Calcitonin gene-related peptide decreases expression of acetylcholinesterase in mammalian myotubes

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Abstract Nerve-derived trophic factors are known to modulate expression of acetylcholinesterase (AChE) in skeletal muscle fibers, yet the precise identity of these factors remains elusive. In the present study, we treated mouse C2 myotubes with calcitonin gene-related peptide (CGRP). Compared to non-treated myotubes, cell-associated AChE activity levels were decreased by $\sim 60\%$ after 48 h of treatment. A parallel reduction in AChE total protein levels was also observed as determined by Western blot analysis. The reduction in AChE activity was due to a decrease in the levels of the G_1 molecular form and to an elimination of G_4. By contrast, levels of secreted AChE remained unchanged following CGRP treatment. Finally, the overall decrease in AChE activity was accompanied by a reduction in AChE transcripts which could not be attributed to changes in the transcriptional rate of the ACHE gene.
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1. Introduction

Several lines of evidence indicate that interactions between motoneurons and their target muscle fibers regulate expression of acetylcholinesterase (AChE) in muscle tissue (for review see [1]). Although previous studies have shown that nerve-evoked electrical activity is a key regulator of AChE in muscle, it appears that nerve-derived trophic factors also play a significant role. In earlier studies for instance, nerve extracts applied to cultures of denervated muscle fibers were shown to maintain total AChE activity [2] whereas disruption of axonal transport with colchicine reduced AChE activity within muscle fibers [3]. In more recent reports, application of tetrodotoxin onto the sciatic nerve, which abolishes nerve action potentials without affecting axonal transport, was shown to lead to lesser reductions in both AChE activity and transcript levels as compared to the effects induced by denervation [4]. Taken together, these findings demonstrate the importance of nerve-derived molecules in directing AChE expression at the neuromuscular junction. Nonetheless, our current knowledge regarding the identity of specific nerve-derived trophic factors regulating AChE expression in muscle as well as the mechanisms by which they act is currently lacking.

Numerous studies focusing on the acetylcholine receptor (AChR) have also highlighted the role of both nerve-derived electrical activity and trophic substances in regulating expression of the genes encoding the various AChR subunits in synaptic versus extrasynaptic compartments of muscle fibers (for reviews see [5,6]). In particular, the calcitonin gene-related peptide (CGRP) has been shown to affect markedly expression of AChR in cultured myotubes [7–9] (for review see [6]). We therefore hypothesized in the present study that this factor may also regulate expression of AChE in mammalian muscle fibers, particularly since it was demonstrated recently that CGRP increased AChE mRNA expression in chick myotubes [10,11].

2. Materials and methods

2.1. C2 cell cultures and CGRP treatment

C2 cells were plated on Matrigel- (Collaborative Biomedical Products, Bedford, MA) coated 60 mm culture dishes and grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Life Sciences/Gibco; Burlington, Ont.) containing 10% fetal bovine serum and L-glutamine. When myoblasts became confluent, the growth medium was replaced with differentiation medium containing low serum. Five days after plating, myotubes were treated with rat CGRP (Sigma, St. Louis, MO) or an equal volume of sterile vehicle solution for 48 h. Since similar effects on AChE expression were observed with CGRP doses of 0.01, 0.05, 0.1 and 1 µM, we chose to treat cultured myotubes with 0.1 µM because several previous studies have in fact used this particular dose (see for example [7,10]). Following the first 24 h, the medium was collected and replaced with fresh differentiation medium containing either CGRP or vehicle solution.

To assess AChE activity secreted from vehicle- and CGRP-treated myotubes, diisopropyl fluorophosphate (DFP; Sigma)-treated horse serum was used to prepare the differentiation medium. Endogenous serum AChE was inactivated by incubating the horse serum with DFP for 48 h then allowing the DFP to degrade for 10 days.

