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Short- and long-term influences of calcitonin gene-related peptide on the synthesis of acetylcholinesterase in mammalian myotubes

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1 The present study analyses the short- (15 min–2 h) and long-term (24–48 h) influences of calcitonin gene-related peptide (CGRP) on acetylcholinesterase (AChE) expression in the rat cultured skeletal muscle and the signal transduction events underlying CGRP actions.

2 To assess the effect of CGRP on AChE synthesis, myotubes were pre-exposed to the irreversible AChE inhibitor diisopropyl fluorophosphate (DFP) and treated with CGRP or forskolin, an adenylyl cyclase (AC) activator. Treatment of myotubes with 1–100 nM CGRP for 2 h increased by up to 42% the synthesis of catalytically active AChE with a parallel increase in the intracellular cyclic AMP.

3 The stimulation of AChE synthesis induced by CGRP was mimicked by direct activation of AC with 3–30 μM forskolin. In contrast, pre-treatment of cultures with 100 nM CGRP for 20 h reduced by 37% the subsequent synthesis of AChE, resulting in a 15% decrease in total AChE activity after 48 h CGRP treatment.

4 Moreover, 24 h treatment of myotubes with 100 nM CGRP reduced by 54% the accumulation of cyclic AMP induced by a subsequent CGRP treatment.

5 These findings indicate that, in skeletal muscle cells, CGRP modulates the AChE expression in a time-dependent manner, initially stimulating the enzyme synthesis through a cyclic AMP-dependent mechanism. The decreased AChE synthesis observed after long-term CGRP treatment suggests that CGRP signalling system is subject to desensitization or down-regulation, that might function as an important adaptative mechanism of the muscle fibre in response to long-term changes in neuromuscular transmission.

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Keywords: Calcitonin gene-related peptide; acetylcholinesterase; adenylyl cyclase; neuromuscular junction; skeletal muscle

Abbreviations: AC, adenylyl cyclase; AChE, acetylcholinesterase; AChR, nicotinic acetylcholine receptor; CGRP, calcitonin gene-related peptide; DFP, diisopropyl fluorophosphate; D-MEM, Dulbecco's Modified Eagle Medium; FCS, foetal calf serum; HBSS, Hanks' balanced salt solution; HS, horse serum; IBMX, 3-isobutyl-1-methylxanthine; Iso-OMPA, tetraisopropyl pyrophosphoramide; PKA, cyclic AMP-dependent protein kinase

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide generated by the alternative splicing of the calcitonin gene in the central and peripheral nervous system (Amara *et al*., 1982; Rosenfeld *et al*., 1983). In motoneurons, electrical stimulation of the nerve induces the release of CGRP which interacts with specific postsynaptic receptors and functions as a regulatory factor (Popper & Micevych, 1989; Uchida *et al*., 1990; Roa & Changeux, 1991). The biological effects of CGRP on the skeletal muscle are mediated by the stimulation of specific G protein coupled-receptors linked to cyclic AMP elevation through the activation of adenylyl cyclase (AC) (Laufer & Changeux, 1987; Uchida *et al*., 1990).

In the vertebrate skeletal muscle, activation of CGRP receptors potentiates neurally evoked muscle contraction (Takami *et al*., 1985; Takamori & Yoshikawa, 1989), increases the synthesis and the insertion of nicotinic acetylcholine receptor (AChR) in the membrane (Fontaine *et al*., 1986; New & Mudge, 1986) and enhances the rate of AChR desensitization (Mulle *et al*., 1988).

Recent studies have also described the influences of CGRP on acetylcholinesterase (AChE, E.C.3.1.1.7) expression. For example, in chick primary myotube cultures, 16–24 h exposure to CGRP substantially increased mRNA and the non-catalytic pool of AChE without significant changes in the enzyme activity (Choi *et al*., 1998). Conversely, mouse myotube cultures exposed to CGRP for 48 h showed a decreased expression of AChE and its transcripts (Boudreau-Larivière & Jasmin, 1999). These divergent effects have been attributed to different regulatory mechanisms involved on skeletal muscle AChE expression, in rodent and avian (Boudreau-Larivière & Jasmin, 1999). However, independently of the animal species studied, these results suggest that long-term exposure to CGRP could alter the functional organization of CGRP receptor-signalling system resulting in either desensitization or down-regulation of the receptor-transduction system.

Taking into account that the actual knowledge regarding the influences of CGRP on the skeletal muscle AChE is based

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mainly on the long-term effects of this neuropeptide and that CGRP receptors might be subjected to desensitization or down-regulation after chronic stimulation (Drake *et al*., 1999), we studied the short- and long-term influences of CGRP on the synthesis of AChE in rat primary cultured myotubes. More importantly, considering that CGRP may regulate AChE biogenesis through adenylyl cyclase activation, we also analysed the short- and long-term effects of CGRP treatment on the intracellular levels of cyclic AMP.

