Antibacterial Activity of Glycosylated and Phosphorylated Chromogranin A-derived Peptide 173–194 from Bovine Adrenal Medullary Chromaffin Granules*

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Recently, we have isolated from bovine chromaffin granules and identified two natural peptides possessing antibacterial activity: secretolytin (chromogranin B 614–626) and enkelyn (proenkephalin-A 209–237). Here, we characterize a large natural fragment, corresponding to chromogranin A 79–431, that inhibits growth of both Gram-positive and Gram-negative bacteria. The aim of the present work was to determine the shortest active peptide located in the 79–431 chromogranin A region. Three peptides, which shared the same 173–194 chromogranin A sequence (YPGPQAKED-SEGPSQGPASREK) but differed in post-translational modifications, including O-glycosylation and tyrosine phosphorylation, were isolated. A detailed study using microsequencing and mass spectrometry allowed us to correlate their antibacterial activity with these post-translational modifications. The chromogranin A precursor fragment (79–431) and the active glycosylated and phosphorylated peptides were, respectively, named prochromacin and chromacin (P, G, and PG for phosphorylated, glycosylated, and phosphorylated-glycosylated form).

Secretory granules from bovine adrenal medullary chromaffin cells contain a complex mixture of secretory products that include low molecular mass constituents such as catecholamines, ascorbate, nucleotides, calcium, enkephalins, and several water-soluble proteins. Among the latter, dopamine-cholamines, ascorbate, nucleotides, calcium, enkephalins, and phosphorylated, glycosylated, and phosphorylated peptides were, respectively, named prochromacin and chromacin. Several of these CGA-derived peptides act predominantly as inhibitors of hormone and neurotransmitter release, either as autocrine or paracrine controls. For example, CGA is the precursor of pancreastatin (248–293), a peptide that negatively modulates insulin secretion from endocrine pancreatic islets (7, 8), amylase release from exocrine pancreas (9), and acid secretion from parietal cells (10). Another CGA-derived peptide, parastatin (347–419), inhibits parathyroid cell secretion (11). As early as 1988, it was established that CGA is the precursor of a peptide that inhibits the activity of chromaffin cells (12). More recently, a peptide corresponding to the sequence 1–113 has been shown to inhibit hormone secretion in the bovine parathyroid gland (13); a homologous peptide named betagamin corresponding to the sequence 1–115 has been isolated from rat pancreas, but its function has not yet been defined (14). Peptides containing the N-terminal domain (1–76), vasostatins, have been characterized in bovine adrenal medulla (15) and have been found to exhibit vasoinhibitory activity of isolated human blood vessels (16, 17).

Recently, we have shown that peptides with antibacterial activity are present as water-soluble components of bovine chromaffin granules and are released during secretion. In material released from stimulated cultured chromaffin cells, we have identified secretolytin (18, 19), a peptide corresponding to the C-terminal sequence (614–626) of bovine chromogranin B. A second antibacterial peptide, enkelyn (20), derived from proenkephalin (200–237), was then identified among the nucoglycin-labeled A. caudatus; MAA, digoxigenin-labeled M. amuren- s; PNA, digoxigenin-labeled A. hypogaea agglutinin; SNA-dig, digoxigenin-labeled S. nigra agglutinin; Sg, secretogranin.

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merorous peptides present in the intragranular matrix. As a continuation of these studies, the present paper reports the antibacterial activity of a large CGA fragment (residues 79–431) generated by natural cleavage at the previously described site 78–79 (6). This CGA fragment was detectable in the intragranular matrix, was released during exocytosis and furthermore increased the growth of both Gram-positive (*Micrococcus luteus*) and Gram-negative (*Escherichia coli*) bacteria.

In view of the large size of this antibacterial CGA fragment, the aim of the present paper was to characterize the shortest active peptide derived from this fragment. After proteolysis of the whole CGA molecule, active peptides located in the 79–431 region were analyzed using a combination of microsequencing and mass spectrometry. We isolated several peptides that shared the same 173–194 sequence but differed by glycosylation and phosphorylation modifications. Their sequence has no homology with the previously described sequences of antibacterial peptides. Structural features and more particularly post-translational modifications are discussed in relation with their antibacterial activity.

