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The tissue distribution of rat chromogranin A-derived peptides: evidence for differential tissue processing from sequence specific antisera

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Summary. The distribution of chromogranin A and related peptides in rat tissues was investigated using sequence specific antisera. N- and C-terminal antisera and a presumptive C-terminal rat pancreastatin antiserum immunostained an extensive neuroendocrine cell population throughout the gastro-entero-pancreatic tract, anterior pituitary, thyroid and all adrenomedullary cells. However, mid- to C-terminal antisera immunostained a subpopulation of chromogranin A positive cells. Most notable of these was with the KELTAE antiserum (R635.1) which immunostained discrete clusters of adrenomedullary cells and antiserum A87A which immunostained a subpopulation of cells in the anterior pituitary and throughout the gastrointestinal tract. The present study has demonstrated the widespread occurrence of chromogranin A and related peptides in rat neuroendocrine tissues and provides evidence of tissue and cell specific processing.

Introduction

The recent determination of the cDNA sequences of bovine (Benedum et al. 1986; Iacangelo et al. 1986), human (Konecki et al. 1987), porcine (Iacangelo et al. 1989) and rat (Hutton et al. 1988a; Parmer et al. 1989) chromogranin A molecules has revealed that there is considerable sequence variation, especially within the pancreastatin domain (Hutton et al. 1988a). However, seven dibasic residue sequences which represent potential proteolytic processing sites are conserved (Hutton et al. 1988a). Sequence analyses of peptides derived from the in vitro processing of bovine chromogranin A have demonstrated that cleavage occurred at the dibasic Lys-Arg (KR) residues 114–115 and 330–331 to generate the N-terminal 21 kDa peptide B-granin, an intervening 40 kDa peptide and a C-terminal 14 kDa peptide (Arden et al. 1991). Isolation of peptides derived from in vivo processing of bovine chromogranin A in gastro-entero-pancreatic tissues revealed that processing also occurred at the dibasic KR residues 314–315 (Watkinson et al. 1991). This lends support to the suggestion that chromogranin A may act as an prohormone for a group of hitherto unknown peptides (Benedum et al. 1986; Iacangelo et al. 1986). Further evidence is that the deduced amino acid sequence of porcine chromogranin A (Iacangelo et al. 1989) contains that of the bioactive peptide, pancreastatin (Tatemoto et al. 1986).

Numerous immunological investigations have demonstrated the widespread distribution of chromogranin A throughout the mammalian neuroendocrine system. However, there is considerable inter- and intra-species variation in both the tissue distribution and cell populations displaying chromogranin A immunoreactivity (Nolan et al. 1985; Rindi et al. 1986; Grube et al. 1986; Buffa et al. 1988; Grube et al. 1989; Hawkins et al. 1989). In view of the fact that chromogranin A is subject to post-translational proteolysis and that most immunocytochemical studies have been performed with sera raised to intact chromogranin A often from another species, such variation is not surprizing.

The present investigation reports the distribution of chromogranin A and related peptides in rat tissues employing rabbit and guinea pig antisera raised to synthetic peptides and fragments of the rat chromogranin A molecule produced as hybrid proteins with beta-galactosidase.

Materials and methods

Antisera production

The following peptide fragments of rat chromogranin A corresponding to residues 316–321, [Tyr°]-Lys-Gly-Gln-Glu-Leu-Glu (hereafter designated KGQELE; single letter amino acid notation) and residues 351–356, [Tyr°]-Lys-Glu-Leu-Thr-Ala-Glu (hereafter

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Table 1. Immunocytochemical tissue distribution of chromogranin A related peptides employing region-specific antisera at their optimal dilutions

Antisera code	Beta-granin	H266	H187	A87A	R607.1	R633.1	H98	R635.1	SP187
Residues	1–128	152–240	241–306	291–319	306–314	316–321	318–349	351–356	376–444
Dilution	1:250	1:400	1:200	1:200	1:200	1:800	1:200	1:200	1:400
Pancreatic islet	+++	++	+++	++	++	+++	−	+	++
Fundus	+++	+++	+++	+++	+++	+	+	−	+
Antrum	+++	+++	+++	++	+++	+	++	++	+++
Duodenum	+++	+++	+++	+	+++	+	−	++	+++
Colon	+++	+++	+++	−	+++	+	−	−	+++
Thyroid	+++	++	+++	++	++	+++	+	++	++
Parathyroid	+++	+	+++	−	+	−	++	+	++
Anterior pituitary	+++/++	+++/++	++/+	++	+++/++	+	+++	++	+++/++
Intermediate pituitary	−	−	−	−	−	+	−	++	−
Posterior pituitary	−	−	−	−	−	−	−	++	−
Adrenal medulla	+++	+	++	−	++	+	+++/++	+++/+	+++