Our present findings indicate that in rat skeletal muscle cultures, CGRP induces a transient increase on the synthesis of AChE that parallels an enhancement in the intracellular cyclic AMP content. We also show evidence that the reduction in AChE activity after long-term treatment of myotubes with CGRP is mediated by a down-regulation of CGRP receptor/AC transduction system. These short- and long-term effects of CGRP on the regulation of protein expression in the neuromuscular junction may be an important mechanism to the skeletal muscle plasticity in response to changes in the physiological demand of this synapse.

Methods

Skeletal muscle cultures

Primary skeletal muscle cultures were obtained from hindlimb muscles of newborn rats. Briefly, the animals were killed under CO_2 anaesthesia, the muscles were removed and the cells dissociated in Hanks' balanced salt solution (HBSS), pH 7.4, containing collagenase type IA (200 u ml^{-1}). The myoblasts (3×10^5 cells ml^{-1}) were grown on collagen-coated 35 mm dishes in 2 ml of Dulbecco's Modified Eagle Medium (D-MEM, Gibco – BRL, Gaithersburg, U.S.A.) supplemented with 10% horse serum (HS), 10% foetal calf serum (FCS) and 40 μg ml^{-1} gentamicin, at 37°C in humidified atmosphere of 90% air and 10% CO_2. The medium was replaced on the third day and every other day with D-MEM supplemented with 10% HS and 2% FCS (complete culture medium). Drug treatments were performed on 6 – 7-day-old cultures. At this stage, myotubes were multinucleated and contracting cells indicating an appropriate maturation of the muscle fibres.

In some experiments, the effect of CGRP was analysed on chicken cultured myotubes obtained from hindlimb muscles of 10-day-old embryos using the same procedure described above.

Effect of drugs on total AChE activity and on the synthesis of catalytically active enzyme

Rat cultured myotubes were incubated with 10 – 1000 nM CGRP in complete culture medium at 37°C in humidified atmosphere of 90% air and 10% CO_2. After 24 or 48 h, the cells were rinsed three times with HBSS, the AChE was extracted and the enzyme activity assayed as described below. To avoid CGRP degradation after 48 h treatment, following the first 24 h CGRP treatment, the medium containing CGRP was replaced with fresh medium containing CGRP as described by Boudreau-Larivière & Jasmin (1999).

To determine whether activation of CGRP receptor or adenylyl cyclase stimulated the ongoing myotube AChE synthesis, 7-day-old muscle cultures were rinsed three times with HBSS, pH 7.4, followed by 10 min incubation with 100 μM diisopropyl fluorophosphate (DFP) to irreversibly inhibit all AChE molecules. The cells were rinsed with HBSS, to remove the unreacted DFP and allowed to synthesize new AChE in complete culture medium, in the presence or absence of CGRP (1 – 100 nM), the adenylyl cyclase activator forskolin (3 – 30 μM) or vehicle solution.

The effects of short- and long-term stimulation of CGRP receptor on the synthesis of AChE was evaluated in cultured myotubes incubated with 10 nM CGRP for 15 – 30 min or 20 h, respectively. Then, the myotubes were treated with DFP and allowed to synthesize AChE in complete culture medium for 2 h.

Extraction of total AChE and enzyme activity assay

AChE was extracted by scraping the cells using 500 μl of 20 mM borate extraction buffer, pH 9.0 (containing 1 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 5 mM *n*-ethylmaleimide, 2 mM benzamidine and 0.7 mM bacitracin). The samples were centrifuged for 30 min at 14,000 r.p.m., at 4°C and total AChE activity from the supernatant was assayed by radiometric procedure (Johnson & Russell, 1975) as described by Rotundo & Fambrough (1979) using ^{3}H-ACh (0.1 μCi, 24 mM; specific activity = 55.2 μCi $mmol^{-1}$) as substrate. The enzyme activity was assayed in the presence of the butyrylcholinesterase inhibitor tetraisopropyl pyrophosphoramide (Iso-OMPA, 10 μM) and the total AChE activity (d.p.m. h^{-1}) was expressed as arbitrary units (U).

cyclic AMP accumulation and adenylyl cyclase activity assay

Cultured myotubes were rinsed two times with Krebs bicarbonate solution, pre-incubated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min and treated with CGRP (1 – 1000 nM), forskolin (3 – 30 μM) or vehicle solutions, at 37°C. After 15 – 60 min, the medium was aspirated and the reaction stopped with 500 μl of cold Krebs solution containing 4 mM EDTA. The samples were transferred to microfuge tubes, boiled for 10 min and centrifuged at 14,000 r.p.m. for 10 min. Cyclic AMP from the supernatant was determined using the cyclic AMP kit [^{3}H]-assay system. The results were expressed as pmol of cyclic AMP per culture dish or per mg of protein. Protein was measured by the method of Bradford (1976).

To analyse the effect of long-term CGRP receptor stimulation on the production of cyclic AMP, cultured myotubes were treated for 24 h with 100 nM CGRP, 10 μM forskolin or vehicle in complete culture medium, rinsed three times with Krebs solution and re-exposed to CGRP for 15 min at 37°C in the presence of 0.1 mM IBMX, as described above.

