Purification and Characterization of a Polypeptide from Chick Brain that Promotes the Accumulation of Acetylcholine Receptors in Chick Myotubes

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Abstract. Acetylcholine receptors (AChRs) are packed in the postsynaptic membrane at neuromuscular junctions at a density of ~20,000/μm², whereas the density a few micrometers away is <20/μm². To understand how this remarkable distribution comes about during nerve–muscle synapse formation, we have attempted to isolate factors from neural tissue that can promote the accumulation of AChRs and/or alter their distribution. In this paper we report the purification of a polypeptide from chick brains that can increase the rate of insertion of AChR into membranes of cultured chick myotubes at a concentration of <0.5 ng/ml. Based on SDS PAGE and the action of neuraminidase, the acetylcholine receptor–inducing activity (ARIA) appears to be a 42,000-D glycoprotein. ARIA was extracted in a trifluoroacetic acid-containing cocktail and purified to homogeneity by reverse-phase, ion exchange, and size exclusion high pressure liquid chromatography. Dose response curves indicate that the activity has been purified 60,000-fold compared with the starting acid extract and ~1,500,000-fold compared with a saline extract prepared from the same batch of brains. Although the ARIA was purified on the basis of its ability to increase receptor incorporation, we found that it increased the number and size of receptor clusters as well. It is not yet clear if the two effects are independent.

The 42-kD ARIA is extremely stable: it was not destroyed by exposure to intact myotubes, low pH, organic solvents, or SDS. Its action appears to be selective in that the increase in the rate of receptor insertion was not accompanied by an increase in the rate of protein synthesis. Moreover, there was no change in cellular, surface membrane, or secreted acetylcholinesterase. The effect of ARIA is apparently independent of the state of activity of the target myotubes as its effect on receptor incorporation added to that of maximal concentrations of tetrodotoxin.

Electrophysiological and α-bungarotoxin (α-BTX) binding studies have shown that motoneurons influence the number and distribution of acetylcholine receptors (ACHR) in developing myotubes. The density of receptors at newly formed synapses in vivo and in vitro is three to tenfold greater than the density in the extrasynaptic membrane, and this difference is evident within a few hours after nerve–muscle contact is first established (see reviews by Fischbach et al., 1979; Fambrough, 1979; Cohen, 1980; Steinbach and Bloch, 1986). Two mechanisms have been implicated in the formation of synaptic clusters. In Xenopus myocytes, aggregation of mobile receptors present in the membrane before nerve–muscle contact plays the major role (Anderson et al., 1977; Kuromi et al., 1985). Receptor aggregation also contributes to synaptic clusters on chick myotubes, but in this system new receptors, inserted after the nerve arrives, make up 60–80% of the total during the first 24 h (Role et al., 1985). Both aggregation and insertion contribute to postsynaptic clusters in rat muscle as well (Ziskind-Conhaim et al., 1984).

Little information is available about factors that might trigger the postsynaptic response. Some evidence that neurons release receptor-inducing factors capable of diffusing over short distances was obtained in chick muscle cultures that contained one or more slices of embryonic spinal cord (Cohen and Fischbach, 1977). Myotubes near the slices (explants) were four to five times more sensitive to inotopically applied acetylcholine (ACh), and they bound more [125I]α-BTX than myotubes located further away in the same culture dish. The number of AChR clusters was also dramatically increased on peri-explant myotubes. Similar observations were made in rat spinal cord–L6 myotube cultures (Podleski et al., 1978). Studies of dissociated spinal cord and peripheral ganglion cells and nerve cell lines suggest that the ability to induce receptor clusters may be unique to cholinergic neurons (Cohen and Weldon, 1980; Role et al.,...
4 d beginning on the fourth day after plating, the maximum receptor clusters on cultured chick myotubes (Jessell et al., 1979). When extracts were added to the cultures daily for 5 d after plating, the increase in receptor number amounted to three to fourfold. The number of receptors and/or the number of receptor clusters did not alter AChR number or distribution. Moreover, the formation of synaptic clusters. A variety of sources has been used and different types of myotubes have been assayed in different ways, so relations between the various factors are not yet clear. It should be possible to compare these factors when they are purified to homogeneity or when specific antibodies are raised and tested in the various assays. In the absence of additional information, we shall refer to all factors that alter the number or distribution of AChRs as acetylcholine receptor-inducing activities (ARIs).

Earlier work in this laboratory showed that saline extracts of embryonic or adult chick brains produced a dose-dependent increase in the number of AChRs and the number of receptor clusters on cultured chick myotubes (Jessell et al., 1979). When extracts were added to the cultures daily for 4 d beginning on the fourth day after plating, the maximum increase in receptor number amounted to three to fourfold. Some degree of tissue specificity was evident in that saline extracts prepared from heart, liver, or cultured fibroblasts did not alter AChR number or distribution. Moreover, the effect of brain extract on AChRs did not seem to be part of a general improvement in the formation or health of the myotubes: there was not a large change in total protein and the activity of the cytoplasmic enzyme creatine kinase did not change at all. Saline brain extract did increase the level of acetylcholinesterase (ACHE) activity to about the same degree as it increased receptor number. This is of interest because the enzyme appears at developing nerve–muscle synapses soon after receptors begin to accumulate.

Our initial attempts to characterize the size of chick brain ARIA by gel filtration indicated that saline extract contained low and high molecular weight species. Acetic acid or HCl/acetone extract of chick brain contained ~40% as much activity as saline extracts, and most of the acid-soluble activity appeared to be <5,000 D in size. Subsequent experiments (Buc-Caron et al., 1983) showed that chick brain ARIA could be extracted in the trifluoroacetic acid (TFA)–containing extraction cocktail described by Bennett et al. (1978), and that this material also appeared to be small. TFA-soluble ARIA was retained on a hydrophobic resin (C$_{18}$), and it could be partially purified by reverse-phase-high pressure liquid chromatography (HPLC). In this second series of experiments, a single addition of crude extract or partially purified fractions was found to produce a measurable effect after only 5 h and a larger effect after 24 h if added on the seventh day rather than the fourth day after plating. In addition, active fractions increased the rate of receptor insertion into the surface membrane as well as the total number of receptors. This was expected as the increase in receptor number is not associated with a decrease in the rate of receptor degradation (Jessell et al., 1979; Buc-Caron et al., 1983).

We have modified the acid extraction protocol and have developed techniques for measuring the rate of receptor insertion in myotube microcultures. The rate assay provided a more sensitive and reproducible index of ARIA after relatively brief exposures than did assays of the total number of receptors. We have also developed several additional chromatographic procedures and report here the complete purification of an ARIA that can increase the rate of receptor incorporation into myotube membranes severalfold at concentrations <1 ng/ml. It also promotes the formation of receptor clusters, but it does not appear to alter the activity or synthesis of AChE. Contrary to our expectation, the chick brain ARIA recovered is a rather large (42,000 D) glycoprotein.

