Proteolytic Cleavage of Chromogranin A (CgA) by Plasmin

SELECTIVE LIBERATION OF A SPECIFIC BIOACTIVE CgA FRAGMENT THAT REGULATES CATECHOLAMINE RELEASE

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Chromogranin A (CgA), the major soluble protein in catecholamine storage vesicles, serves as a prohormone that is cleaved into bioactive peptides that inhibit catecholamine release, providing an autocrine, negative feedback mechanism for regulating catecholamine responses during stress. However, the proteases responsible for the processing of CgA and release of bioactive peptides have not been established. Recently, we found that chromaffin cells express components of the plasminogen system, including tissue plasminogen activator, which is targeted to catecholamine storage vesicles and released with CgA and catecholamines in response to sympatheoadrenal stimulation, and high affinity cell surface receptors for plasminogen, to promote plasminogen activation at the cell surface. In the present study, we investigated processing of CgA by plasmin and sought to identify specific bioactive CgA peptides produced by plasmin proteolysis. Highly purified human CgA (hCgA) was produced by expression in Escherichia coli and purification using metal affinity chromatography. hCgA was digested with plasmin. Matrix-assisted laser desorption/ionization mass spectrometry identified a major peptide produced with a mass/charge ratio (m/z) of 1546, corresponding uniquely to hCgA-(360–373), the identity of which was confirmed by reverse phase high pressure liquid chromatography and amino-terminal microsequencing. hCgA-(360–373) was selectively liberated by plasmin from hCgA at early time points and was stable even after prolonged exposure to plasmin. The corresponding synthetic peptide markedly inhibited nicotine-induced catecholamine release from pheochromocytoma cells. These results identify plasmin as a protease, present in the local environment of the chromaffin cell, that selectively cleaves CgA to generate a bioactive fragment, hCgA-(360–373), that inhibits nicotinic-mediated catecholamine release. These results suggest that the plasminogen/plasmin system through its interaction with CgA may play a major role in catecholaminergic function and suggest a specific mechanism as well as a discrete CgA peptide through which this effect is mediated.

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‡ The abbreviations used are: CgA, chromogranin A; hCgA, human chromogranin A; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; IPTG, isopropyl β-D-thiogalactopyranoside; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; PAGE, polyacrylamide gel electrophoresis; Fmoec, N-9-fluorenylmethoxy carbonyl; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC.
lease from catecholaminergic cells (16). However, the specific peptide(s) released from CgA by plasmin, in particular those that modulate catecholamine secretion, have not been identified.

In the present study, we have focused on the identification of regulatory peptide(s) produced by the interaction of CgA with plasmin. We utilized a combined approach that included development of an expression and purification system for obtaining highly purified human CgA (hCgA) substrate and controlled plasmin-specific proteolysis, coupled with matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, reverse phase HPLC, and amino-terminal amino acid sequencing, to identify specific cleavage sites and specific CgA peptide fragments resulting from the interaction of CgA and plasmin. Our results demonstrate that processing of CgA by plasmin is sufficient to selectively liberate a discrete CgA fragment with a pronounced inhibitory effect on catecholamine secretion. These results suggest that the plasminogen-t-PA system through its interaction with CgA may play a major role in catecholaminergic function and suggest a specific mechanism as well as a discrete CgA peptide fragment through which this effect is mediated. These interactions between catecholaminergic and fibrinolytic pathways thus have important implications for cardiovascular regulation.

