Agrin-induced Acetylcholine Receptor Clustering in Mammalian Muscle Requires Tyrosine Phosphorylation

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Abstract. Agrin is thought to be the nerve-derived factor that initiates acetylcholine receptor (AChR) clustering at the developing neuromuscular junction. We have investigated the signaling pathway in mouse C2 myotubes and report that agrin induces a rapid but transient tyrosine phosphorylation of the AChR β subunit. As the β-subunit tyrosine phosphorylation occurs before the formation of AChR clusters, it may serve as a precursor step in the clustering mechanism. Consistent with this, we observed that tyrosine phosphorylation of the β subunit correlated precisely with the presence or absence of clustering under several experimental conditions. Moreover, two tyrosine kinase inhibitors, herbimycin and staurosporine, that blocked β-subunit phosphorylation also blocked agrin-induced clustering. Surprisingly, the inhibitors also dispersed preformed AChR clusters, suggesting that the tyrosine phosphorylation of other proteins may be required for the maintenance of receptor clusters. These findings indicate that in mammalian muscle, agrin-induced AChR clustering occurs through a mechanism that requires tyrosine phosphorylation and may involve tyrosine phosphorylation of the AChR itself.

THE accumulation of acetylcholine receptors (AChR)[1] beneath the nerve terminal is one of the first discernible steps in synapse formation at the neuromuscular junction (Hall and Sanes, 1993). The concentration of AChRs in the postsynaptic muscle membrane is induced by the nerve and is due both to clustering of the preexisting receptor and to local insertion of the newly synthesized receptor. Agrin is thought to be the nerve-derived factor that induces the initial redistribution of the AChR (McMahan, 1990; Ferns and Hall, 1992), as well as the accumulation of several other synaptic components (Wallace, 1989). Although agrin is expressed in embryonic muscle and in other nonneural tissues, neurons synthesize a specific isoform of agrin, generated by alternative RNA splicing, that is significantly more biologically active than the forms found in muscle (Ruegg et al., 1992; Tsim et al., 1992; Ferns et al., 1993; Hoch et al., 1993). Neurally derived agrin is believed to cluster AChRs by acting on a specific receptor in the myotube membrane. Several groups have recently shown that the predominant agrin-binding protein in muscle is α-dystroglycan (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), a cell surface protein that is part of a transmembrane complex of proteins associated with dystrophin. There is conflicting data, however, as to whether α-dystroglycan is the signaling receptor responsible for the initiation of AChR clusters, and other roles for the protein have been suggested (Sealock and Froehner, 1994; Sugiyama et al., 1994; Fallon and Hall, 1994).

Although the receptor for agrin and its mode of signaling are not yet established, recent evidence suggests that tyrosine phosphorylation may be part of the signaling pathway, at least in chick muscle (Wallace et al., 1991; Qu and Huganir, 1994). Many growth and differentiation factors activate receptor or cytoplasmic tyrosine kinases (Schlessinger and Ullrich, 1992; Kishimoto et al., 1994) whose phosphorylation of specific target proteins then regulates protein–protein interactions that form the signaling cascade (Weiss and Littman, 1994). For example, tyrosine phosphorylation can result in the binding of other proteins via their SH2 domains, leading to the formation of signaling complexes, or to the reorganization of cytoskeletal elements. Such a mechanism may be involved in the agrin-induced immobilization and clustering of the AChR.

We have examined whether agrin-induced AChR clustering in mammalian muscle involves tyrosine phosphorylation. We find that agrin induces a transient tyrosine phosphorylation of the AChR β subunit in mouse C2 myotube cultures. Moreover, tyrosine kinase inhibitors that

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

block this agrin-induced phosphorylation also block clustering. Our results indicate that in mammalian muscle, agrin-induced AChR clustering occurs through a mechanism that requires tyrosine phosphorylation and may involve tyrosine phosphorylation of the AChR itself.

Materials and Methods

Production of Recombinant Agrin

To produce recombinant agrin in soluble form, we expressed the COOH-terminal half of agrin isoforms by transfection in COS cells. We used both the most active, neural-specific isoform (C-$Ag_{12,4,8}$), and the predominant muscle isoform (C-$Ag_{12,0,0}$) (Ferns et al., 1993). COS cells were transiently transfected by the DEAE-dextran method, as described previously (Ferns et al., 1993), and the media containing the secreted agrin was collected. The concentration of agrin was determined using agrin immunoblots, by comparing samples of media to a known standard of purified agrin (Ferns et al., 1993).

Assay of Agrin-induced AChR Clustering

C2C12 mouse muscle cells, and the variant S26 and S27 lines, were cultured as previously described (Gordon and Hall, 1989). All experiments were performed on 2–3-d-old myotubes grown on 8-well chamber slides (Lab-Tek; Miles Laboratories, Inc., Naperville, IL). Myotubes were incubated with neural agrin and stained for the AChR by incubating the live cells with rhodamine-conjugated α-bungarotoxin for 1 h. The cells were then rinsed and fixed with 2% paraformaldehyde for 20 min. The levels of AChR clustering were compared by determining the average AChR cluster number in random fields, at a magnification of 400 with a fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ).

Tyrosine Kinase Inhibitor Experiments

We tested the ability of the following tyrosine kinase inhibitors to block agrin-induced AChR clustering: methyl 2,5-dihydroxycinnamate, lavendustin A, 2-hydroxy-5-(2,5-dihydroxybenzyl) aminobenzoic acid, genistein, reduced carboxamidomethylated and maleylated lysozyme, tyrphostin, herbimycin A (Gibco-BRL, Gaithersburg, MD), and staurosporine (Sigma Chemical Co., St. Louis, MO). Myotubes were preincubated with the inhibitors or a carrier control (DMSO) for 0.5–2 h, and then neural agrin was added at ~500 pM for a further 6–8 h. In separate experiments to examine whether the inhibitors dispersed preformed receptor clusters, myotubes were treated with neural agrin for 24 h, and then treated with the inhibitors for 6–8 h. After these agrin/inhibitor treatments, the myotubes were stained for the AChR, and the levels of AChR clustering were compared, as described above. All cultures were coded and counted blind.

