Calcitonin Gene–related Peptide and Muscle Activity Regulate Acetylcholine Receptor α-Subunit mRNA Levels by Distinct Intracellular Pathways

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Abstract. In cultured chicken myotubes, calcitonin gene–related peptide (CGRP), a peptide present in spinal cord motoneurons, increased by 1.5-fold the number of surface acetylcholine receptors (AChRs) and by threefold AChR α-subunit mRNA level without affecting the level of muscular α-actin mRNA. Cholera toxin (CT), an activator of adenylate cyclase, produced a similar effect, which did not add up with that of CGRP. In contrast, tetrodotoxin, a blocker of voltage-sensitive Na⁺ channels, elevated the level of AChR α-subunit mRNA on top of the increase caused by either CGRP or CT.

L2-O-Tetradecanoyl phorbol-13-acetate (TPA), an activator of protein kinase C, markedly decreased the cell surface and total content of [125I]β-BGT-binding sites and reduced the rate of appearance of AChR at the surface of the myotubes without reducing the level of AChR α-subunit mRNA. Moreover, TPA inhibited the increase of AChR α-subunit mRNA caused by tetrodotoxin without affecting that produced by CGRP or CT. Under the same conditions, TPA decreased the level of muscular α-actin mRNA and increased that of nonmuscular β- and γ-actins mRNA.

These data suggest that distinct second messengers are involved in the regulation of AChR biosynthesis by CGRP and muscle activity and that these two pathways may contribute to the development of different patterns of AChR gene expression in junctional and extrajunctional areas of the muscle fiber.

The nicotinic acetylcholine receptor (AChR) is an integral membrane protein, composed of five transmembrane subunits (α₂βγδ) (8). Under the nerve endings, the number of receptor molecules steadily increases during the maturation of the neuromuscular junction, and then remains very high in the adult junction; in contrast, extrasynaptic receptor density fails to negligible values (9, 15, 29). The decline of extrajunctional AChR has been related to the repression of AChR biosynthesis by the neurally evoked electrical activity of the developing muscle fiber (9, 15, 29). What then are the mechanisms that would account for the persistence of AChR incorporation at the level of the growing subneural domain? Several attempts have been made to identify neural factors that might plausibly be involved in this process, by following the increase of AChR levels in cultured muscle cells caused by extracts of neural tissues and/or by a variety of pharmacological agents (reviewed in 29), including, most recently, the neuropeptide calcitonin gene–related peptide (CGRP) (14, 25).

CGRP is a widely distributed peptide generated by differential RNA processing from a single genomic locus which also codes for calcitonin (28). CGRP exerts several biological actions such as cardiovascular effects, inhibition of gastric secretion, and ingestive behavior (13) and has been proposed to act as a neurotransmitter in the sensory tract (33). Interestingly, CGRP has been localized by immunohistochemistry in chicken motoneurons (14, 25) and in mouse (32) or rat tongue (31) motor end-plates. Moreover, the peptide was found to be colocalized with acetylcholine in rat neurons of several motor systems (31). CGRP may thus serve as a coexisting neuronal messenger (7, 16). Consistent with such a role, CGRP is released from cultures of rat trigeminal cells (21) and enhances skeletal muscle contraction (32). Furthermore, it was recently shown that addition of CGRP to chick muscle cells in primary cultures significantly increases the level of surface AChR (14, 25).

In this paper, we demonstrate that CGRP significantly elevates AChR α-subunit mRNA levels in primary chick myotubes in culture. To further identify the intracellular pathways involved in this effect, a variety of pharmacological agents were tested. We report that cholera toxin (CT), an activator of adenylate cyclase, and L2-O-tetradecanoyl phorbol-13-acetate (TPA), an activator of protein kinase C (6, 27), exert different effects on the levels of AChR and AChR α-subunit mRNA. Similar experiments with CT or TPA were also
performed in the presence of CGRP or tetrodotoxin (TTX), a blocker of muscle electrical activity. The data are interpreted in terms of a model of AChR genes regulation during the development of the neuromuscular junction according to which CGRP and muscle electrical activity might respectively contribute to the maintenance of AChR gene expression in junctional areas and to the disappearance of AChR in extrajunctional areas via distinct intracellular pathways.

