Peptidyl-glycine α -amidating mono-oxygenase activity towards a gonadotropin-releasing-hormone C-terminal peptide substrate, in subcellular fractions of sheep brain and pituitary

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The amidation of a synthetic peptide D-Tyr-Pro-Gly-Gly by sheep hypothalamic and pituitary preparations was measured. This substrate was designed as a glycine-extended C-terminal peptide analogue of gonadotropin-releasing hormone (GnRH) to test the ability of these tissues to convert the product produced by cleavage of the GnRH prohormone into the active amidated decapeptide. An α -amidating activity capable of converting D-¹²⁵I-Tyr-Pro-Gly-Gly into D-¹²⁵I-Tyr-Pro-Gly-NH₂ was identified in crude synaptosomal and neurosecretory-granule fractions from hypothalamus and anterior-pituitary secretory-granule preparations. This activity was stimulated by the addition of Cu²⁺ and reduced ascorbate, and was maximal at neutral pH in sulphonic acid buffers. Highest activity was measured in synaptosomes from the median eminence and medial basal hypothalamus and in pituitary granules. Lower activity was found in synaptosomes prepared from anterior hypothalamic tissue. Negligible activity was measurable in cerebral cortex and none in pineal synaptosomes. Direct comparison of α -amidation with D-¹²⁵I-Tyr-Pro-Gly-Gly and a previously reported substrate D-¹²⁵I-Tyr-Val-Gly showed that, although the latter was 15–20-fold more reactive, the optimal concentration of Cu²⁺ for amidation was similar with both substrates in medial-basal-hypothalamic synaptosomes and pituitary granules. Activity measured with 1 μ M-D-¹²⁵I-Tyr-Val-Gly was inhibited by increasing concentrations of D-Tyr-Pro-Gly-Gly, with 50 % inhibition at 25 μ M-D-Tyr-Pro-Gly-Gly, whereas activity with 3.3 μ M-D-¹²⁵I-Tyr-Pro-Gly-Gly was abolished by addition of 1 μ M-D-Tyr-Val-Gly, evidence that the two substrates were competing for the same enzyme activity. Synaptosomal preparations demonstrated Michaelis–Menten kinetics for D-Tyr-Pro-Gly-Gly as substrate, with values of K_m and V decreasing upon removal of ascorbate. We conclude that D-Tyr-Pro-Gly-Gly-directed α -amidation in sheep hypothalamic synaptosomes resembles the activity with D-Tyr-Val-Gly as substrate, as well as that demonstrated by others with D-Tyr-Val-Gly as substrate in rat hypothalamic and pituitary tissue. Although reactivity towards D-Tyr-Pro-Gly-Gly cannot be assumed to assess amidation solely of GnRH, the negligible D-Tyr-Pro-Gly-Gly-directed activity in the pineal gland and cerebral cortex, areas that are known to synthesize other α -amidated peptides, suggests some substrate specificity in α -amidating enzymes from different tissues.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide secreted from the hypothalamus to stimulate release of the gonadotropins from the pituitary. It has been shown to be synthesized in the rat anterior hypothalamus as a precursor protein (Phillips *et al.*, 1985) that is processed to the bioactive decapeptide by a series of enzymic reactions (Lazure *et al.*, 1983) before secretion from terminals in the median eminence. GnRH and many other bioactive peptides have an α -amidated C-terminus, which protects the hormone from carboxypeptidase action and which is often required for full bioactivity (Eipper *et al.*, 1983).

In all such α -amidated peptides where the amino acid sequence of the precursor has been deduced, the amidated residue is followed by a glycine residue (Eipper *et al.*, 1983). During amidation of a synthetic peptide, C-terminal glycine has been shown to donate its amine group (Bradbury *et al.*, 1982) in an enzymic reaction

requiring O₂ and Cu²⁺ (Eipper *et al.*, 1983). This peptidyl-glycine α -amidating mono-oxygenase (PAM) activity has been characterized previously in rat pituitary (Eipper *et al.*, 1983; Landymore-Lim *et al.*, 1983; Kizer *et al.*, 1984) and hypothalamus (Emeson, 1984), with the synthetic substrate D-Tyr-Val-Gly representing the C-terminus of α -melanotropin.

We have designed for investigation a substrate, D-Tyr-Pro-Gly-Gly, to mimic more closely a glycine-extended C-terminus of the GnRH decapeptide and have characterized PAM enzymic activity acting towards this substrate in subcellular fractions from sheep brain and pituitary.

MATERIALS AND METHODS

Chemicals

Sucrose, Na₂S₂O₈, EDTA, poly(ethylene glycol) 6000 and Folin & Ciocalteu phenol reagent were from BDH

Abbreviations used: GnRH, gonadotropin-releasing hormone (gonadoliberin); PAM, peptidyl-glycine α -amidating mono-oxygenase.

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Chemicals. Synthetic GnRH was from Beckman Instruments, Palo Alto, CA, U.S.A., the anion-exchange resin AG1-X8 was from Bio-Rad Laboratories, Pharmacia Fine Chemicals supplied the SP-Sephadex C-25 and the Percoll density-gradient medium, and bovine fraction II γ -globulin was obtained from Calbiochem-Behring. All other biochemicals and Dowex 50W cation-exchange resin were from Sigma Chemical Co.

