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)[1] 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 inotophoretically applied acetylcholine (ACh), and they bound more [^{125}I]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.,

1. *Abbreviations used in this paper*: α-BTX, α-bungarotoxin; ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptors; ARIA, acetylcholine receptor-inducing activities; BSS, Hank's balanced salt solution; DTT, dithiothreitol; HFBA, heptafluorobutyric acid; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid; TTX, tetrodotoxin.

1985; Nelson et al., 1976; Schubert et al., 1977; Nurse and O'Lague, 1975), so the possibility that ACh is the inducing agent must be considered. However, synaptic clusters form in the presence of saturating concentrations of competitive receptor antagonists (Cohen, 1972; Anderson and Cohen, 1977; Rubin et al., 1980), so the induction cannot be a direct consequence of ACh binding to the nicotinic receptor or the subsequent ionic currents that underlie synaptic transmission and muscle activity.

Several investigators have, therefore, begun to search for receptor-inducing factors in tissue extracts (Jessell et al., 1979; Podleski et al., 1978; Markelonis et al., 1982a; Buc-Caron et al., 1983; Nitkin et al., 1983; Godfrey et al., 1984) or nerve cell–conditioned medium (Christian et al., 1978; Schaffner and Daniels, 1982; Bauer et al., 1981). The general strategy has been to add test fractions to uninnervated myotubes in culture and then, after an interval, to measure the number of receptors and/or the number of receptor clusters. As noted above, both phenomena probably contribute to the formation of synaptic clusters. A variety of sources have 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 (ARIAs).

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 AChR 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 extracts 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 ovotransferrin (40 μg/ml). As reported by others (Ii et al., 1982; Oh and Markelonis, 1982), we found that ovotransferrin 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 redissolved 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 [^{125}I]α-BTX for 1 h at 37°C. The cells were washed twice by immersing the plates in 1 liter Ca^{++}-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 [^{125}I]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 [^{125}I]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 (10^{-7} M 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 [^{125}I]α-BTX at various intervals thereafter.

α-BTX was iodinated by the chloramine-T-catalyzed reaction (Hunter and Greenwood, 1962), and monoiodinated 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 monoiodinated

toxin, estimated by competition with known concentrations of unlabeled α-BTX, ranged between 800 and 1,200 cpm/fmol in different preparations.

AChR Clusters

To facilitate microscopic examination of AChR clusters, myoblasts were plated on collagen-coated polystyrene coverslips cut out to fit in the 96-well plates. In some experiments, the cells were treated with 10^{-5} M cytosine arabinoside for 48 h between the third and fifth day after plating.

Receptor clusters were visualized with rhodamine-derivatized α-BTX prepared by the method of Ravdin and Axelrod (1977). Myotubes were exposed to a 1:100 dilution (in complete medium) of rhodamine-derived α-BTX for 60 min at 37°C, conditions which, judging from inhibition of [^{125}I]α-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 63× objective (1.4 NA Plan Neofluor). 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/270; Comtrol-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 11/44 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 Johnson and Russel (1975) as described by Rotundo and Fambrough (1980*a*) 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 monocholoroacetic acid, 2 M NaCl, and 0.5 M NaOH. The mixture was transferred to counting vials, 4 ml of scintillation cocktail (100 ml isoamyl alcohol, 900 ml toluene, 4 g 2,5 diphenyloxazole, plus 50 mg p-bis(2-[5-phenyloxazdyl])-benzene/liter), was added and the radioactivity was determined in a liquid scintillation counter (model LS3100; Beckman Instruments, Inc., Fullerton, CA). Less than 10% of the esterase activity was inhibited by 10^{-5} M tetraisopropylpyrophosphoramide (Augustinnsson, 1963), suggesting that nearly all of the hydrolysis was due to AChE.

Surface esterase activity was determined by adding the substrate to washed intact cells. To determine total cellular AChE activity, Triton X-100 (0.5%) was added along with the substrate (Rotundo and Fambrough, 1980*a*). Secreted esterase was collected in medium that was treated with 10^{-7} M diisopropylfluorophosphate for 3 h at 37°C at least 5 d before use. In some experiments the rate of reappearance of AChE activity was measured after irreversibly inactivating the enzyme with 10^{-4} M diisopropylfluorophosphate or 10^{-6} M echothiophate (4°C, 15 min), a quaternary ammonium derivative that does not cross the surface membrane (Rotundo and Fambrough, 1980a; Brockman et al., 1982).

Protein Determination

At early stages of the purification, protein was measured by Peterson's modification (Peterson, 1977) of the Lowry method. At later stages when the total mass was small, protein was estimated from the ultraviolet (UV) absorbance trace. The chromatograph, monitored at 210 nm, was photographically enlarged and the area under the peak of interest was integrated with a digitizing tablet. Known amounts of lysozyme and BSA were chromatographed under the same conditions and the areas under the OD traces used to construct a standard curve. As little as 5 ng of protein could be detected in this manner.