To costain for rapsyn (43-kD protein), after fixation the cells were permeabilized in 1% Triton X-100 for 10 min, blocked with 10% horse serum/PBS, and incubated with the anti-rapsyn antibodies (mAb 1234A and mAb 1579A, provided by S. Froehner) and then an FITC-labeled sheep anti–mouse second antibody.

To confirm that the tyrosine kinase inhibitors were not having nonspecific effects, we assayed the level of surface AChR. Control and inhibitor-treated cultures were incubated with 10 nM I^{125} α-bungarotoxin in fusion medium for 2 h to specifically label the surface AChR. The cultures were then rinsed and extracted with 0.1 N NaOH, and the level of bound toxin was determined with a gamma counter.

Extraction and Isolation of the AChR

To assay AChR subunit tyrosine phosphorylation, myotube cultures were treated with agrin/inhibitors, rinsed, scraped off in PBS Ca/Mg-free containing 1 mM sodium orthovanadate, and pelleted. The cells were then extracted in the following buffer containing 1% Triton X-100 (25 mM Tris, 25 mM glycine, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, and the protease inhibitors, PMSF, benzamidine, *N*-ethylmaleimide, and $Na_2S_4O_6$). Insoluble material, such as nuclei and extracellular matrix, was removed by centrifugation at 18,000 *g* for 4 min. The solubilized AChR was isolated by incubating the extracts with α-bungarotoxin conjugated to agarose beads. The proteins isolated on the beads were eluted in SDS loading buffer, separated by electrophoresis on 10% polyacrylamide gels, and transferred to nitrocellulose membranes.

The proteins were probed with a monoclonal antiphosphotyrosine antibody (mAb 4G10), followed by an HRP-conjugated sheep anti–mouse second antibody, and then visualized using the enhanced chemiluminescence method (ECL; Amersham Corp., Arlington Heights, IL). To reprobe the blots, they were first stripped of antibodies by washing with a low pH buffer (200 mM glycine, 0.1% Tween 20, at pH 2.5 for 20 min). They were then reprobed for the AChR subunits, both to identify the phosphotyrosine immunoreactive bands and to confirm that equal amounts of AChR were present in all samples. The α subunit was identified using mAb 210, the β subunit using mAb 124, and the δ subunit using mAb 88b (all gifts of J. Lindstrom, University of Pennsylvania, Philadelphia, PA). The level of β-subunit phosphotyrosine immunoreactivity on blots was quantified with a scanning spectrophotometer (Du-8B; Beckman Instruments, Inc., Fullerton, CA).

Results

Agrin Induces a Transient Tyrosine Phosphorylation of the AChR β Subunit

The mechanism by which agrin induces AChR clustering in mammalian muscle is unclear (Qu et al., 1990). We have examined the possibility that aggregation is regulated by agrin-induced tyrosine phosphorylation of the AChR itself. To address this question, we have taken advantage of a simplified system using C2 mouse myotubes and recombinant isoforms of agrin. The myotubes were treated with a neural-specific isoform of agrin (C-$Ag_{12,4,8}$; Ferns et al., 1993), cell extracts were prepared, and the AChR was partially purified using α-bungarotoxin conjugated to agarose beads. The isolates were separated by PAGE and immunoblotted with an antiphosphotyrosine antibody (4G10). Phosphotyrosine-immunoreactive bands were identified on the basis of size and by reprobing with AChR subunit-specific antibodies.

We find that agrin treatment induces a rapid but transient tyrosine phosphorylation of the β subunit (Fig. 1). In untreated myotube cultures, there was a low, or often undetectable, level of β-subunit tyrosine phosphorylation. After treatment with neural agrin (100 pM), however, there was a dramatic increase of phosphorylation that was evident by 15 min, peaked between 1 and 3 h, and then declined. By 8 h, the level of tyrosine phosphorylation was at 36% of peak level, and by 24 h the level approached, but was still slightly above (7%) that seen in the control cultures (0.5%). In addition, the time course of tyrosine phosphorylation is considerably offset from that of AChR clustering (Fig. 1 *B*). Receptor clusters only begin to form after 2–4 h of agrin treatment and peak in number at 6–8 h. Cluster number then declines slightly, although this appears to be due to the coalescence of small clusters. Agrin-induced β-subunit phosphorylation therefore begins at least 2 h before the first detectable stages of AChR clustering. Similarly, the peak of phosphorylation occurs before discernible receptor clusters form, and well before the peak of cluster number at 6–8 h (Fig. 1 *B*). Phosphorylation of the β subunit thus may be an early, precursor step in the signaling pathway that leads to clustering.