Drugs

Rat alpha-calcitonin gene-related peptide, diisopropyl fluorophosphate, forskolin, 3-isobutyl-1-methylxanthine and tetraisopropyl pyrophosphoramide were from Sigma Chemical Co. (St. Louis U.S.A.), Dulbecco's Modified Eagle Medium, gentamicin, horse serum donor herd and foetal calf serum were from Gibco-BRL (Life Technologies, Grand Island, NY, U.S.A.);

acetylcholine iodide [acetyl-^{3}H] (2.0 GBq mmol^{-1}; 55.2 μCi mmol^{-1}) was from New England Nuclear, (Boston, U.S.A.) and AMP kit [^{3}H]-assay system was from Amersham-Pharmacia Biotech, UK Limited (Buckinghamshire, England).

Statistical analysis

The results were expressed as mean values ± s.e.mean of at least three independent determinations. Differences between means were analysed by Student's *t*-test or one-way analysis of variance followed by Newman Keuls multiple comparison test. The level of significance was set at $P<0.05$.

Results

Developmental changes on myotube AChE activity

During the first 2 days after plating, mononucleate myoblasts proliferated and fused to form multinucleated myotubes. At this stage, the protein content and total AChE activity was 0.190 mg dish^{-1} and 76.1 ± 9.2 U, respectively (Figure 1). From day 2 to 6 after plating, the myotube protein content increased 4.3 times stabilizing at 0.817 ± 0.019 mg dish^{-1} whereas total AChE activity increased by 5.3 times (479.2 ± 28.3 U), reaching 614.4 ± 9.8 U at day 8. Taking

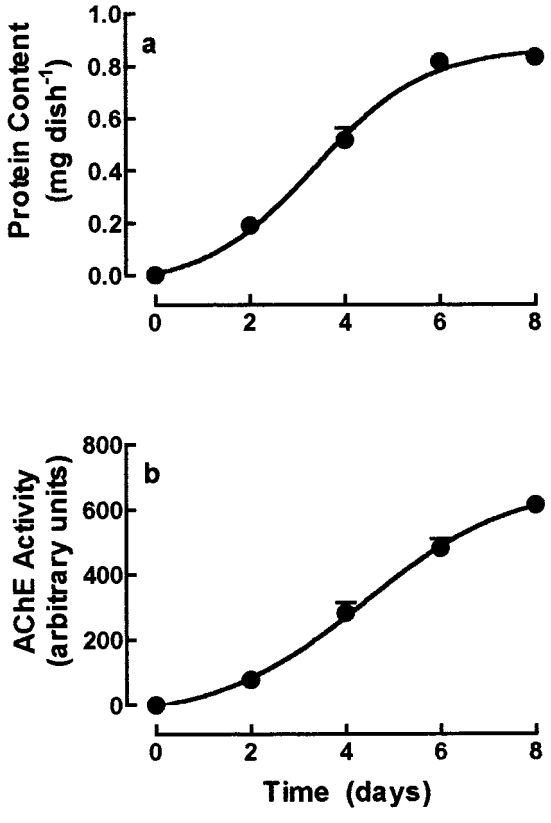

Figure 1 Protein content (a) and total AChE activity (b) of rat skeletal muscle cultures. Myoblasts from newborn rats were platted at day zero. The AChE was extracted on day 2, 4, 6 or 8 and the total enzyme activity assayed using ^{3}H-ACh as substrate. Protein content was analysed as described by Bradford (1976). Each point represents the mean ± s.e.mean; $n=3$.

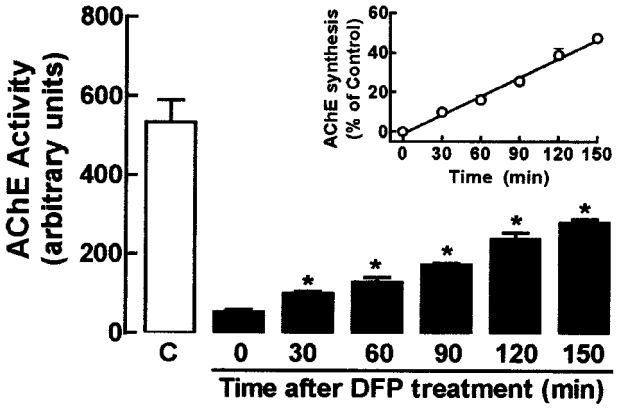

Figure 2 Synthesis of catalytically active AChE in control cultured myotubes. Rat cultured myotubes were treated with 100 μM DFP to irreversibly inhibit all AChE molecules and allowed to synthesize new AChE in complete culture medium. After 30 to 150 min, AChE was extracted and the total enzyme activity assayed using ^{3}H-ACh as substrate. Each column represents the mean ± s.e.mean; $n=3$. *Significantly different from time zero, $P<0.05$. Inset: rate of synthesis of catalytically active AChE expressed as percentage of the total AChE activity in control conditions.

