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Chromostatin, a 20-amino acid peptide derived from chromogranin A, inhibits chromaffin cell secretion

(adrenal medulla/catecholamines/exocytosis/stress)

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ABSTRACT **Chromogranin A (CGA) is a ubiquitous 48-kDa secretory protein present in adrenal medulla, anterior pituitary, central and peripheral nervous system, endocrine gut, thyroid, parathyroid, and endocrine pancreas. Recently, we have demonstrated that the protein could be a precursor of bioactive peptides capable of modulating catecholamine secretion from cultured adrenal medullary chromaffin cells. Here we cleaved CGA purified from bovine chromaffin granules with endoproteinase Lys-C, and we isolated and partially sequenced the peptide inhibiting catecholamine secretion from cultured chromaffin cells. A corresponding synthetic peptide composed of the first 20 N-terminal amino acids produced a dose-dependent inhibition in the 10^{-9} to 10^{-6} M range (with an ID_{50} of 5 nM) of the catecholamine secretion evoked by carbamoylcholine or by potassium at a depolarizing concentration. This peptide affected secretagogue-induced calcium fluxes but did not alter sodium fluxes. It was found to increase desensitization of cell responses and to modify the kinetics of catecholamine release. Our results indicate that the peptide is extracellularly generated from CGA by a calcium-dependent proteolytic mechanism. We suggest that this peptide, named chromostatin, may be an endocrine modulator of catecholamine-associated responses.**

Chromogranin A (CGA), a large acidic protein first found in the catecholamine-storage granules of chromaffin cells (1), is released with catecholamines by exocytosis. Many recent studies indicate that CGA is not confined to adrenal medulla, but it is also present in anterior pituitary, central and peripheral nervous systems, endocrine gut, thyroid, parathyroid, and endocrine pancreas (see ref. 2 for review). However, despite the widespread distribution of CGA, no function has been conclusively established.

The sequence of pancreastatin, a peptide from porcine pancreas inhibiting insulin secretion from rat pancreas (3, 4) and acid secretion from parietal cells (5), is fully contained within the sequence of porcine CGA (6, 7). Recently, we have demonstrated that CGA could be the precursor of peptides which exert a negative feedback control on the secretory activity of chromaffin cells (6). Together these results support the view that CGA is a prohormone (2, 6, 8).

In the present study, we describe the purification of two CGA-derived peptides active on catecholamine secretion from cultured chromaffin cells. A corresponding 20-amino acid peptide named chromostatin was synthesized and was found to completely inhibit catecholamine secretion in the nanomolar to micromolar range. These results further support the potential role of CGA as a precursor of a peptide which might be involved in the regulation of catecholamine secretion.

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MATERIAL AND METHODS

Chromaffin Cell Culture and [^{3}H]Noradrenaline (NAd; Norepinephrine) Release Assay. Chromaffin cells in culture were prepared as previously described (9), except that solutions for washing and disaggregating the cells and culture media were filtered as recommended by Knight (10). Cells were grown on 24-well (16-mm wells) Costar plates. Cells (250,000 per well) were loaded with 125 nM [^{3}H]NAd, washed with Locke's solution (6, 11), and then incubated with peptide at indicated concentrations. Cells were subsequently stimulated with Locke's solution containing either 0.5 mM carbamoylcholine or 59 mM KCl (53.1 mM NaCl replaced by KCl). [^{3}H]NAd secretion was determined as described (6). Net secretory values were obtained by subtracting basal levels in the absence of secretagogues (7.8 ± 0.5%) and were used in the calculation of the percent inhibition produced by chromostatin.

On-Line Measurement of Catecholamine Release from Chromaffin Cells. In some experiments, catecholamine release was measured electrochemically in a perfusion monitoring system (12–14). Freshly isolated chromaffin cells, purified and washed as described previously (9), were suspended in Locke's solution. Cells (10^6) were perfused at a flow rate of 2 ml/min with Locke's solution or with Locke's solution containing 50 μM 1,1-dimethyl-4-phenylpiperazinium ($DMPP^+$) iodide in the presence or absence of chromostatin. Cells were then stimulated with pulses of 250 μM $DMPP^+$. Released catecholamines were measured directly by an electrochemical detector (Metrohm model 641-VA) as described (12). None of the drugs produced electrochemical signals interfering with catecholamine detection.

