Large variations in the proteolytic formation of a chromogranin A-derived peptide (GE-25) in neuroendocrine tissues

Rudolf KIRCHMAIR,* Bernd LEITNER,* Reiner FISCHER-COLBRIE,* Josef MARKSTEINER,† Ruth HOGUE-ANGELETTI‡ and Hans WINKLER*||

*Department of Pharmacology, and †Neurochemical Unit, Department of Psychiatry, University of Innsbruck, A-6020 Innsbruck, Austria, and ‡Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, U.S.A.

We have established a radioimmunoassay for GE-25, a peptide present in the C-terminal end of the primary amino acid sequence of chromogranin A where it is flanked by typical proteolytic cleavage sites. Gel-filtration HPLC was used to characterize the molecular sizes of the immunoreactive molecules. The antiserum recognized not only the free peptide but also larger precursors including the proprotein chromogranin A. The tissues with the highest levels of GE-25 immunoreactivity were in decreasing order: the adrenal medulla, the three lobes of the pituitary gland, intestinal mucosa, pancreas and various brain regions. In adrenal medulla and parathyroid gland most of the immunoreactivity was found to be present as intact chromogranin A and some intermediate-sized peptides, without significant amounts of the free peptide. In anterior pituitary, and even more so in intestine, a shift to smaller peptides was seen. In the posterior and intermediate pituitary and in pancreas the predominant immunoreactive material was apparently represented by the free peptide GE-25. In reverse-phase chromatography this peptide eluted exactly like the synthetic standard, which allows a tentative identification as GE-25. In brain tissue the processing of chromogranin A was intermediate, with significant amounts of immunoreactivity corresponding to GE-25 as well as precursor proteins being present. We suggest that in those organs (endocrine pancreas, intermediate and posterior pituitary) where the major hormones are proteolytically processed there is also a concomitant proteolysis of further susceptible peptides. Since GE-25 is apparently formed *in vivo* and is well conserved between species it seems a good candidate for having specific physiological functions.

INTRODUCTION

Chromogranin A is a member of the acidic proteins, the so-called chromogranins [1], found in large, dense-core vesicles of endocrine and nervous tissues [2]. These proteins also comprise the peptides named chromogranin B [3], secretogranin II [4] and 7B2 [5]. In bovine adrenal chromaffin granules chromogranin A is proteolytically processed to smaller peptides [6,7], a process which can start both from the N- and C-terminal sites [8–10]. The N-terminal peptide [chromogranin A (1–76)], generated *in vivo* by endoproteases, represents a significant component in these organelles [8,9] and is secreted from adrenal medulla [11] and the parathyroid gland [12]. The name vasostatin [13] has been given to this peptide and to an elongated form of this peptide derived by cleavage at the second pair of basic amino acids [chromogranin A (1–114)]. An analogous peptide found in rat pancreas [chromogranin A (1–131)] has been named β-granin [14]. Vasostatin, whose sequence is relatively well conserved between species [2], has been shown to inhibit vasoconstriction induced by endothelin [13] as well as release of hormones from several endocrine cells, i.e. from parathyroid cells [15], and calcitonin release from a lung tumour cell line [16]. The whole chromogranin A precursor molecule was demonstrated to inhibit parathyroid cell secretion [17,18] as well as pro-opiomelanocortin release from AtT20 cells [19].

Another peptide [rat chromogranin A (248–295)] with a defined function is pancreastatin ([20]; for further references see [2]) whose sequence, however, appears much less conserved than that of the N-terminal peptides. This is even more marked for chromatostatin [chromogranin A (123–148)] whose formation *in vivo* has never been demonstrated and for which a claim that it inhibits catecholamine secretion has now been withdrawn [21].