**EXPERIMENTAL PROCEDURES**

**Purification of CGA-derived Peptides 173–194**—Secretory granules were isolated from bovine adrenal medulla (21), and soluble proteins were separated from membranes after lysis and centrifugation (22). CGA was purified by HPLC on a Macherey Nagel Nucleosil 300–5C18 column (4 × 250 mm; particle size: 5 μm and pore size: 100 nm) with the Applied Biosystems HPLC system 140 B as described previously (18).

Then, CGA (10 nmol) was digested for 2 h at 37°C with endoproteinase Lys-C at a protein-to-proteinase weight ratio of 1000:1 in 100 mM Tris-HCl, pH 8.3. Generated peptides were then separated on a Macherey Nagel 300–5C18 column. Absorbance was monitored at 214 nm, and the solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid, 30% water, 69.9% acetonitrile (solvent B). Material was eluted at a flow rate of 0.7 ml/min followed by a gradient of 25–75% over 50 min. Each peak fraction was manually collected and concentrated by evaporation, but not to dryness.

**Sequence Analysis**—The sequence of purified CGA-derived peptides was determined in our laboratory by automatic Edman degradation on an Applied Biosystems 473 A mass spectrometer. Samples (100 pmol) were loaded onto a polyethylene terephthalate and precycled glass-fiber filters (6). In order to identify phosphorylated residues, samples were modified with ethanolamine according to the method previously described (23). Before sequencing, reagents were removed, using the ProSpin sample preparation cartridge (Applied Biosystems, a division of Perkin-Elmer), and the modified peptide was loaded on the ProBlott polyvinylidene difluoride membrane (Applied Biosystems).

**Mass Spectrometry Analysis**—Determination of mass was carried out on a Bruker BIFLEX™ matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF) equipped with the SCOUT™ high resolution optics with X-Y multisample probe, a gridless reflector and the HIMAS™ linear detector. This instrument has a maximum accelerating potential of 30 kV and may be operated either in the linear or reflector mode. Ionization was accomplished with a 335-nm beam from a nitrogen laser with a repetition rate of 5 Hz. The output signal from the detector was digitized at a sampling rate of 250 MHz in linear mode and 500 MHz in reflector mode using a 1-GHz digital oscilloscope (Lecroy model). The instrument control and data processing were accomplished with software supplied by Bruker using a Sun Spare workstation. These studies were realized using as the matrix α-cyano-4-hydroxy-cinnamic acid obtained from Sigma and prepared as a saturated solution in acetone. Aliquots (1–2 μl) of the sample matrix solution were deposited onto probe tips and dried by ambient air. After fast spreading and fast evaporation of the solvent, we obtained a thin layer of matrix crystals (24, 25). A micromolar analyte solution was applied to the matrix and allowed to dry under moderate vacuum. This preparation was washed by applying 1 μl of a 0.5% trifluoroacetic acid in water solution and then flushed after a few seconds. This cleaning procedure often allows an increase in sensitivity and mass accuracy by removing the remaining alkali cations.

**Molar Carbohydrate Composition**—Carbohydrate analysis was performed using gas chromatography with a silicone OV 101 capillary column (0.52 mm x 25 m). Samples were analyzed after methanolysis (0.5 ml hydrochloric acid-methanol for 24 h at 80°C), N-acetylation, and methylation (26).

**Electrophoresis and Blotting with Digoxigenin-labeled Lectins**—Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed under reducing conditions on 4–20% gradient polyacrylamide gels (28) using 10 μg of protein/lane. After migration, proteins were transferred to a nitrocellulose membrane (29). The different lanes were then separated and treated with polyclonal antirabbit IgG (2% in Tris-buffered saline, TBS). Desialylation of one-half of the lanes was performed by treatment with 50 mM fluoride of sialidase from Clostridium perfringens in 50 mM citrate buffer, pH 6.0, 0.9% NaCl, 0.1% CaCl2 for 16 h at 37°C prior incubation with the digoxigenin-labeled lectins. Two lanes (desialylated and not desialylated) were incubated with each lectin: *Amaranthus caudatus* (ACA-dig, 2.5 μg/ml in TBS), *Maackia amurensis* (MAA-dig, 5 μg/ml in TBS), *Arachis hypogaea* agglutinin (PNA-dig, 2 μg/ml in TBS), and *Stemona naga* agglutinin (SNA-dig, 2 μg/ml in TBS). Then, the nitrocellulose strips were incubated for 1 h with anti-digoxigenin alkaline phosphatase-labeled Fab fragments (1 μg/ml in TBS). After washing, labeled glycoproteins were revealed by 4-nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate staining.

