Formation of the Catecholamine Release-inhibitory Peptide Catestatin from Chromogranin A

DETERMINATION OF PROTEOLYTIC CLEAVAGE SITES IN HORMONE STORAGE GRANULES*

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The catestatin fragment of chromogranin A is an inhibitor of catecholamine release, but its occurrence in vivo has not yet been verified, nor has its precise cleavage sites been established. Here we found extensive processing of catestatin in chromogranin A, as judged by catestatin radioimmunoassay of size-fractionated chromaffin granules. On mass spectrometry, a major catestatin form was bovine chromogranin A332–364 identified by the peptide was confirmed by diagnostic Met 346 oxidation. Further analysis revealed two additional forms: bovine chromogranin A333–364 and A343–362. Synthetic longer (chromogranin A332–364) and shorter (chromogranin A344–364) versions of catestatin each inhibited catecholamine release from chromaffin cells, with superior potency for the shorter version (IC50 ~200 nM versus ~350 nM). Radioimmunoassay demonstrated catestatin release from the regulated secretory pathway in chromaffin cells. Human catestatin was cleaved in pheochromocytoma chromaffin granules, with the major form, human chromogranin A340–372 bounded by dibasic sites. We conclude that catestatin is cleaved extensively in vivo, and the peptide is released by exocytosis. In chromaffin granules, the major form of catestatin is cleaved at dibasic sites, while smaller carboxyl-terminal forms also occur. Knowledge of cleavage sites of catestatin from chromogranin A may provide a useful starting point in analysis of the relationship between structure and function for this peptide.

Chromogranin A, the major soluble protein in catecholamine storage vesicles, not only stabilizes the core of such vesicles (1) but also serves as a prohormone cleaved into several biologically active peptides, including the insulin release-inhibitory fragments pancreastatin (2) and β-granin (3–5), the vasodilator vasostatin (6, 7), and the parathyroid hormone release-inhibitory parastatin (8). Recently we described an additional chromogranin A fragment, catestatin (bovine chromogranin A344–364), which acts specifically as a potent (IC50 ~200–400 nM) nicotinic cholinergic antagonist to inhibit catecholamine release (9, 10), suggesting a novel autocrine feedback mechanism controlling sympathochromaffin exocytosis. Initial studies of catestatin relied on synthetic peptides (10); hence, the existence of the peptide in vivo remains unexplored, as do its precise cleavage sites from chromogranin A.

In this study, we explored processing of the catestatin region of chromogranin A in secretory granules of chromaffin cells and sympathetic nerves, as well as in human pheochromocytomas. We documented catestatin cleavage from chromogranin A and determined the precise endogenous cleavage sites binding catestatin in bovine and human chromaffin granules by chromatographic separations coupled with amino-terminal amino acid sequencing, immunoprecipitation, and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. We confirmed catestatin’s regulated secretion by chromaffin cells. A major form of catestatin was processed at dibasic sites (bovine chromogranin A332–364 or human chromogranin A340–372), while a smaller form was also identified (bovine chromogranin A343–362). Both larger and smaller size forms were synthesized; each displayed specific antagonism of nicotinic cholinergic-stimulated catecholamine release, while the smaller form had greater potency of inhibition.

MATERIALS AND METHODS

Preparation of Tissue Fractions—All preparative steps on tissues or protein fractions were conducted at 0–4 °C. Bovine adrenal medullary chromaffin granules were prepared by centrifugation on 0.3 M/1.6 M sucrose density step gradients, as described previously (10). After granule hypotonic lysis and centrifugal removal of granule membranes, the soluble proteins and peptides in the supernatant were size-fractionated on a 2.6 × 80-cm Sephacyr S-300 column (Amersham Pharmacia Biotech), eluting with the volatile buffer 0.3 M ammonium acetate, pH 6.5, as described previously (11). The buffer was removed by lyophilization before further studies. In some experiments, bovine chromaffin granule proteins/peptides (200 μl containing 8 mg of protein) were size-fractionated on a Superdex 75 HR 10/30 FPLC gel filtration column (10 × 300 mm, 24-ml bed volume; Amersham Pharmacia Biotech), eluting at 1 ml/min with 0.3 M ammonium acetate, 1 mM EDTA, pH 7.0, collecting fractions every 0.5 ml (0.5 min). Eluted fractions were analyzed for protein by on-line absorbance at 280 nm (A280) and then lyophilized (to remove the volatile buffer) and resuspended in the same volume of radioimmunoassay (RIA) buffer (50 mM Tris-HCl, pH 8.3, 0.3% bovine serum albumin; 0.1% Triton X-100; see below). Chromaffin granules from human pheochromocytomas were also prepared by centrifugation on 0.3 M/1.6 M sucrose density step gradients, followed by hypotonic lysis and membrane removal by centrifugation, as described

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1 The abbreviations used are: MALDI, matrix-assisted laser desorption ionization; HPLC, high pressure liquid chromatography; RP-HPLC, reverse-phase HPLC; FPLC, fast protein liquid chromatography; RIA, radioimmunoassay; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.
previously (10). Postganglionic sympathetic nerves were obtained by dissection of splenic nerve in samples from the local slaughterhouse. Nerves were placed in ice-cold 0.3 M sucrose at an ~10:1 ratio of tissue to buffer and then minced, homogenized, gauze-filtered, and centrifuged at 1000 × g for 10 min (to remove nuclei and debris). Supernatants were then centrifuged at 10,000 × g for 10 h to pellet a crude fraction of neuronal large dense core vesicles (12), which were lysed by resuspension in 10 mM Hepes, pH 7, and freezing/thawing.