**Materials and Methods**

**Muscle Cultures**

Mononucleated cells were dissociated from pectoral muscles of 10–12-d-old chick embryos (E10) as previously described (Buc-Caron et al., 1983). To reduce the number of fibroblasts, the cells were suspended in complete medium and plated in uncoated 100-mm tissue culture dishes (Falcon Labware, Oxnard, CA) for 30 min at 37°C. Unattached cells were collected and plated in gelatin-coated, 96-well Micro Test culture plates (Falcon Labware) at a density of 50,000/well in 100 µl of Eagle's minimal essential medium supplemented with horse serum (10% vol/vol), glutamine (1 mM), penicillin (50 U/ml), streptomycin (50 µg/ml), and ovaltineferrin (40 µg/ml). As reported by others (Li et al., 1982; Oh and Markelonis, 1982), we found that ovaltineferrin could replace chick embryo extract in supporting myoblast proliferation and myotube survival.

The cells were fed with 100 µl of medium on days 3 and 5. On day 7 they were fed with 60 µl of medium or 50 µl of medium plus 10 µl of a test fraction, and the number of AChRs was measured 24 h later (see below). Aliquots of column fractions to be assayed were dried in a Speed-Vac centrifuge (Savant Instruments, Inc., Hicksville, NY) and redissovled in complete medium. Samples that contained nonvolatile material were first desalted on Sep-Pak C$_{18}$ cartridges (Waters Associates Millipore Corp., Milford, MA). Samples containing <1 µg of protein were supplemented with 10 µg BSA.

**AChR Binding Assay**

To measure the number of surface AChRs the cells were incubated in complete medium containing 5 nM [3H]-α-BTX for 1 h at 37°C. The cells were washed twice by incubating the plates in 1 liter Ca$^{2+}$-free Hank's balanced salt solution (BSS) containing 2% BSA and then solubilized in 150 µl of 1 N NaOH containing sodium deoxycholate (0.5 mg/ml). The amount of [3H]-α-BTX bound was determined with a gamma counter (model 1191; Tracor Atlas, Inc., Houston, TX). Four wells were used for each data point. Nonspecific binding, taken as the amount of [3H]-α-BTX bound in the presence of 10$^{-7}$ M unlabeled α-BTX, was subtracted in each case.

The rate of incorporation of AChRs into the surface membrane was determined as described by Devreotes and Fambrough (1975). All receptors exposed on the muscle surface were blocked with unlabeled α-BTX (80 nM for 1 h at 37°C). The cells were washed thoroughly, returned to the incubator in 100 µl of fresh medium, and the number of new toxin binding sites was assayed with [3H]-α-BTX at various intervals thereafter.

α-BTX was iodinated by the chloramine-T-catalyzed reaction (Hunter and Greenwood, 1962), and moniodinated derivatives were purified by size exclusion (Sephadex G-10; Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and cation exchange (CM-Sephadex; Pharmacia Fine Chemicals) chromatography (Vogel et al., 1972). The specific activity of moniodinated...
toxin, estimated by competition with known concentrations of unlabelled a-BTX, ranged between 800 and 1,200 cpm/ml in different preparations.

**AChR Clusters**

To facilitate microscopic examination of AChR clusters, myotubes were plated on collagen-coated polylysine coverslips cut out to fit in the 96-well plates. In some experiments, the cells were treated with $10^3$ M cysteine arabinoside for 48 h before the third and fifth day after plating. Receptors were visualized with rhodamine-derivatized a-BTX prepared by the method of Ravdin and Axelrod (1977). Myotubes were exposed to a 1:10 dilution (in complete medium) of rhodamine-derivatized a-BTX for 60 min at 37°C, conditions which, judging from inhibition of $[^{3}H]a$-BTX binding, labeled >90% of the AChR. After the cells were washed and fixed (4% formaldehyde in BSS), the coverslips were mounted on glass slides in 90% glycerol/10% PBS (pH 8.0).

The cells were examined with a Leitz Ortholux II microscope equipped with phase-contrast and epifluorescence optics, a 100-W mercury arc light source, rhodamine-selective filters (N2 cube; excitation filter = 530-560 nm, barrier filter = 580 nm), and a 63x objective (1.4 NA Plan Ncofluor). AChR clusters were quantitated in two ways. First, relatively large clusters, >5 μm across, were counted on 20-50 myotube segments that crossed a major axis of the visual field. In the second method, small as well as large clusters were counted in digitized TV images. A silicon-intensified target camera (model 65; Dage-MTI Inc., Wabash, MI) was connected to a Grinnell Image Processor (model GMR/20; Control-3M Image Processing Systems, Pasadena, CA) that digitized the image in a 512 × 512 pixel array with 8 bits of intensity information. The image processor was interfaced with a PDP II/44 computer (Digital Equipment Corp., Marlboro, MA) and operated by Image, a high level language written by J. Voyvodic (Washington University Department of Anatomy and Neurobiology, St. Louis, MO).

Healthy myotubes that spanned the field of view were selected under phase-contrast illumination and outlined with the aid of a digitizing tablet. The intensity of all pixels within the outline was measured under fluorescence illumination, and background values (obtained with the mercury light source switched off) were subtracted. Finally, the number of pixels above the preset threshold was counted and stored. The threshold was selected to include all pixels within identifiable clusters and to exclude fluorescence due to diffusely distributed (extracluster) receptors. At the beginning of each experiment, the gain of the silicon-intensified target camera was set so that the brightest clusters fell well below the upper limit of the image processor.

**AChE**

AChE activity was determined using a modification of the method of Johnsson and Russel (1975) as described by Rotundo and Fambrough (1980a) and Brockman et al. (1982). Either 50 or 100 μl of 0.3 mM $[^{3}H]ACh$ (sp act = 5 Ci/mmol; Amersham Corp., Arlington Heights, IL) in 0.1 M sodium phosphate (pH 7.0) was added to washed cultures kept on ice. After 15-60 min the reaction was stopped by addition of 100 μl of a solution containing 1 M monochloroacetic acid, 2 M NaCl, and 0.5 M NaOH. The mixture was transferred to counting vials, 4 ml of scintillation cocktail (200 μl isomyl alcohol, 900 ml toluene, 4 g 2,5 diphenyloxazole, plus 50 mg p-bis-[5-phenyloxazyl]-benzene/liter), was added and the radioactivity was determined in a liquid scintillation counter (model LS3100; Beckman Instruments Inc., Fullerton, CA). Two 2-ml stirred mixing chambers were connected in series after the 3-ml mixing chamber supplied with the system to minimize baseline noise at high sensitivity. Solutions were prepared with Milli-Q—purified H2O and HPLC grade reagents: acetonitrile and 2-propanol (Burck & Jackson Laboratories Inc., American Hospital Supply Corp., Muskegon, MI); TFA and heptfluorobutyric acid (Pierce Chemical Co., Rockford, IL); phosphoric acid (Fisher Scientific Co., Pittsburgh, PA). All solutions were passed through 0.2 μm nylon filters (Rainin Instrument Co., Inc., Woburn, MA) and degassed before use. Column fractions were collected in siliconized glass tubes or in polypropylene tubes.