**Antibacterial Assays**—Bacteria were grown aerobically at 37°C in yeast extract-free Luria-Bertani medium (1% Bactotryptone and 0.5% NaCl (m/v), pH 7.5). Antimicrobial activity was determined by measuring growth inhibition of *M. luteus* (strain A270), *Bacillus megaterium* (strain MA) and *E. coli* (strain D22) in Luria-Bertani medium (30). Aliquots of peptide extract (200 pmol, 10 μl) were incubated in microtiter plates with 100 μl of a midlogarithmic phase culture of bacteria with starting absorbance at 620 nm of 0.001. Microbial growth was assessed by the increase of OD620 nm after 16 h of incubation at 37°C (*M. luteus* and *E. coli*) or 20°C (*B. megaterium*). The A620 nm value of control cultures grown in the absence of peptide (10 μl of water in place of peptide solution) was taken as 100%.

**Lysis of Erythrocytes**—Bovine erythrocytes were isolated and the buffy coat removed by centrifugation of freshly collected blood at 1000 × g for 5 min and washing three times with 10 ml sodium phosphate, pH 7.5, containing 0.9% NaCl (NaClIP). Erythrocytes (45 μl) were incubated at 37°C for 40 min in NaCl/PIP, with 4 and 8 μl of a mixture of glycosylated/phosphorylated CGA-derived peptides (5 μl). Then, incubation media were centrifuged at 1000 × g for 5 min and aliquots (30 μl) of supernatant were diluted in water (1 ml). The absorbance of the diluted solution was measured at 420 nm. The absorbance obtained after treating erythrocytes with 2% SDS was taken as 100%.

**Peptide Synthesis**—Peptides were synthesized in our laboratory on an Applied Biosystems 432 A peptide synthesizer, SYNERGY, using the stepwise solid-phase synthetic approach and Fmoc (9-fluorenylmethoxycarbonyl) chemistry (31). All residues were incorporated with double coupling. Peptides were further purified by reverse phase HPLC on a Brownlee Aquapore OD-300 (7 μm, 50 × 250 mm) and lyophilized.

**Sequence Comparisons**—Sequence alignment of bovine CGA (173–194) with corresponding fragments of CGA and CGB for different species was performed using the Clustal V multiple sequence alignment program (32) using default parameters. Chromogranin sequences were retrieved from the Swiss-Prot data base.

**RESULTS**

Bovine chromaffin granules contain a large number of chromogranin- and proenkephalin-A-derived peptides. When the soluble intragranular material or the secreted fragments from K+-depolarized chromaffin cells were separated by HPLC and their effects on the growth of *M. luteus* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria) tested, antibacterial activity was detected in several fractions. With soluble granule matrix proteins as starting material (Fig. 1A), active fractions inhibiting the growth of both these bacteria were recovered in a group of peaks (α1 in Fig. 1A1), where proenkephalin-A and CGA-derived peptides were identified. After a second purification step by HPLC, an active fraction (α2) corresponding to a fragment with N-terminal end starting on position 79 in CGA sequence was identified. After monodimensional gel electrophoresis, the apparent molecular mass of this fragment was estimated to 63 kDa. This value corresponds to the sequence of the CGA fragment 79–431 reported previously (6). We also looked for antibacterial activity in material released from K+-stimulated cultured chromaffin cells (Fig. 1B). The same active CGA 79–
431 fragment was isolated (fraction b2). Microsequencing revealed a unique N-terminal end, and electrophoresis separation showed a single component with an apparent molecular mass of 63 kDa. This fragment displays antibacterial activity, inducing a 100% inhibition of bacterial growth at a concentration of 1.5 \( \mu M \).