Calcium-45 and Sodium-22 Uptake Experiments. $^{45}Ca^{2+}$ (10–40 mCi/mg of Ca; $CaCl_2$ salt; Amersham; 1 Ci = 37 GBq) or $^{22}Na^+$ (32 mCi/mg of Na; NaCl salt; Amersham) influx into cells was measured as previously described (15, 16).

Identification and Purification of Active CGA-Derived Peptides. CGA purified from bovine chromaffin granules (6) was digested for 2 hr at 37°C with endoproteinase Lys-C (Endo-LC; Boehringer) at a protein-to-proteinase weight ratio of 1000:1 in 100 mM Tris·HCl, pH 8.5. The generated peptides were then separated on a preparative reverse-phase C_{18} column (ScienceTec, A-303 S-5 120A ODS, 0.46 × 25 cm). Absorbance was monitored at 215 nm. The solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.085% trifluoroacetic acid/49.9% water/50% acetonitrile (vol/vol) (solvent B). Material was eluted at a flow rate of 1 ml/min with a linear gradient. Each peak fraction was manually collected and lyophilized.

Abbreviations: CGA, chromogranin A; $DMPP^+$, 1,1-dimethyl-4-phenylpiperazinium; NAd, noradrenaline (norepinephrine); Endo-LC, endoproteinase Lys-C.

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Further purification of the identified active peak was achieved on an analytical reverse-phase C_8 column (Brownlee RP-300 C8, flow 200 μl/min). The solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.085% trifluoroacetic acid/29.9% water/70% acetonitrile (vol/vol) (solvent B). Material was eluted by using a linear gradient from 0% to 50% solvent B over a period of 80 min. Absorbance was monitored at 205 nm. The primary structures of HPLC-resolved purified peptides were determined by automatic Edman degradation on a gas-phase sequencer (477A, Applied Biosystems).

Peptide Synthesis. Peptide I-20 (chromostatin) and peptide II-24 were synthesized on an NPS 4000 semi-automated multichannel peptide synthesizer (Neosystem, Strasbourg, France) using the stepwise solid-phase synthetic approach of Merrifield (17) and following the protocol described by Van Regenmortel *et al.* (18). Peptides were further purified by HPLC and lyophilized.

RESULTS

Purification of CGA-Derived Peptides Inhibiting Chromaffin Cell Secretion. In the search for the CGA-derived peptides active on catecholamine secretion from chromaffin cells (6), we first probed two previously described CGA-derived peptides, porcine pancreastatin (3) and CAP-14 [a highly conserved peptide localized between amino acids 316 and 329 in the bovine CGA sequence (19, 20)]. However, these two peptides were found to be totally inactive on catecholamine secretion (Table 1). We therefore attempted to purify the active peptides that are generated by digestion of bovine CGA with Endo-LC. The resulting digest was separated by HPLC (Fig. 1*A*). We detected only one peak capable of inhibiting the catecholamine secretion from chromaffin cells. The asymmetrical shape of the peak indicated some heterogeneity and further purification was achieved by HPLC on a microbore column. Three separate peaks were obtained on a C_8 column at a moderate flow rate (200 μl/min), but the inhibiting activity was restricted to the two last peaks, identified as peak 1 and peak 2, with retention times of 43.46 and 44.73 min, respectively (Fig. 1*B*).

The material contained in each active peak was then subjected to structural analysis. Fig. 2 shows the sequence of the first 26 and 30 residues of the N-terminal region of the peptidic material present in peaks 1 and 2, respectively. Comparison with the known bovine CGA sequence indicates that peptide I from peak 1 has its N-terminal serine residue situated at position 124 in the CGA sequence, and peptide II (peak 2) has the N-terminal glutamic residue located at position 120. Therefore the two peptides share the same sequence with the exception of four additional N-terminal residues in peptide II.

