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Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells

(allosteric transitions/receptor modulation/coexisting messengers)

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ABSTRACT **Calcitonin gene-related peptide (CGRP) is a neuropeptide that coexists with acetylcholine in spinal cord motoneurons. The effects of CGRP on the functional properties of the nicotinic acetylcholine receptor (AcChoR) were examined by electrophysiological methods. Using the whole-cell patch-clamp technique and a mouse cell line derived from soleus muscle, we found that CGRP produces a progressive and reversible enhancement of the rapid-decay phase of AcChoR desensitization. Single-channel data further show that CGRP decreases acetylcholine-activated channel opening frequency. This decrease occurs when CGRP and acetylcholine are applied on different cell-surface areas and thus is likely mediated by a second-messenger system. CGRP is also shown to increase cAMP accumulation in this cell line. The effects of CGRP on macroscopic acetylcholine-activated currents are mimicked by external application of forskolin (10 μM) or by internal perfusion of the cell with cAMP (1 mM). In both these cases, further application of CGRP produces no additional enhancement of AcChoR desensitization. These results suggest that, on mouse muscle cells, CGRP regulates AcChoR desensitization by a mechanism that involves, at least in part, cAMP-dependent phosphorylation of the AcChoR.**

The nicotinic acetylcholine receptor (AcChoR) from vertebrate neuromuscular junction is a ligand-gated ion channel that becomes refractory to activation upon prolonged agonist application. The physiological significance of this process, referred to as desensitization (for review, see ref. 1), is still debated but modulation of desensitization and/or its reverse transition by allosteric effectors could be a regulatory mechanism of synapse efficacy at the postsynaptic level (2). Pertinent to such a hypothesis, a thymic hormone, thymopoietin, was recently shown to enhance AcChoR desensitization (3), a process that might contribute to the depressed neuromuscular transmission seen in patients with *myasthenia gravis*.

Moreover, covalent modifications, such as phosphorylation, may affect AcChoR desensitization in an allosteric manner (4–7). For *Torpedo* AcChoR, three different protein kinases phosphorylate the AcChoR molecule *in vitro*, and the action of one of them—the cAMP-dependent protein kinase—has been reported to accelerate desensitization of reconstituted AcChoR in ion flux experiments (5). The same kinase phosphorylates muscle AcChoR in rat primary cultures (8) and in mouse BC3H1 myocytes (9).

In the search for endogenous substances of neural origin that may regulate AcChoR desensitization by means of phosphorylation, neuropeptides coexisting with acetylcholine (AcCho) in motoneurons (10, 11) appeared as plausible candidates. One of them, calcitonin gene-related peptide (CGRP) (12), has been identified in the spinal cord of several vertebrate species and in the motor nerve endings of the rodent neuromuscular junction (13–15). This peptide was shown to stimulate the biosynthesis of AcChoR (14, 15). Furthermore, CGRP increases cAMP synthesis in primary cultures of chick myotubes (16) and thus could play an additional role in the regulation of nerve–muscle interaction by modulating the intrinsic functional properties of the AcChoR through cAMP-dependent phosphorylation.

Using the patch-clamp technique, we examined the effects of CGRP on AcChoR desensitization in a mouse cell line derived from soleus muscle.

METHODS

Culture of Muscle Cells. Primary cultures of soleus muscle were prepared from 4-week-old C3H mice. The cells were cloned by the limiting-dilution technique in 24 multiwell dishes. Experiments were done on one of these clones, Sol8, in which characteristics of growth and differentiation were unmodified after 12 mo in culture (C.P., unpublished results). Myoblasts were plated at low density (50–100 cells/cm^2) and grown at 37°C in MCDB 202 medium (Biochrom, Angoulême, France) supplemented with 20% fetal calf serum and 1 μM dexamethasone. After 2–3 days, differentiation was induced by replacing MCDB 202 medium with Dulbecco's modified Eagle's medium (GIBCO) containing 10 μg of bovine insulin per ml. The cells used 2–4 days later were small and fusiform and generally were mononucleated. They already displayed characteristic features of myogenic cells as, for instance, a high density of surface AcChoR. These small cells (<30 μm in length) were suitable for whole-cell voltage-clamp recordings.