2.2. Extraction and analysis of acetylcholinesterase enzyme activity

Vehicle- and CGRP-treated myotubes were homogenized for 30 s with a Polytron set at low speed in 1 ml of high-salt detergent buffer containing anti-proteolytic agents: 10 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 7.0), 10 mM EDTA, 1 M NaCl, 1% Triton X-100, 2.5 mg/ml aprotinin and 1 mg/ml bacitracin [12]. Samples were then centrifuged at 14 000 rpm for 15 min. The medium was collected after each 24 h period of vehicle or CGRP treatment. Samples were spun at 2000 rpm to remove cell debris. Following centrifugation, the supernatants obtained from the cell extracts and media were stored at −80°C.

Total AChE activity was determined using the spectrophotometric method of Ellman et al. [13] as described elsewhere [12,14]. The activity was measured in the presence of the non-specific cholinesterase inhibitor tetraisopropylpyrophosphoramide (iso-OMPA). The total amount of protein present in the extracts was determined by the bicinchoninic acid assay (BCA; Pierce Laboratories). AChE molecular form profiles were determined by sedimentation analysis according to Jasmin and Gisiger [12]. Creatine kinase activity was assayed using a commercially available kit (Sigma).

2.3. Western blot analysis

Protein extracts from cultured myotubes were denatured at 100°C for 5 min in a buffer containing 0.5% sodium dodecyl sulfate (SDS), 0.5% Triton, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.2 U/ml), 0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl and 0.025% NaN_3.

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Samples were separated by SDS-PAGE in a mini-PROTEAN II Cell apparatus (Bio-Rad, Richmond, CA). After separation, proteins were electroblotted onto a PVDF membrane (Schleicher and Schuell, Keene, NH) and the membrane was then incubated with a mouse monoclonal AChE antibody (Transduction Laboratories, Lexington, KY; 1:2500 dilution) for 1 h followed by a second incubation with a horseradish peroxidase-conjugated secondary antibody for 30 min (1:3000 dilution). The band corresponding to AChE (68 kDa) was visualized by chemiluminescence using an ECL kit (NEN Life Science Products, Boston, MA) and its intensity was quantitated with the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA).

2.4. Reverse transcription and polymerase chain reaction

Total RNA was isolated from myotubes using 2 ml of Trizol (Gibco) per dish. Following isopropanol precipitation, the final pellets were washed with 70% ethanol, air-dried and resuspended in RNase-free water. RNA samples were adjusted to a final concentration of 80 ng/μl using a GeneQuant II RNA/DNA spectrophotometer (Pharmacia). Reverse transcription (RT) of 2 μl of each RNA sample was carried out as previously described [4]. Negative control samples were also prepared by substituting the 2 μl of input RNA by the same volume of RNase-free water.

cDNAs encoding the AChE T subunit and ribosomal RNA (rRNA) were amplified using the polymerase chain reaction (PCR) as described in detail elsewhere [4,15]. Primers for mouse AChE (5'-CTGGGGTGCGGATCGGTGTACCCC, cDNA nucleotides 1175–1198; 3'-TCACAGGTCTGAGCAGCGCTCCTG, cDNA nucleotides 1821–1844) and S12 rRNA (5'-GGAAGGCATAGCTGCTGG, cDNA nucleotides 65–82; 3'-CCTCGATGACATCCTTGG, cDNA nucleotides 415–432) were synthesized on the basis of available sequences [16,17]. For these experiments, several precautions were taken to minimize sample contamination and mRNA degradation (see [18]) and to ensure that all measurements were taken within the linear phase of amplification (see [4,15,19]). Following amplification (AChE, 35 cycles; rRNA, 28 cycles), PCR reaction products were visualized on a 1% ethidium bromide-stained agarose gel. Quantitative PCR experiments were performed by separating PCR products on a Vistragreen (Amersham; Arlington Heights, IL)-stained agarose gel and by scanning the gel with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The intensity of the AChE bands was determined and standardized according to the rRNA PCR products using the accompanying ImageQuant software program.

2.5. Nuclear run-on analysis

Nuclear run-on assays were performed using a procedure described elsewhere [20]. Following in vitro transcription and RQ1 DNase (Promega) treatment, labelled RNA was isolated using Trizol and hybridized for 48 h at 42°C with 10 μg of linearized AChE (2 kb) and β-actin (2 kb) cDNAs and genomic DNA immobilized on Genescreen Plus nylon membrane (DuPont). Following hybridization, membranes were washed thoroughly (1×SSC, 0.1% SDS) at 42°C, and exposed for autoradiography. The intensity of the signals was quantified with a Storm PhosphorImager (Molecular Dynamics). The signals corresponding to AChE were standardized relative to the β-actin signal.