Table 1. Effect of CGA, pancreastatin, synthetic peptide CAP-14, synthetic peptide I-20 (chromostatin), and peptide II-24 on evoked catecholamine secretion from cultured chromaffin cells

Peptide	Net [^{3}H]NAd release, %: Carbamoylcholine (0.5 mM)	Net [^{3}H]NAd release, %: K^+ (59 mM)
None	25.0 ± 0.8	20.2 ± 0.7
CGA, 1 μM	6.3 ± 1.0	11.6 ± 0.9
Pancreastatin, 10 μM	25.2 ± 0.2	20.6 ± 0.4
CAP-14, 10 μM	24.9 ± 0.5	21.9 ± 0.7
I-20, 1 μM	3.8 ± 0.8	9.3 ± 0.9
II-24, 1 μM	4.4 ± 1.0	7.7 ± 0.2

Chromaffin cells were incubated for 10 min with native CGA or with the indicated CGA-derived peptides in Locke's solution and then stimulated for 10 min with Locke's solution containing 0.5 mM carbamoylcholine or 59 mM KCl. Data shown are representative of three separate experiments on different cell culture preparations; each point is the mean of triplicate determinations ± SD.

The presence of a lysine residue in position 25 in peptide II prompted us to further digest the peptides with Endo-LC to determine the part of the molecule having the inhibitory property. Endo-LC (24 hr, 37°C, peptide-to-proteinase ratio of 100:1) did not further cleave peptide I, but peptide II was digested to give the shorter peptide I. This result indicates that the Pro-Lys bond cannot be cleaved by the Endo-LC, in contrast to the Lys-Glu bond. Also, peptides I and II are certainly longer than the 30 residues obtained from sequence analysis, since the next Lys-Glu bond that could be cleaved by the Endo-LC is located in position 179–180 in the bovine CGA sequence (2).

Characterization of Chromostatin and Properties with Respect to Chromaffin Cell Secretion. Two peptides were synthesized: peptide I-20, composed of the 20 residues from position 124 to position 143 in the CGA sequence (Ser-5 to Pro-24 in Fig. 2), and peptide II-24, composed of the 24 residues from position 120 to 143 in the CGA sequence. These synthetic peptides were separated by HPLC but they were not coeluted with the peak containing peptides I and II (data not shown), in agreement with the prediction that peptides I and II are probably longer than 30 amino acid residues. The activity of these two synthetic peptides on catecholamine release was tested on cultured chromaffin cells. As shown in

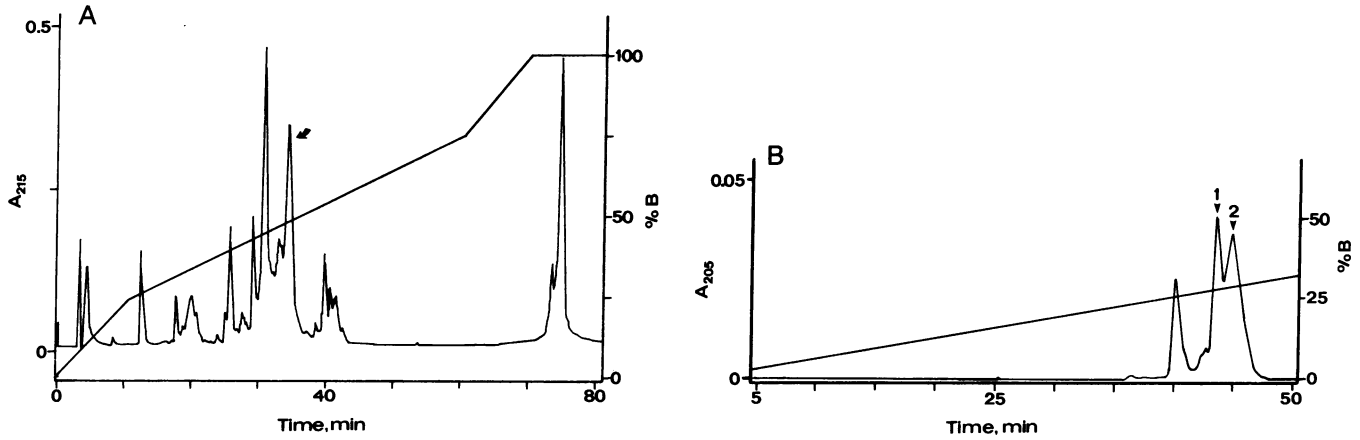

FIG. 1. Identification and purification of the CGA-derived peptides inhibiting chromaffin cell secretion. (*A*) HPLC elution profile of the peptides generated by digestion of bovine CGA with Endo-LC. Peptides were separated on a preparative reverse-phase C_{18} column. The arrow indicates the peak containing the peptide(s) active on catecholamine secretion. (*B*) HPLC rechromatography of the C_{18} active fraction on an analytical C_8 column.

