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Molecular forms of the putative enkephalin precursor BAM-12P in bovine adrenal, pituitary, and hypothalamus

(adrenal medulla/radioimmunoassay/opioid peptide/proenkephalin)

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ABSTRACT **A highly specific radioimmunoassay for one of the putative adrenomedullary [Met]enkephalin precursors, BAM-12P (Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-OH), has been developed. The BAM-12P antibodies recognize the COOH-terminal fragment of the peptide from Arg^7 to Glu^{12} and do not crossreact with [Met^5]- or [Leu^5]enkephalin or any of their COOH-terminal lysine or arginine extended analogs. Specificity for the COOH-terminal Glu-OH is suggested by the 100% crossreactivity with BAM-$12P^{5-12}$ and 0.3% crossreactivity with BAM-$12P^{5-12}$ amide. Using these antibodies, we have measured three forms of BAM-12P-like immunoreactivity in extracts of bovine adrenal medulla, of which the major form (>90%) corresponds to BAM-12P by molecular weight. Extracts of bovine adrenal cortex contain 1% the amount of a BAM-12P-like material ($M_r \approx 1400$; 20 ng per gland), possibly due to crosscontamination with adrenomedullary tissue. The major form of BAM-12P-like material in extracts of bovine neurointermediate pituitaries is of higher molecular weight than authentic BAM-12P ($M_r \approx 4000$); the remaining material (10%) corresponds to BAM-12P by molecular weight. There is no detectable BAM-12P-like immunoreactivity in crude or purified extracts of bovine anterior pituitaries. Extracts of bovine hypothalamic tissues contain small amounts of BAM-12P immunoreactivity (≈2 ng per fragment) which can be detected as one molecular form corresponding to a 1400-dalton molecule. The results indicate that the enkephalin precursor found in the adrenal medulla also may be present in the pituitary and hypothalamus. Furthermore, the processing of this molecule appears to be tissue-specific.**

The detection of enkephalin immunoreactivity in the adrenal medulla has led to the isolation of several enkephalin-related peptides (1–4). Lewis *et al.* (5) have shown that a high molecular weight precursor molecule contains several copies of the pentapeptides [Met]- and [Leu]enkephalin. Presumably, the smaller opioid peptides isolated by Mizuno *et al.* (6, 7) are derived from this precursor. One of these peptides, BAM-12P, is a COOH-terminal extended [Met]enkephalin dodecapeptide that has the structure Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-OH. A synthetic replicate of this peptide was used to raise antibodies in rabbits. We describe here a radioimmunoassay for BAM-12P and report the detection of BAM-12P in extracts of bovine adrenal medulla, adrenal cortex, hypothalamus, and pituitary neurointermediate lobe.

MATERIALS AND METHODS

Peptides. BAM-12P, its fragments, and all other peptides were synthesized by solid-phase methods as described (8, 9).

Induction of Antibodies. Antibodies to BAM-12P were raised by the method of Benoit *et al.* (10). The antisera obtained after the third booster injection of rabbit RB317 were used in subsequent radioimmunoassay (RIA) studies at a final dilution of 1:4000. At this dilution, the antibodies bound 45% of the ^{125}I-labeled BAM-12P (bound cpm/total cpm).

^{125}I Labeling of Peptide. Tyr^1 of BAM-12P was iodinated by the method of Hunter and Greenwood (11). The radioactive peptide was purified on a 0.8 × 14 cm column of Sephadex G-10 (Pharmacia). The fractions containing labeled peptide were diluted 1:10 with 2% bovine serum albumin in saline, and 50-μl aliquots were stored at −20°C. The labeled peptide was not kept beyond 2 weeks.

Radioimmunoassay of BAM-12P. All incubations were done in 10 mM phosphate buffer containing human serum albumin (Sigma, 1 mg/ml), 150 mM NaCl, 25 mM EDTA, and 0.2 M sodium acetate at pH 7.2. Serial dilutions of BAM-12P were added to plastic tubes and brought to 400 μl by the addition of antibody (1:4000) and trace (10,000–15,000 cpm). The tubes were incubated at 4°C for 24 hr or 48 hr, and the bound antigen was separated from free antigen by incubation with goat anti-rabbit gamma globulin or with 1.5 ml of absolute ethanol. The supernatant obtained after centrifugation at 3000 rpm for 30 min was aspirated and the pellets were assayed for 2 min on a Micromedic γ-ray counter. In all experiments, BAM-12P standards as well as synthetic peptides and tissue extracts were run in triplicate.