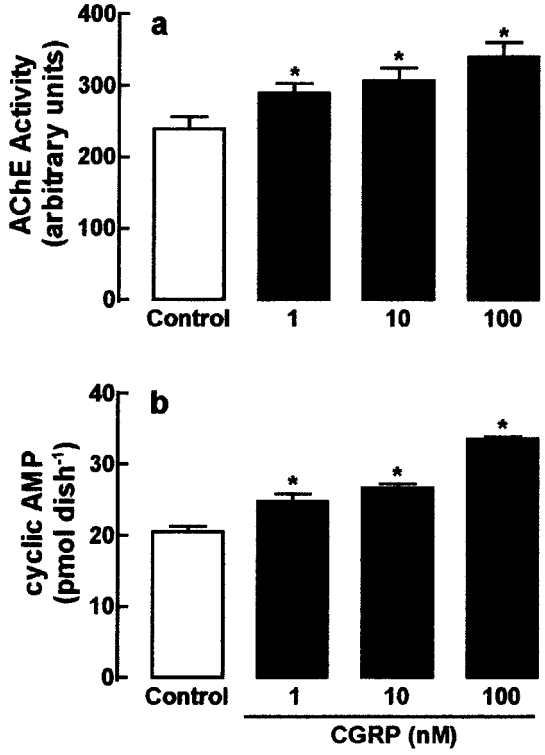

Figure 3 Short-term treatment of myotubes with CGRP stimulates the AChE synthesis and cyclic AMP production. (a) Rat cultured myotubes were treated with 100 μM DFP and allowed to synthesize new AChE in control conditions or in presence of 1, 10 or 100 nM CGRP for 2 h; $n=3$. AChE was extracted and total enzyme activity assayed using ^{3}H-ACh as substrate. (b) myotubes, pre-incubated with 1 mM IBMX (control), were treated with 1, 10, or 100 nM CGRP for 15 min at 37°C. The cyclic AMP was extracted and measured using a cyclic AMP [^{3}H]-assay kit. Each column represents the mean ± s.e.mean; $n=3$. *Significantly different from control myotubes, $P<0.05$.

into account that 5–6-day-old cultures exhibited spontaneous contraction, which indicate an appropriate maturation of the muscle fibres, all drug treatments were performed on 6–7-day-old cultures.

The synthesis of catalytically active AChE was analysed after treatment of 7-day-old myotubes with DFP, an irreversible inhibitor of the enzyme. Immediately after removal of unreacted DFP, the cultured myotubes AChE activity was 52.4±5.7 U which represented less than 10% of the activity of untreated control cultures (534.0±56.3 U). Within 1 and 2 h, the activity of AChE was 24% (129.0±12.5 U) and 45% (239.0±16.9 U) of the total AChE activity from control cultures, respectively (Figure 2). The rate of synthesis, calculated by linear regression analysis, corresponded to 18% of total AChE per hour (Figure 2, inset).

Short-term effects of CGRP treatment on the synthesis of myotube AChE and cyclic AMP production

Two hours incubation of 1, 10 and 100 nM CGRP increased the activity of newly synthesized AChE by 21% (289.0±13.0 U), 28% (306.5±18.0 U) and 42% (339.7±20.5 U) relative to the control values (239.0±16.9 U), respectively (Figure 3a).

Since stimulation of myotube CGRP receptor is known to activate the G protein-coupled adenylyl cyclase, we analysed the effect of CGRP on the cyclic AMP production. As expected, the increased synthesis of AChE induced by CGRP was followed by a proportional increase in the myotube cyclic AMP content (Figure 3b). In presence of 1.0 mM IBMX, the cyclic AMP content in control cultured myotubes was 20.4±0.8 pmol dish^{-1}. Treatment of cells with 1, 10 and 100 nM CGRP for 15 min, increased the basal cyclic AMP level by 22, 31 and 64%, respectively (Figure 3b).

Like CGRP, direct activation of AC stimulated the expression of AChE. In DFP pre-treated cultures, incubation of 3, 10 and 30 μM forskolin for 2 h increased the AChE synthesis by 33% (122.4±8.4 U), 51% (139.3±1.4 U) and 98% (182.1±11.1 U) of control values (92.2±4.7 U, Figure 4a) respectively. This effect was followed by a 35, 415 and 810% increase in the myotube cyclic AMP content (20.4±0.8 pmol dish^{-1}; Figure 4b).