The substrate (D-Tyr-Pro-Gly-Gly) and product (D-Tyr-Pro-Gly-NH₂) peptides for measuring α -amidation activity were synthesized by Dr. D. R. K. Harding by sequential addition of t-butyloxycarbonyl-amino acids to the N-terminus of glycine methyl ester or glycyglycine ethyl ester. The peptides were eluted as single peaks on preparative reverse-phase h.p.l.c. on a Lobar RP-8 (size B) column with a step gradient of methanol/water/formic acid (5:95:2–80:20:2, by vol.) as eluent. The amino acid compositions of these peptides were confirmed by two independent amino acid analyses (Dr. P. F. Davis) and by sequence analysis of D-Tyr-Pro-Gly-Gly (Department of Biochemistry, University of Auckland, Auckland, New Zealand).

A sample of D-Tyr-Val-Gly was generously provided by Professor B. A. Eipper.

Tissue dissection

Brain and pituitary tissue was taken from non-pregnant 2–4-year-old ewes from the local abattoir within 20 min of death, and chilled on ice. Pieces of frontal-lobe cerebral cortex, the pineal body and median eminence were dissected out. The medial basal hypothalamus, defined as the area above the median eminence bounded rostrally by the posterior edge of the optic chiasma and caudally by the posterior edge of the optic chiasma, was approx. 10 mm $\times$ 5 mm. A thin frontal slice of anterior-hypothalamic tissue was cut from the chilled brain, rostral to the anterior edge of the optic chiasma and 2–4 mm thick. This slice was trimmed to remove white matter, leaving a roughly triangular piece. The ventral edge of the slice contained supraoptic and suprachiasmatic nuclei plus the organum vasculosum of the lamina terminalis. The remaining tissue (preoptic area) contained the septal nuclei, preoptic area and medial preoptic nucleus, and parts of the supra-chiasmatic, lateral and anterior-hypothalamic nuclei. The posterior-pituitary and neural tissue were removed from anterior pituitary glands.

Preparation of subcellular fractions

The brain tissue was weighed and homogenized at 0–4 °C in 10 vol. of 0.32 M-sucrose containing 10 μ M-CaCl₂. Tissue was homogenized (Ultra-Turrax homogenizer) in three 10 s bursts between 5 s of cooling. Subfractionation was by the method of Warberg (1982). After centrifugation at 900 g, the supernatant was layered on discontinuous density gradients of equal volumes of 0.8 M- and 1.2 M-sucrose containing 10 μ M-CaCl₂. The density gradients (12 ml) were centrifuged at 100 000 g for 30 min. A pellet (C) and three layers were retained: the upper layer (S2) of clear cellular cytoplasm and two opaque membranous layers at the 0.32 M-/0.8 M-sucrose and 0.8 M-/1.2 M-sucrose interfaces (A and B). GnRH-containing neurosecretory granules were prepared by differential centrifugation of hypo-osmotically shocked homogenates (Barnea *et al.*, 1978; Burrows *et al.*, 1981). Anterior-pituitary secretory granules were prepared

from homogenates by centrifugation on Percoll density gradients (Eipper *et al.*, 1983). Subcellular fractions were either sonicated (30 s at 30 W, in 10 s bursts with cooling at 0–4 °C between) or frozen on solid CO₂ and thawed to 4 °C before assay.

Characterization of subcellular fractions

Lactate dehydrogenase activity was measured spectrophotometrically (Dixon & Webb, 1964), cytochrome oxidase activity by continuous spectrophotometric assay (Bramley & Ryan, 1978), NADPH-cytochrome *c* reductase activity by the method of Dallner *et al.* (1966) and protein by the procedure of Markwell *et al.* (1978) with bovine serum albumin as standard. Fractions were examined by Dr. J. St.J. Wakefield with a Siemens 102 electron microscope after fixation in iso-osmotic buffered half-strength Karnovsky's fixative (Karnovsky, 1965), post-fixation in OsO₄, dehydration, and embedding in Epon 812.

Extraction and radioimmunoassay of GnRH

Samples of the subcellular fractions were extracted in 0.2 M-acetic acid by addition of 2 M-acetic acid directly to the sample at 4 °C, followed by homogenization. The samples were then neutralized and re-centrifuged before radioimmunoassay for GnRH with antiserum CRR11B73 (a gift from Dr. Y. F. Chen and Dr. V. D. Ramirez), which requires the complete decapeptide for binding (King *et al.*, 1985). The final dilution of antiserum was 1:50 000 in 0.02 M-sodium phosphate buffer, pH 7.5, containing 0.15 M-NaCl, 0.1% NaN₃, 0.05 M-EDTA and 0.25% (v/v) normal rabbit serum.