**EXPERIMENTAL PROCEDURES**

**Construction of the Plasmid pET-(hCgA-6his), Encoding Histidine-tagged Mature Human CgA—Human CgA cDNA encoding the mature protein (minus the 18-residue signal peptide) was generated by the polymerase chain reaction using the cDNA clone, pGEM-hCgA (from Dr. Lee Helman, NCI, National Institutes of Health) (17) as template. The 5′ primer, 5′-CATGGCATAGCTCCCTGTAACGCTAT-3′, encoded the N-terminal portion of the mature protein (amino acid residues 1–6) preceded by a NcoI site (underlined). The 3′ primer, 5′-CCGGTCGAGGCCCCCCTGAGTGGTCCCGA-3′, was complementary to the 3′-end of the coding region (encoding amino acid residues 433–439) and contained an XhoI site (underlined). For amplification, 10 ng of cDNA were added to 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-Cl, pH 8.0, 25 mM MgCl2, 0.1% Triton X-100, with the two oligonucleotide primers (each at 50 μM), dNTPs (Life Technologies, Inc.) (each at 25 mM) and 2.5 units of Taq DNA polymerase (Applied Biosystems, PerkinElmer Life Sciences) in a total volume of 100 μl. Twenty-five polymerase chain reaction cycles were carried out in three steps consisting of a 1-min denaturation step at 94 °C, 2-min annealing at 60 °C, and 2-min extension at 72 °C using a PTC-100 Thermal Cycler (MJ Research, Inc., Watertown, MA). The polymerase chain reaction product was digested with NcoI and XhoI (Life Technologies) and gel-purified. The resulting fragments of 1.3 kb were directionally cloned into the expression vector, pET-28b (Novagen, Madison, WI). The final construct was designated pET-hCgA-6his and encoded mature human CgA (439 residues) with the addition of the residues MPW at the amino terminus and the residues LEHHHHHHH at the C terminus (total of 450 residues for the final recombinant protein). The nucleotide sequences of the polymerase chain reaction products and the junction regions between the insert and the vector were verified from both strands using several approaches.**

**Expression and Purification of Recombinant Human Chromogranin A—**One liter of a suspension of *Escherichia coli*, BL21(DE3) cells (Novagen), was transformed with the pET-(hCgA-6his) expression vector, and grown in LB broth containing kanamycin (25 μg/ml) at 37 °C until an *A*$_{600}$ of 0.4 was reached. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 1 mM. Three hours following IPTG induction, the cells were harvested by centrifugation at 7500 × *g* for 5 min at 4 °C. The cells were lysed in 100 ml of buffer A (6 mM guanidine hydrochloride, 0.1 mM sodium phosphate, 0.01 mM Tris-HCl, pH 8.0) by stirring at 4 °C for 18 h. The lysate was centrifuged at 27,000 × *g* for 30 min at 4 °C. The supernatant was loaded onto a N12-nitrio-triacetic acid-Sepharose column (1.5 × 4 cm), equilibrated with 5 mM columns (25 ml) of buffer A. The column was washed at a flow rate of 15 ml/h, with 30 ml of buffer A, followed by 20 ml of buffer B (8 mM urea, 0.1 mM sodium phosphate, 0.01 mM Tris-HCl, pH 8.0) until the effluent had an *A*$_{280}$ of <0.01. The bound proteins were eluted with a pH gradient of 5.9–4.5 (composed of 8 ml of buffer B adjusted to pH 5.9 and 4 ml of buffer B adjusted to pH 4.5) according to the manufacturer's instructions (Qiagen, Santa Clarita, CA). Fractions of 1 ml were collected, and 5 μl of each fraction were electrophoresed on SDS-polyacrylamide gels under reducing conditions followed by Coomassie Blue staining. The affinity-purified recombinant hCgA was identified based on the apparent molecular mass of CgA, 70 kDa (17–21). The peak fractions were pooled and electrophoresed on SDS-polyacrylamide gels under reducing conditions. The band migrating with an apparent molecular mass of 70 kDa was cut out and placed into a sample trap with a 10,000 molecular weight cut-off dialysis membrane. The gel slices were soaked in 10 mM Tris acetate, 1 mM EDTA, pH 7.6. The protein-bound material was recovered from the gel slice in 10 mM Tris acetate, 1 mM EDTA, pH 7.6 following the manufacturer's instructions (ISCO, Inc., Lincoln, NE). After electrophoresis, hCgA was dialyzed into 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA. The purified hCgA was concentrated, and its concentration was determined using the Bradford dye-binding assay (Bio-Rad protein assay).

**Radiodination of Recombinant Human CgA—**Recombinant hCgA was iodinated using a modified chloramine-T procedure as described previously (22) and dialyzed against 0.1 M sodium phosphate, pH 7.4, 0.15 M NaCl (sodium-buffered saline).

**Other Proteins—**Human Glu-plasminogen, the native circulating form of the molecule, was isolated from fresh human blood collected into 3 mM benzamidine, 5 mM EDTA, 100 units/ml aprotinin (Miles, Kankakee, IL), and 100 μg/ml soybean trypsin inhibitor (Sigma). The plasminogen subjected to affinity chromatography on lysine-Sepharose (23) in sodium-buffered saline with 1 mM benzamidine, 0.02% NaN$_3$, and 3 mM EDTA followed by molecular exclusion chromatography on Ultrogel AcA44 (IBF Biotechniques, Villeneuve-la-Garenne, France). The plasminogen concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 16.8 (24). Urokinase (u-PA) was obtained from Calbiochem.