Materials and Methods

Myotube Culture

Myoblasts were obtained from the hind limbs of 11-d-old chick embryos (Centre Avicole de l'Ile de France, Arrapaho) by mechanical dissociation and cultured as described (2). Briefly, the cells were seeded in gelatine-coated plastic dishes at a density of 10^5 cells/mm² and cultured in a 3:1 mixture of MEM (Gibco, Grand Island, NY) and medium 199 (Gibco), containing 10% horse serum (Gibco) and 1% chick embryo extract. From 48 to 96 h of culture, the cells were treated with 10⁻³ M cytosine arabinoside (Sigma Chemical Co., St. Louis, MO) to minimize fibroblast proliferation. Media were then renewed every 2 or 3 d.

Treatments with Pharmacological Agents

The agents were added after 6 d of culture directly into the medium. Synthetic rat CGRP (10⁻³ M final concentration) was purchased from Bachem Feinchemikalien (Bubendorf, Switzerland), CT (25 ng/ml final concentration), a gift of Dr. A. Montenero, was from Makor Chemicals (Jerusalem, Israel) and TTX (10⁻⁴ M final concentration) was from Sigma Chemical Co. After 24-40 h of incubation with cultured myotubes, CGRP-supplemented media were still able to increase the level of AChR when transferred to a non-treated culture dish (data not shown). Moreover, AChR levels did not further increase upon adding a fresh CGRP solution to the culture medium, indicating that CGRP was not or only slightly inactivated during the experiment.

Table 1. Parallel Variation of Total and Surface 
**[^32]I**aBGT-binding Sites in CGRP- or TPA-treated Chick Myotubes in Culture

| Total **[^32]I**aBGT sites* | Surface **[^32]I**aBGT sites† |
|-----------------------------|-----------------------------|
| Control                     |                             |
| CGRP 1.25 ± 0.02             | 1.26 ± 0.05                 |
| TPA 0.60 ± 0.10              | 0.47 ± 0.05                 |

* Results are averages ± SD of two separate experiments with three culture dishes in each experiment.
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For example, in one experiment surface **[^32]I**aBGT-binding sites were ~70 fmol and total **[^32]I**aBGT-binding sites ~135 fmol per control 35-mm dishes.

Results

Effects of CGRP, CT, and TTX on the Levels of AChR and AChR a-Subunit mRNA

As previously described (14, 25), exposure of primary cultures of chick myotubes to 10⁻³ M CGRP increased the level of surface AChR by ~30%. Parallel results were obtained of **[^32]I**aBGT after 1 h of preincubation in the presence of 10⁻⁷ M aBGT. Each sample was then filtered through three stacked DE81 filters (Whatman Inc., Clifton, NJ), which were washed with 5-10 ml of a solution containing 1% Triton X-100, 10 mM NaCl, 10 mM Tris-HCl (pH 7.4).

Total Protein Content and Incorporation of **[^35]S**Methionine

Total protein content was measured in extracts by the Coomassie Blue technique (Bio-Rad Laboratories, Richmond, CA). The rate of incorporation of **[^35]S**Methionine was determined in triplicate 20 ng/ml TPA-supplemented and control dishes. The medium was replaced by medium free of methionine, with TPA where necessary. **[^35]S**Methionine (100 μCi/ml; Amersham Corp.) was then added and incorporation was allowed to proceed for 2-4 h. The cells were washed twice with PBS (pH 7) and extracted in 1 M NaOH containing 5 mM 1-mercaptoethanol. The 10% TCA-insoluble radioactivity was determined in aliquots by a glass fiber filter assay procedure and expressed relative to the total radioactivity measured in the same extract. Results were corrected for the nonspecific binding of **[^35]S**Methionine which was determined at time zero.