We did not detect agrin-induced tyrosine phosphorylation of the other AChR subunits or of coimmunoprecipitating proteins. Agrin-induced tyrosine phosphorylation of the δ subunit has been seen in chick, and is reported to be sensitive to degradation during boiling in SDS loading buffer (Qu and Huganir, 1994). Even using unboiled samples,

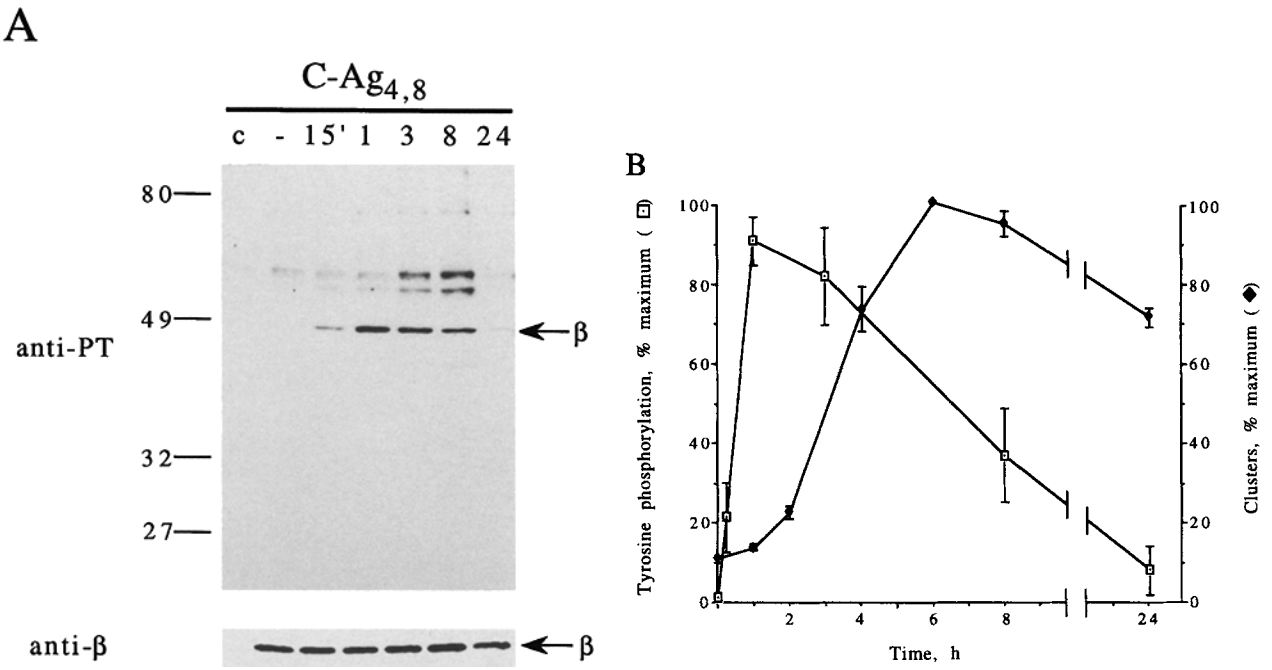

Figure 1. (*A*) Agrin induces a transient tyrosine phosphorylation of the AChR β subunit. AChR from C2 cultures treated with neural agrin (100 pM) over a range of times was isolated on α-bungarotoxin beads and immunoblotted with an antiphosphotyrosine antibody. Agrin induces a transient increase in tyrosine phosphorylation of a 49-kD band. Reblotting with the anti-β-subunit antibody (mAb 124) confirmed that the phosphotyrosine immunoreactive band corresponds to the β subunit, and that equal amounts of the AChR are present in each lane. (*B*) Time course of agrin-induced β-subunit phosphorylation and AChR clustering. The level of phosphotyrosine immunoreactivity for the β subunit was quantified over an agrin time course and is shown averaged from four experiments. Agrin induces a pronounced and rapid increase in tyrosine phosphorylation, which peaks at 1–2 h. The increase is transient, however, and has decreased to 36% of peak at 8 h, and 7% by 24 h. This contrasts with the time course of AChR clustering, where clusters first form at 2–4 h and peak in number at 6–8 h.

however, we found no evidence for phosphotyrosine bands matching those of the mouse δ subunit, which we identified by immunoblotting with a subunit-specific mAb (88b).

Tyrosine Phosphorylation of the β Subunit Correlates with AChR Clustering

To determine how closely β-subunit phosphorylation correlates with receptor clustering, we examined the extent of phosphorylation as a function of agrin concentration. Sets of myotube cultures were treated with concentrations of neural agrin ranging from 1 pM to 333 pM, and assayed either for β-subunit phosphorylation or for receptor clustering. An agrin-induced increase in the level of β-subunit phosphorylation was first observed at 1–3 pM, and increased with agrin concentration up to 333 pM, at which point it appeared to plateau (Fig. 2). As previously observed, increased AChR clustering was first observed with 1–10 pM agrin and continued to increase until a level of 1 nM. The concentration range over which agrin induces AChR phosphorylation thus closely matches that over which it induces AChR clusters.

Similarly, we examined the action of the predominant muscle isoform of agrin ($C\text{-}Ag_{12,0,0}$) on clustering and β-subunit phosphorylation. Muscle agrin only differs from neural agrin in that it lacks two splicing inserts of four and eight amino acids, but it has been found to be >10,000-fold less active in AChR clustering (Ferns et al., 1993). Muscle agrin also fails to block neural agrin-induced clustering (Bowen, D.C., J. Sugiyama, M. Ferns, and Z.W. Hall, manuscript submitted for publication). Consistent with these observations, we find that muscle agrin did not induce β-subunit phosphorylation, even at concentrations up to 10 nM (Fig. 3 *A*), and also failed to induce receptor clustering. This finding thus further suggests that the signaling receptor is highly specific for the neural agrin isoform.

Finally, we examined agrin signaling in two genetic variants of C2 cells that are defective in their ability to cluster AChRs (Gordon and Hall, 1989; Gordon et al., 1993). The S26 and S27 variants have distinct defects in their proteoglycans, but both show significantly reduced AChR clustering when treated with agrin (Ferns et al., 1993). In both S26 and S27 myotubes, we found that neural agrin, even at a concentration of 100 nM, failed to induce AChR β-subunit phosphorylation as well as AChR clustering (Fig. 3 *B*). Moreover, no basal level of β-subunit phosphorylation was detectable in control S26 and S27 cultures, as is sometimes seen in C2 myotubes. Thus, under a variety of experimental conditions, agrin-induced receptor clustering parallels β-subunit phosphorylation.

