Primary Sequence Characterization of Catestatin Intermediates and Peptides Defines Proteolytic Cleavage Sites Utilized for Converting Chromogranin A into Active Catestatin Secreted from Neuroendocrine Chromaffin Cells†

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ABSTRACT: Catestatin is an active 21-residue peptide derived from the chromogranin A (CgA) precursor, and catestatin is secreted from neuroendocrine chromaffin cells as an autocrine regulator of nicotine-stimulated catecholamine release. The goal of this study was to characterize the primary sequences of high molecular mass catestatin intermediates and peptides to define the proteolytic cleavage sites within CgA that are utilized in the biosynthesis of catestatin. Catestatin-containing polypeptides, demonstrated by anti-catestatin western blots, of 54−56, 50, 32, and 17 kDa contained NH_2-terminal peptide sequences that indicated proteolytic cleavages of the CgA precursor at KK↓, KR↓, R↓, and KR↓ basic residue sites, respectively. The COOH termini of these catestatin intermediates were defined by the presence of the COOH-terminal tryptic peptide of the CgA precursor, corresponding to residues 421−430, which was identified by MALDI-TOF mass spectrometry. Results also demonstrated the presence of 54−56 and 50 kDa catestatin intermediates that contain the NH_2 terminus of CgA. Secretion of catestatin intermediates from chromaffin cells was accompanied by the cosecretion of catestatin ($CgA_{344-364}$) and variant peptide forms ($CgA_{343-368}$ and $CgA_{332-361}$). These determined cleavage sites predicted that production of high molecular mass catestatin intermediates requires cleavage at the COOH-terminal sides of paired basic residues, which is compatible with the cleavage specificities of PC1 and PC2 prohormone convertases. However, it is notable that production of catestatin itself ($CgA_{344-364}$) utilizes more unusual cleavage sites at the NH_2-terminal sides of ↓R and ↓RR basic residue sites, consistent with the cleavage specificities of the chromaffin granule cysteine protease "PTP" that participates in proenkephalin processing. These findings demonstrate that production of catestatin involves cleavage of CgA at paired basic and monobasic residues, necessary steps for catestatin peptide regulation of nicotinic cholinergic-induced catecholamine release.

Catestatin is a 21-residue biologically active peptide derived from the chromogranin A (CgA)[1] precursor and is represented by bovine $CgA_{344-364}$. Catestatin peptides are synthesized and stored in secretory vesicles of sympatho-adrenal chromaffin cells (known as chromaffin granules) for subsequent secretion (*1*). Secreted, extracellular catestatin functions as an autocrine regulator of nicotinic-mediated release of catecholamines, potent regulators of blood pressure in the cardiovascular system.

Elucidation of the biosynthetic mechanisms involved in catestatin production requires knowledge of the catestatin-related intermediates and peptides, as well as the proteolytic cleavage sites within CgA that are utilized for generating catestatin-containing products. However, identification of catestatin-related intermediates derived from CgA and deduced cleavage sites for catestatin production has not yet been determined. Therefore, the goal of this study was to characterize the primary sequences of high molecular mass catestatin intermediates to define the CgA proteolytic cleavage sites involved in the production of active catestatin that undergoes secretion.

High molecular mass catestatin-containing polypeptides in chromaffin granules were subjected to primary sequence characterization by NH_2-terminal peptide sequencing and

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[1] Abbreviations: ACN, acetonitrile; α-CHCA, α-cyano-4-hydroxycinnamic acid; CgA, chromogranin; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MS, mass spectrometry; PC, prohormone convertase; PTP, prohormone thiol protease; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; EDTA, ethylenediaminetetraacetic acid; RADARS, rapid, automated data archiving and retrieval software; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; RP-HPLC, reverse-phase HPLC.

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MALDI-TOF mass spectrometry of peptide fragments derived from tryptic digests. Findings from this study indicated that catestatin peptide intermediates result from proteolytic cleavage of bovine CgA (*6*, *7*) at the COOH-terminal sides of paired basic and monobasic residues. However, production of active catestatin, $CgA_{344-364}$, requires unique cleavages at the NH_2-terminal sides of dibasic and monobasic residues of CgA, which differs from more typical prohormone processing at COOH-terminal sides of paired basic and monobasic residues (*8*−*11*). These findings have defined the proteolytic cleavage sites within CgA that are utilized in the production of catestatin intermediates and peptide forms that function in the autocrine regulation of nicotinic cholinergic-stimulated catecholamine release from sympathoadrenal chromaffin cells.

EXPERIMENTAL PROCEDURES

Isolation of Chromaffin Granules (Secretory Vesicles) and Catestatin Immunoblots. Adrenal medullary chromaffin granules (secretory vesicles) were prepared from fresh bovine adrenal medulla by differential centrifugation and discontinuous sucrose density gradient centrifugation, as previously described (*12*). The soluble fraction was prepared from isolated chromaffin granules as previously described (*12*), in the presence of a cocktail of protease inhibitors consisting of 1 mM EDTA, 10 μM pepstatin A, 10 μM leupeptin, 10 μM chymostatin, 10 μM E64c, and 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride]. After incubation of lysed granules with protease inhibitors in isotonic buffer (50 mM sodium acetate, pH 6.0, 150 mM NaCl) at 4 °C on ice for 30 min, samples were subjected to ultracentrifugation (in a Beckman L7-65 ultracentrifuge) at 100000*g* in a SW60 rotor for 30 min at 4 °C, and the resultant supernatant was collected as the soluble granule fraction.

Purified chromaffin granules were prepared for anti-catestatin immunoblots by heating at 95 °C for 5 min in gel loading buffer (10 mM Tris-HCl, 1 mM EDTA, 3% SDS, 20 mM dithiothreitol, 10% glycerol, 0.1% bromphenol blue) and subjected to electrophoresis through SDS−PAGE 10−20% acrylamide gradient gels in the presence of Tris−Tricine buffer (Novex, San Diego, CA). After SDS−PAGE, proteins were electrophoretically transferred to nitrocellulose membranes (BA85; Schleicher and Schuell, Keene, NH), and immunoreactive catestatin was assessed by anti-catestatin immunoblots with rabbit anti-catestatin (at a final dilution of 1:1000 v/v) (*1*), followed by detection with horseradish peroxidase-labeled goat anti-rabbit IgG by the ECL method (ECL kit from Amersham Pharmacia Biotech, Piscataway, NJ).

