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# Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger

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**Recent immunocytochemical studies have shown that calcitonin gene-related peptide (CGRP) coexists with the neurotransmitter acetylcholine in spinal motoneurons of the chick. Moreover, CGRP causes an increase in the number of acetylcholine receptors on the surface of cultured chick myotubes. CGRP might thus serve as one of the signals by which motoneurons regulate endplate development. In a search for the second messengers involved, we now demonstrate that CGRP stimulates accumulation of cyclic AMP (cAMP) in cultured chick myotubes. This effect is, at least in part, mediated by an increase in cAMP synthesis, as the peptide also activates adenylate cyclase in chick muscle membranes. Nanomolar concentrations of CGRP elicit significant increases in both cellular cAMP levels and acetylcholine receptor numbers. The present findings suggest that cAMP is one of the second messengers which mediate the increase in acetylcholine receptor number elicited by CGRP. Furthermore, CGRP might be implicated in other trophic actions mediated by cAMP in skeletal muscle cells.**

*Key words:* acetylcholine receptor/adenylate cyclase/cultured chick myotubes/neuromuscular junction/second messenger

## Introduction

Calcitonin gene-related peptide (CGRP) is a recently discovered neuropeptide of 37 amino acids, which is widely distributed in the nervous system, and is also found in peripheral organs, e.g. the pituitary, adrenal gland, heart and gastrointestinal tract (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). CGRP exerts various peripheral actions, such as potent cardiovascular effects, as well as inhibition of gastric acid secretion and food intake (reviewed by Fischer and Born, 1985). In the central nervous system, CGRP is preferentially localized in the dorsal part of the spinal cord, in sensory ganglia, and in taste and olfactory pathways, suggesting that the peptide may be involved in sensory transmission (Rosenfeld *et al.*, 1983; Tschopp *et al.*, 1985).

CGRP has also been identified in spinal cord motoneurons of several species (Rosenfeld *et al.*, 1983; Gibson *et al.*, 1984; Fontaine *et al.*, 1986; New and Mudge, 1986) and in the nerve terminals of the rodent neuromuscular junction (Takami *et al.*, 1985a,b), raising the possibility that this peptide may act as a neuronal messenger coexisting with the classical transmitter acetylcholine (Hökfelt *et al.*, 1984). Consistent with such a role, it has been shown that CGRP can be released from nerve cells by depolarization in a calcium-dependent way (Mason *et al.*, 1984). The finding that CGRP stimulates accumulation of acetylcholine receptors on the surface of cultured chick myotubes (Fontaine *et al.*, 1986; New and Mudge, 1986) suggests that CGRP may function as a neurotrophic factor, potentially involved in motor endplate development.

One of the characteristic changes that occurs during formation of the neuromuscular junction is the high-density clustering and accumulation of acetylcholine receptors at the level of the endplate. This complex process is regulated by motoneurons in at least two different ways (reviewed by Salpeter and Loring, 1985; Changeux *et al.*, 1987). During innervation of the muscle, the neurally evoked electrical activity of the muscle fibres causes a repression of acetylcholine receptor biosynthesis, which results in the elimination of extrajunctional receptor. At the same time, the number of subneural acetylcholine receptors steadily increases, possibly under the influence of factors released from motoneurons, one of which may be CGRP (Fontaine *et al.*, 1986; New and Mudge, 1986). The CGRP-stimulated increase of acetylcholine receptors in cultured myotubes does not seem to be due simply to reversal of the repression caused by electrical activity. It has been proposed that different signalling pathways are involved in these two regulations (Fontaine *et al.*, 1986). One candidate for a second messenger linking the action of nerve factors to acetylcholine receptor biosynthesis is cyclic AMP (cAMP), as it has previously been shown that analogues of the nucleotide, as well as activators of adenylate cyclase stimulate acetylcholine receptor accumulation in cultured myotubes (Betz and Changeux, 1979; Blosser and Appel, 1980). Even though cAMP has long been suspected to play a regulatory role in the development of a functional motor endplate (Lentz, 1972; Carlsen, 1975), no endogenous factor from neural origin has so far been shown to stimulate adenylate cyclase in muscle cells. We now report that CGRP activates adenylate cyclase in skeletal muscle and evokes a sustained elevation of cAMP levels in cultured chick myotubes. This neuropeptide may thus prove to play an important role in the regulation of nerve-muscle interactions.