**Gel Electrophoresis**

SDS PAGE was performed with a slight modification of the standard Laemmli discontinuous Tris-glycine buffer system (Laemmli, 1979). Dried samples were dissolved in buffer containing 1% SDS, 10 mM Tris (pH 6.8), 10 mM dithiothreitol (DTT), 30% glycerol, and 0.01% bromophenol blue and heated in a boiling water bath for 3 min. Proteins in highly purified fractions were labeled with $[^{125}I]$ in an iodogen-catalyzed reaction (Salicinski et al., 1981) and detected by autoradiography after SDS PAGE. Care was taken to minimize contaminants. Solutions were prepared with Milli-Q—water, and all buffers were passed through Sep-Pak cartridges. Fractions dried in polypropylene microfuge tubes were redissolved in 20 μl of buffer (0.1 M Na phosphate (pH 7.4) 0.1% SDS) and transferred to tubes containing 1 μg of dried iodogen to which 1 μl (100 μCi) $[^{125}]I$Na was added. After 10 min at room temperature, the reaction was stopped by transferring the solutions to tubes containing 10 mM DTT and 10 mM NaF. Partially purified brain extract (10-20 μg) was added as carrier, and the reaction mixture was absorbed on a Sep-Pak cartridge equilibrated in 0.1% TFA. The cartridge was washed with 50 ml of 0.1% TFA, and then eluted with 3 ml 0.1% TFA containing 0.1% 2-propanol. After electrophoresis, the gels were fixed (15% TCA, 3.4% sulfosalicylic acid, and 20% methanol), washed thoroughly, and exposed to Kodak X-AR5 film for 12-72 h at ~90°C using a DuPont Lighting Plus intensifying screen E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT). $[^{3}C]$-Methylated proteins (Amersham Corp. and Searle Medical Products, Dallas, TX) used as molecular weight markers were: phosphorylase (A, Mr 92,000), BSA (Mr 68,000), ovalbumin (Mr 45,000), carbonic anhydrase (M, 31,000), and lysozyme (M, 14,000). In some experiments, purified fractions were labeled by the method of Bolton and Hunter (1973).

In some experiments the activity was eluted from SDS gels. Samples were dissolved in a denaturing solution lacking DTT and heated at 37°C for 15 min. The lane containing the activity was cut into 12 equal segments and each one was placed in a Spectrapor 2 dialysis bag. 2 ml of the dialysis buffer (25 mM Na phosphate (pH 7.0)) was added to each bag and they were dialyzed at 4°C for 24 h against 4 liters of phosphate buffer and then for another 24 h against 4 liters of 10% acetic acid.

**Extractions**

Saline extract was prepared at 4°C as previously described (Jessell et al., 1979). Briefly, adult chick brains were homogenized in 3 vol of Puck's saline G with a Polytron (Brinkman Instruments Co., Westbury, NY). The homogenate was centrifuged at 3,000 rpm (Sorvall SS34 rotor; E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div.) for 10 min and the supernatant was centrifuged again at 15,000 rpm for 30 min in the same rotor. The extract was stored from the ultracentrifuge.

To prepare acid extract 500 frozen chicken brains (1.5 kg wet weight) were crushed in dry ice and delipidated by grinding in acetone (2 ml/g) at

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Results

The Assay

By using high specific activity \(^{125}\text{I}\alpha\)-BTX (800–1,200 cpm/ fmol), we were able to measure the rate of insertion of AChRs in microwell cultures seeded with only 50,000 cells. Because of their small volume, the microwells allowed us to assay small aliquots of many column fractions in quadruplicate. After all exposed receptors were blocked with unlabeled \(\alpha\)-BTX, the number of \(^{125}\text{I}\alpha\)-BTX binding sites increased linearly with time for at least 10 h (Fig. 1). Because the rate of incorporation was constant in both control and extract-treated cultures, we routinely estimated the rate by measuring the number of new receptors at one point, 4 h after the cold toxin block. In many different platings the number of receptors inserted per hour amounted to 3–5% of the surface pool (see Devreotes and Fambrough, 1975). The upper curve illustrates a typical threefold increase in the rate of AChR incorporation produced by a partially purified brain extract (C4 pool, see below).

Brain extract and purified fractions always produced a larger effect (fold increase compared with control) on the rate of receptor insertion, than on the total number of surface receptors. This is the expected result considering that receptor degradation in chick myotubes is a first order process with a half-time of 20–25 h at 37°C. Thus, after the standard 24-h incubation period as many as 50% of the surface receptors were present before ARIA was added. The number of receptors in control cultures varied significantly (up to two-fold) between platings, probably because small differences in the number of myoblasts plated and in the plating efficiency are magnified after 8 d. However, variation in \(^{125}\text{I}\alpha\)-BTX binding between wells within a plating was small (coefficient of variation <10%), so we could detect effects as small as 25%.

Purification

Most of the results described here were obtained from a single extraction of 500 chick brains. However, each of the steps has been repeated at least 10 times and the entire protocol has been replicated three times.

The acid extract was desalted and chromatographed on CM-Sepharose as described in Materials and Methods. The 0.5 M NaCl eluate containing 130 mg protein was brought to pH 3.0 with TFA and pumped onto a semipreparative (1 × 25 cm) Vydac C4 column. ARIA was retained on this hydrophobic support and it appeared as a relatively broad peak when the column was eluted with a gradient of 2-propanol (Fig. 2 A). Attempts to complete the purification by reverse-phase-HPLC alone using a variety of supports and solvents were not successful. However, we found that ARIA could be separated from major contaminating species by cation exchange HPLC. Therefore, after removing the 2-propanol and adjusting the pH to 7.0, the C4 pool was applied to a TSK-SP column equilibrated in 50 mM Na phosphate (pH 7.0) containing 15% acetonitrile. Fig. 2 B shows that ARIA was separated from most of the UV-absorbing material when this column was eluted with a shallow NaCl gradient. The same major peak of activity was observed in other runs in which all fractions up to 1.0 M NaCl were assayed. The small peak of activity in fraction 30 was not observed in other experiments so it was not investigated further. The main ARIA peak eluted at higher NaCl concentration when the pH was lowered to 5.0 but the recovery of activity was reduced significantly.

