Characterisation of N-terminal chromogranin A and chromogranin B in mammals by region-specific radioimmunoassays and chromatographic separation methods

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Abstract

Chromogranin A (CgA) and chromogranin B (CgB) are acidic proteins stored in and released from hormone granules in endocrine and neuroendocrine tissue. The chromogranins are postulated to serve as pro-hormones to generate biologically active peptides, which may influence hormonal release and vascular functions or have antibacterial functions. Although N-terminal and C-terminal regions show some species amino acid homology, the chromogranins as a whole display considerable interspecies differences, which prevents their use in comparative studies of biological functions.

We present four new radioimmunoassays for the measurement of defined N-terminal regions of CgA and CgB. A new radioimmunoassay for measurement of intact bovine CgA has also been developed. With these assays and two previously published ones, we have compared the cross-reactivity of chromogranins from man, cattle, sheep, goat, pig and horse and compared adrenomedullary content and serum levels of CgA from these species. We have also

studied the influence of peptide concentrations and the ionic strength of the mobile phase on molecular weight estimations.

Assays with antibodies directed against the N-terminal parts of CgA and CgB showed sufficient interspecies cross-reactivity to allow comparative quantification of the circulating levels in man, cattle, sheep, goat, pig and horse. Assays measuring the intact human or bovine CgA were not suitable for comparative purposes in samples from sheep, goat, pig and horse. Molecular interactions between vasostatin immunoreactive material and intact bovine CgA were demonstrated in gel permeation studies, suggesting that conclusions about the degree of N-terminal processing from elution profiles should be made with caution.

Reliable interspecies comparison of chromogranins is difficult, but measurements with region-specific assays may be helpful to study concentrations of chromogranins and chromogranin-related peptides.

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Introduction

Chromogranins and secretogranins constitute a family of uniquely acidic proteins that are co-stored with neurotransmitters and peptide hormones in the brain and the diffuse neuroendocrine system (for review see Winkler & Fischer-Colbrie 1992). Structurally these proteins are products of different genes but share some overall properties, such as an abundance of acidic amino acid residues and numerous pairs of basic amino acids as potential positions for post-translational processing (Iacangelo *et al.* 1986). Two members of the family, chromogranin A (CgA) and chromogranin B (CgB), are co-stored and co-released with neuropeptides and hormones in the neuroendocrine cells throughout the body, including the chromaffin cells of the adrenal medulla and the

sympathetic nervous system (Benedum *et al.* 1987, Iacangelo *et al.* 1991).

It has previously been shown that the relative amounts of CgA and CgB may vary in different species (Hagn *et al.* 1986). Furthermore, both proteins have been postulated to serve as pro-hormones for biologically active peptides (Eiden 1987, Helle & Angeletti 1994). Some specific domains of the molecules have been assigned with different biological activity. First, the N-terminal domains of CgA, also called vasostatin, have suppressive effects on vascular contractility (Aardal & Helle 1992, Angeletti *et al.* 1994) and on parathormone secretion (Fasciotto *et al.* 1990, Drees *et al.* 1991, Angeletti *et al.* 1996). Secondly, the central part of CgA, with defined peptide hormones such as pancreastatin, parastatin and catestatin, has paracrine- or autocrine-inhibiting effects on the secretion

of other hormones, such as insulin (Tatemoto *et al.* 1986) and catecholamines (Mahata *et al.* 1997). Thirdly, there are central domains of CgA and C-terminal domains of CgB, termed chromacin and secretolytin respectively, which possess antibacterial effects (Strub *et al.* 1995, Metz-Boutigue *et al.* 1998). There are also a few reports about stimulatory effects on liver glycogenolysis, suggesting endocrine effects of the pancreastatin domain of CgA (Sanchez Margalet & Goberna 1994). However, a further understanding of the physiological relevance of these effects depends on estimations of the circulating levels of these peptides in different animal models. These studies require the use of reliable immunoassays for defined sequences. Such methods have met with problems because of the limited sequence homologies between CgA and CgB in man and other mammalian species.

In this study, we present four newly developed radioimmunoassays for the N-terminal domains of CgA (vasostatin) and CgB with sufficient sensitivity for the estimation of CgA and CgB concentrations in tissue and blood, and complete cross-reactivity for interspecies comparisons. We also present a new radioimmunoassay for the measurement of intact bovine CgA (bCgA) and compare the results with measurement of intact human CgA (hCgA). The results indicate that CgAs circulate at higher levels in mammals such as sheep, goat, horse and pig than in man and cattle. With the aid of these assays, we have also studied the chromatographic behaviour of different peptides covering various sequences in the N-terminal part of CgA. These results are compared with those obtained during purification of chromogranins from biological tissue. Our results show that the chromatographic behaviour of CgA and CgA-related peptides is largely dependent on both the concentration of chromogranin products in the sample and on the buffers used during the separation steps.

Materials and Methods

Chemicals and samples

All chemicals used were of pro-analysis grade (Merck, Darmstadt, Germany). Dilutions in the radioimmunoassays were performed in the assay buffer, which was a 0.05 M sodium phosphate buffer at pH 7.4, with 0.15 M sodium chloride, 0.02% sodium azide, 0.2% bovine serum albumin (BSA) and 0.5% Tween 20. Collection and preparation of samples are described below. All samples were stored at -20°C or -70°C before measurement in the different assays.