## Results

### *CGRP stimulates accumulation of cAMP in cultured chick myotubes*

Two different techniques were used to measure cAMP production in cultured chick myotubes. In the first one, the intracellular ATP pool was labelled by pre-incubation of the cells with [$^3$H]adenine. The radiolabelled precursor was taken up by the cells and steady-state levels were attained after 2 h of incubation at 37°C. At this time, ~25% of the [$^3$H]adenine had entered the myotubes (data not shown). Previous studies with erythrocytes and adipocytes demonstrated that a major part of the radioactivity was incorporated into the intracellular ATP pool (Kuo and DeRenzo, 1969; Humes *et al.*, 1969; Schulster *et al.*, 1978). Figure 1A shows that CGRP stimulates rapid accumulation of [$^3$H]cAMP in chick myotubes that have been prelabelled with [$^3$H]adenine. A new steady-state level is reached within 5 min following addition of the peptide. This level, which is about 3.5 times higher than the basal unstimulated [$^3$H]cAMP content,

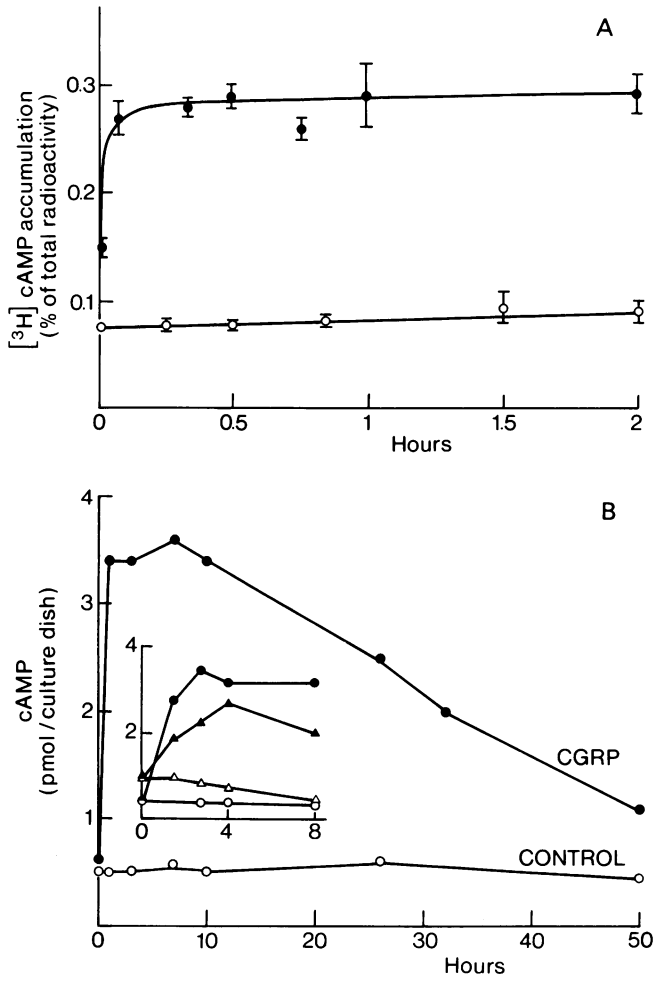


**Fig. 1.** CGRP stimulates accumulation of cAMP in chick myotube cultures. **(A)** At zero time, cells that had been prelabelled with [$^3$H]adenine were incubated with (●) or without (○) 0.1 μM CGRP, and [$^3$H]cAMP formation was determined as described in Materials and methods. Results are expressed relative to the total cellular radioactivity in each culture dish (~ $10^6$ c.p.m.). Each time point represents the mean ± SD of two culture dishes, obtained in one representative experiment. **(B)** At zero time, cells were treated with (●) or without (○) 0.1 μM CGRP, and cAMP content was determined by radioimmunoassay. Each point represents one culture dish. Results are from a single experiment that was repeated several times with similar results. **Inset:** cAMP levels were determined in cultures that had (circles) or had not (triangles) been treated with 10 μM cytosine arabinoside on days 2–4 after plating. Open symbols: control dishes; closed symbols: 0.1 μM CGRP. Data are mean values of two culture dishes.

remained constant for 2 h. Longer incubation times were not tested, since it cannot be assumed that the specific radioactivity of ATP, the substrate for adenylate cyclase, will remain constant during prolonged incubation.

To circumvent this problem, and to confirm the foregoing results by an independent technique, absolute levels of cAMP were determined by radioimmunoassay. As depicted in Figure 1B, addition of CGRP to chick myotube cultures caused a rapid, 7-fold increase in cellular cAMP content, which then slowly declined with time, with a half-life of 30 h. A significant 2-fold elevation over basal cAMP content could still be detected 50 h after addition of the peptide. It should be noted that these experiments were performed in the presence of a mixture of peptidase inhibitors (bacitracin, phosphoramidon, captopril, bestatin and kelatorphan) that have been shown to prevent biological inactivation of various neuropeptides (Horsthemke *et al.*, 1984; Bouboutou *et al.*, 1984; Turner *et al.*, 1985). However, a similar long-lasting effect of CGRP was observed in preliminary experiments that were conducted in the absence of peptidase inhibitors (not shown). These results suggest that CGRP is inactivated slowly, if at all, in chick myotube cultures.

