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Calcitonin Gene-Related Peptide Rapidly Downregulates Nicotinic Receptor Function and Slowly Raises Intracellular Ca^{2+} in Rat Chromaffin Cells *In Vitro*

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Although calcitonin gene-related peptide (CGRP) modulates muscle-type nicotinic acetylcholine receptors (nAChRs) via intracellular second messenger-mediated phosphorylation, the action of this peptide on neuronal-type nAChRs remains unknown. Using neuronal nAChRs of rat chromaffin cells *in vitro* we studied the effect of CGRP, which is physiologically present in adrenal medulla, on membrane currents and $[Ca^{2+}]_i$ transients elicited by nicotine. Our main novel observation was that CGRP (either bath-applied or focally applied for a few seconds or even co-applied with nicotine for a few milliseconds) selectively and rapidly blocked nAChRs (a phenomenon unlikely caused by intracellular messengers in view of its speed) without affecting GABA receptors. The inhibitory effect of CGRP was independent of $[Ca^{2+}]_i$ or membrane potential and not accompanied by baseline current changes. Like the competitive antagonist *N,N,N*-trimethyl-1-(4-*trans*-stilbenoxy)-2-propilammonium, CGRP induced a rightward, parallel shift of the nicotine dose–response curve; during co-application of these blockers the nicotine dose–ratio value was the sum of the values obtained with each antagonist alone. The block by CGRP was insensitive to the receptor antagonist $hCGRP_{8-37}$ but mimicked by $CGRP_{1-7}$. Persistent application of CGRP slowly increased $[Ca^{2+}]_i$, a phenomenon independent from external Ca^{2+}, thus implying Ca^{2+} release from internal stores, and suppressed by $hCGRP_{8-37}$. $CGRP_{1-7}$ had no significant effect on $[Ca^{2+}]_i$. We propose that the 1–7 amino acid sequence of CGRP was responsible for the direct, rapid block of nAChRs, whereas the full-length peptide molecule was necessary for the delayed rise in internal Ca^{2+} potentially able to trigger phosphorylation-dependent modulation of nicotinic receptor function.

Key words: CGRP; CGRP antagonist; neuropeptide; nicotine; calcium imaging; intracellular calcium; receptor modulation

Several neuropeptides can act as neurotransmitters per se as well as neuromodulators of receptors for other transmitters (Hokfelt, 1991; Otsuka and Yoshioka, 1993). In general, it appears that the modulatory role of neuropeptides on fast transmitter-gated channels may comprise at least two distinct processes: (1) an indirect mechanism mediated by the peptide G-protein-coupled receptors that through changes in $[Ca^{2+}]_i$ and other intracellular second messengers control the phosphorylation state of the fast transmitter receptor (Huganir and Greengard, 1990; Levitan, 1994; Smart, 1997), and (2) an incompletely understood effect that involves direct interaction of the neuropeptide with certain subunits of the fast transmitter receptor (Clapham and Neher, 1984; Stafford et al., 1994).

An important insight into the phenomenon of peptide-induced receptor modulation has been provided by studies on muscle-type nicotinic acetylcholine receptors (nAChRs). In particular, the endogenously occurring calcitonin gene-related peptide (CGRP) facilitates nAChR desensitization by phoshorylating certain receptor subunits [Mulle et al. (1988); Miles et al. (1989); but see Lu et al. (1993)] and increases AChR biosynthesis (Changeux et al., 1992). Another endogenous neuropeptide, namely substance P, which is frequently colocalized with CGRP (Bell and McDermott, 1996), inhibits the activity of muscle AChRs (Akasu et al., 1983; Simasko et al., 1985) as well as of neuronal-type AChRs of autonomic ganglia (Simmons et al., 1990; Valenta et al., 1993) or adrenal chromaffin cells (Livett et al., 1979; Clapham and Neher, 1984). Unlike the case of substance P, however, the analysis of the modulatory role of CGRP has been limited to muscle-type nAChR only (Eusebi et al., 1988; Mulle et al., 1988; Miles et al., 1989; Lu et al., 1993). Because in the adrenal medulla CGRP is present in nerve fibers (Costa et al., 1994; Heym et al., 1995) and in the chromaffin cells themselves (Kuramoto et al., 1987), this tissue appeared to be a suitable model for investigating any potential modulatory action of CGRP on neuronal nAChRs.

In the present study we analyzed the action of CGRP on neuronal nAChRs from rat chromaffin cells in culture using an approach based on combined recording of $[Ca^{2+}]_i$ transients and membrane currents elicited by nicotine (Khiroug et al., 1997, 1998). We report that CGRP exerted a dual effect on chromaffin cells: first, very rapid inhibition of nAChR function independent from $[Ca^{2+}]_i$ changes, and second, a slow increase in $[Ca^{2+}]_i$ via CGRP receptor-mediated mechanisms, which are known to control the phoshorylation state of nAChRs and thus their ability to recover from desensitization (Hardwick and Parsons, 1996; Khiroug et al., 1998).

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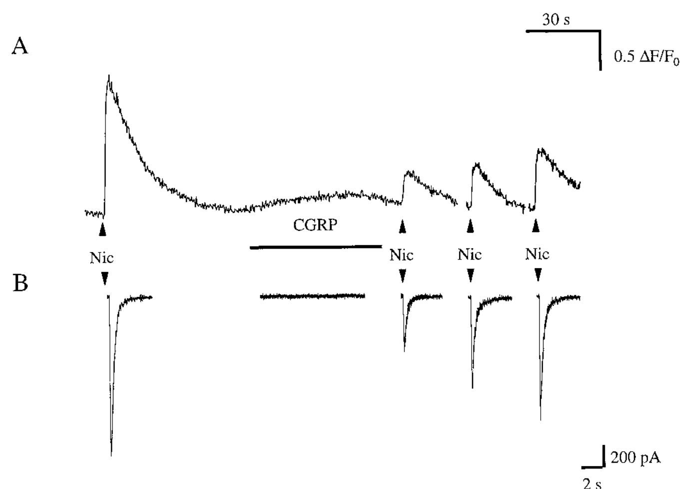

Figure 1. Changes in $[Ca^{2+}]_i$ and membrane current induced by CGRP or nicotine. *A*, $[Ca^{2+}]_i$ fluorescence signals induced by pressure application of nicotine (*Nic*; 20 msec; 0.1 mM pipette concentration; see *arrowheads*) or CGRP (1 min; 1 μM pipette concentration; *horizontal filled bar*). Note that the $[Ca^{2+}]_i$ rise evoked by CGRP is smaller and slower than the one evoked by nicotine but that it induces a lasting depression of nicotine responses evoked at 30 sec intervals. *B*, Membrane currents induced by the same application of nicotine. CGRP did not change baseline current but depressed subsequent responses to nicotine. Note different time scale for *A* and *B*. All traces are from the same cell.