GLU-VAL-GLU-LYS-SER-ASP-GLU-ASP-SER-ASP-GLY-ASP-ARG-PRO-GLN-ALA-SER-PRO-GLY-LEU-GLY-PRO-GLY-PRO-LYS-VAL-GLU-GLU-ASP-ASN-...

1 5 10 15 20 25 30

FIG. 2. Amino acid sequence of first 30 N-terminal amino acids of peptides found in peak I (residues 5–30) and in peak II (residues 1–30). Synthetic peptide I-20 (chromostatin) included the sequence from Ser-5 (arrowhead) to Pro-24 (arrowhead) and peptide II-24 from Glu-1 to Pro-24.

Table 1, both peptide I-20 and peptide II-24 at 1 μM were able to inhibit by 90% the release of NAd evoked by a cholinergic agonist and by 50% the secretion induced by direct depolarization. In a previous paper (6), we reported that CGA at 1 μM inhibited the nicotine-evoked catecholamine secretion by only 40%. This discrepancy is due to modifications that have recently been introduced in our procedure for culturing chromaffin cells: to obtain more reproducible secretory responses we avoided the use of disposable filters, which have been reported to have toxic effects on chromaffin cells (10). This effect of CGA on high-K^+-evoked catecholamine release has also been confirmed on chromaffin cells cultured by another laboratory (D.A. and A. Schneider, unpublished observation).

The dose–response curve obtained with peptide I-20 is illustrated in Fig. 3. Peptide I-20 produced a dose-dependent inhibition of catecholamine release in the 10^{-9} to 10^{-6} M range, with a mean inhibitory dose, ID_{50}, of approximately 5 $\times$ 10^{-9} M. This synthetic 20-amino acid peptide, I-20, was named chromostatin.

Real-time monitoring was used to analyze more precisely the action of chromostatin on catecholamine release. Cells in suspension were stimulated with several pulses of $DMPP^+$ in the presence of 1 μM chromostatin. Repeated pulses with DMPP induced a gradual desensitization in cellular responses (Fig. 4). Chromostatin inhibited by 40% the first transient secretory burst in response to $DMPP^+$ and provoked a complete inhibition of the subsequent secretory responses. Thus chromostatin seems to accentuate the desensitization process of the cholinergic-induced secretory response. When the peptide was removed, secretory responses returned slowly to normal values; after 10 min of washing the response was still only 31% of the control value (data not shown). This result suggests that chromostatin exerts a long-lasting inhibitory effect on chromaffin cell secretory activity, although the possibility that the wash-out procedure has not completely removed the peptide from its binding sites cannot be excluded.

Effects of Chromostatin on Calcium and Sodium Fluxes. Since chromostatin inhibited catecholamine release evoked

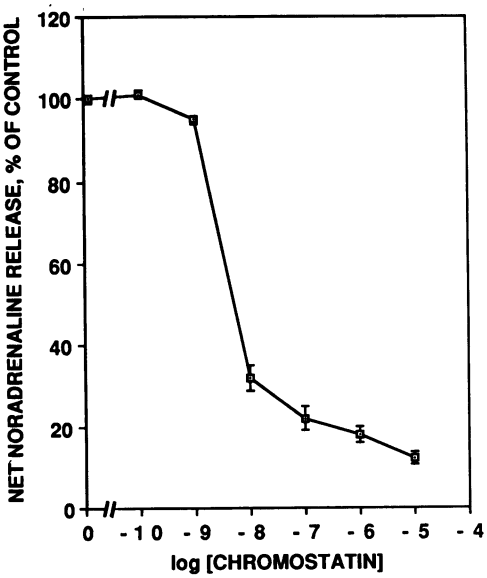

FIG. 3. Effect of chromostatin on catecholamine release from cultured chromaffin cells. Chromaffin cells were incubated for 10 min in Locke's solution containing chromostatin at the indicated concentration (M) and then stimulated for 10 min with 0.5 mM carbamoylcholine. Results are expressed relative to the net [^{3}H]NAd release obtained in the absence of chromostatin (17.7 ± 0.3% of [^{3}H]NAd cell content). Data are given as the mean of triplicate determinations on the same cell preparation ± SD. Similar results were obtained on three different cell preparations.