The C-terminal end of chromogranin A, which just like the N-terminal end is well conserved between species [2], contains six potential cleavage sites likely to be sensitive to the endoproteases PC1 and PC2 which are also found in chromaffin granules [22,23]. However, studies on peptides formed from this chromogranin A stretch have been limited. One peptide flanked by cleavage sites [WE-14: bovine chromogranin A (316–329)] has been shown to occur in carcinoid tumours [24]. A fragment of chromogranin A generated *in vitro* by digestion with the endoprotease Lys-C, starting at position 347 of porcine chromogranin A and spanning to the C-terminus, was shown to inhibit parathyroid cell secretion [25] and was therefore named parastatin. The first 19 amino acids of this fragment representing the C-terminus of the LL 33 peptide (see Figure 1) were equally as potent in biological activity as total parastatin [25]. For the

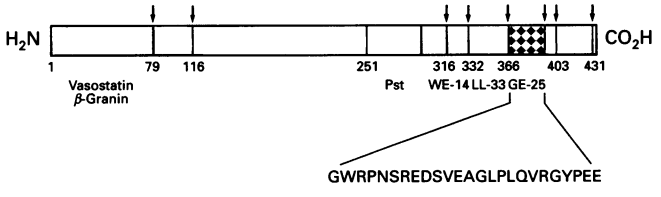

Figure 1 Schematic diagram of the chromogranin A precursor

The sequence of bovine chromogranin A [26,27] is schematically presented. Proteolytic cleavage sites (pairs of basic amino acids) are indicated by arrows. Pst, pancreastatin. The abbreviations of the putative peptides WE-14, LL-33 and GE-25 indicate the first and last amino acid of the peptide given in the single letter code plus the total number of amino acids present.

Abbreviations used: RIA, radioimmunoassay; TFA, trifluoroacetic acid; ACTH, corticotrophin.

|| To whom correspondence should be addressed.

present study we developed a radioimmunoassay (RIA) against one of the C-terminal peptides [bovine chromogranin A (366–391)] of chromogranin A. We tried to answer the following questions. (i) Is this peptide formed in endocrine and nervous tissue? (ii) Does the proteolytic processing of chromogranin A leading to this peptide vary amongst the different tissues? (iii) If so, how can these differences in processing be explained?

MATERIALS AND METHODS

Preparation of tissue extracts

Tissues were obtained from the local slaughterhouse. After dissection of the organs, tissue samples were chilled on ice immediately. Extractions were performed by sonication (10 s) in distilled water (1 ml/100 mg wet weight), followed by immediate boiling for 10 min. Boiled samples were centrifuged for 15 min at 14000 *g* and supernatants used for further determinations. This procedure was chosen to prevent any proteolytic breakdown during extraction (only 10 s elapsed from the beginning of the extraction till boiling, which inactivates any protease activity) and to ensure maximal extraction. This latter aim was tested by extracting the tissue as described above or with 2 M acetic acid (final concentration) or 0.1 M HCl (final concentration). These procedures only yielded 74 and 63% extraction efficiency of immunoreactivity respectively (as measured by RIA) when compared with the above procedure.

HPLC

Extracts were applied to a Superose 12 HR 10/30 gel-filtration column (Pharmacia LKB, Uppsala, Sweden) using a 37.5 mM sodium phosphate buffer, pH 7.4, containing 37.5 mM NaCl as solvent. Proteins were separated at a flow rate of 0.4 ml/min. Fractions (0.4 ml) were collected and analysed by RIA. Some probes were subjected to C_{18} reverse-phase HPLC (Waters) with 0.1% trifluoroacetic acid (TFA) as solvent. Peptides were eluted at a flow rate of 1 ml/min with a 0–70% gradient of acetonitrile containing 0.1% TFA. Fractions (1 ml) were collected and analysed.