**Chromogranin A, Synthetic Peptides, and Antibodies**—Bovine or human chromogranin A was isolated from chromaffin granule soluble core proteins by affinity chromatography (to remove dopamine β-hydroxylase) followed by gel filtration, as described previously (13). Recombinant human chromogranin A was expressed in *Escherichia coli* by a modification of previously described methods (14), except that a His tag was used for affinity purification on a Ni²⁺-nitrilotriacetic acid column (15). Synthetic peptides (20–100-μmol scale) were prepared by the solid phase method, using Fmoc (N-(9-fluorenlymethoxycarbonyl) protection chemistry. Purification was by C-18 reverse-phase HPLC (RP-HPLC). Authenticity of the resulting peptides was confirmed by mass spectrometry, using either MALDI or electrospray ionization. Polyclonal rabbit antisera recognizing the chromatid region of chromogranin A (Fig. 1), either bovine chromogranin A$_{344-364}$ (RSRMLSFRARQFGPGLQL) or human chromogranin A$_{322-372}$ (SSMLKSFRARAYFGPFGPQL), were developed by a modification of protocols previously described for other chromogranin peptides (11, 16). The polyclonal antibody recognizing the chromatid region of chromogranin A was further purified on an Amersham Pharma Biotech Hi Trap protein A column in 0.02 M sodium phosphate (pH 7.0), eluted with 0.1 M sodium citrate (pH 3), and the pH was then adjusted back to 7.0 with Tris-HCl (pH 8.8). A polyclonal rabbit antisera recognizing bovine chromogranin A$_{316-329}$ was obtained from Dr. Marie-France Bader (INSERM U-338, Strasbourg, France), and a polyclonal rabbit antisera recognizing human chromogranin A$_{267-291}$ was obtained from Dr. Reiner Fischer-Colbrie (University of Innsbruck, Austria).

**Immunoprecipitations**—Tissue homogenates, granule soluble core lysates, or gel filtration size-separated fractions were applied to Sep-Pak C-18 cartridges (Waters/Millipore), eluted with 30–40% acetonitrile, lyophilized, resuspended in 500 μl of immunoprecipitation buffer containing protease inhibitors (0.1% Triton X-100, 140 mM NaCl, 0.025% sodium azide, 10 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 mM EDTA, 1 mM N-ethylmaleimide), and then immunoprecipitated by a modification of the protocol of Wang et al. (17). To minimize nonspecific binding results, samples were first incubated with 25 μl of normal (preimmune) rabbit serum with constant rotator mixing at 4 °C for 12–18 h. 60 μl of protein G Plus/protein A-agarose beads were added, and rotational incubation continued for another 3 h, after which the beads were collected by centrifugation, washed three times with immunoprecipitation buffer, and then washed twice with 50 mM Tris-HCl, pH 8 (to remove NaCl and detergent).

**Mass Spectrometric Analyses of Immunoprecipitated Catestatin**—Immunoprecipitated catestatin was eluted from the immune complexes with 20 μl of trisfluoroacetic acid/water/acetonitrile, 1:20:20 (v/v/v) (17). To identify methionine-containing peptides (by oxidation of methionine to methionine sulfoxide, thereby adding the mass of a single oxygen at 16 daltons), 10 μl were oxidized by adding sufficient 3% H₂O₂ to achieve 10 μM final H₂O₂ concentration. 1–2 μl were characterized by MALDI mass spectrometry on a Voyager-Elite mass spectrometer with delayed extraction (PerSeptive Biosystems, Framingham, MA). Samples were embedded in an α-cyano-4-hydroxycinnamic acid matrix (18) and then irradiated with a nitrogen laser at 337 nm, and the ions produced were accelerated with a deflection potential of 30,000 V. Ions were then differentiated according to their mass/charge ratio (m/z) using a time-of-flight mass analyzer. The mass error of this method is characteristic ≤0.1% (i.e., ≤1000 ppm; Ref. 18).