**Table I.** Effect of compounds known to affect acetylcholine receptor biosynthesis on cAMP accumulation in chick myotube cultures

| Drug added | Plateau levels of cAMP (pmol/dish ± SD) | Relative increase over basal level |
|---|---|---|
| None (basal level) | 0.4 ± 0.2 | 1.0 |
| 0.1 μM CGRP | 3.3 ± 0.5 | 8.3 |
| 0.1 μg/ml tetrodotoxin | 0.4 ± 0.1 | 1.0 |
| 1 μM veratridine | 0.4 ± 0.1 | 1.0 |
| 25 ng/ml cholera toxin | 5.1 ± 0.7 | 12.8 |
| 0.1 μM CGRP + 0.1 μg/ml tetrodotoxin | 3.6 ± 0.5 | 9.0 |
| 0.1 μM CGRP + 1 μM veratridine | 3.8 ± 0.2 | 9.5 |
| 0.1 μM CGRP + 25 ng/ml cholera toxin | 13.3 ± 3.5 | 33.3 |

Intracellular cAMP levels were determined by radioimmunoassay. The cAMP levels reached within 1 h of addition of the various effectors remained constant for about 10 h (see Figure 1). The plateau levels shown were averaged from four time points ranging from 1.5 to 8 h. Data are from a typical experiment performed in duplicate culture dishes, which was repeated several times with similar results.

The CGRP-induced accumulation of cAMP in chick myotube cultures is not due to the presence of contaminating fibroblasts. This was demonstrated by comparing cAMP accumulation between sister cultures that had or had not been treated with the antimitotic drug cytosine arabinoside. Even though in the absence of cytosine arabinoside the number of fibroblasts in the cultures was increased by at least 10-fold, as judged by phase-contrast microscopy (not shown), CGRP-stimulated cAMP levels were found to be even slightly lower than in cultures treated with the antimitotic drug (see Figure 1B inset). It is noteworthy that the presence of fibroblasts in chick myotube cultures is also without effect on the CGRP-stimulated increase of acetylcholine receptor levels (New and Mudge, 1986).

*Effect of compounds known to affect acetylcholine receptor biosynthesis*

In accord with a previous report (McManaman *et al.*, 1982), the sodium channel blocker tetrodotoxin was found to be without effect on the basal cAMP levels of cultured chick myotubes. Likewise, tetrodotoxin had no effect whatsoever on the CGRP-stimulated accumulation of cAMP. Similar results were obtained with the membrane depolarizing agent veratridine (see Table I). These results demonstrate that the basal and CGRP-stimulated levels of cAMP do not depend on the electrical activity of the muscle cells.

Cholera toxin was found to elevate cAMP content of cultured chick myotubes to somewhat higher levels than did CGRP (Table I). Accumulation of cAMP in the presence of both effectors was 3–4 times higher than with either compound alone and about 30-fold higher than the cAMP content of untreated cells.

It should be emphasized that the observed increases in cellular cAMP reflect a net stimulatory effect, as they are not counterbalanced by parallel increases in cell mass during the time of the experiments. Indeed, we did not detect any change in protein content of the cultures following treatment with CGRP or