Synthetic GnRH was used as the assay standard and was iodinated by the procedure described by Nett *et al.* (1973). Standards (0–680 fmol/tube) and unknowns (20–100 μ l) were diluted to 320 μ l in radioimmunoassay buffer (0.02 M-sodium phosphate buffer, pH 7.5, containing 0.15 M-NaCl, 0.1% NaN₃, 0.1% gelatin and 1 mM-bacitracin) in polypropylene tubes, and 100 μ l of antibody solution was added before equilibration at 4 °C. Duplicate determinations of non-specific binding were made for all unknowns and standards by omission of antiserum. After 20 h, 80 μ l of ¹²⁵I-GnRH (10 000–50 000 c.p.m.) was added and left to react for a further 24 h. γ -Globulin (1 mg; 20 μ l) in radioimmunoassay buffer was then added to each tube, followed by 1.6 ml of 27% (w/v) poly(ethylene glycol) in 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and 0.1% NaN₃, and the tube contents were well mixed and centrifuged at 2000 g for 10 min at 20 °C. A minimum of 2000 counts was collected. Doses and confidence limits were estimated from the data after correction for non-specific binding by using computer programs described by McIntosh & McIntosh (1980). The inter-assay coefficient of variation (c.v.) for the CRR11B73 antiserum was 20% and the intra-assay c.v. ranged from 5 to 7% in the region of the maximum precision in the assay (5–250 fmol/tube). The limit of detection (least dose significantly different from zero) was 0.2–1 fmol/tube.

Iodination of synthetic peptides

D-Tyr-Pro-Gly-Gly and D-Tyr-Pro-Gly-NH₂ were iodinated by using an adaptation of the chloramine-T method. Briefly, approx. 10 μ g of peptide was dissolved in 20 μ l of 150 mM-Tris/HCl buffer, pH 7.5, and kept at

Table 1. Activities of marker enzymes and GnRH immunoactivity in subcellular fractions of the median eminence of sheep hypothalamus

Specific activity is defined as nmol conversion/min per mg of protein and specific content as pg of GnRH/mg of protein. Percentage recovery is defined as $100 \times (\text{total activity in fraction}) / (\text{total activity in homogenate})$. **Bold** values emphasize maximal values. Results are expressed as the range of values and percentage recoveries obtained from *n* subfractionation experiments.

	Lactate dehydrogenase (<i>n</i> = 2)	Cytochrome oxidase (<i>n</i> = 3)	NADPH- cytochrome <i>c</i> reductase (<i>n</i> = 2)	GnRH (<i>n</i> = 3)
Homogenate	36–55	150–310	1.1–2.3	78–111
A: microsomes/myelin				
Specific activity	21–42	7–30	2.1–5.2	45–125
Percentage recovery	11–16 %	0.4–3 %	31–53 %	8–10 %
B: synaptosomes				
Specific activity	31–37	118–458	0–1.2	270–620
Percentage recovery	6–7 %	13–22 %	0–13 %	70–77 %
C: mitochondria				
Specific activity	0–8	433–2435	0	45–187
Percentage recovery	0–< 1 %	29–33 %	0 %	2–4 %
S2: cytosol				
Specific activity	120–210	0	0.4–1.3	17–283
Percentage recovery	111–122 %	0 %	12–23 %	4–22 %
Neurosecretory granules				
Specific activity	5–26	141–248	Not measured	173–548
Percentage recovery	1–9 %	24–43 %		32–70 %

4 °C. To this was added 0.5 mCi of Na¹²⁵I (5 μ l) and 10 μ l of chloramine-T (1 mg/ml), and the peptide was iodinated for 30 s at 4 °C. The reaction was stopped by addition of 10 μ l of Na₂S₂O₅ (10 mg/ml) and 100 μ l of 150 mM-Mes/NaOH buffer, pH 5.0. The reactants were then eluted on a 2.0 ml column of AG1-8X anion-exchange resin, equilibrated in 50 mM-Mes/NaOH buffer, pH 5.0, containing 0.5 M-NaCl (Chard *et al.*, 1970). Some 15–25 % of the ¹²⁵I was eluted with the peptides.

Separation of D-Tyr-Pro-Gly-Gly and D-Tyr-Pro-Gly-NH₂

Synthetic iodinated D-Tyr-Pro-Gly-Gly and D-Tyr-Pro-Gly-NH₂ were separated on a 1.0 ml column of SP-Sephadex C-25. In five experiments 97 ± 4 % of the uncharged substrate D-Tyr-Pro-Gly-Gly was eluted in 6 ml of 10 mM-NaH₂PO₄, pH 5.0. Subsequently 96 ± 5 % of the product D-Tyr-Pro-Gly-NH₂ was eluted in 4 ml of 50 mM-NaH₂PO₄, pH 5.0, containing 0.5 M-NaCl.

PAM assay

PAM activity was assayed in duplicate, in a final volume of 50 μ l. The method of Eipper *et al.* (1983) was optimized for measuring PAM activity in sheep hypothalamic tissue, with D-Tyr-Pro-Gly-Gly as substrate. Before use the D-¹²⁵I-Tyr-Pro-Gly-Gly was purified by passage through a 1.0 ml column of SP-Sephadex C-25 equilibrated with 10 mM-Mes/NaOH buffer, pH 5.0. Each incubation mixture contained 0.5–1 μ M-D-¹²⁵I-Tyr-Pro-Gly-Gly (50–100 000 c.p.m.), 1–1000 μ M-CuSO₄, freshly prepared ascorbic acid (0.5 mM), 0.1 mg of catalase/ml and prepared tissue (up to 10 μ g of protein) in 150 mM-Tris/HCl buffer, pH 7.0. The buffers Mes/

NaOH (pH 5–6), Tes/NaOH (pH 6–7) and Tris/HCl (pH 6.5–9) were used in optimizing the assay pH.