2.6. Statistical analysis

Paired Student's t-tests were performed to determine the impact of CGRP treatment on AChE expression. The level of significance was set at $P < 0.05$. Data are expressed as means ± S.E.M. throughout. The pattern of AChE molecular forms displayed in Figs. 1 and 3 are representative examples.

3. Results

As shown in Fig. 1A, total AChE activity expressed per culture dish was reduced by approximately 60% ($P < 0.05$) after CGRP treatment. AChE activity expressed per mg of extracted proteins was also reduced to the same extent following CGRP application. Similarly, primary cultures of mouse myotubes treated with CGRP also displayed a significant reduction in AChE expression (data not shown). Consistent
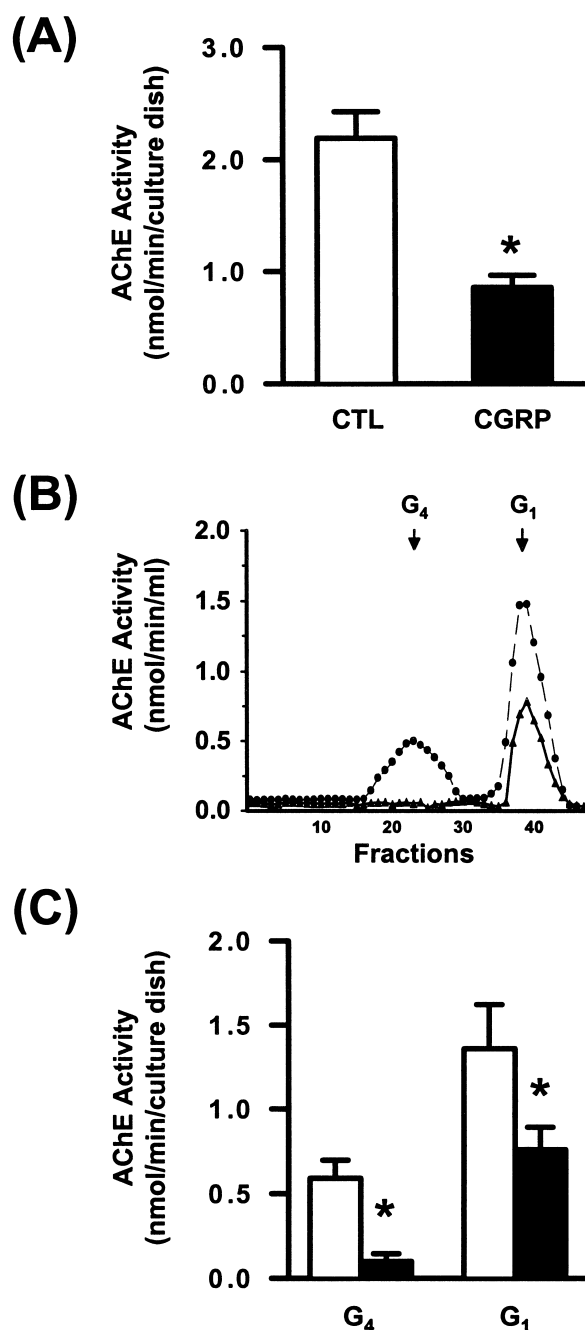

Fig. 1. Effects of CGRP on cell-associated AChE activity and molecular forms in myotubes. A: Analysis of AChE activity per culture dish measured from protein extracts isolated from vehicle- (control; CTL, $n = 7$ independent cell cultures) and CGRP-treated (CGRP, $n = 7$ independent cell cultures) cultured myotubes. B: Representative examples of AChE molecular form profiles obtained from vehicle- (circles) and CGRP-treated (triangles) myotubes. C: Activity of AChE molecular forms in vehicle-control (open bars) and CGRP-treated (closed bars) myotubes. Asterisks denote significant differences ($P < 0.05$).

with these activity data, Western blot analysis of extracts from vehicle- and CGRP-treated myotubes also revealed a significant reduction (control (CTL) = 9244 ± 296, $n = 3$; CGRP = 5037 ± 407, $n = 3$; arbitrary units; $P < 0.05$) in AChE protein levels (Fig. 2).