Radioimmunoassay for human CgA amino acid sequence 1–17 (hCgA1–17)

A peptide covering the amino acid sequence hCgA1–17 (see Table 1), with the amino acid in position 16 replaced

by a tyrosine residue for labelling reasons, was synthesised using Fmoc techniques (Applied Biosystems Model 430, Foster City, CA, USA). This peptide was also used for the production of specific polyclonal antibodies. Before injection into rabbits, the peptide was coupled to Inject malemid-activated keyhole limpet cyanogen according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The antibodies and the synthesised peptide were used to develop a specific radioimmunoassay. For the preparation of tracer, the peptide was labelled with ^{125}I (Amersham International plc, Amersham, Bucks, UK) using the chloramine-T method as previously described (Stridsberg *et al.* 1995). The assay was constructed as follows: standards and unknown samples were incubated with tracer (30 000 c.p.m./tube) and primary antibodies, at a final dilution of 1/12 000, for 3 days at 4°C . All standards and samples were assayed in duplicate. Antibody-bound radioactivity was separated from free tracer by the addition of a second antibody, goat anti-rabbit IgG coupled to a solid phase (decanting suspension 3; Pharmacia Biotech, Uppsala, Sweden). The antibody-bound radioactivity was then measured in a gamma counter (Auto gamma; Wallac, Pharmacia Biotech) and the data were calculated with a logit-log transformation program (Multicalc, Wallac).

Radioimmunoassay for hCgA17–38

A peptide covering the amino acid sequences hCgA17–38 (see Table 1), with the amino acid in position 36 replaced by a tyrosine residue for labelling reasons, was synthesised using Fmoc techniques. As described above, this peptide was also used for production of specific polyclonal antibodies and these reagents were then used to develop a specific RIA. The antibodies were used in a final dilution of 1/120 000.

Radioimmunoassay for hCgB16–37

A peptide covering the amino acid sequences hCgB16–37 (see Table 1), with the amino acid in position 17 replaced by a tyrosine residue for labelling reasons, was synthesised using Fmoc techniques. As described above, this peptide was also used for production of specific polyclonal antibodies and these reagents were then used to develop a specific radioimmunoassay. The antibodies were used in a final dilution of 1/150 000.

Radioimmunoassay for bCgA17–39

The primary antibody was a rabbit polyclonal antibody raised against a synthesised peptide covering the amino acid sequences bCgA17–39 (see Table 1). The peptide, with a closed disulfide bridge, was injected into rabbits with Freund's adjuvant without prior coupling to a carrier protein, and the resulting antibodies were used in a final

Table 1 Amino acid (AA) sequence of the N-terminal parts of CgA from human, bovine and porcine species and CgB from human and bovine species

	Amino acid sequence				
AA number	1	10	20	30	40
Human CgA	LPVNSEPMNKGDEVMKCIIVEVISDTLSKPSMPVVSQECFE				
Bovine CgA	LPVNSEPMNKGDEVMKCIIVEVISDTLSKPSMPVVSKECFE				
Porcine CgA	LPVNSEPMNKGDEVMKCIIVEVISDTLSKPSMPVVSQECFE				
AA number	1	10	20	30	40
Human CgB	MPVDNR NHNEGMVTRCIEVLNLSKSSAPPITPECRQV				
Bovine CgB	MPVDIR NHNEEVVTHCIEVLNALLKSSAPPITPECRQV				

There is a close amino acid sequence homology between human, bovine and porcine CgA, differing only at position 36. There is also a close amino acid sequence homology between human and bovine CgB, with differences at positions 5, 11, 15 and 26. The homology between CgA and CgB is less, differing at 25 positions of the 40 first amino acids, including deletion of one amino acid.

dilution of 1/7500. Standard preparations were prepared from a synthesised peptide covering amino acid sequences bCgA1–40. Tracer preparations were prepared by labelling of the hCgA17–38 synthesised peptide and the assay was constructed as described above, except that the antibody-bound radioactivity was measured in a Cobra II Auto-Gamma (Packard, Meriden, CT, USA) and the data were calculated with another logit–log transformation program, RiaSmart (Packard).

Radioimmunoassay for bCgA

The primary antibody was a rabbit polyclonal anti-bCgA antibody raised against purified bCgA (see below) and used in a final dilution of 1/3000. The purified bCgA was diluted in assay buffer and used as standard, and tracer preparations were prepared by labelling of the bCgA with ^{125}I (Amersham International plc) using the chloramine-T method. The assay was constructed as described above, except that the antibody-bound radioactivity was measured in a Cobra II Auto-Gamma and the data were calculated with the logit–log transformation program, RiaSmart.

Other assays

hCgA and hCgB were measured by previously described assays using antibodies raised against hCgA116–439 and hCgB312–331 (Stridsberg *et al.* 1993, 1995). Protein content was measured by the Bio-Rad Protein Assay (Bio-Rad, Bradford, UK), with BSA as standard.