(+++ intense, ++ moderate and + weak immunoreactivity; − negative)

designated KELTAE) were synthesized. The synthetic peptide fragments (800 µg) and ovalbumin (4 mg) were dissolved in 1 ml 0.1 *M* phosphate buffered saline (PBS) (pH 7.2) and conjugated with 1% glutaraldehyde (1 ml) for 30 min (room temperature) and dialysed (overnight 4° C) against 4 l 0.1 *M* PBS. The conjugate was emulsified in Freund's Complete adjuvant (2 ml) and "New Zealand White" rabbits received a primary boost of 200 $\mu g \cdot ml^{-1}$ peptide/animal, followed by monthly boosts of 100 $\mu g \cdot ml^{-1}$ peptide/animal, emulsified in Freund's Incomplete adjuvant (2 ml). Antisera suitable for immunocytochemical investigatins were produced after the first boost (Table 1). The generation of B-granin (Hutton et al. 1988 b) and presumptive C-terminal rat pancreastatin (Curry et al. 1990) antisera have been described previously.

Restriction fragments of rat chromogranin A (Hutton et al.) generated with the enzymes Alu 1 (A-series), Hae III (H-series), Sau 3A combined with Puv II (SP-series) were subcloned into the pUEX series of expression vectors (Bressan and Stanley 1987) to produce an open reading frame with the C-terminus of β-galactosidase. The expressed recombinant proteins were separated by SDS-PAGE of inclusion bodies prepared from recombinant *E. coli* and the prominent fusion protein bands identified by UV shadowing and excised. The gel pieces were macerated in Complete Freund's adjuvant and administered subcutaneously to "Old English" rabbits (approx. 1 mg protein/rabbit). Boosting with protein in Incomplete Freund's adjuvant (100 µg/animal) was performed thereafter at 4- to 6-week intervals.

Immunocytochemistry

Male Wistar rats (250 g) were killed by cervical dislocation and tissues from the gastro-entero-pancreatic tract, pituitary, adrenal, thyroid and parathyroid glands were rapidly removed and immersion fixed in modified Susa (Johnston et al. 1986), for 4 h at 4° C, placed in a 5% sucrose/0.1 *M* PBS for 24 h at 4° C followed by immersion in 30% sucrose/0.1 *M* PBS for cryoprotection. Cryostat sections (30 µm) were air dried on to gelatin coated slides and incubated simultaneously with the primary guinea pig and rabbit antisera (Table 1), for 24 h at 4° C. After a 10 min wash in 0.1 *M* PBS the sections were incubated (30 min) with the rhodamine conjugated swine anti-rabbit (SWAR) secondary antiserum (Dakopatts, Glostrup, Denmark; dilution 1:40), followed by a 10 min wash in 0.1 *M* PBS. The primary rabbit antisera were reapplied (30 min room temperature) to ensure that any free Fab binding sites on the rhodamine-SWAR were completely bound, washed (10 min) in 0.1 *M* PBS, prior to incubation (30 min) with the fluorescein conjugated rabbit anti-guinea pig (RAGP) (Dakopatts; dilution 1:40), washed in 0.1 *M* PBS (10 min) and mounted in 0.1 *M* PBS/glycerol prior to examination using a Bio-Rad (Richmond, Calif., USA) Lasersharp MRC 500 confocal scanning laser microscope. The confocal scanning laser microscope was equipped with a double channel filter block set with bright excitation lines at 488 nm and 514 nm and emission filters sensitive at 515 nm and 550 nm thus allowing separation and simultaneous collection of the green (fluorescein) and red (rhodamine) images. Immunocytochemical controls included the preabsorption of each antiserum with the appropriate peptide fragment, omission of the primary antisera and replacement with non-immune guinea pig and rabbit serum. The fluorescein and rhodamine channels were sensitive to excitation at the specified wavelengths and were insensitive to any background excitation. Two additional controls were performed to assess any potential non-specific ionic binding of the primary antisera. In the first instance poly-L-lysine (MW 3800) was added to the primary antisera at a concentration of 2 $mg \cdot ml^{-1}$ (Scopsi et al. 1986), in the second one, after the primary incubation, the sections were washed for 1 h in phosphate buffered 0.5 *M* NaCl (Grube 1980).