In order to characterize the shortest active sequence included in this large fragment 79–431, CGA was submitted to proteolysis with endoproteinase Lys-C and after separation by HPLC on a reverse-phase C18 column, the generated fragments were tested for their antibacterial activity, and peptide structure was determined by combination of microsequencing and mass spectrometry analysis. As shown in Fig. 2, peptides inhibiting \( M. \) luteus growth were found in three successive peaks numbered 1 to 3. Complete sequencing of material present in peaks 1–3 indicated that it was the same peptide located in position 173–194 (Fig. 2B). This peptide was also recovered in the neighboring peak 4, but no bacterial activity was found in this fraction. The sequence (173–194) of this peptide was included in the large fragment described previously (Fig. 1), and its elution as four different subspecies suggested post-translational modifications.

**Isolation of Antibacterial CGA-derived Peptides**—The sequencing of peptides present in peaks 1 and 4 showed unambiguously PTH-(Tyr\textsuperscript{173}, Ser\textsuperscript{182}, and Ser\textsuperscript{191}), whereas in peaks 1 and 2, the PTH-Ser\textsuperscript{186} was not detectable, thus indicating a possible O-glycosylation on this residue. According to structure analysis reported by Wilson (33), the statistically significant patterns PS/T and S/TXXP predict O-glycosylation sites, and the sequence around residue Ser\textsuperscript{186} (PSQGP) fits with these two characteristic patterns. In addition, Ser\textsuperscript{182} (KEDSEG) is located in a consensus phosphorylation site by protein kinase C (K/RXXS/T).

To further determine the structure of the 173–194 peptide subspecies, mass spectrometry analyses were initiated using the MALDI-TOF technique.

**Mass Spectra Analysis of the Different Forms of CGA (173–194) Peptide**—The lower mass value (2317 Da) was obtained for the inactive peptide present in fraction 4; this value corresponds to the theoretical mass of the unmodified peptide 173–194. Mass values of 3055, 2974, and 2397 Da were found for peptide species recovered in fractions 1, 2, and 3 respectively. Mass value comparison of predominant peptides present in peaks 1/2 (2974 Da/3055 Da) and in peaks 3/4 (2317 Da/2397 Da) indicated a difference of 80 Da, revealing the presence of a phosphorylated or sulfated residue. Analysis of minor components showed masses of 3070 Da/2763 Da in peak 1 and 2990 Da/2682 Da in peak 2. The differences 3055/2763 and 2974/2682 were similar (292 Da), suggesting that the same moiety has been removed from the two major species in peaks 1 and 2. The difference between the two masses 3070 and 3055 Da in peak 1 and 2990 and 2974 Da in peak 2 was 16 and 15 Da, which corresponds to the oxidation state +1.

In addition, the comparison of the masses corresponding to the 3055- and 2974-Da fragments present in peaks 1 and 2 with the masses of 2397 and 2317 Da fragments in peaks 3 and 4, respectively, revealed a similar difference value of 657 Da.

**Identification of the Phosphorylation Site of the Peptide 173–194**—
FIG. 2. Purification and structural characterization of the four different forms of CGA-derived fragment (173–194) found in fractions 1–4. A, CGA-derived peptides after digestion with endoproteinase Lys-C were separated on a Macherey Nagel reverse-phase C18 column (4 × 250 mm). The elution profile is shown; absorbance was monitored at 214 nm, and elution was performed with a linear gradient as indicated on the right-hand scale. Numbers 1–4 indicate fractions containing the amino acid sequence reported in B. B, sequence determination of the

-P-G-P-Q-A-K-E-D-S-E-G-P-S-Q-G-P-A-S-R-E-K-
Table I reports the relative modified species of 173–194 peptide—structural modifications involving the glycosylation site. It is noteworthy that Ser182 residue, which is a putative tyrosine phosphorylated site, was not phosphorylated in any fraction.