Electrophysiology. Macroscopic currents were recorded using the whole-cell variation of the patch-clamp technique (17). The solution in the patch pipette contained, unless mentioned, 140 mM KCl, 4 mM NaCl, 2 mM $MgCl_2$, 10 mM Hepes, 1 mM K^+-ATP, pH 7.2, and 5 mM BAPTA [bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid] plus 0.5 mM $CaCl_2$ to achieve a final inner concentration of Ca^{2+} equal to 11 nM. The bathing medium contained 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, and 10 mM glucose, pH 7.2. Cells were voltage-clamped at −60 mV.

AcCho was applied externally by means of a fast microperfusion system (18). Other experimental solutions were exchanged by normal bath perfusion. Successive 20- to 30-sec applications of AcCho were followed by 1.5- to 2-min

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; CGRP, calcitonin gene-related peptide; BAPTA, bis(2-aminophenoxy)ethane-*N,N,N′N′*-tetraacetic acid.

washing periods that allowed the AcChoR response to recover from desensitization. Under these conditions, after an initial decline, the peak amplitude of AcCho-activated current was subject to small fluctuations but remained, on the whole, relatively stable over periods of 10–20 min. Single AcCho-activated currents were recorded from cell-attached membrane patches (17). In these experiments, CGRP (100 nM–1 μM) was applied outside the AcCho-containing patch pipette after a control recording period longer than 3 min.

Data were stored on a Racal (Southampton, England) FM analogue tape recorder and then fed into an IBM AT computer for further analysis. Using the Pclamp program of Axon Instruments (Burlingame, CA), the rate of decay of currents evoked by 10 μM AcCho were fitted by the sum of two exponential curves. We also measured the area under the AcCho current trace, which is an index of the total quantity of charge transported across the membrane during AcCho application. BAPTA was obtained from BDH. Other reagents were from Sigma. Numerical results are expressed as mean ± SD.

RESULTS

CGRP Enhances the Decay Rate of AcCho Currents. Fig. 1*A* (control) shows a typical response to fast application of 10 μM AcCho. An inward current rapidly developed (100–200 msec) and then decayed despite the continuous presence of AcCho. The first part of the response has a complex time course determined by diffusion of AcCho, activation of AcChoR, and onset of desensitization; but 200–400 msec after peak amplitude, decay of the current was clearly biphasic in agreement with previous works (refs. 19, 20, see also ref. 1). The time course of desensitization of the response was adequately described by the sum of two exponential components with fast (T_f) and slow (T_s) decay time constants: $I = A_f e^{-T/T_f} + A_s e^{-T/T_s} + C$ where I equals total current, A equals amplitude, and C is a constant (Fig. 1*A Inset*).

Application of AcCho by a fast microperfusion system to small cells (<30 μm in length) avoided limitations encountered in attempting to voltage clamp large myotubes or muscle fibers and thus permitted an efficient analysis of the fast desensitization process. Subsequent numerical results were obtained from experiments with 10 μM AcCho.

In control experiments, after equilibration of the cell with the medium contained in the patch pipette, the absolute values of T_f and T_s were compared 1–3 min after achieving the whole-cell clamp configuration to 10–12 min later. We also studied the relative contribution of the two desensitization phases on the decay of the current by measuring the ratio of the amplitudes A_f/A_s. This parameter was preferred to absolute values of A_f and A_s because the total amplitude of the response to AcCho varied from one cell to another and was subject to spontaneous fluctuations in a given cell. After a slight initial decrease in T_f (16 ± 5%, n = 7) and increase in A_f/A_s (17 ± 12%, n = 7), stable conditions were generally reached within 5 min. Under these conditions values of T_f ranged from 300 to 600 msec (mean 454 ± 120 msec, n = 22) and of T_s from 2 to 7 sec (mean 4.3 ± 1.6 sec, n = 22). The ratio A_f/A_s varied from one cell to another but was very stable under control conditions for a given cell. Most values of A_f/A_s ranged from 5 to 20 (mean 12.7 ± 6, n = 22).

