Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord

(embryonic myotubes/neural factor/nicotinic receptors)

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ABSTRACT Extracts of chicken brain and spinal cord increase the total number of acetylcholine receptors and the number of receptor clusters on uninervated skeletal myotubes in culture. The active component in these extracts may be unique to neural tissue. Spinal cord cells grown in culture contain the active factor and they secrete it into the medium. Most of the activity is associated with a small molecule, possibly a peptide. Such a factor may be responsible for the clustering of receptors at newly formed nerve-muscle synapses.

Acetylcholine receptors (AcChoRs) are distributed over the entire surface of embryonic skeletal myotubes (1–3). Soon after embryonic spinal cord neurites contact myotubes in vitro, synapses form and clusters of AcChoRs can be identified at sites of transmitter release (4, 5). AcChoR clusters are present on uninervated myotubes in vitro, but it is now clear that newly arrived nerve processes induce new AcChoR clusters rather than seek out preexisting ones (4–7). In adult muscle, the density of extrajunctional receptors is 3–4 orders of magnitude lower than in the synaptic membrane (8, 9), so extrasynaptic receptors must disappear soon after innervation. Both the initial clustering of AcChoRs and the subsequent dramatic decrease in the number of extrajunctional receptors have been extensively studied.

Muscle activity is an important determinant of extrajunctional acetylcholine (AcCho) sensitivity in adult and embryonic skeletal muscle (10–12). In contrast, activity does not influence the clustering of AcChoRs at synapses. New clusters appear at nerve-muscle contacts in vitro when cultures are grown in the presence of α-bungarotoxin (α-BTX), curare, or tetrodotoxin (7, 13). This finding has renewed interest in the possibility that a chemical factor released from motor nerves may influence the number of AcChoRs.

Some evidence for such a factor has already been obtained. In chicken spinal cord explant/muscle cocultures, AcCho sensitivity and the density of 125I-labeled α-BTX binding sites on myotubes close to the explant are greater than on myotubes located farther away in the same culture (14). This may reflect the diffusion of a soluble factor from the spinal cord tissue. Rat brain and spinal cord extracts increase AcChoR numbers on L6 myotubes to levels found on primary rat myotubes and also promote the appearance of AcChoR clusters (15). Media conditioned by a hybrid neuroblastoma cell line increase the number of AcChoR clusters but not the total number of receptors on primary mouse myotubes (16).

In this paper we document physiological effects of chicken brain and spinal cord extracts on primary chicken myotubes and describe the partial purification of a factor that increases the number and induces the clustering of AcChoRs.

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MATERIALS AND METHODS

Cell Culture. Muscle cultures were prepared from dissociated myoblasts as described (2). The mononucleated cells were plated at a density of 6 × 10^6 cells per 18-mm collagen-coated Falcon well in 400 μl of Eagle's minimal essential medium supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) embryo extract, and antibiotics. Cultures were fed on the second and fourth days with the same medium but containing only 2% embryo extract. Spinal cord cells were dissociated from chicken 6-day embryos (17) and plated on collagen-coated 100-mm culture dishes at a density of 1.2 × 10^5 cells per dish. Spinal cord cultures were treated with 10 μM 1-β-D-arabinofuranosylcytosine (cytosine arabinoside) for the 24 hr between days 2 and 3 to kill dividing cells. Fibroblasts were obtained from pectoral muscle by collecting rapidly adhering cells (18), allowing them to grow to confluency, and then replating them on collagen-coated 100-mm dishes.

Brain Extracts. Brain extract was prepared by homogenizing chicken 14-day embryo brains with a Dounce homogenizer in 3 vol of Earle's balanced salt solution at 4°C. The homogenate was centrifuged at 1500 × g for 10 min and the supernatant was centrifuged again at 20,000 × g for 60 min (identical results were obtained after centrifugation at 300,000 × g for 60 min). The final supernatant contained 7–8 mg of protein per ml and was stored at −80°C before use. Other methods of extraction and isolation steps are described in the text.

