Muscle Induces Neuronal Expression of Acetylcholinesterase in Neuron-Muscle Co-culture

TRANSCRIPTIONAL REGULATION MEDIATED BY cAMP-DEPENDENT SIGNALING*

Received for publication, June 16, 2003, and in revised form, September 3, 2003
Published, JBC Papers in Press, September 8, 2003, DOI 10.1074/jbc.M306320200

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Presynaptic motor neuron synthesizes and secretes acetylcholinesterase (AChE) at vertebrate neuromuscular junctions. In order to determine the retrograde role of muscle in regulating the expression of AChE in motor neuron, a chimeric co-culture of NG108-15 cell, a cholinergic cell line that resembles motor neuron, with chick myotube was established to mimic the neuromuscular contact in vivo. A DNA construct of human AChE promoter tagged with luciferase (pAChE-Luc) was stably transfected into NG108-15 cells. The co-culture with myotubes robustly stimulated the promoter activity as well as the endogenous expression of AChE in pAChE-Luc stably transfected NG108-15 cells. Muscle extract derived from chick embryos when applied onto pAChE-Luc-expressing NG108-15 cells induced expressions of AChE promoter and endogenous AChE. The cAMP-responsive element mutation on human AChE promoter blocked the muscle-induced AChE transcriptional activity in cultured NG108-15 cells either in co-culturing with myotube or in applying muscle extract. The accumulation of intracellular cAMP and the phosphorylation of MAP-kinase or in applying muscle extract. The accumula- tion of intracellular cAMP and the phosphorylation of CREB, cAMP-responsive element-binding protein (CRE) site located on the human AChE promoter has been shown to regulate the level of AChE transcripts in muscle; these neural factors include calcitonin gene-related peptide (CGRP) that increases the synthesis of cAMP (6–8), ATP that stimulates the accumulation of inositol phosphate and calcium (9, 10), muscular activity (11, 12), and others (13, 14).

Although muscle is the primary source of all forms of AChE at the nmj, two other cell types, presynaptic motor neuron and myelinating Schwann cell, also contribute to the synaptic AChE activity, but to a lower extent (1). The contribution of synaptic AChE from the myelinating Schwann cell, if any, is limited (15). Several lines of evidence indicate that the presynaptic motor neuron is able to synthesize and secrete AChE at the nmj. In frog nmjs, the muscle was permanently removed by x-ray irradiation and all of the synaptic AChE activity was irreversibly blocked by an AChE inhibitor, but the motor axons were kept intact. After a month in the absence of postsynaptic muscle fiber, the AChE activity was recovered, indicating that the motor nerve was capable of producing part of the AChE activity at the synaptic cleft (15). In chicks, a globular form of AChE is expressed in the presynaptic motor neurons, which is down-regulated toward maturity and by nerve denervation (16, 17). In addition, both asymmetric and globular forms of AChE are entirely transported by fast axonal flow in the sciatic nerve of chicken (18). Moreover, AChE at the nerve terminus is secreted in an activity-dependent manner (1, 19). Having the aforementioned evidence, the possibility that motor neuron-derived AChE may play functional roles at the nmj is still an open question.

The transcriptional activity of the AChE gene has been demonstrated to play a role in AChE regulation, and several regulatory elements within the promoter have been reported to regulate the transcriptional activity of AChE gene in Torpedo (20), mice (21, 22), rats (23), and humans (24). Among these possible AChE regulatory elements, a cAMP-responsive element (CRE) site located on the human AChE promoter has been shown to regulate the level of AChE transcripts in muscle (25, 26) and neuron (27). Here, we would like to determine the regulation mechanism of neuronal AChE mediated by the interacting muscle in a co-culture system of neuroblastoma × glioma hybrid cells NG108-15 with chick myotubes; this coculture has been used to resemble the formation of nmjs in vitro previously (14, 28, 29). By using a luciferase-tagged human AChE promoter construct as a tool, the transcriptional

Acetylcholinesterase (AChE, EC 3.1.1.7) is a highly polymorphic enzyme, and its localization at the vertebrate neuromuscular junctions (nmjs) is in different attachment manners (1–3). A single gene encodes this enzyme, and adult mammalian muscles express a single gene variant, corresponding to the catalytic subunit of type T (AChE-T) (1). AChE is restricted to the nmj at a density of ~650–2500 sites/μm² (4) that is mainly contributed by muscle enzyme and mediated by agrin that induces the aggregation of AChE and acetylcholine receptors (AChRs) under the motor nerve (5). The motor nerve provides trophic factors to regulate the postsynaptic AChE expression in muscle; these neural factors include calcitonin gene-related peptide (CGRP) that increases the synthesis of cAMP (6–8), ATP that stimulates the accumulation of inositol phosphate and calcium (9, 10), muscular activity (11, 12), and others (13, 14).

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* This work was supported by Research Grants Council of Hong Kong Grants 6098/02M (to K. W. K. T.) and 4140/98M (to D. C. C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the postdoctoral matching fund from Hong Kong University of Science and Technology.
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1 The abbreviations used are: AChE, acetylcholinesterase; nmj, neuromuscular junction; AChR, acetylcholine receptor; CGRP, calcitonin gene-related peptide; CRE, cAMP-responsive element; DMEM, Dulbecco’s modified Eagle’s medium; Bt2cAMP, dibutyryl cyclic AMP, CREB, cAMP-responsive element-binding protein.

