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The Mechanism of Agrin-Induced Acetylcholine Receptor Aggregation

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The mechanism of agrin-induced acetylcholine receptor aggregation

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SUMMARY

Agrin, a protein isolated from the synapse-rich electric organ of *Torpedo californica*, induces the formation of specializations on myotubes in culture which resemble the post-synaptic apparatus at the vertebrate skeletal neuromuscular junction. For example, the specializations contain aggregates of acetylcholine receptors and acetylcholinesterase. This report summarizes the evidence that the formation of the post-synaptic apparatus at developing and regenerating neuromuscular junctions is triggered by the release of agrin from motor axon terminals and describes results of recent experiments which suggest that agrin-induced tyrosine phosphorylation of the β subunit of the acetylcholine receptor may play a role in receptor aggregation.

INTRODUCTION

At synapses throughout the nervous system certain specialized structures play a crucial role in transmission. For example, active zones at axon terminals are involved in the release of neurotransmitter and transmitter receptors are aggregated in the post-synaptic membranes of target cells. Over the past two decades numerous studies have been directed toward understanding how these specializations form in the embryo, how they are maintained in the adult, and how they reform during regeneration after trauma (Dennis 1981; Schuetze & Role 1987). Much of this effort has focused on the neuromuscular synapse, primarily because of its convenience for study both *in vivo* and *in vitro*. It is now clear that the formation and maintenance of structural specializations at the neuromuscular synapse relies on communication between the axon terminal and muscle fibre. Thus the muscle fibre provides signals that direct the axon terminal to form and maintain active zones and the axon terminal, in turn, provides signals that direct the muscle fibre to organize a post-synaptic apparatus. To understand how synapses form it is necessary to identify these signals, ascertain how their expression is regulated, and determine the mechanisms by which they induce synaptic differentiation.

DISCOVERY OF AGRIN

An important clue to the identity of one such signal came from experiments on regenerating muscles in the adult. The basal lamina in the synaptic cleft of adult junctions was found to contain molecules that cause the formation of synaptic specializations in regenerating axon terminals and muscle fibres (see McMahan & Wallace 1989). For example, the synaptic basal lamina directs regenerating muscle fibres to form junctional folds and aggregates of acetylcholine receptors (AChRs) and acetylcholinesterase (AChE) (Burden *et al.* 1979; McMahan & Slater 1984; Anglister & McMahan 1985). Results of experiments aimed at identifying and characterizing the basal lamina molecules that induce the aggregation of AChRs and AChE led to the discovery of agrin, a protein that appears to play a central role in this communication process.

As a step toward identifying the basal lamina molecules that cause AChR aggregation on regenerating muscle fibres, extracts of the electric organ of *Torpedo californica* were screened for AChR-aggregating activity on cultured chick myotubes (Godfrey *et al.* 1984). The electric organ has a much higher density of cholinergic synapses than muscle, which makes it useful for identifying and characterizing molecules involved in cholinergic synaptic transmission. Basal lamina-containing extracts of electric organ caused a 3- to 20-fold increase in the number of aggregates of AChRs (figure 1) while having little or no effect on myotube size, the total number of AChRs on the myotube surface, or the rate of AChR degradation (Godfrey *et al.* 1984). A library of monoclonal antibodies (mAbs) was made that immunoprecipitated the AChR-aggregating molecules in the electric organ extracts (Reist *et al.* 1987). Each of five different mAbs tested immunoprecipitated polypeptides of 150, 135, 95 and 70 kD (Nitkin *et al.* 1987). Gel filtration chromatography of electric organ extracts revealed two peaks of AChR-aggregating activity: one comigrated with the 150 kD polypeptide, the other with the 95 kD polypeptide. The 135 and 70 kD polypeptides did not cause AChR aggregation. Based on these

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molecular characteristics and on the pattern of staining seen in sections labelled with the mAbs the electric organ AChR-aggregating factor (150 and 95 kD) appeared to be distinct from previously identified molecules at the neuromuscular junction and was named 'agrin' (Nitkin *et al.* 1987).

Agrin also appears to be distinct from other identified molecules that have been shown to cause formation of AChR aggregates on cultured myotubes, including laminin, calcitonin gene-related polypeptide (CGRP), ARIA, sciatin, and ascorbate (for review see Godfrey *et al.* (1984); Nitkin *et al.* (1987)). For example, their molecular weights differ from that of the two forms of agrin, and agrin causes a much more pronounced increase in the number of receptor aggregates. Moreover, CGRP, ARIA, sciatin, and ascorbate cause an increase in the rate of synthesis and insertion of AChRs into the plasma membrane of cultured myotubes while agrin has no effect on AChR synthesis. On the other hand, agrin may be similar to as yet unidentified AChR-aggregating factors others have extracted from rat brain, cultured neurons, neuronal cell lines, and rat diaphragm (see Nitkin *et al.* 1987).

AGRIN-LIKE MOLECULES ARE CONCENTRATED IN SYNAPTIC BASAL LAMINA IN MUSCLE

If agrin is responsible for the aggregation of AChRs at the neuromuscular junction, then it should be present in muscle. Indeed, extracts of *Torpedo* skeletal muscle cause the formation of AChR aggregates in much the same way as do electric organ extracts, and monoclonal antibodies to agrin block and immunoprecipitate the AChR-aggregating activity from muscle (Godfrey *et al.* 1984; Fallon *et al.* 1985). Antibodies to agrin also stain the synaptic cleft at neuromuscular junctions in *Torpedo*, frog, and chicken, and the molecules recognized by the antibodies are stably bound to the basal lamina (Reist *et al.* 1987). Thus muscle contains an AChR-aggregating activity antigenically similar to agrin, but in much lower amounts than the electric organ, as might be expected since electric organ has a much higher concentration of synapses. Moreover, molecules antigenically related to agrin are stably associated with the synaptic basal lamina, where molecules that induce AChR aggregation on regenerating myofibres are known to be.