Tyrosine Kinase Inhibitors Block β-subunit Phosphorylation and AChR Clustering

To examine whether β-subunit tyrosine phosphorylation plays a critical role in the agrin signaling pathway, we tested whether tyrosine kinase inhibitors could block phosphorylation and receptor clustering. As previously

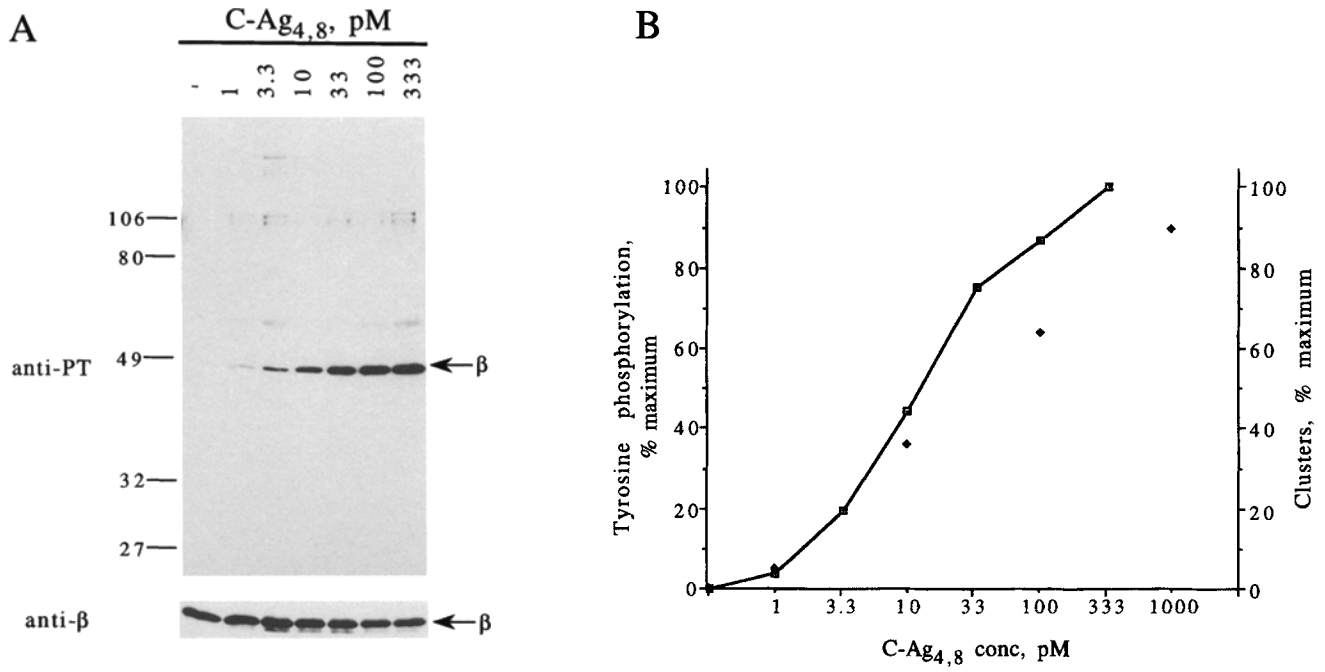

Figure 2. β-subunit tyrosine phosphorylation correlates with agrin concentration. (*A*) Phosphotyrosine immunoblot of AChR from myotubes cultures treated with neural agrin at concentrations ranging from 1 pM to 333 pM. (*B*) The level of phosphotyrosine immunoreactivity for the β subunit (□), and the number of AChR clusters (◆), were quantified over the agrin concentration range. Agrin first increases β-subunit phosphorylation at a concentration of 1–3 pM and approaches maximal phosphorylation from 33 to 100 pM. The level of phosphorylation correlates closely with the relative level of AChR clustering induced by agrin at those concentrations. (The receptor clustering data is taken from Bowen, D.C., J. Sugiyama, M. Ferns, and Z.W. Hall, manuscript submitted for publication, and was obtained using the same batch of recombinant agrin).

described, treatment of myotubes with a soluble form of recombinant neural agrin induces a dramatic increase in the number of AChR clusters over a 6–8-h period (Fig. 4, *A* and *B*) (Ferns et al., 1993). Two tyrosine kinase inhibitors, herbimycin A and staurosporine, efficiently blocked agrin-induced clustering of receptor (Fig. 4, *C* and *D*). Several other tyrosine kinase inhibitors that were tested (see *Materials and Methods*) had no effect. Herbimycin and staurosporine blocked agrin-induced clustering in a dose-dependent manner (Fig. 5). The 9–11-fold increase in the number of AChR clusters induced by agrin was completely inhibited by herbimycin and staurosporine, at concentrations of 0.5 μM and 5 nM, respectively. These results suggest that the agrin signaling pathway in mouse muscle requires tyrosine phosphorylation.

Moreover, we found that treatment of myotubes with herbimycin and staurosporine completely blocked agrin-induced tyrosine phosphorylation of the β subunit. After a 6-h preincubation, both inhibitors blocked the initial peak of β-subunit tyrosine phosphorylation seen after 1 h of agrin treatment (Fig. 6). Similarly, the phosphorylation remaining after 6 h of agrin treatment, when receptor clusters have formed, was absent in the presence of the inhibitors (Fig. 6). As β-subunit phosphorylation is blocked by the inhibitors that block AChR clustering, AChR phosphorylation could be a necessary step in the clustering mechanism.