Binding of 125I-Labeled α-BTX. To measure the specific binding of 125I-labeled α-BTX (125I-α-BTX), the incubation medium was removed and replaced with 250 μl of balanced salt solution containing bovine serum albumin (1 mg/ml) and 5 nM 125I-α-BTX [100–120 Ci/mmol (1 Ci = 3.7 × 10^10 bec- querels); 160–200 cpm/μmol; New England Nuclear]. After 60 min at 37°C, the cells were washed three times with 1 ml of balanced salt solution and dissolved in 250 μl of 1 M sodium hydroxide containing 500 μg of sodium deoxycholate per ml. A 20-μl sample was removed for estimation of protein. Radioactivity in the remaining 230 μl (plus a 500-μl wash) was assayed by gamma counting. Nonspecific binding was estimated by addition of 0.2 mM d-tubocurarine along with the 125I-α-BTX. For autoradiography, the cultures were fixed in 4% formaldehyde in 0.12 M sodium cacodylate buffer (pH 7.2) for 60 min at room temperature. The cells were coated with NTB3 (Kodak) emulsion diluted 1:1 with water and exposed at 4°C for 5 days in a dessicated box. Grains were developed in D-19 (Kodak) for 2.5 min at 20°C.

Electrophysiology. The AcCho sensitivity of control and treated cultures was assayed by recording the change in membrane potential with an intracellular microelectrode while

Abbreviations: AcChoR, acetylcholine receptor; AcCho, acetylcholine; α-BTX, α-bungarotoxin.

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applying AcCho iontophoretically from a microelectrode filled with 1 M AcCho (2). The positive iontophoretic current pulse was 0.5 msec in duration and 5–10 nA in amplitude. High-resistance electrodes (80–100 MΩ) were used with backing currents of 1–2 nA.

RESULTS AND DISCUSSION

AcChoRs. The number of AcChoRs on cultured myotubes was estimated by measuring the specific binding of [125Ι]α-BTX. In control cultures, the number of specific [125Ι]α-BTX binding sites increased over the first 4 days as myoblasts fused to form multinucleated myotubes (Fig. 1). At later times, the number of [125Ι]α-BTX sites remained relatively constant, ranging in different experiments between 9 and 18 fmol per 18-mm culture. Daily addition of saline extracts prepared from embryonic chicken brain to fused myotubes produced a 4.8-fold increase in [125Ι]α-BTX binding after 5 days. A comparable response was observed in more than 30 separate platings. An increase in AcChoR number was apparent 3 days after the first addition of brain extract and the AcChoR number remained increased for at least 3 days after the last addition and return to control medium. The effect was dose-dependent over a concentration range from 20 μg to 380 μg of brain protein added per day. The cultures used in these experiments were not treated with arabinonucleoside so they contained fibroblasts as well as muscle cells (19). Brain extract increased the number of AcChoRs in arabinonucleoside-treated cultures, but the effect was not always as marked.

The increase in [125Ι]α-BTX binding sites reflects an increase in the density of AcChoRs rather than an increase in myotube size and surface area. The protein contents (20) of control and extract-treated cultures were not significantly different (125–160 μg per 18-mm culture). Direct evidence for an increase in AcChoR density was obtained by autoradiography of [125Ι]α-BTX-treated cultures and by measurement of the AcCho sensitivity of individual myotubes. In one experiment the mean grain density on extract-treated myotubes was 3.1-fold greater than the density on untreated myotubes (Fig. 2 A and B); in sister cultures the specific binding of [125Ι]α-BTX increased 3.2-fold. The sensitivity of extract-treated myotubes to iontophoretically applied AcCho ranged between 200 and 1600 mV/nC with a mean of 780 mV/nC (Fig. 2C), whereas the sensitivity of untreated myotubes ranged between 20 and 400 mV/nC with a mean of 118 mV/nC. No difference in resting potential (−60 to −70 mV) or in input resistance (4–6 MΩ) was observed between control and extract-treated cultures.