Velocity sedimentation analysis of extracts obtained from vehicle- and CGRP-treated myotubes revealed that the distri-

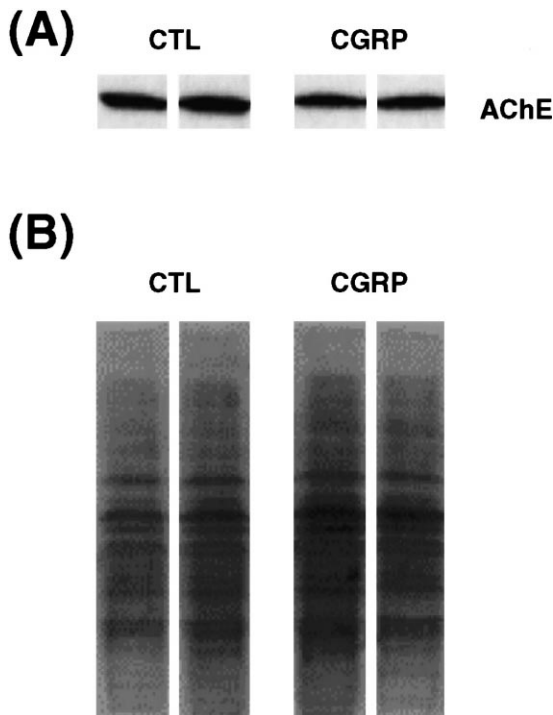**(A)**

CTL   CGRP

AChE

(B)

CTL   CGRP

Fig. 2. Effects of CGRP on AChE protein levels as determined by Western blot analysis. A: Shows representative examples of the immunoreactive 68 kDa AChE protein from vehicle-control (CTL) and CGRP-treated myotube extracts. B: PVDF membranes stained with Ponceau S which reveals that similar levels of proteins were loaded in each lane.

bution of AChE molecular forms was altered following CGRP treatment (Fig. 1B). Consistent with a recent report [21], we observed in control myotubes an elevated level of G_1 as well as a significant peak of G_4. By contrast, levels of G_1 were reduced considerably following CGRP treatment whereas the amount of tetramer was virtually eliminated (Fig. 1C). This latter effect of CGRP on G_4 levels appeared as a specific downregulation since even prolonged incubation (up to 24 h) of extracts from CGRP-treated myotubes with Ellman's assay buffer failed to yield detectable G_4 peaks.

We next determined whether AChE activity and molecular forms in the media were also affected by CGRP. Samples of differentiation medium collected after the first and second 24 h treatment periods were therefore assayed. Interestingly, total activity of the secreted enzyme remained unaltered in CGRP-treated myotubes in comparison to vehicle-treated cells (Fig. 3A; $P > 0.05$). As previously observed (see for instance [22]), a large proportion of secreted AChE was contributed by the globular tetramer, and CGRP did not modify this pattern of molecular form secretion (Fig. 3B,C). When total AChE activity was tabulated per culture dish, thus accounting for both cell-associated and secreted enzyme, we found that CGRP treatment downregulated AChE activity by approximately 20%. Creatine kinase levels, used as a marker of differentiation, were found to be nearly identical in vehicle- versus CGRP-treated myotubes (CTL = 5.07 ± 0.97, $n = 6$; CGRP = 5.32 ± 1.29, $n = 7$; arbitrary units; $P > 0.05$) thereby ruling out the possibility that the observed reductions in AChE levels in response to CGRP were due to differences in the stage of myotube differentiation.