To determine the onset of CGRP effect on AChE synthesis, in another series of experiments rat and chicken myotubes were pre-exposed to CGRP for 15 and 30 min, respectively, treated with DFP for 10 min and allowed to synthesize AChE for 2 h in complete medium. As shown in Figure 5a, short-term treatment of myotubes with CGRP increased the subsequent synthesis of AChE by 17%. This effect was also observed in chick cultured myotubes resulting in an 11 and

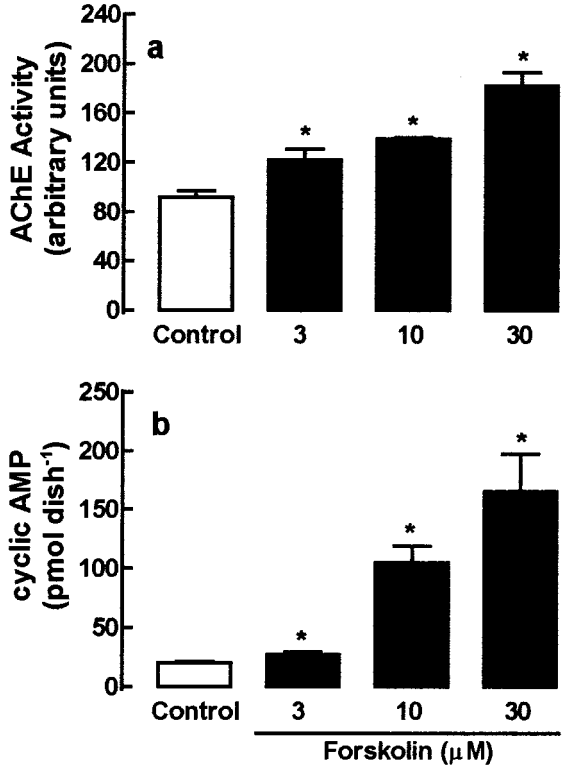

Figure 4 Activation of adenylyl cyclase stimulates the synthesis of myotube AChE. (a) Rat cultured myotubes were treated with 100 μM DFP and allowed to synthesize new AChE in control conditions or in presence of 3, 10 or 30 μM forskolin for 2 h; $n=3$. AChE was extracted and total enzyme activity assayed using ^{3}H-ACh as substrate. (b) myotubes, pre-incubated with 1 mM IBMX (control), were treated with 3, 10 or 30 μM forskolin for 15 min at 37°C. The cyclic AMP was extracted and measured using a cyclic AMP [^{3}H]-assay kit. Each column represents the mean±s.e.mean; $n=3$. *Significantly different from control myotubes, $P<0.05$.

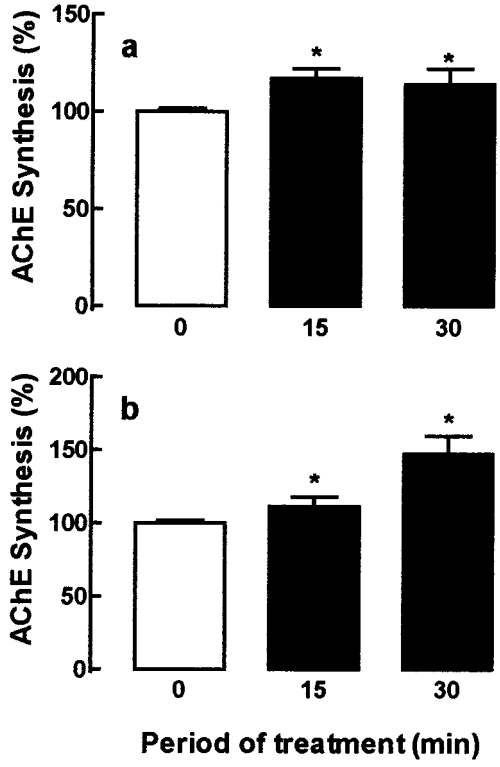

Figure 5 Rat (a) or Chicken (b) cultured myotubes, pre-exposed to 10 nM CGRP for 15 or 30 min were treated with 100 μM DFP for 10 min and allowed to synthesize AChE in complete culture medium for 2 h. AChE was extracted and total enzyme activity assayed using ^{3}H-ACh as substrate. Each column represents the mean±s.e.mean; $n=4$. *Significantly different from control myotubes (time zero), $P<0.05$.

47% increase in AChE synthesis after 15 and 30 min, respectively (Figure 5b).

Long-term effects of CGRP treatment on the synthesis of myotube AChE

The stimulant effect of CGRP on AChE however was not persistent. When rat myotubes were exposed to 10–1000 nM CGRP for 24 h, total AChE activity was not significantly different from the control values (600.8±44.1 U) (Figure 6a). However, application of 100 nM CGRP for 48 h reduced the total AChE activity to 85% (690.7±31.8 U) of control values (790.8±28.4 U) (Figure 6b). As observed with CGRP, treatment of cultures with 3, 10 and 30 μM forskolin for 24 h did not increase the total AChE activity when compared to control values (613.5±59.6 U) (Figure 6c).

The absence of CGRP effect observed on total AChE activity after 24 h treatment could be explained by a reduction of the synthesis observed during prolonged exposure to CGRP. When myotubes were pre-exposed to 100 nM CGRP for 20 h, treated with DFP for 10 min and allowed to synthesize AChE in complete culture medium for 2 h, the synthesis of AChE was reduced by 37% of control values (298.9±11.7 U) (Figure 6d), suggesting that the inhibitory effect of CGRP on AChE synthesis is a time-dependent process.