Several lines of evidence indicate that the inhibition is not due to a nonspecific, toxic effect on the cells. To observe the effect on AChR clustering, myotubes were treated with the inhibitors and agrin for the shortest time possible (6–8 h) to avoid toxicity due to long-term exposure. After both herbimycin and staurosporine treatment, myotube morphology was normal (Fig. 4, *C* and *D*), and spontaneous contraction of myotubes was sometimes observed, as with untreated C2 myotubes, indicating that the health of the cells was not significantly compromised. Consistent with this, the inhibitors did not affect AChR number, as assayed by binding of ^{125}I α-bungarotoxin. Surface levels of receptor were equivalent for the carrier control (84 ± 4 cpm/μg protein [mean ± SEM]) and for the herbimycin- (83 ± 9) and staurosporine-treated cultures (86 ± 7). Furthermore, treated myotubes were able to recover when the inhibitors were removed and to subsequently cluster receptors in response to agrin (data not shown). Finally, some of the other inhibitors that were tested did affect myotube morphology, but did not block receptor clustering.

In double-labeling experiments, we also examined the effect of each inhibitor on the distribution of rapsyn, a cytoplasmic peripheral membrane protein that is precisely codistributed with the AChR in clusters. In all of our experiments, clusters of the AChR were always accompanied by rapsyn and vice versa. Thus, the inhibitors acted to prevent or disperse the clustering of both proteins in concert (data not shown).

Finally, we tested the effect of herbimycin and staurosporine treatment on preformed clusters. Surprisingly, we found that receptor clusters that had been induced by 1–2-d agrin treatment were completely dispersed after 6–8-h treatment with herbimycin and staurosporine at concen-

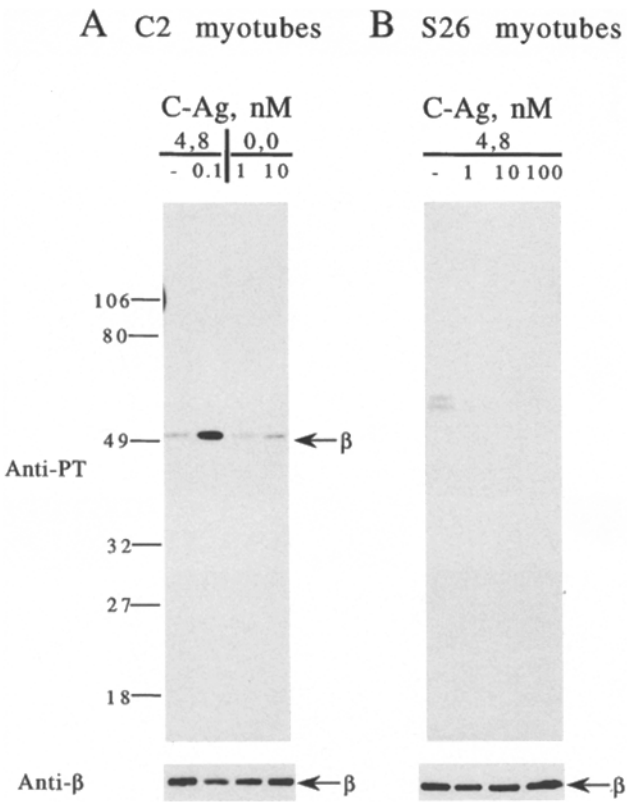

Figure 3. (*A*) Muscle agrin does not induce β-subunit phosphorylation. Phosphotyrosine immunoblot of AChR from C2 myotube cultures treated with muscle agrin ($C\text{-}Ag_{0,0}$). Muscle agrin, even at a concentration of 10 nM, did not induce β-subunit phosphorylation. (*B*) Neural agrin fails to induce β-subunit phosphorylation in S26 myotubes. Phosphotyrosine immunoblot of AChR from S26 myotubes treated with neural agrin ($C\text{-}Ag_{4,8}$). Neural agrin did not increase β phosphorylation when added at concentrations that do not induce receptor clustering (e.g., up to 100 nM).

trations of over 1 μM and 10 nM, respectively (Fig. 7). These results suggest that tyrosine phosphorylation is required not only for the formation but also for the stability and maintenance of agrin-induced AChR clusters.

Discussion

We have investigated the agrin signaling pathway in mammalian muscle and find that it involves tyrosine phosphorylation of the AChR itself. Neural agrin induces a rapid but transient tyrosine phosphorylation of the AChR β subunit. Interestingly, β-subunit phosphorylation begins over 2 h before the first discernible receptor clusters form and peaks 4–6 h before the peak in AChR cluster number. In fact, the level of tyrosine phosphorylation is in marked decline at precisely the time that cluster number is increasing towards its peak. Tyrosine phosphorylation of the β subunit therefore appears to be lost upon receptor aggregation. The transient nature of the phosphorylation provides an explanation as to why previous studies in rat and mouse have not observed tyrosine phosphorylation in association with developing receptor clusters, nor tyrosine phosphorylation of the AChR until 1–2 wk postnatally (Qu et al., 1990; Campanelli et al., 1993).

Studies in chick muscle have also shown that purified agrin, of unknown isoform, induces a rapid tyrosine phosphorylation of the AChR β subunit (Wallace et al., 1991). The phosphorylation is not lost with receptor aggregation, however, and in contrast to mouse muscle, the developing clusters stain with antiphosphotyrosine antibodies. A further difference is that in chick muscle, innervation and agrin also induce tyrosine phosphorylation of the δ subunit (Qu and Huganir, 1994). Despite careful examination, we did not detect δ-subunit tyrosine phosphorylation in C2 mouse myotubes. Our results therefore extend on the findings made in chick and suggest that the agrin-induced phosphorylation of the β subunit is an early, precursor step to aggregation, rather than being involved in the maintenance of clusters.