NH_2-Terminal Peptide Sequencing of Catestatin-Containing Intermediates. The soluble fraction of chromaffin granules contains high molecular mass catestatin-containing intermediates that were subjected to Edman degradation for NH_2-terminal sequencing. The granule soluble fraction was reduced (in NuPAGE SDS sample buffer; Novex, San Diego, CA) and heated at 70 °C for 10 min, subjected to electrophoresis on 12% polyacrylamide SDS−PAGE gels with MES running buffer (Novex, San Diego, CA), and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA). Proteins on the PVDF membrane were visualized with amido black staining. Catestatin-containing bands, identified from parallel catestatin immunoblots, were subjected to NH_2-terminal Edman peptide sequencing on an Applied Biosystems Procise 494 protein sequencer (Harvard Microchemistry and Proteomics Analysis Facility).

Tryptic Digestion and MALDI-TOF Mass Spectrometry of High Molecular Mass Catestatin-Containing Polypeptides. The soluble fraction of chromaffin granules was subjected to SDS−PAGE, anti-catestatin immunoblots, and protein staining by colloidal Coomassie blue (Invitrogen, San Diego, CA). Protein bands corresponding to catestatin immunoreactivity (detected by parallel immunoblots) were excised for "in-gel" tryptic digestion.

The tryptic digestion of samples was performed by the automated robot digester ProGest (from Genomic Solutions) which provides a series of programmed steps to deliver and remove solutions via nitrogen pressure through holes at the bottom of a 96-well plate. Gel pieces (~1.5 mm^2) in a 96-well plate were washed and destained with two cycles of 50 μL of NH_4HCO_3 (25 mM) and 50 μL of ACN. The proteins in gel pieces were then reduced with 40 μL of DTT (10 mM) in NH_4HCO_3 (25 mM) for 10 min at 60 °C, followed by alkylation with 30 μL of iodoacetamide (100 mM) in NH_4HCO_3 (50 mM) for 45 min at room temperature. The reduced and alkylated gel pieces were washed and dehydrated with two cycles of 50 μL of NH_4HCO_3 (25 mM) and 50 μL of ACN prior to trypsin digestion. The digestion was commenced with 10 μL of sequence grade trypsin (250 μg, Promega) dissolved in 1 mM acetic acid mixed with 15 μL of NH_4HCO_3 (25 mM). The reaction was allowed to proceed for 4 h at 37 °C and stopped by the addition of 7 μL of 10% formic acid. The digested peptides were then separated from the gel pieces by pressurizing with nitrogen into a collection plate (96 well).

For mass analyses, each protein digest (0.5 μL) was mixed with α-CHCA matrix (α-cyano-4-hydroxycinnamic acid, 0.5 μL) (Agilent Technologies, Inc.) and spotted onto a MALDI target and air-dried. The mass spectra were acquired on a PE Biosystems Voyager DeSTR MALDI-TOF mass spectrometer with a nitrogen laser, operating in delayed extraction and reflectron mode (*14*, *15*). The spectral analyses were made with internal calibration to masses of trypsin autolysis or external peptide peaks (bradykinin, ACTH fragment 18−39, and angiotensin I), and the resulting peptide mass fingerprints were searched against the NCBI protein database using the Profound search engine within RADARS (*16*) (Proteometrics, New York, NY). Data Explorer software (Applied Biosystems) was also used for generating mass lists for identifying smaller fragments of CgA polypeptides by manually matching against tryptic peptides of CgA.

Secretion of Catestatin from Chromaffin Cells: Gel Filtration, Reverse-Phase HPLC, and Mass Spectrometry of Catestatin Peptides. Primary cultures of bovine chromaffin cells were prepared as previously described (*5*, *17*). Secretion was stimulated during a 15 min period by incubating cells in nicotine (10 μM) or 55 mM KCl for depolarization. Catestatin in the secretion media was measured by radioimmunoassay, as previously described (*1*).

For identification of secreted catestatin peptide(s), gel filtration of the secretion media was performed to obtain a pool of low molecular mass catestatin immunoreactivity, utilizing a Superdex 75 HR 10/30 FPLC gel filtration column (10 × 300 mm, 24 mL bed volume; Amersham Pharmacia

Biotech, Piscataway, NJ) equilibrated with 50 mM β,β-dimethylglutaric acid, pH 7.0 (1 mL/min flow rate, 0.5 mL/fraction). Eluted fractions were analyzed for relative polypeptide content by absorbance at 280 nm (A_{280}), lyophilized, and resuspended in 50 mM Tris-HCl, pH 7.5. Fractions corresponding to the elution position of catestatin peptide standard (bovine $CgA_{344-364}$) were collected and pooled for RP-HPLC.

For RP-HPLC, the low molecular mass pool of catestatin immunoreactivity (obtained by gel filtration) was subjected to RP-HPLC on a 25 × 0.5 cm C18 column equilibrated in 0.1% trifluoroacetic acid (TFA) and eluted with a linear 25−40% gradient of acetonitrile in 0.1% TFA over 30 min at 1 mL/min. Elution of peptides was monitored by absorbance at 214 nm, and fractions were collected at 0.5 min intervals (0.5 min = 0.5 mL). An aliquot of each fraction was lyophilized and resuspended in RIA buffer, and catestatin levels were determined by catestatin RIA, as described previously (*1*).

Aliquots from the RP-HPLC fractions (1−2 μL) were analyzed by MALDI-TOF (matrix-assisted laser desorption ionization time of flight) 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 (*14*, *15*, *18*) and then irradiated with a nitrogen laser at 337 nm, and the ions produced were accelerated with a deflection potential of 30000 V. Ions were then differentiated according to their mass/charge ratio (m/z) using a time-of-flight (TOF) mass analyzer. The mass error of this method is characteristically ≤0.1% (*14*, *15*, *18*). To identify methionine-containing peptides by oxidation of methionine to methionine sulfoxide, which adds 16 Da corresponding to the mass of an oxygen atom, samples (10 μL) were oxidized by addition of H_2O_2 to obtain a final concentration of 10 μM H_2O_2. Molecular masses from MALDI-TOF mass spectra were analyzed, and the primary sequence of catatestin-related peptide fragments was assigned by the program PAWS (Protein Analysis WorkSheet, version 8.1.1, for Macintosh; ProteoMetrics; software available from http://prowl.rockefeller.edu), which assigns average isotopic MH^+ values for catestatin-related peptides (*14*, *15*, *18*).