The active TSK-SP pool was applied to an analytical (0.46 × 25 cm) Vydac C18 column equilibrated with heptafluorobutyric acid (HFBA) and eluted with a 35–50% acetonitrile gradient over 60 min (Fig. 2 C). Although ARIA eluted as a sharp peak that was separated from most of the applied protein, it did not correspond to a discrete UV absorbance peak. The presence of several protein species in the HFBA

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Figure 1. The rate of AChR incorporation in control (open circles) and ARIA-treated (closed circles) myotubes. Surface receptors were blocked by incubating the cells in 10−7 M \(\alpha\)-BTX for 1 h at 37°C. The number of new surface receptors was measured with \(^{125}\text{I}\alpha\)-BTX at the indicated times thereafter. Each point represents one culture well. ARIA was eluted from the semipreparative C4 column (see Fig. 2 legend). Pooled active fractions were added 24 h earlier at 10 μg protein/ml.
Figure 2. Early chromatographic steps in the purification of ARIA. Each panel shows the UV absorbance (solid line), elution gradient (dotted line), and bioassay (hatched bars). Lines above the bars represent SEs (n = 4) and the arrows to the right represent $^{[35]}I$-BTX bound in control, untreated cultures. (A) Semipreparative reverse-phase HPLC. Pooled fractions from the CM-Sephadex column were pumped onto a Vydac C4 (1 x 25-cm) column equilibrated in 0.1% TFA. The flow rate was 2 ml/min, and 5-min fractions were collected. The bioassay used 0.002% of each fraction per 60 µl assay well. Fractions indicated by the horizontal line were pooled. (B) Cation-exchange HPLC. The concentration of 2-propanol in pooled fractions from A was reduced by vacuum centrifugation, and the remaining volume was neutralized by addition of 2 vol 0.05 M Na$_2$HPO$_4$ (pH 7.0) containing 15% acetonitrile. The pool was then loaded onto a TSK-SP column (0.75 x 7.5 cm) (equilibrated with the same buffer) in multiple 1.5-ml injections. The NaCl gradient was run at 0.5 ml/min, and 2-min fractions were collected. 0.04% from each fraction was used per bioassay well. (C) Analytical reverse-phase HPLC in heptfluorobutyric acid. Pooled fractions from B (horizontal line) were adjusted to pH 3.0 with 1.0% TFA and loaded in 1.5-ml injections onto a Vydac C8 column (0.46 x 25 cm) equilibrated in 0.13% HFBA. The acetonitrile gradient was run at 1 ml/min, 1-min fractions were collected, and 0.2% of each fraction was used per assay well.

Figure 3. SDS PAGE of radioiodinated HFBA pool material. HFBA pool was labeled with $^{125}$I in an iodogen-catalyzed reaction, and electrophoresed in a 10-18% gradient polyacrylamide gel. The pool was demonstrated by SDS PAGE after an aliquot was labeled with $^{125}$I. The autoradiogram in Fig. 3 shows five bands ranging in molecular weight from <10,000 to ~40,000. The same result was obtained with HFBA pools from two other extractions.

Considering this range of sizes, aliquots of the HFBA pool were analyzed by gel filtration HPLC. Excellent resolution

Figure 4. Gel filtration HPLC of the HFBA pool (middle trace) and the C$_4$ pool (lower trace). The UV traces are not shown. Each point in the bioassay represents the mean ± SE of four wells. Aliquots (100 µl) of each pool were applied to two TSK-3000 columns connected in series (total dimensions 0.75 x 60 cm) equilibrated in 0.1% TFA plus 30% acetonitrile. The flow rate was 0.5 ml/min and 1-min fractions were collected. Protein standards, BSA (68,000), alpha-chymotrypsinogen (25,000), ribonuclease A (17,600), insulin (7,000), and leutinizing hormone-releasing hormone (1,600) eluted under the same conditions are shown (upper trace).
was achieved when two TSK-3,000 columns were connected in series (0.75 x 60 cm overall dimensions), and equilibrated with 0.1% TFA containing 30% acetonitrile. Although we expected acid-soluble ARIA to be associated with small peptides (Buc-Caron et al., 1983), Fig. 4 (middle trace) shows that ARIA in the HFBA pool eluted between BSA (M, 68,000) and alpha-chymotrypsinogen (M, 25,000). The same result was obtained when material from the C4 pool was applied to the columns (Fig. 4, bottom trace). Whereas it is unlikely that peptides aggregate in the acetonitrile/TFA solvent, the C4 and HFBA pools were also chromatographed in 10% acetic acid and, in some experiments, they were preincubated in 6 M guanidine HCl. In no case did the activity shift to a lower apparent molecular weight. Based on these results, the entire HFBA pool was concentrated from 6 to 0.2 ml and applied to the TSK-3,000 columns in two separate runs. ARIA eluted in the same position as it did in the pilot experiments.

Finally, active TSK-3,000 fractions were diluted with an equal volume of 0.1% TFA and chromatographed on an analytical Vydac C8 column with a shallow acetonitrile gradient (Fig. 5). The ARIA, which eluted at \(~32\%\) acetonitrile, was associated with a small OD_{220} peak. No OD_{220} peaks were observed when the buffer blank (fractions from the preceding TSK-3,000 column that contained no activity or OD_{220} absorbing material) was chromatographed under the same conditions, and monitored at the same high sensitivity (0.1 absorbance units full scale).

Aliquots of C8 fractions 18, 23, 25, and 31 were labeled with ^{125}I in an iodogen-catalyzed reaction and analyzed by SDS PAGE. Fraction 23, one of the most active fractions, contained a single band of slightly lower mobility than the band of fraction 23. No bands were detected in fractions 18 or 30. This is consistent with the flat OD trace, and it also demonstrates that the band detected in fraction 23 is not due to the presence of trace contaminants. Another aliquot from fraction 23 was iodinated with the ^{125}I-Bolton-Hunter reagent. Although this reagent reacts primarily with lysine residues rather than the tyrosine residues labeled by free ^{125}I, a single band at the same position was observed.

OD peak but very little activity, contained a labeled band of slightly lower mobility than the band of fraction 23. No bands were detected in fractions 18 or 30. This is consistent with the flat OD trace, and it also demonstrates that the band detected in fraction 23 is not due to the presence of trace contaminants. Another aliquot from fraction 23 was iodinated with the ^{125}I-Bolton-Hunter reagent. Although this reagent reacts primarily with lysine residues rather than the tyrosine residues labeled by free ^{125}I, a single band at the same position was observed.
The protein content and specific activity of material eluting from the preceding TSK-3,000 gel-filtration step were not determined, so these entries represent net results from both columns.

The net result is a 60,000-fold purification with respect to the acid extract and an apparent recovery of 10% of the activity. Compared with a crude saline extract, the specific activity of ARIA is increased $1.7 \times 10^8$-fold with a recovery of 3%. We estimate that the final C18 pool contains ~400 ng protein. Assuming a molecular weight of 42,000 this represents ~10 pmol.

Characterization of the 42-kD ARIA

As only 10 pmol of the pure polypeptide was obtained from the starting 500 brains, many of the experiments described below were performed with the HFBA pool. Comparing HFBA pool and TFA pool dose-response curves indicates that the 42-kD species represents <10% of the protein in the HFBA pool. On the other hand, the HFBA pool represents a 1,500-fold purification of ARIA present in the initial acid extract. The half-maximal concentration of the HFBA pool

3. Because the earliest curves may have turned down for reasons unrelated to the ARIA, we assume that the maximal effect obtained with purified ARIA represents the maximum for the early curves as well. For example, in Fig. 8 the maximum effect was ~80.5-fold. Therefore the protein concentration that resulted in a 2.75-fold increase in receptor insertion was assumed to be the half-maximal concentration in each case. Despite variation in the amount of toxin bound between platings, half-maximal effects were observed at very similar concentrations of each pool from plating to plating.