RIA

The peptide GE-25 was synthesized by standard t-Boc chemistry to comprise residues 367–391 of the bovine chromogranin A primary amino acid sequence [26,27] and purified by reverse-phase HPLC. New Zealand White rabbits were immunized with the peptide coupled to keyhole limpet haemocyanin via the benzoquinone procedure [28]. GE-25 was iodinated by the chloramine-T method and purified by reverse-phase HPLC. For RIA, GE-25 peptide and iodinated peptide were used as standard and tracer respectively. Antibody (final dilution 1:9000) was incubated with standards and samples for 24 h at 4 °C in RIA buffer (for details see [29]). Then trace (20000 d.p.m./tube) was added and samples and standards incubated for a further 24 h. Bound/free separation was performed by adding 1 ml of dextran-coated charcoal, incubation for 15 min and centrifugation at 3700 *g* for 15 min. The supernatant was counted for radioactivity in a γ-counter. The sensitivity of the assay was 50 fmol. The antibody did not cross-react with other neuropeptides tested, e.g. substance P, neuropeptide Y, neurotensin, calcitonin gene-related peptide, nor with peptides derived from chromogranin A (LE-40, WE-14), from secretogranin II (secretoneurin, EL-17, LF-19, YM-22) or from chromogranin B (PE-11).

Immunocytochemistry

Three bovine pituitary glands were sliced and immediately immersed in cold 4% paraformaldehyde in PBS for 1 week. After rinsing in PBS for 1 day, slices were transferred to 5, 15 and 20% sucrose in PBS for 1 day each. Slices were frozen in isopentane (−45 °C) for 3 min and stored at −70 °C. Serial coronal and transverse sections (60–80 mm) were cut on a cryostat (Reichert, Vienna, Austria). Free-floating sections were processed according to the indirect peroxidase–antiperoxidase technique. The GE-25 antiserum was used at a dilution of 1:750 in Tris/HCl-buffered saline and incubated for 48 h at 4 °C. Controls using no primary antiserum and antisera pre-adsorbed with the synthetic GE-25 peptide (10 mM, 24 h, 4 °C) were included in each experiment.

Immunoblotting

One-dimensional SDS gel electrophoresis providing special resolution in the low molecular mass range was performed as described previously [30]. This was followed by immunoblotting as already described in detail [3]. Antisera dilutions {anti-GE-25 and anti-[human corticotrophin (ACTH)] (Dako)} were 1:200. Labelled proteins were visualized by ^{125}I-Protein A (NEN), excised from nitrocellulose and radioactivity counted in a γ-counter.

RESULTS

Levels of GE-25 immunoreactivity in neuroendocrine tissues

Table 1 gives the GE-25 immunoreactivity levels as measured by RIA in various tissues. By far the highest concentration is found in the adrenal medulla; intermediate levels are found in the various parts of the pituitary gland, the intestine and the pancreas. In brain several regions have similar concentrations. In the pituitary a high concentration was found in the intermediate

Table 1 Levels of GE-25 immunoreactivity in bovine neuroendocrine tissues

Extracts of various tissues were analysed by RIA. The results are expressed as fmol of GE-25 per mg of tissue (mean ± S.E.M.); *n*, number of samples.

Tissue	Amount (fmol/mg)	*n*
Adrenal medulla	30490 ± 610	3
Pituitary gland		
Anterior lobe	1323 ± 180	3
Intermediate lobe	733 ± 170	4
Posterior lobe	146 ± 6	3
Pancreas	45 ± 3	4
Intestine (duodenal mucosa)	459 ± 71	4
Intestine (ileal mucosa)	112 ± 27	4
Brain		
Frontal cortex	13 ± 1	4
Hippocampus	11 ± 1	4
Caudate nucleus	14 ± 2	4
Thalamus	7 ± 1	4
Hypothalamus	16 ± 2	4
Cerebellum	3 ± 0.4	3
Brainstem	6 ± 0.1	3
Spinal cord	1.5 ± 0.1	5