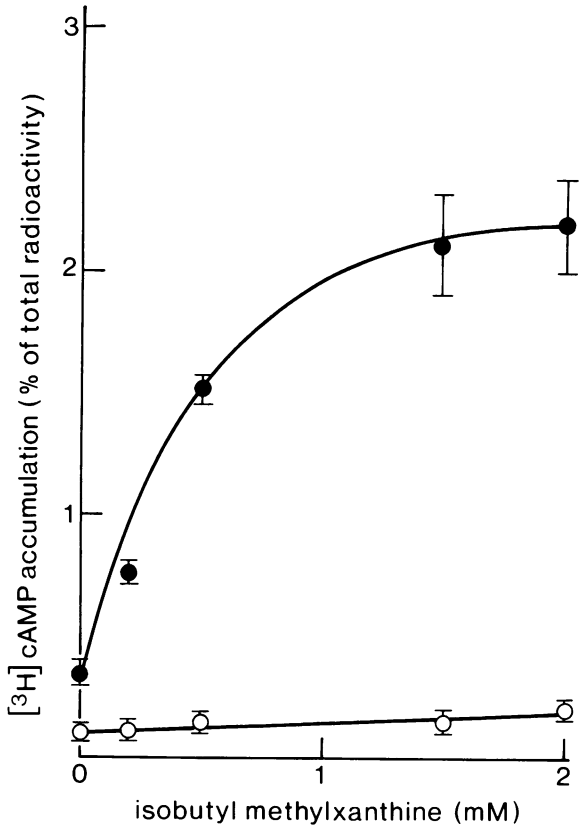


**Fig. 2.** Effect of 3-isobutyl-1-methylxanthine on cAMP accumulation in chick myotube cultures. Formation of [$^3$H]cAMP was determined in cultures previously labelled with [$^3$H]adenine. Cells were incubated for 15 min in the presence (●) or absence (○) of 0.1 μM CGRP, with increasing concentrations of 3-isobutyl-1-methylxanthine . Results are expressed relative to the total cellular radioactivity in each culture dish (~ $10^6$ c.p.m.). Each point is the mean ± SD from two culture dishes.

cholera toxin (data not shown). Moreover, it has previously been shown that neither drug affects total protein synthesis of chick myotubes (Blosser and Appel, 1980; Fontaine *et al.*, 1986; New and Mudge, 1986).

*CGRP stimulates adenylate cyclase in chick muscle membranes*

Since the cAMP levels measured by either of the two techniques described above reflect a steady-state between formation and breakdown of cAMP, it is conceivable that CGRP increases intracellular cAMP content either by stimulating adenylate cyclase or by inhibiting cAMP phosphodiesterase, or both. To assess whether a potent phosphodiesterase activity is present in chick myotube cultures, cells were treated with increasing concentrations of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Figure 2). This compound enhanced the CGRP-stimulated formation of cAMP in a dose-dependent fashion, without greatly affecting basal cAMP levels. The maximal stimulatory effect was obtained at an isobutylmethylxanthine concentration of 2 mM. Under these conditions, the CGRP-stimulated cAMP accumulation was ~7 times higher than in the absence of phosphodiesterase inhibitor, and 11 times higher than the basal cAMP level in the absence of CGRP (Figure 2). Further increasing the concentration of isobutylmethylxanthine (to 5 or 10 mM) was without effect (data not shown). These results suggest that almost all phosphodiesterase activity was inhibited at isobutylmethylxanthine concentrations >2 mM, and that the effect of CGRP observed under these conditions is due to stimulation of adenylate cyclase, leading to increased cAMP synthesis.

**Table II.** CGRP stimulates adenylate cyclase in chick muscle membranes

| Drug added | Adenylate cyclase activity (pmol cAMP/min/mg protein) | Relative increase over basal activity |
|---|---|---|
| None (basal) | 3.9 ± 0.6 | 1.0 |
| 1 μM CGRP | 8.8 ± 1.1 | 2.3 |
| 10 μM GTP | 4.6 ± 0.8 | 1.2 |
| 10 mM NaF | 38.7 ± 0.5 | 9.9 |
| 10 μM GTP + 1 μM CGRP | 15.1 ± 0.1 | 3.9 |

Adenylate cyclase activity was determined as described in Materials and methods, except that GTP was present only where indicated. Results are mean values ± SD of triplicate samples within a single, representative experiment.

To confirm this contention, adenylate cyclase activity was measured in membranes prepared from the leg muscles of 1-week-old chicks. Table II shows that CGRP stimulates adenylate cyclase and that this effect is potentiated by GTP, as in the case of other hormone-responsive adenylate cyclase systems (Salomon, 1979). The activation ratio observed with CGRP is about 40% of that of NaF, which directly activates the guanine nucleotide binding protein associated with the adenylate cyclase (Schramm and Selinger, 1984).

*Dose-dependence of CGRP effects*

CGRP stimulates cAMP formation in a dose-dependent and saturable manner (Figure 3B and C). Half maximal stimulation of adenylate cyclase in chick muscle membranes and of cAMP accumulation in chick myotube cultures was attained at concentrations of 2 and 8 nM, respectively. At nanomolar concentrations, CGRP also elicited significant increases of surface acetylcholine receptor levels in chick myotube cultures. However, the latter effect did not saturate at CGRP concentrations up to $10^{-6}$ M (Figure 3A). It should be noted that the effects of CGRP on adenylate cyclase and cAMP levels were assayed following a 15-min incubation with the peptide, while acetylcholine receptor levels were measured after 24 h. It is thus difficult to compare the shapes of the respective dose–response curves, since CGRP may be partly inactivated and/or adsorbed during prolonged incubation.