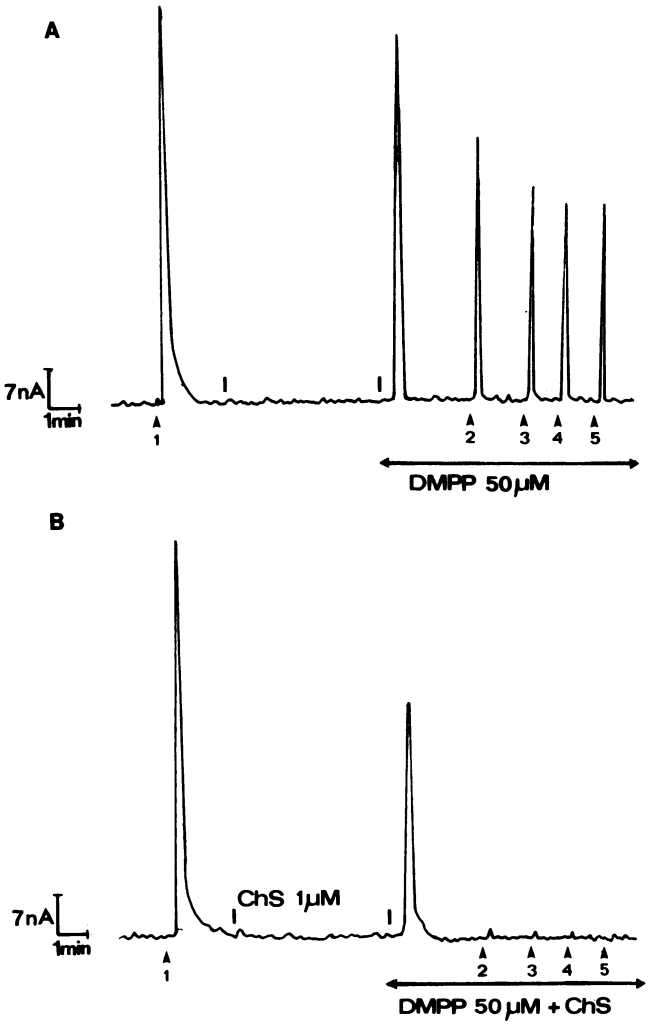

FIG. 4. Effect of chromostatin on $DMPP^+$-evoked secretory response of chromaffin cells in suspension: Continuous recording of electrochemically monitored current-time curve with $DMPP^+$. After exposure to a 250 μM $DMPP^+$ pulsatile stimulation (arrowhead 1), bovine chromaffin cells were washed for 5 min with Locke's solution (*A*) or with Locke's solution containing 1 μM chromostatin (ChS) (*B*). Cells were then perfused continuously with 50 μM $DMPP^+$ in the absence (*A*) or presence (*B*) of peptide. During perfusion, secretory responses to five successive pulses of 250 μM $DMPP^+$ (unmarked and arrowheads 2, 3, 4, and 5) were recorded. The maximum $DMPP^+$ concentration to which cells are exposed on each pulsatile injection is nearly one-fifth of the concentration of the initial injected solution. For continuous perfusion, the maximum concentration in the cell bed is similar to the concentration in the perfusate. Therefore, 250 μM $DMPP^+$ in 50 μl was used for pulsatile stimulation and 50 μM $DMPP^+$ for prolonged stimulation.

by both carbamoylcholine and 59 mM K^+ (Table 1), it may affect a common process of the depolarization-mediated and the receptor-mediated secretory mechanisms—i.e., the rise in internal Ca^{2+} concentration or any step distal to calcium entry. We measured $^{45}Ca^{2+}$ entry into chromaffin cells stimulated with carbamoylcholine or directly depolarized with high potassium in the presence or absence of chromostatin (Fig. 5). Our results show that chromostatin blocked the entry of calcium evoked by carbamoylcholine and high K^+ by 86% and 77%, respectively, suggesting the possibility that the inhibition of catecholamine release might be due to the reduction of calcium influx.