RESULTS

High Molecular Mass Catestatin-Containing Intermediates in Chromaffin Granules. High molecular mass catestatin-containing peptides were detected in isolated chromaffin granules by anti-catestatin immunoblots. Chromaffin granules contained several high molecular mass catestatin-containing polypeptides of ∼66, ∼56, ∼54, and ∼50 kDa, as well as lower molecular mass intermediates of ∼15−35 kDa that were present as soluble components of the granules (Figure 1). Similar forms of these catestatin-containing polypeptides were also detected in membrane fractions of chromaffin granules (data not shown). These high molecular mass catestatin-containing intermediates were secreted from chromaffin cells, demonstrated by their presence in secretion media from cells stimulated by nicotine (10 μM) and by KCl depolarization (Figure 1, lanes 3 and 4). In control, unstimulated cells, secretion of catestatin was not detected. Importantly, the presence of the same high molecular mass forms

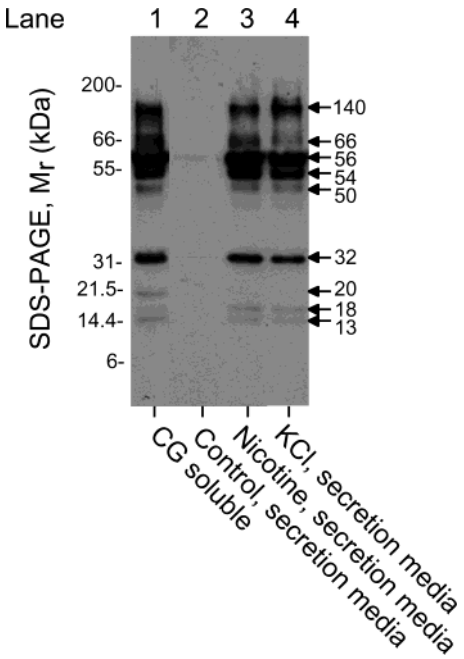

FIGURE 1: High molecular mass catestatin-containing intermediates in chromaffin granules and secretion media of chromaffin cells. Catestatin intermediates in the soluble component of isolated chromaffin granules were detected by anti-catestatin immunoblots (lane 1). Evaluation of catestatin intermediates in secretion media from nicotine and KCl-stimulated chromaffin cells (lanes 3 and 4, respectively), as well as from unstimulated control cells (lane 2), was assessed. Secretion was conducted from chromaffin cells in primary culture at 2 × 10^6 cells/well, with 1 mL of media/well, during a 15 min secretion period; 10 μL aliquots of secretion media were subjected to catestatin immunoblots.

of catestatin-containing peptides in secretion media and in the soluble component of chromaffin granules indicated proportional, all or none (exocytotic) secretion of high molecular mass catestatins from these granules. Thus, the catestatin-containing polypeptides of 50−66 and 15−35 kDa are present within chromaffin granules that store and secrete catestatin-related peptides.

It is noted that a high molecular mass form of catestatin immunoreactivity of ∼140 kDa was observed, suggesting aggregation of chromogranin peptides, which has been reported (*19*, *20*). Alternatively, the high molecular mass CgA form(s) could simply represent the CgA-core proteoglycan of chromaffin granules, as previously characterized (*21*).

NH_2-Terminal Peptide Sequencing of Catestatin-Containing Intermediates. The identities of the high molecular mass catestatin-containing peptides were determined by NH_2-terminal peptide sequencing, combined with MALDI-TOF mass spectrometry of tryptic digests. High molecular mass catestatin-related peptides were separated by SDS−PAGE, electrophoretically transferred to PVDF membranes, and then subjected to Edman degradation for NH_2-terminal peptide sequencing. Results (Table 1) indicated that the ∼66 kDa band contained the NH_2 terminus of CgA represented by the sequence LPVNSPMNKG that represents the NH_2 terminus of the CgA precursor (residues 1−10 of CgA). The ∼54−56 kDa catestatin-containing band(s) contained the NH_2-terminal sequences of both LPVNSPMNKG (residues 1−10) and HSSYEDELSE (residues 79−88, resulting from cleavage after $Lys_{77}Lys_{78}\downarrow$), which indicated the presence of two polypeptides. It was noted that to obtain enough material on

Table 1: Identification of Bovine Catestatin-Containing Polypeptides by NH_2-Terminal Edman Sequencing and MALDI-TOF Mass Spectrometry of Tryptic Peptides[a]

determined apparent molecular mass (kDa)	Edman sequencing	detection of $CgA_{421-430}$ COOH-terminal tryptic peptide by MALDI-TOF	peptide sequence
66	$LPVNSPMNKG^{1-10}$	+	1−430(431)
54−56	$HSSYEDELSE^{79-88}$	+	79−430(431)
	$LPVNSPMNKG^{1-10}$		1−353 or 1−364 (estd)
50	$DDFKEVEKSD^{116-125}$	+	116−430(431)
	$LPVNSPMNKG^{1-10}$		1−318 (estd)
32	$AAPGWPEDGA^{248-257}$	+	248−430(431)
17	$LEGEEEEEED^{332-341}$	+	332−430(431)

[a] Catestatin-containing intermediates were present in chromaffin granules and secretion media (Figure 1). These catestatin-related peptides were subjected to primary sequence analyses by Edman sequencing and MALDI-TOF MS of tryptic digests. It was noted that the ∼54 and ∼56 kDa bands migrated closely on SDS−PAGE gels and, therefore, are referred to as a ∼54−56 kDa band for sequencing studies. The ∼17 kDa band was enriched by a two-dimensional gel to provide adequate amounts of protein for peptide sequencing. Peptide sequencing results are expressed as fragments of intact bovine CgA (residues 1−431). In MALDI-TOF MS experiments, detection of the COOH-terminal tryptic fragment (residues 421−430) is indicated. Since trypsin cleaves between $Arg_{430}\downarrow Gly_{431}$, the presence of Gly_{431} was not experimentally observed, but inferred, since Gly_{431} may be part of the peptide in vivo. Therefore, the deduced COOH terminus may include residue 431 in vivo and is therefore indicated as 430(431). In addition, the COOH termini of 1−353 (or 1−364) and 1−318 are estimated (estd) on the basis of the presence of the NH_2-terminus (residues 1−10), apparent molecular mass on SDS−PAGE, and observed full-length CgA precursor of ∼66 kDa.

the gel for peptide sequencing, the ∼54−56 kDa band appeared merged, compared to distinct ∼54 and ∼56 kDa bands detected with lower amounts of granule sample. The NH_2 terminus of the ∼50 kDa fragment contained the sequences of LPVNSPMNKG (residues 1−10) and DDFKEVEKSD (residues 116−125; with cleavage after $Lys_{114}Arg_{115}\downarrow$), which indicates two polypeptides. The ∼32 kDa band contained the sequence AAPGWPEDGA (residues 248−257, with cleavage at $Arg_{247}\downarrow$) as its NH_2 terminus, and the 17 kDa band contained LEGEEEEEED (residues 332−341, resulting from cleavage at $Lys_{330}Arg_{331}\downarrow$) as its NH_2 terminus. These results indicated that CgA_{1-431} undergoes proteolytic processing at paired basic and monobasic sites consisting of $Lys_{77}Lys_{78}\downarrow$, $Lys_{114}Arg_{115}\downarrow$, $Arg_{247}\downarrow$, and Lys_{330}-$Arg_{331}\downarrow$.