Samples were incubated at 37 °C, in capped tubes, for up to 24 h. The reaction was stopped by addition of 1.0 ml of 10 mM-NaH₂PO₄, pH 5.0, and the newly formed product D-¹²⁵I-Tyr-Pro-Gly-NH₂ was separated from remaining D-¹²⁵I-Tyr-Pro-Gly-Gly by elution from SP-Sephadex C-25. Radioactivities of duplicate samples (400 μ l) from each incubation duplicate were then measured. In each assay one or more of the following controls were included in duplicates: zero-time controls (1.0 ml of 10 mM-NaH₂PO₄, pH 5.0, added before incubation), zero-enzyme controls (enzyme replaced in the assay mix by 0.32 M-sucrose) and boiled-enzyme controls (enzyme placed at 100 °C for 1 min). Zero-time and zero-enzyme controls gave equivalent amounts of radioactivity eluted in the product wash at background levels. Boiled-enzyme controls were always less than 2.5 % of the activity with unheated enzyme. Results are expressed as pmol of product formed/h per mg of protein.

A mixed-substrate experiment was performed to test the specificity of the enzyme for D-Tyr-Pro-Gly-Gly and D-Tyr-Val-Gly. Incubations were carried out for 17 h, and the rate of amidation by median-eminence synaptosomes of 1 μ M-D-¹²⁵I-Tyr-Val-Gly was measured in the presence of 0–30 μ M-D-Tyr-Pro-Gly-Gly and that of 3.3 μ M-D-¹²⁵I-Tyr-Pro-Gly-Gly in the presence of 0–20 μ M-D-Tyr-Val-Gly.

The relative affinity of the enzyme for its substrate was assessed by measuring the rate of amidation of D-Tyr-Pro-Gly-Gly across a range of substrate concentrations, with and without added ascorbate and catalase. The kinetic parameters *K_m* and *V* were calculated from the Michaelis–Menten equation (Dixon & Webb, 1964) by

fitting the data directly by using non-linear-regression analysis (MODFIT program; McIntosh & McIntosh, 1980).

RESULTS

Characterization of subcellular fractions

Subcellular fractions from hypothalamic tissue were characterized by radioimmunoassay for GnRH (a marker for synaptosomes and neurosecretory granules), by cytochrome oxidase activity (a marker for mitochondria), by lactate dehydrogenase activity (cell cytosol), by NADPH-cytochrome *c* reductase activity (microsomal fraction) and by electron microscopy. The results are shown in Table 1. Fraction S2 was enriched in lactate dehydrogenase activity, and fraction A was enriched in NADPH-cytochrome *c* reductase. Electron microscopy revealed that fraction A contained large amounts of myelin, as well as membrane vesicles. Fraction B contained over 70 % of the GnRH immunoactivity that had been applied to the gradient, and electron microscopy confirmed the presence of synaptosomes in this fraction. Fraction C had the enzymic and electron-microscopic characteristics of a mitochondrial fraction. The crude neurosecretory-granule preparation was enriched in GnRH immunoactivity. Electron microscopy revealed the presence of large membranous pieces, as well as identifiable neurosecretory granules of diameter 67–93 nm.

Subcellular distribution of PAM activity

The distribution of Cu^{2+} -stimulated PAM activity measured in freshly prepared sonicated subcellular fractions from single homogenates of median eminence and anterior pituitary are shown in Table 2. PAM activity was enriched 6-fold in the synaptosomal fraction B, which contained 62 % of the total activity recovered.

Table 2. Subcellular distribution of PAM activity in the median eminence and anterior pituitary of the sheep

Neurosecretory granules were prepared from another portion of the same median-eminent homogenate. The activities from median-eminent fractions were determined in the presence of $20 \mu\text{M-CuSO}_4$ and those from anterior pituitary in the presence of $10 \mu\text{M-CuSO}_4$, with $0.5 \mu\text{M-D-Tyr-Pro-Gly-Gly}$ in Tes buffer, pH 7.0. Specific activity is expressed as pmol of product/h per mg of protein and percentage recovery is defined as $100 \times (\text{total activity in fraction}) / (\text{total activity in homogenate})$. Results are expressed as the means $\pm$ S.E.M. This uncertainty is an estimate of assay precision, being calculated from triplicate determinations of enzyme activity on the fractions from one subfractionation.