Several observations point to agrin-induced β-subunit phosphorylation being a critical element in the clustering pathway. First, we observe a precise correlation between the extent of β-subunit phosphorylation and the level of receptor clustering seen over a wide range of concentrations of neural agrin. Second, muscle agrin, which is 10,000-fold less active in inducing receptor clustering than neural agrin, did not induce β-subunit phosphorylation. Third, neural agrin did not induce β-subunit phosphorylation in two proteoglycan mutants that have a significantly reduced clustering response to agrin. Under several different experimental conditions, therefore, the extent of β-subunit tyrosine phosphorylation is correlated with the presence or absence of receptor clustering.

Finally, and most importantly, agrin-induced β-subunit phosphorylation is completely blocked by the inhibitors, herbimycin and staurosporine. As these tyrosine kinase inhibitors also block receptor clustering, phosphorylation of the AChR may thus be a required step for aggregation. Both herbimycin and staurosporine blocked receptor clustering in a dose-dependent manner, and several controls and observations confirm that the block by the inhibitors was not a nonspecific or toxic effect. Treatment with the inhibitors did not adversely affect myotube morphology, contractile activity, or levels of surface AChR. Moreover, after removal of inhibitor, the myotubes were able to recover and form agrin-induced AChR clusters. Our results thus strengthen the finding in chick muscle that both AChR clustering and β-subunit tyrosine phosphorylation are blocked by staurosporine (Wallace, 1994).

A direct test of the role of β-subunit tyrosine phosphorylation in clustering has been made using a nonmuscle cell system. When rapsyn is coexpressed with the AChR in nonmuscle cells, it induces the formation of small receptor aggregates (Froehner et al., 1990; Phillips et al., 1991). Mutation of the relevant tyrosine in the β-subunit intracellular loop (Wagner et al., 1991; Huganir and Miles, 1989) did not prevent this aggregation (Yu and Hall, 1994). The rapsyn-induced clustering in nonmuscle cells, however, may not accurately reflect the regulated clustering that occurs in myotubes. First, tyrosine kinase inhibitors fail to block the AChR/rapsyn aggregation in COS cells (Taylor, R., and Z. Hall, unpublished observations). Second, in noninnervated muscle cells rapsyn and the AChR do not normally form a significant number of spontaneous receptor clusters, but are diffusely distributed at the cell surface. Thus, experiments in muscle cells will be required to de-

