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Chemistry

Mass spectrometric charting of bovine posterior/intermediate pituitary peptides*

(proopiomelanocortin/propressophysin/prooxyphysin/joining peptide/copectin)

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ABSTRACT **The feasibility for charting neuropeptides in neuroendocrine tissues on the basis of the universal property and inherent specificity of their molecular weights was explored. As a model, a comprehensive MS analysis of extractable peptides from bovine posterior/intermediate pituitary was performed. Two suitable MS techniques—namely, plasma-desorption time-of-flight and fast atom bombardment MS—were evaluated, and each method could identify more than 20 peptides, including N-terminally acetylated and C-terminally amidated species. *In toto* these peptides account for almost the entire lengths of propressophysin, prooxyphysin, and proopiomelanocortin. Some of the experimentally determined molecular weights did not match any known peptides. Three of these species were identified as acidic joining peptide(4–24) [proopiomelanocortin(83–103)], C-terminal glycopeptide(22–39) [propressophysin(130–147)], and glycosylated C-terminal glycopeptide(1–19) [propressophysin(109–127)] by conventional sequence analysis.**

The traditional approach of neurochemical pathology—i.e., the attempt to reveal the mechanisms of neurologic and psychiatric disorders by focusing on abnormal concentrations of *single* neuroregulators—has generally failed (1). It is, therefore, desirable to monitor entire neuroregulator profiles and their response to drug treatment, endogenous neuroregulators, stress, and disease. However, a detector capable of measuring all the diverse neuroregulators does not exist. At least 60 neuroregulators are currently known, and most of them belong to the still growing number of neuropeptides (2–5). The common practice of analyzing neuropeptides by RIAs becomes arduous when applied to entire profiles; moreover, this method can only be used for known peptide families.

Recent advances in high-mass MS instrumentation and the introduction of particle-induced desorption–ionization methods for analyzing intact biopolymers (6, 7) have resulted in mass-specific analyzers that can detect peptides regardless of provenance and composition, are fast, do not require prior sequence information, may be used on mixtures, and can be interfaced to HPLC for off- or on-line characterization of complete profiles (8, 9).

In our pursuit to establish a MS method for the charting of peptides in biological tissues, in general, and for neuropeptides, in particular, we devised the general strategy summarized in Scheme 1. This scheme is based on the rationale that molecular weight information is sufficiently specific to tentatively identify all peptides that are either known to be or can be predicted to be in a given tissue (Scheme 1 *B*). This approach reduces the number of peptides that have to be analyzed by more rigorous, but also more time-consuming, methods (Scheme 1 *C* and *D*). Prediction can be on grounds of "classical" precursor processing at consecutive basic amino acids or on grounds of processing at "consensus sequence" sites (10–12). Assignment of peptide candidates aids in choosing the best methods for structure confirmation by either classical methods or special MS techniques, such as MS peptide mapping (13, 14) and tandem MS (MS/MS) (15, 16). [We use the term "peptide charting" when we refer to the componential analysis of peptide/protein mixtures in a given tissue (extract), whereas we use the term "peptide mapping" when we refer to the compositional characterization of individual peptides/proteins by means of chemical or enzymatic digestions.] It also helps to avoid any futile attempt to sequence a N^{α}-acetylated peptide by Edman degradation. Hence, in developing our method we sought to keep sample preparation at a minimum, to determine molecular weights early in analysis, and to use available information on precursor sequence and tissue biochemistry.

In this paper we show the power of MS for charting pituitary peptides. The pituitary was chosen as our first test tissue because this neuroendocrine gland contains peptides from several precursors with a variety of posttranslational modifications. Two different types of mass spectrometers were evaluated: a plasma desorption (PD)-MS instrument because of its wide mass range and excellent sensitivity (17–19) and a double-focusing magnetic sector field instrument with fast atom bombardment (FAB) ionization because of its mass accuracy (20, 21).