The effect of brain extracts on muscle protein was estimated by measuring the incorporation of [^{3}H]leucine into TCA-precipitable material. After a 2-h pulse or after a 24–48-h incubation, the cells were washed (2 liters BSS-BSA), extracted twice for 5 min each time with ice cold 15% TCA (100 μl/well), and solubilized with 150 μl DOC/NaOH. After addition of 100 μl 3 N HCl and scintillation cocktail (RPMI 3a70), radioactivity was determined by liquid scintillation spectrometry.

Chromatography

The HPLC system consisted of two Altex model 110a pumps, a model 421 controller, and a Hitachi model 100–40 variable wavelength spectrophotometer (Beckman Instruments Inc., Altex Scientific Operations, Berkeley, 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 H_2O and HPLC grade reagents: acetonitrile and 2-propanol (Burdick & Jackson Laboratories Inc., American Hospital Supply Corp., Muskegon, MI); TFA and heptafluorobutyric 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, 1970). 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 NaI. 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 40% 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). ^{14}C-Methylated proteins (Amersham Corp. and Searle Medical Products, Dallas, TX) used as molecular weight markers were: phosphorylase (M_r 92,000), BSA (M_r 68,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), and lysozyme (M_r 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 Pucks' 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 at −90°C.

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

−20°C in a blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT). The slurry was collected on filter paper (No. 54; Whatman Chemical Separation Inc., Clifton, NJ), washed with diethylether (−20°C), and stored at −90°C. Subsequent steps in the extraction were performed at 4°C. The material was divided into thirds for ease of handling and homogenized in the blender in a total of 6 liters of extraction cocktail containing: 2% TFA, 1 N HCl, 5% formic acid, 0.1 M NaCl, 0.01% thiodiglycol, 1 μg/ml each of pepstatin, leupeptin, and phenylmethylsulfonyl fluoride, and 10 mM EDTA. After centrifugation at 6,000 rpm for 60 min in a GSA rotor (E.I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div.) the supernatant was filtered through Whatman No. 54 filter paper. The 6 liters of extract was poured in three equal batches through 100 ml of octadecylsilylsilica resin (C_{18}) (removed from a Prep-Pak column, [Waters Associates, Millipore Corp.] and packed in a Buchner funnel) equilibrated in 0.1% TFA. Each batch was washed with 500 ml of 0.1% TFA, and material bound to the resin was eluted with 150 ml of 2-propanol (40%) plus 0.1% TFA (60%). The three batches were pooled, organic solvent was removed by rotary evaporation, and the extract was brought to pH 7.0 with 0.1 N NaOH. A precipitate that formed on neutralization was removed by centrifugation.

The neutralized extract was applied to a CM-Sephadex column (C-25 resin; 5 × 6.5 cm) equilibrated in 50 mM Na phosphate (pH 7.0), washed with 500 ml of the same buffer, and eluted with 500 ml of 0.5 M NaCl in the same buffer. The eluate contained 130 mg of protein. No activity appeared in flow through fractions.

Results

The Assay

By using high specific activity [^{125}I]α-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 α-BTX, the number of [^{125}I]α-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 (C_4 pool, see below).

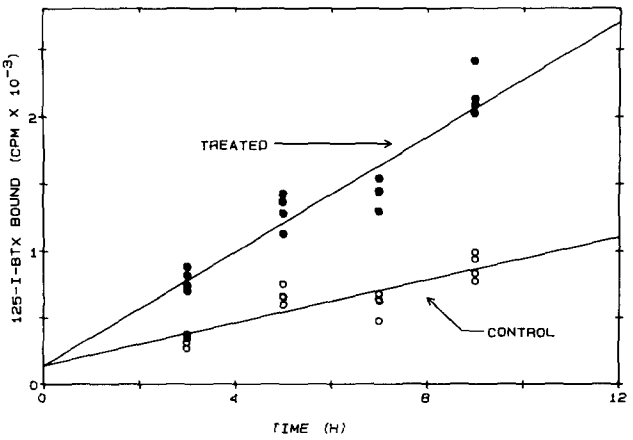

Figure 1. The rate of AChR incorporation in control (open circles) and ARIA-treated (closed circles) myotubes. Surface receptors were blocked by incubating the cells in 10^{-7} M α-BTX for 1 h at 37°C. The number of new surface receptors was measured with [^{125}I]α-BTX at the indicated times thereafter. Each point represents one culture well. ARIA was eluted from the semipreparative C_4 column (see Fig. 2 legend). Pooled active fractions were added 24 h earlier at 10 μg protein/ml.