Further evidence that the band in fraction 23 was the ARIA was obtained by assaying material eluted by dialysis from SDS-polyacrylamide gel slices. In these experiments, the gels were loaded with material purified through the C4 column (which was obtained in relatively high yield) because we expected the recovery of ARIA to be low. After electrophoresis, activity was recovered from only one slice that corresponded to a molecular weight range of 35,000-45,000 (Fig. 7). The same result was obtained in two other experiments. This dialysate represented ~10% of the activity applied to the gel. Control experiments showed that half of the activity was lost after exposure to SDS and subsequent dialysis without electrophoresis.

**Specific Activity and Recovery**

To estimate recoveries and the degree of purification we determined the dose-response relation of each of the active pools. One unit of activity was defined as the amount of material required for a half-maximal effect when dissolved in 1 ml. Typical dose-response curves are shown in Fig. 8 and the results are summarized in Table I. Because of variation in [²⁵¹]ň-BTX-binding between platings, the data are expressed as fold increase over control. In this assay, saline extract (open squares) and acid extract (open triangles) produced less than the maximal effect produced by purified samples. A submaximal effect was consistently observed with acid extract, and in most cases, higher concentrations produced an even smaller effect. The downturn may be due to an inhibitor present in relatively high concentration at this stage, but it was not investigated further. The C18 pool was the first stage at which inhibition at the highest concentrations did not occur.

The acid extract was half-maximally active at 45 µg/ml. Compared with a saline extract of brain tissue, this represents a 30-fold purification with 30% recovery of activity. Only a 1.5-fold purification was achieved by CM-Sephadex chromatography, but this step was valuable because it removed pigmented material that bound tightly to the top of the resin. The active pool eluted from the semi-preparative C4 column was half-maximally active at ~2 µg/ml. There was an apparent recovery from this column of >100%, which probably reflects the removal of an inhibitor or a toxic substance (see above). Cation-exchange HPLC and reverse-phase HPLC on C18 in HFBA each increased the specific activity ~10-fold, and the pooled fractions produced half-maximal responses at 300 and 30 ng/ml, respectively. The recoveries in each case were excellent. Small aliquots of the active fractions from subsequent steps did not contain sufficient protein to measure colorimetrically so we estimated the protein content from the UV absorbance trace (see Materials and Methods). Taken together, the TSK-3,000 column and the final reverse-phase C18 column produced a 40-fold increase in specific activity with 15% recovery of activity. We did not estimate the protein concentration in TSK-3,000 column fractions, however, because the HFBA-containing buffer blank did not produce a flat OD₂₅₄ trace. Hence we do not have a reliable measure of the specific activity or recovery obtained from this column.

The net result is a 60,000-fold purification with respect to the acid extract and an apparent recovery of 10% of the activity. Compared with a crude saline extract, the specific activity of ARIA is increased $1.7 \times 10^8$-fold with a recovery of 3%. We estimate that the final C₁₈ pool contains ~400 ng protein. Assuming a molecular weight of 42,000 this represents ~10 pmol.

**Table I. Purification and Recovery of AChR-inducing Activity**

| Activity          | Protein | Specific activity |
|-------------------|---------|------------------|
| U mg              | U/mg U/mg |
| Saline extract*   | 24,000  | 24,000           | 1 |
| Acid extract      | 6,571   | 227              | 29 |
| CM-Sephadex       | 5,417   | 130              | 42 |
| C-4 HPLC          | 9,000   | 18               | 500 |
| SP IEC-HPLC       | 5,555   | 1.5              | 3,700 |
| C-18 (HFBA)       | 4,560   | 0.11             | 40,000 |
| C-18 (TFA)†       | 666     | 0.0004           | 1,700,000 |

* Saline extract, prepared from 50 brains and expressed as per 500 brains.
† The protein content and specific activity of material eluting from the preceding TSK-3,000 gel-filtration step were not determined, so these entries represent net results from both columns.
The position of the 42-kD band is shifted after digestion with neuraminidase. $^{125}$I-labeled material from fraction 23 of the final C$_8$ column (A) and from the HFBA pool (B) were exposed to 0.1 U/ml neuraminidase (purified from C. perfringens and obtained from Sigma Chemical Co., St. Louis, MO) in 0.1 M Na acetate (pH 5.5) for 2 h at 37°C. Left lanes, buffer alone. Middle lanes, buffer plus enzymes. Right lanes, buffer plus enzyme plus 20 mM 2,3 dehydro-2-desoxy-N-acetylneuraminic acid (Bohringer Mannheim Diagnostics, Inc., Houston, TX). The reaction was terminated by boiling in SDS-denaturing solution and the samples were applied to SDS-10% polyacrylamide gels.

was 30 ng/ml: in most experiments it was used at 300 ng/ml.

42-kD ARIA is a stable Glycoprotein. We routinely measured the rate of AChR incorporation after the cells were exposed to test fractions for 24 h. To determine if activity was lost during this 24-h period in contact with intact myotubes, medium with and without ARIA (HFBA pool) was removed from one set of microcultures after 24 h and transferred to another set. The rate of insertion of receptors measured 24 h later in cultures exposed to transferred ARIA was as high as that in cultures exposed to fresh HFBA/ARIA added and assayed at the same times and greater than control cultures receiving only conditioned medium after 24 h and transferred to another set. The rate of insertion of receptors measured 24 h later in cultures exposed to transferred ARIA was as high as that in cultures exposed to fresh HFBA/ARIA added and assayed at the same times and greater than control cultures receiving only conditioned medium (transferred = 2,231 cpm ± 469; fresh = 2,578 cpm ± 307; control = 1,026 cpm ± 268: mean ± SE, n = 4).

Activity was not destroyed after extraction in strong acid, chromatography in organic solvents, and storage for several months at -20°C. This unusual stability may be conferred by the presence of disulfide bonds or by glycosylation. In fact, partially purified ARIA (C$_4$ pool) was destroyed after sequential reduction with DTT and carboxymethylation with iodoacetic acid in the presence of guanidine HCl (control = 1,245 cpm ± 76; C$_4$ pool = 3,080 cpm ± 68; reduced and carboxymethylated C$_4$ pool = 1,513 cpm ± 175). Treatment of the $^{125}$I-labeled 42-kD polypeptide in the C$_8$ fraction 23 with neuraminidase caused a shift in its mobility on SDS PAGE (Fig. 9 A). This shift was blocked by the sialic acid analogue 2,3 dehydro-2-desoxy-N-acetylneuraminic acid (Meindl and Tuppy, 1969), so it is unlikely to be due to a contaminating protease. A comparable shift in the 42-kD band was observed when radioiodinated HFBA pool material was digested with neuraminidase (Fig. 9 B). That the migration of no other band in this pool was altered provides further evidence against nonspecific proteolysis. Digestion of the ARIA with endoglycosidase F (Elder and Alexander, 1982) caused a similar shift in mobility. In two experiments neuraminidase-digested HFBA-pool ARIA was not decreased when tested at maximal and submaximal concentrations. The ARIA was completely destroyed after incubation with trypsin.