Adrenal glands

Adrenal glands from cattle, sheep, goat, pig and horse were obtained fresh from the local slaughterhouse (Fatland A/S, Bergen, Norway). Normal human adrenal glands and pheochromocytoma tumour tissue were obtained with institutional approval as discarded tissue from patients

undergoing radical nephrectomy at the University Hospital in Bergen (Aardal *et al.* 1996).

Whole extracts of adrenal medullae

One sample of adrenomedullary tissue from each animal was homogenised in ice-cold 0.1% aqueous trifluoroacetic acid (10 ml/0.1–0.15 g wet weight of tissue). The ice-cold homogenate was vortex mixed at intervals for 60 min, thereafter centrifuged for $6 \times 10^5 g_{av}$ min. Aliquots (0.1 ml) were boiled for 10 min before the addition of 0.9 ml 0.1% ice-cold aqueous trifluoroacetic acid. These adrenomedullary extracts were centrifuged before measurements in the radioimmunoassays and for estimation of protein.

Subcellular fractionation of adrenal medullae

Chromaffin cells were prepared from fresh adrenal medullae from cattle, pig, horse, goat, sheep and man. Subcellular fractions were obtained by differential centrifugation of tissue homogenates in 0.3 M sucrose, as previously described for the bovine gland (Helle *et al.* 1990). After removal of cell debris at $6 \times 10^3 g_{av}$ min, the cell-free homogenate (SN1) was centrifuged at $6 \times 10^5 g_{av}$ min in order to separate the large granule fraction (P2) from the cytosol and lighter membranous particles (SN2). The large granule fraction was thereafter resuspended in 0.3 M sucrose and further fractionated by sedimentation through 1.6 M sucrose. The developed gradient was divided into subfractions (F1–F4) according to the scheme previously published (Bolstad *et al.* 1980). The pellet of chromaffin granules (F4) was resuspended in 1 volume of 1.6 M sucrose and used as the source for purification of intact bCgA.

Purification of bCgA

Chromaffin granules were lysed by resuspension in 10 volumes of ice-cold water containing a cocktail

of five protease inhibitors (10 μ M leupeptin, 10 μ M pepstatin, 10 μ M phenylmethylsulphonylfluoride (PMSF), 10 μ M paramercumibenzoate (PMB) and 2 μ g/ml aprotinin) and boiled for 20 min before removal of heat-denatured proteins at 3×10^6 g_{av}/min. The supernatant was dialysed against two shifts of 500 volumes of water to remove sucrose and catecholamines before dialysis against 0.1 M Na-acetate, pH 6.5, in 1 M NaCl also containing 1 mM MgCl₂, MnCl₂ and CaCl₂ in addition to PMSF, PMB and aprotinin (in the same concentrations as above). The dialysed protein solution (2.5 mg protein/ml) was applied to a column (0.9 $\times$ 20 cm) of Concanavalin A-Sepharose 4B (Pharmacia) in order to separate the soluble dopamine β -hydroxylase (DBH) activity from the non-absorbed chromogranins (Aunis *et al.* 1973). The non-absorbed protein was dialysed against 6 mM ammonium acetate, pH 6.5, containing PMSF, PMB and aprotinin, and thereafter lyophilised. N-terminal amino acid sequence and total amino acid analyses were carried out on the protein dissolved in aqueous 0.1% trifluoroacetic acid. An N-terminal sequence corresponding to CgA1–19 was verified by amino acid analysis and the total amino acid composition corresponded to the intact bCgA (Aardal *et al.* 1993). SDS-PAGE electrophoresis was carried out on 16% acrylamide gels. The proteins were immunoblotted to nitrocellulose papers and the immunoreactive components were stained with the bCgA antibodies using the alkaline phosphatase reaction as previously described (Aardal *et al.* 1993). Traces of the N-terminal sequence of the soluble DBH (Skotland & Ljones 1979) were not detected by these analyses and the protein failed to immunostain with a polyclonal antibody to bovine DBH (a kind gift from Dr D Aunis, Strasbourg, France).

Cross-reactivity between chromogranins from different species

To compare the cross-reactivity of chromogranins from different species, the subcellular SN2 fractions from man, cow, sheep, pig and horse were serially diluted in assay buffer and then measured in the different radioimmunoassays.

Gel permeation chromatography

Samples of peptides and tissue extracts were run on a Superdex S-75 column (H10/30; Pharmacia). The mobile phase ($I=0.3$) was the buffered saline (0.05 M sodium phosphate buffer at pH 7.4 in 0.15 M sodium chloride, containing 0.5% Tween 20 and 0.02% sodium azide), i.e. the buffer used for the radioimmunoassays, omitting the BSA. Eluted fractions of 0.25 ml were collected and assayed for chromogranin immunoreactive material.

The following molecular weight standards were used as references for the apparent elution volumes of immunoreactive bCgA17–39 and intact bCgA: Blue dextran 300

(V₀); BSA (V_e 0.07); haemoglobin (V_e 0.16); cytochrome C (V_e 0.56); aprotinin (V_e 0.72).

The molecular weight (MW) distribution of three different CgA peptides were evaluated. A synthesised bCgA17–39 peptide (MW 2300), a synthesised bCgA1–40 peptide (MW 4300) and a recombinant hCgA1–78 peptide (MW 8300, a kind gift from Dr A Corti, Milan, Italy; (Corti *et al.* 1997)) were subjected to gel permeation chromatography. All collected fractions were measured in the bCgA17–39 assay.