Western blot analysis

Insulin secretory granules were prepared from transplantable rat insulinoma according to the method of Hutton et al. (1982). Samples were sonicated (25 W for 15 s, MSE Sonifier) in 125 m*M Tris*/HCl (pH 6.8) containing 2% (w/v) SDS, 1.4% (w/v) sucrose, 65 mM DTT and 0.005% bromophenol blue and the supernatent

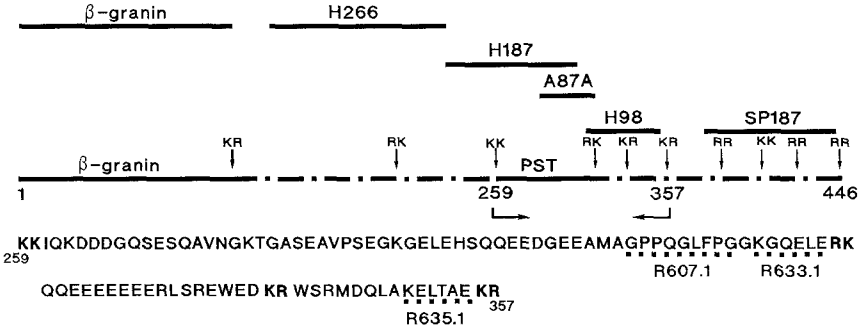

Fig. 1. Diagramatic representation of rat chromogranin A showing the regional distribution of the β-galactosidase hybrid chromogranin A fragments (——), the amino acid sequence of residues 259–357 with the synthetic peptide fragments (– – – –) and their antisera codes. The ten pairs of dibasic residues and the β-granin and presumptive pancreastatin (*PST*) regions are also shown

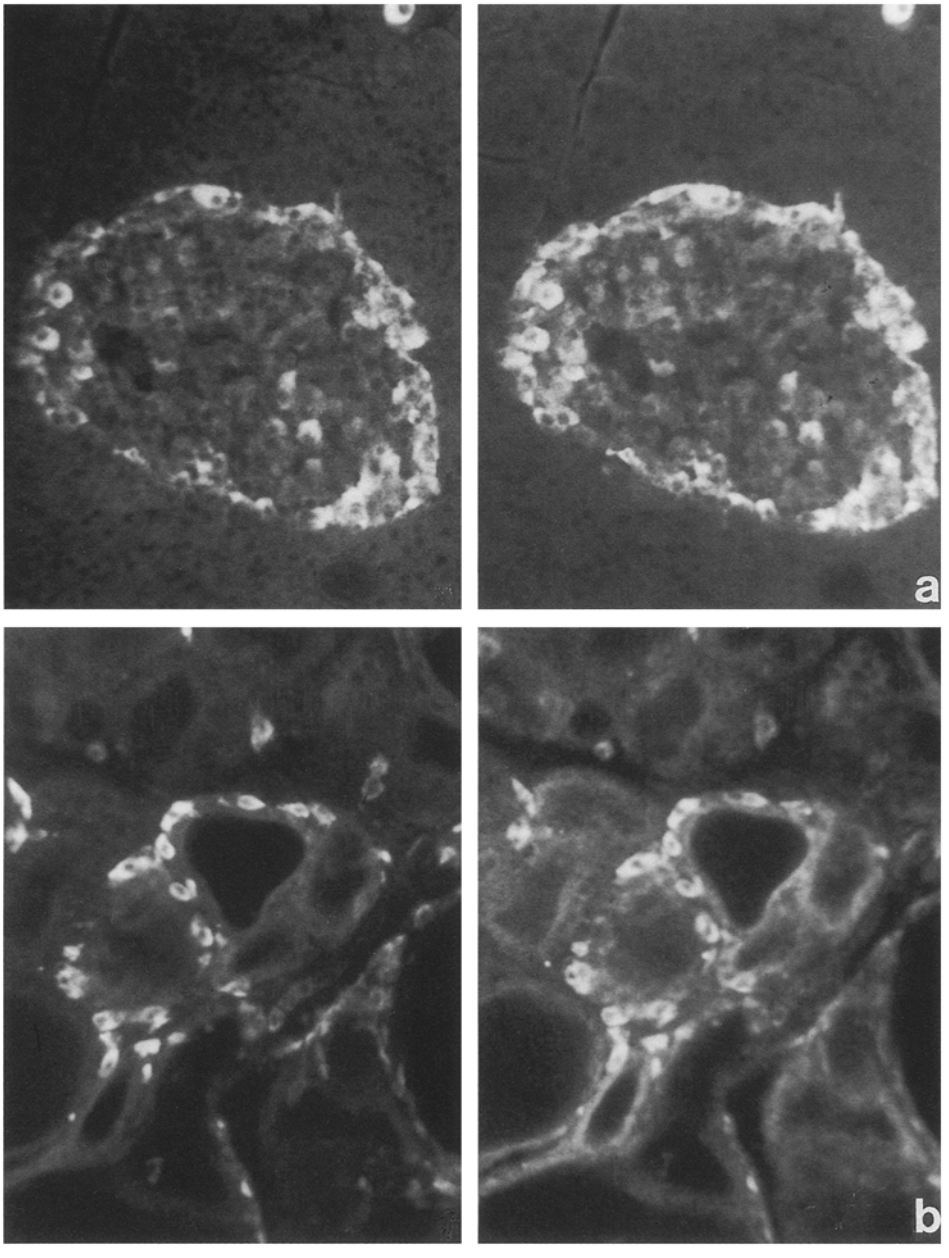

Fig. 2. CSLM of double immunostained tissue sections displaying (**a**) β-granin immunoreactivity (*left*) colocalised with presumptive pancreastatin-like immunoreactivity (*right*) in pancreatic islets and (**b**) SP187 (*right*) and β-granin immunostaining (*left*) in thyroid parafollicular cells. ×460

