Chromostatin Receptors Control Calcium Channel Activity in Adrenal Chromaffin Cells*

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One of the functions of chromogranin A (CGA), the major soluble component of secretory granules in both adrenal medullary chromaffin cells and many other endocrine cell types appears to be that of a prohormone. CGA is the precursor of several peptides including pancreastatin, a 49-residue peptide, and a 20-residue peptide, chromostatin, which have been identified as biologically active peptides. Chromostatin produces a dose-dependent inhibition (IDso of 5 nM) of the secretagogue-evoked catecholamine secretion from chromaffin cells. Here we report that chromostatin potently inhibits L-type calcium currents recorded with the nystatin-perforated patch technique in cultured chromaffin cells. This inhibitory effect of chromostatin on calcium currents was not observed in experiments using the classical patch-clamp whole-cell approach which induces the leakage of cytoplasmic components. Using 125I-chromostatin, we show that chromostatin exhibits a fully reversible and saturable binding to the plasma membrane of cultured chromaffin cells. Analysis of binding experiments at equilibrium indicates the existence of one class of binding sites with a Bmax of 2.7 pmol/mg of chromaffin cell protein and an apparent Kd of 6.5 nM. This high affinity is in good correlation with the half-maximal concentration (IDso 5 nM) of chromostatin inhibiting catecholamine secretion from chromaffin cells. Specificity of the chromostatin binding was further assessed by displacement experiments with unlabeled CGA-related or -unrelated peptides. We found an excellent quantitative correlation between the affinities of the various peptides determined by binding assays and their functional potency tested on catecholamine secretion: bovine chromostatin > human chromostatin > CGA >> rat chromostatin, pancreastatin, CAP-14, substance P, and Leu-enkephalin. Cross-linking experiments reveal that chromostatin associates specifically with an 80-kDa plasma membrane protein. These results together with the patch-clamp experiments support the idea that chromaffin cells possess specific chromostatin receptors and that activation of such receptors leads to the inhibition of L-type voltage-sensitive calcium channels through an intracellular second messenger pathway.

Chromogranin A (CGA) is an ubiquitous 48-kDa secretory protein first described in the adrenal medulla (1) which has subsequently been found in both the central and peripheral nervous systems and in many neuroendocrine cells including thyroid, parathyroid, pancreatic islets, pituitary, and various endocrine cells of the gut (2). At the subcellular level, CGA is stored in the secretory vesicles together with neurotransmitters or hormones characteristic of each endocrine cell type and is released from them by exocytosis after cell stimulation. CGA is the major member of a family of very abundant and very acidic proteins, the chromogranins, which are most concentrated in the adrenal medulla. However, despite their widespread distribution, the function of this class of proteins is still elusive although recent evidence suggests a possible paracrine role for CGA. Cloning of porcine CGA (3) established that CGA is the precursor of pancreastatin, a 49-residue peptide that negatively modulates insulin secretion from endocrine pancreatic islets (4, 5) and Rin m 5F cells (6), amylase release from exocrine pancreas (7), and acid secretion from parietal cells (8). There is also evidence that an amino-terminal CGA-derived peptide differentially regulates the secretion of calcitonin gene products in a human lung tumor cell line (9) and inhibits the contractile response in human blood vessel segments in vitro (31), and a role for CGA as an autocrine inhibitor of pro-opiomelanocortin secretion has been recently proposed (10).

Recently, we have demonstrated that CGA can be the precursor of a 20-residue peptide, chromostatin, which exerts a negative feedback control on the secretory activity of adrenal medullary chromaffin cells and as such may be an endocrine modulator of catecholamine-associated responses (11, 12). The purpose of the present study was to examine the molecular mechanisms underlying the chromostatin inhibitory effect on catecholamine secretion. We demonstrate that bovine chromaffin cells in culture possess specific receptors for chromostatin and show that their activation produces a
marked decrease of calcium entry through voltage-gated L-type calcium channels.