The possibility that chromostatin may alter the potential-dependent activation of the voltage-sensitive calcium channels in response to carbamoylcholine or high-K^+ stimulation by inactivating the voltage-gated sodium channels was examined in the experiment described in Fig. 5. The effect of chromostatin on $^{22}Na^+$ uptake was tested in cells stimulated for 10 min with carbamoylcholine, 59 mM K^+, or veratridine, the latter compound being known to trigger secretion via the activation of voltage-dependent Na^+ channels. Na^+ entry induced by the three secretagogues remained unaffected by 1 μM chromostatin, indicating that the inhibitory action of the peptide is unlikely to involve the voltage-gated Na^+ channels.

Calcium Dependency of CGA Processing. Native CGA provokes inhibition of carbamoylcholine- and high-K^+-evoked catecholamine secretion when the protein is present during an incubation period prior to stimulation (ref. 6; Table 1). Thus, the active peptides are generated during this preincubation period, suggesting that proteolysis occurs in the extracellular space, although the nature of the proteolytic enzymes is not yet known (2, 6, 21). We examined the inhibiting activity of CGA on catecholamine release when calcium was omitted during the preincubation period. As shown in Table 2, the absence of calcium in the external medium during the preincubation period abolished the effect of native CGA on carbamoylcholine-evoked catecholamine release, whereas the inhibiting action of chromostatin was not modified. Thus a calcium-dependent proteolytic enzyme seems to be responsible for the processing of CGA in the extracellular space.

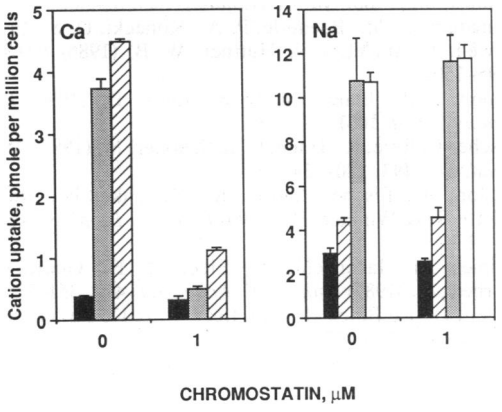

FIG. 5. Effect of chromostatin on secretagogue-evoked $^{45}Ca^{2+}$ uptake and $^{22}Na^+$ uptake. For calcium uptake experiments, cells were incubated for 10 min in Locke's solution containing 1 μM chromostatin, followed by a 30-sec stimulation with Locke's solution (solid bar) or Locke's solution containing either 0.5 mM carbamoylcholine (stippled bar) or 59 mM KCl (hatched bar), in the presence of $^{45}Ca^{2+}$ at 1 μCi/200 μl. For sodium uptake experiments, cells were incubated for 10 min with 1 μM chromostatin and subsequently incubated for 5 min with Locke's solution (solid bar) or stimulated in Locke's solution containing 0.5 mM carbamoylcholine (stippled bar), 59 mM K^+ (hatched bar), or 50 μM veratridine (open bar) in the presence of $^{22}Na^+$ at 1 μCi/200 μl. Data are given as the mean of triplicate determinations on the same cell preparation ± SD. Similar results were obtained on three different cell preparations.

Table 2. Role of extracellular calcium in chromostatin and CGA inhibition of catecholamine secretion

Calcium, mM	Peptide	Net [^{3}H]NAd release, %
0	None	16.7 ± 0.4
0	CGA, 1 μM	15.7 ± 0.2
0	Chromostatin, 1 μM	3.0 ± 0.1
2.5	None	17.6 ± 0.3
2.5	CGA, 1 μM	5.1 ± 0.1
2.5	Chromostatin, 1 μM	3.1 ± 0.1

Cells were incubated for 10 min with purified CGA or peptide I-20 (chromostatin) in either Locke's solution (2.5 mM calcium) or calcium-free Locke's solution containing 5 mM EGTA. Cells were subsequently stimulated for 10 min with 0.5 mM carbamoylcholine in Locke's solution in the absence of CGA or chromostatin.