MOTOR NEURONS CONTAIN AGRIN-LIKE MOLECULES

If agrin is the signal released from axon terminals to induce AChR aggregation at developing neuromuscular junctions, then motor neurons should contain agrin and transport it to their terminals. The cell bodies of motor neurons in the *Torpedo* electric lobe (which innervates the electric organ) and in the spinal cord of *Torpedo*, chick, and frog stain with anti-agrin antibodies (Magill-Solc & McMahan 1988). The staining in motor neurons is localized to the Golgi apparatus, the site where proteins destined for transport to the cell surface are processed. When frog sciatic nerves are ligated, molecules recognized by anti-agrin antibodies accumulate proximal to the ligation (Magill-Solc & McMahan 1989). Extracts of motor neurons and the proximal sections of ligated axons contain AChR-aggregating activity that is immunoprecipitated by anti-agrin antibodies. These results suggest that agrin is synthesized in the cell bodies of motor neurons and transported anterogradely down their axons. Recently, a cDNA clone that encodes agrin was isolated from a cDNA library constructed from electric lobe mRNA. This finding, together with the results of *in situ* hybridization studies which revealed strong labelling of motor neurons in the electric lobe and spinal cord with probes derived from the clone, support the conclusion that agrin is synthesized by motor neurons.

ANTIBODIES TO AGRIN BLOCK NERVE-INDUCED AChR AGGREGATION

When chick motor neurons are grown with chick muscle cells in culture, AChRs aggregate at sites where neurites contact myotubes. Antibodies against immunoaffinity-purified agrin block the formation of such AChR aggregates (Reist & McMahan 1990). Thus nerve-induced AChR aggregation is mediated by agrin-like molecules. It remains to be shown directly, however, that motor neurons release agrin from their axon terminals.

ACTION OF AGRIN ON CULTURED MYOTUBES

The formation of agrin-induced specializations on myotubes in culture resembles events at developing neuromuscular junctions. When agrin is added to the medium bathing chick myotubes, small ($< 4\ \mu m^2$) aggregates of AChRs begin to appear within 2 h and increase rapidly in number until 4 h (Wallace 1988). Over the next 12–20 h the total area occupied by aggregated receptors continues to increase and the mean size of each aggregate grows to approximately $15\ \mu m^2$. Accumulation of AChRs at developing neuromuscular junctions follows a similar timecourse, small clusters of AChRs appear beneath the developing axon terminal and during the ensuing 24 h mature into a large, relatively uniform, and sharply circumscribed patch (Anderson & Cohen 1977; Steinbach 1981). Most of the AChRs that accumulate in agrin-induced specializations and many of the AChRs that accumulate at developing neuromuscular junctions do so by lateral migration of receptors already in the myotube's plasma membrane (figure 1; Anderson & Cohen 1977; Godfrey *et al.* 1984; Ziskind-Conhaim *et al.* 1984; Kuromi *et al.* 1985; Wallace 1988), although there is also evidence for preferential insertion of newly synthesized receptors at synaptic sites (Role *et al.* 1985; Dubinsky *et al.* 1989). Agrin-induced AChR aggregates remain on cultured myotubes as long as agrin is present in the medium; if agrin is removed the number of aggregates declines slowly (Wallace 1988). Similarly, if newly formed neuromuscular junctions *in vivo* are denervated the aggregates of AChRs at the junctions disperse (Slater 1982). The formation and maintenance

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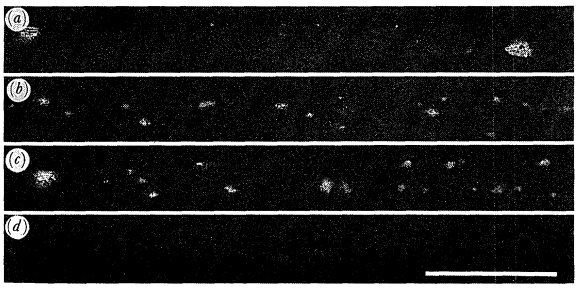

Figure 1. Agrin induces AChR aggregation by lateral migration. Fluorescence micrographs of segments of cultured chick myotubes labelled with rhodamine α-bungarotoxin to reveal distribution of receptors. (*a*, *b*) All AChRs: myotubes labelled at the end of a 6 h incubation with (*a*) saline or (*b*) agrin. (*c*) Pre-existing AChRs: myotube labelled before 6 h incubation with agrin. (*d*) Newly inserted AChRs: Myotube treated with unconjugated α-bungarotoxin to block pre-existing AChRs, incubated for 6 h with agrin, then labelled with rhodamine α-bungarotoxin. Bar, 50 μm.

of both nerve and agrin-induced AChR aggregates requires Ca^{2+} (Bloch & Steinbach 1981; Henderson *et al.* 1984; Wallace 1988). Both nerve- and agrin-induced AChR aggregation are blocked by polyanions such as heparin and heparan sulphate (Hirano & Kidokoro 1989; Wallace 1990).

Other components of the post-synaptic apparatus, such as AChE, butyrylcholinesterase (BuChE), heparan sulphate proteoglycan, and a 43 kD AChR-associated protein, accumulate together with AChRs when myotubes are treated with electric organ extracts (Wallace *et al.* 1985; Wallace 1986, 1989; McMahan & Wallace 1989). Four lines of evidence indicate that agrin causes the accumulation of all of these components: (*a*) the dose dependence for the formation of AChE, BuChE, 43 kD protein, and heparan sulphate proteoglycan aggregates is the same as for AChR aggregates; (*b*) five monoclonal antibodies, recognizing at least four different epitopes on agrin, immunoprecipitate all of the aggregating activities; (*c*) gel filtration chromatography reveals that there are two forms of each activity, one comigrating with the 150 kD agrin polypeptide and the other with the 95 kD agrin polypeptide, and (*d*) immunoaffinity-purified agrin displays all of the aggregating activities.

A characteristic feature of neuromuscular junctions is the accumulation of the A_{12} asymmetric form of AChE, which is believed to be the predominant form of AChE in the synaptic basal lamina (Hall 1973; McMahan *et al.* 1978; see also Massoulie & Bon 1982). Agrin-induced specializations contain both globular and the A_{12} asymmetric forms of AChE and, as at the neuromuscular junction, the A_{12} form in agrin-induced specializations is associated with components of the myofibre's extracellular matrix (Wallace 1989).