Finally, we also examined whether the decrease in AChE

observed following CGRP treatment was accompanied by a concomitant reduction in the abundance of its mRNA. As shown in Fig. 4A,B, AChE transcript levels were reduced by approximately 30% ($P < 0.05$) following CGRP treatment. This decrease in AChE transcript levels is therefore in good agreement with the overall reduction in AChE enzymatic activity (cell-associated plus secreted; see above). Additional

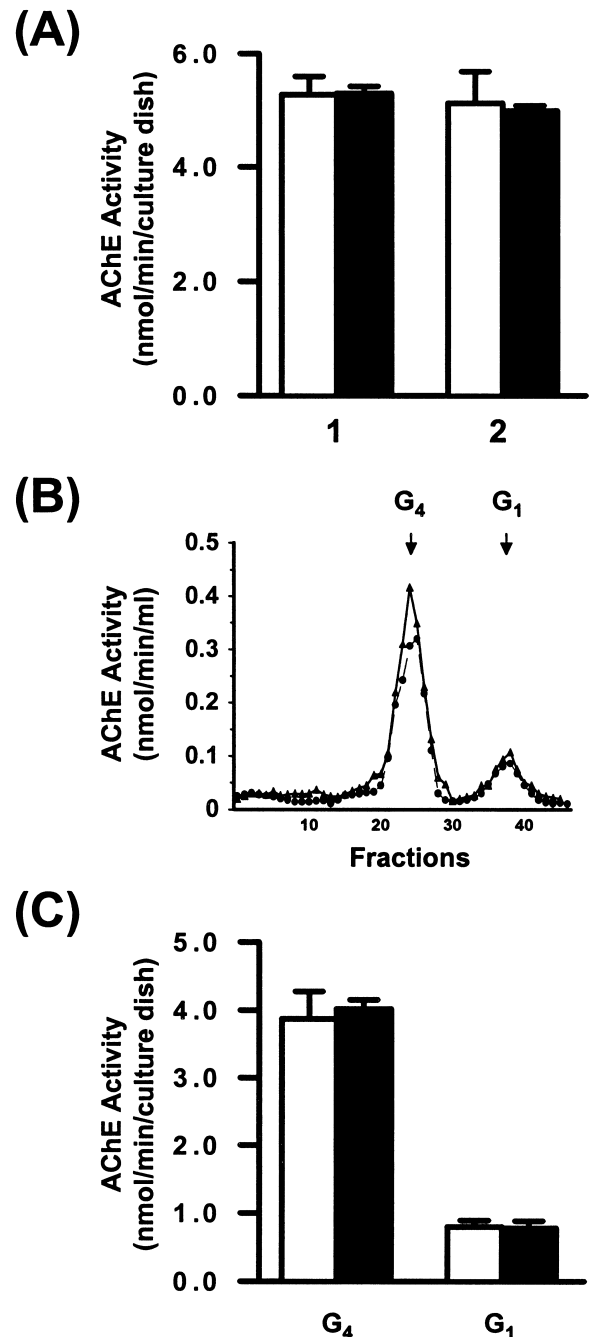**(A)**

(B)

(C)