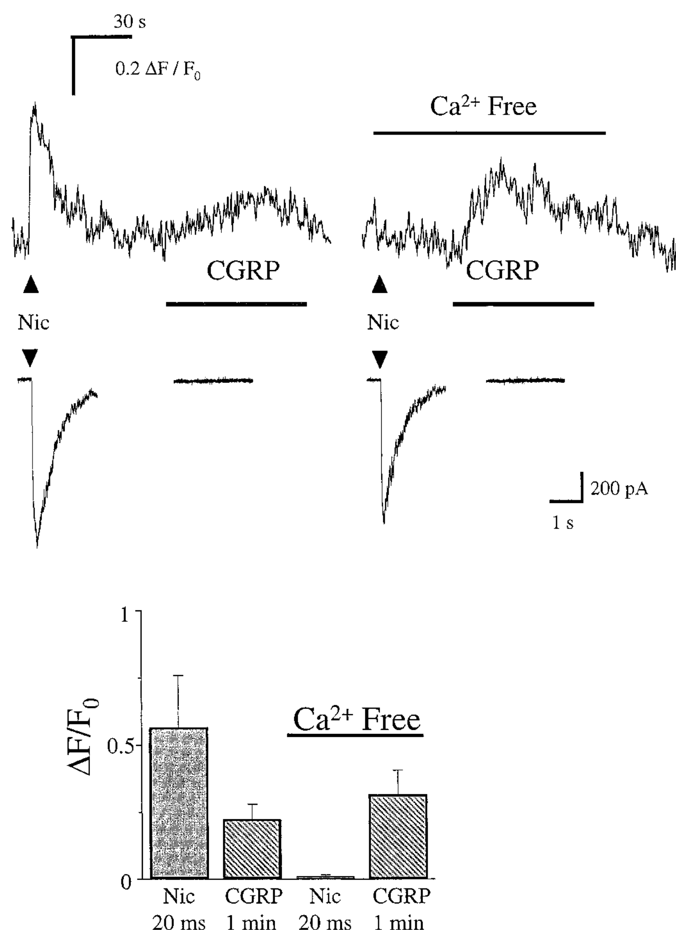

Figure 2. The action of CGRP does not depend on extracellular Ca^{2+}. *Top*, Combined $[Ca^{2+}]_i$ and current records obtained with pressure application of nicotine (20 msec; *arrowheads*) or CGRP (1 min; *horizontal bar*) in control solution (*left*) or in Ca^{2+}-free medium (*right*). Note that in the latter condition the effect of nicotine on $[Ca^{2+}]_i$ is suppressed, whereas the effect of CGRP persists. For further details, see Figure 1 legend. *Bottom*, Histograms of fluorescence signal changes (expressed as fractional increase over baseline) induced by 20 msec nicotine or 1 min CGRP in control solution ($n = 7$) or in Ca^{2+}-free medium ($n = 3$).

MATERIALS AND METHODS

Detailed descriptions of the experimental methods have recently been provided (Khiroug et al., 1997, 1998). In brief, chromaffin cells from ether-anesthetized 25- to 35-d-old rats were dissociated and plated on polylysine-coated (5 mg/ml) Petri dishes and cultured for 1–2 d under a 5% CO_2-containing atmosphere. Cell-containing culture dishes (mounted on the stage of an inverted microscope) were superfused (5–10 ml/min) with control saline solution containing (in mM): NaCl 135, KCl 3.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 10, pH adjusted to 7.4 with NaOH. Patch pipettes were filled with solution containing (in mM): CsCl 120, HEPES 20, $MgCl_2$ 1, Mg_2ATP_3 3, BAPTA 10. When experiments involved confocal $[Ca^{2+}]_i$ imaging, the Ca^{2+}-sensitive dye Fluo-3 was added to this pipette solution, and BAPTA was omitted. The pH of the pipette solution was always adjusted to 7.2 with CsOH. Unless indicated otherwise, cells were voltage-clamped at −70 mV.

For $[Ca^{2+}]_i$ imaging in the visible light range, the Ca^{2+}-sensitive dye Fluo-3 (Minta et al., 1989) was applied via the patch pipette (25 μM) or by preincubation (5 μM; cell permeant AM-ester compound). BAPTA was omitted from the patch pipette. Emission of Fluo-3 was induced by an Ar–Kr laser (488 nm) and detected by the photomultiplier tube of a MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) using a combination of 510 nm high-pass and 530 ± 30 nm bandpass filters. Fluorescence signals were digitized over the whole central optical section as 64 × 32 pixel images in the 32-line rapid scan mode (temporal resolution 320 msec per scan; pixel size 0.6 μm; confocal aperture 200 μm), thus yielding a 38 × 19 μm image. $[Ca^{2+}]_i$ transients were analyzed in terms of fractional amplitude ($\Delta F/F_0$; where F_0 is the baseline fluorescence level, and ΔF is the rise over the baseline). Drugs were usually delivered by pressure application (10–20 psi) from glass micropipettes positioned ~15–25 μm away from the recorded cell. Data are presented as mean ± SEM (n = number of cells), with statistical significance assessed with Wilcoxon test (for nonparametric data) or paired t test (for normally distributed data).

RESULTS

Effects of CGRP or nicotine on $[Ca^{2+}]_i$ and membrane current

Figure 1 shows an example of the contrasting action of nicotine and CGRP on membrane current (*B*) and $[Ca^{2+}]_i$ changes (*A*) of a chromaffin cell (note different time scale in *A* and *B*). A large and rapid $[Ca^{2+}]_i$ rise was first evoked by a 20 msec puffer pulse of nicotine (0.1 mM; applied at the *arrowheads*), which also induced a fast inward current (−1156 pA). After the cell recovered from nicotine, subsequent application of CGRP (applied for 1 min by a pipette containing 1 μM CGRP; indicated by *horizontal bar*) did not change the holding current (Fig. 1*B*) but slowly increased $[Ca^{2+}]_i$ (Fig. 1*A*), which required >10 sec to reach 20% of its peak amplitude. The $[Ca^{2+}]_i$ rise attained a plateau by the end of the application and was smaller than the one observed with nicotine. Nicotine, applied during recovery from CGRP, induced current and $[Ca^{2+}]_i$ responses of lower amplitude (depressed by 67 and 76%, respectively, 30 sec after stopping CGRP) than in control even when the $[Ca^{2+}]_i$ level had returned to baseline. Full recovery of nicotine responses occurred 2 min later (data not shown). On a sample of 11 cells the $[Ca^{2+}]_i$ rise evoked by 0.1 mM nicotine (20 msec pulse) was significantly higher ($p < 0.05$) than the one generated by 1 μM CGRP (1 min), as shown by the histograms in Figure 2. These observations demonstrated not only that CGRP had a slower action than nicotine on $[Ca^{2+}]_i$, but also that after application of CGRP, responses to nicotine were depressed.