Blood samples

Blood samples from cattle, sheep, goat, pig and horse were obtained fresh from the local slaughterhouse (Fatland A/S) and the corresponding sera were prepared in the laboratory. Normal human sera were prepared from peripheral venous blood obtained from healthy volunteers. Aliquots of serum (0.3–1.0 ml) were boiled for 10 min, lyophilised and reconstituted in aqueous 0.1% trifluoroacetic acid, vortex mixed at 2- to 3-h intervals over 24 h at room temperature before centrifugation at 10^4 g_{av}. The resulting supernatants were measured in the intact bCgA and bCgA17–39 assays at three different dilutions.

Statistics

Results are expressed as means $\pm$ S.E.M from measurements of the number of preparations indicated. Intra-assay and total assay variations, expressed as CV%, were calculated from control samples run in each assay.

Ethics

Production of polyclonal antibodies in rabbits was approved by the local animal ethic committee at Uppsala University, Sweden.

Results

Radioimmunoassays

The detection limits were about 10–40 fmol/tube for all peptide radioimmunoassays and about 80 fmol/tube for bCgA assay. Calculated from serial measurements of control samples, the intra-assay variations were generally less than 13% and the total assay variations were less than 16% in all assays.

The N-terminal amino acid sequences of human and bovine CgA and CgB are shown in Table 1 for comparison. As expected from the amino acid sequence homology, the standard preparations used in the hCgA17–38 and bCgA17–39 assays cross-reacted 100% in the respective

assays, but there was less than 1% cross-reactivity to standard preparations of hCgB16–37 and hCgA1–17. Similarly, there was less than 1% cross-reactivity between the different standard preparations when measured in the hCgB16–37 and hCgA1–17 assays. Standard preparations of intact human and bovine CgA cross-reacted to less than 1% in the peptide assays. There was limited (about 30%) interspecies cross-reactivity between standard preparations of human and bovine CgA in their respective assays.

Cross-reactivity of mammalian chromogranins

Dilution curves of chromogranins (cytoplasmic SN2 fractions) from different species were measured in the different radioimmunoassays. The assay for intact bCgA showed limited cross-reactivity to sheep and human CgA and low cross-reactivity to horse and pig CgA (Fig. 1a). The assay for intact hCgA and hCgA fragments (Stridsberg *et al.* 1993) showed limited cross-reactivity to cow and sheep CgA and low cross-reactivity to horse and pig CgA (Fig. 1b). There was, on the other hand, complete cross-reactivity between CgB from humans, cows, sheep, pigs and horses in the previously described hCgB assay (Stridsberg *et al.* 1995) (Fig. 1c). There was also complete cross-reactivity between chromogranins from humans, cows, sheep, pigs and horses in the N-terminally directed hCgA1–17, hCgA17–38, bCgA17–37 and hCgB16–37 assays (Fig. 2).

Naturally occurring chromogranins in the cellular subfractions

The distribution of intact CgA and the N-terminal part of CgA in subfractions of the bovine adrenal medulla were compared by means of the assays for bCgA and bCgA17–39, the latter assay being specific for the N-terminal part of CgA, hereafter referred to as vasostatin. As shown in Table 2, the molar ratio of immunoreactive vasostatin versus immunoreactive CgA was, on average, $1.1 \pm 0.1\%$ in the subfractions, i.e. within the limit of cross-reactivity between bCgA and the peptide standard in the two assays. The chromaffin granule fraction (F4) was the only fraction to differ from this pattern, with a high molar percentage of vasostatin (7.9%), consistent with a marked N-terminal processing in the presence of five protease inhibitors during lysis (Helle *et al.* 1993).

The molecular weight distribution of vasostatin and intact bCgA in the cytoplasmic fraction of the bovine chromaffin cells (SN2) was compared with that of the acid- and heat-stable fraction of the matrix phase of the chromaffin granules (F4) by gel permeation chromatography (Fig. 3). The elution profiles of bCgA corresponded to the patterns of immunoreactive bCgA in the Western blots of SDS-PAGE gels (inserts in Fig. 3). In the cytoplasmic fraction (Fig. 3a), the main peak of CgA recovered in the void volume and the second peak were

Figure 1 Cross-reactivity of CgA and CgB from different species. Serial dilutions of the cytoplasmic fraction (SN2) of adrenal medulla (see text) from different species were measured in three different radioimmunoassays. (a) In the intact bCgA assay, there was limited cross-reactivity to human and sheep CgA and low cross-reactivity to horse and pig CgA. (b) In the hCgA assay, there was limited cross-reactivity to cow and sheep CgA and low cross-reactivity to horse and pig CgA. (c) In contrast, in the hCgB assay, specific for amino acid sequences hCgB312–331, there was complete cross-reactivity between CgB from the human, cow, sheep, pig and horse species.