MALDI-TOF Mass Spectrometry of Tryptic Digests of Catestatin-Containing Intermediates. To further characterize primary structures of catestatin-containing peptides and establish carboxy-terminal sequences, mass spectrometry of tryptic digests was performed. Catestatin intermediates were isolated by SDS−PAGE gels and excised for tryptic digestion for MALDI-TOF mass spectrometry (MALDI-TOF MS). A representative profile of tryptic peptides obtained from the ∼54−56 kDa catestatin band illustrates detection of multiple peptides (Figure 2) derived from CgA. The observed masses of tryptic fragments corresponded with their theoretical masses (Table 2). Importantly, detection of the tryptic peptide at *m*/*z* 1250.8 corresponds to residues 421−430 at the COOH terminus of the CgA precursor. MALDI-TOF MS of tryptic digests of the ∼66, ∼50, ∼32, and ∼17 kDa catestatin-containing bands demonstrated that each of these bands contained the CgA COOH-terminal tryptic peptide (residues 421−430) (Table 1).

Edman Peptide Sequencing and MALDI-TOF MS Define the Predicted Primary Structures of Catestatin-Containing Intermediates. The combined Edman sequencing and MALDI-TOF MS analyses provided predictions of the primary structures of the soluble, secreted catestatin-containing intermediates (Figure 3). The 66 kDa band represents full-length CgA (residues 1−430/431) that contains the NH_2 terminus of LPVNSPMNKG and the COOH-terminal tryptic

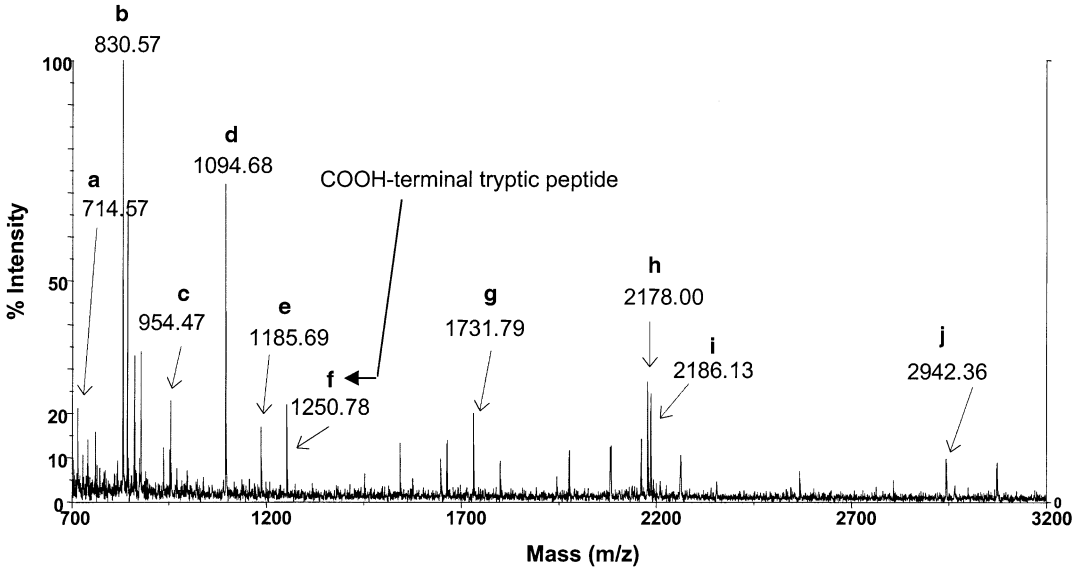

FIGURE 2: MALDI-TOF mass spectrometry (MS) of tryptic peptides derived from high molecular mass catestatin intermediates. MALDI-TOF mass spectrometry of tryptic peptides derived from high molecular mass catestatin-containing peptides detected numerous CgA-derived peptides. A representative MALDI-TOF MS profile of tryptic peptides obtained from the ∼54−56 kDa catestatin immunoreactive band (from immunoblots of the chromaffin granule soluble fraction) is illustrated with masses corresponding to MH^+ ion species. The observed and theoretical masses of CgA-derived tryptic peptides (a−j) are described in Table 2. Notably, the COOH-terminal tryptic fragment of CgA ($CgA_{421-430}$), detected as a peptide with *m*/*z* of 1250.78, indicates the presence of the COOH terminus of the CgA precursor.

Table 2: MALDI-TOF Mass Spectrometry of Tryptic Peptide Fragments of a High Molecular Mass Catestatin-Containing Peptide of ~54−56 kDa[a]

peptide peak	obsd mass	calcd mass	residues of CgA^{1-431}
a	714.57	714.49	48−53
b	830.58	830.49	287−293
c	954.48	954.44	37−43
d	1094.68	1094.60	421−429
e	1185.69	1185.65	60−70
f	1250.78	1250.70	421−430*
g	1731.79	1731.73	331−344
h	2178.00	2177.96	203−219
i	2186.14	2186.08	402−430
j	2942.37	2942.37	145−172

[a] Tryptic peptide fragments derived from the ~54−56 kDa catestatin-containing peptide were identified by MALDI-TOF mass spectrometry as peptide fragments of bovine chromogranin A (CgA). The observed and calculated masses of tryptic peptides are illustrated with their corresponding peptide sequences of bovine CgA (1−431). The COOH-terminal tryptic peptide of CgA corresponds to fragment 421−430 (*). Similar tryptic peptide fragments derived from other high molecular mass catestatin-containing peptides were also detected by MALDI-TOF MS.

fragment of CgA. It is noted that the 66 kDa band may contain the COOH-terminal residue 431 in vivo, but it was not actually detected by mass spectrometry since trypsin cleaves at the NH_2-terminal side of the basic Arg residue at position 430. Therefore, the identity of the ~66 kDa band is designated as 1−430(431) of CgA in this study.