MATERIALS AND METHODS

Tissue Extraction and HPLC Fractionation. Bovine pituitaries from a local slaughterhouse were dissected on-site into anterior and posterior/intermediate lobes and stored at −20°C until extraction. For the initial charting experiments (Fig. 1*A*) two posterior/intermediate lobes (≈1 g) were homogenized in 2 ml of 1% aqueous trifluoroacetic acid with a Polytron (Brinkmann). The homogenate was centrifuged (1000 × *g*, then 15,000 × *g*; 15 min each) to yield ≈550 μl of supernatant that was immediately fractionated by reversed-phase-HPLC (octadecylsilyl silica as the solid phase, 5 μm, 4.6 × 250 mm; ≈140 μl per injection) by using a Hewlett–

Abbreviations: pOMC, proopiomelanocortin; pPP, propressophysin; pOP, prooxyphysin; AJP, acidic joining peptide; CLIP, corticotropin-like intermediate lobe peptide; MSH, melanotropin; CPP, C-terminal glycopeptide of pPP; VP-NP and OT-NP, vasopressin- and oxytocin-neurophysin, respectively; PD-MS, plasma desorption MS; FAB-MS, fast atom bombardment MS.

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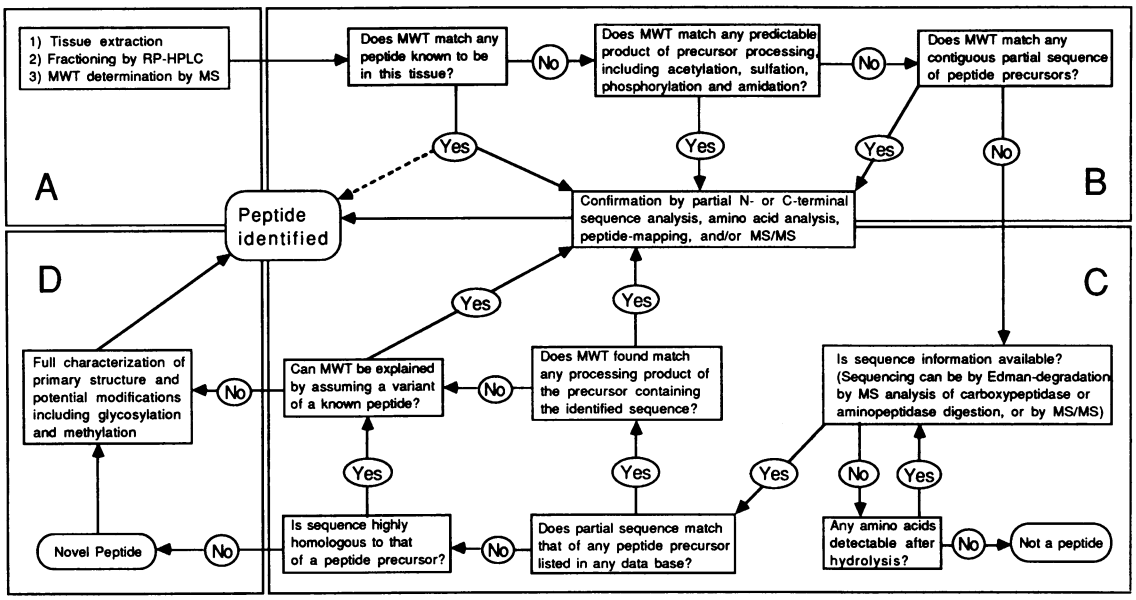

Scheme 1. Strategy used for the identification of neuropeptides.

Packard 1090M work-station, a linear gradient of 0–50% (vol/vol) acetonitrile in 0.1% aqueous trifluoroacetic acid over 50 min, a flow rate of 1 ml/min, and detection at 215 nm (22). One-millimeter fractions were collected into 1.5-ml polypropylene tubes and dried in a vacuum centrifuge. Samples for PD-MS, FAB-MS, and sequence analysis were obtained from equivalent work-ups. Isolation of fractions corresponding to individual HPLC peaks was not attempted, except for follow-up studies of the glycopeptides and the putative novel processing products and for examining "suppression effects" in the analysis of peptide mixtures. For the latter, pooled fractions corresponding to the more complex regions of the screening chromatogram were rechromatographed with shallower acetonitrile gradients for improved resolution; fractions corresponding to individual UV-absorbing peaks were collected in glass tubes; all other conditions were as above.

PD-MS. PD overview spectra were acquired on a Bio-Ion 10 instrument (Bio-Ion Nordic, Uppsala, Sweden) using an acceleration voltage of 16 kV, a time window of 8 or 16 μsec (corresponding to a M_r range of $\approx$7000 or 28,000, respectively), a resolution of 1 nsec, and a calibration of individual spectra based on the flight times of H^+ and Na^+. Dried HPLC fractions were redissolved in 10 or 15 μl of 12.5% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid, and 20–50% of the solution was applied to a nitrocellulose target by spin-drying (23).