Northern Blot Analysis

Total RNA was prepared from pools of four 100-mm culture dishes. After two washings with PBS (pH 7) and one with 0.25 M NaCl-10 mM Tris-HCl (pH 7.5)-10 mM MgCl₂, the cells were scraped with a rubber policeman, and lysed was performed in 4 M urea-2 M LiCl by slowly pipetting through a syringe. Precipitation of RNA by LiCl was allowed to proceed for 6-24 h at 0°C. After centrifugation, the pellet was dissolved in 10 mM Tris HCl (pH 7.5) - 0.5% SDS-1 mM ethylenedinitrilotetraacetic acid, extracted once with phenol and once with phenol-chloroform-isomylalcohol (25:24:1) before ethanol precipitation (ia). Yields varied between 10 to 30 μg of RNA per dish. RNA samples (8-12 μg) were subjected to electrophoresis in 1.2% formaldehyde-agarose gels and transferred onto a nylon membrane (Hybond N; Amersham Corp.) by standard procedures (20). Hybridization and final washes of the blots were performed according to manuals of membrane suppliers. The AChR α-subunit single-stranded probe (3) was synthesized from a DNA fragment of 500 nucleotides containing 120 nucleotides of exon P2 of the chicken AChR α-subunit gene (27) and labeled by α[^32]P-dATP (800 Ci/mmole; Amersham Corp.). The actin probe (gift of Dr. S. Alonso) was a 1,150-bp mouse cDNA insert in plasmid pLaLi derived from cytoskeletal β-actin mRNA, as determined from its 3' untranslated sequence (1, 24). It was labeled according to the Amersham multiprime procedure by α[^32]P-dCTP (Amersham Corp.; 800 Ci/mmole). Autoradiograms of the hybridized blots were scanned with a densitometer using appropriate exposures to obtain calibration curves.

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Effect of CGRP on AChR α-subunit and α-actin mRNA levels compared with its effect on surface $[^{125}]\text{I}\alpha$BGT-binding sites in cultured chicken myotubes. After 6 d in culture, chick myotubes were treated with CGRP (10^{-7} M), TTX (0.1 µg/ml), CT (25 ng/ml), or combinations of these agents. After 30 h of incubation, total RNA was extracted and Northern blot analysis was performed as described in Materials and Methods. Size markers were chicken rRNAs. Each track contained 10 µg of total RNA. In the experiment shown, relative scanning of the control track was performed with a more exposed autoradiogram and normalized back to the other tracks. Results are expressed as fractions of AChR α or α-actin mRNA levels in control cultures. Measurements of surface $[^{125}]\text{I}\alpha$BGT-binding sites were conducted in parallel and averaged on two culture dishes (deviations were negligible in the experiments shown), and expressed as fractions of the sites present in control cultures. Surface $[^{125}]\text{I}\alpha$BGT-binding sites were usually in the range of 80–160 fmol per 35-mm dish.

When total AChR content was measured instead of surface AChR (Table I), the total content of AChR α-subunit mRNA in cultured myotubes was assessed by Northern blot analysis with a chicken genomic probe as described in Materials and Methods. As shown in Figs. 1 and 2, CGRP increased the level of AChR α-subunit mRNA by about threefold without changing α-, β-, and γ-actins mRNA levels. Similar results were obtained with 25 ng/ml CT. When CT was added together with CGRP, no further increase of AChR α-subunit mRNA took place (Figs. 1 and 2 A). When added to CGRP- or CT-treated cultures, 0.1 µg/ml TTX increased the total content of AChR α-subunit mRNA on top of the increase caused by CGRP or CT alone (Figs. 1 and 2 A). Under the same experimental conditions, levels of surface $[^{125}]\text{I}\alpha$BGT were measured: their variations were qualitatively similar to those of AChR α-subunit mRNA levels. However, the magnitude of the latter were at least twofold higher than the increases in surface AChR (Fig. 1).

Effects of TPA on AChR Biosynthesis

Addition of TPA to cultured embryonic chick myotubes caused a significant decrease of the number of surface $[^{125}]\text{I}\alpha$BGT-binding sites. No significant effect was noticed at TPA concentrations below 1 ng/ml, and the response was maximal at 10 ng/ml TPA after a 40-h incubation period (Fig. 2).
Figure 3. Effect of TPA on surface AChR. (A) Dose–response curve of the effect of TPA on [125I]αBGT-binding sites in cultured myotubes after a 40-h incubation period. Number of surface AChR sites are expressed as the percentage of [125I]αBGT sites in parallel untreated cultures. (B) Time course of the evolution of [125I]αBGT-binding sites in TPA (20 ng/ml)-treated and control cultures. Each of these experiments was repeated at least twice. Each point is a mean of two different culture dishes, with the bar indicating the variation between the two.

