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# ARIA, a protein that stimulates acetylcholine receptor synthesis, also induces tyrosine phosphorylation of a 185-kDa muscle transmembrane protein

(synaptogenesis/tyrosine kinase/trophic factor/neuromuscular junction)

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ABSTRACT **Motoneurons promote the accumulation of acetylcholine receptors (AChRs) at developing neuromuscular synapses. The AChR-inducing activity protein ARIA, which is purified from chicken brain and increases the synthesis of AChRs in chicken myotubes, may play a crucial role in this process. Here we show that ARIA induces the rapid tyrosine phosphorylation of a $M_r$ 185,000 protein (p185) in muscle cells. Phosphorylation of p185 correlates with AChR induction at each stage of ARIA purification. Moreover, medium conditioned by spinal cord motoneurons stimulates AChR synthesis and p185 phosphorylation. Studies with membrane-impermeant reagents and $^{125}$I-labeled ARIA indicate that p185 is a transmembrane ARIA–receptor tyrosine kinase. Our data suggests that muscle AChR synthesis can be regulated through tyrosine phosphorylation.**

During formation of the neuromuscular junction, embryonic motor nerve terminals promote the accumulation of acetylcholine receptors (AChRs) and other synaptic proteins in the postsynaptic muscle membrane and cytoplasm. The increase in postsynaptic AChR density is due primarily to an increase in local AChR synthesis (1) and to the aggregation of receptors already present in the membrane at the time of nerve–muscle contact (2). There is strong evidence that a form of the protein agrin plays a role in AChR aggregation (3, 4). Less is known about factors that induce AChR gene expression. We have purified a 42-kDa glycoprotein from chicken brain that stimulates AChR synthesis in cultured embryonic myotubes (5, 6). Our hypothesis is that this protein, named ARIA for its *a*cetylcholine *r*eceptor *i*nducing *a*ctivity, regulates receptor synthesis at developing neuromuscular junctions. ARIA stimulation of AChR synthesis is associated with an increase in the level of the $\alpha$-subunit mRNA in chicken muscle (7). In mouse muscle a prominent effect on the $\varepsilon$-subunit mRNA was observed (8). The local increase in AChR synthesis is remarkable because it occurs despite a dramatic decrease in AChR synthesis in extrasynaptic regions of the same muscle cells, as synaptic transmission drives muscle electrical/mechanical activity.

We have begun to study the mechanism by which ARIA regulates AChR subunit gene expression. In this paper we present evidence suggesting that ARIA regulates muscle AChR synthesis by activating a receptor tyrosine kinase.

## MATERIALS AND METHODS

**Tissue Culture.** Chicken myoblasts were dissociated from embryonic day 11 (E11) pectoral muscle and seeded in 96- or 48-well culture dishes (20,000 and 80,000 cells per well, respectively) in Eagle's minimum essential medium (MEM)/10% horse serum/2% chicken embryo extract. L6 cells were grown in Dulbecco's MEM (DMEM)/10% fetal bovine serum. Motoneurons were dissociated from chicken spinal cords (E5) and purified through metrizamide gradients (9) before plating. Cells were plated in DMEM/F-12 (GIBCO), supplemented with glucose to 4 g/liter, 10% fetal bovine serum, and chicken muscle extract at 10 $\mu$g/ml (9).

**ARIA Purification.** ARIA was purified by a modification of our published protocols (5, 6, 10), which include a step of heparin chromatography introduced primarily to separate ARIA from the chicken prion-like protein. Frozen pulverized chicken brains were delipidated with acetone and ether and extracted in an acid mixture (5, 6). ARIA was then purified by chromatography on CM-Sepharose equilibrated in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes, pH 6) and eluted with a gradient of NaCl (50–1000 mM); Vydac $C_4$ (22 × 250 mm column) equilibrated in 0.1% trifluoroacetic acid eluted with a gradient of isopropyl alcohol (15–27.5%); heparin-5PW (TosoHAAS, Philadelphia) equilibrated with phosphate-buffered saline, and eluted with a gradient of NaCl (150–1500 mM); Vydac $C_4$ (10 × 250 mm column) equilibrated in 0.13% heptafluorobutyric acid eluted with a 30–45% gradient of isopropyl alcohol. Active fractions from the last column were pooled (2 ml), dried, and redissolved in culture medium (20 ml) before addition to muscle cultures (60 $\mu$l per well in 96-well plates, 200 $\mu$l per well in 48-well plates).