## Discussion

The results of the present study demonstrate that CGRP enhances accumulation of cAMP in cultured chick myotubes by stimulating sarcolemmal adenylate cyclase. CGRP has been recently reported to activate adenylate cyclase in other peripheral tissues, such as the rat heart and spleen (Sigrist *et al.*, 1986), indicating that some of the actions of the neuropeptide may be mediated by cAMP. In the rat kidney, CGRP has been proposed to stimulate adenylate cyclase by interaction with calcitonin receptors rather than CGRP receptors. However, while the latter effect is observed at rather high, micromolar concentrations of CGRP (Goltzman and Mitchell, 1985), significant increases of both surface acetylcholine receptor numbers and cAMP levels in cultured chick myotubes are elicited by nanomolar concentrations of the peptide (see also New and Mudge, 1986). Both of these actions seem therefore to be mediated by receptors, whose affinity for CGRP resembles that of the CGRP receptor in rat heart (Sigrist *et al.*, 1986). Furthermore, it has previously been shown that surface acetylcholine receptor levels of cultured chick myotubes increase upon treatments that elevate intracellular cAMP (Betz and Chang-

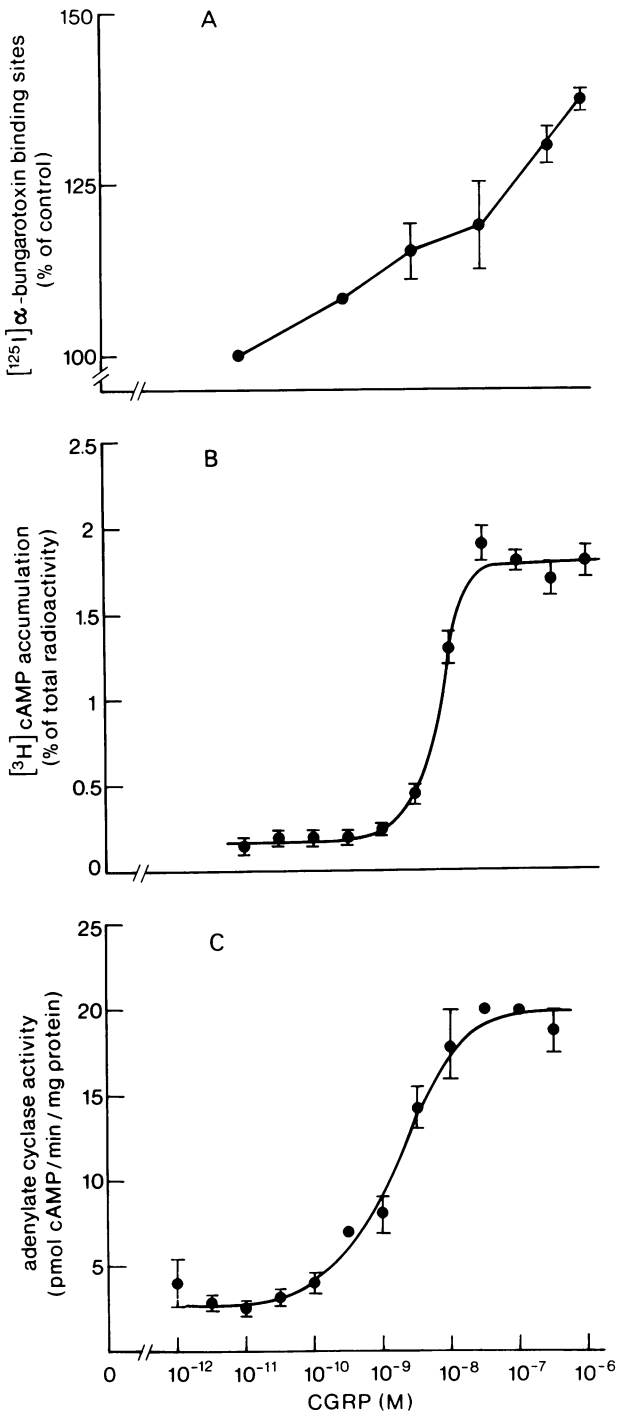


**Fig. 3.** Dose-dependence of CGRP-stimulated effects. **(A)** CGRP-induced increase of acetylcholine receptor numbers on the surface of cultured chick myotubes. Cells were incubated with increasing concentrations of CGRP for 24 h and specific [$^{125}$I]α-bungarotoxin binding sites were then assayed as previously described (Betz and Changeux, 1979). Results are expressed as a percentage of binding sites detected in parallel, untreated cultures. Each point is the mean ± SD of three culture dishes. **(B)** CGRP-induced stimulation of cAMP accumulation in chick myotube cultures. Cells that had been prelabelled with [$^{3}$H]adenine were incubated with increasing concentrations of CGRP, in the presence of 2 mM 3-isobutyl-1-methylxanthine , for 15 min at 37°C. Similar results were obtained in the absence of isobutylmethylxanthine (not shown). Each point is the mean ± SD of two culture dishes. **(C)** CGRP-induced stimulation of adenylate cyclase in chick muscle membranes. Adenylate cyclase activity was determined as described in Materials and methods. Results are mean values ± SD from duplicate determinations.

eux, 1979; Blosser and Appel, 1980). It is thus likely that cAMP serves as a second messenger mediating the CGRP-induced increase of acetylcholine receptor numbers in skeletal muscle cells, even though CGRP may conceivably exert additional, cAMP-independent effects.