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 twofold) 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}I]α-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-Sephadex 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 C_4 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 C_4 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 C_{18} column equilibrated with heptafluorobytyric acid (HFBA)[2] 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

2. The retention times of many peptides are increased and shifted with respect to one another in HFBA compared with TFA (Bennett et al., 1981, 1982) that was used with the preceding C_4 column and with a subsequent C_{18} reverse phase column. HFBA was used early rather than in a later stage of the purification because it exhibits a higher OD at 210 nm than does TFA.

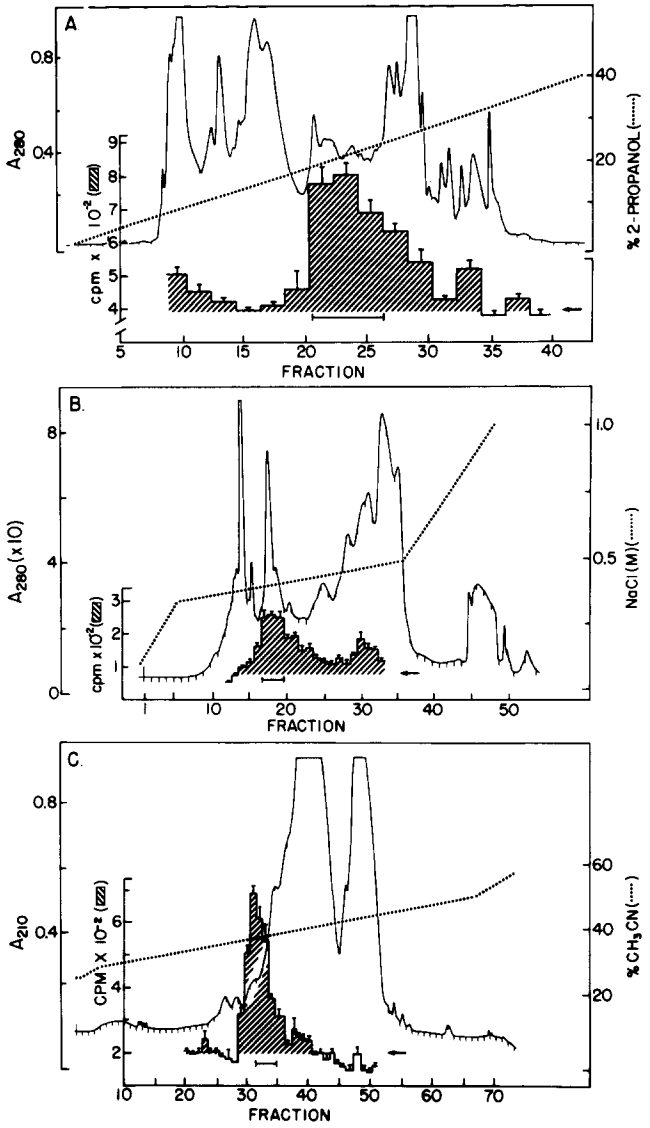

Figure 2. Early chromatographic steps in the purification of ARIA. Each panel shows the UV absorbance (solid line), elution gradient (dotted line), and bioassay (hatched bars). Lines above the bars represent SEs (n = 4) and the arrows to the right represent [^{125}I]α-BTX bound in control, untreated cultures. (*A*) Semipreparative reverse-phase HPLC. Pooled fractions from the CM-Sephadex column were pumped onto a Vydac C_4 (1 × 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-propranol 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_2HPO_4 (pH 7.0) containing 15% acetonitrile. The pool was then loaded onto a TSK-SP column (0.75 × 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 heptafluorobutyric 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 C_{18} column (0.46 × 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.

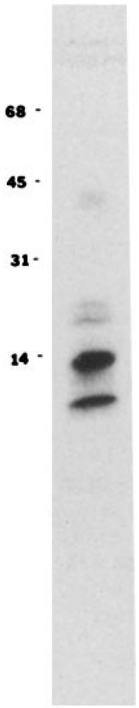

Figure 3. SDS PAGE of radioiodinated HFBA pool material. HFBA pool was labeled with ^{125}I in an iodogen-catalyzed reaction, and electrophoresed in a 10–18% gradient polyacrylamide gel.

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

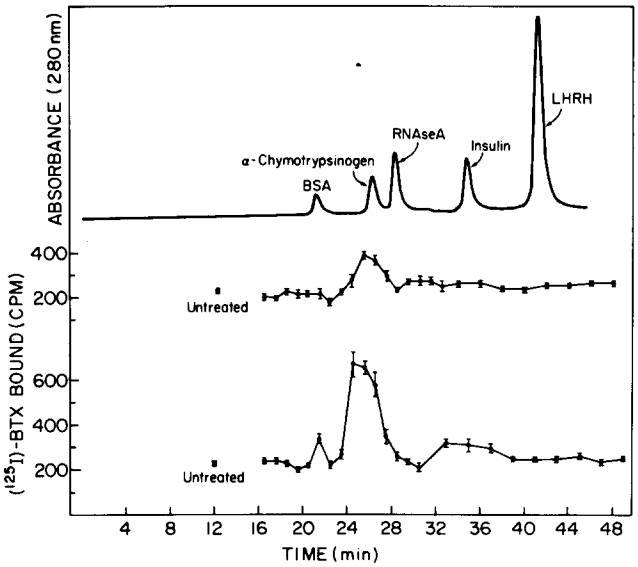

Figure 4. Gel filtration HPLC of the HFBA pool (*middle* trace) and the C_4 pool (*lower* trace). The UV traces are not shown. Each point in the bioassay represents the mean ± SE of four wells. Aliquots (100 µl) of each pool were applied to two TSK-3000 columns connected in series (total dimensions 0.75 × 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).