CGRP was added to the perfusion medium after a control recording session longer than 5 min. CGRP by itself did not activate any detectable conductance. As illustrated in Fig. 1*A*, CGRP (500 nM) did not affect the peak current amplitude but progressively accelerated the decay of AcCho-activated currents. Total charge transported after a 15-sec AcCho application decreased by 16 ± 3% 3–5 min after CGRP application and by 34 ± 2% after more than 8 min (n = 10).

This global decrease resulted from the alteration of at least two variables. (*i*) Time constant of the rapid phase of desensitization (T_f) decreased (net decrease 28 ± 3%, range 14–48%, 8-min CGRP application; n = 10), whereas the time constant for the slow phase of desensitization (T_s) did not alter significantly. (*ii*) Acceleration of decay of the current also resulted from enhancement of the fast desensitization process relative to the slow one; this effect was analyzed in terms of an increase in A_f/A_s (net increase 72 ± 30%, range 18–118%, n = 10; after 8-min CGRP application) (Fig. 2*B*) and was due to both an increase in A_f and a decrease in A_s. These changes occurred progressively and stabilized after >5 min. CGRP affected desensitization at concentrations as low as 100 nM, and the observed effect appeared reversible (Fig. 2*A*). However, under conditions suitable for quantitative measurements, exposure to CGRP generally lasted >10 min, and complete reversibility was seldom achieved before the end of a recording session. An effect of CGRP on AcChoR desensitization was still seen at AcCho concentrations as low as 2 μM (Fig. 1*B*).

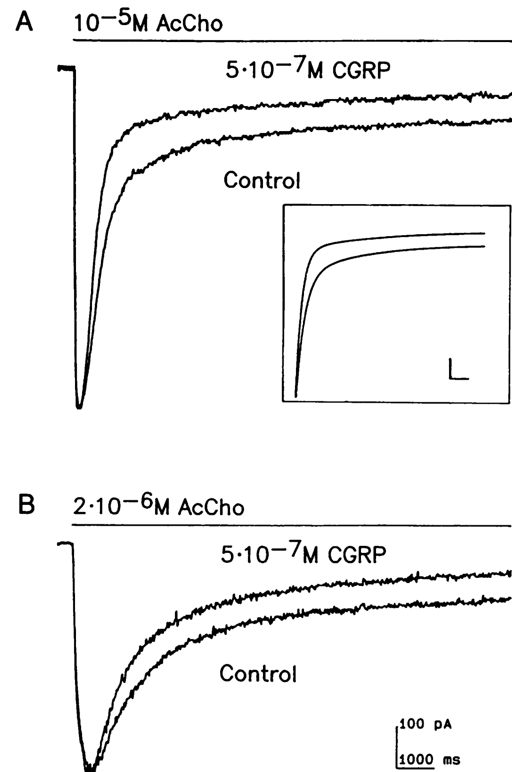

FIG. 1. Enhancement of the desensitization rate of AcChoR by CGRP. Macroscopic currents were recorded from cells voltage-clamped at −60 mV (*A* and *B*). Two superimposed traces show recordings obtained before and 5 min after perfusion with 500 nM CGRP. In *A* the cell was exposed to 10 μM AcCho. Inward current reached a maximum in <200 msec and then decayed in the continuous presence of AcCho. Current decay is described by the sum of two exponential curves with fast (amplitude A_f; time constant T_f) and slow (amplitude A_s; time constant T_s) components. Zero point time for fitting was determined by the onset of the current, and start of fitting region was set between 200 and 400 msec after the current peak. Exponential fits are illustrated in *inset* for control (T_f = 530 msec; T_s = 3800 msec; A_f/A_s = 7) and after 5 min in the presence of 500 nM CGRP (T_f = 395 msec; T_s = 3800 msec; A_f/A_s = 14). For *Inset*, calibration was 100 pA × 1 sec. In *B* the cell was exposed to 2 μM AcCho, at which concentration the slow desensitization process was prominent, and the parameters of the two phases of decay were less accessible to quantitative analysis.