The stimulation of cyclic AMP production induced by the CGRP was also transient. Figure 7 shows the time course of CGRP effect on cyclic AMP accumulation. Addition of 100 nM CGRP increased the cyclic AMP content to a maximum of 64–68% (37.9±0.4 to 39.9±1.8 pmol dish^{-1}) of control values after 15–30 min stimulation, respectively. However, after 60 min stimulation, the cyclic AMP content (22.2±2.3 pmol dish^{-1}) returned to the levels of non-stimulated cultures. The basal level of cyclic AMP, determined in the presence of 1 mM IBMX alone, was not significantly changed from 15–60 min (20.9±2.9 to 23.8±1.4 pmol dish^{-1}, respectively).

Long-term exposure of myotubes to CGRP desensitize the cellular response to the neuropeptide

The lack or even the inversion of CGRP effect on the AChE expression after 24 or 48 h treatment suggests that the receptor-signalling system could be restrained by long-term exposure to activators. To analyse the influence of long-term CGRP treatment on the activation of AC, cultured myotubes were pre-exposed to 100 nM CGRP for 24 h. The cells were then rinsed with HBSS and immediately afterwards re-exposed to the same concentration of CGRP for 15 min in the presence of IBMX. Figure 8a shows that treatment of myotubes with CGRP increased by 63% (6.7±0.3 pmol mg protein^{-1}) the basal intracellular cyclic AMP (4.1±0.1 pmol mg protein^{-1}). However, pre-treatment of myotubes with CGRP attenuated by 54% the production of cyclic AMP (5.3±0.4 pmol mg^{-1} protein^{-1}) induced by a subsequent CGRP-treatment.

To evaluate if long-term activation of AC could also interfere on CGRP-induced AC stimulation, the cyclic AMP

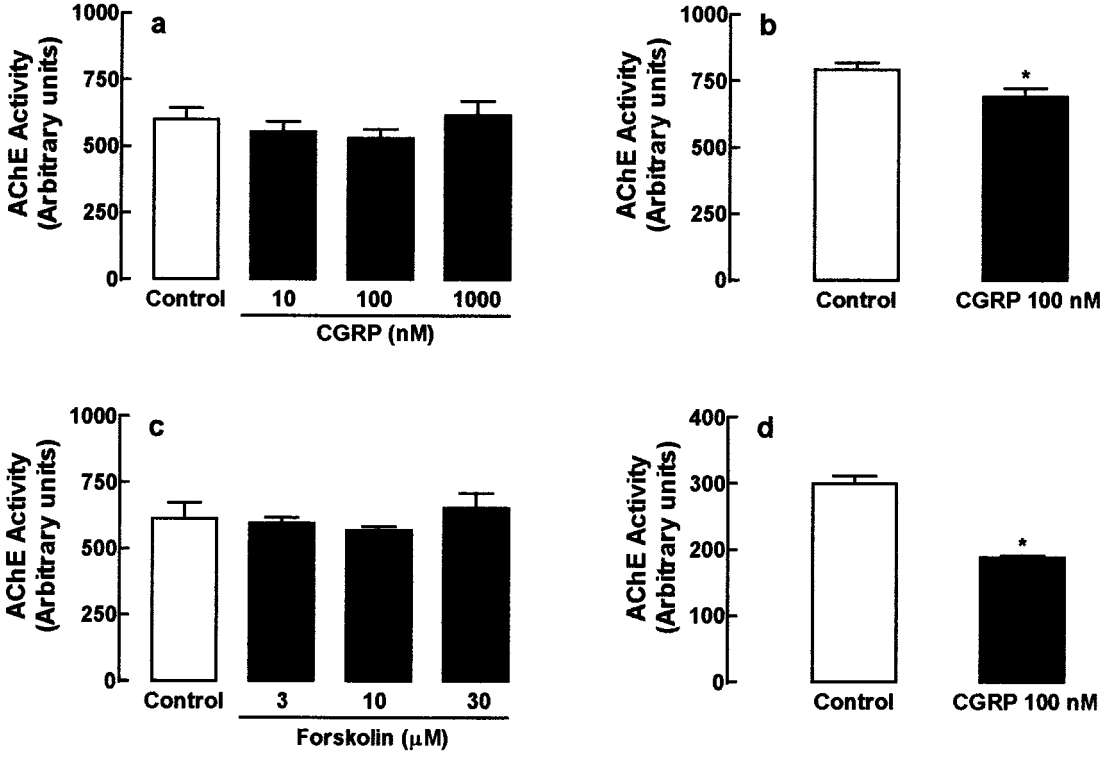

Figure 6 Long-term effect of CGRP and forskolin on myotube AChE. Myotubes were incubated with 10–1000 nM CGRP (a) for 24 h or (b) for 48 h or with 3–30 μM forskolin (c) for 24 h in complete culture medium, $n=3-4$. (d) Myotubes pre-exposed to 100 nM CGRP for 20 h were treated with DFP for 10 min and allowed to synthesize AChE in complete culture medium for 2 h; $n=5$. AChE was extracted and total enzyme activity assayed using ^{3}H-ACh as substrate. Each column represents the mean±s.e.mean. *Significantly different from control, $P<0.05$.

production was evaluated in myotubes pre-exposed or not to 10 μM forskolin for 24 h. As shown in Figure 8b, treatment of myotubes with CGRP alone for 15 min increased by 28% the basal cyclic AMP content (4.3 ± 0.1 pmol dish^{-1}). However, CGRP was unable to stimulate the production of cyclic AMP when myotubes were pre-treated with forskolin for 24 h. In fact, the cyclic AMP content (3.7 ± 0.2 pmol dish^{-1}) was reduced by 12%, indicating that CGRP lost its ability to stimulate cyclic AMP production.