**Immunoblotting**—After suspension of bovine chromaffin granule protein samples in loading buffer (10 mM Tris-HCl, 1 mM EDTA, 3% SDS, 20 mM diethiothreitol, 10% glycerol, 0.1% bromphenol blue), 0.8–50 μg of protein were electrophoresed through SDS-PAGE 10–20% acrylamide gradient gels, in the presence of Tris-Tricine buffer (Novex, San Diego, CA). This gradient gel system resolves peptides as small as 2 kilodaltons (19). Human pheochromocytoma chromaffin granule samples were electrophoresed on 10% non-reduced acrylamide gels. After SDS-PAGE, gels were stained for total protein with 0.1% Coomassie Brilliant Blue R250 in water/methanol/acetic acid (50:40:10%) (20), followed by destaining in water/methanol/acetic acid (82.5:10:7.5%). Parallel gels were electroeluted onto nitrocellulose paper (BA85; Schleicher and Schuell, Keene, NH), followed by immunoreactive catestatin staining by immunoblotting (21). The primary immunoblotting antibodies were rabbit anti-bovine catestatin (chromogranin A$_{344-364}$; RSMRLSFRRARQFGPGLQL; titer 1:1000 (v/v)), rabbit anti-bovine chromogranin A$_{316-329}$ (titer 1:500), rabbit anti-bovine chromogranin A$_{387-392}$ (titer 1:500 (v/v)), or rabbit anti-human catestatin (chromogranin A$_{352-372}$; SSMLKSFRARAYFGPFGPQL; titer 1:2000 (v/v)) (10). Second antibody staining was visualized by either enhanced chemiluminescence (horseradish peroxidase-labeled goat anti-rabbit IgG, titer 1:4000 (v/v); Amersham Pharmacia Biotech ECL kit) or color development (goat anti-rabbit IgG alkaline phosphatase conjugate, titer 1:2000 (v/v); Bio-Rad).

After RP-HPLC of bovine chromaffin granule peptides (see below), 50 μl of each 500-μl HPLC fraction were vacuum-dried, resuspended in 100 μl of water, adsorbed by vacuum filtration onto a nitrocellulose membrane using a slot-blotting device (MiniFold II; Schleicher and Schuell), and immunoblotted using the anti-bovine catestatin primary antibody (at 1:500 (v/v)) and peroxidase-conjugated anti-rabbit secondary antibody (at 1:7000 (v/v)) with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech).

**Densitometry Analysis**—Densitometry analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet).

**RP-HPLC**—Gel filtration size-fractionated bovine chromaffin granule peptides were further separated by RP-HPLC using a 25 × 0.5-cm C-18 column, equilibrated in 0.1% trifluoroacetic acid, and eluted with a linear 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid, over 60 min, at 1 ml/min. The elution was monitored by A$_{214}$ (peptide bond absorbance), and fractions were collected at 0.5-min (0.5-ml) intervals. 50-μl aliquots from each fraction were vacuum-dried and subjected to anti-catestatin slot immunoblotting (see above), and slot-blot positive fractions were further subjected to amino-terminal microsequencing (100 μl; see below) and MALDI mass spectrometry (1–2 μl; see above).

**Amino-terminal Amino Acid Microsequencing**—Eluted HPLC fractions (100-μl aliquots from 500–500 fractions) in 0.1% trifluoroacetic acid/acetonitrile/H₂O column buffer were analyzed for amino-terminal sequence by automated Edman microsequencing (10–100 pmol; ABI 494 Procise® sequencing system with ABI 610 data analysis system; Applied Biosystems/Perkin Elmer).

**Mass Spectrometric and Sequencing Analyses**—Molecular weights from MALDI mass spectra were interpreted, and peptide fragments within the chromogranin A primary structure were assigned by the program PAWS (Protein Analysis WorkSheet, version 8.1.1, for Macintosh; ProteoMetrics; freeware available on the Internet), assigning average isotopic MH⁺ values for chromogranin A peptides (18). Sequencing results were analyzed at each Edman cycle by the algorithm “Hydrosites” (for Macintosh) to deconvolute multiple amino-terminal sequences derived from the same parent molecule at a given cycle (22).

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**FIG. 1. Amino acid sequences in the catestatin (bovine chromogranin A$_{344-364}$) region of the primary structures of bovine and human chromogranin A.** The regions between dibasic cleavage sites ([KR] and [RR]) are shown. Previous studies (10) have established the catecholamine release-inhibitory activity of synthetic bovine chromogranin A$_{344-364}$. Previous studies (10) have established the catecholamine release-inhibitory activity of synthetic bovine chromogranin A$_{344-364}$.
Catestatin Processing

22907

FIG. 2. Size separation of bovine adrenal medullary chromaffin granule soluble core proteins and peptides. After chromaffin granule isolation and lysis, soluble proteins and peptides were fractionated by gel filtration on a 2.6 × 80-cm column of Sephacryl S-300 (Amersham Pharmacia Biotech), equilibrated and eluted with the volatile buffer 0.3 M ammonium acetate, pH 6.5. Low molecular weight (LMW) peptide fractions 42–52, devoid of chromogranin A by SDS-PAGE analysis, were used for further studies. For Coomassie Blue staining of SDS-PAGE, the low molecular weight fraction molecular mass values ranged from <55 kDa to the dye front.

