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In Vitro and *in Vivo* Evidence that the C-Terminus of Preproenkephalin-A Circulates as an 8500-Dalton Molecule (41799)

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Abstract. One of the major immunoreactive components of the enkephalin precurser BAM-22P in extracts of adrenal medulla is a molecule of ~8500 Da. A similar component is detected, after gel-permeation chromatography, in extracts of bovine plasma and in culture medium harvested from acetylcholine-stimulated bovine adrenomedullary cells in culture. Simultaneous detection of the C-terminal epitome of preproenkephalin-A: Met-enkephalin-Arg^6-Phe^7-OH in this secreted high-molecular-weight zone suggest that the ~8500-Da form of BAM-22P is the C-terminal of preproenkephalin A.

Following the isolation of several enkephalin-related peptides from the adrenal medulla (1–11), the predicted sequence of their putative precursor, preproenkephalin-A, was deduced by cloning and sequence analysis of its mRNA (1–3). One of the peptides derived from this precursor, BAM-22P (9) has been shown to exist both immunohistochemically (12, 13) and chemically (14, 15) in peripheral tissues and in the central nervous system. The amino acid sequence of this molecule is completely conserved in bovine and human species (1–3) and it is considerably more potent than either Met- or Leu-enkephalin in either the guinea pig ileum assay (9) or in its ability to induce analgesia *in vivo* (15). Numerous peptides in extracts of adrenal medulla and brain show immunological properties similar to BAM-22P (15) suggesting that any one of a number of these enkephalin-related peptides may exist in the peripheral circulation. In this study we describe the detection of an 8500-Da peptide that is the major form of BAM-22P-like immunoreactivity, both secreted by the adrenal medulla *in vitro* and detected in extracts of bovine plasma.

Materials and Methods. *Peptides.* BAM-22P, its fragments and all other peptides used in these studies were synthesized in this laboratory by solid-phase methods described elsewhere (16, 17).

Extraction of tissues. Fresh bovine adrenals and plasma were collected at a local slaughterhouse. The adrenals were immediately dissected free of fat and adrenocortical tissue and frozen in liquid nitrogen. Bovine adrenal medullae were extracted in boiling water (1:10, wt/vol), cooled to 4°C, centrifuged at 20,000*g* for 45 min, and the supernatant defatted with petroleum ether and lyophilized for further analysis. Acetone powders of bovine plasma were prepared by successive transfers through 200 ml each of acetone, hexane, and acetone at −20°C. The powder was extracted twice with 0.3 *N* HCl containing pepstatin (10 mg/liter) and phenylmethylsulfonyl fluoride (PMSF, 10 mg/liter) centrifuged at 27,000*g* for 45 min, and the supernatant lyophylized.

Cell culture. Isolated bovine chromaffin cells were prepared as described by Kilpatrick *et al.* (19) and cultured in Hepes-buffered Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum on collagen-coated tissue-culture dishes. On the fifth day of culture, the cells were incubated with 4 m*M* acetylcholine in Locke's buffer (19) for 5 min. The incubation medium was collected in a tube precooled in liquid nitrogen and lyophylized for further analysis.

Gel-permeation chromatography. The lyophilisate of each tissue extract was dissolved in 30% acetic acid, centrifuged at 20,000*g* for 30 min, and the supernatant applied to a column of Sephadex G-75 preequilibrated with 30% acetic acid. Aliquots of column fractions were removed, dried *in vacuo* in the presence of 100 μg of human serum albumin, reconstituted in radioimmunoassay buffer (14), and the amounts of BAM-22P-like immunoreactivity or Met-enkephalin-Arg^6-Phe^7-OH immunoreactivity determined by radioimmunoassay.

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Radioimmunoassays. Antibodies to BAM-22P were raised by the methylated-BSA method described by Benoit *et al.* (18). The antiserum obtained after the fourth injection of rabbit RB334 was used at a final dilution of 1:400,000 and the radioimmunoassays were performed as described for BAM-12P (14). The radioimmunoassay for Met-enkephalin-Arg^6-Phe^7-OH was performed under identical conditions (final dilution 1:5000) using an antibody (generously supplied by Dr. E. Weber, Stanford University, Palo Alto, Calif.) which shows no cross-reactivity with Met- or Leu-enkephalin.