The induction of AChR aggregates by agrin is not prevented by inhibitors of protein synthesis (figure 2; Wallace 1988), consistent with our finding that agrin-induced accumulation of AChRs occurs by lateral migration of receptors already on the surface of the myotube (Godfrey *et al.* 1984; Wallace 1988). The accumulation of components of the extracellular matrix, such as heparan sulphate proteoglycan, would seem less likely to occur by lateral migration and so might require release of newly synthesized proteins. Indeed, formation of aggregates of heparan sulphate proteoglycan is prevented by inhibitors of protein synthesis (figure 2; Wallace 1989). Thus there are differences in the mechanisms by which different components of the post-synaptic apparatus accumulate in agrin-induced specializations.

Agrin-induced AChR aggregation is prevented by inhibitors of energy metabolism (Wallace 1988). The dependence on metabolic energy shows that agrin-induced AChR aggregation is a more complex process than simple cross-linking of surface receptors by a multivalent ligand (such as occurs during patching on lymphocytes), a process which does not rely on energy metabolism. Moreover, we estimate that for every molecule of agrin we add to the culture medium, 160 AChRs are induced to aggregate (Nitkin *et al.* 1987).

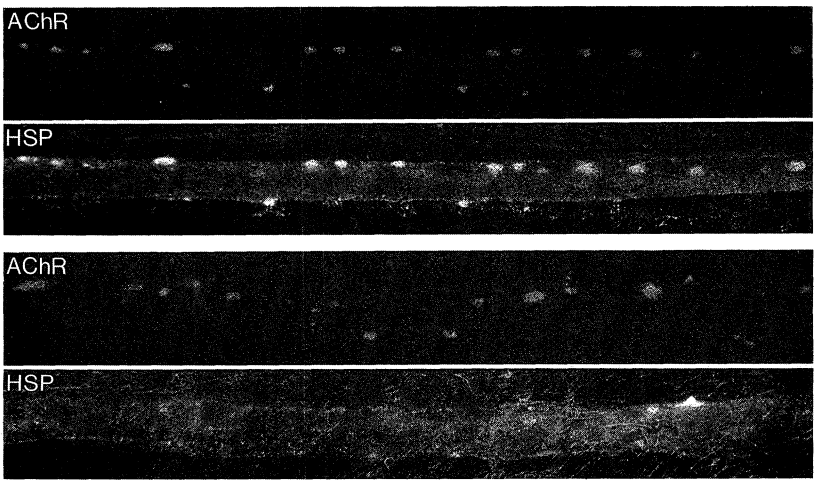

Figure 2. Inhibitors of protein synthesis block formation of agrin-induced aggregates of heparan sulphate proteoglycan but not of AChRs. Fluorescence micrographs of segments of myotubes from agrin-treated cultures labelled with rhodamine α-bungarotoxin and fluorescein-conjugated antibodies to heparan sulphate proteoglycan. Upper panel: normal medium. Lower panel: cycloheximide (0.1 mg ml^{-1}) was added 3 h before addition of agrin.

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This stoichiometry, together with the finding that agrin-induced specializations contain high concentrations of several other components of the post-synaptic apparatus, suggests that agrin acts catalytically, for example by binding to a receptor on the surface of myotubes and triggering a cascade of intracellular events that results in the formation of a post-synaptic specialization.

PHOSPHORYLATION OF AChRs

As mentioned above, the accumulation of AChRs into agrin-induced specializations does not require protein synthesis. Therefore, agrin-induced AChR aggregation must be mediated by post-translational modification(s) of existing proteins. A common post-translational regulatory mechanism is protein phosphorylation. AChRs are themselves substrates for protein kinases; when AChRs are isolated from the electric organ of *Torpedo californica* each of the four receptor subunits is phosphorylated (Vandlen *et al.* 1979). Moreover, several protein kinases that phosphorylate AChRs are found in post-synaptic membranes isolated from the electric organ: a cAMP-dependent protein kinase, protein kinase C, and a tyrosine-specific protein kinase (Huganir & Greengard 1983; Huganir *et al.* 1984). Phosphorylation of AChRs has been shown to increase the rate of receptor desensitization (Huganir *et al.* 1986; Hopfield *et al.* 1988; see Schuetze & Role 1987; Huganir & Miles 1989). However, receptor phosphorylation may have other consequences. For example, the formation of agrin-induced AChR aggregates is blocked by phorbol 12-myristate 13-acetate (TPA), an activator of protein kinase C (Wallace 1988). TPA also causes spontaneous and agrin-induced AChR aggregates to disperse (Wallace 1988; Ross *et al.* 1988), and at the same time causes AChRs to be phosphorylated (Ross *et al.* 1988). These findings suggest that phosphorylation of AChRs might play a role in regulating receptor distribution.

AGRIN CAUSES AChR PHOSPHORYLATION

When myotube cultures are incubated overnight with [^{32}P]inorganic phosphate the β, γ, and δ AChR subunits are labelled (figure 3). The incorporation of ^{32}P into the β subunit is always much less than into γ and δ. Overnight incubation with agrin causes a three-fold increase in phosphorylation of the β subunit (figures 3 and 4, table 1). Agrin also causes an increase in phosphorylation of the γ and δ subunits that is of similar absolute magnitude but proportionately much smaller (table 1, figures 3 and 4). On the other hand, agrin treatment causes little or no change in the overall pattern of protein phosphorylation; analysis of myotube extracts shows no detectable change in either the incorporation of ^{32}P into TCA-precipitable material (agrin-treated = 100.7 ± 3.1 % of control, mean ± s.e.m. [$n = 9$]) or in the pattern of phosphoproteins revealed by 2D isoelectric focusing-SDS polyacrylamide gel electrophoresis. Thus agrin-induced phosphorylation of AChRs is selective and not part of a general increase in protein phosphorylation.

Table 1. *Agrin-induced increase in phosphorylation of AChR subunits*

subunit	control[a]	agrin-treated	difference[b]	p[c]
β	145	429	284 ± 129	< 0.0005
γ	1071	1366	295 ± 311	< 0.025
δ	1000	1190	190 ± 291	< 0.05

[a] Autoradiogram densities were normalized to label in the δ subunit from control myotubes.
[b] Mean ± s.d., $n = 9$.
[c] Students t-test, one-sided paired comparisons.