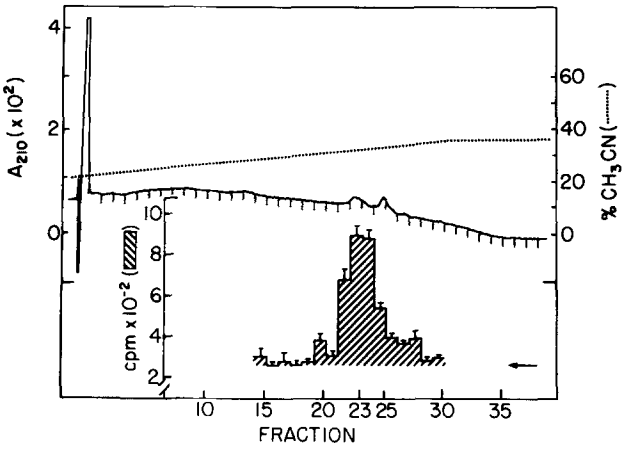

Figure 5. Reverse-phase HPLC on C_{18} in TFA. Active fractions from the TSK-3,000 columns representing half the 500 brain preparation (see text) were diluted with an equal volume of 0.1% TFA and applied to a Vydac C_{18} column (0.46 × 25 cm). Initial conditions were 0.1% TFA plus 18% acetonitrile. The column was operated at 1 ml/min and monitored at 210 nm, 0.1 absorbance unit full scale. 2-min fractions were collected and 0.4% per assay well was used for the bioassay. Similar results were obtained with the second half of the preparation.

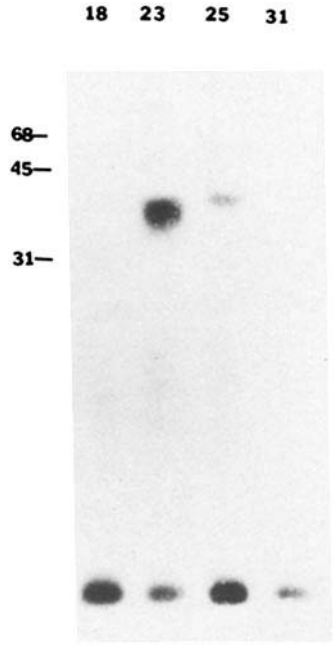

Figure 6. SDS PAGE of selected fractions from the C_{18} column shown in Fig. 5. Aliquots of the indicated fractions were radioiodinated in an iodogen-catalyzed reaction, and applied to a 12% polyacrylamide gel. A significant amount of the applied radioactivity ran at the dye front in this and all experiments where small quantities of protein were iodinated.

was achieved when two TSK-3,000 columns were connected in series (0.75 × 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_r 68,000) and alpha-chymotrypsinogen (M_r 25,000). The same result was obtained when material from the C_4 pool was applied to the columns (Fig. 4, bottom trace). Whereas it is unlikely that peptides aggregate in the acetonitrile/TFA solvent, the C_4 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 C_{18} column in 0.1% TFA with a shallow acetonitrile gradient (Fig. 5). The ARIA, which eluted at ~32% acetonitrile, was associated with a small OD_{210} peak. No OD_{210} peaks were observed when the buffer blank (fractions from the preceding TSK-3,000 column that contained no activity or OD_{210} absorbing material) was chromatographed under the same conditions, and monitored at the same high sensitivity (0.1 absorbance units full scale).

Aliquots of C_{18} 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 (Fig. 6). The band is centered at 42 kD, but it is broad compared with standards run in the same gel. Although only approximate, this estimate based on electrophoresis is consistent with the size of the ARIA determined by gel filtration. Fraction 25, which showed a distinct 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.