heated for 5 min at 100° C. Electrophoresis was performed using the discontinuous buffer system of Laemmli (1970) on slab gels (15 × 15 × 0.15 cm) polymerised from 15% (w/v) acrylamide and 0.25% N,N′-methylene bisacrylamide. The electrophoresed proteins were transferred electrophoretically onto nitrocellulose paper for 1 h using the semi-dry electrophoretic method described previously (Hutton et al. 1988b). Affinity purified antisera was used at a dilution equivalent to 1:250–1:500 of the original sera and binding was visualised with horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulin G (Dakopatts, Copenhagen,

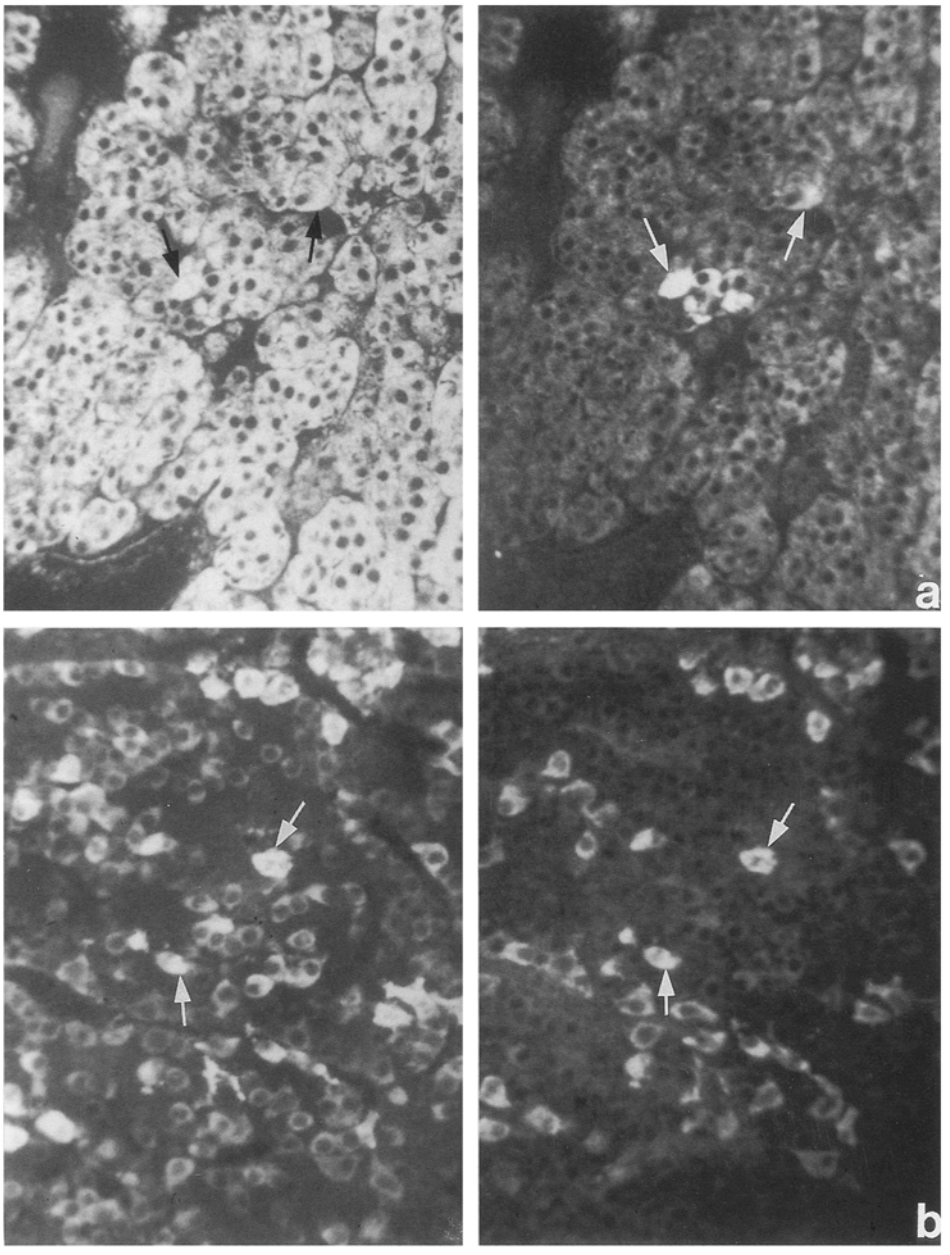

Fig. 3. Double immunostaining employing CSLM of (**a**) adrenomedullary cells displaying intense β-granin immunoreactivity (*left*), with KELTAE immunostaining (*right*) occurring in discrete clusters of cells (*arrows*) and (**b**), A87A immunostaining (*right*) in a subpopulation of β-granin positive cells (*left*) scattered throughout the anterior pituitary (*arrows*). ×460

Denmark; 1:500 dilution) and 3-amino-9-ethylcarbazole as a substrate. Calibration of molecular size was achieved with phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme (Bio-Rad).