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This paper is available online at http://www.jbc.org

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element that mediated the muscle-induced neuronal AChE expression in the neuron-muscle co-culture was elucidated. The gene activation was demonstrated to be mediated by a cAMP-dependent signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Tissue Cultures**—Eggs of New Hampshire chicks were purchased from a local farm and hatched in the University Animal Care Facility. In primary chick myotube cultures, hind limb muscles dissected from 11-day-old chick embryos were minced and then dissociated by trypsinization, stirring, and centrifugation (29). Muscle cells were routinely cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, 2% chick embryo extract, 1 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell culture medium, fetal bovine serum, and other cell culture reagents were from Invitrogen. The cultures were incubated at 37 °C in a water-saturated incubator of 95% air and 5% CO₂. About 3 × 10⁵ C₂C₁₂ myoblasts were added at different cell density onto the 4–5-day-old chick myotubes or C₂C₁₂ myotubes. The co-culture was carried out for 2–3 days, and the medium was changed daily. For control cultures, equal amount of NG108-15 cells were added onto a 12-well plate in the myotube culture medium but without myotubes; the myotube medium showed a weak effect on the neuronal differentiation of cultured NG108-15 cells. To determine the effect of neuron-muscle contact, a cell culture insert with pore size of 0.4 μm (BD Biosciences Clontech) was employed. In brief, chick myotubes were allowed to grow inside the cell culture insert and placed onto a 6-well plate that contained NG108-15 cells. The numbers of NG108-15 cell in both control and co-culture groups were counted by a microscope before the cell collection. 20 different views were randomly selected for each well.

**cDNA Transfection**—The ~2.2-kb DNA fragment of human AChE promoter (24) and the CRE site mutated human AChE promoter (25) were subcloned into BglIII and HindIII sites of pGL3 basic vector (Promega, Madison, WI) having a downstream tagged luciferase, which was named as pAChE-Luc (27) and pAChE<sub>CRE</sub>-Luc (25), respectively. This ~2.2-kb regulatory element was shown to reflect the genuine situation of AChE regulation in a C₂C₁₂ cell; the activity of this promoter was in parallel with the endogenous AChE expression during the myogenic differentiation of C₂C₁₂ myotube (26). The 2.1-kb mouse AChE promoter, generated by PCR using the published sequence as described in Li et al. (21), was tagged with luciferase and designated as pAChE<sub>μ</sub>-Luc. The DNA construct of CRE sequence tagged with a luciferase (pCRE-Luc), AP-1 sequence tagged with a luciferase (pNFκB-Luc) were from BD Biosciences Clontech. Double-stranded oligonucleotides containing four repeats of the intact CRE sequence (~2195CAC GTC A~2185, 5’ to 3’) derived from human AChE promoter were subcloned into pTA-Luc luciferase reporter vector (BD Biosciences Clontech) to form pCRE<sub>μ</sub>-Luc. For pAβP-2-Luc construction, double-stranded oligonucleotides con-
taining four repeats of the intact AP-2 binding sequences (5′-GACC GGCA GCC-3′, 5′-GAGC CAGG-3′) derived from human AChE promoter were subcloned into pTA-Luc vector. The construct of β-galactosidase gene under a cytomegalovirus promoter (pCMV-β-gal) from BD Biosciences Clontech was used as a control vector. For the construction having AChE as a reporter, the human AChE promoter was tagged upstream of a cDNA encoding the chick AChE catalytic subunit (14) to form the pAChE-emAChE construct. DNAs encoding various constructs were purified by a Qiagen column, and they were stably transfected into NG108-15 cells by calcium phosphate precipitation method (29). All of the expression plasmids contained a G418-resistant gene as described by the supplier. G418-resistant stable cell lines were selected in medium containing 400 μg/ml G418 (Genetin; Invitrogen) and tested for the expression of luciferase. Normally, four or five stable cell lines were generated for each DNA construct. For transient transfection, pCMV-β-gal was co-transfected with others, serving as a control, in determining the transfection efficiency that was normally over 50% in the cultured NG108-15 cells.

Preparation of Muscle Extract—1 g of hind limb muscle dissected from 19-day-old chick embryos was homogenized in 5 ml of ice-cold DMEM containing 10 mM HEPES, pH 7.5, and 1 mM phenylmethlysulfonyl fluoride by a Polytron homogenizer (Ultra-Turrax T25; Labortechnik) with 24,000 rpm for 1 min three times, followed by centrifugation at 2,000 × g for 10 min at 4 °C. Phenylmethlysulfonyl fluoride was excluded when the cultured cells were being used for the AChE enzymatic assay. The supernatant was filtered by a 0.2-μm filter and freshly used within the same day. NG108-15 cells were treated with the extracts at different protein concentrations from 0.125 to 1.0 mg/ml culture medium.