**Mass Spectrometric Analysis—**Purified hCgA was digested with plasmin, and the reaction mixtures were analyzed by MALDI mass spectrometry (25, 26). The plasmin digests were dissolved in 0.1% trifluoroacetic acid to a concentration of 10 μM. Either a-cyano-4-hydroxycinnamic acid or sinapinic acid was used as the UV laser desorption matrix depending on the mass ranges included in the analysis. Samples of 5–10 pmol were loaded onto the mass spectrometer sample probe and dried at ambient temperature. Mass spectra were acquired using a Voyager-Elite mass spectrometer (Perseptive Biosystems, Houston, TX).

**Mass spectra obtained from the MALDI analyses were compared with values generated from a theoretical digest of mature CgA by a plasminogen-t-PA system through its interaction with CgA.**, **C′**

**Reverse-phase High Pressure Liquid Chromatography (RP-HPLC)—**Fractions containing digested hCgA were separated on a Supelcosil LC-318 column (25 cm × 4.6 mm; Supelco). The column was equilibrated in 0.1% trifluoroacetic acid/H$_2$O (eluent A), and the peptides were eluted with a linear gradient of 0–80% acetonitrile, 0.1% trifluoroacetic acid (eluent B). The effluent was monitored at 214 nm, and fractions were collected at 0.5-min intervals. Peak fractions were lyophilized and stored at −20 °C.

**Protein Sequence Analysis—**Amino-terminal sequence analysis was performed using automated Edman microsequencing (Applied Biosystems Precise Sequencer, model 494, PerkinElmer Life Sciences).**

**Peptide Synthesis—**Peptides were synthesized at 10–100-μmol scale using Fmoc protection chemistry and purified to >95% homogeneity by RP-HPLC on a C-18 column. Authenticity and purity of the peptides were verified by electrospray ionization or MALDI mass spectrometry.

**Cell Culture—**PC12 cells (28) (at passage number 8) were grown at 37 °C, 6% CO$_2$, in Dulbecco's modified Eagle's medium/high glucose medium supplemented with 5% fetal bovine serum, 10% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin as described previously (15, 29).

**Catecholamine secretion experiments were performed as described previously (15, 29). Briefly, PC12 cells were plated on poly-D-lysine-coated polystyrene dishes (Falcon, Franklin Lakes, NJ) and labeled with $[^3H]$norepinephrine (PerkinElmer Life Sciences) at 1 μCi/ml in the PC12 cell culture medium. After a 3-h incubation, the cells were washed twice with release buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 10 mM HEPES, pH 7.0) and further incubated in release buffer at 37 °C for 30 min in either the...
Expression and Purification of Recombinant Human CgA—We sought to obtain highly purified CgA in order to precisely characterize the peptide products generated by plasmin proteolysis. Results of expression and purification of recombinant hCgA using the construct pET-(hCgA-6his) are shown in Fig. 1. Aliquots from cultures of E. coli strain BL21(DE3) harboring plasmid pET-(hCgA-6his) were removed at the indicated times following IPTG induction, and total cellular proteins were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A protein band, with an apparent molecular mass of 70 kDa, corresponding to the recombinant hCgA was observed 30 min after IPTG induction (Fig. 1, lane 1). The samples were lysed, total cellular proteins were analyzed on SDS-PAGE under reducing conditions, and gels were stained with Coomassie Blue. Three hours after IPTG induction, cell pellets were solubilized in 6 M guanidine HCl, pH 8.0, and applied to a Ni²⁺-nitrilotriacetic acid-Sepharose column, and the bound proteins were eluted with a pH gradient as described under “Experimental Procedures.” Lane 6 shows 50 μg of the peak fraction containing purified recombinant hCgA. Lane 7 shows 2.5 μg of purified recombinant hCgA after electroelution.

presence or absence of the secretagogue, nicotine (60 μM). The release buffer was aspirated, and the cells were lysed in release buffer containing 0.1% Triton X-100. The amounts of [³H]norepinephrine in the medium and cell lysates were measured by liquid scintillation counting. Results were expressed as percentage of secretion (amount released/ (amount released + amount in cell lysate)) × 100. Net release is secretagogue-stimulated release minus basal release, where basal norepinephrine release is typically 5.8 ± 0.36% of total cellular [³H]norepinephrine released over 30 min (n = 10 separate secretion assays).