Any difference in the action by nicotine or CGRP after changing external Ca^{2+} was examined next. An example is shown in Figure 2 (top) in which on the same cell $[Ca^{2+}]_i$ increases were evoked by 0.1 mM nicotine (20 msec) or 1 μM CGRP (1 min), the latter without any associated variation in holding current (bottom traces). After replacing external Ca^{2+} with equimolar Mg^{2+}, the same test pulse of nicotine elicited an inward current without any $[Ca^{2+}]_i$ rise, whereas CGPR retained its ability to elevate $[Ca^{2+}]_i$. The histograms of Figure 2 show that on average in Ca^{2+}-free medium 0.1 mM nicotine (20 msec) was unable to raise $[Ca^{2+}]_i$, whereas 1 μM CGRP (1 min) maintained its effect essentially unchanged. These results indicate that although the $[Ca^{2+}]_i$ rise elicited by nicotine was caused mainly by influx of this cation via activated nicotinic receptors (Mulle et al., 1992; Vernino et al., 1994; Khiroug et al., 1997, 1998), the $[Ca^{2+}]_i$ rise induced by CGRP was presumably caused by release from internal stores, confirming that not only the time course but also the source of the $[Ca^{2+}]_i$ transients differed when nicotine or CGRP was applied.

Modulation of nicotine responses by CGRP

The depression of nicotine-induced responses observed after application of CGRP (Fig. 1) raised the question of how the peptide might modulate nicotinic receptors, especially because CGRP raised $[Ca^{2+}]_i$, which is thought to control nicotinic receptor activity (Lena and Changeux, 1993; Amador and Dani, 1995). Thus, it was interesting to examine issues such as how quickly this phenomenon could take place, its dependence on $[Ca^{2+}]_i$, and the receptor mechanisms involved. The first two issues were studied by applying nicotine in the presence of CGRP and by buffering $[Ca^{2+}]_i$ with 10 mM BAPTA (which strongly chelated $[Ca^{2+}]_i$, as shown previously by the lack of any change in $[Ca^{2+}]_i$ fluorescence) (Khiroug et al., 1997, 1998). Figure 3*A* shows an example of the early depression of nicotine responses by CGRP. The test response to 20 msec nicotine (−1144 pA) was readily depressed (by 56%) when CGRP was applied for 15 sec before nicotine, indicating a relatively rapid onset of this block, with partial return of the response appearing 45 sec after the CGRP application was stopped. The downregulation of nicotine responses by CGRP did not depend on the low $[Ca^{2+}]_i$ because analogous results ($p > 0.05$) (Fig. 3*B*, histograms) were obtained when 25 μM Fluo-3 [which per se does not interfere with endogenous $[Ca^{2+}]_i$ buffering (Khiroug et al., 1997, 1998)] was used instead of 10 mM BAPTA. The use of Fluo-3 also allowed us to investigate the reduction in nicotine-elicited $[Ca^{2+}]_i$ rises during CGRP application. In this case the increase in $[Ca^{2+}]_i$ (after subtracting any slight increment induced by CGRP itself) was 7 ± 3% of control ($n = 6$).

Comparable reductions in nicotine-induced currents were observed when 1 μM CGRP was continuously applied via the bathing solution as shown by the examples of Figure 3*C* (in which control traces or those in the presence of CGRP are superimposed), suggesting that the blocking action of CGRP was not an artifact caused by the pressure-pulse application. Mixing CGRP and nicotine inside the same pressure pipette or in the extracellular space theoretically might have reduced the amount of nicotine available to the cell receptors if the peptide had chemically bound the agonist. For this purpose we activated approximately equivalent numbers of nAChRs, as judged from equi-amplitude inward currents, by applying presumably the same dose of nicotine from a pipette containing either 0.1 mM concentration (50 msec pulse duration; left panel) or a 10-fold higher concentration (1 mM) for a shorter time (10 msec; right panel). On five cells on which these two applications were tested, the 0.1 mM nicotine response was equally sensitive (48 ± 8% depression) as the 1 mM nicotine response (50 ± 12% depression) to CGRP ($p > 0.05$). These observations thus indicate that the depression of nicotine currents by CGRP was not caused by some interaction between these two substances. The onset of such nicotine currents (measured as rise time from baseline to peak) apparently did not change in the presence of CGRP because it was 142 ± 26 and 130 ± 28 msec, respectively ($p > 0.05$). Furthermore, the time constant of monoexponential current decay (to 10% of peak amplitude) was not significantly affected, being 736 ± 108 msec in control and 552 ± 89 msec in the presence of CGRP, respectively ($p > 0.05$), making unlikely major underlying changes in activation and deactivation of nAChRs.