Results. Standard curves generated with the BAM-22P antiserum (RB 334B4) permitted the detection of <15 pg of synthetic antigen per tube. The ED_{50} for these radioimmunoassays (RIA) was 150 pg. The cross-reactivity of various peptides with the antiserum is shown in Table I. The antigenic determinant recognized by these antibodies extends from Gly^9 to Gly^{22}. The importance of residues Pro^{11}, Glu^{12}, and the C-terminal Gly^{22} is demonstrated by the low cross-reactivity (1%) of BAM-22P (13–22) and BAM-22P (9–21) in contrast to that (100%) of BAM-22P (11–22) and BAM-22P (9–22). Extension of the C-terminal (as for example in the case of Peptide E in Table I) decreased cross-reactivity to 10%. The displacement of the BAM-22P trace with synthetic Peptide E was parallel and otherwise indistinguishable from that obtained with BAM-22P.

Figure 1 shows that the distribution of BAM-22P-like immunoreactivity in the bovine adrenomedullary extract was divided into four major components. The first corresponds to an estimated molecular weight of 8500 Da (8.5K), while the second, third, and fourth have estimated molecular weights of 3200 (3.2K), 2500 (2.5K), and 1400 (1.4K) Da, respectively. Similar results were obtained upon examination of a rat adrenal extract (results not shown).

The pattern of BAM-22P-like immunoreactive peptides secreted by bovine adrenomedullary cells in culture is shown in Fig. 2. The first form of BAM-22P-like immunoreactivity is of higher molecular weight than any of the species detected in tissue extracts and has an estimated molecular weight of 14,000 Da (14K). The second has an estimated molecular weight that corresponds to the 8500-Da species detected in tissue extracts. The third and fourth forms of BAM-22P-like immunoreactivity correspond to synthetic BAM-22P (2500 Da) and to an estimated molecular weight of 1400 (1.4K) Da. Measurements of Met-enkephalin-Arg^6-Phe^7-OH immunoreactivity in this same column effluent is shown in Fig. 2B. We detected a high-molecular-weight form of immunoreactive Met-enkephalin-Arg^6-Phe^7-OH that coeluted with the ~8500-Da form of BAM-22P. All other peaks of Met-enkephalin-Arg^6-Phe^7-OH immunoreactivity eluted independent of the BAM-22P immunoreactivities shown in Fig. 2A.

The distribution of BAM-22P-like immunoreactive peptides in an extract of bovine plasma is shown in Fig. 3. The major form of detectable BAM-22P-like immunoreactivity

TABLE I. CROSS-REACTIVITY OF SYNTHETIC PEPTIDES WITH ANTISERUM RB334B4 RAISED AGAINST SYNTHETIC BAM-22P

Peptide		Cross-reactivity (%)
BAM-22P (1–22)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-OH	100
(1–5)	[Met-enkephalin]	<0.01
(1–7)		<0.01
(1–12)		<0.01
(5–12)		<0.1
(5–11)		<0.1
(7–12)		<0.1
BAM-22P-$Gly^{23}Phe^{24}Leu^{25}$-OH (Peptide E)		10
(9–22)		100
(11–22)		100
(13–22)		1
(9–19)		0.1
(9–21)		1

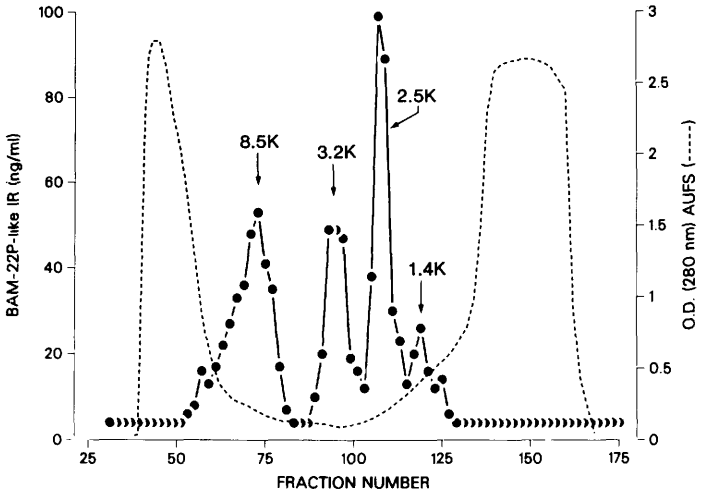

FIG. 1. Gel-permeation (G75 fine) chromatography of extracts of 50 bovine adrenal medullas was performed as described in the text. Estimated molecular weights (8.5K: 8500 Da; 3.2K: 3200 Da; 2.5K: 2500 Da; and 1.4K: 1400 Da) were obtained from calibrations with bovine serum albumin, cholecystokinin, γ_3-melanotropin 1–18, γ_3-melanotropin 1–8, [D-^{2}Trp]Leu-enkephalin, and NaCl and are indicated in the figure.