FAB-MS. FAB-MS data were obtained with a VG-ZAB-SE instrument (VG Analytical, Manchester, U.K.) and the multichannel analyzer of a VG-11/250 data system (calibrated with CsI). Typically, dried HPLC fractions were redissolved in 2–3 μl of 1% trifluoroacetic acid. For FAB overview spectra the solutions were applied to 1–2 μl of *m*-nitrobenzylalcohol (24) (Aldrich) and analyzed by scanning exponentially from m/z 12,000 to 1000 at a rate of 20 sec per decade and a resolution of 500. Unit resolution spectra were acquired by using a thioglycerol matrix and an exponential scan from m/z 3700 to 650 at a rate of 80 sec per decade and a resolution of 3000.

Mass Analysis. For data analysis we used software written for an IBM PC (program GPMA; P.H., copyright). Molecular weights of known or predicted peptides are calculated and compared with the experimental data. Data inexplicable in this direct manner are analyzed by a "find mass" program for

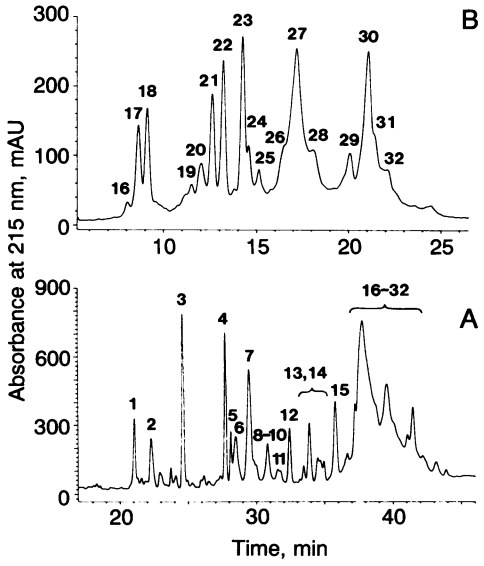

FIG. 1. Sequential reversed-phase-HPLC chromatograms. (*A*) Typical result from the initial charting of bovine posterior/intermediate pituitary peptides (linear gradient of 0–50% acetonitrile in 0.1% aqueous trifluoroacetic acid over 50 min; an extract aliquot equivalent to 0.5 pituitary was used). (*B*) Rechromatography of pooled fractions 40–42 (equivalent to 0.8 pituitary) with a gradient of 28–40% acetonitrile over 30 min [because of peak tailing, peptides corresponding to fractions 38–42 (*A*) were seen]; (apparent) molecular ions for compounds 24–26, 28, 29, and 32 were only detected after rechromatography with the shallower gradient but not in the initial charting. Peak numbering in *A* and *B* corresponds to the following peptides: 1, acidic joining peptide (AJP) (4–24); 2, AJP(1–24); 3, vasopressin; 4–6, C-terminal glycopeptide of propressophysin (CPP) (22–39), oxytocin, and Lys-γ_3-melanotropin (MSH); 7, β-MSH; 8–10, corticotropin-like intermediate lobe peptide (CLIP) (1–21), β-endorphin(1–16), and Des-Ac-α-MSH; 11, CLIP(1–20); 12, α-MSH; 13 and 14, Lys-γ_1-MSH and Ac-α-MSH; 15, CLIP; 16, CPP(1–19); 17–23, N fragment of proopiomelanocortin (pOMC), Ac-β-endorphin(1–17), and neurophysins {17, [Val^{89}]-VP-NP; 18, [Ile^{89}]-VP-NP; 23, putative oxytocin-neurophysin (OT-NP) (1–91)}; 24 and 25, not identified; 26, β-endorphin(1–25); 27, β-endorphin(1–27); 28 and 29, not identified; 30, pOMC(1–49); 31, Ac-β-endorphin(1–27); 32, not identified. Assignments based on data compiled in Table 1 plus Edman-type sequencing and additional MS experiments for *B* including PD mapping for the neurophins (data not shown). mAU, milli-absorbance units.

a match with any contiguous sequence within appropriate precursors (examples of analysis reports appear in ref. 25).