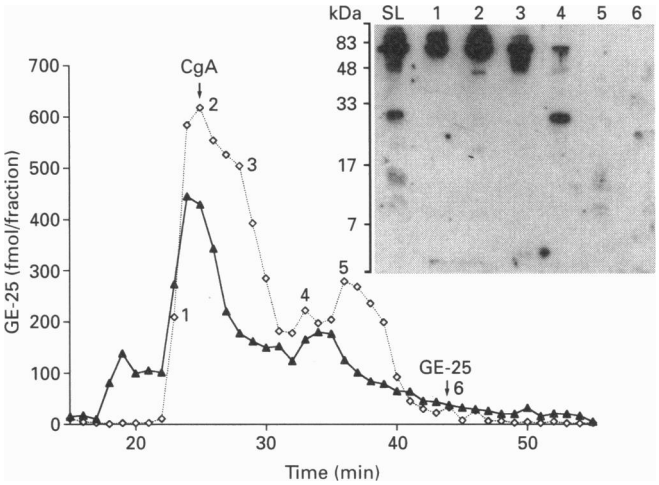

Figure 2 HPLC of extracts from bovine adrenal medulla (◇) and parathyroid gland (▲)

Extracts were subjected to molecular-sieve chromatography. The eluted fractions were analysed by RIA. The elution positions of intact chromogranin A (CgA) and of the free peptide GE-25 are indicated. The inset presents immunoblotting patterns of the total extracts of adrenal medulla (SL) and of the fractions eluted from the column (numbered 1–6).

lobe. Since it is difficult to completely separate this lobe from the posterior one by dissecting, additional experiments were performed to ascertain that the posterior lobe actually contained chromogranin A. Immunoblot analysis (results not shown) for ACTH, a typical component of the intermediate lobe, revealed that the posterior lobe preparation was at most 5 % contaminated with intermediate lobe tissue. Immunohistochemistry established that both the posterior and intermediate lobes displayed a significant density of GE-25-like immunoreactivity. Control sections treated with either pre-immune sera or pre-absorbed sera were free of reaction (results not shown).

Characterization of GE-25 immunoreactivity by HPLC

Figure 2 demonstrates the results obtained with an extract of bovine adrenal medulla separated by gel-filtration chromatography. In the position where the free peptide GE-25 is eluted no significant immunoreactivity was found with the RIA. Thus most of the immunoreactivity was attributable to larger peptides. Analysis by SDS gel electrophoresis and immunoblotting revealed that the major immunoreactive peak (the fraction numbered 2 and lane 2 of the immunoblot in Figure 2) corresponds to chromogranin A. Higher-molecular-mass material present in the earlier fractions of this peak probably represents the proteoglycan form of chromogranin A [31]. In the fraction numbered 3 (in Figure 2) smaller immunoreactive bands are present, in fraction 4 there is one strongly staining band of molecular mass 30 kDa and in fraction 5 two rather diffusely migrating bands. In the position where the free peptide GE-25 elutes (fraction 6) there is no immunoreactive band. In the parathyroid gland, intact chromogranin A is apparently the most abundant chromogranin A species (Figure 2).

Extracts of the pituitary yielded quite different results. In the anterior pituitary, medium and small sized immunoreactive peptides were prominent, whereas in the intermediate lobe the major immunoreactive peak eluted exactly in the position of the free peptide GE-25 (Figure 3a). Also in the posterior lobe the free peptide appeared to be the dominant form, whereas in the pituitary stalk intermediate peptides were also prominent (Figure 3b). The synthetic GE-25 peptide eluted in fraction 42 by gel-filtration chromatography and in some experiments in fraction 43, due to slight variations in the system. In order to ascertain that the peptide eluting in the position of GE-25 actually represented this peptide reverse-phase chromatography was performed. As shown in Figure 4 the GE-25 standard and the pituitary peptide eluted in exactly the same position.

In extracts from the intestinal tract, intermediate size peptides predominated (see Figure 3c) whereas in the pancreas the main immunoreactive material corresponded to the free peptide GE-25 (see Figure 3c). When this peak material was subjected to reverse-phase chromatography it eluted exactly in the position of the GE-25 standard (see Figure 4).

In brain extracts (see Figure 3d) elution patterns comparable to that of the anterior pituitary (cf. Figure 3a) were found. Apparently little intact chromogranin A was present. Mainly intermediate-sized peptides and, especially in the hypothalamus (and also frontal cortex; not shown), free GE-25 was prominent (Figure 3d).