CGRP Decreases the Frequency of Channel Openings. In another series of experiments, AcCho-activated single-channel currents were recorded in the cell-attached mode from a membrane patch while the remainder of the cell was perfused with control external solution and later with a

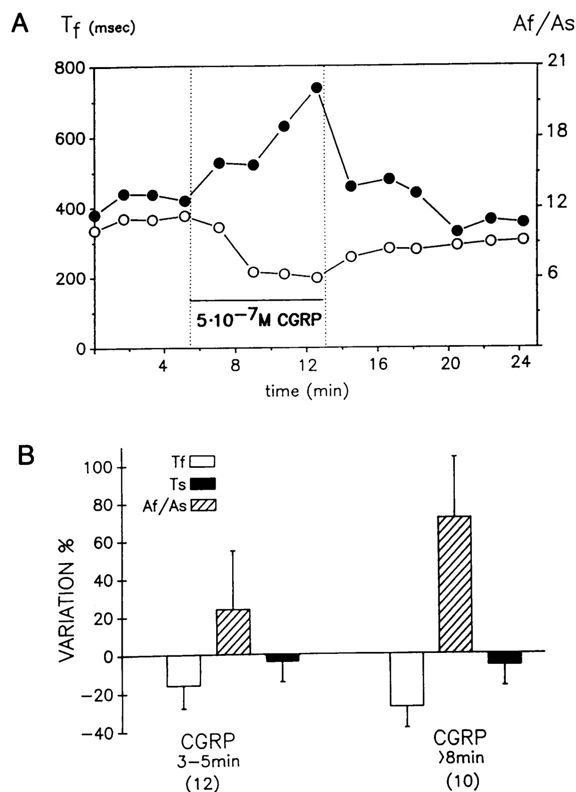

FIG. 2. Quantitative effects of 500 nM CGRP on AcChoR desensitization. AcCho was applied for 20 sec at regular intervals before and during CGRP application. In *A* the graph shows the evolution of the fast time constant T_f (○) and of A_f/A_s (●), the relative amplitude of the fast-to-slow component of desensitization. In *B* are summarized the relative variations of T_f, T_s, and A_f/A_s after relatively short (3–5 min) or longer (>8 min) applications of 500 nM CGRP (values are given as mean ± SD). In these experiments intracellular Ca^{2+} was held at 11 nM using BAPTA as a Ca^{2+} chelator.

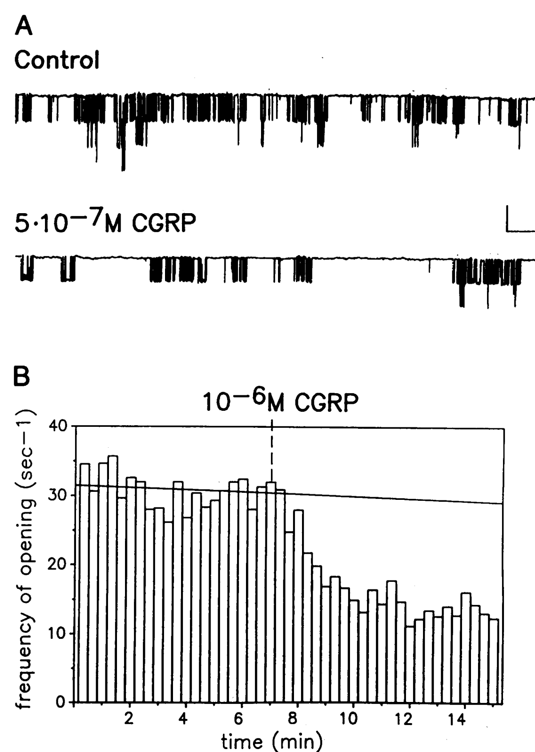

FIG. 3. Single AcChoR channel-opening frequency is decreased upon application of CGRP outside the cell-attached patch pipette. In *A* and *B* the patch pipette contained 2 μM AcCho. Pipette potential was held at +40 mV. (*A*) Recordings obtained before (upper trace) and after (lower trace) application of 500 nM CGRP. Note that the amplitude of single-channel events did not change. Frequency of opening decreased by ≈40%. Calibration bars, 3 pA and 1 sec. (*B*) By use of an event detector with multiple threshold levels, frequency of channel openings was measured in a patch where more than five AcChoR-activated channels could open simultaneously. Regression line shows that frequency of opening decreased spontaneously at a very slow rate in control conditions.