The ~54−56 kDa band included two catestatin-containing fragments. One fragment consists of residues 79−430(431) of CgA, based on the presence of the NH_2-terminal sequence of HSSYEDELSE and the COOH-terminal tryptic peptide, $CgA_{421-430}$. The other 54−56 kDa catestatin-containing fragment begins with the NH_2 terminus of CgA represented by the sequence LPVNSPMNKG and may possess a COOH truncation near residue 353 or 364, predicted by its apparent molecular mass of ~54−56 kDa and the observed apparent molecular mass of the CgA precursor of 66 kDa, as well as basic residue processing sites within CgA.

The ~50 kDa band included two catestatin-containing fragments. One fragment represents residues 116−430(431), based on Edman sequencing and the detection of the COOH-terminal tryptic peptide of CgA by MALDI-TOF MS. A second predicted fragment contains the NH_2 terminus of CgA (LPVNSPMNKG) and a truncated COOH terminus that may be estimated near residue 318, representing predicted processing at a monobasic site.

These results demonstrate that catestatin-containing intermediates are generated by proteolytic processing at the COOH-terminal sides of paired basic and monobasic processing sites of CgA.

Identification of Secreted Catestatin Peptides Utilizing RP-HPLC and MALDI-TOF Mass Spectrometry. Catestatin peptides have been identified in isolated chromaffin granules (*1*), and its stimulated secretion is required in support of its predicted role as an extracellular, autocrine regulator of catecholamine release. However, stimulated secretion of the active form of catestatin has not been demonstrated. Therefore, catestatin peptide(s) in secretion media was (were) positively identified in this study utilizing RP-HPLC for isolation and MALDI-TOF MS for identification.

Catestatin peptides in secretion media from chromaffin cells stimulated by KCl depolarization were size-fractionated by gel filtration to obtain a pool of low molecular mass fractions that coelute with synthetic catestatin $CgA_{344-364}$. This low molecular mass pool was subjected to RP-HPLC that monitored absorbance of eluted peptides at 214 nm (Figure 4a), and eluted fractions were analyzed by RIA for catestatin, $CgA_{344-364}$ (Figure 4b). Fractions 20 and 21 contained catestatin immunoreactivity that coeluted with synthetic $CgA_{344-364}$ and were therfore analyzed by MALDI-TOF mass spectrometry (MS). In both fraction 20 and

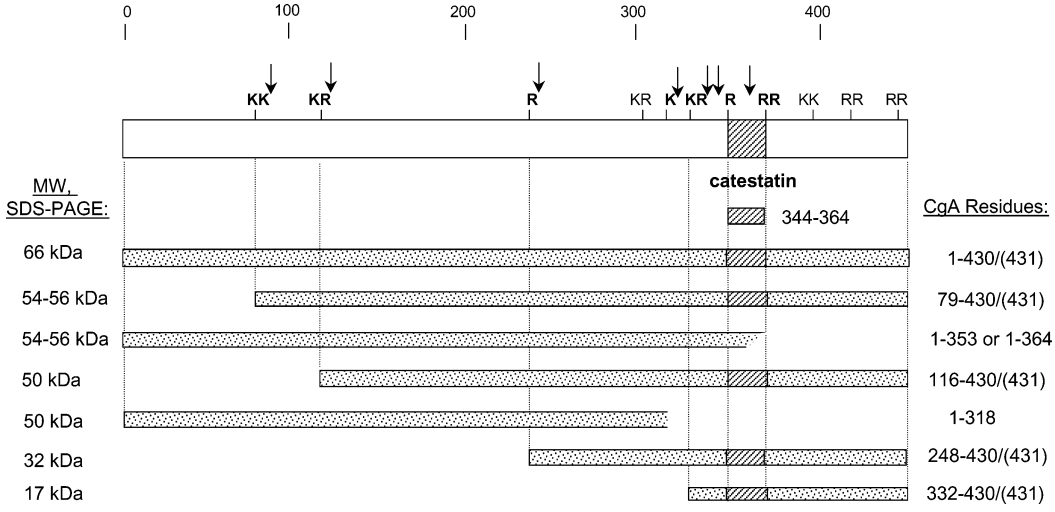

FIGURE 3: High molecular mass catestatin intermediates derived from bovine chromogranin A. High molecular mass catestatin-containing peptides in chromaffin granules were identified by NH_2-terminal Edman sequencing and MALDI-TOF MS of tryptic digests. High molecular mass forms of catestatin-related peptides were demonstrated as (i) full-length CgA, residues 1−430(431), (ii) NH_2-terminally truncated forms of catestatin-related peptides consisting of residues 79−430(431), 116−430(431), 248−430(431), and 332−430(431), and (iii) COOH-terminal truncated forms predicted as 1−353 or 1−364 and 1−318. Arrows indicate cleavage of CgA at dibasic and monobasic residues (bold letters). Chromaffin granules contain active catestatin peptide, $CgA_{344-364}$, that inhibits nicotinic-stimulated catecholamine release (*1*). It is noted that the COOH-terminal tryptic fragment, $CgA_{421-430}$, does not contain residue 431, but residue 431 may exist as part of the peptide sequence in vivo (prior to in vitro trypsin cleavage). Therefore, the COOH-terminal residue(s) of CgA fragments is (are) illustrated as terminating at residue 430, with the possibility that residue 431 was present in vivo, designated as 430(431).