**AChR Binding Assay.** The rate of incorporation of AChRs into the surface membrane was measured in chicken muscle cells plated in 96-well culture dishes, as described in Usdin and Fischbach (5). Briefly, cells were treated with test samples for 20 hr. Thereafter, all exposed receptors were blocked with unlabeled $\alpha$-bungarotoxin (BTX) ($10^{-7}$ M for 1 hr at 37°C). After the plates were washed, fresh medium containing 5 nM iodinated $\alpha$-BTX ([$^{125}$I]BTX) was added, and plates were returned to the incubator for 5 hr. Then cells were washed and solubilized, and radioactivity was counted in a $\gamma$ counter. Nonspecific binding of [$^{125}$I]BTX was measured in the presence of 1.2 $\mu$M unlabeled $\alpha$-BTX. Each sample was assayed in quadruplicates.

**Immunoblotting.** Cells were washed with phosphate-buffered saline, solubilized in 40 $\mu$l of Laemmli sample buffer (2×) and heated to 95°C for 3 min. Aliquots were electrophoresed in 5% SDS/polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes (Millipore). The transferred proteins were probed with a monoclonal antibody (mAb) raised against phosphotyrosine residues (mAb 4G10; provided by B. Druker and T. Roberts of the Dana–Farber Cancer Institute, Boston). Bound mAb 4G10 was visualized

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Abbreviations: AChR, acetylcholine receptor; ARIA, AChR-inducing activity protein; TTX, tetrodotoxin; DTSSP, 3,3′-dithiobis-(sulfosuccinimidyl propionate); BTX, $\alpha$-bungarotoxin; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; WGA, wheat germ agglutinin; mAb, monoclonal antibody.

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with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Boehringer Mannheim) and enhanced chemiluminescence (ECL; Amersham).

**Affinity Precipitations.** Cells were lysed in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 1 mM *o*-vanadate, and protease inhibitors. Insoluble components were removed by centrifugation, and the supernatants were incubated for 3–4 hr with either antiphosphotyrosine antibodies (mAb 4G10) conjugated to agarose beads, monomeric avidin coupled to agarose beads (Pierce), or wheat germ agglutinin (WGA) coupled to Sepharose beads (Sigma). After centrifugation, the beads were washed three times with lysis buffer, and the proteins were eluted with Laemmli sample buffer.

**Biotinylation of Cell Surface Proteins.** Proteins exposed on the surface of L6 cells were biotinylated by adding sulfosuccinimidyl–biotin (Pierce) at a final concentration of 0.1 mg/ml in Tyrode's saline solution and incubated for 30 min at 4°C. Before biotinylation the cells were treated with ARIA for 30 min in Tyrode's saline solution at 4°C.

**Crosslinking.** ARIA-treated L6 cells were exposed to 2 mM 3,3′-dithiobis-(sulfosuccinimidyl propionate) (DTSSP, Pierce) for 15 min at 4°C. Cells were lysed in SDS sample buffer without reducing agent. Immunoblots with mAb 4G10 were done as above. The most active fraction eluting from the final $C_4$ column described above was used. The preparation used for iodination was not subjected to heparin-affinity chromatography. Chemiluminescence was allowed to decay for 3 days, and an autoradiogram of the blot was made (one screen, −70°C, 15 days).

## RESULTS

Recent experiments have shown that ARIA binds to heparin with moderate affinity, eluting between 500 and 700 mM NaCl (D.L.F., unpublished observations). Because many growth/differentiation factors that activate tyrosine kinase receptors bind to heparin (11–13), we tested the effect of ARIA on protein-tyrosine phosphorylation.

Immunoblots probed with antiphosphotyrosine antibodies showed that ARIA did induce protein-tyrosine phosphorylation. A 185-kDa band (p185) was evident in lysates of chicken myotubes treated with ARIA but was not seen in untreated controls (Fig. 1*A*). Primary muscle cultures contain fibroblasts as well as myoblasts and myotubes, so it is significant that no phosphorylation of p185 was observed in ARIA-treated cultures of chicken fibroblasts that did not contain muscle cells (data not shown).