Results

Immunocytochemistry

Table 1 summarises the tissue distribution of chromogranin A-related peptide immunoreactivities employing the region specific antisera at their optimal working dilution. A diagrammatic representation of the regional specificity of each antiserum is shown in Fig. 1.

Confocal scanning laser microscopy (CSLM) of double immunostained tissue sections indicated that β-granin immunoreactivity was colocalised with H266, H187, SP187 and presumptive C-terminal nonapeptide pancreastatin (R607.1) immunoreactivities in an extensive neuroendocrine cell population occurring throughout the gastro-entero-pancreatic system, thyroid, parathyroid, anterior pituitary and all adrenomedullary cells. Mid- to C-terminal antisera (A87A, H98, R633.1 [KGQELE] and R635.1 [KELTAE]) immunostained a subpopulation of B-granin positive cells in the majority of the tissues examined (Figs. 2–5).

Moderate to intense immunoreactivity was observed with each antiserum in peripheral pancreatic islet cells, with weak immunoreactivity in a limited number of cells in the islet core (Fig. 2a). However, only peripheral islet cells displayed KELTAE-like (R635.1) immunoreactivity, while H98 was uniformly negative. All antisera immunostained thyroid parafollicular (Fig. 2b) and para-

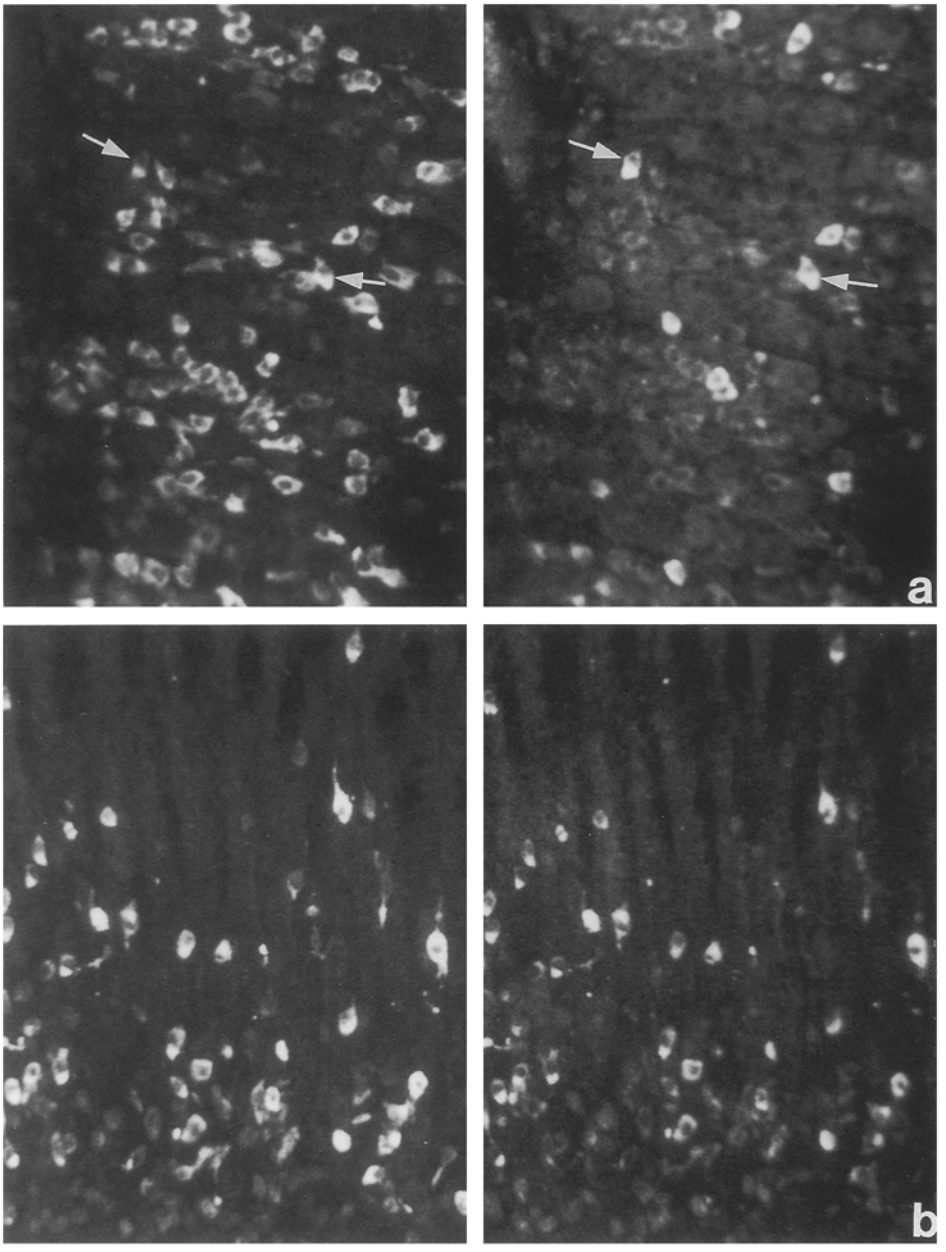

Fig. 4. Double immunostained CSLM displaying (**a**) A87A immunoreactivity (*right*) in a subpopulation of β-granin positive cells (*left*) in the fundus (*arrows*), and (**b**) β-granin (*left*) and SP187 (*right*) immunoreactivity occurring in a similar neuroendocrine cell population in the antrum. $\times 460$