The autoradiographs also revealed a striking change in the distribution of AcChoRs on extract-treated cultures. After 8 days, there was 0.2 ± 0.04 cluster per mm of myotube (mean ± SEM; n = 15 fields) in control cultures (Fig. 3A). There was a 44-fold increase in the number of clusters (5.8 ± 0.3 clusters per mm of myotube) in sister cultures treated with brain extract (Fig. 3 B and C). The density of AcChoRs within clusters was at least 5000 sites/μm².

The increase in the number of AcChoR clusters did not precede the increase in total AcChoR number. No significant increase was observed for the first 48 hr after addition of brain extract. By the third day there were 6.1 ± 0.5 clusters per mm of myotube (n = 20 fields) in extract-treated cultures and 0.5 ± 0.1 clusters per mm of myotube (n = 20 fields) in untreated cultures. Thus, there does not appear to be a redistribution of existing surface receptors before the total number of surface AcChoRs increases.

Tissue Specificity of AcChoR Stimulation. The factor that increases AcChoR number is present in the spinal cord and subcortical brain and, to a lesser extent, in the cerebellar cortex. No activity was observed in extracts prepared from the retina, heart, or liver (Fig. 4). Spinal cord cells grown in culture contained the factor (Fig. 4). Medium conditioned for 24 hr by spinal cord cells produced a 2.1 ± 0.1-fold increase in AcChoR number (Fig. 4). Thus, the activity is released by intact cells. In contrast, cultured fibroblasts did not contain significant amounts of the factor and none was released into the medium. Extracts of cultured fibroblasts that had been treated with brain extract for 4 days produced no change in AcChoR number. It is likely, therefore, that brain extracts exert a direct effect on muscle cells rather than an indirect one mediated by fibroblasts.

All neurons in the avian ciliary ganglion are cholinergic, and many innervate skeletal muscle fibers in vivo and in vitro (21–24). Neurons dissociated from 8-day embryonic ciliary ganglia (24) and added to cultures of multinucleated myotubes produced a 2.2 ± 0.2-fold (mean ± SEM, n = 4) increase in AcChoR number after 4 days. This increase
cannot be accounted for by the number of $^{125}$I-αBTX binding sites on the ciliary neurons themselves. In contrast, the same number of dorsal root ganglion neurons added to muscle cultures produced no change in AcChoR number after 4 days. The factor may therefore be unique to cholinergic neurons.

**AcChoR Metabolism.** An increase in AcChoR number might result from stimulation of receptor synthesis or from decrease in the rate of receptor degradation. The loss of bound $^{125}$I-αBTX (or the appearance of $^{125}$I in the culture medium) is an accurate measurement of the rate of AcChoR degradation (25–27). By this measurement, diffusely distributed and clustered AcChoRs on innervated and on uninnervated myotubes are degraded with a half-life of 22–30 hr (3, 25, 28, 29). Brain extract-treated cultures exhibited a 4.2-fold increase in AcChoR number; however, the rate of AcChoR degradation was not decreased (Fig. 5). In fact, the apparent turnover rate in treated cultures ($t_{1/2}$, 18.5 hr) was slightly greater than in control cultures ($t_{1/2}$, 28 hr). It is likely, therefore, that the brain extract–induced increase in AcChoR number reflects an enhanced rate of AcChoR synthesis.