Discussion

The efficiency of the neuromuscular transmission depends on the adequate expression of AChE and AChR which in turn is modulated by the electromechanical activity of the skeletal muscle and by nerve-derived trophic substances (for review, see Massoulié *et al*., 1993; Duclert & Changeux, 1995). Several studies have indicated that CGRP might play an important role in the development of the neuromuscular synapse by regulating the expression of AChR at embryonic and adult muscle fibre. The presence of CGRP high affinity binding sites in skeletal muscle membrane has been demonstrated in mature fibres and in tissue cultured myotubes (Roa & Changeux, 1991; Jennings & Mudge, 1989). In the skeletal muscle fibre, these receptors are coupled to heterotrimeric G protein (Gs) and mediate an increase in cyclic AMP production through activation of adenylyl cyclase (van Rossun *et al*., 1997; review).

The present report shows, for the first time, that in rat primary skeletal muscle cultures, the CGRP modulation of AChE synthesis is a time-dependent process. Short-term stimulation of CGRP receptors increases the intracellular content of cyclic AMP and induces a proportional enhancement of AChE synthesis. Since direct stimulation of adenylyl cyclase with forskolin also increases AChE synthesis, we concluded that cyclic AMP signalling pathway is involved in CGRP effects. Also, 15–30 min exposure to CGRP stimulated the synthesis of chick myotube AChE, indicating that the primary effect of CGRP in avian and in mammalian species is the increase of AChE synthesis. This result is consistent with recent studies indicating that high levels of cyclic AMP stimulate the synthesis of AChE in avian myotubes (Choi *et al*., 1998). Moreover, cyclic AMP responsive element (CRE) sequences were found either in mouse (Atanasova *et al*., 1999) or human AChE genes (Ben Aziz-Aloya *et al*., 1993). Similarly, the human and chick AChE gene promoters were shown to be activated by cyclic AMP-dependent pathway (Wan *et al*., 2000; Choi *et al*., 2000), suggesting the involvement of CRE in mammals and avian AChE transcription.

The present results showed that 20 h sustained stimulation of CGRP receptors reduced the synthesis of catalytically active AChE (Figure 6d). This decreased AChE synthesis is probably responsible for the reduction of total myotube AChE activity after 48 h CGRP treatment (Figure 6b) and might be related to the inability of CGRP to maintain elevated levels of cyclic AMP after prolonged periods of receptor stimulation.

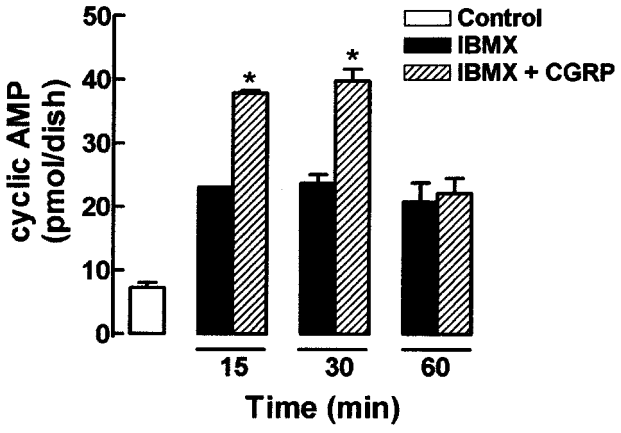

Figure 7 Time course of CGRP-dependent cyclic AMP production in tissue-cultured skeletal muscle. Rat cultured myotubes, pre-incubated with 1 mM IBMX, were treated with 100 nM CGRP for 15, 30 and 60 min at 37°C. The cyclic AMP was extracted and measured using a cyclic AMP [^{3}H]-assay kit. Each column represents the mean ± s.e.mean; $n = 3$. *Significantly different from IBMX, $P < 0.05$.