Fig. 3. Effects of CGRP on AChE activity and molecular forms secreted in the media. A: Analysis of total secreted AChE activity following the first (1) and second (2) 24 h period of vehicle (open bars; $n = 4$ independent cell cultures) or CGRP (closed bars; $n = 4$ independent cell cultures) treatment. B: Representative examples of molecular form profiles obtained from the media of vehicle- (circles) and CGRP- (triangles) treated myotubes. C: Activity of AChE molecular forms in vehicle- (open bars) and CGRP-treated (closed bars) myotubes.
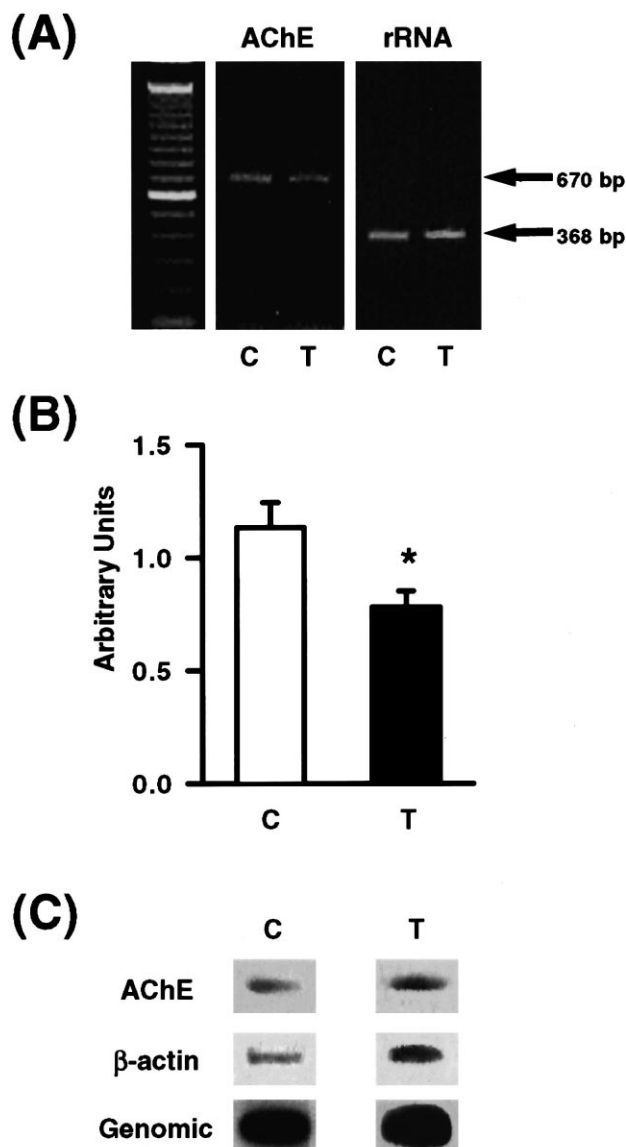
Fig. 4. Effects of CGRP on AChE mRNA levels in cultured mouse myotubes. A: Examples of ethidium bromide-stained agarose gels displaying AChE and rRNA PCR products from vehicle- (control; C) and CGRP-treated (T) samples. Left lane is the 100 bp molecular mass marker (Gibco, BRL). B: Quantitation of AChE mRNA levels in vehicle- (control, C; $n = 5$ independent cell cultures) and CGRP-treated (T; $n = 5$ independent cell cultures) myotubes standardized to rRNA. Asterisk denotes a significant difference ($P < 0.05$). C: Effects of CGRP on the transcriptional rate of the ACHE gene in cultured myotubes. This representative run-on assay was performed with nuclei isolated from vehicle- (control; C) and CGRP-treated (T) cultured myotubes. See text for quantitation.

experiments revealed that this reduction in AChE mRNA levels was not due to detectable changes in the transcriptional activity of the ACHE gene (Fig. 4C; CGRP 108% ± 13 of control; $n = 3$; $P > 0.05$) suggesting that CGRP likely exerts its effects at the post-transcriptional level.

4. Discussion

CGRP is a neuropeptide expressed by spinal cord motor neurons that has received considerable attention recently for its potential function at the neuromuscular junction (for re-

view see [6]). Until now however, the focus of most studies has been to characterize the role of CGRP in the regulation of AChR expression. For instance, CGRP has been shown to increase the number of cell surface AChR by approximately 1.5-fold [8]. In addition, CGRP selectively increases the level of transcripts encoding the AChR α-subunit by approximately 3-fold [7,9]. In the present investigation, we demonstrate that CGRP also exerts trophic effects on AChE expression by reducing enzyme activity as well as the abundance of AChE transcripts in mouse myotubes.

Modulations in AChE enzyme activity has been shown previously to correlate well with changes in AChE immunoreactivity in mammalian tissue. For instance, analysis of rat denervated muscle revealed a parallel reduction in both AChE activity and AChE protein levels [23]. Consistent with these findings, our data show a similar reduction in AChE activity and AChE protein levels in CGRP-treated cultured myotubes. Similarly, correlations between modifications in AChE enzyme activity and mRNA levels have also been reported. Following denervation of the rat extensor digitorum longus muscle for example, AChE activity is downregulated by 90% while AChE transcripts undergo a 10-fold reduction [4] (see also [19]). For these reasons, we hypothesized that the decrease in total AChE enzyme activity seen in response to CGRP treatment would be parallelled by a similar reduction in AChE transcript levels. Our findings support such a correlation.