3 A). 3 h after the addition of TPA (20 ng/ml), this effect was already noticeable, and it reached its maximum after 24 h of treatment (Fig. 3 B). No further decrease was found between 30 and 40 h (not shown). The reduction in the number of [125I]αBGT-binding sites caused by TPA averaged 45% (25–60% of control values in nine separate experiments). A similar but smaller effect was obtained on total AChR (Table I).

The rate of insertion of AChR into the sarcolemmal membrane was measured by following the time course of the increase of [125I]αBGT-binding sites after blocking preexisting surface AChR by unlabeled αBGT. Insertion of new surface AChR proceeded linearly for several hours in control and in TPA- or CGRP-treated cultures (Fig. 4 A). However, TPA decreased the rate of insertion of surface AChR to about one-third of control. In contrast, this rate was increased by one-third relative to control in cultures pretreated with 10^{-7} M CGRP (Fig. 4 A). These results correlate well with the observed variations in surface [125I]αBGT-binding sites (Fig. 1).

In the absence of cytosine arabinoside, the density of mononucleated cells (partly fibroblasts) increased by over 10-fold (as judged by phase-contrast microscopy). Yet, no modification of the effect of TPA on the level of surface AChR was observed (data not shown). The presence of contaminating fibroblasts thus does not modify the effect of TPA on surface AChR content.

In TPA-treated cells and in the presence of cytosine arabinoside, the total protein content decreased by 15 ± 10% (SD; n = 3) compared to untreated cultures and [35S]methionine incorporation was reduced by 20 ± 5% (n = 2). Since, under these culture conditions, mononucleated cells did not account for more than ~5% of the total population of cells (as judged by phase-contrast microscopy), the myotubes appear to be the main target of this ~20% inhibition of protein synthesis. Yet, this effect does not suffice to account for the average 45% decrease of surface AChR caused by TPA.

To test for a possible effect of TPA on AChR degradation rate, the kinetics of degradation of the [125I]αBGT-AChR complex were measured in control cultures and in cultures preincubated for 24 h with TPA (20 ng/ml). No significant difference was observed (Fig. 4 B). TPA thus primarily affects the biosynthesis of the AChR rather than its degradation.

Effects of CGRP, CT, and TTX on AChR Metabolism in TPA-treated Cells

As mentioned above, CGRP and CT caused increases of surface AChR which are additive to the increase caused by TXX. Interestingly, CT still increased the surface AChR...
A). In contrast, the level of \( \alpha \)-actin mRNA decreased by CGRP, a neuropeptide which coexists with acetylcholine in skeletal muscle (14, 25). The present results further demonstrate that, collectively, CGRP increases (by about threefold) the level of AChR \( \alpha \)-subunit mRNA without significantly affecting the level of \( \alpha \)-actin mRNA, a major muscular component. Such an increase may result from a specific stimulation of \( \alpha \)-subunit gene transcription or from a stabilization of the \( \alpha \)-subunit mRNA (II). Nuclear run-on experiments should decide in favor of one of these two possibilities.

The amplitude of this increase in mRNA levels appears significantly larger (about twofold) than the increase of total and surface \( \alpha \)BGT-binding sites as well as of the rate of AChR appearance at the surface of the myotubes. A similar difference between AChR \( \alpha \)-subunit mRNA and AChR protein content has already been noticed in the case of TTX blockade of the spontaneous electrical activity of cultured myotubes (17). Posttranscriptional events which include the covalent modification and conformational maturation of AChR subunits, their assembly into the \( \alpha \)2\( \beta \)\( \gamma \) pentamer, and their transport to the cell surface via the Golgi apparatus may account for this difference (23).