	Specific activity	Recovery (%)
Median eminence		
Homogenate	0.84 ± 0.03	
A: microsomes/myelin	0.84 ± 0.24	13 ± 1
B: synaptosomes	5.12 ± 0.18	62 ± 6
C: mitochondria	2.40 ± 0.11	26 ± 3
S2: cytosol	0.84 ± 0.05	23 ± 2
Neurosecretory granules	3.28 ± 0.15	16 ± 8
Anterior pituitary		
Homogenate	0.21 ± 0.01	
Secretory granules	1.70 ± 0.08	167 ± 9

Activity was enriched in the neurosecretory-granule preparation from the whole homogenate of median eminence nearly 4-fold. Only 16 % of the activity in the homogenate was recovered in the neurosecretory granules, however, and the hypo-osmotic supernatant from the neurosecretory-granule preparation contained no

Fig. 1. Separation of PAM activity and GnRH immunoactivity in pituitary secretory granules separated on a Percoll density gradient

PAM activity (■) and GnRH immunoactivity (△) were measured in 0.5 ml fractions of the 12 ml Percoll density gradient (—) used to prepare pituitary secretory granules. PAM activity was measured with $2 \mu\text{M-Cu}^{2+}$ and $0.1 \mu\text{M-D-Tyr-Pro-Gly-Gly}$ in Tes buffer, pH 7.0.

Fig. 2. Time course of PAM activity and product degradation by median-eminence synaptosomes

Time courses are shown of (a) PAM activity and (b) degradation of D-¹²⁵I-Tyr-Pro-Gly-NH₂ by median-eminence synaptosomes, determined with 20 μ M-Cu²⁺ and 0.5 μ M-D-Tyr-Pro-Gly-Gly in Tes buffer, pH 7.5. Individual results are shown from duplicate determinations at each time point in single experiments.

measurable activity. Total activity recovered in all neurosecretory-granule fractions was 31 % of the activity measured in the median-eminence homogenate before hypo-osmotic dilution.

Anterior-pituitary secretory granules contained about 8 times the PAM activity of whole-pituitary homogenate. The increase in total activity after subfractionation suggested the presence of inhibitors in the homogenate. When the Percoll-gradient fractions were analysed for GnRH the peak of immunoactivity did not coincide with the peak of PAM activity, but was contained in less dense membrane fractions, as shown in Fig. 1.

Linearity of activity with time and amount of enzyme added

Median-eminence synaptosomal preparations were used to characterize PAM activity further. The production of amidated peptide was linear with time of incubation for up to 24 h at 37 °C after an initial lag of about 2 h (Fig. 2a). In order to determine if the product was being degraded by any other peptidases, synthetic D-¹²⁵I-Tyr-Pro-Gly-NH₂ was incubated under identical conditions, and the increase in the radioactivity appearing in the first wash with 10 mM-Na₂HPO₄ was measured. Cleavage of D-¹²⁵I-Tyr-Pro-Gly-NH₂ at Pro-Gly or D-Tyr-Pro would have led to radioactive products without an amidated C-terminus, which would not have been bound to the cation-exchange column. Fig. 2(b) shows that about 10 % of the D-¹²⁵I-Tyr-Pro-Gly-NH₂ was degraded after 24 h at 37 °C. No correction was made to measurements of PAM activity for other enzyme activity

Fig. 3. Variation of PAM activity with pH

PAM activity in median-eminence synaptosomes was measured at various pH values, with 5 μ M-Cu²⁺ (□) and 20 μ M-Cu²⁺ (■) and 0.5 μ M-D-Tyr-Pro-Gly-Gly in two experiments, and expressed as a fraction of the maximal activity measured. Individual results are shown from duplicate determinations at each pH.

acting either on the substrate or on newly formed product.

Enzyme activity after a 16 h incubation was linear with amount of enzyme added for up to 10 μ g of protein (0.2 mg of protein/ml).

Optimal pH and inhibitors

PAM activity in median-eminence synaptosomes was measured over the range pH 5–9 in two experiments (Fig. 3). The activity appeared maximal between pH 7 and pH 9, but was inhibited when the pH was lowered to below 6.5. Addition of 1 mM-sodium phosphate to the incubation decreased PAM activity to 43 %, and 100 mM-phosphate or 100 μ M-EDTA completely abolished it. Only 1–10 % of the activity remained in the absence of CuSO₄ or when 0.01 % NaN₃ was added. The GnRH peptidase inhibitor *p*-hydroxymercuribenzoate (100 μ M) only minimally affected PAM activity in preoptic area synaptosomes (73 $\pm$ 16 % activity in duplicate incubations), and addition of 10 μ M synthetic GnRH to the incubation had no effect on the activity.

Optimal conditions for cofactors

The synaptosomal enzyme activity, like the reported pituitary activity (Eipper *et al.*, 1983), was sensitive to Cu²⁺ concentration. The optimal CuSO₄ concentration appeared to vary between tissues and with the different subfractions prepared (Fig. 4). Emeson (1984) noted a changed optimal Cu²⁺ requirement when PAM was assayed in purified neurosecretory granules rather than in synaptosomes.