Immunoblotting and Phosphorylation Assay—Neuron-muscle co-cultures or muscle extract-treated NG 108-15 cell cultures in 12-well culture plates were homogenized in 0.3 ml of ice-cold lysis buffer containing 10 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mg/ml bacitracin, and 1 μM NaCl, followed by centrifugation at 12,000 × g for 5 min at 4 °C. Protein samples were denatured in 100 °C for 5 min in a buffer containing 1% SDS and 1% dithiothreitol and separated by SDS-polyacrylamide gel electrophoresis. In phosphorylation analyses of cAMP-responsive element-binding protein (CREB), cultures were washed with 1× Na2VO3/phosphate-buffered saline, followed by the addition of 200 μl of lysis buffer containing 50 mM dithiothreitol, 1 mM Na3VO4 (pH 8.0), 5 mM phenylmethlysulfonyl fluoride, 1% SDS for lysis. Lysates were collected and centrifuged at 14,000 rpm for 2 min at 4 °C. Fifty μl of supernatant was used in SDS-PAGE. Proteins from the gel were electroblotted (Bio-Rad Mini-Protein II transblot system) onto nitrocellulose paper for 16 h (16). The blot was absorbed with blocking agent of 5% nonfat milk powder in phosphate-buffered saline and collected in 1 ml of 4 mM EDTA containing 50 μg/ml of lysis buffer. The anti-neurofilament 200 and anti-tubulin antibodies were from Sigma and were used at a 1:5,000 dilution. The anti-phospho-CREB and total CREB antibodies (New England Biolabs, Beverly, MA) were in a 1:1,000 dilution. The peroxidase-conjugated secondary antibodies (1:5,000) were from Cappel (Cochranville, PA). The chemiluminescence method was used according to the ECL protocol provided by the supplier (Amersham Biosciences). The anti-neurofilament 200 and anti-tubulin antibodies (clone 73-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the antibody was purified from hybridoma medium using protein G-Sepharose 4B (Amersham Biosciences). The working concentration of protein-G purified primary antibody for AChE was 5–10 μg/ml. The anti-neurofilament 200 and anti-tubulin antibodies were from Sigma and were used at a 1:5,000 dilution. The anti-SNAP-25 monoclonal antibody (BD Biosciences) was used at a 1:20,000 dilution. The anti-phospho-CREB and total CREB antibodies (New England Biolabs, Beverly, MA) were in a 1:1,000 dilution. The peroxidase-conjugated secondary antibodies (1:5,000) were from Cappel (Beverly, MA). The immunocomplexes were visualized using the ECL method (Amersham Biosciences). The intensities of the bands were compared on an image analyzer, using in each case a calibration plot constructed from a parallel gel with serial dilutions of one of those samples. The intensity of the recognized protein band was determined by a densitometer.

Sucrose Density Gradient—Separation of various molecular forms of AChE was performed by sucrose density gradient analysis according to previous study (30). 5–20% sucrose gradients were prepared in 0.5 ml of sucrose or in 12-ml polyallomer tubes. Sucrose gradients were in 10 mM HEPES, pH 7.5, 0.5% Triton X-100, 1 μM NaCl. Cell extract (0.1 ml) was loaded onto the gradient. Centrifugation was run for 1.5 h at 38,000 rpm by a Sorvall T111 441 rotor at 4 °C. Sedimentation markers (catalase, 11.4 S; β-galactosidase, 16 S) were used for calibration of the gradient. About 40 fractions were collected for AChE enzymatic activity determination.

cAMP Accumulation Assay—The NG108-15 cell cultures were cultured in 35-mm culture dishes. After a 30-min treatment of chick muscle extract, or CGRP, the cell cultures were washed with phosphate-buffered saline and collected in 1 ml of 4 mM EDTA containing 50 μM 1-methyl-3-isobutylxanthine followed by boiling for 4 min, and the

![Fig. 2. Activation of human AChE promoter in NG108-15 cell by co-culturing with myotube.](http://www.jbc.org/)

Luciferase reporter was tagged downstream of the AChE promoter of either humans (pAChE-Luc) or mice (pAChEm-Luc). The construct of β-galactosidase under a cytomegalovirus promoter (pCMV-β-gal) served as control. These DNAs were stably transfected into NG108-15 cells and co-cultured with chick myotubes. Stably transfected NG108-15 cells at different amounts were co-cultured with chick myotubes (A), and 104 of these cells were co-cultured with chick myotubes for different time periods (B) or co-cultured with different cell types (C). Values are expressed per well of cells and as the ratio of the treated NG108-15 cells to the basal (equal amount of NG108-15 cell without the co-culture, incubated in parallel) activity in a final lysate, and in all cases values are mean ± S.E. for five independent experiments, each with triplicate samples.
Fig. 3. Activation of intracellular signals in NG108-15 cell by co-culturing with myotube. A, multiple copies of DNA sequences of CRE, AP-2, AP-1, and NF-xB were tagged with luciferase, as pCRE-Luc, pAP-2-Luc, pAP-1-Luc, and pNF-xB-Luc, respectively. These constructs were stably transfected into cultured NG108-15 cells and then co-cultured with myotubes as in Fig. 2. Luciferase activity was determined after 2 days of co-culture. B, a schematic diagram shows the constructs of pCREHC-Luc and pCRE-Luc. C, NG108-15 cells stably transfected with pCREHC-Luc or pCRE-Luc at different amounts were co-cultured with myotubes for 2 days. D, NG108-15 cells (10^6) stably transfected with pCREHC-Luc or pCRE-Luc were co-cultured with myotubes at different time periods. The values are presented as in Fig. 2.

cell debris was removed by centrifugation. 50 µl of the supernatant was assayed for cAMP using the cAMP assay kit from Amersham Biosciences. Assays were performed according to the manufacturer’s instructions.

Drug Treatments—To determine the role of muscle in affecting the transcription and translation of ACHE synthesis, cultured NG108-15 cells were pretreated with 10 µM cycloheximide (a translation inhibitor) and 20 µM cycloheximide (a translation inhibitor; both from Sigma) for 1 h, followed by the treatment of 1 mg/ml muscle extract for 10 h. Rat αCGRP and αCGRP anti-37 (an antagonist of CGRP receptor) were purchased from Sigma. They were stored at 0.5 mM in H2O. 1 mM to 10 mM of CGRP was used to induce the cyclic AMP accumulation and the CREB phosphorylation in cultured NG108-15 cells. 30 µM CGRP anti-37 was used to block the phosphorylation of CREB, which was induced by 3 µM CGRP or 0.5 mM muscle extract, respectively. In the blocking experiment, NG108-15 cells were pretreated with CGRP anti-37 for 1 h before the application of CGRP or muscle extract.