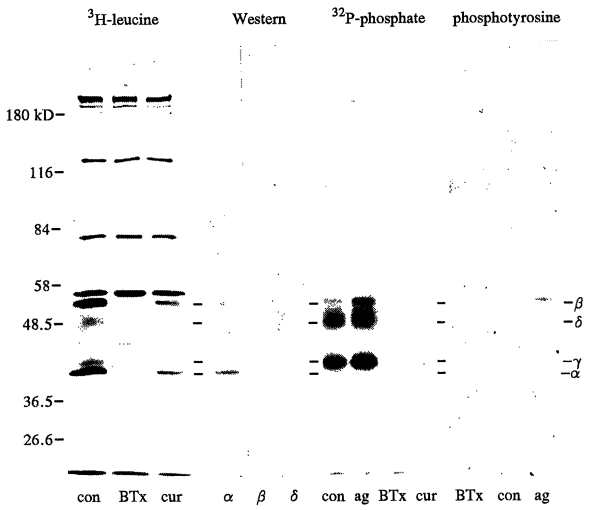

Figure 3. Agrin induces phosphorylation of AChRs in cultured chick myotubes. AChRs were isolated from chick myotubes and the subunits separated by SDS-polyacrylamide gel electrophoresis. [^{3}H]leucine: Fluorograms of AChRs isolated from myotubes radiolabelled with [^{3}H]leucine. Isolation of AChR subunits of 40, 42, 48, and 52 kD (*con*) is specifically blocked by α-bungarotoxin (*BTx*) or curare (*cur*). Western: Western blot identification of AChR α, β, and δ subunits. AChRs were isolated, transferred to nitrocellulose, and stained by using subunit specific monoclonal antibodies. The 42 kD subunit, which did not bind any of the antibodies tested, is tentatively identified as the γ subunit. [^{32}P] phosphate: Autoradiogram of AChRs isolated from myotube cultures labelled with [^{32}P]H_3PO_4. The β, γ, and δ subunits were labelled in control cultures (*con*). Overnight incubation with agrin caused an increase in phosphorylation of the γ, δ, and especially β subunits (*ag*). Specificity was confirmed by blocking the isolation of AChR subunits with α-bungarotoxin (*BTx*) or curare (*cur*). Phosphotyrosine: Autoradiogram of AChRs isolated, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies and [^{125}I]Protein A. The only subunit labelled was the β subunit, and agrin caused a marked increase in the level of phosphotyrosine it contained. (*BTx*): Sample from cultures in which isolation of AChRs was specifically blocked with α-bungarotoxin. (*con*): AChRs isolated from control myotubes. (*ag*): AChRs isolated from myotubes treated overnight with agrin.

PROPERTIES OF AGRIN-INDUCED AChR PHOSPHORYLATION

H-7 (1-[5-isoquinolinylsulfonyl]-2-methylpiperazine) has been shown to be taken up into cells in culture and to inhibit a variety of protein kinases, including cAMP-dependent kinase, cGMP-dependent kinase, and protein kinase C (Hidaka *et al.* 1984).

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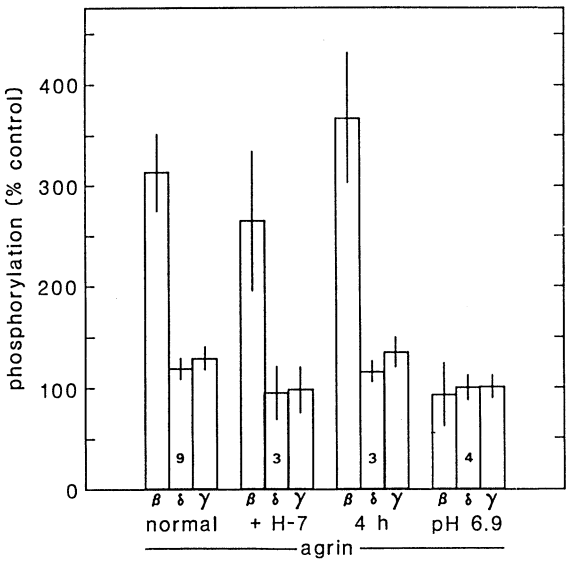

Figure 4. Characterization of agrin-induced AChR phosphorylation. Agrin caused phosphorylation of the β, γ and δ subunits (*normal*). Agrin-induced phosphorylation of the γ and δ, but not the β, subunits was blocked by H-7, an inhibitor of protein kinase C and cyclic nucleotide-dependent protein kinases (H-7). Agrin-induced AChR phosphorylation occurred within 4 h of adding agrin (4 h) and was prevented in cultures incubated at pH 6.9 (pH 6.9). The incorporation of [^{32}P] into the AChR subunits, calculated as a percentage of the incorporation in control cultures, is expressed as the mean ± s.e.m., the number within each set of columns shows the number of observations.

0.2 mM H-7 blocks agrin-induced phosphorylation of the γ and δ AChR subunits (figure 4), indicating that phosphorylation of these subunits might be due to the activity of protein kinase C or a cyclic nucleotide-dependent protein kinase. On the other hand, H-7 does not block agrin-induced phosphorylation of the β subunit (figure 4), nor does H-7 prevent agrin-induced AChR aggregation.

Agrin-induced AChR aggregation is blocked by TPA and by lowering the pH of the culture medium to 6.9 or below (Wallace 1988). Figure 4 shows that agrin-induced phosphorylation of AChRs is also blocked in myotubes incubated at pH 6.9. TPA appears to prevent agrin-induced AChR phosphorylation as well, although interpretation of this result is complicated by the fact that TPA itself causes AChR phosphorylation (Ross *et al.* 1988). Thus treatments that prevent agrin-induced AChR aggregation also prevent agrin-induced receptor phosphorylation, suggesting that these two effects of agrin might be related.