DISCUSSION

Despite the ubiquitous localization of CGA in many endocrine, nervous, and nonnervous tissues and its abundance in secretory granules of adrenal medulla, the physiological function of CGA remains unknown. However, recent speculations favor the idea that CGA may be a putative precursor of biologically active peptide fragments (2, 22). This view is supported by the discovery that pancreastatin, a peptide controlling insulin release (3, 4), is contained in the CGA sequence (8) and that tryptic digestion of CGA generates peptides which modulate catecholamine secretion (6).

The present data further support this concept. We have purified and partially sequenced two CGA-derived peptides (I and II) which are active on catecholamine release, and we have shown that a synthetic peptide composed of the first 20 amino acids of peptide I inhibits catecholamine secretion. This peptide, named chromostatin, is likely to correspond to the active sequence of the natural CGA-derived peptide modulating catecholamine release, although the natural peptide remains to be fully characterized. The proteolytic enzyme processing CGA into smaller fragments is not yet known. It is generally accepted that CGA is processed within secretory granules. However, recent studies failed to demonstrate an active processing in cultured chromaffin cells (21), and characterization of proteolytic enzymes in purified granules has shown the occurrence of contaminating lysosomal enzymes (23). Thus the possibility of extracellular processing of CGA should be considered. Accordingly, we have previously observed that CGA can be processed extracellularly (6, 21), and we describe here a calcium-dependent proteolytic processing of CGA in the extracellular space (Table 2) leading to the production of CGA-derived fragments inhibiting the cell secretory response.

Comparison of the bovine chromostatin sequence with human, porcine, and rat sequences shows that 45%, 30%, and 30%, respectively, of residues are conserved. Interestingly, there is a conserved region composed of two neighboring groups separated by a variable amino acid, a first group of two residues, Asp-Gly, and a second group of four residues, Arg-Pro-Gln-Ala. This region has the structural feature of a calcium-binding pocket (E-F hand loop region; ref. 24). The meaning of this property is not yet known, but it could be of significance with regard to structural/functional relationships of chromostatin. The C-terminal side of chromostatin, which is quite hydrophobic compared to the hydrophilic N-terminal side, displays considerable sequence variability from one species to another (2).

The current characterization of the CGA gene (A. Iacangelo and L. Eiden, personal communication) provides an

opportunity to examine the correspondence between exons and structural and functional units of the CGA protein. The bovine CGA gene has eight exons. Chromostatin and pancreastatin are localized on different exons: chromostatin corresponds to a region of the CGA gene on the 5′ side of exon 6, while the CGA gene region encoding pancreastatin straddles the 3′ terminus of exon 6 and the 5′ terminus of exon 7. The different localizations of chromostatin- and pancreastatin-encoding regions favor the idea of different functions; this appears to be the case, since pancreastatin was found to be inactive on catecholamine secretion from cultured chromaffin cells (Table 1). However, porcine pancreastatin was tested on bovine chromaffin cells and problems due to species differences cannot be excluded.

Chromostatin is able to completely inhibit catecholamine release when chromaffin cells are stimulated with nicotine, $DMPP^+$, or carbamoylcholine and partially when cells are directly depolarized. In the present paper, we have found that chromostatin reduces calcium fluxes through voltage-sensitive calcium channels without affecting voltage-gated sodium channels. Similarly, incubation of chromaffin cells with native CGA decreased secretagogue-evoked calcium entry (D.A., J. P. Simon, and A. Schneider, unpublished observation). Chromostatin was also found to enhance the desensitization process of the cholinergic-evoked secretory response. Surprisingly, after removal of the peptide from extracellular medium, the cell response recovery was very slow, indicating a long-lasting effect on catecholamine secretion. This property may indicate either that chromostatin has a rather high affinity for its receptor, which remains to be characterized, or that it has long-lasting effects on intracellular mechanisms.