Other Assays—The protein concentration was measured routinely by Bradford’s method (31) with a kit from Bio-Rad. The luciferase assay was performed according to the kit supplied (Tropix Inc., Bedford, MA). In brief, cell cultures were washed with phosphate-buffered saline and resuspended in 0.2% Triton X-100, 1 mM dithiothreitol, and 100 mM potassium phosphate buffer (pH 7.8). 30 µl of lyase per sample was used in luciferase assay. The luminescent reaction was quantified by Tropix TR717 microplate luminometer (Tropix), and the activity was expressed in absorbance per mg of protein. The activity of ACHE was assayed by the method of Ellman et al. (32) in a medium containing 0.1 mM tetrathiolephosphoramide as an inhibitor of butyrylcholinesterase as described (30). Values of ACHE or of luciferase were calculated per single well of cells in the co-culture or per µg of protein in the stable cells. Statistical tests were made by the PRIMER program (60), and differences from basal or control values (as shown in the plots) were classified as highly significant (**) where p < 0.001.

RESULTS

Induction of Neuronal ACHE Expression in Neuron-Muscle Co-culture—The co-culture of cholinergic NG108-15 cells with chick myotubes could resemble the formation of nmjs in vitro. Having co-cultured with myotubes, NG108-15 cells were induced to differentiate, and neurite outgrowth was revealed (Fig. 1A). Both myotube and NG108-15 cells are able to express ACHE in the co-culture. In order to discriminate the source of ACHE in the neuron-muscle co-culture, a mammal-specific anti-ACHE antibody was used, which recognized ACHE from NG108-15 cells. A molecular mass of ~68 kDa on SDS gel electrophoresis (Fig. 1B) corresponding to the catalytic subunit of mouse ACHE was revealed only from NG108-15 cells (14, 27); the muscle-derived ACHE (chick) was not recognized by the mammal-specific antibody (Fig. 1B). ACHE in chick myotube, recognized by the chick-specific antibody, had a molecular mass of ~105 kDa. By using the species-specific anti-ACHE antibody, the expression of neuronal ACHE was found to increase over 4–6-fold by co-culturing with chick myotubes. In parallel, the expressions of neurofilament at ~200 kDa (NF-200) and SNAP-25 at ~25 kDa, specific markers of NG108-15 cell, were increased over 3–6-fold in the co-culture (Fig. 1, C and D), indicating the muscle-induced neuronal differentiation of the cultured neurons. The amount of myoblasts used in the co-culture was optimized here to ensure a sufficient number of myoblasts formed; a higher number of myoblasts showed inhibition to the growth of added NG108-15 cells (data not shown). Various amounts of NG108-15 cells were co-cultured with a constant amount of chick myotubes for 2 days in order to optimize the co-culture condition that could trigger higher induction of neuronal ACHE (Fig. 1, C and D). A higher amount of NG108-15 cells did not show a better induction of neuronal ACHE. When the intensities of stained bands were analyzed by a densitometer, the level of neuronal ACHE expression was increased over 4-fold by co-culturing with 10,000 NG108-15 cells (Fig. 1D). In view of the possibility that the induced ACHE expression could be due to rapid division of NG108-15 cells in the co-culture, the cell number of the NG108-15 cells was determined with or without the co-culture, which, however, did not show any significant variation (Fig. 1D). In contrast, the co-culture of NG108-15 cells with myotubes by using a culture insert of 0.4-µm pore size did not show any induction of these neuronal proteins (Fig. 1, C and D). Thus, a close proximity of neuron-muscle contact was required for activation of specific gene expression in NG108-15 cells.

To further investigate the effect of muscle in inducing neuronal ACHE expression, NG108-15 cells stably transfected with a construct of human ACHE promoter (~2.2 kb in length) tagged with luciferase (pACHE-Luc) were co-cultured with
chick myotubes. Over 4-fold induction of luciferase activity was revealed when pAChE-Luc-expressing NG108-15 cells were co-cultured with myotubes; similar fold induction was revealed at different concentrations of NG108-15 cell (Fig. 2A). The coculture effect was also revealed when NG108-15 cells were transfected with a construct of luciferase-tagged mouse AChE promoter (−2199 to −2184), namely pAChEm-Luc (Fig. 2A). The muscle-induced AChE expression in NG108-15 cell was time-dependent; the induction reached a maximum at −5-fold for both human and mouse AChE promoters after 2 days of co-culture (Fig. 2B). As a control, stable NG108-15 cells transfected with a β-galactosidase gene driven under a cytomegalovirus promoter were co-cultured with myotubes; however, the expression of β-galactosidase remained unchanged (Fig. 2A), which indicated a specific effect of muscle on AChE promoter activity. The induction of AChE expression in the co-culture was specific for muscle regardless of the origin from chick or mouse, and the co-culture of pAChE-Luc stably expressed NG108-15 cells with COS-7 or HEK293 cells showed no induction effect (Fig. 2C).