RESULTS

Expression and Purification of Recombinant Human CgA—We sought to obtain highly purified CgA in order to precisely characterize the peptide products generated by plasmin proteolysis. Results of expression and purification of recombinant hCgA using the construct pET-(hCgA-6his) are shown in Fig. 1. Aliquots from cultures of E. coli strain BL21(DE3) harboring plasmid pET-(hCgA-6his) were removed at the indicated times following IPTG induction, and total cellular proteins were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A protein band, with an apparent molecular mass of 70 kDa, corresponding to the recombinant hCgA was observed 30 min after IPTG induction (Fig. 1, lane 1). The samples were lysed, total cellular proteins were analyzed on SDS-PAGE under reducing conditions, and gels were stained with Coomassie Blue. Three hours after IPTG induction, cell pellets were solubilized in 6 M guanidine HCl, pH 8.0, and applied to a Ni²⁺-nitrilotriacetic acid-Sepharose column, and the bound proteins were eluted with a pH gradient as described under “Experimental Procedures.” Lane 6 shows 50 μg of the peak fraction containing purified recombinant hCgA. Lane 7 shows 2.5 μg of purified recombinant hCgA after electroelution.

and doubly charged signals at m/z of 50,976 and 25,495 (see below; Fig. 3, A–C). The observed mass value of 50,976 ± 1 for the singly charged signal (or 25,495 × 2 for the doubly charged signal) is consistent with the molecular mass calculated from the primary sequence of the 450-residue recombinant hCgA.³ These results (Fig. 3, A–C) demonstrated that the hCgA substrate obtained was highly purified (without contaminating small peptides) for subsequent proteolytic studies.

Proteolysis of Recombinant hCgA by Plasmin—We examined whether recombinant hCgA could be processed by plasmin. Plasmin readily digested the recombinant hCgA, and the extent of the digestion was dose-dependent (Fig. 2). Major products were sequentially produced with apparent molecular masses of 65, 55, and 43 kDa, as detected using SDS-PAGE.

Identification of a 1546 m/z CgA Peptide Derived from Plasmin Proteolysis—To identify the peptides produced by plasmin proteolysis of hCgA, mass spectrometric analysis was applied. The mixture of proteolytic products of hCgA after digestion with plasmin for 4 h was analyzed by MALDI mass spectrometry. The resulting mass spectra are shown in Fig. 3 (D–F). The most prominent peak was observed at m/z = 1546. A time course of proteolysis of hCgA by plasmin was performed, and the proteolytic products were analyzed on the mass spectrometer (Fig. 4). The 1546 m/z peptide was the predominant species observed at 15 min (Fig. 4A) and was still the predominant species with an even stronger signal after incubation for 45 and 120 min (Fig. 4, B and C). Additional time course experiments demonstrated the appearance of this peptide at even earlier time points (5 min) and also demonstrated that this peptide was stable to further cleavage even after prolonged incubation for 8 h (data not shown). In control experiments, MALDI analysis of plasminogen plus urokinase alone incubated for 2 h at 37 °C and incubation of hCgA alone for 2 h at 37 °C gave no peaks in the m/z range of 500–5000, showing that neither of these control incubations generated any detectable peptides (data not shown). Thus, the observed 1546 m/z peak was specifically generated by the interaction between plasmin and hCgA.

To investigate the identity of the 1546 m/z hCgA peptide produced by the interaction of plasmin and hCgA, these data were analyzed and compared against specific mass information