Activity of Synthetic Catestatins—Peptides were subjected to a test of activity by inhibition of secretagogue-stimulated norepinephrine release from [3H]norepinephrine-prelabeled PC12 pheochromocytoma cells, over a 30-min secretion period, as described previously (10, 23). The stimuli to catecholamine release were either nicotinic cholinergic (50 μM nicotine) or membrane depolarization (55 mM KCl). In some control experiments, the peptide was immunoadsorbed overnight (4 °C, 1:100 antibody titer in secretion buffer) prior to test of secretion.

Bovine Chromaffin Cell Isolation, Culture, and Stimulation of Secretion—Primary cultures of bovine chromaffin cells were prepared as described previously (24). Secretion was stimulated over a 15-min period—(60 μM nicotine) or membrane depolarization (by 55 mM KCl). In some experiments, the peptide was immunoadsorbed overnight (4 °C, 1:20 antibody titer (v/v)) and carrier antiserum (2% bovine serum albumin) were incubated in culture medium prior to addition of peptide. After 24 h at ambient temperature, 500 μl of rabbit anti-bovine chromogranin A antibody and subjected to MALDI mass spectrometry (Fig. 3). The initial spectrum revealed a major peak at m/z = 3829 (Fig. 3D), which, within the 431-amino acid bovine chromogranin A primary structure (25, 26), corresponds uniquely to chromogranin A333–343 (LE-GEEEEEEDPDRSMFLRAGYGRFRPGLQ; calculated m/z = 3827.2); the 0.047% difference between experimental and calculated m/z values is within the range of acceptable error of the MALDI method (0.1% m/z; Ref. 18). Preimmune serum did not recognize a peak of this mass (Fig. 3A). Since mass spectra of peptides are susceptible to shifting by formation of peptide-cation (such as Na+ ) adducts, we repeated the experiment on gruel norepinephrine peptides that had been desalted by adsorption to a C-18 matrix (Fig. 3C); an m/z = 3827 peak remained the principal component, with some diminution in surrounding minor peaks. To confirm that this peak represents catestatin, the granule peptide sample was gently oxidized with H2O2 to convert any methionine residues into methionine sulfoxide, as described by Wang et al. in studies of β-amyloid processing by mass spectrometry (17); since bovine catestatin contains a single methionine residue (bovine chromogranin A Met346; Fig. 1), this procedure provides a further specific diagnostic for the identity of the catestatin region. After oxidation, the major peak was now found at m/z = 3844 (Fig. 3D), a mass shift corresponding to the added 16-dalton oxygen atom in Met346-sulfoxide.

Isolation and Characterization of Catestatin Fragments: RP-HPLC, Mass Spectrometry, and Amino-terminal Amino Acid Sequencing—In a second approach to determine the boundaries of endogenous catestatin cleavage, the chromaffin granule low molecular weight peptide fraction (gel filtration fractions 42–52; Fig. 2) was first separated by RP-HPLC (Fig. 4, bottom), and fractions were immunoblotted with anti-bovine catestatin. HPLC fraction 27, containing intense catestatin immunoreactivity (Fig. 4, middle), was then analyzed (Fig. 4, top) by both MALDI mass spectrometry and amino-terminal amino acid sequencing. MALDI revealed two peaks, at m/z = 3718 and 2300 (Fig. 4, top). In the catestatin region of bovine chromogranin A (Fig. 1), m/z = 3718 is compatible with chromogranin A333–343 (EGEEEEEEDPDRSMFLRAGYGRFRPGLQ), while m/z = 2300 is compatible with chromogranin A342–352 (DRSMFLRAGYGRFRPGL). Fraction 27 was amino-terminally sequenced over 10 residue cycles, with the result suggesting two peptides (22): (a) EGEEEEEEDP . . . , corresponding to bovine chromogranin A333–342, the first 10 amino acids of the 3718 m/z peptide EGEEEEEEDPDRSMFLRAGYGRFRPGLQ (chromogranin A333–343), and (b) DRS . . . , corresponding to bovine chromogranin A342–345, the first three amino acids of the 2300 m/z peptide DRSFLRAGYGRFRPGL (chromogranin A342–343).