DISCUSSION

The RIA detected not only GE-25, against which the antiserum was raised, but also the intact proprotein chromogranin A and intermediate peptides derived from it. It is therefore not surprising that the tissue levels for GE-25 immunoreactivity agreed well with previous studies [32] using antisera against chromogranin A. By far the highest concentration of this peptide is found in adrenal medulla with lower levels present in the pituitary, the intestinal mucosa and the pancreas. With these two latter organs one has of course to consider that the chromogranin A-expressing cells, the endocrine pancreatic cells and the intestinal endocrine cells, constitute only a small proportion of the total tissues. Thus, the chromogranin A concentration in these cells and in their large, dense-core vesicles is obviously much higher. However, whereas in chromaffin granules chromogranin A is the major peptidic component [2], in insulin granules it represents only about 1 % of the secretory proteins [33].

GE-25 immunoreactivity was found in all three parts of the pituitary. For the anterior and intermediate lobe this is in agreement with previous studies on several species [2], whereas for the posterior lobe of sheep a lack of immunostaining for chromogranin A was reported [34]. It may be significant that these negative data were obtained with an antiserum against intact chromogranin A, since in this organ chromogranin A seems to be completely processed (see below). In fact, antisera against pancreastatin strongly stained the posterior lobe of pig pituitary [35,36] and by RIA significant chromogranin A levels were found in bovine posterior lobe [37]. The present study confirms by RIA and immunohistochemistry that the bovine posterior lobe contains chromogranin A immunoreactivity.

In brain, GE-25 immunoreactivity was found to be widely distributed, which is in agreement with previous immunochemical [7,35,38,39], immunohistochemical [40] and immunoblotting [41] data and also with the distribution of chromogranin A mRNA [42,43].

The degree of proteolytic processing of chromogranin A varies widely between different neuroendocrine organs In adrenal medulla, and even more so in the parathyroid, this processing is very limited (for previous data see [2]); no significant amounts of immunoreactivity corresponding to free GE-25 are found in these organs. In the anterior pituitary the endogenous breakdown of chromogranin A is higher, with significant levels of such immunoreactivity. This contradicts previous data [9], apparently