³ m/z, the mass/charge ratio, depends on the number of charges on the protein generated by ionization, such that a given protein generates more than one ion signal detected by the mass spectrometer. Thus, the detected m/z ratios of 50,976 and 25,495 represent the 450-residue recombinant hCgA with charge states of 1+ and 2+, respectively.
Fig. 3. MALDI-MS of purified recombinant hCgA and its proteolytic products detected after plasmin digestion. Five pmol of the recombinant hCgA dissolved in 0.1% trifluoroacetic acid were analyzed on a Voyager-Elite mass spectrometer using α-cyano-4-hydroxycinnamic acid (A) and sinapinic acid (C) as matrices. The entire spectra from mass range of 1000 to 80,000 Da (A → C) showed only [M + H]⁺ and [M + 2H]²⁺ signals at m/z of 50,976 and 25,495. Human CgA (at 10 μM) was incubated with plasminogen (2 μM) and urokinase (10 μM) at 37°C for 4 h. The reaction was stopped by the addition of aprotonin (2.5 μM). Five pmol of the reaction mixture were loaded onto the mass spectrometer sample probe, and the same mass range was scanned using matrices of α-cyano-4-hydroxycinnamic acid (D and E) and sinapinic acid (F). A prominent peak was observed at an m/z of 1546.

Fig. 4. MALDI-MS of recombinant hCgA digested with plasmin for various times. Purified recombinant hCgA (10 μM/reaction) was incubated at 37°C with plasminogen (2 μM) and urokinase (10 μM) in buffer (0.01 M Tris–Cl, pH 8.0, 0.15 M NaCl) for various times, and the reaction was stopped by the addition of aprotonin (2.5 μM). Five pmol of digested recombinant hCgA were analyzed on the mass spectrometer using α-cyano-4-hydroxycinnamic acid as matrix. A predominant 1546 m/z peak was observed after a 15-min incubation with plasminogen and u-PA (A) and was present with a stronger signal after longer incubations of 45 min (B) and 120 min (C). A prominent peak corresponded to the mass of the synthetic 14-mer, hCgA-(360–373). This same eluted RP-HPLC fraction was subjected to amino-terminal microsequencing. Using Edman degradation microsequencing over 7-residue cycles, the first 7 amino-terminal residues were determined to be ARAYGFR, precisely matching the N-terminal sequence of the 14-mer, hCgA-(360–373).

Under conditions where plasmin had generated products uniquely to the 14-mer, hCgA-(360–373), the N-terminal sequence was ARAYGFRGPGPQL, which is unique to this 14-mer (Fig. 4). We identified this 14-mer, hCgA-(360–373), as the major product generated by plasmin treatment of hCgA due to its prominent MALDI-MS peak (Fig. 3).

Generated from a theoretical digest, using the program Sherpa (27). This program calculates molecular weight and the charge state m/z values for all possible fragments generated from a theoretical proteolytic digest and orders them by molecular mass. Based on the known primary sequence of mature hCgA, a theoretical digest was carried out, cleaving at basic (arginine or lysine) residues (known recognition sites for plasmin (30)) and after incubation with plasmin. Before plasmin treatment, both peptides were subjected to MALDI-MS analyses before incubation with plasmin. Before plasmin treatment, the theoretical proteolytic digest and orders them by molecular mass (determined by MALDI-MS) of the peptide recovered from our plasmin digest of hCgA (Figs. 3 and 4).
each spectrum showed a clear peak, with m/z of either 1546 or 2326, corresponding to the 1546 peptide (Fig. 6A) or the hCgA-(352–372) (21-mer) (Fig. 6C), respectively. After plasmin digestion, the spectrum in Fig. 6B showed no difference from that in Fig. 6A (single peak at m/z = 1546), whereas the spectrum in Fig. 6D contained a new peak at an m/z of 1389. By analysis of the mass spectrometric data using the Sherpa program, the 1389 m/z peak was identified as ARAYGFRGPGPQL, corresponding to hCgA-(360–372). Thus, hCgA-(352–372) was selectively cleaved by plasmin at Arg359 within the 21-mer hCgA sequence (hCgA-(360–372), SSMKLSFR 359 ARAYGFRGPGPQL) to yield hCgA-(360–372), ARAYGFRGPGPQL (see Fig. 6). No cleavage at either Arg361 or Arg366 within the 21-mer catestatin peptide was observed.