Cleavage of the Catestatin Region of Chromogranin A in Neurons: Mass Spectrometry—Bovine splenic nerve was homogenized, desalted by adsorption to/elution from a C-18 matrix (SepPak), immunoprecipitated by an anti-catestatin (bovine chromogranin A344–364) antibody, and subjected to MALDI mass spectrometry. MALDI revealed a peak at m/z = 3832 (Fig. 3B) corresponding to bovine chromogranin A344–358 in RIA buffer (50 mM Tris-HCl, pH 8.3, 0.3% bovine serum albumin, 0.1% Triton X-100) at a titer of 1:1000 (v/v), and after chromaffin granule soluble core proteins and peptides. After chromogranin A333–342, the first three amino acids of the 2300 (top) EGEEEEEEDP... . , corresponding to bovine chromogranin A333–343, the first 10 amino acids of the 3718 m/z peptide EGEEEEEEDPDRSMFLRAGYGRFRPGLQ (chromogranin A333–343), and (b) DRS . . . , corresponding to bovine chromogranin A342–345, the first three amino acids of the 2300 m/z peptide DRSFLRAGYGRFRPGL (chromogranin A342–343).
5, top), which, in the cestatin region of bovine chromogranin A (Fig. 1), is within 0.125% of 3827.2, the calculated MH$^+$ of chromogranin A$^{332–364}$ (LEGEEEEEEDPDRSMRLSFRAGYGFRGPQLQ). After oxidation by H$_2$O$_2$, the $m/z = 3832$ peak diminished, while an $m/z = 3843$ peak became more prominent (Fig. 5, bottom), a mass shift corresponding to the added 16-dalton oxygen atom in Met$^{346}$-sulfoxide in chromogranin A$^{332–364}$ (predicted MH$^+$ = 3843.2).

**Determination of Cestatin Cleavage Sites in Human Chromogranin A: Mass Spectrometry**—After anti-cestatin immunoprecipitation of human pheochromocytoma chromaffin granules, $m/z$ values of 3770–3771 were noted (Fig. 6), corresponding uniquely within the chromogranin A primary structure to chromogranin A$^{340–372}$ (KRLEGQEEEEDNRDSSMKLSFRARYGFRGPQLLR; calculated $m/z =$ 3771.1), which is bounded on either side by dibasic recognition sites for prohormone cleavage (underlined) (Fig. 1). Upon H$_2$O$_2$ oxidation, in each case these $m/z$ 3770–3771 peaks shifted to $m/z =$ 3787, consistent with the addition of an oxygen (16 daltons) to form Met$^{354}$-sulfoxide. Other peaks in this region did not shift upon oxidation. Experimental and calculated $m/z$ values are well within the 0.1% expected experimental error of MALDI mass determination (18).

**Reproduction of Cestatin Activity in Synthetic Peptides Corresponding to Cleavage Sites: Potency and Specificity of Nicotinic Cholinergic Antagonism**—To test the activity and specificity of the bovine cestatin peptides predicted by MALDI mass spectrometry and amino-terminal sequencing (Figs. 3 and 4), we synthesized both longer (chromogranin A$^{332–364}$: LEGEEEEEEDPDRSMRLSFRAGYGFRGPQLQ) and shorter (chromogranin A$^{344–364}$: RSMRLSFRARYGFRGPQLQ) versions of cestatin as well as whole chromogranin A and evaluated their effects on catecholamine release from PC12 pheochromocytoma cells (Fig. 7).

As previously reported for the shorter version of cestatin (10), the longer version also selectively inhibited catecholamine release evoked by nicotinic cholinergic stimulation but not that evoked by membrane depolarization (Fig. 7A). Both peptides and chromogranin A showed concentration-dependent inhibition of nicotine-induced secretion (Fig. 7B): intact chromogranin A had an IC$_{50}$ of $\sim$4.2 $\mu$M, chromogranin A$^{344–364}$ had an IC$_{50}$ of $\sim$0.35 $\mu$M, and the chromogranin A$^{332–364}$ IC$_{50}$ was $\sim$2.01 $\mu$M. At high dose (10 $\mu$M) each peptide completely blocked nicotinic-stimulated secretion, while whole chromogranin A only partially blocked secretion.

A peptide corresponding to the cestatin region (chromogranin A$^{352–372}$: SSMKLSFRARYGFRGPQLQ) of human chromogranin A (see Fig. 1) also inhibited nicotinic cholinergic-stimulated catecholamine secretion (by 74% at 10 $\mu$M peptide), and the inhibition was specifically reversed by a rabbit antiserum directed toward the cestatin region of human chromogranin A (chromogranin A$^{352–372}$: SSMKLSFRARYGFRGPQLQ), although not by preimmune serum (Fig. 7C).

**Cestatin Immunoreactivity Has a Variety of Size Forms in**

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**Fig. 3.** MALDI mass spectrometry identification of cestatin in immunoprecipitated bovine adrenal medullary chromaffin granules. Low molecular weight chromaffin granule peptides, devoid of chromogranin A (fractions 42–52 from gel filtration; Fig. 2), were examined. Aliquots (200 $\mu$l) of the size fraction were immunoprecipitated (20 $\mu$l of antiserum) and then subjected to MALDI mass spectrometry (1–2 $\mu$l). A, immunoprecipitation by preimmune serum. B, immunoprecipitation by rabbit anti-bovine chromogranin A$^{344–364}$ (RSMRLSFRARYGFRGPQLQ). C, immunoprecipitation by rabbit anti-bovine chromogranin A$^{332–364}$ followed by adsorption and elution from a C-18 (Sep-Pak) cartridge. D, immunoprecipitation by rabbit anti-bovine chromogranin A$^{344–364}$ after Met$^{346}$ oxidation by 10 $\mu$M H$_2$O$_2$. 