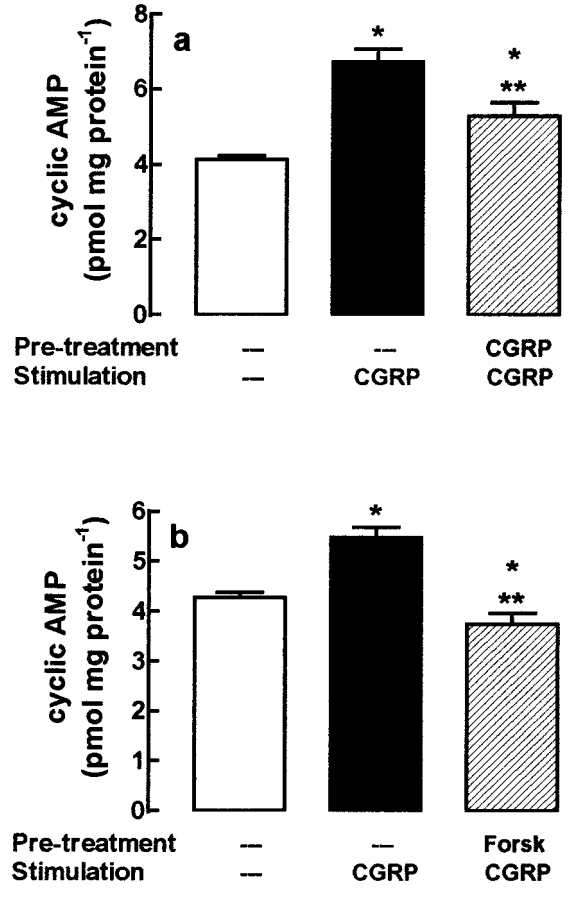

Figure 8 Long-term stimulation of CGRP receptors or adenylyl cyclase down regulates the cellular response. Rat cultured myotubes were submitted or not to pre-treatment with 100 nM CGRP (a) or 10 μM forskolin (b) in complete culture medium for 24 h at 37°C. The cells were then rinsed with Krebs bicarbonate buffer, pre-incubated with 100 μM IBMX and stimulated with the same concentration of CGRP, for 15 min at 37°C. The cyclic AMP was extracted and measured using cyclic AMP [^{3}H]-assay kit. Each column represents the mean ± s.e.mean; $n = 4$. *Significantly different from non-stimulated group; **Significantly different from stimulated group, $P < 0.05$.

Two possible reasons might explain the long-term effects of CGRP on the synthesis of myotube AChE. First, the receptor desensitization or down regulation. This hypothesis is supported by the limited effectiveness of CGRP to stimulate AC after long-term receptor stimulation (Figure 8a). Quick desensitization of G protein-coupled receptors has already been described after persistent receptor activation (Morris & Malbon, 1999; Bunemann *et al*., 1999). The best example is the β2-adrenoceptor known to be phosphorylated by cyclic AMP-dependent protein kinase (PKA) and uncoupled from G protein (Lohse *et al*., 1990). Our results strengthen the desensitization hypothesis since the maximal cyclic AMP increase after CGRP receptor stimulation was observed within 15–30 min but it returned to basal levels after 60 min. This putative desensitization of CGRP receptor is further supported by recent findings showing a significant loss of CGRP receptor responsiveness in SK-N-MC cells, after 20 min incubation of the peptide (Drake *et al*., 1999). In addition, Aiyar *et al*. (2000) reported that pre-treatment of HEK-293-PR cells with 30 nM CGRP for 30 min induced a decrease in CGRP-mediated adenylyl cyclase activity that is accompanied by a 40% decrease in [^{125}I]CGRP binding sites. CGRP binding analysis in cultured myotubes may be helpful to corroborate the desensitization hypothesis, herein presented.

A second possibility that might explain the long-term effect of CGRP on AChE is the down-regulation of downstream molecules involved in CGRP signalling pathway (e.g. adenylyl cyclase). Skeletal muscle fibre expresses at least four different AC, all of them activated by the α subunit of G protein-coupled receptors. In addition, as described for AChE, the expression of the skeletal muscle AC isoforms is influenced by motoneuron. For example, after denervation of mouse gastrocnemius muscle, there is an increase in AC2 and AC9 mRNA whereas the transcripts of AC6 and AC7 are decreased (Suzuki *et al*., 1998). Also, Torgan & Kraus (1996) reported a time-dependent adjustment of AC isoform expression in response to changes in the physiological demands of the skeletal muscle. An adaptative change of AC activation or isoform expression could explain the reduced generation of cyclic AMP observed after 24 h stimulation of CGRP receptor and the inability of CGRP stimulate AC after long-term treatment of myotubes with forskolin (Figure 8b).

In summary, an important finding from our study is that CGRP increases the AChE synthesis but the continuous stimulation of CGRP receptor does not maintain the effect. Oscillation of the intracellular levels of cyclic AMP could be a physiological mechanism of skeletal muscle to control AChE synthesis. After long-term stimulation (24–48 h) of CGRP receptors, a non-operational signalling system is probably the cause for the decreased synthesis of AChE. Simultaneous modification of both CGRP receptor and downstream signalling system cannot be discarded though.

Taking into account that CGRP released in the synaptic cleft of vertebrate neuromuscular junction stimulates the muscle cyclic AMP production (Uchida *et al*., 1990) our results suggest that, under increased nerve output, CGRP might increase the rate of AChE synthesis to down regulate the excessive stimulation of AChR. The decline of CGRP effects after prolonged treatment of myotubes agrees with the actual evidences of fast receptor-G protein-effector uncoupling (Bunemann *et al*., 1999), an important mechanism to adapt muscle fibre to long-lasting changes in neuromuscular transmission.

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