Agrin-induced phosphorylation of AChRs could be a cause or a consequence of receptor aggregation. For example, protein kinases, such as are found in post-synaptic membranes purified from *Torpedo* electric organ (Huganir & Greengard 1983; Huganir *et al.* 1984), might accumulate together with AChRs in agrin-induced specializations, phosphorylating receptors as they aggregate. Alternatively, phosphorylation of diffusely distributed AChRs might trigger their aggregation. As shown in figure 4, within 4 h of adding agrin to the culture medium AChR phosphorylation reaches its final level. As described above, AChR aggregation can not be detected until 2 h after addition of agrin and continues for at least the next 16 h; at 4 h AChR aggregation has reached less than 40% of its final value, as measured by the total area occupied by aggregated receptors (Wallace 1988). Thus agrin-induced AChR phosphorylation reaches its maximum hours before AChR aggregation is complete. This suggests that phosphorylation is not a result of aggregate formation but rather may play a role in the aggregation process itself.

Table 2. *Agrin-induced tyrosine phosphorylation of AChRs. Isolated AChRs labelled with anti-phosphotyrosine antibodies and [^{125}I]Protein A*

treatment	cpm[a]	Δcpm	(%)	(%) aggregation[b]
blank	2689 ± 502	—	—	—
control	25147 ± 787	—	—	—
control + TPA	14496 ± 766	—	—	—
control @ pH6.9	26018 ± 126	—	—	—
agrin	57556 ± 403	32409	100	100
agrin + TPA	19170 ± 1170	4674	14	−2.8
agrin @ pH6.9	38172 ± 702	12154	37	14.6

[a] Mean and range for duplicate samples, each sample was composed of AChRs isolated from two 35 mm myotube cultures.
[b] Number of AChR aggregates per myotube segment, expressed as percentage of control.

AGRIN ACTIVATES A TYROSINE PROTEIN KINASE

The pattern of subunits phosphorylated by agrin, the distribution of potential phosphorylation sites predicted by sequence analysis (Huganir & Miles 1989), and the effects of the kinase inhibitor H-7 suggest that agrin-induced phosphorylation of the β subunit might be the result of a tyrosine-specific protein kinase. This was confirmed by using anti-phosphotyrosine antibodies (Qu *et al.* 1990). The only polypeptide that binds anti-phosphotyrosine antibodies on Western blots of surface AChRs isolated from myotube cultures is the β AChR subunit, and agrin causes a five-fold increase its labelling (figure 3). Based on these findings a rapid and simple assay for agrin-induced phosphorylation was devised in which isolated toxin–receptor complexes are labelled with anti-phosphotyrosine antibodies and [^{125}I]Protein A. By using this assay the inhibitory effects of TPA and low pH were confirmed (table 2). Thus, in addition to regulating AChR desensitization, phosphorylation may play a crucial role in regulating AChR distribution during the formation and maintenance of the post-synaptic apparatus at the neuromuscular junction.

AGRIN HYPOTHESIS

The results described above show that (*a*) agrin induces the formation of specializations on myotubes in culture that resemble the post-synaptic apparatus,

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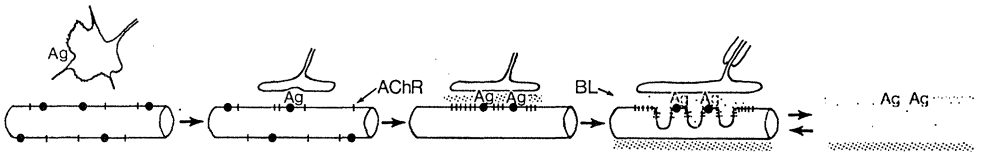

Figure 5. Schematic representation of the role of agrin in the formation, maintenance, and regeneration of the postsynaptic apparatus at the neuromuscular junction. (*Ag*): agrin. (*BL*): basal lamina. (●), agrin receptors.

both in the components they contain and the manner in which they form; (*b*) molecules recognized by anti-agrin antibodies are concentrated in the cell bodies of motor neurons and in the synaptic basal lamina at the neuromuscular junction; (*c*) motor neurons contain and transport down their axons agrin-like molecules that cause AChR aggregation, and (*d*) agrin-like molecules mediate nerve-induced AChR aggregation *in vitro*. These results are consistent with the hypothesis that agrin is synthesized in the cell bodies of motor neurons, is transported along motor axons to their terminals, and is released from their terminals to direct the formation of post-synaptic specializations at developing neuromuscular junctions. According to this scheme (figure 5), developing myofibres have receptors for agrin scattered along their plasma membrane and axon terminals release agrin as they grow over the myofibre's surface. When agrin binds to its receptor it triggers a cascade of intracellular events leading to the formation of a specialization on the surface of the myofibre at which AChRs, AChE, and other components of the post-synaptic apparatus accumulate, including components of the synaptic basal lamina such as heparin sulphate proteoglycan. Agrin becomes associated with this nascent synaptic basal lamina and thus is bound at the synaptic site. Release of agrin from motor nerve terminals at adult neuromuscular junctions and its incorporation into synaptic basal lamina would help maintain the post-synaptic apparatus and induce differentiation of post-synaptic specializations on myofibres regenerating in the absence of nerve terminals. Release of agrin by regenerating axons would account for their ability to induce a post-synaptic apparatus at ectopic sites on denervated myofibres.

Formation of a mature neuromuscular junction involves signals in addition to agrin (see Nitkin *et al.* 1987; Wallace 1989). For example, electromechanical activity is known to regulate the extent to which AChE accumulates at synaptic sites (Dennis 1981). There is also evidence that molecules such as ascorbate, ARIA, and CGRP may be released by axon terminals to increase the rate of synthesis of AChRs (see Schuetze & Role 1987). On the other hand, molecules antigenically similar to agrin are not restricted to motor neurons and the synaptic basal lamina. For example, agrin-like molecules are found in the extrajunctional region of slow muscle fibres, on smooth muscle fibres of blood vessels, and at nodes of Ranvier (Reist *et al* 1987; Godfrey *et al.* 1988*a*; Magill-Solc & McMahan 1988), locations where they clearly cannot be involved in AChR aggregation. Molecules recognized by anti-agrin antibodies also accumulate spontaneously together with AChRs on muscle fibres in aneural limbs of chick embryos (Fallon & Gelfman 1989), although there is no reason to think that these molecules are responsible for the AChR aggregates; no agrin-like AChR-aggregating activity has been detected in non-synaptic regions of muscle fibres. The location and properties of these agrin-like molecules suggest that they are in each case associated with basal lamina (Reist *et al.* 1987; Godfrey *et al.* 1988 *a*, *b*; Magill-Solc & McMahan 1988). One hypothesis to account for these observations is that agrin is a member of a family of basal lamina-associated proteins that induce the aggregation of membrane components, such as sodium channels at nodes of Ranvier and potassium channels in astrocytic endfeet along capillaries in the brain (Reist *et al.* 1987; Magill-Solc & McMahan 1988). Such proteins may possess some AChR-aggregating activity; molecules that are recognized by anti-agrin antibodies and cause aggregation of AChRs on cultured myotubes have been extracted from *Torpedo* heart and gut (Godfrey *et al.* 1988*a*). The extent to which these agrin-like molecules resemble those in motor neurons and in the synaptic basal lamina remains to be determined.