Structural Characterization of the Glycosylation of the 173–194 Peptide—The molar carbohydrate composition was performed on the 173–194 peptide isolated from peak 2 using a gas chromatography. The carbohydrates NeuAc, Gal, and GaINAc were detected in a molar ratio of 1:1:1. Using similar experimental conditions, a similar molar ratio was also obtained for the whole purified CGA protein, suggesting a unique structure for the different glycans distributed along the protein. To obtain more structural information, the strategy was to electrophorese purified CGA onto nitrocellulose sheet and to use a panel of lectins, the specificity of which is detailed in Fig. 3A. As shown in Fig. 3B, CGA was not recognized by SNA-dig lectin (lane 1), whereas a 70-kDa band was revealed with MAA-dig lectin (lane 2); the binding of this lectin to CGA is abolished with sialidase treatment (lane 6). This result indicated that sialic acid residues are in a α2–3 linkage and that no α2–6-linked sialic acid residues are present in CGA glycans.

In addition, ACA-dig lectin decorated a similar band prior to or after sialidase treatment (lanes 4 and 8, respectively), whereas PNA-dig lectin only bound to the glycoprotein after desialylation (lane 7). The two lectins recognized the T-antigen (Galβ1–3GalNAcα1–O-Ser) with the difference that ACA lectin is also able to bind to the cryptic T-antigen. Desialylation clearly indicated that all the Galβ1–3GalNAcα1–O-Ser sequences in CGA are sialylated. Taken together these data suggest that the different glycosylation sites in CGA are on serine residues and are composed of the trisaccharide NeuAcα2–3Galβ1–3GalNAcα1–O-linked to a serine residue. In the glycopeptide 173–194 this glycan moiety is linked to Ser186. The calculated molecular mass of this glycan is 655 and corresponds to the experimental value determined from mass spectrometry (Fig. 2).

Structural Characterization of the Different Post-translational Modified Species of 173–194 Peptide—Table I reports the detailed mass spectra analysis of fractions 1–4 (Fig. 2C): (i) mass spectra analysis of fraction 1 revealed the presence of a major component corresponding to the tyrosine phosphorylated form of the CGA 173–194 glycopeptide carrying in addition the 40-kDa moiety (3055 Da) characteristic sequence pattern PSQGP surrounds the Ser186 proline residues, especially at positions –1 and +3 relative to the glycosylated residue. In the glycopeptide 173–194, a characteristic sequence pattern FGSGP surrounds the Ser186 O-glycosylation site.

**Antibacterial Activity of CGA 173–194 Subspecies—** As shown in Fig. 4, CGA peptides present in fractions 1–3 inhibited the growth of *M. luteus* (strain 270), but were inactive toward *E. coli* (strain D22). *B. megaterium* (strain M A) was affected only by the peptide present in fraction 1. Complete inhibition of bacteria growth was reached for a peptide concentration around 1.5 μM. In contrast, unmodified CGA 173–194 from fraction 4 was totally inactive. These results suggest the importance of these post-translational modifications for antibacterial activity. As a control, in order to demonstrate that the antibacterial activity was not due to nonpeptidic material present in the fractions, we digested extract aliquots (10 μl) of the endogenous material present in fraction 1 to proteolysis with trypsin (enzyme/peptide ratio of 1:50) for 24 h at 37 °C. The resulting mixture of tryptic peptides (YPQPAK, EDSEG-
PSQGPASR, and EK as determined by microsequencing) had no effects on *M. luteus* and *B. megaterium* growth (Fig. 4), showing that the N-terminal moiety 173–179 (phosphorylated or nonphosphorylated) and the two C-terminal residues (E, K) are essential for antibacterial activity.

Antibacterial peptides corresponding to the phosphorylated/ glycosylated forms of CGA 173–194 did not cause hemolysis of bovine erythrocytes. We propose to name these antibacterial peptides chromacin: chromacin-P, chromacin-G, and chromacin-PG, respectively, for phosphorylated, glycosylated, and phosphorylated-glycosylated form. Since this sequence is included in a larger CGA fragment (79–431), this precursor, which has inhibitory effects on *M. luteus* and *E. coli*, could be named prochromacin.