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Catestatin Processing
FIG. 4. Isolation and further characterization of catestatin size forms in bovine chromaffin granules by RP-HPLC, microsequencing, and mass spectrometry. Low molecular weight bovine chromaffin granule peptides, devoid of chromogranin A (fractions 42–52 from gel filtration; Fig. 2), were separated on a 0.5 x 25-cm C18 RP-HPLC column (bottom panel), and eluted fractions were tested for catestatin immunoreactivity by slot-blotting (middle panel) with rabbit anti-bovine chromogranin A<sub>244-364</sub> (titer 1:2000). The fraction with catestatin immunoreactivity (fraction 27, 1–2 µL) was then subjected to two analyses (top panel): amino-terminal amino acid sequencing and MALDI mass spectrometry.
Bovine Chromaffin Granule Radioimmunoassay—Chromaffin granule soluble core proteins were size-fractionated on a standard calibrated gel filtration column (Fig. 8), and fractions were analyzed by radioimmunoassay. Four major peaks of catestatin (bovine chromogranin A332–364) were eluted in fractions 35–40 (Fig. 8). Peak 1 contained 71.7 nmol of catestatin immunoreactivity and 54% of total, peak 2 contained 19.1 nmol and 13.2% of total, peak 3 contained 28.2 nmol and 21.4% of total, and peak 4 contained 7.5 nmol of catestatin immunoreactivity and 8% of total catestatin immunoreactivity. Of note, peak 4 elutes in the size position (fractions 37 and 38) of chromogranin A344–364, the small, potent form of catestatin (Fig. 7B). Synthetic peptides corresponding to chromogranin A332–364 and chromogranin A344–364 were size-fractionated on the same column, eluting in fractions 35 and 36 and fractions 37 and 38, respectively. A standard curve of the gel filtration elution was created using bovine serum albumin (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa), a larger form of catestatin (chromogranin A332–364; 3827 kDa), and a smaller form of catestatin (chromogranin A344–364; 2541.3 kDa).

Cleavage within the Catestatin Region of Bovine Chromogranin A: Immunoblots of Chromaffin Granules with Antisera Directed to Flanking Peptides—Chromaffin granule soluble core proteins were separated by SDS-PAGE and then immuno-blotted with not only an antibody directed against the catestatin region (bovine chromogranin A344–364) but also antibodies directed against peptide regions that are bounded by dibasic cleavage sites and lie either directly amino-terminal (bovine chromogranin A316–329) or directly carboxyl-terminal (bovine chromogranin A367–391) to catestatin (Fig. 9). Each antibody recognized not only intact chromogranin A (at a molecular mass of ~60–70 kDa) but also several lower molecular mass chromogranin A fragments, ranging from ~10 to 50 kDa. All of the lower molecular mass chromogranin A fragments are prominently recognized by all three antisera, with the exception of a ~19-kDa fragment (Fig. 9, arrow), which is visualized by anti-chromogranin A316–329 and anti-chromogranin A344–364, al-
though not by anti-chromogranin A367–391. Thus, the ~19-kDa fragment probably represents the peptide just amino-terminal to a dibasic cleavage at Arg365–Arg366 in bovine chromogranin A.

When the low molecular weight chromaffin granule peptides (Fig. 2, fractions 42–52) were subjected to catestatin immunoblotting, followed by densitometry of the immunoreactive bands, more than half of the catestatin immunoreactivity was found in fractions of lower molecular mass than chromogranin A: 23.7% in a ~34-kDa band and 27.1% in a ~15 kDa band.

**Chromogranin A Processing to Catestatin in Human Pheochromocytoma Chromaffin Granules: Immunoblot**—The anti-human catestatin antibody (rabbit anti-human chromogranin A362–373) recognized intact human chromogranin A, at M, ~70 kDa (Fig. 10, lane 1) in pheochromocytoma chromaffin granule immunoblots. All pheochromocytomas studied exhibited at least some processing of the catestatin region. Low molecular weight peptides, migrating near the tracking dye and bearing the catestatin epitope, are readily apparent in two pheochromocytomas (Fig. 10, lanes 4 and 5), and the overall pattern of catestatin processing from human chromogranin A was strikingly similar in two of five pheochromocytomas (Fig. 10, lanes 4 and 5). In three of the five tumors, more than 50% of the catestatin immunoreactivity still resided in the intact chromogranin A parent molecule (Fig. 10, lanes 4–6). Similar processing was noted on catestatin immunoblots of chromaffin granules from eight additional pheochromocytomas (data not shown).