ARIA also promoted tyrosine phosphorylation of a 185-kDa band in cultures of the rat L6 cell line (Fig. 1*A*) and in primary cultures of mouse and human muscle (data not shown). The ARIA-induced band was much more intense in L6 cells and mouse muscle primary cultures than in chicken or human cultures. Therefore, many of our results are demonstrated by using L6 cells. Most experiments were done simultaneously on chicken myotubes and L6 cells, and the results were identical.

Several polypeptide growth/differentiation factors promote the phosphorylation of tyrosine residues in proteins that range between 170- and 190-kDa molecular size. We found that epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) promoted tyrosine phosphorylation of chicken muscle proteins, but in each case the bands could be distinguished from p185 (Fig. 1*B*). Insulin increased the tyrosine phosphorylation of a 90-kDa protein, but no band in the 180- to 190-kDa range was detected. We detected no effect of fibroblast growth factor, colony stimulating factor-1, or nerve growth factor on muscle protein-tyrosine phosphorylation. None of the proteins tested increased the synthesis of AChRs.

Agrin did not promote detectable phosphorylation of any protein >80 kDa. Although this protein stimulates receptor aggregation, we have confirmed earlier reports (14) that it does not increase AChR synthesis. Agrin does promote the tyrosine phosphorylation of the AChR $\beta$ subunit (15). Tetrodotoxin (TTX) increases AChR synthesis in chicken myotubes, presumably by inhibiting muscle activity (16, 17). It is significant that no effect of TTX on tyrosine phosphorylation was detected (data not shown).

ARIA-induced p185 phosphorylation was rapid (Fig. 1*C*) and transient. A band was detectable 1 min after ARIA addition to intact myotubes. The band was most intense after 1 hr, and it was undetectable 6–9 hr later despite the continued presence of the factor.

At each stage of the ARIA purification, p185 tyrosine phosphorylation correlated highly with AChR-inducing activity, suggesting that the same protein causes both p185 phosphorylation and the increase in AChR synthesis. Fig. 2 illustrates this match in the final step of a purification that included ion exchange, heparin affinity, and reverse-phase chromatography. Previously we had shown that a chicken prion protein homolog copurifies with ARIA (10). It is noteworthy that this protein was separated from the ARIA by the heparin chromatography (D.L.F., unpublished observations).

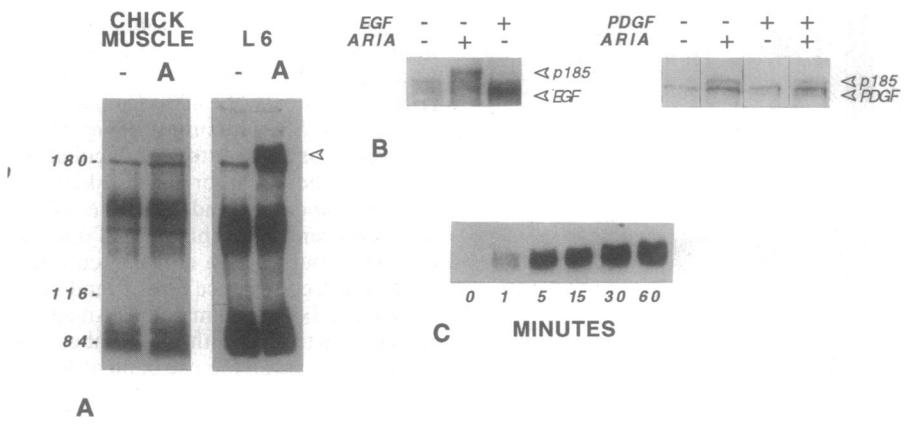


FIG. 1. ARIA promotes the tyrosine phosphorylation of a 185-kDa protein in chicken and rat (L6) muscle cells. (*A*) Several bands are evident in immunoblots of muscle extracts probed with an antiphosphotyrosine mAb, but only one (arrowhead) is specific for cells treated with ARIA (A) for 1 hr. Note that the p185 band is broader and more intense in L6 cells than in chicken myotubes. (*B*) The ARIA-stimulated tyrosine phosphorylation of p185 is not mimicked by EGF (100 ng/ml) or $PDGF_{BB}$ (20 ng/ml). EGF and PDGF phosphorylate tyrosine residues of other proteins (see arrowheads). (*C*) Early time course of ARIA-induced p185 phosphorylation in L6 cells.