MATERIALS AND METHODS

Chromaffin Cell Culture and [3H]Noradrenaline Release Assay—Chromaffin cells were isolated and cultured as previously described (12, 13). Cells were grown on 24-well (16-mm wells) Costar plates (Costar) at a density of 250,000 cells/well. 4-7-day-old cultured cells were loaded with [3H]noradrenaline (Amersham, United Kingdom), washed with Locke’s solution (11), and then incubated for 10 min in Locke’s solution containing the indicated concentrations of peptides. Cells were subsequently stimulated with 0.5 mM carbamylcholine in Locke’s solution. [3H]Noradrenaline secretion was calculated as the percentage of total radioactivity present in the cells at the beginning of the experiment assessed from the amount released plus the amount remaining in the cells. Net secretory values obtained by subtracting basal release in the absence of secretagogue (7.8 ± 0.5%) were used in the calculation of the percent inhibition produced by chromostatin or other peptides.

Peptide Synthesis and Preparation of [125I]-Chromostatin—Bovine chromostatin (SDEDSGDRQAPGSLGCAPGP), homologous rat chromostatin (DAFEGTTTEQRPAPGQPEPQKES), human chromostatin (SCEATGDQAPQLEPMPQES), and CAP-14 were synthesized by standard solid-phase t-BOC chemistry and purified by an acetonitrile/water gradient. The solvent system consisted of 19.9% water, 80% acetonitrile (solvent B). The biological activity of [125I]-chromostatin was tested on carbamylcholine-evoked catecholamine secretion from cultured chromaffin cells and was found to be unchanged compared to unlabeled chromostatin.

Binding Studies—For binding experiments, chromaffin cells (250,000 cells/well) were extensively washed with Locke’s solution and preincubated with [125I]-chromostatin (9.5 × 10⁵ ± 3.0 × 10⁴ cpm) in the presence of various concentrations of unlabeled chromostatin or in the presence of various concentrations of the indicated CGA-derived peptides. The assays were performed in 0.5 ml of Locke’s solution containing 0.25% bovine serum albumin. Steady state was achieved by incubation for 2 h at 15°C. The binding buffer was removed to terminate the reaction, cells were washed three times with Locke’s solution, and the remaining radioactivity associated with the cells was measured in a LKB 1260 gamma counter.

Electrophysiology—Chromaffin cells were bathed in a solution to which the following composition (in mM/liter): choline C1, 140; KCl, 5; CaCl₂, 2.5; MgCl₂, 1; HEPES, 10; glucose, 11 (pH 7.4). A high capacitive transient was subtracted using a P/8 procedure (17). The protocols were under computer control, and current transients were acquired at 40-ps sampling intervals. The linear component of the current was obtained before addition of the peptide (Fig. 1A). The amplitude and kinetic characteristics of this Ca²⁺ current did not change appreciably with time up to 30 min after establishing electrical continuity between the cell interior and the pipette solution. Following application of chromostatin (1 μM), there is a substantial, time-dependent reduction in the amplitude of the inward current that reached a value of around 50% of the control value obtained before addition of the peptide (Fig. 1B). The peptide was added to the bath in aqueous solution using a micropipette. Addition of a similar volume of fluid without the peptide did not affect chromaffin cell Ca²⁺ currents (data not shown). The time dependence of the inhibitory action of chromostatin is related to the time required for diffusion of the peptide from the point of application in the bath to the cell. Current-voltage relationships for a control cell, taken 9 min apart, indicate that, using the nystatin-perforated patch, there is no significant decrease in Ca²⁺ current amplitude during this period (Fig. 1C). However, the addition of chromostatin (1 μM) to the bath in aqueous solution resulted in a large decrease in the peak current at all membrane potentials studied (Fig. 1D). As a result, the chord conductance decreased from 5.8 nS, in the absence of the peptide, to 3.2 nS, in the presence of chromostatin. While data reported in Fig. 1, C and D were obtained in individual cells, the average values of the peak Ca²⁺ current for all the cells studied were (for the initial family of depolarizing pulses): −214.9 ± 32.2 pA (n = 6; minimum and maximum values of the current: −138.1 and −327.5 pA) for control cells and −245.2 ± 54.9 (n = 4; minimum and maximum values of the current: −112 and −336.1 pA) for cells that were treated later with chromostatin. These data demonstrate that both groups of either control or treated cells are similar concerning depolarization-induced inward Ca²⁺ currents in non-treated conditions. Data also indicate that to study the buffer. The cross-linking reaction was stopped by washing the cells rapidly with a solution of 50 mM Tris (pH 7.6) and 160 mM NaCl. Cells were then scraped off the plate, homogenized, and centrifuged at 55,000 rpm for 30 min to separate membrane and soluble fractions. Samples were solubilized in sodium dodecyl sulfate sample buffer and analyzed on 12% polyacrylamide gel electrophoresis. The dried gel was exposed to Amersham Hyperfilm MP with an intensifying screen at −70°C. The radioactivity was quantitated on the autoradiograph by scanning densitometry using an LKB 2202 Ultrascan laser densitometer at 633 nm.