42-kD ARIA does Not Affect Total Protein Synthesis. In the experiment illustrated in Fig. 10, HFBA pool material was added to cells for 48 h, and the total number of $[^{3}H]$a-BTX binding sites and the amount of $[^{3}H]$leucine incorporated into TCA-precipitable material were then assayed. There was a dose-dependent increase in the number of surface AChRs that reached 1.6-fold at 150 ng/ml, but there was no significant increase in $[^{3}H]$leucine incorporation even at the highest ARIA concentration. Because the $t_1/2$ for degradation is ~24 h under our culture conditions, most of the AChRs exposed on the cell surface were synthesized during the 48-h incubation with the HFBA pool.

The experiment summarized in Table II shows that crude saline brain extract did produce a significant effect on the rate of protein synthesis and on the accumulation of total protein. Material that promotes overall protein synthesis was lost early in the purification. After the second chromatographic procedure (C$_4$ pool), ARIA was purified ~500-fold relative to saline extract, and no effect on $[^{3}H]$leucine incorporation could be detected at concentrations that significantly increased the rate of insertion and the total number of AChRs.

42-kD ARIA Acts on Inactive Myotubes. Electrical ac-

Figure 9. The position of the 42-kD band is shifted after digestion with neuraminidase. $^{125}$I-labeled material from fraction 23 of the final C$_8$ column (A) and from the HFBA pool (B) were exposed to 0.1 U/ml neuraminidase (purified from C. perfringens and obtained from Sigma Chemical Co., St. Louis, MO) in 0.1 M Na acetate (pH 5.5) for 2 h at 37°C. Left lanes, buffer alone. Middle lanes, buffer plus enzymes. Right lanes, buffer plus enzyme plus 20 mM 2,3 dehydro-2-desoxy-N-acetylneuraminic acid (Bohringer Mannheim Diagnostics, Inc., Houston, TX). The reaction was terminated by boiling in SDS-denaturing solution and the samples were applied to SDS-10% polyacrylamide gels.

Figure 10. 42-kD ARIA does not increase protein synthesis. Serial dilutions of the HFBA pool were added to 7-d-old cultures along with $[^{3}H]$leucine (1 μCi/ml). After 48 h the amount of $[^{3}H]$leucine incorporated into material precipitated by cold TCA (15%) was determined. The total number of surface AChRs was measured with $[^{125}]$a-BTX in sister cultures at the same time.

4. All samples were dissolved in 25 mM Na phosphate (pH 7.0) plus 6 M guanidine HCl and incubated at 37°C. Some samples were reduced with 20 mM DTT for 30 min and alkylated by exposure to 30 mM iodoacetic acid for 30 min.
Table II. Effect of 24-h Incubation with Saline Extract or C-4 Pool on AChR and Protein Accumulation

|                      | [\(^{125}\)I]to-BTX binding | [\(^3\)H]Leucine Incorporation |
|----------------------|-----------------------------|---------------------------------|
|                      | Rate*                       | Total†                         | Rate§†                       | Total‡†                       |
| Control              | 172 ± 22                    | 1,278 ± 134                    | 4,329 ± 513                   | 9,050 ± 809                   |
| Saline extract (3.3 mg/ml) | 473 ± 60                    | 2,136 ± 112                    | 7,054 ± 419                   | 15,446 ± 956                   |
| C, pool (5 μg/ml)    | 419 ± 56                    | 1,894 ± 121                    | 4,651 ± 413                   | 9,085 ± 690                   |

All entries are mean cpm ± SE; n = 4.
* [\(^{125}\)I]to-BTX binding 4 h after block with cold α-BTX.
† [\(^{125}\)I]to-BTX binding without preceding cold toxin block.
§ [\(^3\)H]Leucine incorporation during final 2 h of incubation (5 μCi/ml added).
‡ [\(^3\)H]Leucine incorporation during 24-h incubation (1 μCi/ml added).

Table III. Steady-state and Newly Synthesized Acetylcholinesterase Activity after 48 h of Incubation with Brain Extract or HFBA-pool

|                      | Cellular* | Surface‡ | Secreted§ |
|----------------------|-----------|----------|-----------|
|                      | Total     | per 3 h  | Total     | per 11 h  | Total     | per 11 h  |
| Control              | 383 ± 27  | 166 ± 40 | 121 ± 6.7 | 63.4 ± 4.7 | 2,106 ± 136| 412 ± 48  |
| Saline extract (3.3 mg/ml) | 467 ± 21† | 219 ± 15‡ | 116 ± 11 | 68 ± 12   | 2,140 ± 212| 596 ± 40‡ |
| HFBA-Pool (300 ng/ml) | 371 ± 27  | 160 ± 19 | 123 ± 21 | 63 ± 11   | 1,804 ± 38 | 360 ± 76  |

Entries = mean cpm hydrolyzed/min ± SE; n = 4.
* Measured with 0.5% Triton X-100 present.
† Measured without detergent.
‡ Measured in medium removed from cells.
§ 3 h after block with DFP.
‖ 11 h after block with echothiophate.
★★ 11 h after addition of fresh medium.
** Significantly (P < 0.05) different from control.

Acetylcholine receptors are an important determinant of AChR number in chick myotube cultures (Cohen and Fischbach, 1973) as it is in vivo (Lomo and Rosenthal, 1972; Linden and Fambrough, 1979; Hall and Reiness; 1977). Tetrodotoxin (TTX), which paralyzes muscle by blocking Na-dependent action potentials, produced an increase in the rate of AChR incorporation of the same order as that produced by the ARIA. However, the effect of HFBA pool ARIA and TTX were additive, even when TTX was added at saturating conditions (Fig. II). This suggests that the effect of ARIA is not mediated by muscle paralysis.

42-kD ARIA Does Not Affect AChE Activity. We found no effect of HFBA pool ARIA on intracellular, surface membrane, or secreted AChE after incubations for 24 or 48 h. One experiment is summarized in Table III. The relative amounts of AChE in the three pools were comparable to values reported by Rotondo and Fambrough (1980a,b). Saline brain extract increased cellular AChE by ~20% but did not affect the amount of surface or secreted activity. Small increases in intracellular AChE were observed in two other experiments.

If the pools of AChE are relatively large and their rates of turnover are low (cf. Rotondo and Fambrough, 1980b), a significant increase in the rate of enzyme synthesis or appearance on the cell surface might have been missed. Therefore, we estimated the rate of AChE synthesis by measuring the reappearance of activity 3 h after blocking all active sites with diisoproprylfluorophosphate. The rate of AChE appearance on the cell surface was estimated by measuring the surface activity 11 h after exposed sites were blocked with echothiophate, a nonpermeable organophosphate. No effect of the HFBA pool was evident on the rate of AChE synthesis, incorporation, or secretion (Table III). Brain extract did increase the rate of synthesis and secretion of the enzyme, but it produced no change in the rate of appearance of active sites on the extracellular surface.
Figure 12. Clusters of AChRs labeled with rhodamine-α-BTX in untreated myotubes grown in the presence (a and b) or absence (c and d) of fibroblasts. Note that the clusters range in size from 0.5-2 μm speckles to large patches ~10 μm across. a and c, phase-contrast; b and d, fluorescence illumination; N₂ barrier filter. Bar, 50 μm.