**Activity-Independent AcChoR Induction.** Electrical or mechanical activity plays a significant role in determining the AcCho sensitivity of skeletal muscle in situ and in culture (10–12). Inactive myotubes grown in tetrodotoxin are more sensitive to iontophoretically applied AcCho and contain more AcChoR clusters than do control myotubes (12). It seems unlikely, however, that the effects of brain extract are mediated by changes in muscle activity. In contrast to the delay observed in extract-treated cultures, the tetrodotoxin-induced increase in AcChoR number was evident after only 24 hr. Furthermore, a far greater increase in AcChoR number was observed with brain extracts than with maximal concentrations of tetrodotoxin. Tetrodotoxin at 0.3 μM produced a 2.4 ± 0.14-fold (mean ± SEM, n = 4) increase in AcChoR number and a 4.0 ± 0.2-fold (mean ± SEM, n = 20 fields) increase in the number of AcChoR clusters per mm of myotube. In the same experiment, 780 μg of brain protein added each day produced a 4.6 ± 0.45-fold increase in AcChoR number and a 44.0 ± 1.5-fold increase in the number of AcChoR clusters per mm of myotube. The effects of tetrodotoxin and brain extract were additive. Cultures grown in tetrodotoxin (0.3 μM) and brain extract (780 μg/day) produced an 8.1 ± 0.27-fold increase in AcChoR number and a 60.0 ± 3.0-fold increase in the frequency of AcChoR clusters.

**Extracts Affect Acetylcholinesterase and Fibroblast Proliferation.** In 8-day myotube cultures, total acetylcholinesterase activity (30) was 33.8 ± 6.6 pmol of acetylthiocholine hydrolyzed per μg of protein per min (mean ± SEM, six separate platings). In parallel cultures treated for 4 days with 200–250 μg of brain extract protein per day, the esterase activity increased to 132.1 ± 33.1 pmol of acetylthiocholine hydrolyzed per μg of protein per min. This 3.9-fold increase was comparable to the 3.3 ± 0.5-fold increase in AcChoR number in parallel cultures treated with the same concentration of brain extract. Brain extracts did not increase the activity of all enzymes. In extract-treated cultures, activity of the soluble cytoplasmic enzyme creatine kinase (31) (0.26 ± 0.01 μmol of ATP formed per min per mg of protein) was not significantly different from that in control cultures (0.26 ± 0.02 μmol).

Brain extracts also stimulated the proliferation of mononu-
Induction of AcChoR by extracts prepared from different tissues and from cells in culture. Tissue extracts were prepared from chicken 14-day embryos and from cultured cells and added to myotubes (75 μg/day for 4 days) in 25 μl. Medium conditioned by spinal cord or fibroblast cells was diluted 2:1 with fresh 2% minimal essential medium, and 500 μl of this diluted medium was used to feed 4-day myotube cultures every 24 hr for 4 days. In control cultures, half of the medium (250 μl) was removed and replaced with fresh 2% minimal essential medium each day. Extracts of cultured spinal cord (sc) and fibroblast (f) cells were added to myotubes at a concentration of 35 μg of protein per day. Each column represents the mean ± SEM of four to eight determinations. c, Control; sc, spinal cord; f, subcortical brain; cb, cerebellum; ret, retina; liv, liver; h, heart; bsa, bovine serum albumin.

Initial Characterization of the Factor. Filtration of brain extracts through a Bio-Gel P-150 column equilibrated with phosphate-buffered saline (pH 7.4) revealed two distinct peaks of biological activity. The higher molecular weight peak coeluted with bovine serum albumin (68,000 daltons) and the lower molecular weight peak coeluted with neurotensin (1674). Activity in the higher molecular weight peak was retained after dialysis, whereas the lower molecular weight component was lost. The same two peaks of activity were evident after extraction of embryonic brains in 2 M acetic acid at 4°C followed by gel chromatography through Bio-Gel P-150 (Fig. 6). The high molecular weight component contained 12.2% of the recovered activity; the low molecular weight component contained 87.8%. Compared with saline extracts of embryonic brain, the acid-stable low molecular weight component contained 1.68% of original protein and 42.8% of the original biological activity. With 1 unit defined as the amount of material required to produce a 10-fmol increase in specific [125I]-αBTX binding, the activity of saline brain extracts in the experiment shown in Fig. 6 was 18 units/mg of protein. The activity in the pooled low molecular weight peak was 409 units/mg of protein; in the high molecular weight peak it was 56 units/mg of protein. Dose–response curves using material from fraction 37 (Fig. 6) showed a 71-fold increase in specific activity (1280 units/mg of protein) compared with saline extracts of embryonic brain.