Following addition of CGRP to chick myotube cultures, cAMP levels maximally increase within a few minutes, and slowly decline thereafter, with a half-life of 30 h. In comparison, CGRP was shown to stimulate the rate of insertion of newly synthesized acetylcholine receptors into the sarcolemmal membrane after 4 h of treatment (New and Mudge, 1986). Maximal receptor accumulation was attained after 24 h, after which time it slowly decreased (Fontaine *et al.*, 1986). In chick myotubes, the insertion of mature acetylcholine receptors into the membrane occurs ~3.5 h after synthesis of the individual polypeptide subunits (Devreotes *et al.*, 1977; Merlie, 1984). Our results are thus consistent with the hypothesis that CGRP acts via a rapid increase in intracellular cAMP, which in turn stimulates some early step(s) of receptor biosynthesis. Further support for this view comes from the fact that the stimulation of acetylcholine receptor levels in chick myotube cultures by dibutyryl cAMP follows a time course similar to that observed with CGRP (Betz and Changeux, 1979).

The present results lend support to the previous proposal (Betz and Changeux, 1979), that acetylcholine receptor biosynthesis is regulated by two different mechanisms, which utilize calcium ions and cAMP as intracellular second messengers, respectively. The regulation of acetylcholine receptor synthesis by electrical activity of the muscle has been shown to operate by a calcium-dependent, cyclic nucleotide-independent mechanism (Pezzementi and Schmidt, 1981; McManaman *et al.*, 1982). Accordingly, neither tetrodotoxin, which blocks $Na^+$ channels and stimulates acetylcholine receptor biosynthesis, nor veratridine, which activates sodium channels and decreases acetylcholine receptor biosynthesis (Betz and Changeux, 1979; Klarsfeld and Changeux, 1985), had any effect on basal or CGRP-stimulated cAMP levels in chick myotube cultures. On the other hand, cholera toxin, which has previously been reported to increase acetylcholine receptor synthesis (Blosser and Appel, 1980; Fontaine *et al.*, 1986), elevated cAMP levels in myotubes, and potentiated the effect of CGRP. This result is in accordance with the known action of cholera toxin as a blocker of adenylate cyclase deactivation (Cassel and Selinger, 1977). However, the finding that cholera toxin potentiates the CGRP-stimulated accumulation of cAMP seemingly disagrees with the non-additivity of the actions of CGRP and cholera toxin on acetylcholine receptor biosynthesis (Fontaine *et al.*, 1986). A likely explanation for this apparent discrepancy might be that the effect on acetylcholine receptor biosynthesis is already saturated at the intracellular cAMP levels attained following maximal CGRP stimulation of adenylate cyclase. Further elevating cAMP, by blocking the deactivation pathway of adenylate cyclase with cholera toxin, would then be without effect on acetylcholine receptor biosynthesis. It should be noted that physiological effects mediated by cAMP can be expected to saturate at low concentrations of the nucleotide, since they usually involve a chain of catalytic reactions resulting in high amplification factors (Cohen, 1985).

The mechanism by which cAMP regulates the number of surface acetylcholine receptor sites remains to be established. In the case of other eukaryotic proteins whose expression is regulated by cAMP, both transcriptional and post-transcriptional control mechanisms have been proposed (see for example Noguchi *et al.*, 1982; de Bustros *et al.*, 1986). A first possibility is that cAMP affects the maturation and assembly of receptor subunits (Merlie,

1984), their transit and incorporation into the cytoplasmic membrane in subneural areas. Yet, little evidence exists in favour of this hypothesis. Another possibility is that cAMP regulates gene transcription by directly binding to, or stimulating the phosphorylation of a protein which interacts with specific DNA sequences, as demonstrated in prokaryotes (de Combrugghe *et al.*, 1984). In any case, it is noteworthy that highly conserved cAMP-regulated DNA elements have been demonstrated to flank several eukaryotic genes such as those for phosphoenolpyruvate carboxykinase, tyrosine aminotransferase, corticotropin releasing factor, somatostatin and proenkephalin (reviewed by Comb *et al.*, 1986; de Bustros *et al.*, 1986). The 5′ end and promoter region of the chicken acetylcholine receptor $\alpha$-subunit unit has been isolated recently and functionally characterized (Klarsfeld *et al.*, 1987). It will thus be possible to establish whether similar cAMP-regulated sequences control the expression of acetylcholine receptor genes.