RESULTS

Effect of Chromostatin on Voltage-dependent Calcium Current—Previous work from our laboratory has shown that chromostatin, a peptide located between positions 124 and 143 in the bovine CGA sequence (12), produces a dose-dependent inhibition of the catecholamine secretion from chromaffin cells evoked either by cholinergic agonists or by direct depolarization with potassium (12). This peptide also affects secretagogue-induced Ca²⁺ uptake in chromaffin cells but does not alter Na⁺ uptake (13). The simplest explanation for these effects is the inhibition of the voltage-dependent Ca²⁺ currents. However, initial experiments using the classical patch-clamp whole-cell approach (19) failed to show any effect of chromostatin on chromaffin cell Ca²⁺ currents (data not shown). Therefore we used the nystatin-perforated patch technique (20) to record whole cell Ca²⁺ currents in chromaffin cells. This new technique does not provoke the leakage of cytoplasmic small metabolites and proteins, and membrane currents remain stable for more than 1 h. Depolarization of chromaffin cells, from a holding potential of −80 mV, in 10 mV depolarizing steps induces activation of voltage-dependent Ca²⁺ channels and a clear inward Ca²⁺ current was observed (Fig. 1A). This current showed a single non-inactivating component as previously described for chromaffin cells (18, 21). The amplitude and kinetic characteristics of this Ca²⁺ current did not change appreciably with time up to 30 min after establishing electrical continuity between the cell interior and the pipette solution. Following application of chromostatin (1 μM), there is a substantial, time-dependent reduction in the amplitude of the inward current that reached a value of around 50% of the control value obtained before addition of the peptide (Fig. 1B). The peptide was added to the bath in aqueous solution using a micropipette. Addition of a similar volume of fluid without the peptide did not affect chromaffin cell Ca²⁺ currents (data not shown). The time dependence of the inhibitory action of chromostatin is related to the time required for diffusion of the peptide from the point of application in the bath to the cell. Current-voltage relationships for a control cell, taken 9 min apart, indicate that, using the nystatin-perforated patch, there is no significant decrease in Ca²⁺ current amplitude during this period (Fig. 1C). However, the addition of chromostatin (1 μM) to the bath produced a large decrease in the peak current at all membrane potentials studied (Fig. 1D). As a result, the chord conductance decreased from 5.8 nS, in the absence of the peptide, to 3.2 nS, in the presence of chromostatin. While data reported in Fig. 1, C and D were obtained in individual cells, the average values of the peak Ca²⁺ current for all the cells studied were (for the initial family of depolarizing pulses): −214.9 ± 32.2 pA (n = 6; minimum and maximum values of the current: −138.1 and −327.5 pA) for control cells and −245.2 ± 54.9 (n = 4; minimum and maximum values of the current: −112 and −336.1 pA) for cells that were treated later with chromostatin. These data demonstrate that both groups of either control or treated cells are similar concerning depolarization-induced inward Ca²⁺ currents in non-treated conditions. Data also indicate that to study the
Fig. 1. Effect of chromostatin on L-type Ca\(^{2+}\) currents in chromaffin cells. Panel A, family of recordings showing inward and tail Ca\(^{2+}\) currents in a bovine chromaffin cell. The numbers on some of the recordings indicate membrane potential (in mV). Panel B, similar family of recordings obtained from the same cell 9 min after addition of chromostatin (1 \(\mu\)M) to the vicinity of the cell. Panel C, current-voltage relationships for two families of recordings obtained 9 min apart in a control cell. Open circles, current obtained 16 min after gigaseal formation; triangles, current obtained 25 min after gigaseal formation. Panel D, current-voltage relationship for the families of recordings shown in panels A and B. Open circles, current obtained in the absence of peptide 16 min after gigaseal formation; chromostatin (1 \(\mu\)M) was added at min 17; closed circles, current-voltage relationship obtained 25 min after gigaseal formation (8 min after addition of chromostatin to the bath). Panel E, time course of the effect of chromostatin (1 \(\mu\)M) on peak Ca\(^{2+}\) current in chromaffin cells. Peak current was determined 10 min after gigaseal formation and then every 3 min. Data represent mean ± S.E. for both control (open circles, \(n = 6\)) and treated (closed circles, \(n = 4\)) cells. The peptide was added to the treated cells group at the point shown by the arrow (*, \(p < 0.05\), as compared to control).
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effect of specific compounds on chromaffin cell L-type Ca\(^{2+}\) inward currents it is necessary to normalize inward Ca\(^{2+}\) currents to avoid individual cell variation. Two procedures can be used for normalization: either to relate the peak current to cell capacitance, used as index of cell size (18) or to relate peak currents obtained during consecutive families of depolarizing pulses to the peak current obtained in the same cell during the initial family of depolarizing pulses in the absence of treatment. The later method is specially useful when time inactivation of Ca\(^{2+}\) currents is minimized as it occurs using the nystatin-perforated patch technique (20). This procedure of normalization is shown in Fig. 1E where the time course of the inhibitory action of the peptide was studied by repeating I-V curves every 3 min for both control and peptide-treated cells. The ratio between the maximal inward current obtained at each time and the maximal inward current obtained 3 min after whole cell configuration (10 min after cell-attached seal) was investigated. This ratio is a good index of the decrease of the peak Ca\(^{2+}\) current with time. In control, non-treated cells the ratio \(I_{peak}/I_{peak}\) was not substantially modified and remained close to 1 (Fig. 1E). However, addition of chromostatin (1 \(\mu\)M) caused a marked decrease in this ratio, indicating that chromostatin blocks L-type voltage-dependent Ca\(^{2+}\) currents in chromaffin cells (Fig. 1E).