The findings that (*a*) agrin selectively induces phosphorylation of AChRs; (*b*) agrin-induced AChR aggregation and phosphorylation of the β subunit are both blocked by low pH and by TPA, but not by the kinase inhibitor H-7; (*c*) agrin-induced AChR phosphorylation reaches a maximum hours before receptor aggregation is complete, and (*d*) agrin-induced phosphorylation of the β AChR subunit occurs on tyrosine residues suggest that phosphorylation of the β subunit of the chick AChR by a tyrosine protein kinase is an early step in agrin-induced receptor aggregation. Our working hypothesis for the intracellular events leading to the accumulation of AChRs is that the combination of agrin with its receptor on the myotube surface activates a tyrosine protein kinase that phosphorylates AChRs (and perhaps other components of the post-synaptic apparatus as well). As a consequence of phosphorylation the properties of AChRs change such that they tend to become attached to the cytoskeleton and so are immobilized in the vicinity of the activated kinase, forming an aggregate. The activity of protein phosphatases would account for the requirement that agrin be present for aggregates to be maintained and might tend to sharpen the boundaries of aggregates. The agrin-activated kinase might also affect secretory pathways within the myotube such that new receptors, as well as other components of the post-synaptic apparatus, are preferentially inserted at synaptic sites.

This hypothesis is consistent with our observations that agrin-induced aggregation of AChRs utilizes

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AChRs already in the myotube membrane and requires metabolic energy but not protein synthesis. It is also consistent with the evidence that AChR immobilization is mediated by attachment to the cytoskeleton (Bloch 1986; Froehner 1986; Anthony *et al.* 1988; Bloch & Morrow 1989; Marazzi *et al.* 1989), and with photobleach-recovery measurements that indicate that not only are AChRs in agrin-induced aggregates immobile, but agrin also immobilizes a fraction of the receptors in the diffuse phase (Dubinsky *et al.* 1989). Interestingly, attachment of AChRs to the cytoskeleton is thought to occur through an interaction of the β subunit with the 43 kD AChR-associated protein and actin filaments (Burden *et al.* 1983; Walker *et al.* 1984; Froehner 1986), and it is the β subunit that is phosphorylated by agrin. Myotubes transformed with the Rous sarcoma virus do not have spontaneously occurring AChR aggregates nor do they respond to aggregating factors such as agrin. These effects are mediated by the sarc gene product, a tyrosine protein kinase, and are thought to be due to changes in the cytoskeleton (Anthony *et al.* 1984, 1988; Marazzi *et al.* 1989). However, tyrosine phosphorylation of AChRs by diffusely distributed sarc protein kinase might also reduce aggregate formation by immobilizing diffusely distributed AChRs. Tyrosine phosphorylation of the AChR β subunit has also been shown to occur *in vivo* during formation of the rat neuromuscular junction; although, in this case phosphorylation appears to take place after AChRs aggregate (Qu *et al.* 1990). Experiments are in progress to establish the relationships between agrin-induced AChR phosphorylation and aggregation in the chick and to determine how AChRs and other components of the post-synaptic apparatus at the neuromuscular junction are aggregated and immobilized.