**A cAMP-dependent Signaling Mediates Muscle-induced Neuronal AChE Expression**—In order to determine the transcriptional element(s) that is responsible for the muscle-induced neuronal AChE expression, multiple copies of DNA sequences of different transcriptional elements, including CRE, AP-2, AP-1, and NF-κB, were tagged with luciferase in a mammalian expression vector as pCRE-Luc, pAP-2-Luc, pAP-1-Luc, and pNFκB-Luc, respectively. These constructs were stably transfected into NG108-15 cells and subsequently co-cultured with chick myotubes for 2 days. Only the DNA binding sequences of CRE and AP-2 showed robust induction (−3-fold) of luciferase activity after co-cultured with myotubes (Fig. 3A). Both CRE and AP-2 sites of the human AChE promoter have been reported to respond to the challenge of cAMP; however, the responsiveness of the AP-2 site to cAMP showed a lower magnitude than that of the CRE site (26, 33). Moreover, this CRE sequence of human AChE promoter was reported to mediate the cAMP-induced AChE expression in cultured NG108-15 cells (27). Therefore, the CRE site was chosen for further analysis. A construct having four repeats of CRE sequence derived from human AChE promoter was placed upstream of a luciferase reporter, namely pCRE4-Luc (Fig. 3B), and it stably transfected into NG108-15 cells. The CRE-driven luciferase activity was determined in the co-culture and compared with a similar CRE construct (pCRE-Luc) from a commercial source (Fig. 3D). Similar to pAChE-Luc stably transfected NG108-15 cells, the activity of pCRE4-Luc was induced over 3-fold by co-culturing with myotubes (Fig. 3C). The muscle-induced luciferase activity was in a time-dependent manner and reached a maximum after 2 days of co-culture (Fig. 3D); this time course was very similar to that of pAChE-Luc as in Fig. 2B. The responsiveness of pCRE4-Luc in the co-culture was in line with that of pCRE-Luc, but at weaker magnitude.

In order to further confirm the role of the CRE site on the promoter in regulating the muscle-induced neuronal AChE expression, the CRE-mutated human AChE promoter having a tagged luciferase reporter in an expression vector, namely pAChEACRE-Luc, was constructed (Fig. 4A); the mutation was shown to block the binding to CREB previously (25, 26). In stably transfected NG108-15 cells, luciferase activity driven by the human AChE promoter (pAChE-Luc) was markedly increased, at least 3−4-fold, by the application of Bt2cAMP or forskolin. However, the Bt2AMP- or forskolin-induced promoter activity was inhibited over 60% when the mutated promoter (pAChEACRE-Luc) was used in the stably transfected NG108-15 cells (Fig. 4B). Moreover, this blocking effect on pAChEACRE-Luc stably transfected NG108-15 cells was also revealed in the co-culture with chick myotubes (i.e. a drop of over 50% in the induction of reporter activity as compared with pAChE-Luc-expressing NG108-15 cells) (Fig. 4C). The complete inhibition by the CRE site mutation could be due to other cAMP-responsive regulatory elements on the human AChE promoter (26, 33), such as AP-2, revealed here in Fig. 3A.

**Muscle Extract Stimulates the Phosphorylation of CREB in Cultured NG108-15 Cells**—Muscle extracts from embryonic chick muscles were shown to block the binding to CREB previously (25, 26). In order to further confirm the role of the CRE site on the promoter in regulating the muscle-induced neuronal AChE expression, multiple copies of DNA sequences of different transcriptional elements, including CRE, AP-2, AP-1, and NF-κB, were tagged with luciferase in a mammalian expression vector as pCRE-Luc, pAP-2-Luc, pAP-1-Luc, and pNFκB-Luc, respectively. These constructs were stably transfected into NG108-15 cells and subsequently co-cultured with chick myotubes for 2 days. Only the DNA binding sequences of CRE and AP-2 showed robust induction (−3-fold) of luciferase activity after co-cultured with myotubes (Fig. 3A). Both CRE and AP-2 sites of the human AChE promoter have been reported to respond to the challenge of cAMP; however, the responsiveness of the AP-2 site to cAMP showed a lower magnitude than that of the CRE site (26, 33). Moreover, this CRE sequence of human AChE promoter was reported to mediate the cAMP-induced AChE expression in cultured NG108-15 cells (27). Therefore, the CRE site was chosen for further analysis. A construct having four repeats of CRE sequence derived from human AChE promoter was placed upstream of a luciferase reporter, namely pCRE4-Luc (Fig. 3B), and it stably transfected into NG108-15 cells. The CRE-driven luciferase activity was determined in the co-culture and compared with a similar CRE construct (pCRE-Luc) from a commercial source (Fig. 3D). Similar to pAChE-Luc stably transfected NG108-15 cells, the activity of pCRE4-Luc was induced over 3-fold by co-culturing with myotubes (Fig. 3C). The muscle-induced luciferase activity was in a time-dependent manner and reached a maximum after 2 days of co-culture (Fig. 3D); this time course was very similar to that of pAChE-Luc as in Fig. 2B. The responsiveness of pCRE4-Luc in the co-culture was in line with that of pCRE-Luc, but at weaker magnitude.