solution containing CGRP (100 nM–1 μM). Under control conditions, with 1–2 μM AcCho contained in the patch pipette, frequency of channel opening decreased spontaneously but at a very slow rate ($T_{1/2}$ ranged from 600–1000 sec). After a 3- to 6-min control recording session, CGRP (100 nM–1 μM) caused a further decrease of frequency by ≈40% of the initial value within 3 min (value corrected for the spontaneous decrease) (Fig. 3 *A* and *B*). At all CGRP concentrations tested, there was no significant change in single-channel mean open time (control, 9.8 ± 1.0 msec; CGRP, 9.3 ± 1.0 msec, for a pipette potential of +40 mV; n = 11), nor of elementary current amplitude (control, 2.9 ± 0.3 pA and CGRP, 2.9 ± 0.4 pA).

The decrease of channel-opening frequency could be due to decreased affinity of the activable state of the AcChoR for AcCho. If such were the case, CGRP should cause a decrease of the peak amplitude of the AcCho response recorded in whole-cell experiments. We rather suggest that the decreased rate of single-channel opening resulted from enhanced inactivation of AcChoR by modification of the parameters of desensitization. Because in these experiments AcChoR activity was monitored in an area of the cell surface physically isolated from that where CGRP was added, modulation of AcChoR desensitization by CGRP was expected to occur by means of a second-messenger system.

CGRP Increases cAMP Content in Muscle Cell Line Sol8. Recently it has been shown that CGRP increased the cAMP level in primary culture of chick myotubes (16) and in isolated mouse diaphragm (21). We measured absolute levels of cAMP in the Sol8 cell line by radioimmunoassay in triplicate experiments as described (16). In the presence of 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, 100 nM CGRP (15-min application) increased cellular cAMP content 3.5-fold.

CGRP Effects Are Mimicked by Increases in Cellular cAMP Content. Recently, two groups using standard electrophysiological techniques and iontophoretic application of AcCho showed that forskolin, a potent activator of adenylate cyclase, increased the apparent rate of desensitization of the AcChoR in rat soleus endplate (6, 7), probably as a result of AcChoR phosphorylation by cAMP-dependent protein kinase.

Now, using whole-cell configuration again, we examined the effect of forskolin on cells from mouse Sol8 cell line under experimental conditions that allow direct measurement of both phases of desensitization. After 5- to 10-min perfusion with 10 μM forskolin, decay rate of the response to a pulse of AcCho increased (Fig. 4*A1*). Similar to that observed with CGRP, T_f progressively decreased by ≈30%, and the ratio of amplitudes of the fast-versus-slow phases of desensitization (A_f/A_s) increased by >50% (n = 8).

In another series of experiments, cells were loaded with 1 mM cAMP and 1 mM theophylline, a phosphodiesterase inhibitor. As illustrated in Fig. 4*B1*, this procedure progressively enhanced the rate of desensitization far beyond that seen under long control recordings. Of seven cells loaded with cAMP and theophylline and studied for >12 min, five cells showed a significant progressive change of response to AcCho, similar to that seen with CGRP. A decrease in T_f (net decrease 25 ± 4%, n = 5) occurred together with a marked