In attempts to gain insight into the molecular mechanisms underlying these reductions in AChE mRNA levels, we performed run-on assays with nuclei isolated from control and CGRP-treated myotubes. Within the range of sensitivity of this assay, we failed to observe any significant change in the rate of transcription of the ACHE gene with CGRP treatment. Although there is evidence indicating that CGRP regulates transcription of different genes via the cAMP pathway, the contribution of this signalling cascade in the regulation of mRNA stability has also been documented [24–26]. Our results, in fact, fit nicely with the recent observations showing that stability of existing transcripts is the primary mechanism by which AChE mRNA levels are controlled in differentiating myotubes [27], neurons [28] and hematopoietic cells [29] maintained in culture. Taken together, these data indicate, therefore, that CGRP likely exerts its effects on AChE expression at the post-transcriptional level.

Analysis of AChE molecular forms further indicated that CGRP exerts its effects on the pattern of molecular forms expressed by these cultured myotubes. More specifically, CGRP significantly reduced the levels of G_4 in agreement with recent in vivo studies [30,31]. Since CGRP also reduced the amount of G_1, the absence of G_4 could therefore imply that CGRP simply downregulates translation of G_1 catalytic precursors necessary for the assembly of the tetramer. Analysis of the secreted forms of the enzyme, however, clearly demonstrates that CGRP-treated myotubes are still capable of assembling tetramers destined for secretion thereby indicating that the reduction of the cell-associated G_4 pool represents a specific effect.

Previous studies have provided compelling evidence that in skeletal muscle, levels of G_4 are regulated independently from the other molecular forms [12,32,33]. These studies have demonstrated in fast muscle, the presence of a separate pool of G_4 which can be increased substantially in response to high-fre-

quency neuromuscular activation. Such specific regulation has, in turn, led to the suggestion that the AChE tetramer fulfils a function altogether distinct from that assumed by the synaptic forms of AChE (see for instance [12]). Specifically, it has been suggested that G_4 may be responsible for the hydrolysis of excess acetylcholine released during high-frequency activation thereby preventing desensitization of the AChR. Interestingly, CGRP has previously been shown to directly increase the rate of desensitization of the AChR by modulating its phosphorylation status [34,35]. CGRP may therefore exert its effects both directly and indirectly by phosphorylating AChR and reducing G_4 levels, respectively, in order to ultimately increase the rate of AChR desensitization thereby modifying the efficacy of neurotransmission. Although the physiological relevance of increased AChR desensitization may be unclear, it appears reasonable to envisage that desensitization at the neuromuscular junction may provide a myoprotective mechanism necessary during periods of elevated phasic neuromuscular activity.

In a recent study, the effects of CGRP on AChE expression were also examined in cultured chick myotubes [10,11]. In this latter study, levels of AChE transcripts increased approximately 3-fold following CGRP treatment with no change in AChE activity. These data which stand in contrast to the results of the present investigation, are not entirely unexpected especially if we consider the dramatic differences already reported concerning the effects of muscle denervation on AChE expression in rodent versus chick. Specifically, whereas AChE activity levels are reduced substantially in denervated rat muscle, enzyme activity in avian muscle appears to increase [1]. Taken together with our current results, these findings provide further evidence that AChE levels in muscle are differentially regulated in response to the influence of neural factors in small rodents versus avian species.

References

[1] Massoulié, J., Pezzementi, L., Bon, S., Krejci, E. and Vallette, F.-M. (1993) Prog. Neurobiol. 41, 31–91.

[2] Davey, B., Younkin, L.H. and Younkin, S.G. (1979) J. Physiol. 289, 501–515.

[3] Fernandez, H.L. and Inestrosa, N.C. (1976) Nature 262, 55–56.

[4] Michel, R.N., Vu, C.Q., Tetzlaff, W. and Jasmin, B.J. (1994) J. Cell Biol. 127, 1061–1069.

[5] Hall, Z.W. and Sanes, J.R. (1993) Cell/Neuron 72, 1099–1121.

[6] Duclert, A. and Changeux, J.-P. (1995) Physiol. Rev. 75, 339–368.