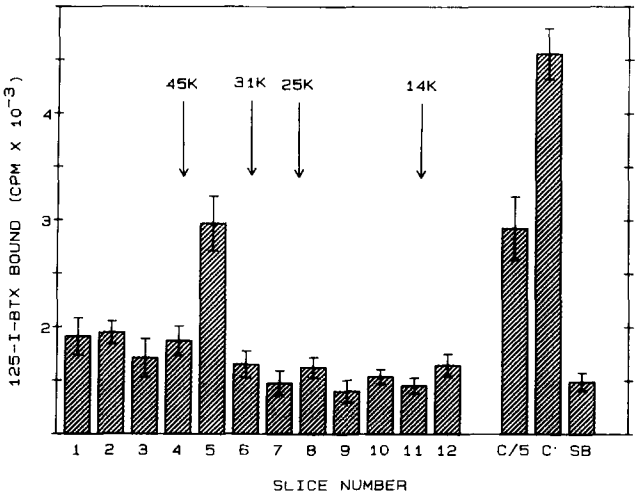

Figure 7. Recovery of ARIA from slices of an SDS-10% polyacrylamide gel. An aliquot of the C_4 pool was heated at 37°C for 15 min in sample buffer (1% SDS, 20 mM Tris HCl [pH 6.8] 30% glycerol) and applied to one lane of the gel. The position of Coomassie Blue-stained protein standards (ovalbumin, carbonic anhydrase, alpha-chymotrypsinogen, and lysozyme) run in a second lane are indicated. The AChR incorporation rate in wells that received dialyzed sample buffer (SB), a denatured and dialyzed but not electrophoresed C_4 sample (C), and a fivefold dilution of that sample (C/5) are shown at the right.

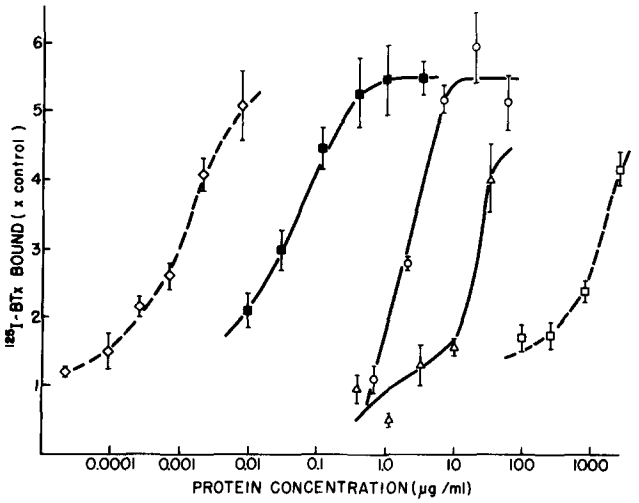

Figure 8. Dose-response curves of saline brain extract (open squares), acid extract (open triangles), C_4 pool (open circles), HFBA pool (solid squares), and the final C_{18} pool (open diamonds). The data, obtained from two different platings (solid and dashed curves), are plotted as the fold increase over control. Each point represents the mean ± SE of four wells.

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 C_4 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 [^{125}I]α-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 affect 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 C_4 pool was the first stage at which inhibition at the highest concentrations did not occur.[3]

Table I. Purification and Recovery of AChR-inducing Activity

	Activity	Protein	Specific activity
	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 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 C_4 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 C_{18} 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 C_{18} 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 OD_{210} 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 × 10^6-fold with a recovery of 3%. We estimate that the final C_{18} 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 ~5.5-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.

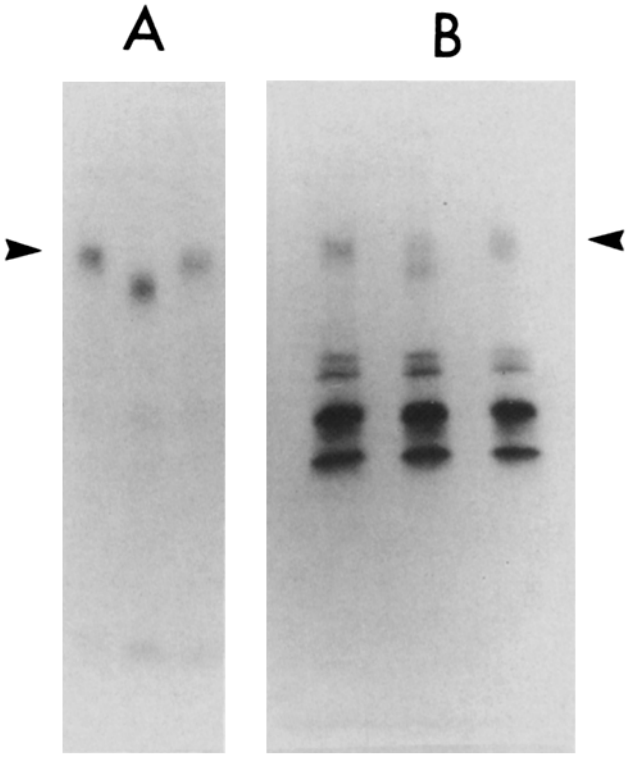

Figure 9. The position of the 42-kD band is shifted after digestion with neuraminidase. ^{125} I-labeled material from fraction 23 of the final C_{18} column (*A*) and from the HFBA pool (*B*) were exposed to 0.1 U/ml neuraminidase (purified from *C. perfringens* and obtained from Sigma Chemical Co., 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 (Bohreinger 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 (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°. 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 ± 171).[4] Treatment of the ^{125}I-labeled 42-kD polypeptide in the C_{18} 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 [^{125}I]α-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 $t_{½}$ 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-