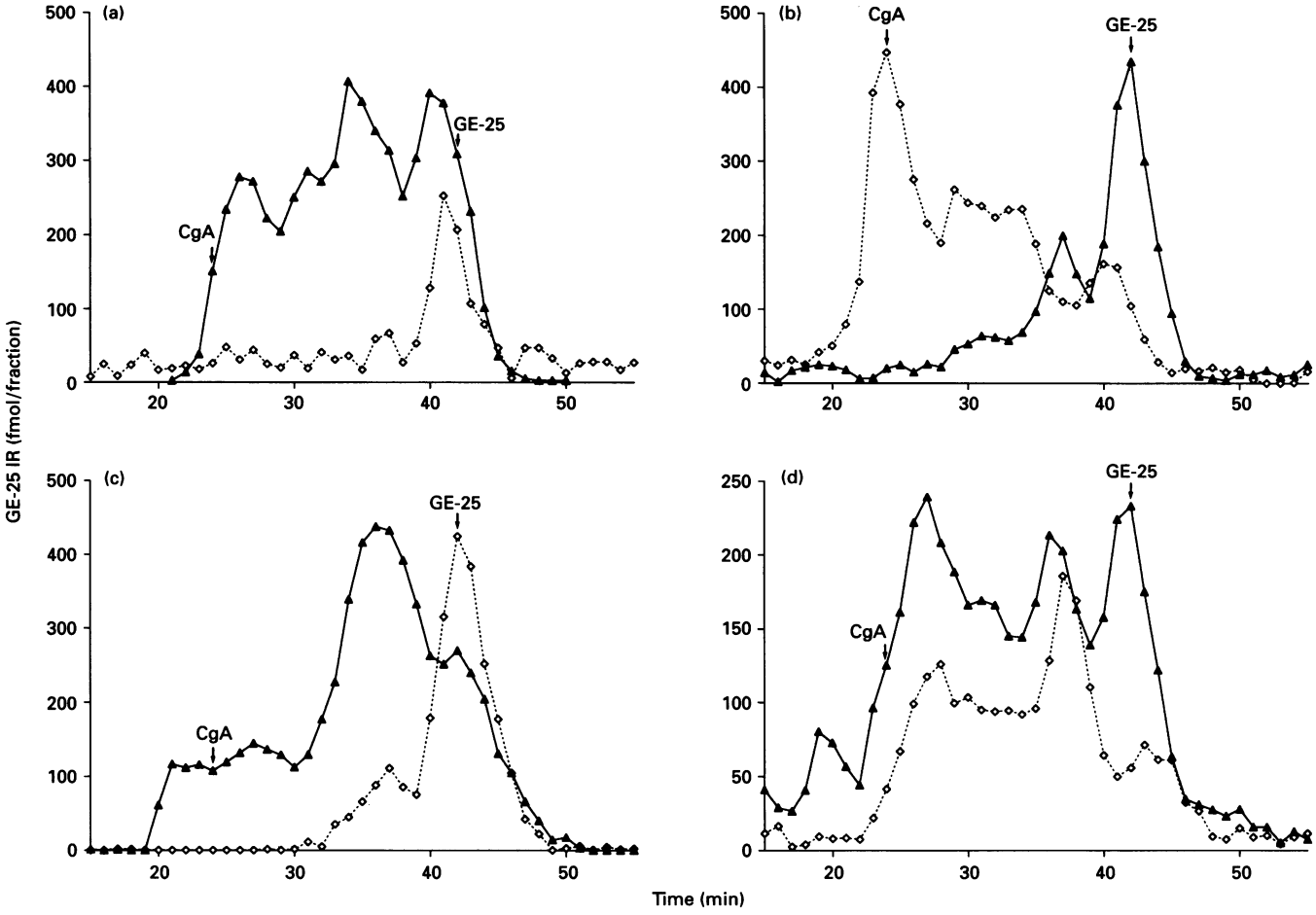

Figure 3 HPLC of extracts from various tissues subjected to molecular-sieve chromatography

(**a**) Bovine pituitary anterior (▲) and intermediate (◇) lobes; (**b**) pituitary stalk (◇) and posterior lobe (▲); (**c**) pancreas (◇) and duodenal mucosa (▲); and (**d**) brain frontal cortex (▲) and hippocampus (◇). The elution positions of chromogranin A (CgA) and of the free peptide GE-25 are indicated.

demonstrating a lower processing in anterior pituitary when compared with adrenal medulla. However, their conclusion was based on immunoblotting of extracts, a method which misses smaller peptides. In the intestine and especially in the pancreas, chromogranin A was processed to a high degree. This is in agreement with previous studies employing an RIA against bovine pancreastatin and related peptides [44] (for rat tissues see also [45–47] and for porcine pancreas [48]). Watkinson et al. [44] described a practically complete breakdown of chromogranin A in the pancreas and considerable breakdown in the intestine, whereas only traces of free pancreastatin could be detected in the adrenal medulla (compare absence of GE-25 in this organ; see above). For the endocrine pancreas the fast processing of chromogranin A (half time 30 min) was also established in a pulse-label study [47,49]. A nearly complete processing of chromogranin A was also found in the present study for the intermediate and posterior pituitary. When the pituitary stalk was analysed a more limited but still considerable breakdown of chromogranin A was detected, indicating that processing of this peptide occurs already during axonal transport in the hypothalamo–pituitary neurons, which is also the case for the vasopressin precursor [50], for prosomatostatin [51] and for secretogranin II [52]. For posterior pituitary and for pancreas the immunoreactivity corresponding to the free peptide GE-25 after molecular-sieve chromatography was further characterized by reverse-phase chromatography. The immunoreactive material eluted exactly in the position of GE-25. This allows the tentative conclusion that the GE-25 immunoreactive material eluting in molecular-sieve chromatography as the free peptide GE-25 actually represents this peptide.

