChR phosphorylation.

Tyrosine Phosphorylation and AChR Aggregation

In chick myotubes agrin induces increased phosphorylation of the β, γ, and δ AChR subunits (Wallace et al., 1991) (Figs. 6 and 7; Table I). Some, if not all, of the phosphorylation of the β subunit occurs on tyrosine residues (Wallace et al., 1991). We have not detected binding of antiphosphotyrosine antibodies to the γ and δ subunits on Western blots of AChRs isolated from either control or agrin-treated cultures. This suggests that these subunits, which contain consensus sequences for both tyrosine and serine phosphorylation sites (Huganir and Miles, 1989), are phosphorylated primarily on serine residues. Consistent with this hypothesis is the observation that H-7, a protein serine kinase inhibitor, blocks agrin-induced phosphorylation of the γ and δ subunits, but not of the β subunit (Wallace et al., 1991) (Figs. 6 and 7, Table I). These findings suggest that agrin might activate both protein serine and tyrosine kinases, or inhibit the corresponding phosphatases. An alternative possibility is that the γ and δ subunits contain phosphotyrosine residues in a context that is not recognized by the antiphosphotyrosine antibodies we have used. In this regard it is worth noting that in our studies of the phosphotyrosine content of the β subunit of isolated chick AChRs we detected binding of only one of several antiphosphotyrosine antisera and monoclonal antibodies we tested. In either case, since H-7 does not block agrin-induced AChR aggregation but does inhibit phosphorylation of the γ and δ subunits, we conclude that agrin-induced phosphorylation of the γ and δ subunits is not a necessary step in receptor aggregation.

On the other hand, agrin-induced phosphorylation of tyrosine residues on the AChR β subunit appears to play a role in receptor aggregation. Agrin-induced AChR aggregation

and tyrosine phosphorylation have the same dose dependence, and both responses are inhibited by polyanions, phorbol ester, and low pH (Wallace et al., 1991, 1992). Here we report that the protein kinase antagonist staurosporine selectively inhibited agrin-induced AChR aggregation and phosphorylation of the β subunit (Figs. 6 and 7; Table I). This provides the most direct evidence to date that agrin-induced tyrosine phosphorylation of the AChR β subunit is a necessary step in receptor aggregation.

Despite the many parallels between agrin-induced AChR aggregation and tyrosine phosphorylation of the β subunit, the possibility must be considered that aggregation of AChRs is not dependent on receptor phosphorylation. As noted above, it appears likely that proteins in addition to AChRs are phosphorylated in response to agrin treatment, although direct evidence for this has yet to be obtained. Phosphorylation of any of these proteins might mediate or regulate aggregation of AChRs, or play a role in the accumulation of other components of the postsynaptic apparatus in agrin-induced specializations. The results reported here demonstrate that in chick myotubes only those phosphorylation events that are inhibited by staurosporine, but not by H-7 or any of the other tyrosine kinase antagonists tested, are likely to be directly involved in AChR aggregation.

Phosphorylation and AChR Aggregate Stability

The finding that staurosporine caused AChR aggregates in both control and agrin-pretreated cultures to disperse suggests that similar mechanisms underlie the formation and stability of both spontaneously occurring and agrin-induced aggregates. Consistent with this idea are the findings that adding staurosporine to control cultures caused both phosphorylation of the AChR β subunit and AChR aggregation to decrease to a similar extent (Table I). Moreover, we have observed that when agrin is added to myotube cultures it not only induces formation of new AChR aggregates, but also induces an increase in the size and receptor density of preexisting aggregates (unpublished observation).

AChR Aggregation and Attachment to the Cytoskeleton

AChRs accumulate in agrin-induced aggregates by moving laterally in the myotube membrane (Godfrey et al., 1984; Wallace, 1988). Several lines of evidence suggest that AChRs are held within aggregates by attachment to the cytoskeleton, and that this linkage occurs through binding of the AChR β subunit to a 43-kD receptor-associated protein (Froehner, 1993). For example, measurements of AChR mobility in rat myotubes indicate that, while many nonaggregated receptors are free to diffuse laterally in the plane of the membrane, AChRs in spontaneously occurring aggregates are relatively immobile (Axelrod et al., 1976). In addition, AChRs in aggregates appear more resistant to extraction with mild detergents (Prives et al., 1982; Podleski and Salpeter, 1988). Treating myotubes with agrin also causes AChRs to become more resistant to detergent extraction (Wallace, 1992) (Fig. 8). The change in detergent extractability occurs with the same time course as agrin-induced AChR phosphorylation. The finding that staurosporine, but not H-7, prevented the agrin-induced change in detergent extractability (Figs. 7 and 8, Table I) suggests that, as is the case for aggregation, the decrease in detergent extractability is a consequence of tyrosine phosphorylation of the β subunit and not phosphorylation of the γ and δ subunits. Likewise, addition of staurosporine to control cultures decreased selectively phosphorylation of the β subunit and appeared to increase slightly the rate at which AChRs were extracted into detergent solution (Table I). (The increase in detergent extractability in control cultures treated with staurosporine, although not statistically significant, was consistent, occurring in six of seven experiments.) This observation suggests that phosphorylation of the β subunit also regulates resistance to detergent extraction in control cultures and provides further support for the conclusion that similar mechanisms underlie the formation and stability of both spontaneously occurring and agrin-induced aggregates. In light of the results presented in this report one might speculate that agrin-induced tyrosine phosphorylation of the AChR β subunit modifies in some way the interaction of the receptor with the 43-kD receptor-associated protein and, in turn, with the cytoskeleton, resulting in a decrease in detergent extractability and leading to the formation of AChR aggregates.

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