**DISCUSSION**

Bovine CGA from adrenal medulla is a glycoprotein containing 5.4% carbohydrate (1). It has been reported previously that sugars are mainly present as O-glycosidically-linked tri- and tetrasaccharides composed of N-acetylgalactosamine, galactose, and sialic acid (38). In addition, it has also been established that adrenal CGA is a phosphoprotein containing five phosphoserine residues per molecule (39) and that it also in
lished that adrenal CGA is a phosphoprotein containing five
it appearing bound to carbohydrates.

The present paper reports the location of two post-translational modifications on bovine CGA isolated from adrenal medulla, since we clearly demonstrate that tyrosine 173 and serine 186 are, respectively, phosphorylation and O-glycosylation sites. The glycan moiety bound to serine 186 was shown to be NeuAc

**TABLE I**

| Fraction number | Molecular mass (Da) | Phosphorylated peptide | Glycosylated peptide | Oxidation state |
|-----------------|---------------------|------------------------|----------------------|-----------------|
| 1               | GP-chromacin        | +                      | +                    | 0               |
| 2               | G-chromacin         | +                      | +                    | 0               |
| 3               | P-chromacin         | +                      | +                    | 0               |

**FIG. 4. Antibacterial activity of the four different forms of the CGA-derived fragment 173–194.** Material contained in peaks 1–4, and the tryptic digest of the glycosylated-phosphorylated form (fraction 1) were tested against *M. luteus* (*Ml*) and *B. megaterium* (*Bm*). Microbial growth was assessed by measuring the increase in A 620 after incubation at 37 °C (closed column, *Ml*) or 20 °C (dashed column, *Bm*) for 24 h. Values found with control cultures grown in the absence of peptide were taken as 0%. Data are typical of several experiments and are given ± 10%.

bonds (175–176, 184–185, 188–189) will induce conformational constraints on the peptide chain: the side chain is probably bent back onto the backbone amide position. Inside an α-helix, proline residues can no longer establish hydrogen bonds with the preceding turn, thereby introducing a kink. Furthermore, the specific properties of proline render its presence in nature of the corresponding antibacterial chromacin-P, -G, and -PG species. This property contrasts with the idea that antibacterial peptides need to be positively charged in order to bind to bacterial surfaces; however, we reported previously that enkelytin, which is also a highly negatively charged peptide derived from proenkephalin-A, has potent antibacterial activity (20). In CGA 173–194 sequence, the total proportion of charged residues is 32%, and its hydropathy profile corresponds to a very hydrophilic peptide in contrast with the amphipathic structure generally described for antibacterial peptides.

In the chromacin peptide, the 4 proline residues located in positions 174, 176, 185, 189, and the three glycine-proline bonds may function as receptor subunits or transporters (42). In addition, functioning as a built-in signal, proline residues in polypeptide chain may function as a contributing to a diversity of processes (43) such as immunomodulation (44–46), coagulation (47), homeostasis (43), inflammation, and microbial/viral infections (48, 49). Many neuro- and vasoactive peptides have proline residues in their sequences. An examination of the amino acid sequences of proteins registered in international data banks reveals that numerous cytokines and growth factors share an X-proline sequence at their amino terminus (i.e., interleukins IL-1β, IL-2). Alignment of bovine CGA peptide 173–194 with homologous fragments from several species (Fig. 5) shows the conservation of this X-proline sequence at the N-terminal end of bovine, porcine, and human CGA as well as human CGB. Proline may not only determine secondary structural properties necessary for their biological activity, but may also hinder nonspecific proteolytic modifications such as C-terminal amidation, acetylation, or N-terminal cyclization to pyroglutamic acid. The striking degree of conservation seems to reflect an evolutionary pressure toward this X-proline motif. Alternatively, proline-containing motifs may serve as recognition sites for specific peptidases (46).

Some other inducible antibacterial peptides can also be described as proline-rich peptides (2–3 kDa) and glycine-rich peptides (9–30 kDa). It is important to point out that the chromacin sequence is different from these antibacterial molecules, including apidaecin (50), abaecin (51), drosocin (30), pyrrhocoricin (52), diptericin (53), and lebecin (54).

Drosocin, pyrrhocoricin, diptericin, and lebecin carry O-gly-
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prochromacin (CGA 79–431) has antibacterial activity directed against both Gram-positive and Gram-negative bacteria. This double activity is lost in chromacin-P, -G, and -PG, suggesting that other domains in prochromacin are responsible for the antibacterial activity directed against Gram-negative bacteria.