In order to further confirm the role of the CRE site on the promoter in regulating the muscle-induced neuronal AChE expression, the CRE-mutated human AChE promoter having a tagged luciferase reporter in an expression vector, namely pAChEACRE-Luc, was constructed (Fig. 4A); the mutation was shown to block the binding to CREB previously (25, 26). In stably transfected NG108-15 cells, luciferase activity driven by the human AChE promoter (pAChE-Luc) was markedly increased, at least 3−4-fold, by the application of Bt2cAMP or forskolin. However, the Bt2AMP- or forskolin-induced promoter activity was inhibited over 60% when the mutated promoter (pAChEACRE-Luc) was used in the stably transfected NG108-15 cells (Fig. 4B). Moreover, this blocking effect on pAChEACRE-Luc stably transfected NG108-15 cells was also revealed in the co-culture with chick myotubes (i.e. a drop of over 50% in the induction of reporter activity as compared with pAChE-Luc-expressing NG108-15 cells) (Fig. 4C). The complete inhibition by the CRE site mutation could be due to other cAMP-responsive regulatory elements on the human AChE promoter (26, 33), such as AP-2, revealed here in Fig. 3A. **Muscle Extract Stimulates the Phosphorylation of CREB in Cultured NG108-15 Cells**—Muscle extracts from embryonic chick myotubes. Over 4-fold induction of luciferase activity was revealed when pAChE-Luc-expressing NG108-15 cells were co-cultured with myotubes; similar fold induction was revealed at different concentrations of NG108-15 cell (Fig. 2A). The coculture effect was also revealed when NG108-15 cells were transfected with a construct of luciferase-tagged mouse AChE promoter (−2199 to −2184), namely pAChEm-Luc (Fig. 2A). The muscle-induced AChE expression in NG108-15 cell was time-dependent; the induction reached a maximum at −5-fold for both human and mouse AChE promoters after 2 days of co-culture (Fig. 2B). As a control, stable NG108-15 cells transfected with a β-galactosidase gene driven under a cytomegalovirus promoter were co-cultured with myotubes; however, the expression of β-galactosidase remained unchanged (Fig. 2A), which indicated a specific effect of muscle on AChE promoter activity. The induction of AChE expression in the co-culture was specific for muscle regardless of the origin from chick or mouse, and the co-culture of pAChE-Luc stably expressed NG108-15 cells with COS-7 or HEK293 cells showed no induction effect (Fig. 2C).
chicks were prepared and applied onto pAChE-Luc stably transfected cultured NG108-15 cells. The expression of endogenous mouse AChE at 68 kDa, recognized by species-specific antibody, was increased 2-3-fold by the application of muscle extract (Fig. 5A). The enzymatic activity of AChE was also increased (2-fold) in muscle-treated NG108-15 cells (Fig. 5A). The induction of AChE enzymatic activity was lower than that of AChE protein. This discrepancy could be due to the complication of AChE biosynthesis and assembly. Indeed, the cAMP/CGRP-induced AChE expression in chick myotubes has been reported to be biologically inactive (7). Fig. 5A shows that the cell extract (from mouse NG108-15 cells) did not contain any chick AChE, as indicated by the chick-specific anti-AChE antibody. Thus, the increase in AChE enzymatic activity was not due to contamination of the applied chick muscle extract in the preparation of muscle-treated NG108-15 cells. Other methods had been used in preparing the muscle extracts including cell disruption by sonication or by having different buffer or salt concentration; however, none of them was working as well as the current method. In addition, the extracts should be freshly prepared before the experiment, which preserved better the AChE induction activity (data not shown). The molecular form of AChE in muscle-treated NG108-15 cell cultures was determined, and the distribution of AChE molecular forms did not show significant variation (Fig. 5B). In contrast, application of Bt2cAMP induced an up-regulation of G1 form AChE. This suggested that although Bt2cAMP and muscle extract were able to induce AChE expression in cultured NG108-15 cells, their induction mechanism could be similar but not identical, at least in terms of their mechanism in inducing the distribution of different AChE molecular forms.

The role of muscle extract on the CRE-driven luciferase activity was determined in pCRE-Luc and pCRE HP-Luc stably transfected NG108-15 cells. The activity of pCRE HP-Luc or pCRE-Luc was induced over 3-fold maximum level by the treatment of muscle extract, and the induction was in a dose-dependent manner (Fig. 5C). Similarly, the luciferase activity driven by human AChE promoter in pAChE-Luc stably transfected NG108-15 cells was induced in a dose-dependent manner to over 2-fold the maximum after application of 1 mg/ml muscle extract. In contrast, the muscle-mediated transcriptional activity was markedly blocked by the CRE mutation on human AChE promoter (i.e. pAChE CRE-Luc stably transfected NG108-15 cells showed only an induction of <50% in the treatment of muscle extract) (Fig. 5D).

Overproduction of neuronal AChE has been reported to induce multileveled aberrations in the nmjs (34, 35). In order to mimic the in vivo situation, the human AChE promoter was...
tagged upstream of a chick AChE cDNA, namely pAChE-
cAChE (Fig. 5E). The choice of using chick AChE as a reporter was to distinguish that from the endogenous mouse enzyme being expressed by cultured NG108-15 cells. Similar to the case in using luciferase reporter, the expression of chick AChE driven by the human AChE promoter was induced to 3–4-fold the basal level by treating the cultured neurons with muscle extract, Bt2cAMP, or forskolin; this induction was in parallel to that of the endogenous enzyme (Fig. 5, E and F). The result indicated that the muscle-induced neuronal AChE overproduction did not show any feedback effect in regulating AChE expression in the cultured neurons.

Post-transcription regulation has been reported to be involved in neuronal AChE regulation (36, 37). Thus, the role of muscle extract in regulating the post-transcription gene expression was analyzed by using transcription and translation blockers. The muscle-induced AChE protein, recognized by antibody at ~68 kDa, was significantly blocked by actinomycin D or cycloheximide (Fig. 6, A and B), which indicated that the primary role of transcription in regulating the gene expression. However, the post-transcriptional regulation of AChE in these neurons could not be completely eliminated. Serving as a control here, the expression of α-tubulin was not affected by these treatments.