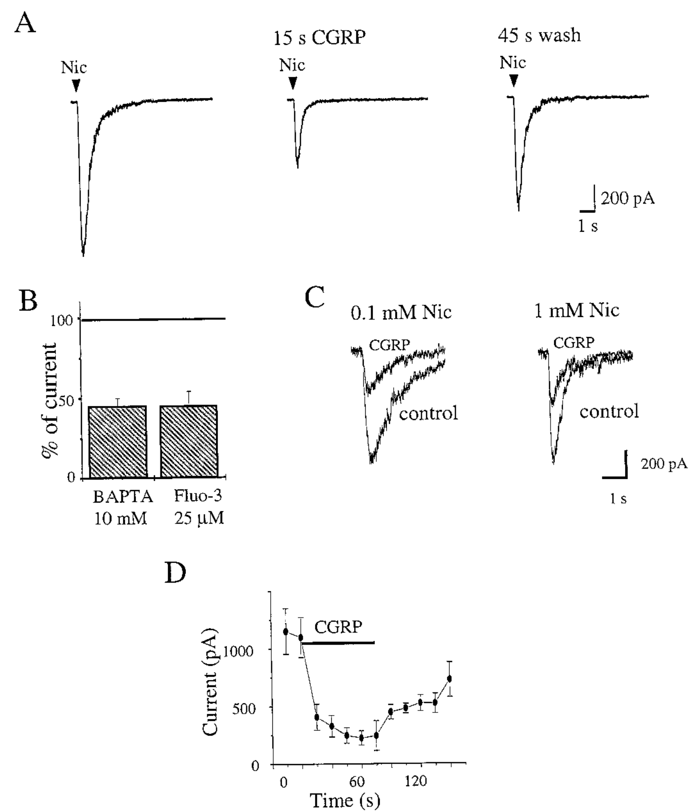

Figure 3. Rapid downregulation of nicotine-induced responses by CGRP. *A*, Current records (recorded with a 10 mM BAPTA-containing pipette) obtained with 20 msec nicotine (0.1 mM pipette concentration; *left*), 15 sec after starting pressure application of CGRP (1 μM pipette concentration; *middle*) and 45 sec after washout of CGRP. Note reduction in nicotine current amplitude. *B*, Histograms of 20 msec nicotine current responses (as percentage of control ones) after 30 sec application of CGRP. The same depression ($p > 0.05$) was observed regardless of the presence of 10 mM BAPTA ($n = 20$) or 25 μM Fluo-3 ($n = 11$) in the recording pipette. Other details as in *A*. *C*, Similar amplitude currents induced by 50 msec nicotine (0.1 mM pipette concentration; *left*) or 10 msec nicotine (1 mM pipette concentration; *right*) before and during bath application of CGRP (1 μM; 5 min). Note analogous depression of either response. Control and CGRP data are superimposed for sake of comparison. Records are obtained with Fluo-3-containing pipettes. *D*, Time course of CGRP depression of nicotine currents (20 msec applications; 0.1 mM pipette concentration) after pressure application of CGRP (1 μM pipette concentration) to eight cells. Other details as in *C*.

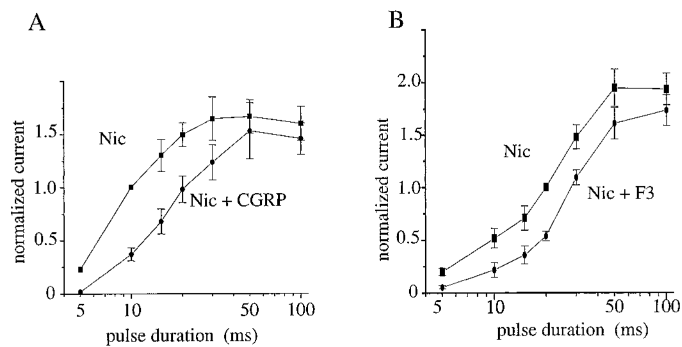

Figure 4. Plot of nicotine current amplitudes versus increasing duration of nicotine pressure pulses in control solution, in the presence of CGRP or F3. *Ordinate*, Current amplitude normalized with respect to the response evoked by 10 or 20 msec nicotine in control solution for each cell. *Abscissa*, Pulse duration of nicotine (0.1 mM) applications. *A*, CGRP (1 μM pipette concentration) was applied for ~15 sec before each nicotine response (n = 9 cells). *B*, F3 (8 nM pipette concentration) was applied for ~15 sec before each nicotine response (n = 8–19 cells). Note rightward shift of either plot without decrease in maximal response. All records were obtained with BAPTA-containing patch pipettes.

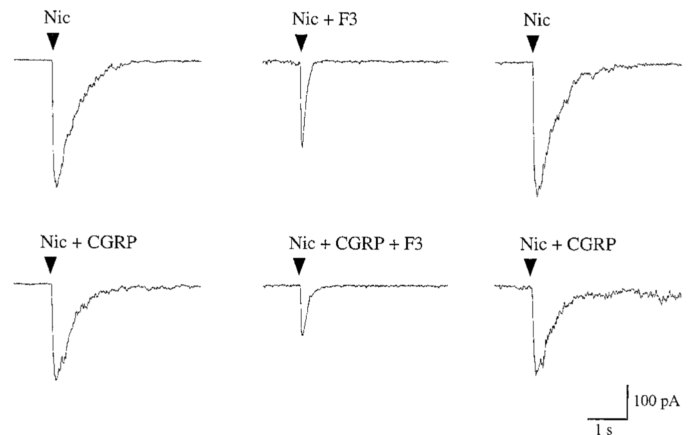

Figure 5. Depression of nicotine-evoked currents by F3, CGRP, or a combination of them. *Traces* are inward currents evoked by nicotine (20 msec pulse; 0.1 mM pipette solution) in control solution, in the presence of F3 (15 sec application; 8 nM pipette concentration), or of CGRP (0.5 μM bath application for about 1 min), or during combined application of F3 and CGRP. All records were obtained from the same cell with a BAPTA-containing patch pipette.

Dynamics of nicotine current block by CGRP

Figure 3*D* shows the time course of CGRP depression for eight cells recorded with a pipette containing BAPTA. It is noteworthy that the extent of depression did not progressively increase during CGRP application and that recovery was achieved 3 min later. Similar data were also obtained when the recording pipette contained Fluo-3 (data not shown). Because the protocol used in the experiments of Figure 3*D* relied on low-frequency applications of nicotine pulses, it might have caused underestimation of more rapid processes underlying the action of CGRP such as desensitization or channel block, which are manifested as fast, use-dependent depression. This issue was explored with paired-pulse (20 msec) delivery of nicotine (interpulse interval = 2 sec). In this case, in control solution the nicotine current evoked by the second pulse was 84 ± 10% (n = 5) of the first one, and in the presence of 10 μM CGRP the second current amplitude was 102 ± 23% of the first current ($p > 0.05$), indicating that the extent of CGRP block was not augmented by previous activation of nAChRs. The relatively fast onset of the blocking effect of CGRP prompted further tests to determine whether this phenomenon could also occur on a more rapid time scale. For these experiments we used one puffer pipette filled with 0.1 mM nicotine and a second puffer pipette (filled with 0.1 mM nicotine plus 1 μM CGRP) immediately adjacent to the first one. For 20 msec applications, CGRP decreased the nicotine current to 50 ± 10% of control ($n = 7; p < 0.01$). This drug delivery protocol therefore suggested that the onset of such a phenomenon was particularly fast. In summary then, these experiments revealed that CGRP induced a $[Ca^{2+}]_i$-independent, strong, and rapid depression of nicotinic receptors of chromaffin cells apparently unrelated to the slow action of CGRP on $[Ca^{2+}]_i$.