In one experiment the PAM activity of two median-eminence synaptosomal preparations, in the presence of 5 μ M-CuSO₄ but without added ascorbic acid and catalase, was 20–23 % of the activity with these factors added. Replacement of catalase alone or ascorbic acid

Fig. 4. Variation of PAM activity with Cu^{2+} concentration

PAM activity was measured in two median-eminenence synaptosomal preparations ($\square$ and $\blacksquare$) and two preparations of anterior-pituitary secretory granules ($\circ$ and $\bullet$) as a function of $[\text{Cu}^{2+}]$. No activity was observable without added Cu^{2+} . Activity was determined with $0.5 \mu\text{M}$ -D-Tyr-Pro-Gly-Gly at pH 7.5. Individual results are shown from duplicate determinations at each $[\text{Cu}^{2+}]$ value.

alone restored activity to 55–60% and 62–66% of control values respectively.

Anatomical distribution of PAM activity in the sheep brain and pituitary

The anatomical distribution of activity was determined with synaptosomes from the brain areas studied and the purified secretory-granule preparation from the anterior pituitary (Eipper *et al.*, 1983). The PAM activity was measured in fresh preparations that had been sonicated or freeze-thawed and the results are given in Fig. 5. The highest levels of PAM activity were consistently measured in median-eminenence and medial-basal-hypothalamic synaptosomes. Activity was present in the anterior hypothalamus, but was not measurable in synaptosomes from the pineal gland or cerebral cortex.

PAM activity with an alternative substrate

Table 3 shows the results obtained when PAM activity was measured with either D-Tyr-Pro-Gly-Gly or D-Tyr-Val-Gly as substrate ($0.5 \mu\text{M}$) in median-eminenence synaptosomes and in pituitary secretory granules. Nearly 20 times as much activity was measured with the shorter substrate, but there were no significant differences in the activity ratios between tissues. Each tissue preparation had a similar optimal Cu^{2+} concentration for both substrates (results not shown). The results in Table 3 also show a higher activity in the synaptosomes than in pituitary secretory granules, with D-Tyr-Val-Gly as substrate, suggesting that the same enzyme could be amidating both substrates.

To analyse further the substrate specificity of the enzyme activity, two mixed-substrate experiments were carried out, where activity of synaptosomes from the median eminenence towards each substrate was determined in the presence of increasing concentrations of the other.

Fig. 5. Anatomical distribution of PAM activity in sheep brain and anterior pituitary

PAM activity was measured in two to four sonicated or freeze-thawed synaptosomal preparations from areas of brain and secretory-granule preparations from the anterior pituitary. Activity was assayed in the presence of $50 \mu\text{M}$ - Cu^{2+} for all synaptosomal preparations and of $10 \mu\text{M}$ - Cu^{2+} for pituitary granules, in Tes buffer, pH 7.0. The optimal $[\text{Cu}^{2+}]$ for PAM activity was assumed to be similar in all the brain areas tested. Analysis of variance showed that variation between preparations was not significantly greater than within duplicate or triplicate determinations; consequently, results are expressed as the means and S.E.M. for the numbers of pooled samples given in parentheses. The lower limit of sensitivity of the PAM assay (-----) under the conditions used was 0.2 pmol of product/h per mg of protein. Supraoptic/OVLT, organum vasculosum of the lamina terminalis/supraoptic nucleus.

Table 3. Comparison of PAM activity with two substrates

The specific activity was measured with $0.5 \mu\text{M}$ -D-Tyr-Val-Gly or -D-Tyr-Pro-Gly-Gly, 0.5 mM -ascorbic acid, 0.1 mg of catalase/ml and $5 \mu\text{M}$ - (anterior pituitary) or $50 \mu\text{M}$ - (median eminenence) CuSO_4 . A $5\text{--}10 \mu\text{l}$ portion of a sonicated or freeze-thawed preparation was used for each incubation. The mean ratios were calculated from the individual ratios of pairs of determinations made in parallel incubation. Results are expressed as the means $\pm$ S.E.M. for the numbers of determinations given in parentheses.

Substrate	PAM activity (pmol of product/h per mg of protein)	
	Median-eminenence synaptosomes	Pituitary secretory granules
D-Tyr-Val-Gly (A)	91.1 ± 4.6 (4)	29.6 ± 2.7 (6)
D-Tyr-Pro-Gly-Gly (B)	5.0 ± 0.7 (4)	1.5 ± 0.1 (6)
Activity ratio A/B	18.8 ± 1.9 (4)	19.9 ± 1.6 (6)

Fig. 6. PAM activity in the presence of competing substrates

PAM activity in median-eminence synaptosomes was measured in duplicate in two experiments, with $1 \mu\text{M}$ -D- ^{125}I -Tyr-Val-Gly, $50 \mu\text{M}$ -Cu $^{2+}$ and 0 – $30 \mu\text{M}$ -D-Tyr-Pro-Gly-Gly (□). Activity in the absence of competing D-Tyr-Pro-Gly-Gly was 80 ± 3 pmol/h per mg of protein in one experiment and 67 ± 4 pmol/h per mg of protein in the other. In another single experiment PAM activity was determined with $3.3 \mu\text{M}$ -D- ^{125}I -Tyr-Pro-Gly-Gly, $50 \mu\text{M}$ -Cu $^{2+}$ and 0 – $10 \mu\text{M}$ -D-Tyr-Val-Gly (■). Activity in the absence of competing D-Tyr-Val-Gly was 3.7 ± 0.2 pmol/h per mg of protein). Results are expressed as the mean $\pm$ S.E.M. percentages of the activity measured in the absence of competing substrate.