**Catestatin Is Released by Chromaffin Cells, in Secretagogue-regulated Fashion**—Bovine chromaffin cells in primary culture were stimulated by either the nicotinic cholinergic agonist nicotine (100 μM) or membrane depolarization (by 55 mM KCl), each in the presence of extracellular calcium (1 mM) (Fig. 11A). Under basal (unstimulated) circumstances, cells released 90.7 ± 9.7 nmol/ml of catestatin, rising to 20,700 ± 7850 nmol/ml after nicotine (~228-fold stimulation) or 4570 ± 1130 nmol/ml after membrane depolarization (~50-fold stimulation).

We also tested the calcium-dependence of catestatin secretion (Fig. 11B); in the presence of extracellular calcium (1 mM), membrane depolarization (by 100 mM KCl) stimulated catestatin release by ~40-fold (from 31.5 ± 5.6 to 1260 ± 19.4 nmol/ml), while in the absence of extracellular calcium this stimulation was abolished (back to 18.7 ± 1.0 nmol/ml). Qualitatively similar results were observed for enkephalin secretion (Fig. 11B); in the presence of extracellular calcium, membrane depolarization (by 100 mM KCl) stimulated enkephalin release by ~27-fold (from 546 ± 104 to 15,000 ± 3710 nmol/ml), while in the absence of extracellular calcium this stimulation was virtually abolished (back to 626 ± 430 nmol/ml). Using this radioimmunoassay, we detected catestatin immunoreactivity in fetal bovine serum (at 2.71 nm) and adult equine serum (at 22.2 nm).

**Discussion**

The catestatin region of chromogranin A (bovine chromogranin A 326–364) is a potent and specific inhibitor of chromaffin cell catecholamine release when triggered by nicotinic cholinergic stimulation, the physiologic pathway for such cells (10); catestatin also antagonizes nicotinic desensitization of the process (27). In these studies, we documented cleavage of the catestatin region in normal chromaffin granules (Figs. 3, 4, 8, and 9) and sympathetic nerve catecholamine storage vesicles (Fig. 5) as well as chromaffin granules from human pheochromocytoma (Figs. 6 and 10). Indeed, cleavage of the catestatin region occurs at high frequency in chromogranin A: ~46% of catestatin immunoreactivity in chromaffin granules was of a lower molecular size form than intact chromogranin A (Fig. 8A, peaks 19 and 22).
2–4), and ~8% of catestatin immunoreactivity co-eluted with the small peptide bovine chromogranin A\textsubscript{344-364} (Fig. 8A, peak 4). Furthermore, catestatin is subject to calcium-dependent, secretagogue-regulated release by chromaffin cells (Fig. 11).

Size separation of chromaffin granule peptides (Fig. 2), followed by immunoprecipitation and MALDI mass spectrometry.
(Fig. 3), revealed a major catestatin form (bovine chromogranin A332–364) cleaved, as expected, at dibasic sites: KRLEGEEE-EEEDPDRSMRLSFRARGYGFRGPGLQLRR. Recognition of dibasic recognition sites by prohormone convertases is well described in chromogranin A; we (28) and others (29) have shown that chromogranin A is a substrate in vivo for prohormone convertases 1 and 2 as well as furin. Chromogranin B and secretogranin II are also cleaved by prohormone convertases (30). Other proteases recognizing dibasic sites may also be active in chromaffin granules (31). Since proteases recognize dibasic sites cleave to the carboxyl-terminal side of such sites (32), the lack of the carboxyl-terminal residues Arg365-Arg366 suggests, in addition, carboxypeptidase B (33, 34) removal of the remaining Arg365-Arg366 residues after initial cleavage by the prohormone convertase(s). Further resolution of chromaffin granule peptides on reverse-phase chromatography (Fig. 4), followed by MALDI mass spectrometry and amino-terminal sequencing, revealed two further forms: a long form (EGEEEEEEDPDRSMRLSFRARGYGFRGPGLQL; chromogranin A333–364) lacking the amino-terminal Leu332 of the form previously identified (Fig. 3) and a shorter form (DRSMRLSFRARGYGFRGPGL; chromogranin A344–362). Radioimmunoassay confirmed the secretion and physiological relevance of catestatin.

Both longer (chromogranin A332–364; LEGEEEEEEDPDRSMRLSFRARGYGFRGPGLQL) and shorter (chromogranin A344–364; RSMRLSFRARGYGFRGPGLQL) synthetic forms of bovine catestatin showed specific antagonism of nicotinic cholinergic-stimulated catecholamine release (Fig. 7A), although the shorter form had superior potency (IC50; 0.35 versus 2.01 μM; Fig. 7B), suggesting that cleavage of the longer to the shorter version may remove an inhibitory domain (LE-GESEEEDP; bovine chromogranin A332–342) from catestatin.
although the enzymology of such further internal cleavage is uncertain. Whatever the cleavage mechanism, removal of the acidic amino terminus (LENEEEDDEPD) seems to delete an inhibitory domain, thus potentiating the biological activity (IC50) of the peptide. Finally, cathepsin cleavage from intact chromogranin A magnifies its potency by ~12-fold (IC50 ratio, 4.2±0.35 μM; Fig. 7B).