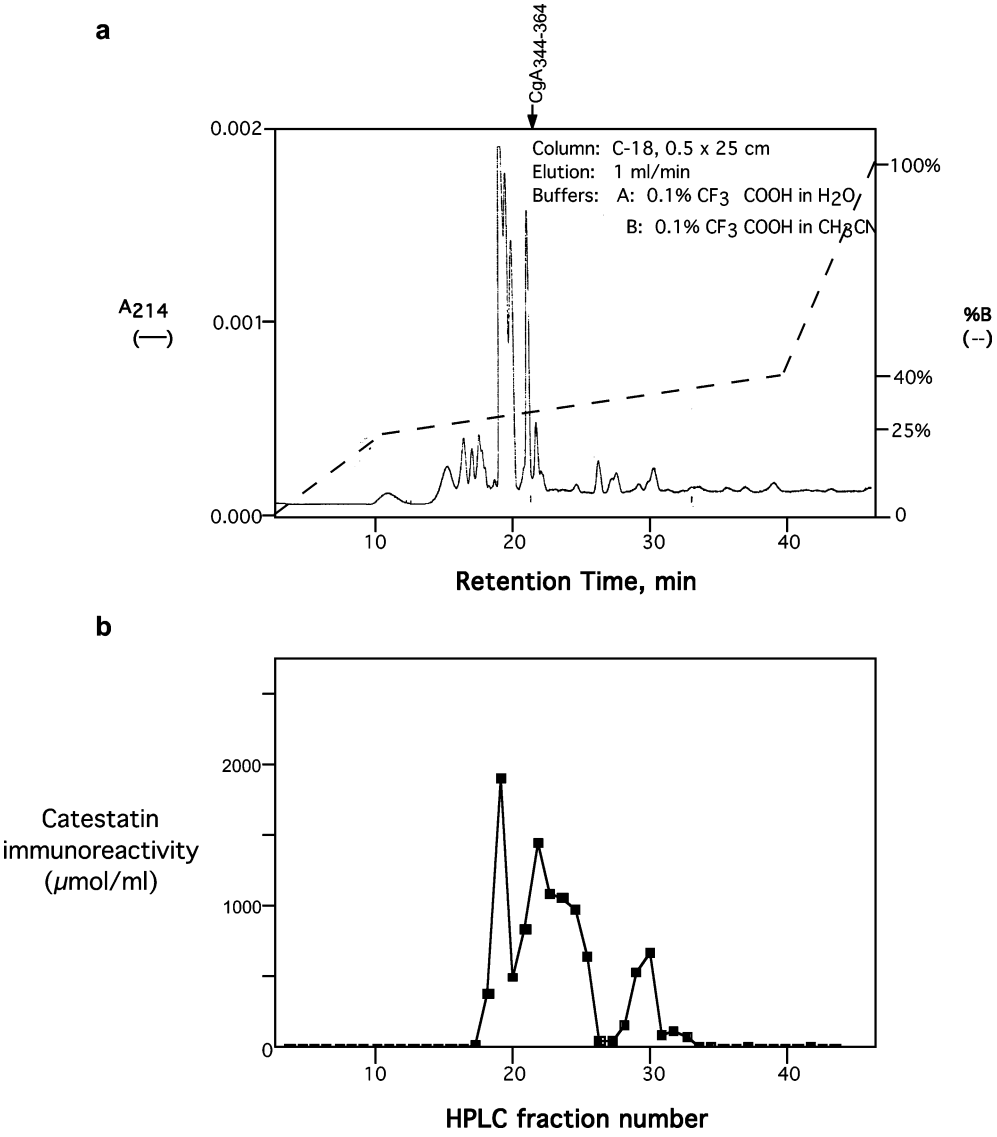

FIGURE 4: RP-HPLC of catestatin peptide immunoreactivity secreted from chromaffin cells. (a) RP-HPLC of the low molecular mass pool of secretion media. The low molecular mass pool of catestatin immunoreactivity (1−3 kDa) obtained by gel filtration of secretion media of chromaffin cells stimulated by KCl was subjected to chromatography on a 0.5 × 25 cm C18 RP-HPLC column, with elution of peptides monitored by absorbance at 214 nm (A_{214}). The standard elution position of synthetic catestatin peptide, bovine $CgA_{344-364}$, is shown by the arrow. (b) RP-HPLC of catestatin immunoreactivity. The content of catestatin immunoreactivity ($CgA_{344-364}$) in eluted fractions from the C18 RP-HPLC column was measured by RIA. Results are expressed as μmol/mL, with 1 mL per fraction.

fraction 21, MALDI-TOF MS revealed a peak with m/z = 2426 [Figure 5, peak a in A(i) and B(i)], which is consistent with the calculated mass of catestatin corresponding to $CgA_{344-364}$ (RSMRLSFRARGYGFRGPGLQL; calculated m/z = 2425.8).

Prior to RP-HPLC, these catestatin-containing samples were also oxidized with H_2O_2 to convert methionine residues into methionine sulfoxide. Catestatin contains a single methionine residue (bovine CgA-Met-346) whose mass is expected to increase by 16 Da upon oxidation. In both samples after oxidation, the peak at m/z = 2426 disappeared, and peaks at m/z = 2443−2444 appeared [Figure 5, peak a in A(ii) and B(ii)]. These shifts in masses correspond to addition of oxygen (16 Da) as Met-346 sulfoxide.

Fraction 20 also contained two lower molecular mass peaks at m/z = 3098 and 3473 [Figure 5, peaks b and c in A(i)] that disappeared upon oxidation, with appearance of peaks at m/z = 3115 and 3492 [Figure 5, peaks b and c in A(ii)]. The masses of these peaks are consistent with two extended catestatin forms consisting of $CgA_{343-368}$ (DRSMRLSFRARGYGFRGPGLQLRRGW; calculated m/z = 3096.6) and $CgA_{332-361}$ (LEGEEEEEEDPDRSMRLSFRARGYGFRGPG; calculated m/z = 3472.7), respectively (Table 3).

These peptide sequencing results indicated that secreted catestatin, $CgA_{344-364}$, is generated by proteolytic processing at the NH_2-terminal sides of $\downarrow Arg_{344}$ and $\downarrow Arg_{365}Arg_{366}$ cleavage sites (Figure 6). These cleavage specificities for the NH_2-terminal side of the dibasic or monobasic cleavage sites are distinct from the more usual prohormone processing sites at the COOH-terminal sides of paired basic or monobasic residues (*8*−*11*). The variant form of catestatin ($CgA_{344-364}$) represented by $CgA_{343-368}$ may be generated by cleavage at $Pro_{342}\downarrow Asp_{343}$ and $Trp_{368}\downarrow Arg_{369}$; the $CgA_{332-361}$ catestatin form may be generated by cleavage at $Arg_{331}\downarrow Leu_{332}$ and $Gly_{361}\downarrow Leu_{362}$ (Figure 6). Thus, the production of catestatin and variant catestatin peptides may involve proteolytic cleavage of CgA at basic residues, as well as at nonbasic residue sites.