[7] Fontaine, B., Klarsfeld, A. and Changeux, J.-P. (1987) J. Cell Biol. 105, 1337–1342.

[8] New, H.V. and Mudge, A.W. (1986) Nature 323, 809–811.

[9] Osterlund, M., Fontaine, B., Devillers-Thiery, A., Geoffroy, B. and Changeux, J.-P. (1989) Neuroscience 32, 279–287.

[10] Choi, R.C.Y., Leung, P.W.Y., Dong, T.T.X., Wan, D.C.C. and Tsim, K.W.K. (1996) Neurosci. Lett. 217, 165–168.

[11] Choi, R.C., Yung, L.Y., Dong, T.T., Wan, D.C., Wong, Y.H. and Tsim, K.W. (1998) J. Neurochem. 71, 152–160.

[12] Jasmin, B.J. and Gisiger, V. (1990) J. Neurosci. 10, 1444–1454.

[13] Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95.

[14] Gisiger, V. and Stephens, H.R. (1988) J. Neurosci. Res. 19, 62–78.

[15] Jasmin, B.J., Lee, R.K. and Rotundo, R.L. (1993) Neuron 11, 467–477.

[16] Forster, E., Otten, U. and Frotscher, M. (1993) Neurosci. Lett. 155, 216–219.

[17] Legay, C., Huchet, M., Massoulié, J. and Changeux, J.-P. (1995) Eur. J. Neurosci. 7, 1803–1809.

[18] Hubatsch, D.A. and Jasmin, B.J. (1997) Am. J. Physiol. (Cell Physiol.) 273, C2002–C2009.

[19] Sveistrup, H., Chan, R.Y.Y. and Jasmin, B.J. (1995) Am. J. Physiol. (Cell Physiol.) 269, C856–C862.

[20] Ray, A., Kyselovic, J., Leddy, J.L., Wigle, J.T., Jasmin, B.J. and Tuana, B.S. (1995) J. Biol. Chem. 270, 25837–25844.

[21] Luo, Z.D., Camp, S., Mutero, A. and Taylor, P. (1998) J. Biol. Chem. 273, 28486–28495.

[22] Rubin, L.L., Chalfin, N.A., Adamo, A. and Klymkowsky, M.W. (1985) J. Neurochem. 45, 1932–1940.

[23] Brimijoin, S., Hammond, P. and Rakonczay, Z. (1987) J. Neurochem. 49, 555–562.

[24] Oddis, C.V., Simmons, R.L., Hattler, B.G. and Finkel, M.S. (1995) Am. J. Physiol. (Heart Physiol.) 269, H2044–H2050.

[25] Danner, S., Frank, M. and Lohse, M.J. (1998) J. Biol. Chem. 273, 3223–3229.

[26] Tian, D., Huang, D., Short, S., Short, M.L. and Jungmann, R.A. (1998) J. Biol. Chem. 273, 24861–24866.

[27] Fuentes, M.E. and Taylor, P. (1993) Neuron 10, 679–687.

[28] Coleman, B.A. and Taylor, P. (1996) J. Biol. Chem. 271, 4410–4416.

[29] Chan, R.Y.Y., Adatia, F., Krupa, A.M. and Jasmin, B.J. (1998) J. Biol. Chem. 273, 9727–9733.

[30] Hodges-Savola, C.A. and Fernandez, H.L. (1995) Neurosci. Lett. 190, 117–120.

[31] Fernandez, H.L. and Hodges-Savola, C.A. (1996) J. Appl. Physiol. 80, 357–362.

[32] Fernandez, H.L. and Donoso, J.A. (1988) J. Appl. Physiol. 65, 2245–2252.

[33] Boudreau-Larivière, C., Gisiger, V., Michel, R.N., Hubatsch, D.A. and Jasmin, B.J. (1997) Am. J. Physiol. (Cell Physiol.) 272, C68–C76.

[34] Mulle, C., Benoit, P., Pinset, C., Roa, M. and Changeux, J.-P. (1988) Proc. Natl. Acad. Sci. USA 85, 5728–5732.

[35] Miles, K., Greengard, P. and Huganir, R.L. (1989) Neuron 2, 1517–1524.