In conclusion, CGA appears to be a hormone precursor of at least two active peptides (2, 8). One of these peptides, pancreastatin, is able to control insulin release from pancreatic islets, while the recently discovered one, chromostatin, modulates catecholamine release from adrenal chromaffin cells. Interestingly, the reported values for CGA concentration in human plasma range between 0.19 to 0.35 mg/liter (25), representing a circulating concentration of 3.9–7.3 nM. The presence and the actual concentration of a chromostatin-like peptide in plasma require further investigation. However, the concentrations of chromostatin required to produce inhibition of catecholamine release (ID_{50} = 5 nM) are quite compatible with the estimated plasma CGA concentration, suggesting that the inhibition of catecholamine secretion can occur under physiological conditions. Therefore we propose that chromostatin may be the active sequence of an endocrine effector that could have an important function in regulating hormonal changes in catecholamine-involving situations, such as stress.

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1. Blaschko, H., Comline, R. S., Schneider, M., Silver, M. & Smith, A. D. (1967) *Nature (London)* **215,** 58–59.
2. Simon, J. P. & Aunis, D. (1989) *Biochem. J.* **262,** 1–13.
3. Tatemoto, K., Efendic, S., Mutt, V., Makk, G., Feistner, G. & Barchas, J. D. (1986) *Nature (London)* **324,** 476–478.
4. Efendic, S., Tatemoto, K., Mutt, V., Quan, C., Chang, D. & Ostenson, C.-G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7257–7260.
5. Lewis, J. J., Goldenring, J. R., Asher, V. A. & Modlin, I. M. (1989) *Biochem. Biophys. Res. Commun.* **163,** 667–673.
6. Simon, J. P., Bader, M. F. & Aunis, D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1712–1716.
7. Iacangelo, A. L., Fischer-Colbrie, R., Koller, K. J., Brownstein, M. J. & Eiden, L. E. (1989) *Endocrinology* **122,** 2339–2341.
8. Eiden, L. E. (1987) *Nature (London)* **325,** 301.
9. Perrin, D. & Aunis, D. (1985) *Nature (London)* **315,** 589–592.
10. Knight, D. E. (1990) *Nature (London)* **343,** 218.
11. Bader, M. F., Sontag, J. M., Thiersé, D. & Aunis, D. (1989) *J. Biol. Chem.* **264,** 16426–16434.
12. Castro, E., Oset-Gasque, M. J. & Gonzalez, M. P. (1989) *J. Neurosci. Res.* **23,** 290–296.
13. Green, D. J. & Perlman, R. L. (1981) *Anal. Biochem.* **110,** 270–276.
14. Kumakura, K., Karoum, F., Guidotti, A. & Costa, E. (1986) *J. Neurochem.* **46,** 1851–1858.
15. Artalejo, C. R., Garcia, A. G. & Aunis, D. (1987) *J. Biol. Chem.* **262,** 915–926.
16. Sontag, J. M., Sanderson, P., Klepper, M., Aunis, D., Takeda, K. & Bader, M. F. (1990) *J. Physiol. (London)* **427,** 495–517.
17. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85,** 2149–2154.
18. Van Regenmortel, M. H. V., Briand, J. P., Muller, S. & Plaué, S. (1988) in *Synthetic Polypeptides as Antigens*, Laboratory Techniques in Biochemistry and Molecular Biology (Elsevier, Amsterdam), Vol. 19, pp. 1–227.
19. Iacangelo, A. L., Affolter, H. U., Eiden, L. E., Herbert, E. & Grimes, M. (1986) *Nature (London)* **323,** 82–86.
20. Benedum, U. M., Baeuerle, P. A., Konecki, D. S., Frank, R., Powell, J., Mallet, J. & Huttner, W. B. (1986) *EMBO J.* **5,** 1495–1502.
21. Simon, J. P., Bader, M. F. & Aunis, D. (1990) *Biochim. Biophys. Acta* **1051,** 123–130.
22. Fischer-Colbrie, R., Hagn, C. & Schober, M. (1987) *Ann. N.Y. Acad. Sci.* **493,** 120–134.
23. Laslop, A., Fischer-Colbrie, R., Kirschke, H., Hogue-Angeletti, R. & Winkler, H. (1990) *Biochim. Biophys. Acta* **1033,** 65–72.
24. Grimes, M., Iacangelo, A., Eiden, L. E., Godfrey, B. & Herbert, E. (1987) *Ann. N.Y. Acad. Sci.* **493,** 351–378.
25. Dillen, L., De Block, J., Van Lear, L. & De Potter, W. (1989) *Clin. Chem. N.Y.* **35,** 1934–1938.