Sequence Analysis. Automatic Edman degradation was done on a gas-phase sequencer (Applied Biosystems model 470A) with 0.5–2 nmol of peptide and Polybrene as adsorbent.

RESULTS AND DISCUSSION

Method Evaluation. Before MS, only minimal sample preparation was necessary. The simple acidic extraction and the single stage of reversed-phase gradient chromatography used were sufficient; Fig. 1*A* shows a typical chromatogram, and Fig. 2 shows representative mass spectra. With few exceptions, the same peptides were seen with both MS methods. The nonscanning time-of-flight mass analyzers used in PD-MS are total integrating devices that detect nearly all ions produced, whereas the scanning sector analyzers of FAB-MS are differential devices that use only a very small fraction of the total ion current for analysis. As expected, we generally experienced 5 to 10 times greater sensitivity in the PD-MS mode than in the FAB-MS mode. From the sequencing yields we estimate that the absolute amounts of peptides used for PD-MS analysis were in the 50- to 600-pmol range, whereas for FAB-MS analysis they were in the 0.2- to 2-nmol range.

Even below mass 3 kDa the PD-MS measurements were only reliable to ±3 Da (Table 1, column 6); more accurate molecular mass data were obtained with FAB-MS (Table 1, columns 7–10). This has direct bearing on the efficiency of the "find mass" program, which is inversely proportional to the number of precursors that must be considered and to the experimental error of the mass measurement. A smaller search window results in fewer matches and therefore less ambiguity in identification. With tissues, such as the pituitary, that contain multiple precursors, mass accuracy becomes especially important. For example, FAB-MS was needed to unambiguously distinguish between CLIP and AJP, which have nearly the same mass.

Improvement of HPLC resolution (example in Fig. 1*B*) revealed additional peptides, but apparently all the major ones had already been identified in the initial charting. Multiple cationization with H^+, Na^+, and K^+ was noted throughout the study; for the peptides this posed no problem, but for the small proteins it somewhat complicated data interpretation. For example, due to cluster formation of varying composition, considerable mass variance for the molecular ion of the N terminus of pOMC was seen between different batches (Fig. 2*D*). In PD-MS (but not FAB-MS) interfering cations can, to some extent, be removed from the target by washing (23); however, small peptides are sometimes lost during this procedure. Thus involatile salts should be excluded from samples as much as possible by choosing salt-free matrices and avoiding glassware, for example.

Confirmation of Known Peptides. The overview spectra revealed 50–60 significant ions, about half of which were identified as molecular ions of peptides. The molecular weight data (Table 1, columns 5 and 7) immediately pinpointed 14 of the reported processing products of pPP, pOP, and pOMC (27, 30) (marked with a † in Table 1) and, with the exception of the glycopeptides Lys-γ_3-MSH and CPP [also coined copectin (35)], ultimately accounted for the entire lengths of pOMC, pPP, and pOP.

Because molecular ions of glycopeptides are not always seen in MS (36) and because the carbohydrate structures of the posterior/intermediate pituitary glycopeptides are not known, the identification of these compounds was not straightforward. Nevertheless, Lys-γ_3-MSH was identified upon Edman degradation of fraction 29 during an attempt to explain an ion at m/z 1008.5 not due to oxytocin. Similarly, CPP(1–*N*) was identified during an attempt to explain an ion of mass 3814.9 seen for fraction 37 (see below).

The largest molecules we encountered were the neurophysins with molecular masses >9 kDa; they are comprised of two classes (VP-NP and OT-NP), which in themselves are microheterogenous (Ile/Val at residue 89 of VP-NP and Leu/Gln at residue 93 of OT-NP). In addition, the neurophysins may be truncated (32, 33). The initial mass analysis was obviously performed on a mixture of such species. Together with multiple cationization and partial oxidation of methionines and reduction of cystines, the number of species with different, but closely related, masses was too high and the mass resolution too low for distinguishing individual molecular ions. However, investigations in our laboratories indicate that individual neurophysins may be conveniently identified by peptide mapping (unpublished data).