Binding Properties of Chromostatin on the Chromaffin Cell Plasma Membrane—Using \(^{125}\)I-labeled synthetic chromostatin, we examined the binding characteristics of chromostatin on the plasma membrane of intact cultured chromaffin cells. Experiments were performed at 15 °C to reduce the possibility of endocytotic uptake that may occur in living cells. As illustrated in Fig. 2, binding curves indicated the presence of a single site with an apparent \(K_D\) of 6.51 ± 0.73 nM, a value which is comparable to the half-maximal concentration of chromostatin which inhibits catecholamine release, and a \(B_{max}\) of 2.7 ± 0.4 pmol/mg protein (determined on six different cell preparations).

\(^{125}\)I-Chromostatin exhibited a high degree of specific binding to chromaffin cells but not to endothelial- or fibroblast-like cells present in the culture, since removal of chromaffin cells by treatment with 6-hydroxydopamine (22) or by differ-

![Fig. 2. Equilibrium binding of \(^{125}\)I-chromostatin to intact chromaffin cells maintained in culture. A representative experiment performed in triplicate is shown. Solid line represents total binding (+); dotted lines represent saturable (○) and non-saturable (▲) components obtained by nonlinear regression analysis using the LIGAND program (30). Inset, Scatchard plot of the saturable binding component. Scatchard analysis of six experiments on different cell preparations indicates an average binding capacity \(B_{max}\) of 2.7 ± 0.4 pmol/mg protein with an apparent \(K_D\) of 6.51 ± 0.73 nM.](image1)