Pheochromocytoma provides an accessible source of human chromaffin granules (35, 36). Synthetic human cathepsin (chromogranin A352–372) specifically inhibited chromaffin cell catecholamine release (Fig. 7C), and liberation of cathepsin from human chromogranin A between dibasic sites at residues of chromogranin A350–372 (KRELQGEEEDNNRSDMMKLSFRAYGFGRPGLQPLRR) was demonstrated by mass spectrometry (Fig. 6).

Previous studies established several cleavage sites in the cathepsin region of bovine chromogranin A. Within bovine chromaffin granules, Metz-Boutigue et al. (37) found peptides with amino termini at chromogranin A323 (Leu323, i.e. after dibasic site Lys320–Arg321), chromogranin A351 (Arg351), and chromogranin A354 (Gly354); upon secretion into the extracellular space, cleavage was also detected before Gly359. Sigafoos et al. (38) also detected a fragment with the amino terminus chromogranin A342 (Pro342). Evidence for cleavage at the dibasic site Arg365–Arg366 in chromogranin A has not heretofore been obtained. Such previous studies used amino-terminal amino acid sequencing but occurred before the widespread availability of protein and peptide mass spectrometry; thus, carboxy-terminal boundaries of the detected peptides could only be deduced imprecisely.

Studies on the formation of peptides flanking cathepsin, i.e. bovine chromogranin A316–329 (referred to as WE14 (39)) and bovine chromogranin A350–372 (referred to as GE25 (40)), also provide evidence of cleavage in the cathepsin (bovine chromogranin A344–364) region. Our region-specific immunoblots of the cathepsin region (Fig. 9) indicate that chromogranin A fragments bearing the cathepsin epitope are at least as prominent as fragments bearing the WE14 or GE25 epitopes and document dibasic cleavage at Arg365–Arg366 in bovine chromogranin A.

What prompted cleavage of amino-terminal Leu323 from bovine chromogranin A323–364 (Fig. 3), to yield chromogranin A323–364 (GELEEEEEDP) (Fig. 4)? Chromaffin granules contain lysine- and arginine-aminopeptidase activities (41), although an aminopeptidase recognizing aliphatic hydrophobic residues (such as Leu323) has not been described in chromaffin cells; if present, the exopeptidases leucine aminopeptidase or aminopeptidase M could accomplish this cleavage (32).

Likewise, the enzymology giving rise to endopeptidolytic cleavage between bovine chromogranin A residues Pro342 to Asp343, to yield chromogranin A342–362 (DRSMLSFRA-RGYGFGRPGLQPLLR) (Fig. 4), is not certain, although a post-proline-cleaving enzymatic activity (at Pro342) would suffice (42, 43). Asp–Pro peptide bonds are unstable in acidic solution (42), but acidic cleavage at Asp341 to Pro342 would still yield a peptide (chromogranin A342–362) with amino-terminal Pro342 (DPSMLSRFRAYGFGRPGLQPLLR), unlike chromogranin A343–362 (DPSMLSRFRAYGFGRPGLQ).

What prompted cleavage of carboxy-terminal Gln363–Leu364 from bovine chromogranin A343–364 (DPSMLSRFA-RGYGFGRPGLQ), to yield chromogranin A343–362 (DPSMLSRFA-RGYGFGRPGLQ) (Fig. 4)? Chromaffin granules do contain carboxypeptidase B (34), but carboxypeptidase B cleaves preferentially at basic amino acids (Arg, Lys); carboxypeptidase types P or Y (32) could catalyze sequential removal of Gln363–Leu364, but such carboxypeptidases have not been described in chromaffin granules. Cleavage at chromogranin A residues Leu362 Gln363 may represent a chymotrypsin-like cleavage, at the hydrophilic residue Leu362; chymotrypsin-like enzymatic cleavages are known to occur in neuroendocrine peptides (44), and a chymotrypsin inhibitor has been isolated from chromaffin granules (45).

Since peptide fractions characterized in these experiments came from sucrose density gradient-purified chromaffin granules, artificial proteolysis is unlikely to be problematic here. We detected cathepsin immunoreactivity in bovine and equine serum (see “Results”). Using chromogranin A radioimmunoassays directed to regions overlapping the cathepsin portion of human (chromogranin A344–374) Ref. 46 or rat (chromogranin A359–389; Ref. 47) chromogranin A, Yanaihara and co-workers (46–49) also found cathepsin region immunoreactivity in the circulation (46, 47) as well as in saliva, where its release was triggered by autonomic stimulation (48, 49). Since cathepsin administration into the bloodstream exerts profound effects upon blood pressure (50), detection of cathepsin in serum has implications for control of the circulation.

Thus, what prompted cleavage of chromogranin A is formed by endogenous proteolytic cleavage in vivo. Such authentic cleavage sites provide a useful starting point in analysis of the relationship between structure and function for this potent and specific catecholamine release-inhibitory peptide (10).

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Catestatin Processing

22915

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