Identification of Unexpected Peptides. The usefulness of the "find mass" program for the recognition of unexpected peptides was demonstrated in at least two cases, namely for AJP(4–24) and CPP(22–39). These peptides were the most favorable matches for the ions seen for fractions 22 and 28

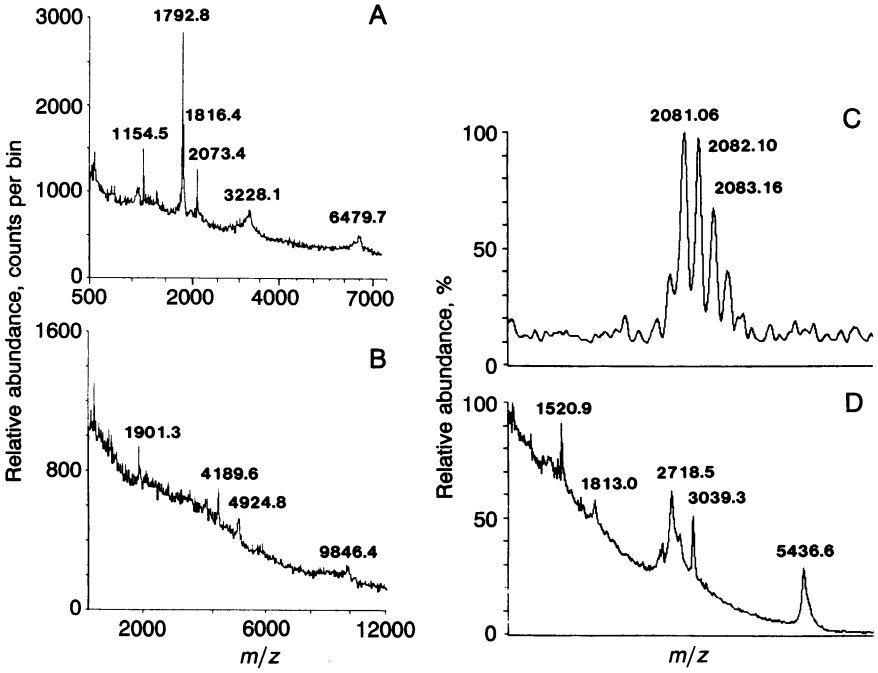

FIG. 2. Representative selection of mass spectra. (*A*) Partial PD overview spectrum of fraction 28 showing dominant molecular ions of CPP(22–39) at m/z 1792.8 ($[M+H]_c^+$) and 1816.4 ($[M+Na]_c^+$), respectively; other compounds obviously contained in this fraction have not yet been identified. (*B*) Partial PD overview spectrum of fraction 38 containing Ac-β-endorphin(1–17) (1901.3), pOMC N fragment (4189.6), and vasopressin-neurophysins (VP-NPs) (singly and doubly charged, 9846.4 and 4924.8, respectively). (*C*) Molecular ion region of the unit resolution FAB spectrum of AJP(4–24). (*D*) Partial FAB overview spectrum of fraction 42 showing molecular ions of the N terminus of pOMC at m/z 5436.6 ($[(M-H)+K+Na]_c^+$), 2718.5 ($[M+K+Na]_c^{2+}$), and 1813.0 ($[M+H+K+Na]_c^{3+}$) and of Ac-β-endorphin(1–27) at m/z 3039.3 ($[M+H]_c^+$) and 1520.9 ($[M+2H]_c^{2+}$). Note that although the two peptides are desorbed out of the same matrix the N terminus is preferentially cationized by Na^+ and K^+, whereas Ac-β-endorphin(1–27) is predominantly cationized by H^+.

Table 1. Summary of mass and sequence data obtained for bovine posterior/intermediate pituitary peptides