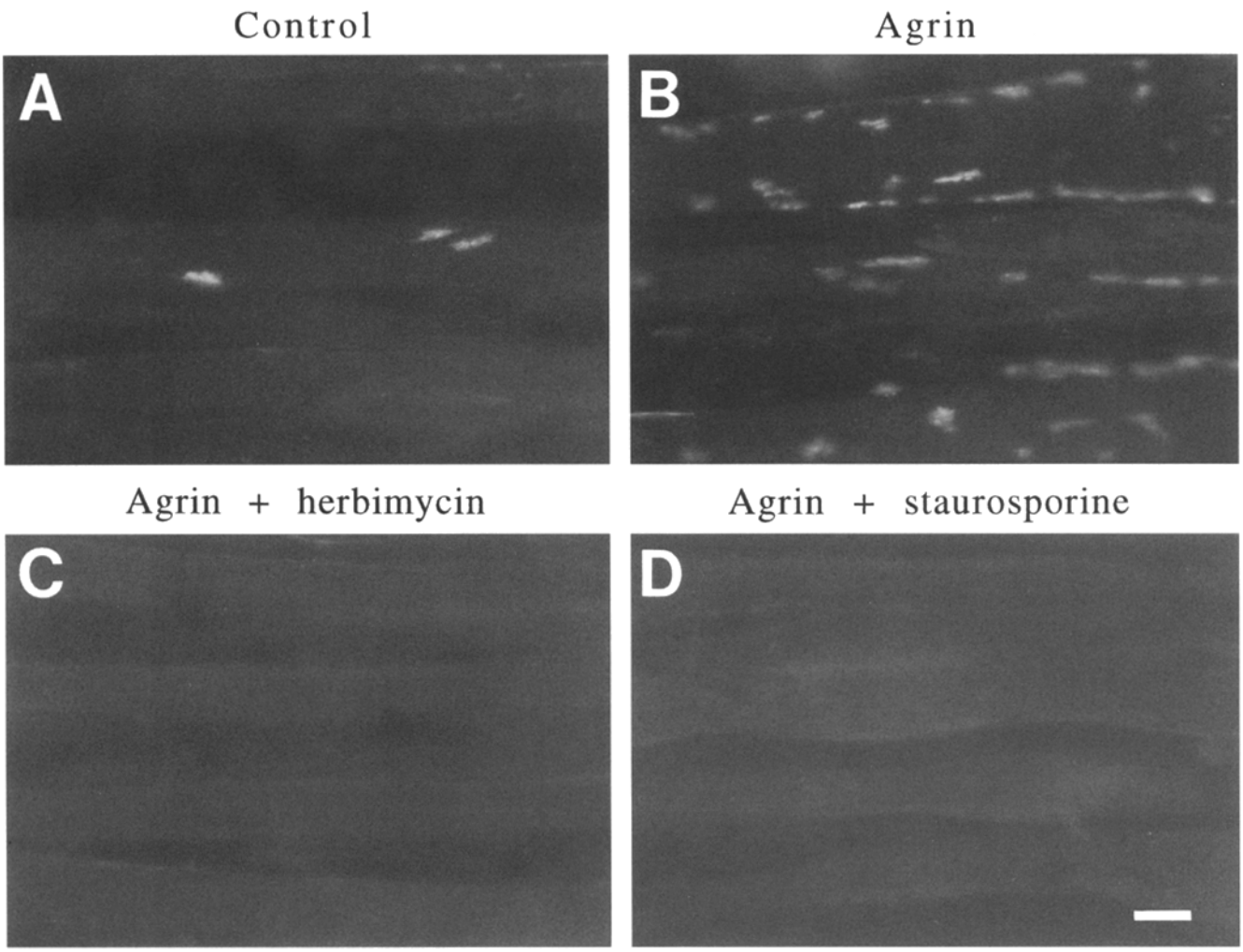

Figure 4. Inhibition of agrin-induced AChR clustering by herbimycin and staurosporine. C2 myotubes either were untreated (*A*), or were treated with agrin in the presence of a carrier control (*B*), 1 μM herbimycin (*C*), or 10 nM staurosporine (*D*). Cultures were stained with rhodamine-conjugated α-bungarotoxin to assay receptor clustering. Herbimycin and staurosporine both completely inhibited agrin-induced AChR clustering. Bar, 10 μm.

termine if β-subunit phosphorylation is required for agrin-induced clustering of the AChR.

The agrin-induced tyrosine phosphorylation of the β subunit and the effect of the inhibitors suggests that the agrin signaling pathway in mammalian muscle involves activation of tyrosine kinases. Potentially, the pathway could require a receptor tyrosine kinase activated by agrin binding, a cytoplasmic tyrosine kinase associated with a nonkinase receptor, or a kinase involved in a downstream signaling event. As the known specificities of the two effective

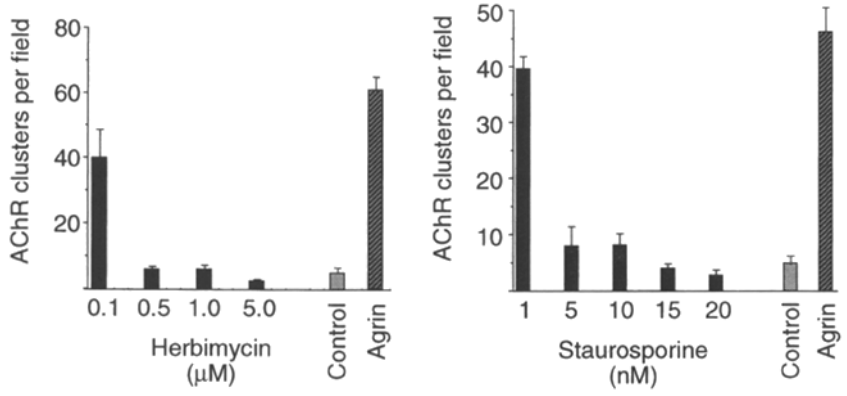

Figure 5. Dose dependence of inhibition of clustering by herbimycin and staurosporine. The number of AChR clusters per field was determined for myotube cultures treated with agrin alone or with agrin in the presence of a range of concentrations of herbimycin and staurosporine. Agrin alone induced a 10-fold increase in the number of receptor clusters. The increase in clustering was inhibited by both herbimycin and staurosporine in a dose-dependent manner.

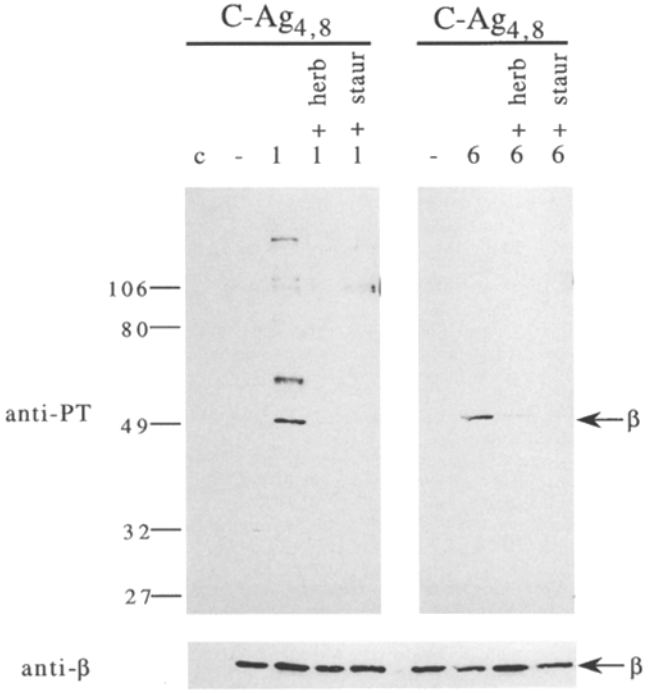

Figure 6. Agrin-induced β-subunit phosphorylation is blocked by herbimycin and staurosporine. Phosphotyrosine immunoblot of AChR from myotube cultures treated with a carrier control, agrin alone (100 pM), or agrin in the presence of 1 μM herbimycin or 10 nM staurosporine. The agrin-induced increase in β phosphorylation is blocked by both inhibitors, under the same conditions in which they block agrin-induced AChR clustering.