CGRP blocking action depended on nicotine dose but not on membrane potential

Further tests were performed to characterize the mechanism underlying the depression by the peptide of nicotinic receptors. Figure 4*A* shows that on nine cells recorded with a BAPTA-filled electrode, increasing the duration (5–100 msec) of 0.1 mM nicotine pulses yielded progressively larger current amplitude with apparent saturation at 50 msec pulses. When comparable applications were repeated in the presence of 1 μM CGRP (15 sec puffer pipette preapplication), currents induced by 5–30 msec nicotine pulses were blocked, whereas responses induced by 50–100 msec pulses were not affected. Thus, the plot was shifted to the right, whereas the analogous maximum response was retained. Taking average responses at approximately the midpoint of the curve (20 msec) before and after the peptide application gave a 55 ± 5% depression with the 1 μM CGRP dose (n = 20), a value not different from the one observed with 10 μM CGRP (60 ± 5%; n = 8), whereas the 0.1 μM dose elicited a smaller depression (19 ± 6%; n = 5). These data suggest that the reduction in nicotine-induced currents was already maximal by focally applied 1 μM CGRP and that smaller amplitude currents were more sensitive than larger ones to this action of the peptide.

This pattern of antagonism outlined the possibility of a competitive interaction by CGRP with the nicotine-binding site of the receptor. Demonstrating that CGRP acted competitively on nicotinic receptors was difficult in view of the fast agonist applications (necessary to avoid receptor desensitization), which did not allow agonist equilibrium conditions to be reached. Direct comparison of CGRP with known competitive antagonists is made difficult by the fact that most nAChR blockers act noncompetitively on autonomic ganglia (for review, see Colquhoun et al., 1987). For instance, preliminary tests with mecamylamine in concentrations as low as 1 nM indicated the noncompetitive nature of its antagonism (data not shown). Nevertheless, the 4-oxystilbene derivative *N,N,N*-trimethyl-1-(4-*trans*-stilbenoxy)-2-propilammonium iodide (Gotti et al., 1998) (F3; preapplied for at least 15 sec from 8 nM pipette solution) induced a parallel, rightward shift of the dose–response curve (Fig. 4*B*). The dose of F3 was selected to induce a degree of antagonism comparable in entity to the one evoked by pressure-applied 1 μM CGRP (Fig. 4*A*). The blocking action of F3 had rapid onset with full recovery of nicotine responses after 15–30 sec from the end of the F3 application (data not shown). These data thus indicate that F3 and CGRP had an analogous blocking effect on nAChRs of rat chromaffin cells. To test whether these substances shared a common site of action, we applied them in low concentrations either alone or in combination. Figure 5 shows an example of this

Table 1. Additive antagonism of nAChRs by low doses of simultaneously applied F3 and CGRP

Cell	Observed sum of percentage depression attributable to CGRP + F3	Calculated sum of percentage depression attributable to CGRP + F3
1	80	80
2	46	55
3	50	58
4	81	98
5	60	60
6	81	90

Data are expressed as percentage reduction in 20 msec nicotine-induced peak currents observed experimentally (left column) or predicted by simple addition of individual blocking action by each antagonist (right column).

approach. Application of F3 (8 nM pipette solution for 15 sec) caused a 34% reduction in the peak current induced by 20 msec nicotine (0.1 mM); after washout, bath application of 0.5 μM CGRP reduced the same nicotine current by 26%. Subsequent application of F3 in the continuous presence of CGRP decreased the control nicotine response by 60%, showing additive antagonism. Table 1 shows that on six cells the observed depression of the nicotine current amplitude was very similar to the one calculated by adding the individual antagonism values. According to standard receptor theory (Barlow, 1980), combining two competitive antagonists should produce an agonist dose–ratio (*DR*) value (i.e., the ratio between agonist doses required to reproduce the same amplitude response before and after antagonist application) given by the equation $DR = DR_1 + DR_2 - 1$ where DR_1 and DR_2 are the *DR*s observed with each one of the antagonists applied separately. This approach has been used in the past to study the site of action of various blockers against, for instance, glutamate (Evans et al., 1982; Martin and Lodge, 1985) or GABA (Simmonds, 1982) receptors. In the present experiments, precise quantification of *DR* values was made difficult by the use of pressure application and nonequilibrium agonist responses; thus, data obtained with this approach allow only an estimate of the antagonist site of action. Notwithstanding this limitation, when taking the nicotine pulse duration as agonist "dose" (20 msec) for six cells, CGRP produced a $DR_1 = 1.75$, whereas F3 induced a $DR_2 = 1.40$. Combining CGRP with F3 gave a *DR* value of 2.50, which is similar to the calculated one of 2.15, suggesting that these two blockers were likely to have a similar site of action.

To analyze further the mechanism of action of CGRP on nAChRs, we explored the possibility that its antagonism is altered when the cell membrane potential is changed, as would be expected in the case of channel block. Figure 6*A* shows that on the same cell recorded with a BAPTA-containing electrode and held at −120 or −70 mV, CGRP elicited a similar reduction in nicotine current amplitude (49 and 41%, respectively). On average the depression at −120 mV was 61 ± 8% ($n = 6$), a value thus not significantly different ($p > 0.05$) from the 55 ± 5% observed at −70 mV. Application of a rapid membrane potential ramp (from −120 to 0 mV) at the peak of the nicotine current allowed us to obtain an *I–V* plot (after leak subtraction; see Fig. 6*B*) with apparent reversal near 0 mV. CGRP (1 μM) reduced the nicotine response uniformly throughout this potential range, with no detectable change in extrapolated current reversal potential. This was also apparent by scaling the plot obtained in the presence of CGRP to the one in control and superimposing them as shown in the inset to Figure 6*B*. These data therefore confirm that the block by CGRP of nicotinic receptor-mediated responses was voltage independent and not caused by a negative shift in current reversal. Thus, the action of CGRP was unlikely to involve channel block of activated nicotinic receptors.