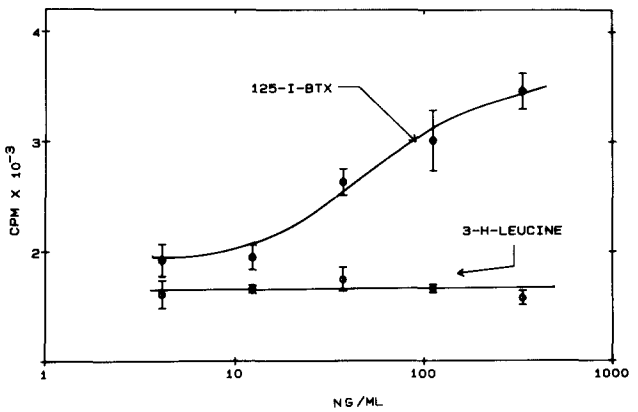

Figure 10. 42-kD ARIA does not increase protein synthesis. Serial dilutions of the HFBA pool were added to 7-d-old cultures along with [^{3}H]leucine (1 μCi/ml). After 48 h the amount of [^{3}H]leucine incorporated into material precipitated by cold TCA (15%) was determined. The total number of surface AChRs was measured with [^{125}I]α-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]α-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_4 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]α-BTX binding 4 h after block with cold α-BTX.
‡ [^{125}I]α-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	Rate‖	Total	Rate¶	Total	Rate**
		per 3 h		*per 11 h*		*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.

tivity is 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. 11). This suggests that the effect of ARIA is not mediated by muscle paralysis.

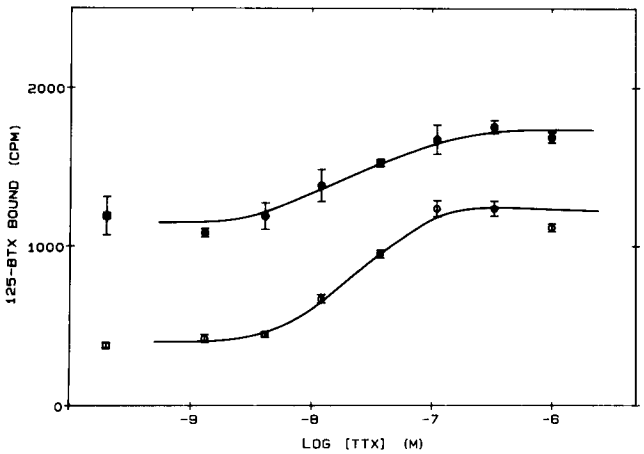

Figure 11. The effect of HFBA pool ARIA and of TTX on AChR incorporation rate are additive. The indicated concentrations of TTX were added to myotube cultures in the absence (open circles) or presence (closed circles) of one concentration of HFBA pool (300 ng/ml). The rate of AChR incorporation was estimated 24 h later. Squares to the left indicate wells that received no TTX.

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 (1980*a*,*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, 1980*b*), 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.