also of high molecular weight. In the granule lysate, there was a shift of material from the void volume to the second peak and, in addition, peaks of lower molecular weights were conspicuous. The molar percentage of immunoreactive vasostatin to bCgA in these eluograms was <1%, indicative of a predominance of unprocessed CgA in the SN2 fractions. N-terminal processing was indicated in the granule lysate, notably in the parallel peak of immunoreactive CgA, and vasostatin material eluted with an apparent MW of 20 kDa. Weakly stained bands in this position were also evident in the Western blot (insert in Fig. 3b). The elution profiles of both the cytoplasmic and granule lysate fractions revealed peaks of vasostatin material preceding bCgA in the void volume fractions. These profiles were consistent with higher degrees of N-terminal processing of CgA in the lysates of chromaffin granules than in the cytoplasmic fractions, as shown in Table 2. Also, the molar percentage of vasostatin immunoreactivity to bCgA immunoreactivity in the SN2 and F4 fractions (Table 2) corresponds to the molar percentage of vasostatin and bCgA in the void volume peaks of vasostatin in Fig. 3. Thus, the peaks of vasostatin preceding the void volume peaks may represent aggregated forms of processed vasostatin.

The molecular weight distribution of N-terminal immunoreactivity in the adrenomedullary cytoplasm (SN2) of human material is shown in Fig. 4, comparing the vasostatin profiles for a normal human gland and a metastatic human pheochromocytoma. Closely similar patterns were obtained with higher levels of immunoreactive material in the tumour than in the normal gland. The main material was eluted as high molecular weight forms, indicating similar, relatively low degrees of N-terminal processing in both the normal and the neoplastic tissue fractions.

Molecular weight distributions of N-terminal bCgA peptides (vasostatin)

Gel permeation chromatography of the three vasostatin peptides at pH 7.4 revealed abnormal distribution patterns relative to the expected mobilities. The elution patterns of synthetic bCgA1-40 at I=0.3 was dependent on the protein concentration (Fig. 5a), revealing three peaks of apparent molecular weights (MW_{app}) >70 kDa, 36 kDa and 20 kDa respectively, when applied to the column at

Figure 2 Cross-reactivity of N-terminal CgA and CgB from different species. Serial dilutions of the cytoplasmic fraction (SN2) of adrenal medulla (see text) from different species were measured in four different radioimmunoassays. There was complete cross-reactivity between chromogranins from humans, cows, sheep, pigs and horses in all assays. (a) Dilution curves for the bCgA17-39 assay, (b) dilution curves for the hCgA1-17 assay, (c) dilution curves for the hCgA17-38 assay and (d) dilution curves for the hCgB16-37 assay.

Table 2 Concentrations of immunoreactive CgA (as measured in the bCgA assay) and vasostatin immunoreactive peptides (as measured in the bCgA17–39 assay) in subcellular fractions of bovine chromaffin cells

		bCgA (nmol/mg protein)	bCgA17–39 (nmol/mg protein)	bCgA17–39/bCgA (%)
Homogenate subfractions	P2 Subfractions			
	SN1	26.4	0.16	0.6
	SN2	17.6	0.08	0.5
P2 (large granules)		57.5	0.79	1.4
	F1 (supernatant in 0.3 M sucrose)	24.7	0.43	1.7
	F2 (0.3 M/1.6 M sucrose interphase)	16.4	0.10	0.6
	F3I (upper layer of 1.6 M sucrose)	53.9	0.67	1.2
	F3II (lower layer of 1.6 M sucrose)	131.0	0.7	0.6
	F4 (granule pellet in 1.6 M sucrose)	90.3	7.12	7.9

Results from subfractionation of bovine chromaffin cells (see text). The molar ratio of immunoreactive vasostatin versus immunoreactive CgA was on average $1.1 \pm 0.1\%$ in the subfractions, i.e. within the limit of cross-reactivity between bCgA and the peptide standard in two assays. The chromaffin granule fraction was the only fraction to differ from this pattern. In the lysed granule fraction there was a higher molar percentage of vasostatin (7.9%), consistent with a marked N-terminal processing by the lysis procedure.

1 μM concentration. These elution volumes corresponded to molecular weights many times higher than the actual mass (MW 8.3 kDa, 4.3 kDa and 2.3 kDa respectively). Reduction of the ionic strength of the mobile phase had dramatic effects on the elution patterns for 1 μM CgA1–40, shifting the eluted peptide towards the void volume and enhancing the relative amounts of immunoreactive material. At $I=0.03$ (Fig. 5b), a large proportion of immunoreactive material recovered in the void volume, while the elution volume of the low molecular weight form corresponded to MW_{app} 28 kDa of the globular protein standards. At further reduction in ionic strength of the mobile phase, to $I=0.003$ and $I=0.0003$, a proportionally larger fraction of CgA immunoreactivity was shifted towards the void volume peak and progressively less immunoreactivity was recovered in a low molecular form (data not shown). The protein concentration was also found to markedly affect the elution of the synthetic CgA1–40. At 0.1 μM concentration at $I=0.03$, the peptide was eluted in a main peak corresponding to MW_{app} of 30 kDa (Fig. 5b). The anomalous elution behaviour was not limited to bCgA1–40. By comparison, the gel permeation patterns for 1 μM aliquots of CgA17–39 (MW 2300) and the recombinant hCgA1–78 (MW 8300) at $I=0.3$ (Fig. 5c) revealed elution volumes corresponding to MW_{app} 12 kDa and 50 kDa for the peptides with molecular masses 2.3 kDa and 8.3 kDa (Fig. 5c). These elution patterns indicated that the synthetic N-terminal CgA peptides, containing the 'loop' sequence of vasostatin, behaved as non-ideal molecules in solution, being markedly dependent on the ionic strength at a given protein concentration.