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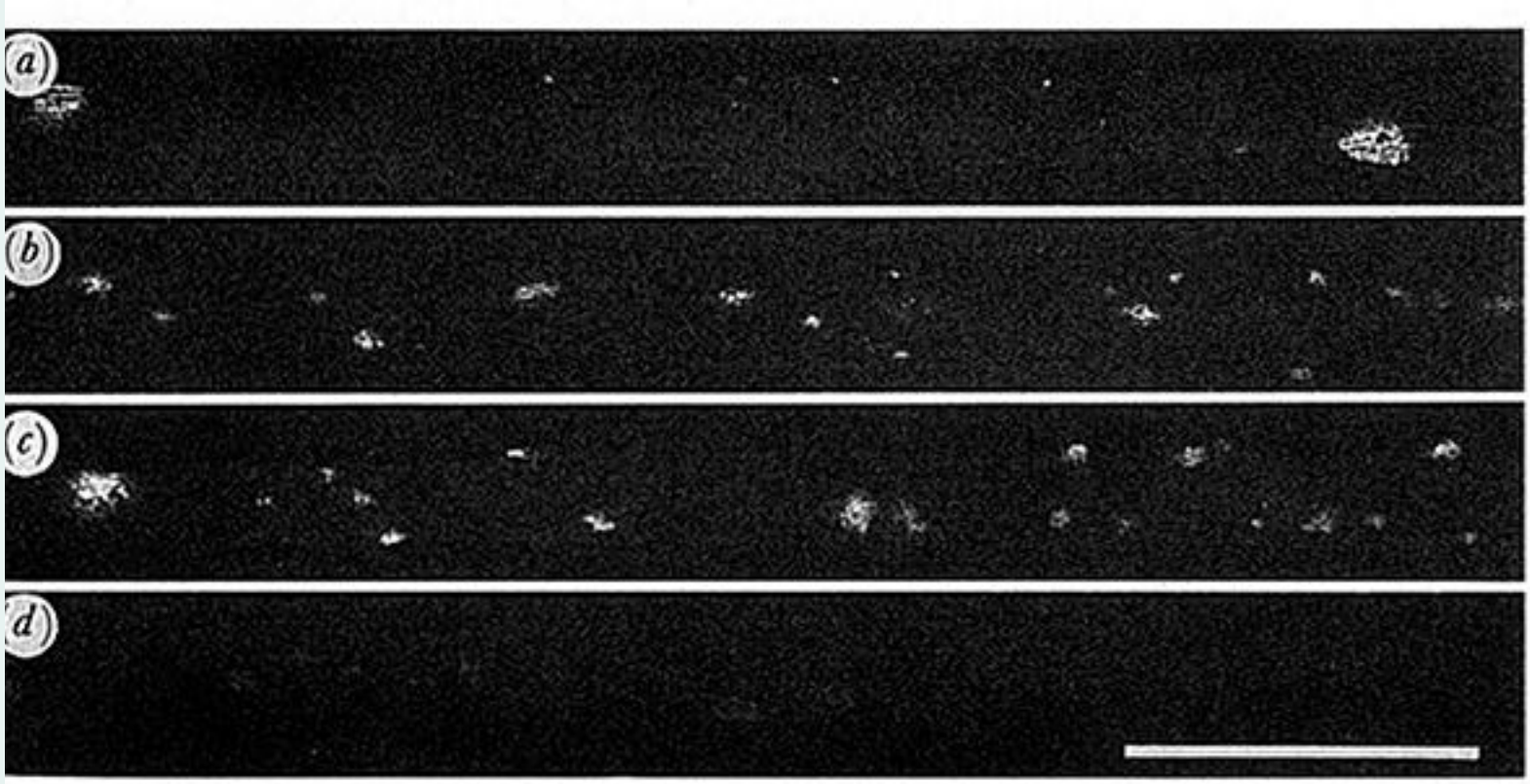
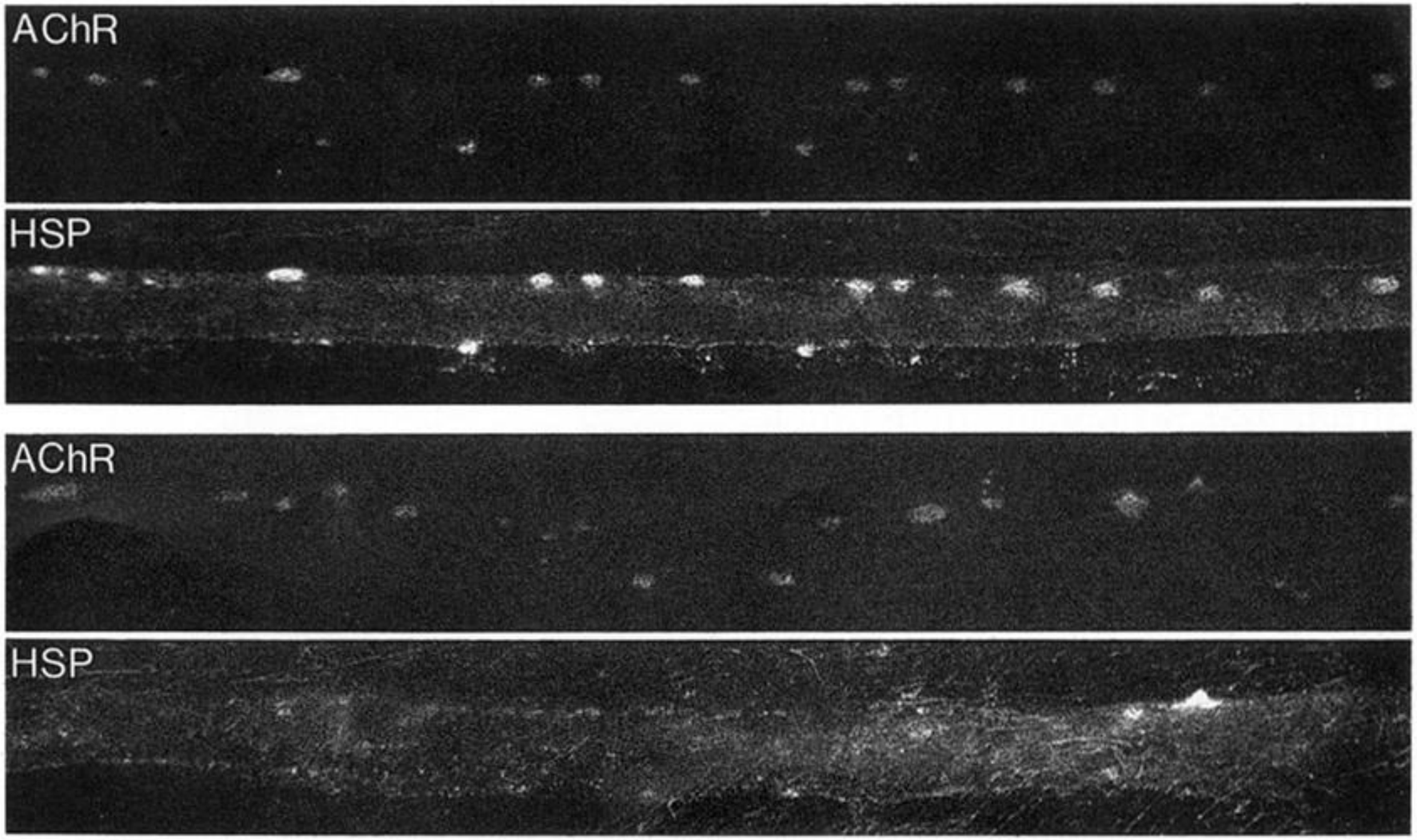


gure 1. Agrin induces AChR aggregation by lateral gration. Fluorescence micrographs of segments of cultured ick myotubes labelled with rhodamine α-bungarotoxin to veal distribution of receptors. (*a*, *b*) All AChRs: myotubes belled at the end of a 6 h incubation with (*a*) saline or (*b*) rin. (*c*) Pre-existing AChRs: myotube labelled before 6 h cubation with agrin. (*d*) Newly inserted AChRs: Myotube ated with unconjugated α-bungarotoxin to block pre-isting AChRs, incubated for 6 h with agrin, then labelled th rhodamine α-bungarotoxin. Bar, 50 μm.



gure 2. Inhibitors of protein synthesis block formation of agrin-induced aggregates of heparan sulphate proteoglycan it not of AChRs. Fluorescence micrographs of segments of myotubes from agrin-treated cultures labelled with odamine α-bungarotoxin and fluorescein-conjugated antibodies to heparan sulphate proteoglycan. Upper panel: rmal medium. Lower panel: cycloheximide (0.1 mg ml^{-1}) was added 3 h before addition of agrin.