42-kD ARIA Promotes the Formation of AChR Clusters.
In this series of experiments the cells were plated on polystyrene coverslips, and some cultures were treated with cytosine arabinoside to reduce the number of fibroblasts. When receptors were labeled with rhodamine-derived α-BTX, bright patches of fluorescence revealed the position of AChR clusters (Fig. 12). Patches were present on the top, bottom, and edges of striated and unstriated myotubes, and they varied in size from small speckles that appeared to be 0.12-0.5 μm in diameter to large aggregates 10-20 μm across. Some of the large patches appeared to be made up of many speckles packed closely together. We noted no obvious difference between myotubes in cytosine arabinoside-treated and untreated cultures in regard to the size or distribution of receptor clusters or the effect of ARIA (see below).

To measure the area occupied by clustered receptors we analyzed digitized images of the Rh-BTX-labeled myotubes. Myotubes were selected under phase-contrast illumination (Fig. 13, top). An image of the same field viewed under rhodamine-selective illumination was then digitized (Fig. 13, middle), and all pixels above a preset threshold intensity were counted. The initial choice of the threshold was subjective: a high value was selected to minimize the contribution of dim clusters and diffusely distributed receptors. The adequacy of the threshold in selecting obvious clusters of all sizes was checked by displaying pixels above the chosen value (Fig. 13, bottom). Once determined, the same threshold was used for all myotubes within a given experiment. This technique insures that contributions of both large and small “hotspots” are appropriately weighted and that exactly the same criteria are used for all myotubes within an experiment.

Data from an experiment in which myotubes were exposed to crude saline extract or HFBA pool material for 24 h is shown in Table IV. The HFBA pool caused a threefold increase in the average area (number of pixels) occupied by

Table IV. The Area of AChR Clusters on Myotubes Treated with Saline Brain Extract or Purified AChR-inducing Activity

|                | Experiment 1 | Experiment 2 |
|----------------|--------------|--------------|
| No. fibers     | Mean cluster area* |
| Control        | 50           | 36           |
| Saline extract | 45           | 24           |
| (3.3 mg/ml)    | 26 ± 5.9     | 246 ± 76     |
| HFBA pool      | 58           | 59           |
| (300 ng/ml)    | 43 ± 7.2     | 130 ± 39     |

Data are expressed as square micrometers ± SE (1 pixel = 0.067 μm²).
* Mean cluster area is the average area per fiber length (~200 μm) occupied by AChR aggregates.
clustered receptors. In this experiment, saline extract caused a twofold increase in hotspot area. To be sure that the 42-kD species is responsible for the increase in aggregate area, the effect of the C₈ pool was also evaluated. The completely purified activity produced a nearly threefold increase in the area occupied by AChR aggregates (Table IV).

In some experiments an effect on AChR cluster area was immediately evident and could be documented by simply counting the number of large clusters. Here again, healthy myotubes were selected under phase-contrast illumination. Only those fluorescent patches >5 μm across were counted. In one experiment including 111 myotube segments there was a twofold increase in the number of clusters per segment in cultures incubated with 0.4 ng/ml of the completely purified activity (control = 0.9 ± 0.1, n = 78 vs. treated = 1.8 ± 0.2, n = 33).

**Discussion**

We have purified a polypeptide of ~42,000 D that can increase the rate of insertion of AChR into the membrane of cultured chick myotubes two- to sixfold at a concentration on the order of 0.5 ng/ml. It is an extremely stable molecule that is not irreversibly denatured at pH 3, or by exposure to SDS, organic solvents, or intact myotubes. Because it migrates more rapidly in polyacrylamide gels after digestion with neuraminidase, we assume that it is a sialoglycoprotein. Sialic acid residues are apparently not, however, necessary for biological activity.

We recovered <0.5 μg in the final active fractions, so it is difficult to be certain that the ARIA has been purified to homogeneity. The following arguments suggest that it has. Activity was associated with a single, nearly symmetric, OD₂₅₀ absorbance peak on the final reverse-phase C₁₈ column. The peak was broad, but this is not unexpected for a glycoprotein eluted with a shallow acetonitrile gradient (0.28%/min). More significantly, only one band was detected by autoradiography after SDS PAGE of the most active C₁₈ fraction. Activity was eluted from slices of other gels that correspond to this band so it undoubtedly contains an ARIA. The band is centered at 42 kD, but it is relatively wide. Whereas this is also consistent with the presence of carbohydrate residues, the possibility that the band contains more than one polypeptide must be considered. However, that the entire band was shifted after digestion with neuraminidase argues against the presence of more than one species. It is unlikely that polypeptides of different molecular weight were present in the final C₁₈ fraction, but were not detected by PAGE. If this were the case, either they cannot be iodinated on tyrosine or lysine residues or they represent <10% of the 42-kD band. The final C₁₈ pool produced a clear effect at <1 ng/ml so it is difficult to imagine that ARIA is associated with minor contaminants at this stage.

A few comments about the purification protocol are warranted. The TFA-containing extraction cocktail was adopted with a small peptide in mind (see below), and in retrospect it may seem inappropriate for extraction of a 42-kD protein. However, the 42-kD ARIA is extremely stable at low pH and

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**Figure 13.** The method used to assay the area occupied by AChR clusters. All photographs are of digitized images displayed on a television monitor. The cells were labeled with R-BTX, fixed, and mounted in glycerol. (Top) A myotube was selected under phase contrast illumination. (Middle) The same field viewed with rhodamine-selective fluorescence optics. The histogram displays the fluorescence intensity of individual pixels included within the outlined area. (Abscissa = intensity; ordinate = log number of pixels.) Only those pixels above threshold (long vertical bar) were counted. (Bottom) A display of all selected (suprathreshold) pixels. Note that small (arrow) as well as large (arrowhead) clusters were included. Bar, 15 μm.
The 42-kD ARIA was purified on the basis of its ability to increase the rate of AChR incorporation, but we found that it increased the size and number of receptor clusters as well. These effects may be related in that some clusters may represent sites of preferential receptor insertion. In addition, since diffusely distributed receptors can enter established clusters (Stya and Axelrod, 1983), a small increase in receptor number might lead to a significant increase in cluster size. On the other hand, the two effects might be independent. The 42-kD ARIA might increase the mobility of receptors or promote their "trapping." More data are needed to determine the precise topography of receptor insertion and the rate of receptor migration in treated cells.