corresponds to a molecular weight of ~8500 Da (8.5K). A second peak of immunoreactive BAM-22P is detected in an area compatible with the 2500-Da species detected in extracts of the bovine adrenal medulla (2.5K, Fig. 1). Two other species of immunoreactive BAM-22P were detected with estimated molecular weights of 1400 (1.4K) and 700 (0.7K) Da, respectively. Unfortunately, insufficient materials precluded analysis for heptapeptide immunoreactivity. In similar extracts of human and rat plasma, the major form of BAM-22P-like immunoreactivity was of 8500 Da (results not shown).

Discussion. The recent technological advances in molecular biology have enabled the complete characterization of the mRNA encoding adrenomedullary preproenkephalin-A (1–3). These results, however, while suggesting points of enzymatic cleavage, provide little information on the *in situ* post-translational processing of the enkephalin precursor. It is to this end that we have investigated the distribution of BAM-22P-like immunoreactive peptides in plasma and tissue extracts.

Of particular interest in the studies reported here is the detection of an 8500-Da molecule with BAM-22P-like immunoreactivity which appears to be a major form in plasma and the major form secreted by the bovine chromaffin cells in culture. While at this point in time it is difficult to ascribe a full sequence to this molecule using the predicted DNA sequence, the detection of Met-enkephalin-Arg^6-Phe^7-OH immunoreactivity in the secreted form of immunoreactive BAM-22P suggests the existence of a C-terminal extension of BAM-22P to the C-terminus of preproenkephalin-A (Fig. 4). This extension, coupled to an N-terminal extension of BAM-22P is the only extension that can account for the existence of an 8500-Da molecule with Met-enkephalin-Arg^6-Phe^7-OH immunoreactivity, since examination of the sequence derived from the mRNA to preproenkephalin-A shows that this molecule must also carry the epitome recognized by anti-BAM-22P antisera.

An analysis of the ~8500-Da C-terminal fragment of preproenkephalin-A shows that there are only four amino acid differences between the human and bovine sequences. Moreover, a further extension of this molecule toward the N-terminal of preproenkephalin-A introduces a sequence of amino acids with 80% divergence in the human and bovine sequences. We are currently determining the extent of the NH_2-terminal extension of BAM-22P by classical techniques of protein chemistry. These results, coupled to the isolation and identification by Kilpatrick *et al.* (22) of the N-terminal 18,200-Da fragment of preproenkephalin, suggest that the ~8500-Da form of BAM-22P described here may be generated by the first enzymatic cleavage of the