thyroid cells, except antisera A87A and R633.1 which failed to immunostain the parathyroid gland. All adrenomedullary cells displayed moderate or intense immunoreactivity when immunostained with β-granin, H-266, H187, SP187 and R607.1 antisera. Antisera H98 and R635.1 (Fig. 3a) displayed intense immunoreactivity in discrete clusters of adrenomedullary cells. In addition antiserum H98 displayed moderate to weak immunoreactivity in the remaining medullary cells, while weak diffuse KELTAE (R635.1) immunoreactivity was only evident in a limited number of the remaining medullary cells. A major population of cells displaying intense and moderate immunoreactivities were scattered throughout the anterior pituitary when immunostained with N-terminal (β-granin, H266, H187) and C-terminal (SP187) chromogranin A and presumptive C-terminal pancreastatin antisera. Antisera raised to the mid molecular region of chromogranin A; A87A (Fig. 3b), H98, R633.1 and R635.1 immunostained a subpopulation of cells in the anterior pituitary, these cells also display the most intense immunoreactivity with the N- and C-terminal chromogranin A antisera. All intermediate pituitary cells displayed weak to moderate KGQELE and KELTAE immunoreactivity respectively. Fibres displaying KELTAE immunoreactivity were detected in the posterior pituitary. Beta-granin, H266 (Fig. 5b), H187 (Fig. 5a), SP187 (Fig. 4b) and presumptive C-terminal pancreastatin antisera immunostained a typical mucosal endocrine cell population throughout the gastro-intestinal tract. A subpopulation of these cells were immunostained by A87A (Fig. 4a), H98, R633.1 and R635.1 antisera.

Complete preabsorption of the antisera were achieved at 10 $ng \cdot ml^{-1}$ to 10 $\mu g \cdot ml^{-1}$ of their respective peptide fragments. Rat chromogranin A was not available to