The results are shown in Fig. 6. Activity measured with $1 \mu\text{M}$ -D- ^{125}I -Tyr-Val-Gly was increasingly inhibited by 10 – $30 \mu\text{M}$ -D-Tyr-Pro-Gly-Gly, with 50% inhibition occurring at about $25 \mu\text{M}$ -D-Tyr-Pro-Gly-Gly. Conversely, activity determined with $3.3 \mu\text{M}$ -D- ^{125}I -Tyr-Pro-Gly-Gly was greatly inhibited by only $1 \mu\text{M}$ -D-Tyr-Val-Gly. This was further evidence that the two substrates compete for the same enzyme or enzymes, and showed that the value of K_m of the synaptosomal activity was greater for D-Tyr-Pro-Gly-Gly than for D-Tyr-Val-Gly.

Kinetic studies

Table 4 shows estimates of V and K_m obtained from data for sonicated medial-basal-hypothalamic and anterior-pituitary-granule enzyme preparations, both with and without ascorbate/catalase. Addition of ascorbic acid to medial-basal-hypothalamic synaptosomes increased the K_m nearly 9-fold and the V 25-fold. There was no increase in the K_m measured with pituitary secretory granules when ascorbic acid was added, but the V increased 12-fold.

DISCUSSION

Amidation of the C-terminus occurs as a post-translational modification in a number of peptide hormones, for example α -melanotropin, gastrin, vasopressin, oxytocin, corticotropin-releasing hormone (corticoliberin), thyrotropin-releasing hormone (thyroliberin), cholecystokinin and calcitonin, as well as GnRH.

Table 4. Kinetic parameters calculated for D-Tyr-Pro-Gly-Gly PAM activity in the presence and in the absence of ascorbic acid/catalase

PAM activity was determined with 0.2 – $100 \mu\text{M}$ -D-Tyr-Pro-Gly-Gly (five concentration points) and $5 \mu\text{M}$ -CuSO $_4$ for the pituitary preparation or $50 \mu\text{M}$ -CuSO $_4$ for the medial basal hypothalamic (MBH) one. + Ascorbate/catalase, assayed with ascorbic acid (0.5 mM) and catalase (0.1 mg/ml). – Ascorbate/catalase, assayed without ascorbic acid and catalase. Results are expressed as the means $\pm$ S.E.M. of the values, estimated by non-linear-regression analysis, from data obtained with a single sonicated preparation.

	K_m (μM)	V (pmol of product/h per mg of protein)
MBH synaptosomes		
+ Ascorbate/catalase	289 ± 48	1296 ± 136
– Ascorbate/catalase	33 ± 4	51 ± 2
Pituitary secretory granules		
+ Ascorbate/catalase	75 ± 12	176 ± 15
– Ascorbate/catalase	68 ± 30	15 ± 6

We have investigated glycine-directed amide formation in a synthetic substrate designed to mimic a glycine-extended C-terminus of GnRH. The characteristics of the amidating enzyme activity observed were similar to those of the peptidyl-glycine α -amidating mono-oxygenase (PAM) activity first described in rat pituitary by Bradbury *et al.* (1982), then in rat brain by Kizer *et al.* (1984) and Emeson (1984). These authors used peptide substrates imitating the C-terminus of either α -melanotropin (D-Tyr-Val-Gly) or a glycine-extended thyrotropin-releasing hormone (<Glu-His-Pro-Gly). The similarities between the α -amidating enzyme activity measured by us in sheep tissues and the activities previously reported included its subcellular localization and dependence on Cu $^{2+}$, cofactors and pH. Activity measured with D-Tyr-Pro-Gly-Gly as substrate was inhibited by low concentrations of D-Tyr-Val-Gly, suggesting that the α -amidating activity measured here was not specific for the GnRH terminal peptide. Results obtained with D-Tyr-Pro-Gly-Gly therefore do not allow conclusions on the anatomical distribution of pro-GnRH-processing events alone.

PAM activity with D-Tyr-Pro-Gly-Gly as substrate was linear with time for up to 24 h after an initial lag of about 2 h. A similar lag phase has been shown with D-Tyr-Val-Gly as substrate for enzymes from rat anterior pituitary (Glembotski *et al.*, 1984), bovine intermediate pituitary (Eipper *et al.*, 1983) and rat hypothalamus (Emeson, 1984). The lag before product formation may be caused by the presence of endogenous substrates in the lysed synaptosomal preparations assayed (Dixon & Webb, 1964). Measurement of PAM activity *in vitro* after disruption of tissue containing a variety of peptidergic neuron types may contribute to an artificially increased concentration of other endogenous substrates, not seen *in vivo*, when the α -amidating enzyme(s) might be expected to be compartmentalized with its specific prohormone (Lazure *et al.*, 1983).

PAM activity was almost undetectable in whole

homogenates, but activity was enriched in neurosecretory granules and synaptosomal preparations, as reported for rats by Emeson (1984). This is consistent with the proposed subcellular location of hypothalamic post-translational prohormone-processing events. The enrichment of PAM activity in the mitochondrial fraction may indicate contamination of this fraction with free neurosecretory granules from lysed synaptosomes.