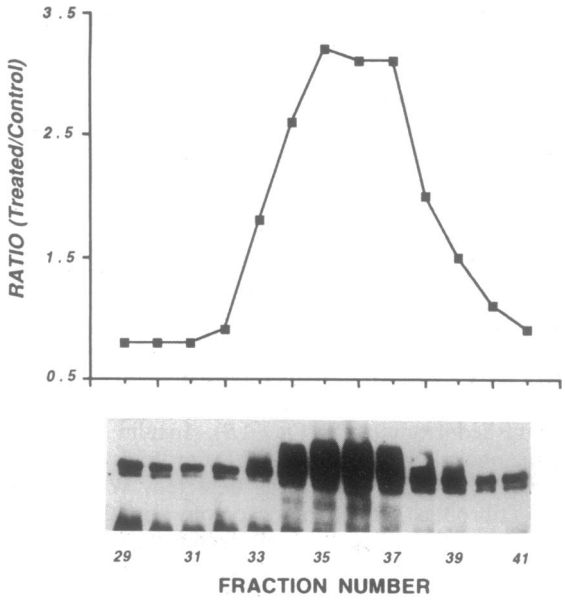


FIG. 2. Phosphorylation of p185 correlates with ARIA. (*Lower*) p185 tyrosine phosphorylation after 1-hr treatment of L6 cells with material eluted from the $C_4$ heptafluorobutyric acid column. The same result was obtained with chicken myotubes. (*Upper*) Relative rate (compared with controls) of AChR incorporation into the surface membrane of chicken myotubes treated with the same dilution of the same fractions.

In addition to the correlation during ARIA purification, the dose–response curves for ARIA-induced AChR synthesis and p185 phosphorylation were nearly identical (data not shown). Further, suramin, a drug known to interfere with the binding of other polypeptide growth factors (18), blocked both the ARIA-induced AChR synthesis and p185 phosphorylation with the same dose dependence (Fig. 3). Suramin had no effect on the enhanced AChR synthesis that follows blockade of electrical activity with TTX.

Several drugs reported to block the catalytic activity of tyrosine kinases had no effect on ARIA-induced p185 phosphorylation or on AChR synthesis at nontoxic doses. We tested tyrphostin RG50864 (19), genistein (20), Lavendustin A

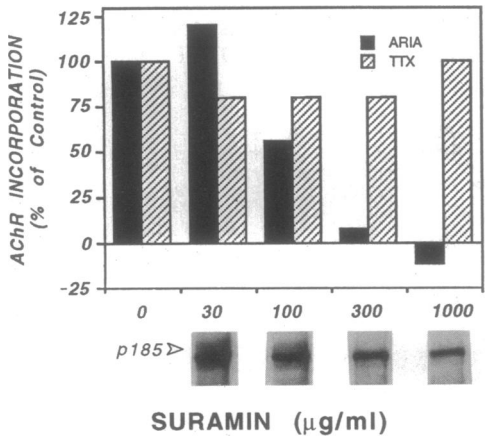


FIG. 3. Suramin blocks ARIA-induced AChR synthesis and p185 phosphorylation in chicken myotubes. (*Upper*) Bar graph shows that suramin blocked the effect of ARIA on AChR synthesis but did not block that of TTX (1 μM). Controls in this experiments were muscle cultures exposed to suramin (at the indicated concentration) without added ARIA or TTX. The cells were preincubated in suramin for 2 hr before adding ARIA or TTX. (*Lower*) Suramin also blocks the ARIA-induced p185 phosphorylation over the same dose range. Phosphorylation was assayed 1 hr after ARIA addition.

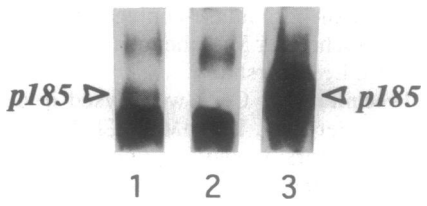


FIG. 4. Motoneuron-conditioned medium induces p185 phosphorylation. Phosphotyrosine immunoblots of L6 cells treated for 1 hr with motoneuron-conditioned medium (lane 1), fresh medium (lane 2), and ARIA dissolved in fresh medium (lane 3). The dose of ARIA used in lane 3 was 5-fold higher than that required for maximal ARIA-induced AChR synthesis.