Gel permeation chromatography of the purified bCgA revealed a main peak of immunoreactive bCgA that was eluted close to the void volume, corresponding to MW_{app} 70 kDa, in accordance with the expected size (not shown).

The SDS-PAGE electrophoresis pattern (Fig. 3 inserts) consistently revealed the same doublet of immunoreactive material throughout the peak of eluted material.

N-terminal CgA (free vasostatin) in mammalian adrenals and sera

The adrenomedullary content of vasostatin was determined in the bCgA17–39 assay. As shown in Table 3, the vasostatin content ranged between 4.1 and 12.7 nmol/g wet weight, similarly low in sheep and goat and highest in the horse, with closely similar, intermediate values for cattle and pig.

Vasostatin immunoreactivity in sera of the domestic mammals (Table 4) varied from below detection levels (<0.2 nM) in cattle to 2.3 nM in the pig. In cattle, the molar per cent of vasostatin to intact CgA was the same ($<3\%$) as in the adrenomedullary extracts, indicating a dominance of non-degraded CgA in the bovine circulation. This was in apparent contrast to the sheep, goat, horse and pig circulation, with serum levels of free vasostatin in the 2 nM range. In porcine serum, the concentration of vasostatin was of the same order as that detected in the preoperative serum sample of a patient suffering from a metastatic pheochromocytoma (patient number 5 in Aardal *et al.* 1996).

Discussion

The need for reliable quantification of circulating levels of CgA and CgB in clinical studies has spurred the development of suitable radioimmunoassays. The first chromogranin radioimmunoassay (O'Connor & Deftos 1986), which was based on intact CgA purified from human pheochromocytoma as antigen and the corresponding

Figure 3 Distribution of N-terminal and intact CgA in the adrenomedullary cytoplasm (SN2 fraction) and in the core fraction of the chromaffin granules. (a) Gel permeation chromatography of the cytoplasmic fraction (SN2) and (b) in the lysate of the purified chromaffin granules (F4) was performed on a Superdex S-75 column at $I=0.3$ (see Materials and Methods). The eluted fractions were analysed for immunoreactive material in the intact bCgA (■) and bCgA17–39 (□) assays. The fractions collected after the column were measured for intact bCgA at 200 times dilution, whereas the bCgA17–39 (vasostatin) was measured without prior dilution. The apparent molecular weight (in kDa), as indicated for the vasostatin peaks, was obtained from standard elution volumes for globular protein markers. V_0 denotes the void volume of the column. Inserts: SDS-PAGE electrophoresis of (a) the SN2 fraction and in (b) the lysate of the F4 fraction of purified chromaffin granules, immunostained with the anti-bCgA antibody. Molecular weights of globular proteins are indicated.

Figure 4 Distribution of N-terminal CgA in the adrenomedullary cytoplasm (SN2 fraction) of the human adrenal gland. Gel permeation chromatography of the cytoplasmic fractions (SN2) of a normal adrenal gland (□) and a pheochromocytoma (■) was performed on a Superdex S-75 column at $I=0.3$ (see Materials and Methods). The eluted fractions were analysed for immunoreactive material in the bCgA17–39 assay. The apparent molecular weights (in kDa), as indicated for the vasostatin peaks, were obtained from standard elution volumes for globular protein markers. V_0 denotes the void volume of the column.

polyclonal antibodies, reported serum levels of 57–129 ng/ml, corresponding to about 1–2 nM of intact CgA. Since then, other assays for measurements of hCgA (Dillen *et al.* 1989, O'Connor *et al.* 1989, Eriksson *et al.* 1990, Bender *et al.* 1992, Stridsberg *et al.* 1993, 1995, Syversen *et al.* 1994, Corti *et al.* 1996) and bCgA have been reported (Dillen *et al.* 1989, Kawakubo *et al.* 1989). Region-specific assays for the determination of CgA and CgB have also been published (Tateishi *et al.* 1989, Iguchi *et al.* 1990, McGrath-Linden *et al.* 1991, Kirchmair *et al.* 1994, Syversen *et al.* 1994, Stridsberg *et al.* 1995). So far these assays have not been used for comparative purposes.