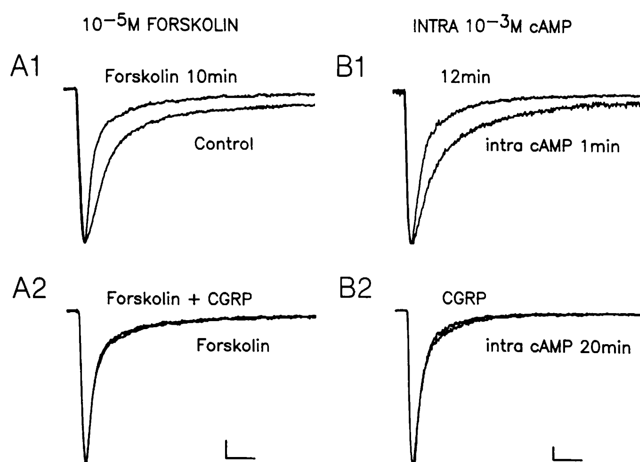

FIG. 4. Enhancement of AcChoR desensitization by external application of forskolin (*A*), or by addition of cAMP to the pipette filling solution (*B*). As for Fig. 1, macroscopic currents were evoked by application of 10 μM AcCho. In *A1*, after a 5-min control session, external application of forskolin (10 μM) progressively enhanced the decay rate of AcChoR-activated currents. In *A2* the two superimposed traces, recorded from the same cell as in *A1*, demonstrate that CGRP (500 nM, 8-min application) had no additional effect on the rate of desensitization after forskolin treatment. (Calibration bars, 500 pA and 1 sec.) In *B*, the pipette solution was supplemented with cAMP (1 mM). In *B1*, responses to application of AcCho 1 min and 12 min after rupture of the patch membrane are compared. cAMP caused a progressive increase of the rate of desensitization far beyond that seen in control experiments (see text). Traces in *B2* are from the same cell. CGRP (500 nM, 8-min application) had no additional effect after extensive intracellular perfusion with cAMP. (Calibration bars, 200 pA and 1 sec.)

increase in the ratio A_f/A_s (135 ± 33%, n = 5, after 12-min recording). Again, no significant modification of T_s was noticed. These effects were also seen when the pipette solution was supplemented with cAMP alone (n = 4), as illustrated in Fig. 4*B1*. The nonhydrolyzable permeant cAMP analogue 8-bromo-cAMP (1 mM) applied outside the cell caused similar, although weaker effect, but 8-bromo-cGMP (1 mM) did not produce any effect (data not shown).

After perfusion with 10 μM forskolin for 10 min or more (Fig. 4*A2*) or once cAMP added in the pipette solution had produced extensive enhancement of AcChoR desensitization (Fig. 4*B2*), no additional increase in the rate of desensitization by CGRP (500 nM) was ever seen.

In addition to cAMP, Ca^{2+} is another potential second messenger that could mediate modulation of AcChoR desensitization. Its putative role could be either direct or indirect by activating intracellular Ca^{2+}-dependent enzymes like Ca^{2+}-phospholipid dependent protein kinase. To prevent any transient rise of intracellular Ca^{2+} that might take place during CGRP application, BAPTA, a potent chelator of Ca^{2+} was systematically added to the intracellular medium. In another series of experiments, the level of intracellular Ca^{2+} was elevated to 300 nM. The effects of CGRP did not significantly differ from those observed when $[Ca_i^{2+}]$ = 11 nM (T_f decreased by 23 ± 5% and A_f/A_s increased by 50 ± 13%; n = 6). Thus, CGRP modulation of AcChoR desensitization does not appear dependent on intracellular Ca^{2+} activity.

DISCUSSION

The results show enhancement of the rate of AcChoR desensitization by CGRP in a mouse muscle cell line. Recording of macroscopic currents on small muscle cells with the whole-cell patch-clamp technique and fast AcCho application allowed an evaluation of the parameters of the fast and slow phases of desensitization (19, 20, for review see ref. 1). CGRP alone did not cause AcChoR desensitization (no change in peak amplitude of the response) but rather modified the properties of desensitization caused by AcCho; CGRP produced a progressive and reversible enhancement of the rapid-decay phase of desensitization. Single-channel data further indicated that this effect was not accompanied by any decrease of mean open time or of unitary current amplitude. However, when CGRP was added outside the patch pipette under the cell-attached mode, a decrease in channel-opening frequency occurred. This effect correlated with the decrease caused by CGRP of the quasi-stationary conductance level reached after a few seconds of AcCho application seen in whole-cell experiments. The decrease in single-channel-opening frequency did not result from a change of affinity of the activable state of the AcChoR for AcCho, which would decrease the peak amplitude of the macroscopic current elicited by AcCho in whole-cell experiments. The decrease of channel-opening frequency, most likely, results from enhancement of desensitization. Additional information on the desensitization properties affected by CGRP will be gained by extensive analysis of single-channel data (as in refs. 20, 22).