(21), 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid (22), herbimycin A (23), and k-252a (24). No effect of these agents on p185 phosphorylation was observed, even when the muscle cells were homogenized, so the negative results are probably not due to limited cell membrane permeation.

Earlier observations showed that spinal cord explants secreted a substance into the cultured medium that stimulated AChR synthesis in cocultured myotubes (ref. 25; see also refs. 26 and 38). We found that medium conditioned by chicken spinal cord motoneurons induced tyrosine phosphorylation of p185 when applied (undiluted) to L6 cells (Fig. 4). The only change in tyrosine phosphorylation detected after treatment with motoneuron-conditioned medium was in the 185-kDa band. The same conditioned medium was tested in our AChR induction bioassay. When applied (undiluted) to chicken myotubes, the conditioned medium stimulated AChR synthesis to half the maximal ARIA response in the same bioassay (data not shown).

The molecular weight of p185, the time course of its phosphorylation, and the fact that it is the most conspicuous protein phosphorylated by ARIA treatment are consistent with the idea that p185 is an ARIA receptor. Several experiments support this hypothesis. First, p185 is a transmembrane protein. It was phosphorylated on tyrosine residues after addition of ARIA to intact cells, so it must have an intracellular domain. p185 could be labeled by sulfosuccinimidyl–biotin (27), a membrane-impermeant biotinylating reagent (Fig. 5*A*), indicating it has an extracellular domain. p185 could be precipitated specifically by WGA (Fig. 5*B*), suggesting that it is a glycoprotein and supporting the conclusion that p185 is exposed on the cell surface.

Evidence that p185 binds ARIA was obtained by use of the membrane-impermeant, bifunctional, coupling reagent DTSSP (29). After incubating intact muscle cells with ARIA and then adding the crosslinker, a distinctive phosphotyrosine band centered at ≈400 kDa appeared on immunoblots, accompanied by a proportionate decrease of the p185 band (Fig. 5*C*, lane 3). The 400-kDa band was absent when either ARIA or DTSSP was omitted (Fig. 5*C*, lanes 1 and 2) or when suramin was added to the incubation medium (data not shown). Other receptor tyrosine kinases are known to dimerize upon binding ligand (30). The 400-kDa broad band probably represents a combination of one, two, or zero molecules of ARIA coupled to a crosslinked dimer of p185.

More direct evidence for the binding of ARIA was obtained by using radiolabeled material. An autoradiogram of a muscle preparation treated with an $^{125}$I-labeled ARIA preparation and DTSSP showed an intense band at 400-kDa and a fainter band at 220 kDa (Fig. 5*D*, lane 2). The less intense 220-kDa band presumably represents ARIA crosslinked to p185 monomers.

## DISCUSSION

We suggest a pathway for the regulation of muscle AChR gene expression that begins with activation of a receptor