A further understanding of the physiological relevance of circulating CgA and CgB and their processed peptides depends on their quantification in a wide range of mammals. The radioimmunoassays using antibodies raised against the whole chromogranin molecule cannot be used for accurate measurements in other species, due to the limited interspecies cross-reactivity. This is clearly demonstrated in the present study, where the radioimmunoassay based on antibodies raised against the purified intact bCgA revealed variable degrees of limited interspecies cross-reactivity towards adrenal tissue extracts and the cytoplasmic fractions. Similar results were also found with the previously described assay for measurements of hCgA116–439 (Stridsberg *et al.* 1993), in which samples from horse, pig, sheep and cow did not reveal sufficient cross-reactivity to allow reliable quantification (Fig. 1b). Nonetheless, this study is the first to report on circulating levels of bCgA, although two assays have previously reported on satisfactory cross-reactivity between human

and bovine CgA. One was a region-specific assay, based on the synthetic bCgA 367–391 (bGE-25) sequence, being immunoreactive against the free sequence as well as hCgA and bCgA in brain and cerebrospinal fluid samples (Kirchmair *et al.* 1994). The other was an enzyme-linked

Table 3 Immunoreactive CgA (as measured in the bCgA assay) and vasostatin immunoreactive peptides (as measured in the bCgA17–39 assay) in the adrenal medulla of mammals. Results are given as means $\pm$ S.E.M. for the number of glands shown in parentheses

Species	bCgA assay (nmol/g wet weight)	bCgA17–39 assay (nmol/g wet weight)
Cattle	531 $\pm$ 52 (4)	8.4 $\pm$ 0.6 (6)
Sheep	*	4.2 $\pm$ 0.3 (5)
Goat	*	4.1 $\pm$ 0.3 (6)
Horse	*	12.7 $\pm$ 1.7 (3)
Pig	*	8.4 $\pm$ 0.6 (5)

These whole adrenomedullary extracts were boiled before analysis at appropriate dilutions (1/10 for bCgA17–39 RIA and 1/160 for the bCgA RIA).

*CgA levels are not given due to limited interspecies cross-reactivity in the bCgA radioimmunoassay

immunosorbent assay with an antibody raised towards purified bCgA (Dillen *et al.* 1989).

In the present study we also present four newly developed region-specific radioimmunoassays directed towards the highly conserved N-terminal sequences of CgA and CgB. Our aim was to develop assays for comparative purposes and to estimate circulating levels of CgA in different mammals. According to our hypothesis, a set of region-specific radioimmunoassays directed to the highly homologous domains of the N-terminus (see Table 1) might show sufficient interspecies cross-reactivity to allow reliable quantification for comparative purposes. We have shown that these assays have sufficient interspecies cross-reactivity to allow comparison of CgA and CgB in mammalian adrenal tissue extracts and sera. We have also compared interspecies cross-reactivity with a previously published assay for CgB specific for the amino acid sequence hCgB312–331 (Stridsberg *et al.* 1995) and found complete cross-reactivity also with this assay. Consequently, all these assays can be used for comparison of chromogranin levels in the present selection of mammals. Whether these assays are also applicable to more distantly related species, such as rodents, canines and felines, remains a challenge for future investigations.

Evidently the region-specific assays for the N-terminal domain was specific for the vasostatin, as these assays did

Figure 5 Effects of different ionic strength and peptide concentrations on the elution of synthetic N-terminal CgA peptides from a Superdex S-75 column. The elution profiles of CgA1–40 in 1 μ M (●) and 0.1 μ M (○) concentrations at (a) $I=0.3$ and (b) $I=0.03$. In (c) the ionic strength was $I=0.3$ and the concentrations were 1 μ M for the two peptides bCgA17–39 (□) and hCgA1–78 (■). The molecular masses for the synthetic peptides bCgA17–39, bCgA1–40 and hCgA1–78 were 2.3, 4.3 and 8.3 kDa respectively. The apparent molecular weights (in kDa), were obtained from standard elution volumes for globular protein markers. V_0 denotes the void volume of the column.

Table 4 Serum concentrations of immunoreactive CgA (as measured in the bCgA assay) and vasostatin immunoreactive peptides (as measured in the bCgA17–39 assay) in mammals. Results are given as means ± S.E.M. of the numbers (n) of glands and sera

Species	n	bCgA RIA (nM)	bCgA17–39 RIA* (nM)
Cattle	6	6.9 ± 0.6	<0.2
Sheep	6	**	1.3 ± 0.2
Goat	6	**	1.5 ± 0.05
Horse	5	**	1.9 ± 0.3
Pig	5	**	2.3 ± 0.3

*The serum dilutions were 0.33 for the sheep, 0.5 for the pig and 1 for cattle, horse and goat.
**CgA levels are not given due to limited interspecies cross-reactivity in the bCgA radioimmunoassay.
In human serum vasostatin immunoreactivity was 2.8 nM in the preoperative serum sample of a patient with metastatic pheochromocytoma (patient 5, Aardal *et al.* 1996), while <0.1 nM in the post-operative sample was consistent with serum concentrations of <0.1 nM for healthy volunteers (n=6).

not quantify more than <2% of the total of the intact CgA in the bovine adrenomedullary and SN extracts of adrenal medulla and in serum. A reason for this may be differences in the three-dimensional structure of the intact CgA and the vasostatins in solution, seemingly impairing recognition of the specific binding site on the intact protein by the specific epitopes on the antibodies. Similar results have been shown earlier when an antibody, highly specific for the amidated C-terminal of pancreastatin, was used for binding studies of different CgA preparations. These antibodies did not recognise the intact CgA, but did partly bind to larger fragments of CgA and bound completely to the pancreastatin molecule (Stridsberg *et al.* 1995). Analogously, we have presently demonstrated that standard preparations of intact bCgA and hCgA do not cross-react in the region-specific assays. Thus, it is evident that the region-specific assays presented in this study quantify the free N-terminal domain, i.e. the vasostatin part, and only to a minor degree (<2%) the intact CgA. We can also conclude that comparison of measurements in bCgA17–39 and bCgA assays provides relevant information on the degree of N-terminal processing in a given sample of bovine origin.