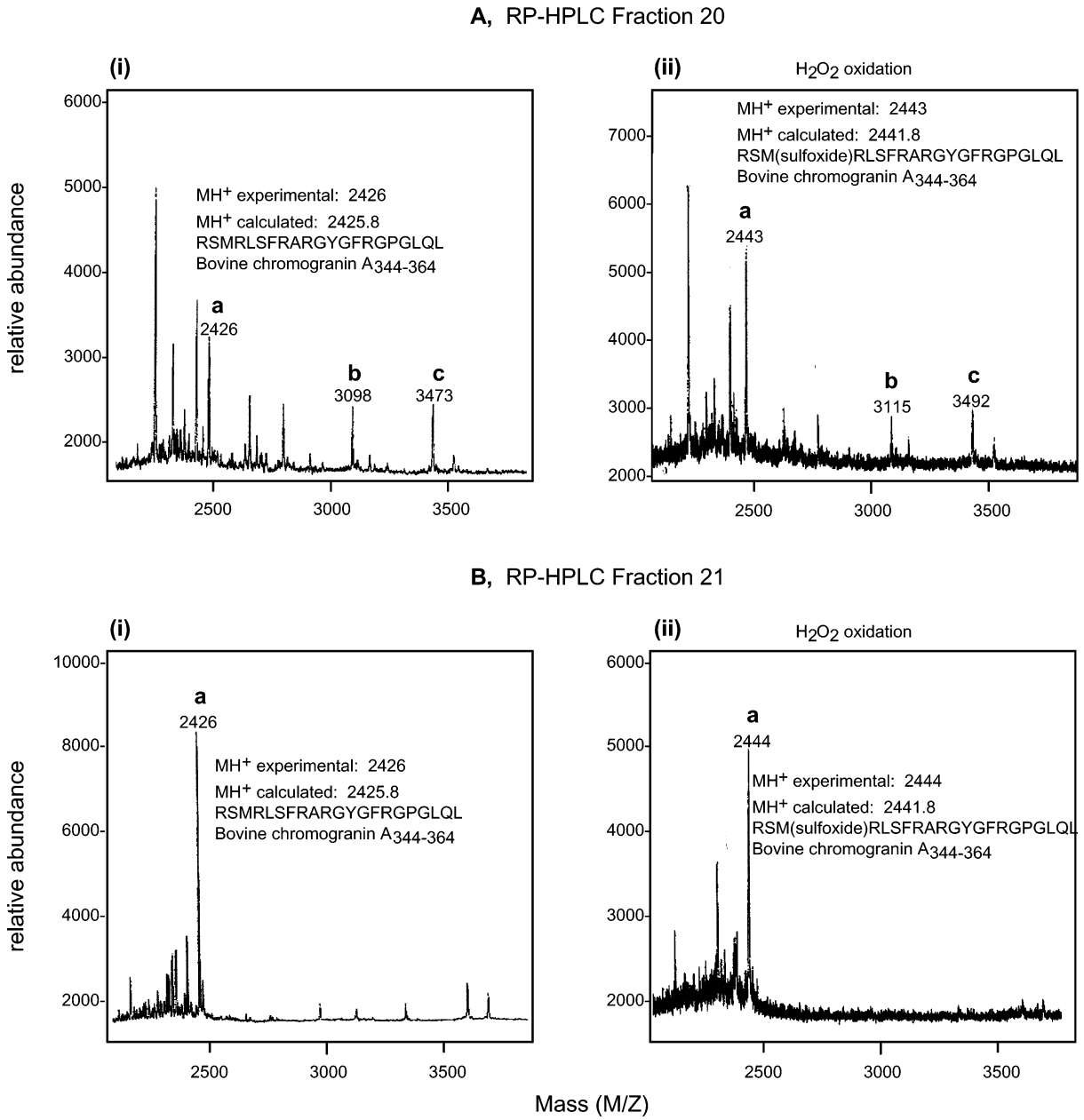

FIGURE 5: MALDI-TOF mass spectrometry of secreted catestatin peptides. Eluted RP-HPLC fractions 20 and 21 (from Figure 4), which coelute with synthetic bovine $CgA_{344-364}$, were subjected to MALDI-TOF mass spectrometry. (A) Mass spectrometry of fraction 20 from RP-HPLC. Mass spectrometry indicated the presence of peaks a, b, and c. Masses of these peaks were analyzed without oxidation (i) and after oxidation with hydrogen peroxide (H_2O_2) (ii). (B) Mass spectrometry of fraction 21 from RP-HPLC. Mass spectrometry indicated the presence of peak a, which was analyzed without oxidation (i) and after oxidation by hydrogen peroxide (ii).

DISCUSSION

The biosynthesis of the biologically active catestatin peptide ($CgA_{344-364}$) requires proteolytic processing of its CgA precursor within secretory vesicles of neuroendocrine chromaffin cells (chromaffin granules). To define the proteolytic cleavage sites required in the production of active catestatin peptide in this study, primary sequence characterization of catestatin-containing intermediates and secreted catestatin peptides was achieved by NH_2-terminal peptide sequencing combined with MALDI-TOF mass spectrometry. High molecular mass catestatin intermediates of 54−56, 50, 32, and 17 kDa contained NH_2-terminal peptide sequences that indicated cleavage of the CgA precursor at KK↓, KR↓, R↓, and KR↓ basic residue sites, respectively; these intermediates contained the COOH terminus of CgA. Production of these catestatin intermediates by cleavage at the COOH-terminal sides of paired basic residues is consistent with the cleavage specificities of the subtilisin-like family of processing proteases that include PC1 and PC2 that are present in chromaffin granules (*8*−*11*, *22*). The secreted catestatin was identified by MALDI-TOF MS as $CgA_{344-364}$, which indicates utilization of unique cleavage sites at the NH_2-terminal sides of ↓R and ↓RR basic residue sites of CgA. These cleavage sites at the NH_2-terminal sides of dibasic and monobasic residues are similar to those utilized by the proenkephalin cleaving cysteine protease known as "prohormone thiol protease" (PTP) present in chromaffin granules (*23*−*25*). Secretion of catestatin from chromaffin cells was accompanied by the cosecretion of high molecular mass catestatin intermediates. In addition, secretion of related

FIGURE 6: Secreted catestatin peptide forms derived from chromogranin A. Secreted catestatin peptides derived from cleavage of bovine CgA are illustrated. Mass spectrometry demonstrated that secreted catestatin corresponded to $CgA_{344-364}$. Also, variant forms of catestatin were identified as $CgA_{343-368}$ and $CgA_{332-361}$. Predicted cleavages within CgA to generate the identified catestatin peptides are illustrated by arrows.