In cultured NG108-15 cells, application of muscle extract stimulated a transient increase of intracellular cAMP level with a peak of activation after 10 min (Fig. 7A). The accumulation of cAMP synthesis in muscle-treated NG108-15 cells was in a dose-dependent manner, and a maximum stimulation of ~40% increase was revealed at ~1 mg/ml muscle extract (Fig. 7B). The downstream signaling of the cAMP-dependent pathway was elucidated. CREB belongs to a group of transcription factors that is known to be activated by cAMP. In order to determine the relationship of CREB and AChE regulation, the extent of CREB phosphorylation was determined in NG108-15 cell cultures. Application of muscle extract onto cultured NG108-15 cells increased the phosphorylation of CREB. Fig. 7C shows that the anti-CREB antibody recognized a protein at ~45 kDa corresponding to total CREB, which remained unchanged in the presence of muscle extract, or Bt2cAMP, or forskolin. However, the extent of CREB phosphorylation, recognized by anti-phosphorylated CREB antibody, was increased to a peak of ~7-fold in the treatment of muscle extract. The muscle-stimulated CREB phosphorylation was transient, which returned to normal level after 1 h of activation (Fig. 7, C and D). The cAMP inducers, Bt2cAMP and forskolin, stimulated the cAMP accumulation over 6-fold; however, the inductions were sustained for over 5 h after the challenge (Fig. 7D). Similar to the muscle-induced cAMP accumulation, the phosphorylation of CREB induced by muscle extract in cultured NG108-15 cells showed a dose-dependent manner, having a maximum induction of ~7-fold above the background (Fig. 7, E and F).

CGRP Mimics the Effect of Muscle—CGRP is found at the nmjs and regulates the expression of postsynaptic muscle AChE in a cAMP-dependent manner (7, 8, 25). The possible role of CGRP in this muscle-induced gene activation therefore was tested. In cultured NG108-15 cells, application of CGRP stimulated the accumulation of intracellular cAMP in a dose-dependent manner (Fig. 8A). This dose-dependent effect was in parallel with the induction of CREB phosphorylation (Fig. 8B, left panel). In addition, the CGRP-induced CREB phosphorylation in the cultured neurons was transient, having a peak of induction after ~30 min of CGRP application (Fig. 8B, right panel). When the amount of endogenous AChE protein in CGRP-treated NG108-15 cells was probed by the specific antibody, the amount of AChE protein at ~68 kDa was increased over 3-fold. The enzymatic activity was also significantly induced by application of CGRP, although the extent of induction was not robust as that of AChE protein (Fig. 8C). The control protein α-tubulin at ~55 kDa remained unchanged.

Although CGRP could mimic the effect of muscle extract, the existence of CGRP in muscle has not been demonstrated. By using the phosphorylation of CREB as an assay system, the existence of CGRP or CGRP-like substance(s) in the muscle extract can be accounted for by the existence of CGRP or CGRP-like substance(s) in muscle extract. In contrast, CGRP4–37 did not show any blockade effect on the phosphorylation induced by Bt2cAMP (Fig. 9, A and B).

DISCUSSION

The primary objective of our laboratory is to determine the regulation and functional role of motor neuron AChE played during the formation of nmjs. Whereas DNA manipulation in cultured motor neurons technically is not feasible, NG108-15 cell could be an alternative neuronal cell model. Many aspects of NG108-15 cells are similar to the motor neuron: (i) they are both cholinergic in nature, secret acetylcholine, and form functional neuromuscular synapses when co-cultured with muscle.
(ii) both contain agrin as the primary AChR-aggregating factor that induces AChR aggregation in co-culturing with myotubes (29, 38); and (iii) the motor neuron-derived AChR-inducing activities, such as neuregulin, have also been demonstrated to be expressed in NG108-15 cells and play similar functions as that in motor neurons (39).

By using a chimeric co-culture of NG108-15 cells with chick myotubes in resembling the neuromuscular contact in vitro, we are hypothesizing that trophic factor(s) derived from the postsynaptic muscle acts on the innervating presynaptic motor neuron, which subsequently stimulated the expression of neuronal AChE by a cAMP-dependent signaling pathway. The stimulation is predominantly via the activation of the promoter activity. The transcriptional activation in the muscle-induced gene expression in cultured NG108-15 cells is mediated, at least being shown here, by a CRE site located on the AChE promoter. On the other hand, the post-transcriptional regulation of AChE has been proposed to play an important role in the increase of AChE transcripts during neuronal differentiation of P19 carcinoma induced by retinoic acid (36) and of PC12 cells induced by nerve growth factor (37). The incomplete blockage of muscle-induced AChE expression by using actinomycin D and cycloheximide suggested the possible effect(s) of muscle in affecting the post-transcriptional regulation. Whether the post-transcriptional regulation could be involved a cAMP-dependent pathway in the neuroblastoma cells, as described here, has not been determined. Nevertheless, the role of muscle in maintaining high level of AChE expression in the presynaptic neurons could be crucial during the formation and maintenance of vertebrate nmjs.