Our finding that a peptide contained in spinal motoneurons stimulates adenylate cyclase of skeletal muscle is consonant with the important role cAMP seems to play in neuromuscular function. For instance, Nirenberg and co-workers (1984) have shown that elevating neuronal cAMP content promotes synapse formation between neuroblastoma-hybrid cells and myotubes in culture. In skeletal muscle cells, the surface number of voltage-sensitive sodium channels (Sherman *et al.*, 1985) and calcium channels (Schmid *et al.*, 1985), as well as the activity of acetylcholinesterase (Lentz, 1972), increase upon treatments that elevate intracellular cAMP. This suggests that the nucleotide may coordinately control the expression of several synaptic proteins at the neuromuscular junction. Further evidence for a possible regulatory role of cAMP at different stages of muscle differentiation comes from experiments showing characteristic changes in cAMP levels, as well as adenylate cyclase, cAMP-dependent protein kinase and cAMP phosphodiesterase activities during myoblast differentiation (Zalin and Montague, 1974; Moriyama *et al.*, 1976), muscle disuse or denervation (Carlsen, 1975; Smith *et al.*, 1978) and postnatal muscle development (Smith, 1979, 1984; Smith and Clark, 1980). The regulatory subunit of cAMP-dependent protein kinase has been shown to undergo a denervation-induced modulation of its autophosphorylative properties, which seems to involve a neurotrophic signal other than acetylcholine (Held *et al.*, 1986). Furthermore, the rate of desensitization of the muscular acetylcholine receptor seems to be regulated by a cAMP-dependent phosphorylation mechanism (Huganir *et al.*, 1986; Albuquerque *et al.*, 1986; Middleton *et al.*, 1986). It is tempting to speculate that CGRP might be one of the so far elusive 'anterograde' factors which have been proposed to regulate cAMP levels in skeletal muscle (Lentz, 1972) and may thereby control the biogenesis and maturation of the endplate postsynaptic membrane.

## Materials and methods

*Materials*

Dulbecco's modified Eagle's medium was obtained from Flow Laboratories. Other culture media, horse serum and antibiotics were from Gibco. Rat $\alpha$-CGRP was obtained from Bachem. Stock solutions were made in water containing 0.1 mg/ml bovine serum albumin and stored at −20°C. Fresh dilutions were prepared in the same diluent on the day of experiment. [$\alpha$-$^{32}$P]ATP, [$^{32}$P]cAMP, [2,8-$^{3}$H]cAMP and [2-$^{3}$H]adenine were from Amersham, and [$^{125}$I]succinyl cAMP tyrosine methyl ester from Institut Pasteur Production. A rabbit antiserum to cAMP was kindly donated by Drs Roxane Predeleanu and Agnès Ullmann (Institut Pasteur, Paris). Kelatorphan and captopril were kind gifts from Dr B. Roques (Paris). All other chemicals were purchased from Sigma.

*Myotube cultures*

Myoblasts were obtained from the hind limbs of 11-day-old chick embryos by mechanical dissociation, and seeded at a density of $3-5 \times 10^5$ cells/well in gelatine-coated 24-multiwell dishes. They were cultured in a 3:1 mixture of modified Eagle's medium and medium 199 containing 10% horse serum, 1% embryo extract, 100 units/ml penicillin and 100 $\mu$g/ml streptomycin (Betz and Changeux, 1979). Media were changed every 2−3 days. To eliminate fibroblasts, $10^{-5}$ M cytosine arabinoside was present in the first change of medium. The concentration of horse serum was lowered to 4% after cytosine arabinoside treatment.

*Determination of [$^{3}$H]cAMP formation in myotube cultures.* After 6−7 days in culture, cells were incubated in 0.5 ml of assay medium (Dulbecco's modified Eagle medium containing 4% horse serum, 100 units/ml penicillin and 100 $\mu$g/ml streptomycin) for at least 1 h at 37°C. Experiments were started on the same, or on the following day (6−8 days after plating) by incubating the cells in 0.25 ml of fresh medium containing 10 $\mu$Ci (2 $\mu$M) of [2-$^{3}$H]adenine for 2 h at 37°C. The plates were then washed twice with fresh medium; 0.25 ml of assay medium supplemented with 15 mM Hepes, pH 7.4, 100 $\mu$M bestatin, 50 $\mu$g/ml bacitracin, 1 $\mu$M phosphoramidon and 1 $\mu$M captopril was added to each culture dish. Following addition of various effectors, the cells were incubated at 37°C for different times, as specified in Results. Reactions were stopped by addition of 0.5 ml of 70% ethanol and 0.2 ml of a solution containing 5000 c.p.m. of [$^{32}$P]cAMP as recovery indicator, as well as 0.1 mM each of ATP, ADP, AMP, cAMP, adenosine and adenine. The cell extracts were centrifuged at 10 000 *g* for 1 min and [$^{3}$H]cAMP content of the clear supernatants was determined by sequential chromatography on columns of Dowex AG 50W-X8 and aluminium oxide (Salomon, 1979).