Extraction of Bovine Tissues. Fresh bovine hypothalamus, pituitaries, and adrenals were collected at a local slaughterhouse. The glands were immediately dissected into anterior and neurointermediate lobes or adrenocortical and adrenomedullary tissue and flash frozen in liquid nitrogen. All dissected tissues were stored at −80°C until processed. The frozen tissues were extracted in a mixture of 1 M HCl/5% (vol/vol) formic acid/1% trifluoroacetic acid/1% NaCl, as described by Bennett *et al.* (12). Peptides were extracted from the tissue homogenates by reverse-phase peptide extraction on a column of octadecasilyl silica (LRP-2, Whatman, 2 × 25 cm) as described (12, 13).

Gel Permeation Chromatography. The crude peptide fractions obtained from the octadecasilyl silica column were dissolved in 30% acetic acid and applied to a 2 × 75 cm column of Sephadex G-50 (Pharmacia; bed volume, 250 ml) preequilibrated with 30% acetic acid. The sample was eluted with 30% acetic acid at room temperature at a flow rate of 12 ml/hr. The column was calibrated with bovine serum albumin, insulin, somatostatin-28, luteinizing hormone-releasing factor, and NaCl as standards and later with synthetic BAM-22P and BAM-12P.

RESULTS AND DISCUSSION

Standard curves generated with the BAM-12 antiserum (RB-317) permitted the detection of ≤50 pg of antigen per tube. The

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Abbreviations: RIA, radioimmunoassay; IR, immunoreactive.

Table 1. Crossreactivities of synthetic peptides with antiserum RB317 raised against BAM-12P

Peptide	Cross-reactivity, %
Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-OH	100
Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-OH	100
Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-NH_2	0.3
Met-Arg-Arg-Val-Gly-Arg-Pro-OH	<0.01
Arg-Val-Gly-Arg-Pro-Glu-OH	100
Gly-Arg-Pro-Glu-OH	0.3
Tyr-Gly-Gly-Phe-Met-OH ([Met]enkephalin)	<0.01
Tyr-Gly-Gly-Phe-Met-Arg-OH	<0.01
Tyr-Gly-Gly-Phe-Met-Arg-Arg-OH	<0.01
Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH	<0.01
Tyr-Gly-Gly-Phe-Met-Lys-OH	<0.01
Tyr-Gly-Gly-Phe-Leu-OH ([Leu]enkephalin)	<0.01
Tyr-Gly-Gly-Phe-Leu-Arg-OH	<0.01
Tyr-Gly-Gly-Phe-Leu-Arg-Lys-OH	<0.01
Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-OH	<0.01
Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-OH (BAM-22P)	<0.01
Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH (dynorphin 1-13)	<0.01

ED_{50} for these RIAs was 1.2 ng. The crossreactivity of various peptides with this antiserum is shown in Table 1; no crossreactivity was observed for [Met]enkephalin, [Leu]enkephalin, or any of their COOH-terminal extended analogs. The antigenic determinant is clearly the COOH terminus of BAM-12P. The importance of Glu^{12}-OH in this reaction can be illustrated by the crossreactivity of BAM-$12P^{5-12}$-OH (100%), the low crossreactivity of BAM-$12P^{5-12}$-NH_2 (0.3%), and the absence of crossreactivity of BAM-$12P^{5-11}$-OH and BAM-22P (<0.01%).

Fig. 1 shows the serial dilution curves obtained from extracts of bovine hypothalamus, anterior and neurointermediate pituitaries, adrenal cortex, and adrenal medulla. No immunoreactivity was detected in the anterior pituitary extract. In the other extracts, however, a dose-dependent and parallel displacement of the BAM-12P tracer was observed. Using these curves we estimated that there is 1.5 μg of BAM-12P-like immunoreactivity in one gland-equivalent of adrenal medulla, 12 ng in one gland-equivalent of adrenal cortex, 10 ng in one gland-equivalent of neurointermediate pituitary, and 2 ng in the hypothalamus. Gel permeation chromatography of these different extracts is shown in Figs. 2–5.