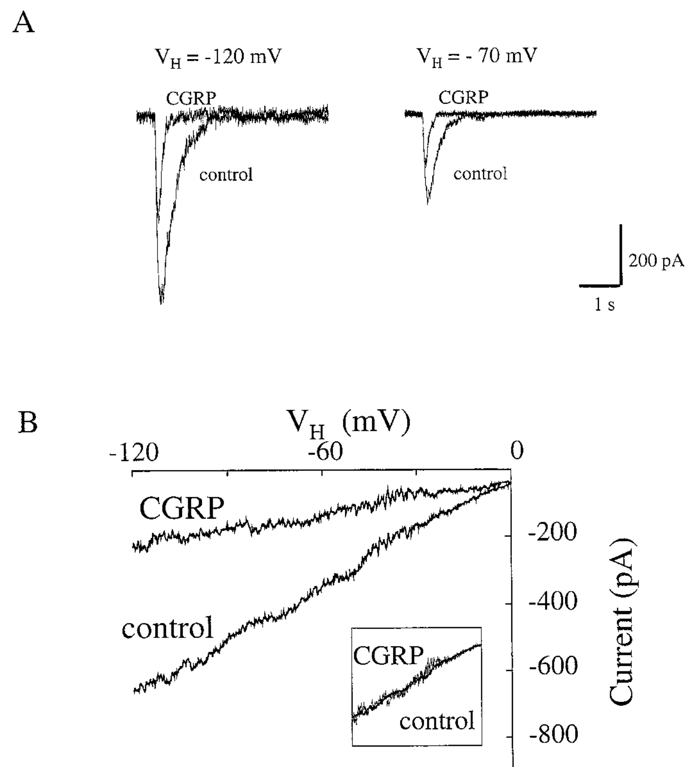

Figure 6. CGRP-induced depression of nicotine currents is voltage independent. *A*, Comparison of currents induced by 20 msec nicotine (0.1 mM pipette concentration) in control solution or during pressure application of CGRP (1 μM pipette concentration) at −120 mV (*left*) or −70 mV (*right*) holding potential. Data are superimposed for comparison. Note that the extent of peak current depression was similar for either holding potential. *Traces* are from the same cell recorded with a BAPTA-containing pipette. *B*, *I–V* plot obtained at the peak of 50 msec nicotine-induced current in control solution or in the presence of CGRP (1 μM pipette concentration). All data are leak-subtracted. Note lack of apparent change in current reversal and that in the presence of CGRP the plot was uniformly reduced. Scaling of the latter graph and superimposing it on the control one (see *inset*) show similar *I–V* properties. Ramp voltage was from −120 to 0 mV at 0.5 mV/msec.

Specificity of CGRP blocking action

We examined this issue by determining whether CGRP could modify responses to another fast-acting neurotransmitter such as GABA (Peters et al., 1989) or whether other neuropeptides could affect nicotine-mediated responses. In the first case, bath-applied CGRP (1 μM) depressed peak currents elicited by nicotine (0.1 mM; 20 msec pulse) by 46 ± 9%, whereas it left unchanged (96 ± 5%) the similar amplitude currents evoked by GABA (1 mM; 50 msec pulse) on the same cells ($n = 5$). Thyrotropin-releasing hormone (TRH) (1 μM by pressure application for 1 min) did not change (98 ± 2%; $n = 6$) nicotine-evoked currents. Similarly, dynorphin A (1 μM; pressure-applied for 1 min) left the nicotine currents unchanged (95 ± 2%; $n = 17$).

Effect of a CGRP receptor antagonist

If CGRP acted directly on nicotinic receptors, this effect presumably would have not been mediated by conventional G-protein-

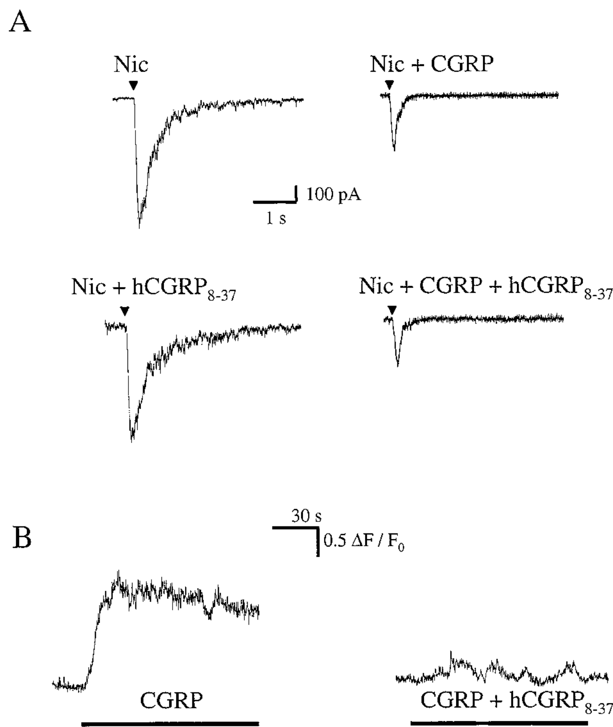

Figure 7. Effect of the CGRP receptor antagonist $hCGRP_{8-37}$ on nicotine or CGRP responses. *A*, Inward current evoked by 20 msec nicotine (0.1 mM pipette concentration; *top left*) is depressed by CGRP (1 μM pipette concentration; *top right*). Subsequent bath application of $hCGRP_{8-37}$ (1 μM; 5 min) does not change nicotine response (*bottom left*) or the depressant action of CGRP on the nicotine current. All data from the same cell recorded with Fluo-3-containing pipette. *B*, $[Ca^{2+}]_i$ increase induced by 2 min application of CGRP (1 μM pipette concentration; *left*) is suppressed in the presence of bath-applied $hCGRP_{8-37}$ (1 μM; *right*). Data are from a cell loaded with Fluo-3 AM.

coupled CGRP receptors, which are known to exist on chromaffin cells (Mazzocchi et al., 1996) and trigger synthesis of cAMP to activate PKA (Bell and McDermott, 1996). This was tested by using human $CGRP_{8-37}$ ($hCGRP_{8-37}$), a pharmacological antagonist selective against G-protein-coupled CGRP receptors (Bell and McDermott, 1996) or the selective PKA inhibitor Rp-cAMPS (de Wit et al., 1984; Khiroug et al., 1998). Figure 7*A* shows that on the same cell in which pressure-applied CGRP (1 μM) depressed the inward current induced by 20 msec nicotine (0.1 mM), bath application of 1 μM $hCGRP_{8-37}$ for ~5 min failed to change the nicotine current or the baseline current and did not prevent the depressant effect of CGRP. On three cells, the CGRP depression observed in the presence of $hCGRP_{8-37}$ was 94 ± 6% of the one found before superfusion with $hCGRP_{8-37}$ ($p > 0.05$), indicating insensitivity of this phenomenon to $hCGRP_{8-37}$. Nevertheless, $hCGRP_{8-37}$ fully prevented the slow $[Ca^{2+}]_i$ rise (measured after transmembrane loading of the cells with Fluo-3 AM) induced by 2 min pressure application of CGRP (as exemplified in Fig. 7*B*). Pooling data from 24 cells showed that CGRP (1 μM) increased $[Ca^{2+}]_i$ by 41 ± 11%, whereas in the presence of $hCGRP_{8-37}$ (1 μM) this action of CGRP was suppressed (11 ± 5%; $p < 0.01$ vs control). On 12 cells pretreated with 10 μM Rp-cAMPS for 20 min, the $[Ca^{2+}]_i$ rise evoked by CGRP was also suppressed (18 ± 10; $p < 0.05$ vs control). These results indicate that the slow $[Ca^{2+}]_i$ rise induced by CGRP was mediated by CGRP receptors and involved PKA activity.