Several lines of evidence from previous studies support the aforementioned hypothesis. In developing chick spinal cord, the expression of globular AChE changed during development; the peak of expression was at the embryonic stage that overlapped with the critical period of the nmj formation (16). AChE expression in chick spinal cord was markedly reduced (over 50%) after the sciatic nerve denervation (17). Furthermore, preliminary studies from our laboratory suggested that the muscle extract described here, when applied onto cultured chick spinal motor neurons, was able to induce the AChE expression. These studies in chick spinal cord therefore strongly suggested the possible trophic role of postsynaptic muscle in maintaining the expression of presynaptic neuronal AChE. The cAMP-induced AChE expression in NG108-15 cells was also supported by the analyses on the promoter elements of the human AChE gene. Although the CRE site of human AChE promoter was suppressed by intracellular cAMP when the pro-
motor was expressed in muscle, the same CRE site when expressed in NG108-15 cells showed an activation effect (27). This reciprocal regulation between muscle and neuron was due to steric interference of the binding of regulatory element(s) onto CRE and E-box sites on the promoter (26).

We are hypothesizing that CGRP could act as an AChE-inducing factor for both muscle and motor neuron at the nmjs. CGRP is known to regulate AChE expression in cultured myotubes by a cAMP-dependent mechanism (7, 8, 9, 40). In addition, CGRP could mimic the effect of muscle-induced CREB phosphorylation as well as AChE induction in cultured NG108-15 cells. Although CGRP is known to be released by the motor neurons at the nmjs, whether muscle is able to make CGRP has not been demonstrated. Nevertheless, even in the absence of muscle-released CGRP, the synaptic CGRP released by the presynaptic nerve at the nmj could serve an autocrine function. Moreover, the contribution of CGRP at the cleft by the Schwann cell has not been determined. Previous studies have demonstrated the trophic actions of CGRP in cultured spinal neurons as well as the expression of its corresponding receptors. For example, the application of CGRP in spinal cord neuron cultures induced specific gene expression that was mediated by a cAMP-dependent signaling (41, 42).

Antagonist CGRP$_{8-37}$ was not able to block completely the muscle-induced CREB phosphorylation in cultured NG108-15 cells indicating that other trophic factor(s) in muscle should be considered. A number of neurotrophic factors such as BDNF, NT-3, NT-4, and GDNF are produced in muscle during synaptic development of the nmjs and retrograde-transported to the cell bodies of motor neurons, which therefore are able to regulate the presynaptic functions (43). In an in vivo analysis, muscle-derived NT-4 could act as a retrograde trophic signal for adult motor neurons to modulate the maintenance of the nmjs (43). In Xenopus neuron-muscle cultures, the batch-applied neurotrophins modulated the level of synaptic activity and efficacy at developing nmjs (44). Moreover, application of neurotrophic factors increased the expression of neuregulin in spinal cord neurons in both in vitro (45) and in vivo (46) analyses. In NG108-15 cell-muscle co-cultures, NT-3 derived from muscle was able to stimulate the formation of AChR aggregates, which could be mediated indirectly by Trk receptors expressed on NG108-15 cells (47). In axotomized rat facial motor neurons,
the denervation-induced down-regulation of AChE expression could be prevented by the infusion of NT-4/5 and BDNF, which are probably derived from muscles (48). Thus, neurotransmitter released by muscle and localized at the nmj is a possible candidate in regulating the expression of the presynaptic AChE, which now merits determination by using the established pAChE-Luc stably transfected NG108-15 cells.

ATP is an additional potential AChE-inducing factor at the nmj. ATP is co-stored in and constantly co-released quantitatively with acetylcholine from the nerve terminals or released by the muscle at the nmjs (49). Under normal physiological conditions, ATP concentration in the synaptic cleft could be in a range of 0.1–1 μM; these concentrations are adequate to obtain nearly maximal effects on its receptors in vitro preparations. The transcriptional activity of AChE in muscle has been shown to be mediated by the synaptic ATP (9). The ATP-induced AChE expression is mediated by P2Y1 receptors localized on the postsynaptic muscle membrane, which subsequently act through a mitogen-activated signaling pathway (10). Different types of ATP receptors (P2X and P2Y) are expressed by the presynaptic motor neurons, which are able to respond to the challenge of ATP at the nmjs (50, 51). However, its role in inducing the expression of AChE in motor neuron is not known yet.

The functional role of AChE expressed by motor neuron, particularly during the early stage of development, is an open question. In developing nmjs, the overexpression of exogenous AChE in Xenopus embryos could result in an increase of the length and the infolding of the postsynaptic membrane of nmjs (52, 53). Furthermore, the neurite growth-promoting activity of AChE has been demonstrated in cultured Xenopus spinal neurons. Spinal cord neurons from AChE-overexpressed Xenopus embryos grew ~3-fold faster and had a greater total neurite length by comparison with the control neurons (54). We are favoring the synaptogenic functions of motor neuron AChE during the formation and/or the maintenance of nmjs, and its expression is induced by the innervated muscle. Indeed, the expression of agrin, a synapse-inducing protein, was up-regulated by overexpression of AChE in cultured neurons, has been correlated with stages of neurite outgrowth (55). In addition to in the nmj, the transient expression of AChE during development of different brain regions, or cultured neurons, has been correlated with stages of neurite outgrowth (56, 57), and with the differentiation of neurons (27, 36, 37). In addition, the neurogenetic functions have also been suggested for AChE. The AChE expression in neuroblasts of Xenopus embryos could result in an increase of the

**Acknowledgments**—We are grateful to Tina Dong and H. Y. Choi from our laboratory for expert technical assistance.

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Muscle Induces Neuronal Expression of Acetylcholinesterase in Neuron-Muscle Co-culture: TRANSCRIPTIONAL REGULATION MEDIATED BY cAMP-DEPENDENT SIGNALING

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J. Biol. Chem. 2003, 278:45435-45444.
doi: 10.1074/jbc.M306320200 originally published online September 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306320200

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