![Fig. 3. Panel A, specificity of \(^{125}\)I-chromostatin binding to cultured bovine chromaffin cells. Representative curves are shown for the concentration-dependent inhibition of \(^{125}\)I-chromostatin binding by various CGA-derived peptides. Results shown are the mean of triplicate determinations and are expressed relative to the specific binding obtained in the absence of unlabeled peptides. Specific binding was calculated by subtracting the nonspecific binding (binding in the presence of 1 \(\mu\)M chromostatin) from the total binding. Nonspecific binding was ±2% of the added radioactivity. Panel B, effect of chromostatin and the various CGA-derived peptides on carbamylcholine-evoked catecholamine release from cultured chromaffin cells. Results are expressed relative to the net \([\text{H}]\)noradrenaline release obtained in the absence of chromostatin (17.7 ± 0.3%). Data are given as the mean of triplicate determinations on the same cell preparation ± S.D. Similar results were obtained on three different cell preparations. ChS, chromostatin.](image2)
CAP-14, nor rat chromostatin were able to modulate catecholamine release from bovine chromaffin cells whereas both chromostatin and the chromostatin-related peptide strongly inhibited secretion (Fig. 3B). Interestingly, human chromostatin, which is 50% identical to bovine chromostatin and which has a highly conserved six-amino-acid region in its internal sequence, displaced bound $^{125}$I-chromostatin with an IC$_{50}$ of 50 nM (Fig. 3A), a value which correlates well with the ID$_{50}$ of 30 nM calculated from its biological inhibitory effect on catecholamine secretion from cultured bovine cells (Fig. 3B). Furthermore, as illustrated in Fig. 3 native CGA which has a biological activity approximately 100 times less than that of chromostatin on catecholamine release (ID$_{50}$, 600 nM), displaced bound $^{125}$I-chromostatin with a 100-fold less efficiency (IC$_{50}$, 480 nM) than chromostatin. $^{125}$I-Chromostatin binding was neither affected by CGA-unrelated peptides (substance P, Leu-enkephalin) nor by 1,1-dimethyl-4-phenylpyrazinium$^*$ and apomorphine (data not shown), excluding the possibility that chromostatin may interfere with the nicotinic or the dopaminergic receptors present on chromaffin cells (24).

Cross-linking of $^{125}$I-Chromostatin to Chromaffin Cell Plasma Membranes—Cross-linking studies were performed to determine the molecular mass of the chromostatin-binding site. $^{125}$I-Chromostatin was cross-linked to cultured chromaffin cells using the water-soluble coupling agent (25) EDC and the labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since 125I-chromostatin had its amino groups blocked, coupling is likely to take place through the six carboxylic groups present along the peptide (4 Asp, 1 Glu, 1 Pro) enhancing the probability of the reaction. As illustrated in Fig. 4, a unique band corresponding to a component of apparent molecular mass 80 kDa was detected. This band was not detectable when cells were preincubated with an excess of native chromostatin before incubation with $^{125}$I-chromostatin. Moreover, the presence of chromostatin in the extracellular medium in concentrations which displace the specific high affinity binding of $^{125}$I-chromostatin (see Fig. 3A), progressively decreased the labeling of the 80-kDa band.

Mild treatment of the cells with trypsin under conditions which do not detach the cultured cells led to the complete disappearance of the radioactive 80-kDa band, confirming that the binding site for $^{125}$I-chromostatin is a plasma membrane protein. This putative chromostatin receptor is specifically located on the chromaffin cell membrane since the selective removal of chromaffin cells with 6-hydroxydopamine prior to the binding reaction completely inhibited the labeling of the 80-kDa protein (data not shown).