Effect of the 14-Mer, hCgA-(360–373), on Secretagogue-stimulated Catecholamine Secretion from PC12 Cells—To test whether the 14-mer, hCgA-(360–373) (ARAYGFRGPGPQLR), could inhibit secretagogue-stimulated catecholamine release, the effect of synthetic hCgA-(352–372) was evaluated on nicotine-induced norepinephrine secretion from PC12 pheochromocytoma cells (Fig. 7). (Nicotine acts at nicotinic cholinergic receptors, as does acetylcholine, the major physiologic stimulus mediating catecholamine release from chromaffin cells.) The peptide, ARAYGFRGPGPQLR (hCgA-(360–373)), inhibited

FIG. 5. Isolation and identification of the major peptide from the plasmin digestion of hCgA by reverse phase HPLC, MALDI-MS, and amino-terminal sequence analysis. Fifty micrograms of hCgA (10 μM) were incubated at 37 °C with plasminogen (2 μM) and u-PA (10 nM) in 0.01 M Tris-Cl, pH 8.0, 0.15 M NaCl for 2 h, and the reaction was stopped by the addition of aprotinin (2.5 μM). The reaction mixture was chromatographed on a Supelcosil LC-318 column (25 cm × 4.6 mm). The column was equilibrated in 0.1% trifluoroacetic acid/H2O (eluent A), and the peptides were eluted in a linear gradient of 0–80% acetonitrile, 0.1% trifluoroacetic acid (eluent B). The effluent was monitored at 214 nm, and fractions were collected at 0.5-min intervals (see A). Approximately 5 pmol of recovered peptide from fraction 40 (retention time 19.5–20 min, marked with an arrowhead in A) was analyzed by MALDI-MS (B), which revealed a single peptide peak consistent with the 14-mer peptide, ARAYGFRGPGPQLR. Amino-terminal sequence analysis of the same HPLC fraction revealed that the first 7 residues were ARAYGFR, matching the aminoterminal sequence of hCgA-(360–373).

TABLE I
| Bovine synthetic catestatin | Bovine CgA-(344–364) | RSMRLSFRARGYFGPGPQLQL |
|-----------------------------|----------------------|-------------------------|
| Human synthetic catestatin  | hCgA-(352–372)       | SSMKLSFRARAYGFRGPGPQL  |
| Plasmin-generated 1546 m/z CgA peptide | hCgA-(360–373) | ARAYGFRGPGPQLR |

Relationship of human CgA-(360–373) to human and bovine synthetic catestatin
Bovine and human synthetic catestatin sequences are from Ref. 11.

see Fig. 6). No cleavage at either Arg361 or Arg366 within the 21-mer catestatin peptide was observed.
nicotine-induced catecholamine release in a dose-dependent fashion with marked inhibition of catecholamine release in the micromolar range (IC$_{50}$ 3.0 ± 0.046 μM). In control experiments with the reverse peptide, RLQPGPGRFGYARA, no inhibition was detected (Fig. 7).

**DISCUSSION**

In this study, we have demonstrated that proteolysis of CgA with the major fibrinolytic enzyme, plasmin, results in the production of a discrete and stable peptide, ARAYGFRGPGPQLR, corresponding to human CgA-(360–373), which markedly inhibits secretagogue-stimulated catecholamine release from catecholaminergic cells. These results suggest important interactions between catecholaminergic and fibrinolytic systems that may have a profound influence on cardiovascular regulation.

In earlier studies done to demonstrate that CgA may function as a prohormone, catecholamine release-inhibitory activity was generated by incubation of CgA with the serine proteases, trypsin (9), and the bacterial enzyme, endoproteinase Lys C (10), proteases that are not present in the environment of the chromaffin cell. More recently, we demonstrated that CgA is readily processed by plasmin, which also results in generation of a peptide mixture with catecholamine release-inhibitory activity, significantly inhibiting nicotine-mediated catecholamine release from PC12 cells and primary bovine adrenal chromaffin cells (16). However, the specific peptide(s) that inhibited catecholamine release had not been identified. In a previous study (11), we screened synthetic peptides spanning 80% of the linear sequence of mature bovine CgA. Using this screening approach, we identified a peptide domain approximated by the region corresponding to bovine CgA-(344–364) (human CgA-(352–372)), referred to as “catestatin,” which inhibited secretagogue-stimulated catecholamine release. Interestingly, the peptide ARAYGFRGPGPQLR, produced by plasminolytic cleavage of human CgA and identified in the current study, is contained within the catestatin sequence with the addition of an Arg at the carboxyl terminus of the peptide (Table I). Thus, using two independent approaches, this region of the CgA molecule has been demonstrated to harbor catecholamine secretion-inhibitory activity.