In Fig. 5, the alignment of chromacins (59) with corresponding peptides of chromogranins raises several points. First, comparison of these different CGA peptides shows a relatively high degree of identity between porcine CGA (60) (66.7%), human CGA (61) (70.8%), and also mouse (62) and rat CGA (63) (45.8%). Interestingly, some similitude with CGB sequence and more particularly with human and mouse CGB (64, 66) (33.3 and 25%, respectively) was also observed, but only a weak identity (12, 5%) was obtained with bovine CGB (65). Second, the simultaneous presence of phosphorylated tyrosine and O-glycosylated serine occurs only in the bovine CGA sequence. A potential O-glycosylation site is present in the porcine CGA sequence, while the N-terminal sequence including the first tyrosine residue is identical in human CGA. Third, the N-terminal domains (residues 173–183 in bovine CGA) seem to be more conserved than the C-terminal region 184–194. A ratio of 90% is obtained for comparison with human CGA. In addition, alignment of the N-terminal sequence 173–183 with sequences contained in data banks show 100% of homology with a short peptide (YPAPQGRE) of metavinulin (VINC_XENLA), a protein known to be localized in the subplasmalemmal region in many cells and to be involved in the binding of cytoskeletal filaments to membranes.

Finally, our studies indicate that the different antibacterial peptides isolated from bovine chromaffin granules, enkelytin, secretolytin, and prochromacin are co-released with catecholamines and other neuropeptides during exocytosis following K⁺ stimulation of cultured chromaffin cells. In view of the widespread distribution of the chromogranins and proenkephalin-A, these peptides may also be present and secreted from other endocrine and neuroendocrine chromogranin-containing cells. The identification of different classes of antibacterial peptides in diverse range of organisms, including prokaryotes, insects, frogs, and mammals, suggest they play a potentially important role in the host defense against microbial infections. Experiments currently in progress in our laboratory aim to establish the physiological relevance of this bovine chromaffin granule antibacterial activity.

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REFERENCES
1. Simen, J. P. & Aunis, D. (1989) Biochem. J. 262, 1–13
2. Helle, K. B. (1990) Neurochem. Int. 17, 165–175
3. Huttner, W. B., Gerdes, H. H. & Rossi, P. (1991) Trends Biochem. Sci. 16, 27–30
4. Winkler, H. & Fischer-Colbrie, R. (1992) Neurosci. Res. 19, 497–528
5. Dileen, L., Mosezer, B., Zalay, M., Aunis, D. & De Potter, W. (1993) Neurochem. Int. 22, 315–352
6. Metz-Begueille, M. H., Garcia-Sablone, P., Hugue-Angelleti, R. & Aunis, D. (1993) Eur. J. Biochem. 214, 659–676
7. Tatemoto, K., Efendic, S., Mutt, V., Makk, G., Feistner, G. & Barchas, J. D. (1986) Nature 324, 476–478
8. Efendic, S., Tatemoto, K., Mutt, V., Quan, C., Chang, D. & Ostenzoo, C. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7267–7270
9. Ishizuka, J., Asada, I., Posten, G., Lluis, F., Tatemoto, K., Gereley, G. & Thompson, J. (1989) Pancreas 4, 277–281
10. Lewis, J. A., Geldenring, J. R., Asher, V. A. & Modlin, I. M. (1989) Biochem. Biophys. Res. Commun. 163, 667–673
11. Fasciotti, B. H., Trauss, C. A., Gereley, G. H. & Cohn, D. V. (1993) Endocrinology 123, 461–466
12. Simon, J. P., Enderle, M. F. & Aunis, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1712–1716
13. Dree, B. M., Rouse, J., Johnson, J. & Hamilton, J. W. (1994) Endocrinology 128, 3581–3587
14. Hutton, J. C., Pesharia, M., Johnstone, C. F., Razzola, M. & Orci, L. (1988) Endocrinology 122, 1014–1020
15. Helle, K. B., Marley, P. D., Angeletti, R. H., Aunis, D., Galindo, E., Small, D,