**DISCUSSION**

CGA was first characterized about 25 years ago and it was thought that this protein was limited to chromaffin granules of the adrenal medulla (26). It is now clear that CGA is actually widely distributed in mammalian endocrine and neuroendocrine cells and in nervous tissues. Since it was discovered, CGA has been extensively studied; its physicochemical properties have been analyzed in detail (for review, see Ref. 2), the complete amino acid sequence has been described (27, 28) but no physiological function has been conclusively established. We have recently demonstrated that proteolytic processing of CGA generates a peptide, chromostatin, which is able to exert an autocrine modulation of catecholamine secretion from chromaffin cells (11, 12), and we have therefore suggested that CGA may be a prohormone precursor of biologically active peptides. This view is strongly supported by the discovery that pancreastatin, a peptide controlling insulin release (4, 5) is also contained in the CGA sequence (3) and that CGA-derived peptides appear to regulate secretion of pro-opiomelanocortin (10) and of calcitonin gene products (9). Thus, CGA seems to play an important role in homeostasis.

To further support the proposed hormonal function of CGA-derived peptides, it was essential to investigate the molecular basis of their action on the secretory activity of target cells. The purpose of the present studies was to determine the molecular mechanisms by which chromostatin modulates catecholamine secretion. Chromostatin is able to completely inhibit catecholamine release when chromaffin cells are stimulated with cholinergic agonists (12) and partially when cells are directly depolarized with veratridine$^*$ or potassium (12). Since the common mechanism of action of these secretagogues involves an elevation of the cytosolic free calcium, we investigated the possibility that chromostatin may alter the secretagogue-induced calcium fluxes. The present results clearly indicate that chromostatin is able to decrease the activity of the L-type voltage-gated calcium channels present in the plasma membrane of medullary chromaffin cells. Since we have previously observed that chromostatin also reduces the calcium uptake in stimulated cells (12), it is reasonable to assume that the mechanism by which chromostatin inhibits exocytosis in chromaffin cells is based on the ability of the peptide to alter the influx of calcium following cell stimulation. Interestingly, initial experiments using the classical patch-clamp whole-cell approach failed to show any effect of chromostatin on chromaffin cell calcium currents. In contrast, using the nystatin-perforated patch technique which prevents dialysis of second messengers, chromostatin was found to produce a marked decrease in the L-type voltage-dependent calcium currents. Thus, it is unlikely that chromostatin directly inhibits the calcium current by an open

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$^*$E. Galindo, M. Mendes, M.-F. Bader, and D. Aunis, unpublished results.
channel blocking effect or through a putative regulatory site on the calcium channel itself. Indeed our observation is consistent with the notion that a specific receptor for chromostatin exists on chromaffin cells and that its activation by chromostatin leads to the inhibition of L-type calcium channels through a coupling mechanism involving a diffusible second messenger.

Radiolabeled chromostatin was used to demonstrate the existence of specific receptors for chromostatin on chromaffin cells grown in primary culture. We have found that chromostatin exhibits a fully reversible and saturable binding specific to chromaffin cells, since it was not observed in other cell types present in the culture after removal of chromaffin cells. Analysis of binding experiments at equilibrium indicates the existence of one class of binding sites with a capacity of 2.7 pmol/mg of chromaffin cell protein and an apparent $K_d$ of about 6.5 nM. This low value correlates well with the half-maximal concentration ($D_{50}$, 5 nM) of chromostatin inhibiting catecholamine secretion from chromaffin cells. Specificity of the chromostatin binding was further assessed by displacement experiments with unlabeled CGA-related or -unrelated peptides. We found an excellent quantitative correlation between the affinities of the various peptides determined by binding assays and their functional potency tested on catecholamine secretion from chromaffin cells after removal of the peptide. The observation that chromostatin inhibits Ca$^{2+}$ currents from chromaffin cells after removal of the peptide from the incubation bath (12), indicates that chromostatin leads to the inhibition of L-type calcium channels through a coupling mechanism involving a diffusible messenger.

Furthermore, chromogranin A is present not only in catecholaminergic systems but also in many other neurotransmitter secretory vesicles. Thus, chromostatin regulation may be involved in controlling calcium-dependent secretion in various neuroendocrine cell types. More detailed studies on the processing and half-life of the peptide in vivo are now required to substantiate this hypothesis.

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