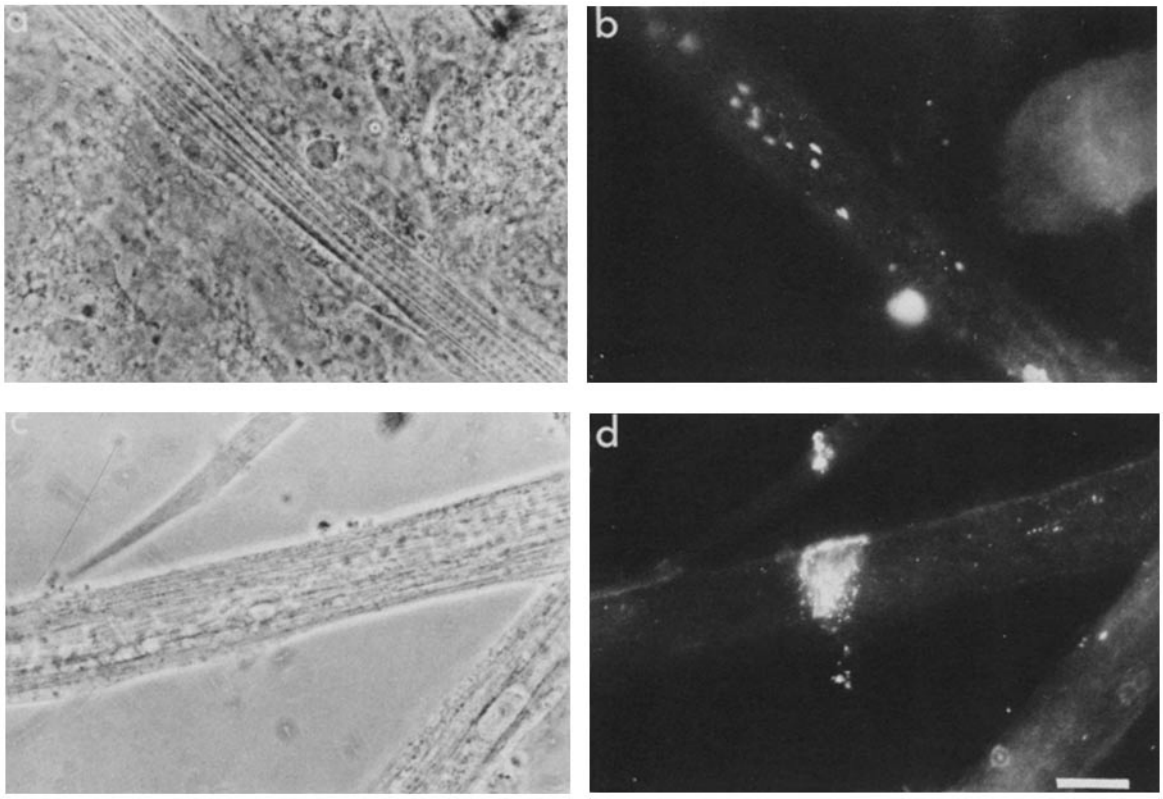

Figure 12. Clusters of AChRs labeled with rhodamine-α-BTX in untreated myotubes grown in the presence (*a* and *b*) or absence (*c* and *d*) of fibroblasts. Note that the clusters range in size from 0.5–2-μm speckles to large patches ~10 μm across. *a* and *c*, phase-contrast; *b* and *d*, fluorescence illumination; N_2 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 Rα-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	
	No. fibers	Mean cluster area*
Control	50	15 ± 2.1
Saline extract (3.3 mg/ml)	45	26 ± 5.9
HFBA pool (300 ng/ml)	58	43 ± 7.2
	Experiment 2	
Control	36	49 ± 17.5
Saline extract (3.3 mg/ml)	24	246 ± 76
C_{18} pool (10 ng/ml)	59	130 ± 39

Data are expressed as square micrometers ± SE (1 pixel = 0.067 μm^2).
* Mean cluster area is the average area per fiber length (~200 μm) occupied by AChR aggregates.