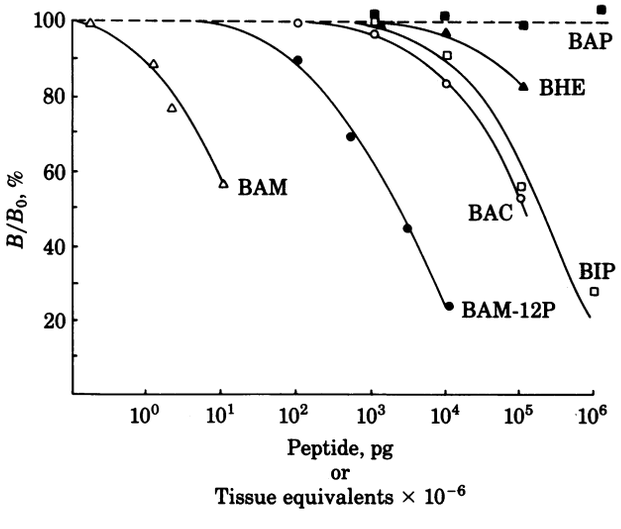

FIG. 1. Serial dilutions of bovine adrenal, pituitary, and hypothalamic extracts. The amount of BAM-12P-like immunoreactivity in the crude extracts of bovine adrenal medulla (BAM, △), adrenal cortex (BAC, ○), neurointermediary (BIP, □), anterior pituitary (BAP, ■), and hypothalamus (BHE, ▲) was measured in the octadecasilyl silica column-retained fraction. The amount of BAM-12P-like immunoreactivity per gland presented in the text represents an extrapolation from the amount of material that gives 50% and 60% B/B_0.

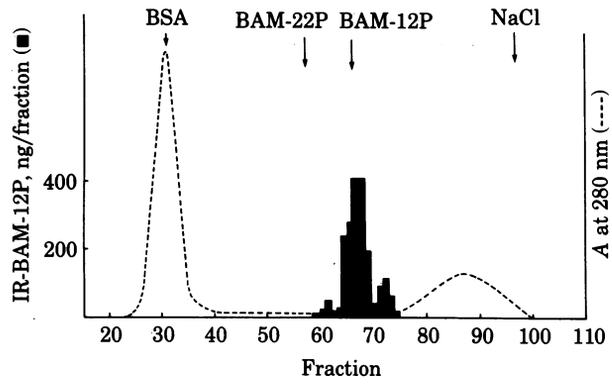

FIG. 2. Gel permeation chromatography of bovine adrenal medulla extract. The sample (76.4 mg) was dissolved in 5 ml of 30% acetic acid, loaded onto a column of Sephadex G-50 fine (2 × 100 cm), and eluted with 30% acetic acid at a flow rate of 12 ml/hr. Column fractions (2.1 ml) were collected and aliquots were dried under reduced pressure with 100 μl of 0.1% human serum albumin. The dried fractions were reconstituted in RIA buffer and the amounts of BAM-12P-like immunoreactivity were measured with antiserum RB317. The column was subsequently calibrated with bovine serum albumin (BSA), BAM-22P, BAM-12P, and NaCl.

Three peaks of immunoreactive BAM-12P-like material were detected in adrenal medulla extracts (Fig. 2). The major peak (K_{av} = 0.55; $M_r \approx$ 1500) eluted in a zone corresponding to the predicted elution volume of synthetic BAM-12P (K_{av} = 0.59; M_r = 1380). The minor forms of BAM-12P immunoreactivity may represent an endogenous COOH-terminal fragment of the native molecule (K_{av} = 0.62; M_r = 1000) formed in the putative processing of BAM-12P to [Met]enkephalin. Due to the specificity of the antibody, the larger form (K_{av} = 0.45; $M_r \approx$ 2000) must represent crossreactivity with an NH_2-terminal extended precursor or a glycosylated form of the molecule. The sum of adrenomedullary BAM-12P-like immunoreactive material recovered after gel permeation was 1.8 μg per gland.

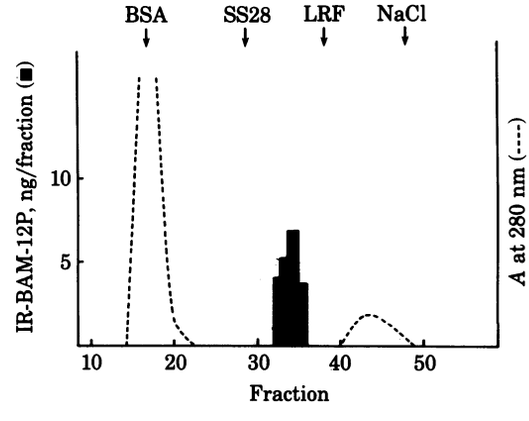

FIG. 3. Gel permeation chromatography of bovine adrenocortical extract. The sample (50.0 mg) was dissolved in 4 ml of 30% acetic acid and treated as described for Fig. 2. The column had previously been calibrated with bovine serum albumin (BSA), somatostatin-28 (SS28), luteinizing hormone-releasing factor (LRF), and NaCl.