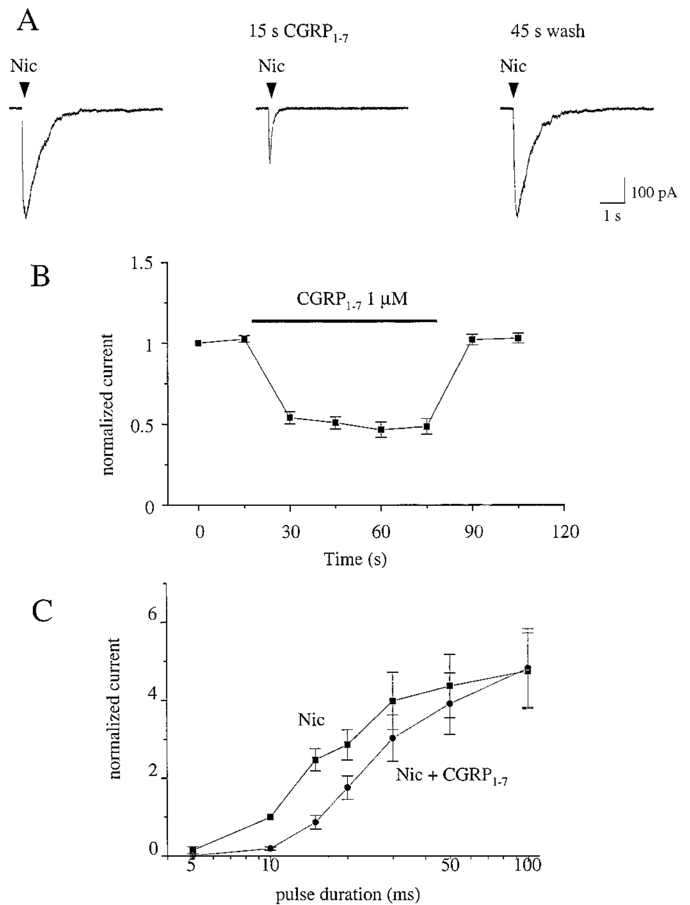

Figure 8. Depression of nicotine currents by the $CGRP_{1-7}$ fragment. *A*, Inward current induced by nicotine (20 msec; 0.1 mM pipette concentration) is largely and reversibly depressed by $CGRP_{1-7}$ (15 sec pulse; 1 μM). *B*, Time course of depression of nicotine currents by pressure-applied $CGRP_{1-7}$. Data are from 8–17 cells. Note rapid onset and offset of current depression and absence of use-dependent block. *C*, Plots of normalized nicotine current amplitude versus increasing duration of nicotine pressure pulses in control solution or in the presence of CGRP1–7 (1 μM; pressure applied; $n = 4$–18 cells). For further details see legend to Figure 4.

$CGRP_{1-7}$ blocks nicotine-mediated currents without changing $[Ca^{2+}]_i$

Because $hCGRP_{8-37}$ had no effect on nicotine currents, it seemed likely that the nicotine receptor blocking action was mediated by the 1–7 terminal region of CGRP. For this purpose we used rat $CGRP_{1-7}$ (custom-synthesized by American Peptides, Sunnyvale, CA) to test its action on nicotine-mediated response. Figure 8*A* shows representative records of nicotine-induced (0.1 mM; 20 msec) currents before and during the pressure application of $CGRP_{1-7}$ (1 μM), which depressed by 48% the nAChR-mediated inward current with rapid recovery after washout. Figure 8*B* depicts the time course of $CGRP_{1-7}$ blocking action, which was already fully developed at the first pulse of nicotine in the continuous presence of this antagonist, thus showing lack of use-dependent block. Prompt recovery of nicotine responses was attained at the end of $CGRP_{1-7}$ application. The profile of pharmacological antagonism by $CGRP_{1-7}$ was characterized by a rightward shift of the nicotine plot with unchanged maximum (Fig. 8*C*), a phenomenon thus similar to the one reported above for native CGRP (Fig. 4*A*). In particular, for a 20 msec test pulse of nicotine the depression by $CGRP_{1-7}$ (1 μM intrapipette solu-

tion) amounted to 46 ± 5% (n = 15; $p < 0.01$). Conversely, $CGRP_{1-7}$ did not significantly increase $[Ca^{2+}]_i$ ($\Delta F/F_0$ = 0.2 ± 0.1; n = 9; $p > 0.05$), whereas application of 20 msec nicotine (0.1 mM) raised $[Ca^{2+}]_i$ ($\Delta F/F_0$=3.1 ± 1.1; $p < 0.01$) in the same group of cells (n = 9). These observations suggest that $CGRP_{1-7}$ interacted with nAChRs but did not activate the G-protein-coupled CGRP receptors responsible for the $[Ca^{2+}]_i$, thus indicating that distinct regions of the peptide sequence were responsible for fast nicotine current modulation and slow metabotropic changes on chromaffin cells.

DISCUSSION

The principal finding of the present study is the novel, very fast modulation by CGRP of neuronal nAChRs on rat chromaffin cells. This was manifested as a rapid onset and agonist-surmountable block of inward currents (and associated $[Ca^{2+}]_i$ transients) evoked by pulse applications of nicotine. Such a phenomenon was distinct from the slow rise in $[Ca^{2+}]_i$ induced by CGRP via its G-protein-coupled receptors in view of its sensitivity to $hCGRP_{8-37}$ antagonism or intracellular BAPTA. Because of the endogenous occurrence of CGRP in the adrenals (Kuramoto et al., 1987; Costa et al., 1994; Heym et al., 1995), such a quick and reversible downregulation of nicotinic receptors suggests that CGRP may play an important modulatory role in fast signaling of these cells, before any Ca^{2+}-dependent modulation of nAChRs could develop.

Characteristics of the fast action of CGRP on nicotine-mediated responses

When CGRP was focally applied to a chromaffin cell for up to 15 sec before nicotine application, it evoked no change in baseline current but strongly depressed the inward currents and $[Ca^{2+}]_i$ rises induced by nicotine. The extent of the block did not intensify during continuous application of CGRP and was unrelated to $[Ca^{2+}]_i$ buffering by BAPTA. In fact, we often used BAPTA in the recording pipette to eliminate the slow $[Ca^{2+}]_i$ rise elicited by CGRP to avoid a possible contamination of the early CGRP action. The similarity of blocking action with puffer- or bath-applied CGRP excluded the possibility of a drug delivery artifact or a large underestimation of the peptide potency. Future experiments using an ultra-fast perfusion system should help to provide a more direct quantification of the potency of this substance. When CGRP and nicotine were co-applied from the same pipette, the ensuing current response was decreased even if the application time was only 20 msec long. This finding shows that CGPR could act on a very rapid time-scale, as fast in fact as nicotine itself, raising the possibility that CGRP interacted directly with the nicotinic receptors. This effect of CGRP did not extend to the ionotropic receptors opened by GABA nor was it mimicked by other neuropeptides such as dynorphin A (which modulates NMDA receptors of central neurons) (Zhang et al., 1997) or TRH. These observations concur to assign specificity to the blocking action of CGRP on nAChRs. The recent description of a discrete, direct blocking action by substance P against certain subunits of nAChRs suggests that a similar phenomenon is not a peculiarity of CGRP action (Stafford et al., 1994).