In the various brain regions only small amounts of the intact chromogranin A molecule but higher amounts of the first major breakdown product were present (apparent molecular mass 67 kDa; see Figure 3). These two components have previously been detected by immunoblotting [40,41]. In addition, intermediate chromogranin A peptides and a considerable amount of immunoreactivity corresponding to free GE-25 were apparently found. Previous studies on chromogranin A peptides in neurons are limited; however, the apparent formation of pancreastatin in porcine hypothalamus and posterior pituitary has been reported [35], which is in good agreement with our present results, demonstrating the presence of immunoreactivity corresponding to the free peptide GE-25.

What causes the great differences in proteolytic processing amongst the various neuroendocrine organs? It appears significant that in the three organs with the highest degree of chromogranin A processing, the major secretory constituents of the large, dense-core vesicles, i.e. proinsulin, vasopressin–

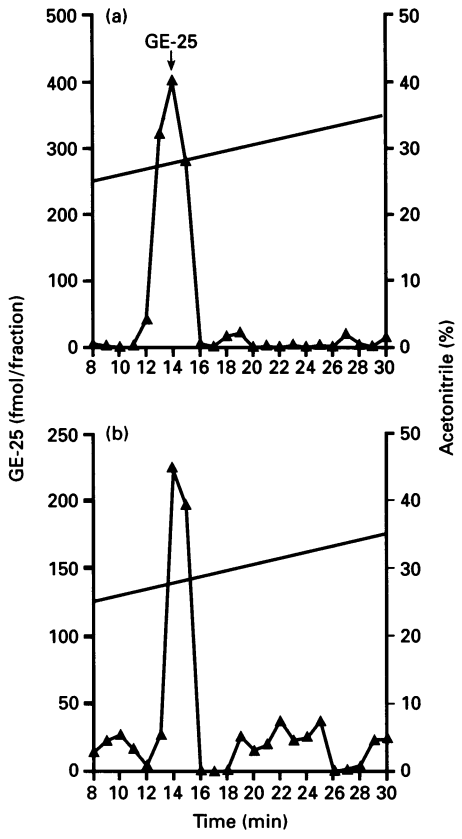

Figure 4 Reverse-phase chromatography of pancreas (a) and of pituitary extracts of the posterior lobe (b)

The extracts were first subjected to molecular-sieve chromatography. The respective peak fractions corresponding to the free peptide [cf. Figures 3(b) and 3(c)] were then subjected to reverse-phase chromatography. The eluted fractions were analysed by RIA. The elution position of the synthetic standard (GE-25) is indicated.

neurophysin and pro-opiomelanocortin, have to be proteolytically processed. On the other hand, the major hormones of the anterior pituitary are not processed in this way and accordingly chromogranin A breakdown is limited. We have recently shown that in posterior pituitary also, another chromogranin, i.e. secretogranin II, is completely broken down, leading to the formation of the neuropeptide secretoneurin [52]. The proteases likely to be responsible for this processing are the endoproteases PC1 and PC2 [53]. We have shown that the degree of secretogranin II processing in large, dense-core vesicles of sympathetic nerves and adrenal medulla correlates with the concentration of these proteases in their secretory content [54]. High concentrations of these enzymes are also found in the endocrine pancreas [55] and the intermediate and posterior pituitary lobes [23,56]; however, at least for the endocrine rat pancreas, the most recent evidence indicates that PC2 appears to be much more active than PC1 in processing chromogranin A [47]. In any case, we can propose the following concept: vesicles whose major hormones have to be proteolytically processed contain a high concentration of PC1 and PC2. All peptides within these vesicles having cleavage sites susceptible to these endoproteases are processed concomitantly. Therefore in such vesicles all proproteins with susceptible cleavage sites are processed to a high degree.

We have presented evidence that GE-25 is formed from chromogranin A in several tissues. Since this peptide is well conserved between species it is a good candidate for having a biological function. Like pancreastatin it is not found in any significant amount in the adrenal medulla, therefore a possible function of GE-25 is unlikely to be connected with chromaffin cells. The endocrine pancreas, the intermediate pituitary or neurons are more likely candidates.

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