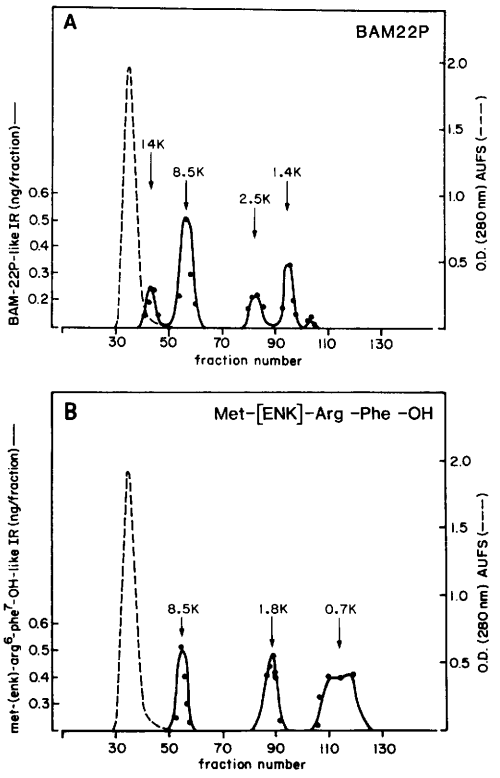

FIG. 2. Gel-permeation chromatography of the secreted products of adrenal chromaffin cells in culture. Column fractions were processed for the radioimmunoassay of BAM-22P (Panel A) and Met-enkephalin-Arg^{6}Phe^7-OH (Panel B) as described in the text. Estimated molecular weights (14K: 14,000 Da; 8.5K: 8500 Da; 2.5K: 2500 Da; 1.8K: 1800 Da; 1.4K: 1400 Da; and 0.7K: 700 Da) were obtained from calibrations with bovine serum albumin, cytochrome C, cholecystokinin, γ_3-melanotropin 1–18, γ_3-melanotropin 1–8, [D-^{2}Trp]Leu-enkephalin, and NaCl and are indicated in the figure.

precursor at the sequence Met-Arg-Gly-Leu (See * in Fig. 4).

Unfortunately, because the antibodies used in these studies crossreact partially with C-terminal extended forms of BAM-22P (Table I), it is difficult to quantify the relative amounts of each peptide with BAM-22P-like immunoreactivity in either plasma or extracts. As such, the amounts of the 8500-Da form(s) of BAM-22P are at least 10-fold higher than those measured here (see Table I). The resolution of gel-permeation chromatography precludes any identification of the smaller-molecular-weight immunoreactive forms of BAM22P. The detection of immunoreactivity reflects the presence of a family of BAM22P-related pep-

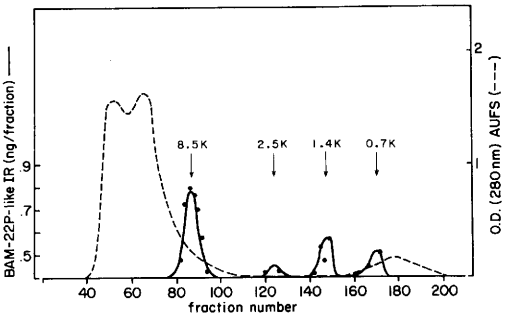

FIG. 3. Gel-permeation chromatography (Sephadex G-75) of an extract of 50 ml of bovine plasma. Estimated molecular weights (8.5K: 8500 Da; 2.5K: 2500 Da; 1.4K: 1400 Da; and 0.7K: 700 Da) were obtained from calibrations with bovine serum albumin, cholecystokinin, γ_3-melanotropin 1–18, γ_3-melanotropin 1–12, γ_3-melanotropin 1–8, [D-^{2}Trp]Leu-enkephalin, and NaCl and are indicated in the figure.

tides generated from preproenkephalin-A, including BAM22P, Peptide E, and Peptide I (see Fig. 4), all of which have been previously characterized (7, 9–11, 15).

The presence of a large circulating enkephalin may be the homeostatic mechanism through which potent opioid activity (as for example that of BAM-22P when compared to Met-enkephalin) is delivered to target tissues

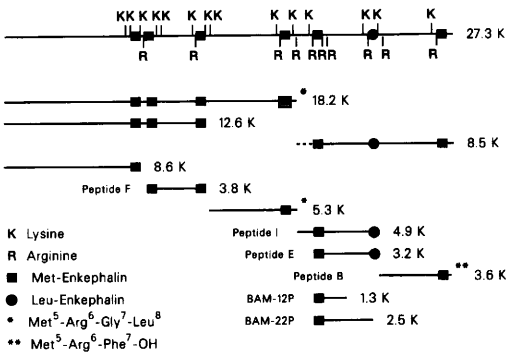

FIG. 4. The known fragments of preproenkephalin-A that have been isolated and characterized from extracts of adrenomedullary tissues. The coelution of 8500-Da BAM-22P with Met-enkephalin-Arg^{6}Phe^7-OH immunoreactivity suggests the existence of a novel fragment presented as the 8.5K species. The *N*-terminal extension of BAM-22P in this molecule has not yet been chemically determined. The proposed cleavage site at Met-Arg-Gly-Leu (see * in figure) would yield two fragments: the 18,200-Da N-terminal (18.2K) and an 8500-Da fragment (8.5K) containing both immunoreactive Met-enkephalin-Arg^6-Phe^7-OH as well as immunoreactive BAM-22P.

without significant loss of biological activity. Of course, it will be important to determine whether the 8500-Da form of circulating BAM-22P immunoreactivity has biological activity heretofore unaccountable by either Met- or Leu-enkephalin themselves.

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