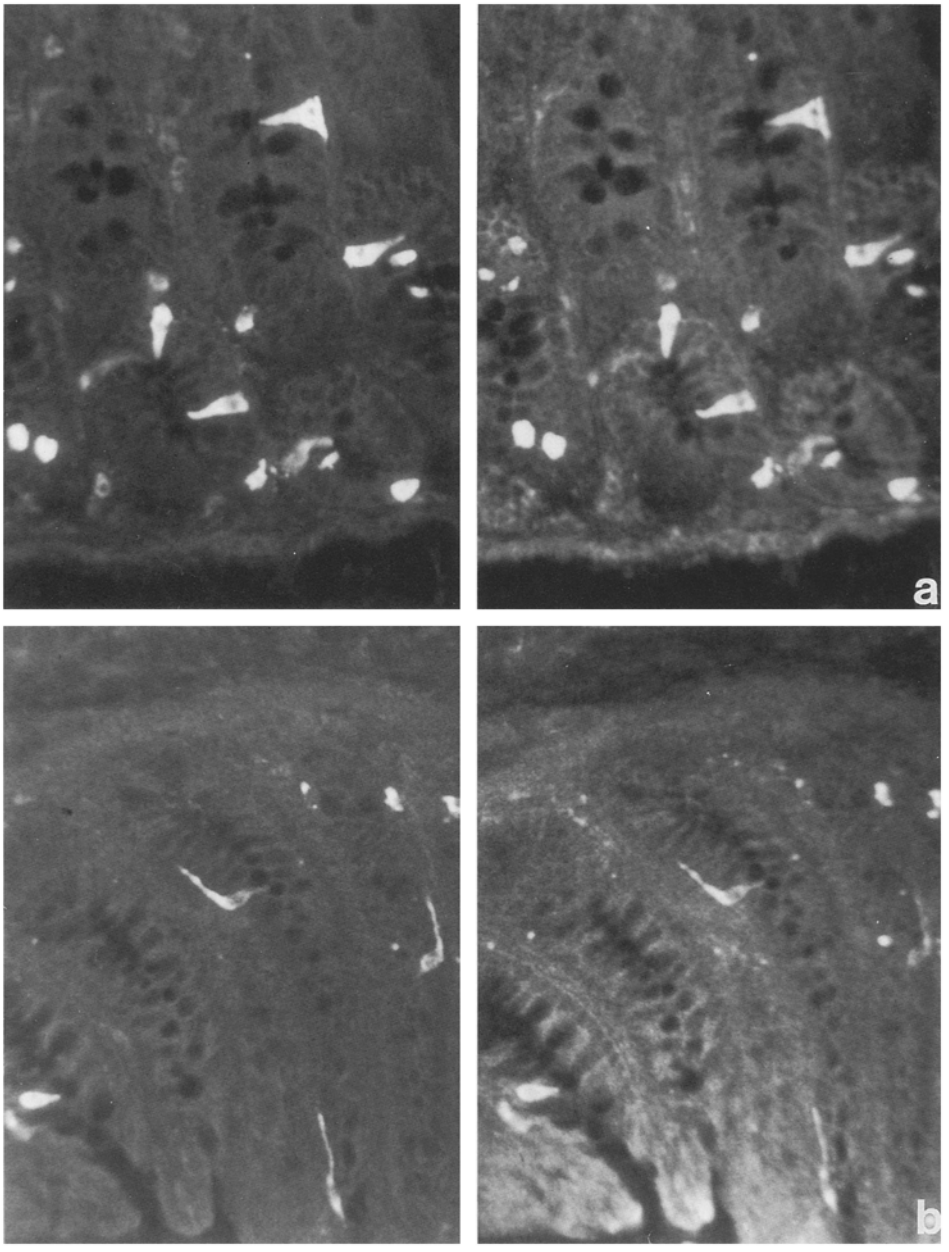

Fig. 5. CSLM of duodenal cells (**a**) displaying β-granin (*left*) and H187 (*right*) immunoreactivity and (**b**) colonic cells double immunostained with β-granin (*left*) and H266 (*right*) antisera. ×460

assess the degree of cross-reactivity of these antisera with it. The techniques employed to eliminate any ionic interaction of the primary antisera did not modify the intensity or cellular distribution of immunoreactivity of any of the antisera.

Western blot analysis

Western blot analysis of insulin secretory granules using the different antisera revealed a number of characteristic patterns of staining (Fig. 6). Sera raised to the N-terminal 21 kDa peptide β-granin showed marked staining of this peptide and of chromogranin A which migrates on these gels at an anomalous molecular size of 100 kDa. As previously shown, the relative intensity of the reactivity of the 21 kDa and 100 kDa bands reflects differences in their abundance rather than major differences in immunoreactivity. The sera H187 and SP187 also decorate the 100 kDa protein to a similar extent as did serum H266 (data not shown). By contrast sera H98 did not decorate this protein though they did react with other peptides resolved by SDS-PAGE. Sera A87A and R635.1 were not reactive on Western blots. The failure of the last two groups of sera to recognize chromogranin A was confirmed by Western blot analysis of adrenal samples; a tissue which contains considerably greater amounts of unprocessed chromogranin A than pancreatic B-cells. The titre of these sera determined with their recombinant antigens were similar and the observed differences in reactivity are attributed to the fact that, on Western blot at least, they recognize conformational epitopes which are not present in the intact chromogranin A molecule.