Electrical activity of muscle fibers in culture and in vivo is known to repress AChR biosynthesis (9, 15, 29). In cell cultures, CGRP causes an increase in AChR number which appears independent of the electrical activity of the muscle fibers, suggesting that CGRP and electrical activity regulate AChR biosynthesis via different intracellular pathways (14). These results have now been extended to the AChR \( \alpha \)-subunit mRNA level. Moreover, in our initial observations (14), it was noticed that CT, an activator of adenylyl cyclase, also increased AChR number (as already described with dibutyryl cyclic AMP [2] and with CT [4, 19]), but this effect was not found additive with that of CGRP. Interestingly, the same observations hold at the mRNA level, further supporting a contribution of cyclic AMP to the regulation of AChR biosynthesis by CGRP. Consistent with such a mechanism, CGRP has recently been shown to increase cyclic AMP level in cultured myotubes (18) and in mouse diaphragm (30), and to activate adenylyl cyclase in chick muscle membranes (18).

To investigate the possible involvement of protein kinase C in AChR biosynthesis, the phorbol ester TPA, an activator of protein kinase C (6, 27), was applied to chick myotube cultures. TPA has already been shown to exert a pleiotropic action on cultured myotubes (10) and to modulate their terminal differentiation. This effect could account for the observed increase of nonmuscular \( \beta \)- and \( \gamma \)-actin mRNA levels and for the decrease of muscular \( \alpha \)-actin mRNA level. An increase of \( \beta \)- and \( \gamma \)-actin mRNA levels has also been recently reported in BC3H1 cells exposed to other mitogenic factors: 20% FCS and fibroblast growth factor (35). Of particular interest for the present study, TPA caused a specific decrease of surface and total AChR content in cultured myotubes as well as a correlative decrease of the rate of AChR insertion into the sarcolemmal membrane. Yet, general protein synthesis was hardly diminished; AChR lifetime did not change and AChR \( \alpha \)-subunit mRNA level remained constant (or slightly increased). Under these culture conditions, TPA thus primarily affects posttranscriptional events in the maturation of AChR. Posttranscriptional regulation of AChR biosynthesis has also been observed with differentiated BC3H-1 cells in culture. When forced back into the cell cycle by refeeding with 20% FCS, AChR \( \alpha \)-subunit mRNA level increased although AChR expression at the cell surface was completely blocked (23). Also, an inhibition of AChR assembly by electrical activity has been suggested in primary cultures of embryonic rat muscle cells (5).

It is noteworthy that muscle electrical activity has been proposed to stimulate phosphatidylinositide metabolism (34), and, thus, may enhance protein kinase C activity via the resulting production of diacylglycerol (27). Exposure of the cells to TPA might then reproduce the effect of electrical stimulation on AChR biosynthesis. Although TPA did not significantly modify the level of AChR mRNA in cells where AChR expression is already repressed by spontaneous electrical activity (9, 15, 29), TPA did completely block the increase of AChR biosynthesis caused by TTX. In contrast, the accumulation of AChR \( \alpha \)-subunit mRNA elicited by CGRP and CT persisted in TPA-treated myotubes. Therefore, the present data do not rule out the possible involvement of protein kinase C in linking muscle electrical activity to AChR \( \alpha \)-subunit mRNA content. Moreover, calcium has also been shown to play a significant role in the regulation of AChR biosynthesis by muscle activity (15, 29), and the interactions between calcium and protein kinase C are now well documented (27). In any case, these data further strengthen the conclusion that CGRP and electrical activity regulate AChR gene expression via different second messenger systems (14).

Our results are consistent with a model (9) according to which, \((a)\) in the adult muscle, the sarcolemmal nuclei that underlie the endplate are in a state of differentiation different from those located in extrajunctional cytoplasm (22), and \((b)\) the development and maintenance of this pattern involves distinct intracellular signaling pathways in extrajunctional and junctional areas. Tentatively, one may thus suggest that, during development, CGRP, co-released with acetylcholine by the motor nerve ending, would increase the expression of the genes coding for the AChR via the cyclic AMP cascade in subneural areas while, in extrajunctional nuclei, muscle membrane electrical activity would repress their transcrip-
by an intracellular pathway involving calcium (19) and, more speculatively, the activation of protein kinase C. Whereas the in vivo role of muscle electrical activity in AChR gene regulation is now well established, the actual relevance of CGRP to the development of the neuromuscular junction remains to be demonstrated. Interestingly, a possible contribution of cyclic AMP to synapse stabilization has been proposed on the basis of in vitro results (26) and CGRP appears to be one candidate “anterograde factor” (7, 9) which, among others, could mediate this regulation.

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