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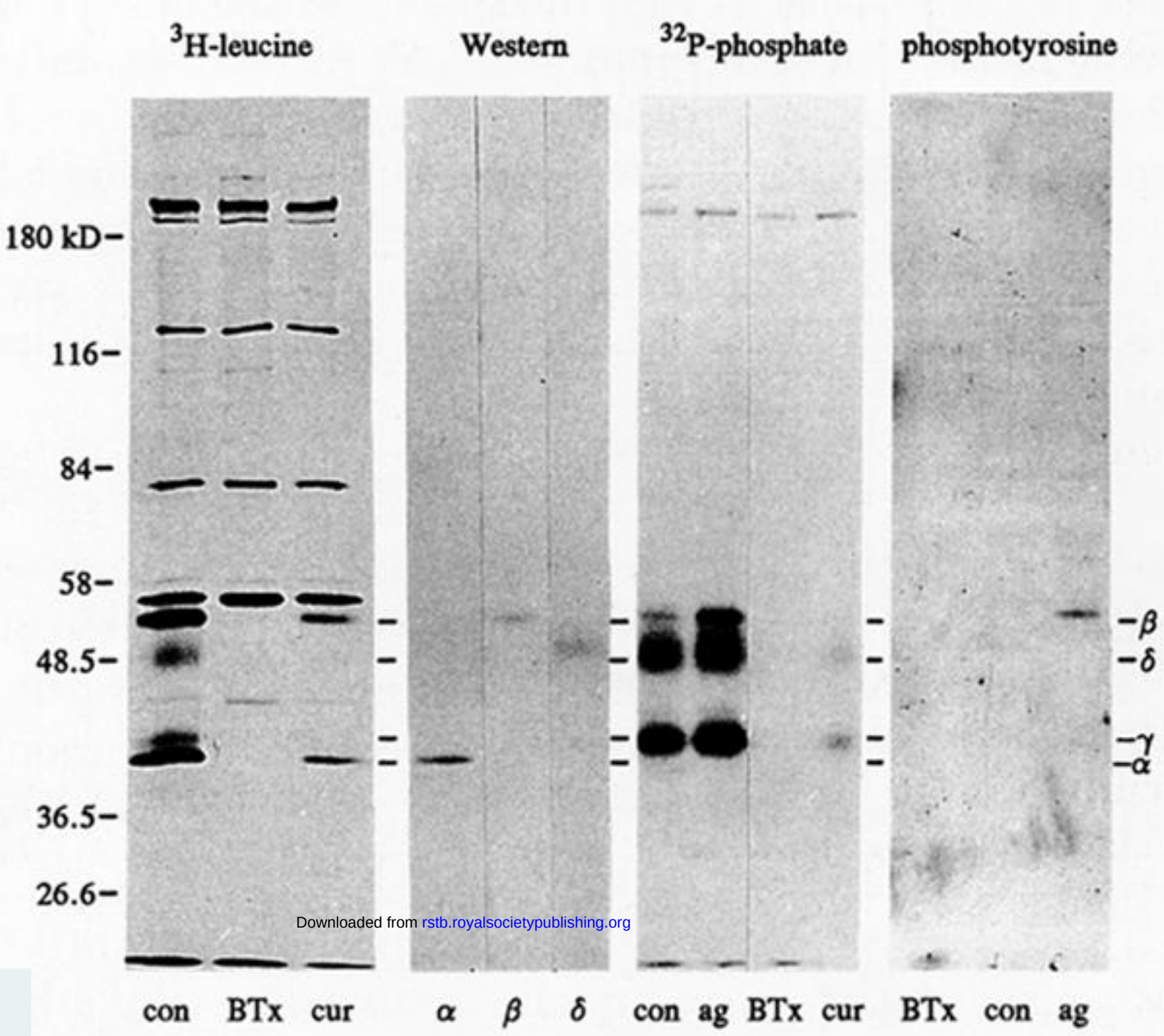

gure 3. Agrin induces phosphorylation of AChRs in ltured chick myotubes. AChRs were isolated from chick yotubes and the subunits separated by SDS-polyacrylamide l electrophoresis. [^{3}H]leucine: Fluorograms of AChRs olated from myotubes radiolabelled with [^{3}H]leucine. olation of AChR subunits of 40, 42, 48, and 52 kD (*con*) is ecifically blocked by α-bungarotoxin (*BTx*) or curare (*cur*). estern: Western blot identification of AChR α, β, and δ bunits. AChRs were isolated, transferred to nitrocellulose, d stained by using subunit specific monoclonal antibodies. ne 42 kD subunit, which did not bind any of the antibodies sted, is tentatively identified as the γ subunit. [^{32}P] osphate: Autoradiogram of AChRs isolated from myotube ltures labelled with [^{32}P]H_3PO_4. The β, γ, and δ subunits re labelled in control cultures (*con*). Overnight incubation th agrin caused an increase in phosphorylation of the γ, δ, d especially β subunits (*ag*). Specificity was confirmed by ocking the isolation of AChR subunits with α-bungarotoxin *Tx*) or curare (*cur*). Phosphotyrosine: Autoradiogram of ChRs isolated, transferred to nitrocellulose, and probed th anti-phosphotyrosine antibodies and [^{125}I]Protein A. ne only subunit labelled was the β subunit, and agrin used a marked increase in the level of phosphotyrosine it ntained. (*BTx*): Sample from cultures in which isolation of ChRs was specifically blocked with α-bungarotoxin. (*con*): ChRs isolated from control myotubes. (*ag*): AChRs isolated m myotubes treated overnight with agrin.