Extracts of adult chicken brains also induced an increase in AcChoR number. The specific activity (between 5 and 10 units/mg of protein in saline extracts) was comparable to that of embryonic brain. Extraction of adult brains with acetone/HCl by the method of Chang and Leeman (34) eliminated almost all high molecular weight proteins. The low molecular weight material that remained soluble in acetone/HCl retained 37.9% of the activity present in saline extracts. This purified

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material increased the number of receptor clusters as well as the total number of receptors.

Tryptic digestion (TPCK trypsin, Worthington; 0.1 mg/ml) of the low molecular weight, acetone/HCl-soluble material followed by inactivation with soybean trypsin inhibitor (0.1 mg/ml) destroyed 86% of the activity. The single Bio-Gel P-150 peak could be resolved into several components by filtration through a Bio-Gel P-4 column; however, biological activity was not confined to a single protein peak. The specific activity of pooled fractions eluted from a cation exchange column (sulfoethyl-Sepharose, Pharmacia) was 1500–2000 units/mg of protein, but only 20% of the activity applied to the column was retained.

CONCLUSIONS

The concept that trophic interactions occur between motor nerves and muscle fibers is not new, but little is known of the underlying molecular mechanisms. We have found that a small molecule, possibly a peptide, in brain and spinal cord stimulates the synthesis and clustering of AcChoRs in embryonic myotubes. Although attention is currently focused on peptides as modulators of synaptic function and neuronal excitability, a role for peptides in long-term regulation of postsynaptic targets is certainly possible. Throughout this report we have referred to the low molecular weight peak of activity as a single factor although it is possible that the peak contains more than one active component. Further purification and characterization should resolve this issue and greatly facilitate studies of cellular and subcellular localization within neural tissue, mechanism of release, and precise effects on target myotubes.

Recent studies of neuronal extracts suggest that larger molecules can alter the distribution of AcChoRs (15, 16). We found only about 10% of the recovered AcChoR-inducing activity associated with high molecular weight material. This component may be an active precursor or a carrier of the smaller molecule. Alternatively, the two molecules may be entirely unrelated.

Brain and spinal cord extracts added to the culture medium increased the number of AcChoRs over the entire myotube surface membrane, but this does not imply that the factor is ordinarily involved in regulation of extrajunctional receptors. Local release from intact motor nerve terminals would probably increase AcChoR sensitivity only in the immediate vicinity of synaptic contact. We have shown that the factor is, in fact, released from intact embryonic spinal cord cells. It remains to be determined if the release is restricted to synaptic nerve–muscle contacts. Protein is released along with AcCho from stimulated adult motor nerve terminals (35), and recent experiments have demonstrated that stimulated motor axons release a substance that maintains AcCho esterase at denervated end plates (36).

The increase in AcCho esterase in extract-treated cultures is consistent with earlier reports that neural extracts slow the loss of AcCho esterase in denervated muscle (36–38). AcCho receptors cluster at newly formed synapses in the absence of synaptic function and muscle activity (7, 13), but some degree of muscle activity is required for the accumulation of AcCho esterase at sites of transmitter release (13). Thus, a neurally derived factor is probably not sufficient to ensure the accumulation of AcCho esterase at synapses. It may be significant in this regard that we have not found an increase in the high molecular weight form of AcCho esterase (19.5 S) in extract-treated cultures. This, of course, does not diminish the possibility that synthesis of AcChoRs and AcCho esterase is coordinately controlled, and it will be important to determine if the synthesis of both is stimulated by the same molecule.

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