We investigated processing of CgA by plasmin because plasminogen circulates at a high concentration (2 μM (13)) and is present in the local chromaffin cell environment. In addition, our previous studies revealed that chromaffin cells express the major activator of this system, t-PA, which is targeted to catecholamine storage vesicles and is co-released along with catecholamines and the substrate CgA, from this subcellular stor-
TABLE II

| Species          | Sequence comparison |
|------------------|---------------------|
| Human CgA-(360–373) | FR (ARAYGFRGPQPLR) RG |
| Bovine CgA-(352–365) | FR (ARGYGFGRGPQPLR) RG |
| Porcine CgA-(351–364) | FR (APAYGFRGPQPLR) RG |
| Rat CgA-(375–388)   | FR (ARAYGFRGPQPLR) RG |
| Mouse CgA-(376–389) | FR (TRAYGFRGPQPLR) RG |

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CgA sequences are from the following references: human CgA from Ref. 20, bovine CgA from Ref. 19, porcine CgA from Ref. 39, rat CgA from Ref. 21, mouse CgA from Ref. 40. Arrows indicate plasmin cleavage sites at human CgA Arg^359 and Arg^373. Numbering refers to the 14-mer peptide produced by plasmin cleavages at Arg^359 and Arg^373 for human CgA (human CgA-(360–373)) and corresponding residues for the other species (e.g. bovine CgA-(352–365)).

age pool in response to chromaffin cell stimulation (15). Moreover, these cells express high affinity, high capacity cell surface binding sites for plasminogen (16) as a means for localizing and concentrating the activity of this protease system at the cell surface, where plasminogen is rapidly activated to plasmin by t-PA released from intracellular secretory vesicular stores during chromaffin cell stimulation. These results, then, suggested the presence of a local, chromaffin cell, plasminogen/t-PA system, through which chromaffin cells have the ability to concentrate and spatially organize plasmin activity in the local environment into which CgA is secreted, providing a logical microanatomic and physiologic rationale for a mechanism for local extracellular proteolytic processing of CgA.

In other previous experiments, overexpression of t-PA in PC12 cells resulted in markedly diminished secretagogue-stimulated catecholamine release. Conversely, when plasmin activity was inhibited using a monoclonal antibody against the catalytic domain of plasmin, nicotine-stimulated catecholamine secretion was substantially increased in these cells (16). Thus, both positive and negative modulation of the local chromaffin cell plasminogen/t-PA system resulted in marked alterations in secretagogue-mediated exocytotic release of catecholamines, suggesting a major role for fibrinolytic molecules in the regulation of catecholamine secretion. We therefore sought to identify regulatory peptide(s) produced when CgA interacts with plasmin, in particular focusing on regulatory CgA fragments with catecholamine release-inhibitory activity, as a mechanism through which the effects of the plasminogen/t-PA system might mediate regulatory effects on catecholamine secretion.

To examine the proteolytic effects of plasmin on CgA, we prepared highly purified recombinant human CgA and exposed this highly purified substrate to plasmin in controlled proteolytic studies. Recombinant human CgA was readily processed by plasmin in a dose-dependent fashion. We used a combined approach that included mass spectrometry, RP-HPLC, and amino-terminal microsequencing to confirm the identity of the specific peptides formed from the interaction of CgA with plasmin. This approach utilizes specific mass information of the peptides generated and the known primary sequence of the CgA substrate. Such an approach has been shown to be highly effective in the identification of peptide fragments from complex, heterogeneous samples, including those generated from proteolytic digests (25, 26, 31–37). Examination of the peptide mixture produced by plasmin-mediated cleavage of CgA by MALDI mass spectrometry identified a major peptide peak at 1546 m/z.

These data were compared against specific mass information generated from a theoretical digest (with cleavages at basic residues) of the known primary sequence of mature human CgA. This analysis revealed that the observed 1546 m/z peak corresponded uniquely to the mass of the peptide, ARAYGFRGPQPLR, a 14-mer corresponding to hCgA-(360–373), results that were hence consistent with proteolytic cleavage on the C-terminal side of residues Arg^359 and Arg^373 and also consistent with the known proteolytic substrate specificity of plasmin (cleavage at the C terminus of basic residues) (30).