One obvious possibility is that CGRP blocked receptor channels opened by nicotine in analogy with the results obtained with other substances such as local anesthetics (Neher and Steinbach, 1978), especially because this process has been shown to occur with substance P (Clapham and Neher, 1984). This mechanism seems unlikely under the present conditions, however, because the block was not use dependent either during continuous application of CGRP or with the paired-pulse protocol. Furthermore, there was no voltage dependence of the block, which appeared to be uniform throughout a wide range of membrane potential.

The use of nonequilibrium responses to nicotine and the puffer-application protocol precluded strictly quantitative pharmacological data to analyze in detail the nature of the CGRP antagonism. Even with these constraints it was apparent that CGRP preferentially blocked small (and short) responses to nicotine and that increasing the amount of nicotine delivered to the cell counteracted the inhibitory effect of CGRP. In fact, the graph plotting the fractional response amplitude versus the amount of nicotine delivered by pressure pulse showed a rightward shift in the presence of CGRP. This observation is consistent with an apparently competitive antagonism of CGRP on nicotinic receptors, especially because the competitive antagonist F3 elicited a very similar type of antagonism. Co-application of rather low doses of CGRP and F3 produced antagonism summation. When the extent of the rightward shift of the nicotine plot was analyzed in terms of agonist *DR* values to reproduce equivalent responses, it was apparent that the *DR* value in the combined presence of CGRP and F3 was the sum of the individual values for each blocker alone. This is regarded as indicative of competitive antagonism on the basis of the standard receptor theory (Barlow, 1980), although the present experiments cannot identify the discrete receptor structure to which CGRP would bind to exert its effect.

The residual nicotine currents (left despite the wide range of CGRP concentrations tested) might have reflected heterogeneity in nAChR sensitivity to CGRP antagonism. Because nAChRs of chromaffin cells are known to comprise various subunit assemblies with predominantly $\alpha_3\beta_4$ composition but also α_5 and α_7 (Campos-Caro et al., 1997; Lopez et al., 1998), it seems conceivable that CGRP preferentially blocked only some of them. Future experiments with substances such as α-conotoxin AuIB, which has very recently been reported as a highly selective blocker of the $\alpha_3\beta_4$ subunits (Luo et al., 1998), should help to resolve this issue.

Several other processes appeared unable to account for the blocking effect of CGRP. For example, facilitation of desensitization (as proposed for substance P) (Clapham and Neher, 1984; Simmons et al., 1990; Valenta et al., 1993) also appeared improbable because responses induced by large doses of nicotine, which are more prone to desensitization (Valenta et al., 1993; Khiroug et al., 1997, 1998), were relatively spared by CGRP. Allosteric modulation of nAChRs by binding to a discrete region of the nicotinic receptor distinct from the agonist site would also depress nicotinic responses, as amply investigated in the case of substance P (Livett et al., 1979; Stafford et al., 1994). Although a similar action by CGRP is not excluded, this should be accompanied by a downward shift of the agonist dose–response curve (Akasu et al., 1983; Stafford et al., 1994), a prediction not borne out by the present findings. A more direct examination of these possibilities, however, will require future studies based on single-channel recording and site-directed mutagenesis of recombinant receptors in expression systems.

Characteristics of the slow action of CGRP

CGRP is known to be present in adrenal tissue (Kuramoto et al., 1987; Costa et al., 1994; Heym et al., 1995) where it binds predominantly to the $CGRP_1$ receptor subclass (Mazzocchi et al., 1996) preferentially antagonized by the peptide fragment $hCGRP_{8-37}$ (Bell and McDermott, 1996). CGRP receptors are

G-protein-coupled units that trigger slow metabolic reactions typically mediated by a rise in intracellular cAMP and PKA activity (Bell and McDermott, 1996). In keeping with this general property, we also observed that CGRP elicited a delayed increment in $[Ca^{2+}]_i$ blocked by $hCGRP_{8-37}$ or the PKA inhibitor Rp-cAMPS. The CGRP-induced slow $[Ca^{2+}]_i$ rise presumably involved release of this divalent cation from internal stores because it persisted in Ca^{2+}-free solution, whereas the nicotine-induced $[Ca^{2+}]_i$ transients, which are caused mainly by transmembrane influx (Mulle et al., 1992; Vernino et al., 1994; Khiroug et al., 1997, 1998), were abolished. It is interesting that $hCGRP_{8-37}$ per se had no effect on $[Ca^{2+}]_i$, suggesting that this substance was not a partial agonist on these cells (Bell and McDermott, 1996).

Structural determinants for the action of CGRP

$hCGRP_{8-37}$ did not affect nicotine-induced currents, yet this compound differs from CGRP for missing only a terminal sequence series of amino acids. This consideration suggests that CGRP actually interacted with nicotinic receptors through the amino acid sequence missing from the antagonist molecule. This possibility was supported by the direct demonstration that $CGRP_{1-7}$ acted like full-length CGRP to rapidly block nAChRs with comparable effectiveness. At the same time, even sustained applications of $CGRP_{1-7}$ failed to raise $[Ca^{2+}]_i$ significantly, indicating that one terminal sequence of the peptide was responsible for rapid block of nAChRs, whereas the full-length molecule was necessary for the slow $[Ca^{2+}]_i$ increase presumably mediated by metabotropic CGRP receptor activation. This secondary, albeit small, increase in $[Ca^{2+}]_i$ induced by CGRP might be expected to control the phosphorylation state of the nAChRs because it has been shown to occur in the case of CGRP on muscle-type nAChRs (Mulle et al., 1988; Miles et al., 1989; Lu et al., 1993) or of substance P on neuronal-type nAChRs (for review, see Huganir and Greengard, 1990). Thus, on chromaffin cells a slow rise in $[Ca^{2+}]_i$ might have important functional consequences because it regulates the rate of recovery of nAChRs from desensitization by controlling the balance between their phosphorylation/dephosphorylation (Khiroug et al., 1997, 1998), or it would directly enable tonic catecholamine release (Rosenfeld et al., 1992). In summary then, on chromaffin cells CGRP may regulate nAChRs via a dual action consisting of fast, direct receptor interaction and a slow, indirect receptor modulation mediated by $[Ca^{2+}]_i$ rise.

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