Table 3: Secreted Catestatin Peptides Identified by MALDI-TOF Mass Spectrometry[a]

HPLC fraction no.	mass (Da) without oxidation obsd mass	mass (Da) without oxidation calcd mass	mass (Da) after H_2O_2 oxidation obsd mass	mass (Da) after H_2O_2 oxidation calcd mass
fraction 20				
peak a, bCgA 344−364	2426	2425.8	2443	2441.8
peak b, bCgA 343−368	3098	3096.6	3115	3112.6
peak c, bCgA 332−361	3473	3472.7	3492	3488.7
fraction 21				
peak a, bCgA 344−364	2426	2425.8	2444	2441.8

[a] Comparison of the masses of peptides identified by MALDI-TOF mass spectrometry in bovine chromaffin cell secretion media with calculated masses of catestatin-related peptides derived from bovine chromogranin A (bCgA). In vitro diagnostic oxidation of Met^{346} to Met^{346} sulfoxide (adding 16 Da) was accomplished by H_2O_2.

peptide forms of catestatin, $CgA_{343-368}$ and $CgA_{332-361}$, were also identified. These results have defined the primary sequence characteristics of high molecular mass catestatin intermediates in secretory vesicles, which have indicated the proteolytic cleavage sites of CgA utilized in the production of active, secreted catestatin that regulates nicotine-stimulated catecholamine release.

Identification of NH_2- and COOH-terminal peptide sequences of catestatin intermediates was important for defining the identities of the high molecular mass catestatin intermediates. Direct NH_2-terminal peptide sequencing defined the NH_2 termini of these intermediates (Table 1). Results indicated proteolytic processing of CgA at paired basic and monobasic sites consisting of $Lys_{77}Lys_{78}\downarrow$, $Lys_{114}Arg_{115}\downarrow$, $Arg_{247}\downarrow$, and $Lys_{330}Arg_{331}\downarrow$. These sites represent typical prohormone processing sites at basic residues (*8*−*11*, *26*). Identification of the COOH termini of the catestatin-containing intermediates was achieved by MALDI-TOF mass spectrometry, which detected the presence of the COOH-terminal tryptic peptide of CgA corresponding to $CgA_{421-430}$. In addition, COOH-terminally truncated forms of CgA of ∼54−56 and ∼50 kDa catestatin forms were identified. These peptide sequence analyses of catestatin-containing polypeptides provided evidence for proteolytic processing of full-length CgA at amino- and carboxyl-terminal domains, demonstrating bidirectional processing of CgA (*21*, *27*) for the production of active catestatin.

The soluble high molecular mass catestatin intermediates identified in chromaffin granules were cosecreted with catestatin peptides from chromaffin cells. The secretion media contained full-length CgA of ∼66 kDa, and high molecular mass catestatin intermediates of ∼56, 54, and ∼50 kDa with several less abundant catestatin-containing polypeptides of ∼15−35 kDa. Thus, secretion of several forms of catestatin present in secretory vesicles demonstrates the coordinate storage and secretion of a family of high molecular mass catestatin intermediates and catestatin-related peptides.

Importantly, results demonstrated stimulated secretion of the active catestatin peptide, bovine $CgA_{344-364}$, identified by MALDI-TOF mass spectrometry. The $CgA_{344-364}$ form of catestatin results from proteolytic processing of CgA at $\downarrow Arg_{343}$ and $\downarrow Arg_{366}Arg_{367}$, which indicates unique cleavages at the NH_2-terminal sides of dibasic and monobasic sites. Cleavages at these sites could be achieved by the cysteine protease known as prohormone thiol protease (PTP) that participates in proenkephalin processing in chromaffin granules (*23*−*25*). The cleavage specificity of PTP differs somewhat from that of the subtilisin-like PC1 and PC2 enzymes (PC = prohormone convertase) (*8*−*11*) and the aspartyl protease PCE (POMC converting enzyme) (*11*, *28*−*30*), which show preferences for cleavages at the COOH-terminal side of paired basic residues. The predicted cleavage sites necessary for production of catestatin, $CgA_{344-364}$, suggest involvement of PTP as a candidate processing enzyme for producing catestatin.

In addition to $CgA_{344-364}$, related peptide forms of catestatin were also secreted, identified as bovine $CgA_{332-361}$ and $CgA_{343-368}$ by MALDI-TOF MS. These secreted forms of catestatin-related peptides contain the core sequence (residues 344−358) that is known to be sufficient for biological activity (*3*). It will be of interest in future studies to evaluate the relative biological activities of these catestatin peptides for modulating catecholamine release.

It is noted that the secreted forms of catestatin identified in this study differ from those previously detected in purified chromaffin granules. Chromaffin granules represent regulated secretory vesicles of bovine chromaffin cells (*1*). It must be recognized that isolated chromaffin granules from bovine adrenal medulla tissue provide a population of immature, mature, and aged secretory vesicles that contain both peptide precursors such as chromogranin A and proenkephalin and small biologically active peptides that include catestatin and (Met)enkephalin (*8*, *17*). However, the secreted catestatin peptides represent only those peptides secreted from the pool of mature secretory vesicles that are designated for regulated

secretion. Recent studies demonstrate that chromaffin cells contain different vesicle pools, and each pool undergoes selective and differential release by different secretagogues (*34*). The specific pools of these secretory vesicles that contain the different forms of secreted catestatin peptides identified in this study, compared to forms of catestatin peptides found in isolated chromaffin granules (*1*), are not yet known. Importantly, however, results from this study demonstrate the specific forms of active catestatin peptides that are secreted in a regulated manner from chromaffin cells.

In summary, primary sequence characterization by NH_2-terminal sequencing and MALDI-TOF mass spectrometry have defined the cleavage sites within CgA that are utilized to generate high molecular mass catestatin intermediates and active catestatin peptides. It is predicted that cleavage at the COOH-terminal sides of paired basic residues are utilized for generating catestatin intermediates. These cleavage sites are compatible with the specificities of PC1 and PC2 processing enzymes (*8*−*11*, *22*). PC1 and PC2 have been implicated in proteolysis of CgA (*31*−*33*). However, production of catestatin ($CgA_{344-364}$) by cleavage of CgA at the NH_2-terminal sides of $\downarrow Arg_{344}$ and $\downarrow Arg_{365}Arg_{366}$ residues is compatible with the cleavage specificities of PTP as a candidate processing enzyme that cleaves at the NH_2-terminal side of basic residues of proenkephalin (*23*−*25*). It is predicted that catestatin in other neuroendocrine tissues may be generated in similar fashion and may be investigated in future studies. Results from this study have provided knowledge of the high molecular mass catestatin intermediates and CgA cleavage sites that are involved in the biosynthesis of secreted catestatin that regulates nicotinic-stimulated catecholamine release.

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