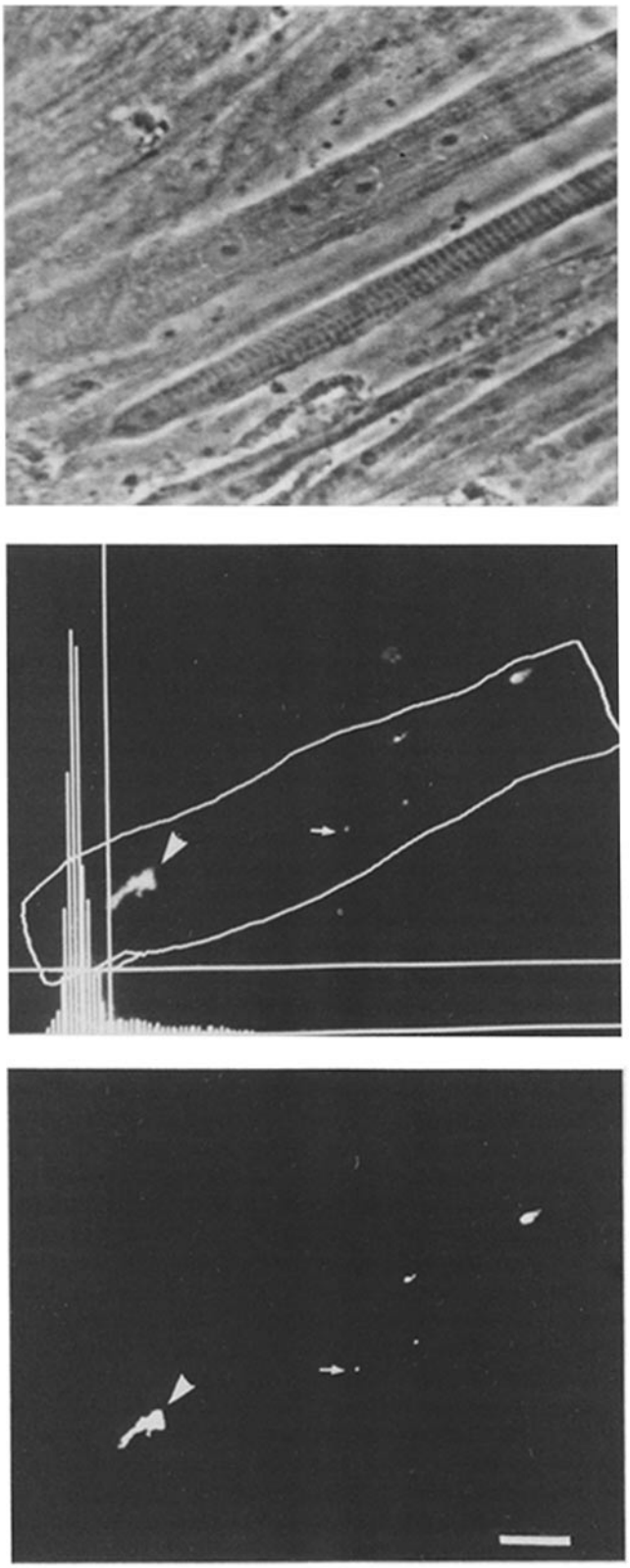

Figure 13. The method used to assay the area occupied by AChR clusters. All photographs are of digitized images displayed on a television monitor. The cells were labeled with R-BTX, fixed, and mounted in glycerol. (*Top*) A myotube was selected under phase contrast illumination. (*Middle*) The same field 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.

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 C_{18} 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 sialic acid containing glycoprotein. 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, OD_{210} absorbance peak on the final reverse-phase C_{18} 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 C_{18} 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 C_{18} 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 C_{18} 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

enough activity was solubilized to allow its complete purification while 99% of the protein was precipitated. In this sense the acid cocktail was extremely useful. Dose-response curves indicate that TFA extracts contain ~30% as much ARIA as saline extracts prepared from the same batch of frozen brains, so acid extraction is also relatively efficient. This comparison must be interpreted with some caution, however, because we do not know the efficiency of saline or acid extraction with regard to the 42-kD protein and we do not know if the 42-kD species accounts for all of the activity in either preparation.

The overall recovery of 10% of the ARIA present in the starting acid extract is remarkable considering the number of steps involved. Recoveries from the CM-Sephadex, Vydac C_4, TSK-SP, and the first Vydac C_{18} columns were excellent. Together, they resulted in a 1,500-fold purification with an apparent loss of only 50% of the activity. Although we tested several columns and solvent systems on an empirical basis, the ones adopted have in fact been used by others concerned with maximizing the resolution and recovery of relatively large polypeptides (Hearn, 1982; Pearson et al., 1982; Regneir and Gooding, 1980; Regnier, 1983; Bennett et al., 1981, 1982). On the other hand, the final two steps that produced a further 40-fold increase in specific activity were less efficient: only 15% of the activity applied to the TSK-3,000 columns was recovered after subsequent reverse-phase HPLC on C_{18}. Preliminary results suggest that most of this loss occurred during the gel filtration step. This might be the inevitable cost of reducing the sample volume 6 ml to 0.2 ml or of chromatographing micrograms of protein on this type of support.

The mechanism by which the 42-kD ARIA increases the rate of AChR insertion remains to be determined. It is reasonable to assume that the effect is due to an increased rate of receptor synthesis because insertion parallels synthesis in untreated myotubes (Devreotes and Fambrough, 1975; Devreotes et al., 1977). An intracellular pool of AChRs is present in embryonic chick myotubes, but it amounts to only 10% of the number present on the surface (Devreotes and Fambrough, 1975). The ARIA-stimulated rate of insertion reached a level of 7–10% of the surface complement per hour, and this rate was sustained for at least 24 h, so an increased rate of insertion of preformed receptors can be ruled out. However, it is possible that ARIA affects AChR subunit assembly rather than, or in addition to, subunit synthesis. Recent studies of transformed mouse muscle cells ($BC3H_1$) have shown that assembly of the mature, 9S, AChR is a complex process associated with cotranslational and posttranslational modifications of the four component subunits (Merlie et al., 1984). When the rate of proliferation of $BC3H_1$ cells is slowed, the number of surface receptors increases 100-fold, but the level of translatable α-subunit mRNA increases only fourfold (Olsen et al., 1983, 1984) suggesting that, in this case, the rate of assembly of receptor subunits as well as their rate of synthesis determines the appearance of receptors in the surface membrane. A disparity between synthetic capacity and the accumulation of mature receptors also occurs in primary cultures of inactive (TTX-treated) chick and rat myotubes (Klarsfeld and Changeux, 1985; Carlin, et al., 1986). Although it is unlikely that ARIA and TTX act in the same way (see Fig. 6), the relative contribution of subunit synthesis and assembly must be explored.

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., 1980*a*). 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 [^{125}I]α-BTX binding sites and after 7 d there was none at all (Markelonis et al., 1982*a*). We did not detect an effect of the HFBA pool on [^{3}H]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 (Ii et al., 1982; Markelonis et al., 1982*b*). 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, 1980*a*,*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 diisopropylfluorophosphate 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 C_{18} 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.

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