If PAM activity is associated with neuropeptide-containing neurosecretory granules, its anatomical distribution probably then reflects the concentration of these granules in the tissue. High levels of activity were expected and found in the median eminence, where many α -amidated neuropeptides are released into the portal blood system or are present in the terminals of infundibular neurons. The major anterior-pituitary hormones, on the other hand, are not α -amidated, although the gland does produce α -amidated α -melanotropin, oxytocin and vasopressin (Eipper *et al.*, 1983).

Negligible PAM activity was detected by us in sheep cerebral cortex or pineal synaptosomes. If PAM activity is postulated to be involved in the formation of neuropeptides from their prohormones, its undetectable activity in these areas implies that α -amidated peptides are synthesized or released here in very low concentrations. There is, however, evidence for the presence of α -amidated cholecystokinin in adult rat (250 pmol/g of tissue; Goltermann *et al.*, 1981) and pig (600 pmol/g of tissue; Rehfeld, 1978) cerebral cortex. Similarly, the pineal gland has been reported to contain comparable amounts of a number of α -amidated neuropeptides, including vasopressin and oxytocin (Dogterom *et al.*, 1980; Fernstrom *et al.*, 1980), somatostatin (Pelletier *et al.*, 1975), α -melanotropin (Vaudry *et al.*, 1978; Oliver & Porter, 1978) and substance P (Powell *et al.*, 1977). The lack of PAM activity, with D-Tyr-Pro-Gly-Gly as substrate, in sheep cerebral-cortex and pineal synaptosomes may imply that several PAM enzymes exist, with differences in specificity for different substrates. Kizer *et al.* (1984) separated two α -amidating activities from sonicated whole-cell preparations of rat brain that differed in their pH maxima, their affinities to different substrates and their behaviours towards various proteinase inhibitors.

Although the tetrapeptide D-Tyr-Pro-Gly-Gly reacted much more slowly with the PAM activity than did the tripeptide D-Tyr-Val-Gly containing only one neutral amino acid less, the tetrapeptide might be expected to be more comparable with the longer natural substrates that both are designed to mimic.

The mixed-substrate experiment, in which PAM activity towards D-Tyr-Pro-Gly-Gly and D-Tyr-Val-Gly was measured in the presence of increasing concentrations of the other, showed that the tripeptide competed for the same enzyme active site as the tetrapeptide in synaptosomes of the sheep median eminence, and at least partial competition occurred in the reverse situation. A higher concentration of D-Tyr-Pro-Gly-Gly was required to displace D-Tyr-Val-Gly from the binding site than was necessary in the reverse case, as expected from the higher value of K_m calculated for D-Tyr-Pro-Gly-Gly in another experiment reported here, in comparison with the value of K_m for D-Tyr-Val-Gly found by others in reaction with the rat brain enzyme (Emeson, 1984).

PAM activity was markedly stimulated by Cu^{2+} and totally inhibited by EDTA and NaN_3 . The optimal

concentration of Cu^{2+} required varied somewhat among subfractionations, which may have been related to the proportions of synaptosomes and free neurosecretory granules in each preparation (Emeson, 1984). Hypothalamic PAM appeared to require higher Cu^{2+} concentrations than pituitary PAM for optimal activity. This concentration was similar to that noted by Emeson (1984) for hypothalamic synaptosomes (25 μM). Emeson (1984) and others (Eipper *et al.*, 1983; Glembotski *et al.*, 1984) have also noted the variability of optimal Cu^{2+} concentrations with tissue preparations. Similar variation has been described for dopamine β -hydroxylase (Duch *et al.*, 1968; Orcutt & Molinoff, 1976), an enzyme that shares many characteristics with PAM, such as Cu^{2+} -dependence, stimulation by ascorbic acid, inhibition by NaN_3 and dependence on O_2 .

PAM activity with D-Tyr-Pro-Gly-Gly was maximal between pH 7 and 8 and fell below pH 6.5. Similar pH-activity profiles for PAM activity have been noted by others (Bradbury & Smyth, 1983; Eipper *et al.*, 1983; Glembotski *et al.*, 1984; Emeson, 1984; Mains *et al.*, 1984; Kizer *et al.*, 1984). Isolated neurosecretory granules are, however, thought to have an internal pH of 5.5–6.0 (Russell & Holz, 1981; Carty *et al.*, 1982). Assay of PAM activity *in vitro*, in lysed neurosecretory-granule preparations, may increase its pH optimum, as noted for catheptic activity in lysosomes (Szego & Pietras, 1984). However, there is some evidence (Scherman & Nordmann, 1982) that the internal pH of secretory granules is higher in newly formed granules than in mature ones. It is therefore possible to speculate that the pH optimum of PAM could be a mechanism whereby newly formed prohormone is more rapidly processed, so that amidation is diminished in mature granules ready for release. Further studies on the purification and characterization of this and other neurosecretory-granule-associated enzymes are needed to elucidate the precise mechanisms of prohormone processing and maturation.

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