The identity of the peptide produced from the interaction of CgA with plasmin was further established by several independent criteria. First, when the digestion mixture of CgA and plasmin was separated by RP-HPLC, a major peak was noted at a retention time corresponding to the retention time of the synthetic peptide, ARAYGFRGPQPLR (corresponding to hCgA-(360–373)) Second, MALDI mass spectrometric analysis of the peak HPLC fraction and of the synthetic peptide, ARAYGFRGPQPLR, yielded identical molecular mass determinations of 1546 m/z (identical to the experimental mass, determined by MALDI-MS, of the peptide recovered from plasmin digests of CgA). Third, amino-terminal microsequencing of the peak HPLC fraction, revealed an amino-terminal sequence of ARAYGFR, precisely matching the amino-terminal sequence of the 14-mer, hCgA-(360–373). The observed 1546 m/z peptide was specifically liberated by the interaction between hCgA and plasmin and not observed in control experiments, including experiments with hCgA substrate alone or with plasminogen plus plasminogen activator without substrate.

We found that ARAYGFRGPQPLR (corresponding to hCgA-(360–373)) exhibited bioactivity. In functional secretagogue-mediated release studies, hCgA-(360–373) markedly inhibited nicotine-induced catecholamine secretion from pheochromocytoma cells. In control experiments, the reverse peptide, RLQPGFRGPQGYARA, had no effect on nicotine-mediated catecholamine release, demonstrating the specificity of this effect for hCgA-(360–373). These results suggest that the interaction between CgA and plasmin is sufficient to liberate a specific peptide from the CgA catestatin domain, which is bioactive, with pronounced effects on catecholamine secretion.

Our results also demonstrated that cleavage of CgA by plasmin occurred in a selective fashion, with processing at specific internal cleavage sites within the CgA sequence. Multiple digestion experiments and time course studies revealed that the 14-mer, hCgA-(360–373), was consistently and selectively liberated from CgA. In addition, the peptide was released from the parent CgA protein at early time points, and the peptide fragment formed was quite stable, with no evidence of further internal cleavages (for example, at Arg^361 or Arg^366) within the hCgA-(360–373) sequence, even after prolonged exposure to plasmin. The stability of hCgA-(360–373) and the selectivity of the cleavage site at Arg^359 were also demonstrated in experiments in which plasmin was incubated with the synthetic peptides, hCgA-(360–373) and hCgA-(352–372) (Fig. 6). These findings (i.e., early, robust cleavage of the peptide from the CgA substrate and stability (resistance to further internal cleavages by the protease responsible for its liberation)) are also consistent with an important physiological role for this peptide fragment. Thus, taken together, these results suggest that plasmin may indeed act upon CgA in a highly selective fashion (cleaving CgA at the basic residues Arg^359 and Arg^373 but not cleaving at Arg^361 and Arg^366) to liberate a specific bioactive peptide, hCgA-(360–373), with catestatin activity.

The cleavage sites at Arg^359 and Arg^373 are particularly noteworthy in light of recent studies utilizing substrate phase display and peptide substrates, which revealed that several primary sequence determinants in the P2P1 | P1′P2′ positions may enhance substrate cleavage specificity of plasmin. These include a basic residue (Arg or Lys) at the cleavage site (at the
CgA in the local extracellular space may be as high as 0.4 mM (Scripps Research Institute, La Jolla, CA).

Also of note, interspecies comparison of the primary sequence of CgA (including human (20), bovine (19), porcine (39), rat (21), and mouse (40) sequences) reveals that the cleavage sites at Arg<sup>359</sup> and Arg<sup>373</sup> (numbering according to the human sequence) are completely conserved, and the additional preferential substrate P2P1↓P1′P2′<sup>+</sup> sequence characteristics are also well conserved across species (Table II). In addition, the amino acid sequence of the bioactive 14-mer hCgA-(360–373), liberated from CgA by plasmin, is highly conserved with observed sequence homologies of 86–93% (12/14 residues for bovine/human, porcine/human, or mouse/human comparisons and 13/14 residues comparing the rat with the human sequence) (Table II).

Finally, we examined a range of substrate and enzyme concentrations likely to occur physiologically in the milieu of the chromaffin cell (nanomolar to micromolar range) and noted extensive processing of CgA by plasmin. In addition, the concentration of CgA within the chromaffin vesicle is extremely high, ~4 mM, so that following exocytosis, the concentration of CgA in the local extracellular space may be as high as 0.4 mM (41), much greater than the concentrations examined here. In addition, plasminogen circulates at high concentration (2 RG), exhibit three out of four of these preferential sequence characteristics, which may thus contribute to the selective nature of the proteolytic action of plasmin upon CgA.

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