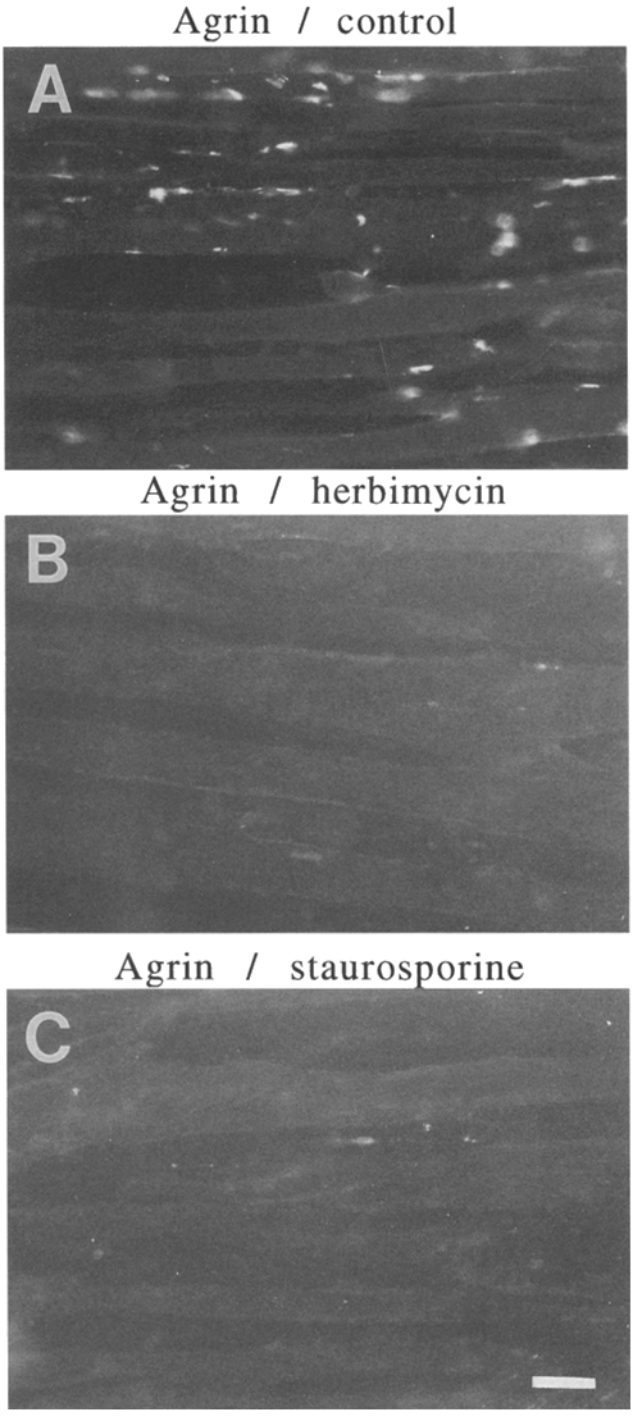

Figure 7. Dispersal of preformed AChR clusters by herbimycin and staurosporine. Myotube cultures that had been previously treated with agrin to induce AChR clusters were treated with a carrier control (*A*), 2 μM herbimycin (*B*), or 20 nM staurosporine (*C*). Preformed clusters were dispersed by both inhibitors after 6–8 h. Bar, 10 μm.

inhibitors differ, the identity of the critical kinase(s) is unclear. Herbimycin is reported to be a relatively specific inhibitor of protein tyrosine kinases, especially of the src family. Staurosporine is less specific, however, and is known to inhibit not only some receptor tyrosine kinases, but also protein kinase C and cyclic nucleotide-dependent kinases. Another possibility is that the two inhibitors may not act on the same kinase, but may affect distinct kinases at different points in the signaling pathway.

Agrin signaling may well involve tyrosine phosphorylation of proteins in addition to the AChR. Consistent with this possibility, the maintenance of receptor clusters was found to be dependent on tyrosine phosphorylation, even though β-subunit phosphorylation is lost by that time and is unlikely to be involved. The ability of herbimycin and staurosporine both to block cluster formation and to disperse clusters suggests that the same tyrosine kinase may be involved in each case. One possibility is that an inhibitor-sensitive kinase phosphorylates the β subunit and is recruited to clusters along with the AChR, where it may then act on other proteins. A number of proteins that are concentrated at the neuromuscular junction contain tyrosine phosphorylation consensus sites, or have been shown to be tyrosine phosphorylated in Torpedo electric organ. These include integral membrane proteins (β-dystroglycan [Ibraghimov-Beskrovnaya et al., 1992]), membrane-associated proteins (rapsyn and the 58-kD protein [Froehner, 1991; Adams et al., 1993]), and cytoskeletal elements (the 87-kD protein and paxillin [Wagner et al., 1993; Gumbiner, 1993]). Agrin-induced phosphorylation of such proteins may also be required for cluster formation or maintenance.

Given the complexity of the postsynaptic structure, it seems likely that the agrin signaling pathway governing its

assembly will be complex and will involve regulatory steps in addition to tyrosine phosphorylation. This is supported by the fact that clustering can be blocked not only by tyrosine kinase inhibitors, but also by phorbol esters, which activate protein kinase C (Wallace et al., 1991), and by orthovanadate, a phosphatase inhibitor. Orthovanadate is interesting in that it increases the level of β-subunit phosphorylation, yet blocks agrin-induced clustering in both chick (Wallace, 1995) and mouse myotubes (Ferns and Hall, 1992) (data not shown). Thus, β-subunit tyrosine phosphorylation is not sufficient, in itself, to produce clustering of receptor. Clearly a coordinated interplay of tyrosine kinase and phosphatase activity is required for aggregation.

Overall, our findings provide strong correlative evidence linking β-subunit phosphorylation and receptor clustering. Our findings suggest the following general model for agrin-induced receptor clustering. In uninnervated muscle, the AChR is diffusely distributed and is not tyrosine phosphorylated. Upon innervation, neural agrin binds its receptor, inducing the activation of tyrosine kinases that phosphorylate the AChR and possibly other proteins. Tyrosine phosphorylation then regulates the association of the AChR with proteins that link it to the cytoskeleton and result in its recruitment into clusters. These proteins may include the utrophin-associated complex. After aggregation, tyrosine phosphorylation of the AChR is lost in mammalian muscle, but the tyrosine phosphorylation of other proteins required for the further development and maintenance of clusters is maintained. An important future area of interest will be to define the protein interactions of the AChR that are regulated by tyrosine phosphorylation.

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