*Determination of cAMP accumulation in myotube cultures.* After 6 days in culture, cells were incubated in medium supplemented with 50 $\mu$g/ml bacitracin, 1 $\mu$M captopril and 1 $\mu$M kelatorphan. Various effectors were added and incubation continued at 37°C. Reactions were stopped by rapid aspiration of the medium and addition of 0.5 ml of 0.1 N ice-cold HCl. Following neutralization, dilution and acetylation of the extracts, cAMP content was determined by radioimmunoassay as described (Brooker *et al.*, 1979). The lower limit of detection (corresponding to 15% displacement of bound [$^{125}$I]succinyl cAMP tyrosine methyl ester) was 2 fmol of cAMP, and 50% displacement was obtained with 14 fmol of cAMP.

*Preparation of chick muscle membranes.* The leg muscles of 1-week-old chicks were dissected on ice, weighed and placed in ice-cold buffer A (0.32 M sucrose, 0.1 mM EGTA, 5 mM $MgCl_2$, 2 mM $\beta$-mercaptoethanol, 10 mM Tris−HCl, pH 8). All further operations were performed at 4°C. The tissues were minced with scissors and homogenized for 3 × 15 s in 5 vol of buffer A with a Polytron blender operating at half-maximal speed. The homogenate was diluted 4-fold and filtered twice over two layers of surgical gauze. The filtrate was centrifuged at 2000 *g* for 10 min and the resulting pellet was washed three times with buffer A. The final pellet was resuspended in buffer A and 4.2 vol of buffer B (2.4 M sucrose, 0.1 mM EGTA, 5 mM $MgCl_2$, 2 mM $\beta$-mercaptoethanol, 10 mM Tris, pH 7.5) was added to give a sucrose concentration of 2 M. This suspension was centrifuged at 12 000 *g* for 10 min and the sticky precipitate that formed along the walls of the tube was discarded. The supernatant and loose pellet were diluted 8-fold with buffer C (buffer A without sucrose; pH 7.5) and centrifuged at 31 000 *g* for 10 min. The pellet was suspended in buffer D (same composition as buffer A; pH 7.5) at a protein concentration of 10 mg/ml, frozen in liquid nitrogen and stored at −70°C. Prior to use in an adenylate cyclase assay, membranes were thawed, washed with 50 mM morpholinopropanesulfonic acid, pH 7.5, containing 5 mM $MgCl_2$, 0.2 mM EGTA and 2 mM $\beta$-mercaptoethanol and suspended to a protein concentration of 1−2 mg/ml.

*Adenylate cyclase assay.* Assays were performed in borosilicate glass tubes containing in a final volume of 110 $\mu$l: 1−3 $\mu$Ci [$\alpha$-$^{32}$P]ATP, 0.7 mM ATP, 7 mM $MgCl_2$, 1 mM cAMP, 0.2 mM theophylline, 40 $\mu$M GTP, 0.2 mM EGTA, 10 $\mu$M propranolol, 5 mM $\beta$-mercaptoethanol 10 mM creatine phosphate, 58 units/ml creatine phosphokinase, 40 $\mu$g/ml bacitracin, 1 $\mu$M captopril, 1 $\mu$M kelatorphan, 50−100 $\mu$g membrane protein and 70 mM morpholinopropanesulfonic acid, pH 7.5. The tubes were incubated at 37°C for 15 min. The reaction was terminated by addition of 100 $\mu$l of a solution containing 40 mM ATP, 1.4 mM cAMP and 15 000 c.p.m. of [$^{3}$H]cAMP as recovery indicator, immediately followed by heating at 90°C for 10 min. The samples were then centrifuged at 1000 *g* for 10 min and the [$^{32}$P]cAMP content of the clear supernatants was determined by the method of Salomon (1979).

*Protein determination.* Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard. Protein content of cultured myotubes was determined following solubilization of the cells by 1% Triton, 0.1 N NaOH.

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## Note added in proof

Takami *et al.* (1986) recently reported that CGRP increases cAMP levels in isolated mouse diaphragm.