Because CGRP affected the frequency of channel openings under conditions where the patch pipette physically isolated the recorded AcChoR from the CGRP-containing extracellular medium, CGRP does not interact directly with the AcChoR. Rather, CGRP modulates AcChoR desensitization by means of a second-messenger system. Even though evidence is incomplete, our results together with recent data from other laboratories support the conclusion that the second messenger involved is cAMP. (*i*) CGRP stimulates accumulation of cAMP in mouse Sol8 muscle cells as previously reported in isolated mouse diaphragms (21) and in cultured chick myotubes (16). In this latter case the elevation occurred rapidly and reached a steady-state level within 5 min, a time scale that fits with CGRP effects seen in our experiments. (*ii*) Effects of CGRP were mimicked by external application of forskolin as well as of the permeant cAMP analogue 8-bromo-cAMP. Further, loading the cells with cAMP produced similar effects. These procedures prevented any additional effect of CGRP on AcChoR desensitization.

At the forskolin concentrations used in our experiments, enhancement of AcChoR desensitization, in agreement with previous work done on rat soleus endplate (6, 7), was not caused by a direct effect on AcChoR conformational states as seen with some noncompetitive blockers (1). Forskolin and permeant cAMP analogues are known to stimulate the phosphorylation of AcChoR 20-fold over basal levels in rat myotubes (8) and BC3H1 mouse cells (9) with a time course compatible with the physiological effects. Furthermore, a direct link between cAMP-dependent phosphorylation of AcChoR and the enhancement of its fast desensitization rate has been demonstrated *in vitro* on *Torpedo* AcChoR reconstituted into phospholipid vesicles (5). Altogether, these data suggest that CGRP acts by activating a cAMP-dependent protein kinase, which phosphorylates the AcChoR, thereby increasing its rate of desensitization.

The contribution of other second-messenger systems cannot be excluded at this stage. For instance, it has been suggested that protein kinase C, which phosphorylates *Torpedo* AcChoR (4), might regulate AcChoR function in chick myotubes (23) and enhance the rate of nicotinic AcChoR desensitization in sympathetic ganglion (24). Protein kinase C activation is expected to depend on the level of intracellular Ca^{2+} (for review, see ref. 25), but in our experiments the CGRP effect on AcChoR desensitization was not modified when the intracellular Ca^{2+} concentration was increased.

The physiological stimuli responsible for activating the protein kinases that phosphorylate the AcChoR have not

been identified. Preliminary reports suggest that the neurotransmitter itself, via nicotinic receptor stimulation could indirectly activate protein kinase C (26–28). Neural signals distinct from AcCho, like neuropeptides that coexist with AcCho (10), may also regulate AcChoR phosphorylation and desensitization. The present data support the idea that at the motor endplate CGRP is one of the physiological signals that regulate AcChoR function through its phosphorylation by a cAMP-dependent protein kinase. Substance P, through activation of protein kinase C, appears a plausible candidate for a similar function at some other cholinergic synapses (22, 24, 29–31). This type of receptor–receptor interaction (35) represents an original mechanism for the regulation of ligand-gated channels.

In addition, cAMP may play a long-term regulatory role in the development of cholinergic synapses (31–34). At the motor endplate, CGRP is one among other neural factors (36) that increase biosynthesis of subneural AcChoR (14–16) by means of a mechanism tentatively involving cAMP (16). CGRP, as a neuropeptide that coexists with AcCho in motor nerve endings (10), may thus contribute to both short- and long-term regulation of synapse properties (11) by activating cAMP-dependent protein kinases.

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