Another way to study processing of chromogranins has been to apply the samples to gel permeation chromatography and measure CgA in the eluted fractions with both assays. Surprisingly, we found that vasostatin fragments in the tissue extracts, measurable in the bCgA17–39 assay, were eluted at high molecular weight positions preceding that of intact bCgA. In the granule lysate fraction, in which the molar ratio of vasostatin to CgA was 16-fold higher than in the cytoplasmic fraction, both high and low molecular weight forms of vasostatin were increased, implicating electrostatic interactions between the pro-

cessed and unprocessed molecules. This assumption is supported by the complex elution patterns obtained with the synthetic peptides and the recombinant vasostatin I (Fig. 5). Only the ‘loop’ peptide CgA17–39 eluted as a single peak at 1 µM concentration, but at apparent molecular weights many fold higher than the molecular mass. In contrast, under similar conditions, the N-terminal extended peptide CgA1–40 was distributed over three peaks, none of them corresponding to the molecular mass of the peptide. Furthermore, the eluted positions were dependent on both the concentrations of the peptide and the salt content of the mobile phase. Chromogranins are acidic and hydrophilic proteins, properties which result not only in a three-dimensional structure far from the globular proteins used as molecular weight standards but apparently also in protein–protein and protein–peptide interactions (Helle *et al.* 1990, Yoo & Lewis 1992, Corti *et al.* 1997, Thiele & Huttner 1998). Both recombinant vasostatins CgA1–78 and CgA1–115 have been shown to form dimers (Corti *et al.* 1997). Also, a homodimerisation between CgA1–60 and the intact molecule, being sensitive to pH, has been demonstrated to involve the disulphide-bonded loop as well as the C-terminus of CgA (Thiele & Huttner 1998), in addition to a pH- and Ca²⁺-sensitive dimerisation of the intact bCgA (Corti *et al.* 1997). From the present study we may conclude that the N-terminal part of vasostatin (CgA1–40) and the ‘loop’ itself (CgA17–39), both containing the disulphide bridge, also behave abnormally in solutions. Notably, CgA1–40 appeared sensitive to both protein concentration and ionic strength at pH 7.4, suggesting complex electrostatic interactions involving the N-terminal end of the molecule. Thus, conclusions from elution patterns in gel permeation experiments on the degree of processing of chromogranins must be drawn with caution. Consequently, processing of chromogranins is best studied by quantitative measurements of the different chromogranin peptides generated in tissue extracts and sera, using region-specific assays with defined interspecies cross-reactivity.

We found the lowest content of vasostatins in the adrenal medulla from sheep and goat (Table 3). In the adrenal medulla from cattle and pig the amounts were twice as high and even higher in the horse. In bovine tissue extract and serum, the molar ratio of vasostatin to bCgA was 2% and <3% respectively, indicating similar, limited N-terminal processing in both phases. Normal serum levels of intact CgA in man are usually less than 4 nM (Stridsberg *et al.* 1995). The limited interspecies cross-reactivity in the bCgA assay makes it difficult to measure the true serum levels of intact CgA from the different species. However, the serum concentrations of vasostatin were about tenfold higher in sheep, goat, horse and pig than in cattle and man. This makes it reasonable to assume that the contents of CgA in the adrenomedullary tissue and sera from sheep, goat, horse and pig may be higher than in cattle and man. Alternatively, N-terminal

processing into vasostatins may be considerably higher in sheep, goat, horse and pig than in cattle and man.

The concentration of vasostatin in cattle was comparable to the level measured in one human patient after a successful operation for pheochromocytoma, where the serum level is likely to be 'normal' (Aardal *et al.* 1996). Hence, these data can be interpreted in two ways, either that the serum concentrations of CgA, or the degree of N-terminal processing to yield free vasostatin, differ among these mammals. Synthetic vasostatin peptides, covering the sequences bCgA1–40 and bCgA1–76, have previously been demonstrated to possess inhibitory actions in the 10–100 nM range in human blood vessels (Aardal & Helle 1992, Angeletti *et al.* 1994) and on bovine parathyroid cells (Fasciotto *et al.* 1990, Drees *et al.* 1991, Angeletti *et al.* 1996). These concentrations of vasostatin are more likely to arise in the circulation of the sheep, goat, horse and pig than in cattle and man. Thus, vasostatin may also have significant regulatory functions in mammals other than cattle and man. Analogously, quantification of CgB by the two radioimmunoassays presently compared (CgB16–37 and CgB312–331) in the adrenomedullary tissues of the domestic mammals and man can be expected to yield important information on the contents and degree of N-terminal processing in the circulation of these species.

In conclusion, reliable interspecies comparison of chromogranins can be obtained from measurements with region-specific assays of sufficiently high interspecies cross-reactivity, as presently demonstrated for the assays directed towards the N-termini of CgA and CgB.

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