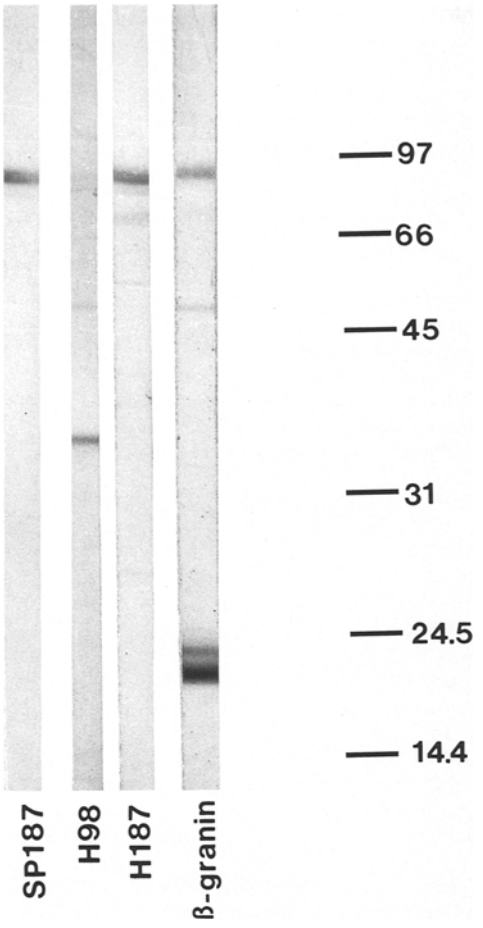

Fig. 6. Western blot analysis. Insulin secretory granule proteins (70 µg protein/cm) were resolved by SDS polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose and reacted with the indicated sera at a 1:100 dilution. The migration of the molecular size marker proteins is indicated

Discussion

The current immunochemical investigation has demonstrated the tissue distribution of chromogranin A-related peptides in rat employing sequence specific antisera raised to chromogranin A sequences of rat origin (Fig. 1). Immunoreactivity to N-terminal (β-granin, H266, H187) and C-terminal (SP187) chromogranin A and presumptive C-terminal pancreastatin antisera (R607.1), were distributed in a parallel manner in all the tissues examined. The distribution and number of cells immunostained with these antisera are comparable with previous investigations of other mammalian neuroendocrine tissues employing either polyclonal or monoclonal antisera to the complete chromogranin A molecule (Grube et al. 1989). A common feature of most of the β-galactosidase hybrid antibodies was their recognition of the intact chromogranin A molecule on Western blots and it cannot be precluded that these antisera detect chromogranin A rather than derived peptides. Nevertheless, with respect to β-granin it is clear that the molecular form detected by immunoblots and pulse-chase experiments varies in a tissue specific manner (Hutton et al. 1988b). Similarly, gel permeation of presumptive rat pancreastatin-like immunoreactivity displays a tissue specific profile (Curry et al. 1990). At best it can be concluded that although the chromogranin A molecule may be extensively processed, the epitopes recognised by these antisera can not be significantly altered by the processing of the primary sequence or any conformational changes occurring in the generation of the peptide fragments. The antigenic fragments corresponding to B-granin, H266 and H187 encompass regions which do not contain any Lys-Arg (KR) and Arg-Arg (RR) dibasic sequences which represent common cleavage sites of peptides from their prohormones (Andrews et al. 1987). However, the antigenic fragment SP187 possesses several RR sites, consequently the parallel immunoreactivity observed with this antiserum may indicate that few if any of these sites are cleaved, though the presence of a dominant antigenic determinant cannot be dismissed.

The observations made with the mid- to C-terminal antisera (A87A, H98, R633.1 and R635.1) in contrast to the above, show considerable variation in each tissue. These antigenic fragments (A87A, H98 and KGQELE) represent the rat pancreastatin-like domain and a region, WE-14 (R635.1), adjacent to its C-terminus. These observations appear to indicate that this region is subject to tissue and cellular specific processing. This conclusion is supported by the recent isolation of a C-terminally extended 8 kDa pancreastatin-like molecule (bovine chromogranin A 248–313) from bovine gastric corpus and small intestinal tissues and a 3 kDa peptide (bovine chromogranin A 297–313) from gastric antral and pancreatic extracts (Watkinson et al. 1991). Therefore, it is possible that the subpopulation of gastro-intestinal cells displaying A87A and KGQELE immunoreactivities may be generating peptides comparable to those isolated from bovine gastro-entero-pancreatic tissues. The failure of the presumptive C-terminal rat pancreastatin antiserum (R607.1) to display a similar pattern of immunoreactivity to that observed with adjacent (KGQELE) and overlapping (A87A) antigenic fragments, may indicate that this epitope is only accessible in a cleaved pancreastatin peptide or the intact chromogranin A molecule and that it may be rendered inaccessible due to conformational changes resulting from the generation of larger peptide fragments.

The finding that the KELTAE and H98 sera displayed tissue-specific distributions and were not immunoreactive with chromogranin A suggests that a peptide corresponding to the residues detected by these antisera may be produced in some neuroendocrine tissues. An obvious candidate is the peptide, WE-14, which is flanked by dibasic KR residues which are known to be sites of proteolysis. It is of interest that the WE-14 sequence shows a very high degree of sequence conservation which is consistent with a potential role as a bioactive peptide.

The availability of region specific antisera will permit further investigation of the extent and variability of cleavage of the chromogranin A molecule. The isolation and characterisation of the specific peptide fragments

resulting from the tissue specific processing of chromogranin A will permit the generation of additional selectively chosen region-specific antisera. The application of these antisera in two site assays should provide an insight into the heterogeneity of peptides and the regulation of their production from the diverse range of neuroendocrine tissues which synthesise the chromogranin A molecule.

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