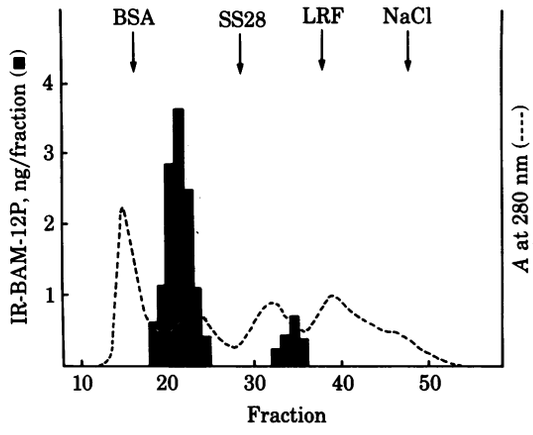

FIG. 4. Gel permeation chromatography of extract of bovine neurointermediate pituitary. The sample (20 mg) was dissolved in 4 ml of 30% acetic acid and treated as described for Fig. 2. The column had previously been calibrated with bovine serum albumin (BSA), somatostatin-28 (SS28), luteinizing hormone-releasing factor (LRF), and NaCl.

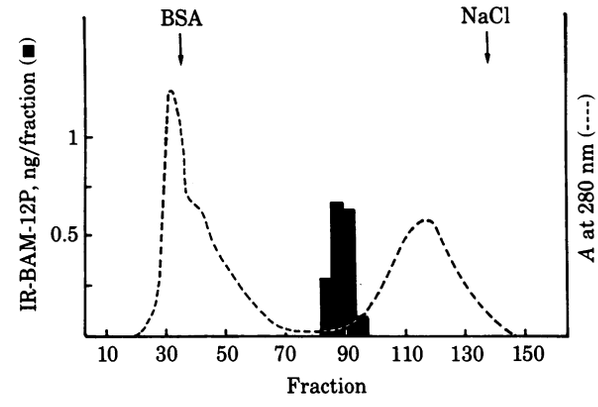

FIG. 5. Gel permeation chromatography of bovine hypothalamic extract. The sample (76 mg) was dissolved in 5 ml of 30% acetic acid and treated as described for Fig. 2. The column had previously been calibrated with bovine serum albumin (BSA), somatostatin-28 (SS28), luteinizing hormone-releasing factor (LRF), and NaCl.

The adrenocortical extract contained one form of BAM-12P-like immunoreactivity with an apparent M_r of 1500 (Fig. 3). The detection of low amounts of BAM-12P-like material (12–19 ng per gland) may represent contamination of the cortical tissue by adrenal medulla. The total amount of BAM-12P-like immunoreactive material recovered after gel permeation was 19 ng per gland.

The extract of neurointermediate pituitary (Fig. 4) contained two forms of BAM-12P-like immunoreactivity. The major form (90%) was of higher molecular weight than BAM-12P; the minor form (10%) eluted similarly to BAM-12P. The sum of BAM-12P-like immunoreactive material recovered after gel permeation was 13 ng per gland. It is conceivable that the amount of peptide present in the extract is considerably underestimated because the major form of BAM-12P immunoreactive material is of higher molecular weight.

Unlike the extract of neurointermediate pituitary and adrenal medulla, the hypothalamic extract contained only one form of BAM-12P-like immunoreactivity (Fig. 5). The material (2 ng per gland) eluted with the predicted elution volume of authentic BAM-12P ($M_r \approx 1400$) and the recovery after column chromatography agreed well with the estimated amounts predicted by serial dilution (Fig. 1).

In a separate series of experiments, we attempted to compare the forms of BAM-12P-like immunoreactive material found in these different bovine tissues with those in the rat. We were unable to detect any BAM-12P immunoreactivity in extracts of rat adrenal, neurointermediate pituitary, or hypothalamus (data not shown). It is not clear whether the absence of BAM-12P immunoreactivity in these tissues is due to total absence of the peptide or whether the antigenic determinant of BAM-12P of rat origin is different from that of its bovine counterpart. The application of these antibodies to immunohistochemical studies will have to be restricted to bovine tissues.

The development of a radioimmunoassay for BAM-12P has enabled us to establish that the peptide is present in extracts of the bovine adrenal, the neurointermediate lobe of the pituitary, and the ventral hypothalamus. The presence of BAM-12P-like immunoreactivity in the bovine hypothalamus suggests that the same multivalent proenkephalin originally isolated from the adrenal medulla (2–5) exists in the central nervous system. Indeed, Lewis *et al.* (14) have demonstrated the presence of such a large opioid-containing peptide in the bovine, rat, and guinea pig striata. Tissue-specific processing of this molecule (as suggested by the different molecular species of BAM-12P found in the medulla, pituitary, and hypothalamus) would enable any given tissue to generate a series of enkephalin-related peptides. The presence of the multivalent proenkephalin precusor in the brain would allow different cells to express the same gene with the production of different enkephalin-related peptides. This process would be completely independent of the biosynthesis of β-endorphin.

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