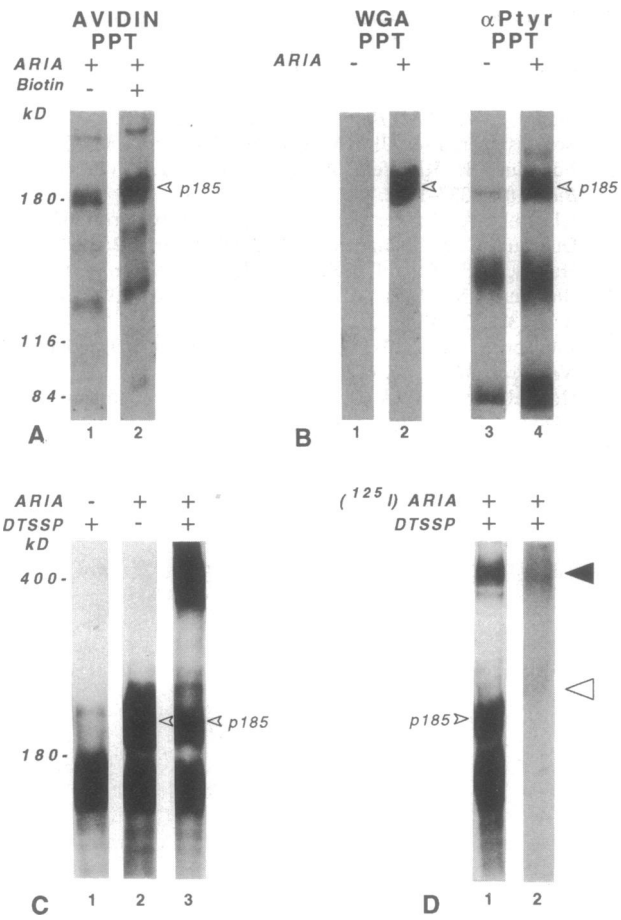


FIG. 5. Evidence that p185 is a transmembrane ARIA-binding protein. (*A*) Avidin–agarose beads precipitate phosphorylated p185 (detected with mAb 4G10) from L6 cells treated with ARIA and sulfosuccinimidyl–biotin (lane 2). No phosphorylated p185 was precipitated when sulfosuccinimidyl–biotin was omitted (lane 1). Phosphorylated p185 was detected in the supernatant corresponding to lane 1 but was not detected in lane 2. (*B*) Phosphorylated p185 was precipitated by WGA beads from extracts of ARIA-treated L6 cells (lane 2) but was not precipitated from untreated L6 cells (lane 1). No phosphorylated p185 was precipitated by WGA beads in the presence of 300 mM *N*-acetyl-D-glucosamine (data not shown). Precipitation with antiphosphotyrosine-coupled beads showed that the WGA precipitation was specific to p185 (compare lanes 2 and 4). (*C*) A phosphotyrosine band of ≈400-kDa appears after treatment with ARIA and DTSSP (lane 3). The 400-kDa band was absent from samples not treated with ARIA (lane 1) or DTSSP (lane 2). Note that the amount of phosphorylated p185 is decreased in crosslinked preparations (compare lanes 3 and 2). (*D*) Lane 2 is an autoradiogram of samples from cells treated with $C_4$-active fractions radioiodinated by the chloramine T method (28) [($^{125}$I)ARIA] and DTSSP. The 400-kDa band is heavily labeled, and a more lightly labeled band is evident at ≈220 kDa. The same lane probed with mAb 4G10 is shown in lane 1.

tyrosine kinase. Certainly, phosphorylation of a $M_r$ 185,000 transmembrane protein occurs rapidly after exposure to ARIA. Moreover, a radioiodinated component in highly purified ARIA preparations, presumably the 42-kDa species, can be crosslinked to what appear to be p185 monomers and dimers.

Previous studies have implicated other second-messenger cascades in the regulation of muscle AChR synthesis. Protein kinase A is involved in the induction of AChR synthesis by calcitonin gene-related peptide (CGRP) (31). Protein kinase C has been implicated in the down-regulation of AChR synthesis by electrical activity (32, 33). Changes in the levels of intracellular calcium have also been shown to influence muscle AChR synthesis (32, 34). The action of ARIA probably does not involve changes in cAMP and/or $Ca^{2+}$ levels. ARIA does not increase intracellular cAMP levels in chicken myotubes (35), and pharmacological experiments using calcium channel blockers, ionophores, and ryanodine suggest that ARIA does not act by an overall change in intracellular $Ca^{2+}$ (G.C. and G.D.F., unpublished data).

It will be important to determine whether the cascades initiated by different kinase classes interact in the regulation of AChR expression, and if the action of ARIA leads to tyrosine phosphorylation of AChR subunits (cf. refs. 15 and 36).

Phosphorylation of a $M_r$ 185,000 protein by medium conditioned by spinal cord motoneurons indicates that brain-derived ARIA is similar, if not identical, to the molecule secreted by motoneurons. This phosphorylation is also consistent with a physiological role for ARIA at developing neuromuscular junctions.

ARIA joins a growing list of polypeptide receptor tyrosine kinase ligands. This group now includes neurotrophins, EGFs, fibroblastic growth factors, PDGFs, and heregulins. The many effects of these pleiotropic growth and differentiation factors depend on the time and location of their expression. We can, therefore, expect additional effects of ARIA to emerge. In muscle, we already know that ARIA can regulate different AChR subunits that appear early and late during the course of nerve–muscle synapse formation (7, 8). ARIA also increases the number of muscle voltage-gated sodium channels (37). Further analysis of p185 and of ARIA itself will facilitate studies of trophic interactions at developing nerve–muscle synapses.

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