Other polypeptides that increase the number and/or alter the distribution of AChRs in chick myotubes have been partially or completely purified. While none, including the 42-kD ARIA described here, have been characterized in great detail, a few comparisons can be made. Sciatin, an 84,000-D protein extracted from sciatic nerves, was purified on the basis of its ability to promote the proliferation of mononucleated muscle precursor cells and the survival of multinucleated myotubes (Markelonis and Oh, 1979; Markelonis et al., 1980a). When added to muscle cultures at the time of plating, it produces a large and sustained increase in protein synthesis (Markelonis et al., 1980b). As expected, sciatin does increase the number of AChRs, but after 4 d there was only a small increase in the specific activity of [3H]α-BTX binding sites and after 7 d there was none at all (Markelonis et al., 1982a). We did not detect an effect of the HFBA pool on [3H]leucine incorporation at concentrations that increased receptor insertion severalfold. Therefore, the mechanism of sciatin's effect appears to be quite different from the selective, rapid effect of 42-kD ARIA. Recent studies have shown that sciatin is the crucial myotrophic protein in chick embryo extract, and that it is virtually identical to transferrin (Li et al., 1982; Markelonis et al., 1982b). Ovotransferrin is a component of our control medium (at 40 μg/ml), so the 42-kD ARIA effect is superimposed on this general trophic support.

A polypeptide extracted in high salt from basement membrane–enriched fractions of Torpedo electric organ promotes the aggregation of chick myotube AChRs (Nitkin et al., 1983; Godfrey et al., 1984). Monoclonal antibodies that block the effect of the partially purified Torpedo factor label an 80-kD band on nitrocellulose blots of SDS gels (Nitkin et al., 1983; Fallon et al., 1984). This polypeptide may be responsible for the remarkable ability of the persistent synaptic basal lamina to induce the accumulation of AChRs on regenerating muscle fibers (Burden et al., 1979) because the same antibodies label mature Torpedo neuromuscular junctions (Fallon et al., 1984). Although the time of appearance of basal lamina at developing synapses is uncertain, the Torpedo factor must be considered a strong candidate for an ARIA at developing junctions. The Torpedo factor is larger than the chick brain ARIA, and it apparently does not increase the total number of receptors, but it is premature to conclude that they are entirely unrelated.

Low molecular weight ARIAs have also been described. Earlier reports from this laboratory described the induction of AChRs by trypsin-sensitive molecules that were included on Biogel P-4 and P-2 and on Sephadex G-25 columns (JesSELL et al., 1979; Fischbach and Jessell, 1980; Buc-Caron et
al., 1983). We did not detect peptide ARIAs in the current experiments but our protocol is not identical to those used previously, and one or more of the changes might account for the apparent discrepancy. For example, small peptides might have been lost when the frozen brains were delipidated with acetone. In addition, if peptide ARIAs represented <25% of the total activity, they might not have been detected at early stages because of the inhibition of AChR incorporation observed at high concentrations of relatively impure material. In later steps, only the most active fractions were pooled: discarded “side” fractions may have contained peptide ARIAs. It is also possible that the active peptides observed previously were proteolytic fragments of the 42-kD ARIA. Four protease inhibitors were included in the current extraction cocktail whereas only two were used in the earlier studies. Finally, it is possible that the activity may have appeared small in the earlier work because it absorbed to the Biogel and Sephadex matrices employed even though the running buffers contained 10% acetic acid.

Low molecular weight material increases the number of receptors on L5 rat myotubes and the responsible agent is apparently ascorbic acid (Knaak and Podleski, 1985). It is extremely unlikely that ascorbic acid contributed to the ARIA described here. This compound would probably not survive the extraction and purification protocol or remain active after prolonged storage without reducing agents. Moreover we did not detect an effect on the rate of receptor insertion when ascorbic acid was added to chick myotubes over a concentration range from 1 ng/ml to 5 mg/ml.

Factors extracted from rat brain (Podleski et al., 1978; Salpeter et al., 1982) and neuron-conditioned medium (Christian et al., 1978; Bauer et al., 1981; Schaffner and Daniels, 1982) increase the number of AChR aggregates on primary rat and mouse myotubes. These factors appear to be much larger than the ARIA described here but the data do not permit more detailed comparison.

We do not mean to imply that the action of the 42-kD ARIA is limited to AChRs. Other proteins are concentrated at or restricted to the synaptic cleft (Sanes and Chiu, 1983), the postsynaptic membrane (Beam et al., 1985), or the subjacent myoplasm (Froehner et al., 1981; Bloch and Hall, 1983). It is attractive to think that the same neural factor regulates the synthesis or accumulation of several synapse-specific molecules.

AChE is of particular interest because it accumulates at embryonic endplates in vivo and in vitro soon after AChRs, and it is synthesized in embryonic myotubes at about the same rate as AChRs (Rotondo and Fambrough, 1980a,b). Moreover, both molecules are transported to the cell surface in coated vesicles (Bursztajn and Fischbach, 1984; Benson et al., 1985). Although we found no effect of HFBA pool ARIA on AChE synthesis ( reappearance of activity after dioxopropylfluorophosphate blockade), the conclusion that the 42-kD ARIA has no effect on AChE is not warranted. The asymmetric, collagen-tailed form of AChE that is concentrated at endplates in several species makes up only 1–2% of the total AChE activity in chick muscle cultures. A significant effect of the 42-kD ARIA on the accumulation of this species would not have been detected. Finally, ARIA may be required but not sufficient in regard to AChE. Crude saline brain extract does increase the synthesis and secretion of the enzyme (Table III; Jessell et al., 1979), and a crucial cofactor may have been lost during the purification. It may also be significant in this regard that the appearance of AChE at newly formed junctions in chick cultures is influenced by the level of activity of the innervated myotubes (Rubin et al., 1980; Siegel, 1981). It will be important to determine if 42-kD ARIA adds to this effect. Finally, it is possible that ARIA promotes the aggregation of AChE in the absence of a significant effect on the number of enzyme molecules. The 80-kD, Torpedo ARIA does increase the number of AChE patches (Wallace et al., 1985).

In sum, we have completely purified a polypeptide that can selectively stimulate the incorporation of nicotinic AChRs into chick myotube membranes and can promote the formation of receptor clusters. Both phenomena occur during de novo nerve–muscle synapse formation in chick cultures (Role et al., 1985), but it remains to be shown that the 42-kD polypeptide is the necessary signal. This will undoubtedly require preparation of specific blocking antibodies, and/or partial amino acid sequence information. We estimate that ~400 ng (10 pmol) of the 42,000 species were recovered from the final C18 column. Although this estimate is subject to some uncertainty, it is clear that accurate amino acid sequence analysis will require more material. We must alter the extraction procedure, modify one or more of the chromatography steps, or scale up the existing protocol.

We have focused on the neuromuscular junction, but considering that the polypeptide was isolated from brain extracts, it is worth speculating that it might also affect the number or distribution of AChRs on neurons in the central and peripheral nervous system. It might modulate other types of receptors as well.

We are grateful to Ms. Nancy Lancaster for technical assistance in the biochemical procedures and to Ms. Rebecca Dill-Dorov for maintaining the muscle cultures and the tissue culture facility. Joseph Leykam offered valuable suggestions concerning HPLC columns.

This work was supported by grants to Gerald D. Fischbach from the United States Public Health Service (NS No. 18458) and the Muscular Dystrophy Association. Ted Usdin was supported by MSTP grant No. 5T32GM07200.

Received for publication 24 February 1986, and in revised form 17 April 1986.

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