Fraction	Peptide	Source	Calculated $[M+H]_c^+$	PD-MS $R \approx 500$	Δ	FAB-MS $R = 500$	Δ	FAB-MS $R = 3000$	Δ	Calculated $[M+H]_m^+$	Ref.
22	AJP(4–24)	pOMC(83–103)	2082.1	2081.0	−1.1	2081.2	−0.9	2081.1	+0.1	2081.0	This study
23	AJP(1–24)	pOMC(80–103)	2469.5	2468.5	−1.0	2468.4	−1.1	2468.3	+0.2	2468.1	26
25	Vasopressin†	pPP(1–9)NH_2	1085.2	1083.7	−1.5	1084.7	−0.5	1084.5	+0.1	1084.4	27
28	CPP(22–39)	pPP(130–147)	1795.0	1792.8	−2.2	1794.8	−0.2	NM	NM	1793.9	This study
29	Oxytocin†	pOP(1–9)NH_2	1008.2	1006.2	−2.0	1007.6	−0.6	1007.4	±0	1007.4	27
	K-γ_3-MSH‡	pOMC(50–77)-CHO	ST								28
30	β-MSH†	pOMC(189–206)	2135.4	2132.9	−2.5	2134.9	−0.5	2134.1	+0.1	2134.0	29
31	CLIP(1–21)	pOMC(123–143)	2317.1	2318.6	+1.5	2318.2	+1.1	2317.3	+0.2	2317.1	
	β-End(1–16)†	pOMC(209–224)	1747.0	NO	NO	1746.8	−0.2	1745.8	±0	1745.8	30
	Des-Ac-α-MSH†	pOMC(106–118)NH_2	1623.8	1622.9	−0.9	1623.5	−0.3	1622.9	+0.1	1622.8	30
32	CLIP(1–20)	pOMC(123–142)	2189.4	2189.0	−0.4	NO	NO	2188.2	+0.1	2188.1	
33	α-MSH†	Ac-pOMC(106–118)NH_2	1665.8	1664.6	−1.2	1665.3	−0.5	1664.9	+0.1	1664.8	30
34	K-γ_1-MSH†	pOMC(50–61)NH_2	1641.9	1640.8	−1.1	1641.5	−0.4	1640.6	−0.2	1640.8	31
35	Ac-α-MSH†	Ac_2-pOMC(106–118)NH_2	1707.8	1706.4	−1.4	1707.8	±0	1706.6	−0.2	1706.8	30
36	CLIP	pOMC(123–144)	2465.7	2467.4	+1.7	2465.4	−0.3	2464.4	+0.2	2464.2	29, 30
	β-End(1–17)†	pOMC(209–225)	1860.1	1860.6	+0.5	1860.0	−0.1	NO	NO	1858.9	30
37	CPP(1–19)‡	pPP(109–127)	ST								This study
38	N fragment†	pOMC(147–186)	4188.5	4189.6	+1.1	4187.7	−0.8	NM	NM	4186.0	29
	Ac-β-End(1–17)†	Ac-pOMC(209–225)	1902.1	1901.3	−0.8	1902.1	±0	1901.0	+0.1	1900.9	30
39	VP-NP§	pPP(13–107)	ST	9846.4	ST	9903	ST	NM	NM		27, 32, 33
40	OT-NP§	pOP(13–105)	ST	9383.3	ST	9414	ST	NM	NM		27, 32, 33
	β-End(1–31)†	pOMC(209–239)	3439.0	NO	NO	3439.8	+0.8	NM	NM	3436.8	29, 30
41	β-End(1–27)†	pOMC(209–235)	2997.5	NO	NO	2996.8	−0.7	NM	NM	2995.6	30
42	N terminus¶	pOMC(1–49)	5375.9	5382.9	+7.0	5377.0	+1.1	NM	NM		34
	Ac-β-End(1–27)†	Ac-pOMC(209–235)	3039.5	3044.3	+4.8	3039.2	−0.3	NM	NM	3037.6	30

$[M+H]_c^+$ and $[M+H]_m^+$, chemical and monoisotopic masses of protonated molecular ions, respectively; pPP, propressophysin; pOP, prooxyphysin. *R*, mass resolution; End, endorphin; Ac, acetyl; NM, not measured; NO, not observed; ST, see text. †Tentative assignment immediately following M_r determination. ‡Only seen in the glycosylated form. §Mixture of closely related species; see text. ¶Extensive alkali attachment seen; see text.

($[M+H]_m^+$ 2081 and $[M+H]_c^+$ 1795, respectively). All other peptides suggested by the program were judged to be poor candidates either because they contained dibasic cleavage sites not expected to survive in the intermediate pituitary or because their processing would have required the unlikely cleavage of an Xaa-Pro or a Pro-Xaa bond. AJP(4–24) and CPP(22–39) were confirmed by Edman degradation. The computer analysis also suggested C-terminally truncated CLIPs as candidates for peptides found in fractions 31 and 32 (see Table 1); CLIP(1–21) has been isolated from the rat (37, 38).

With fraction 37 we encountered an example where the "find mass" program proposed a possible, but nevertheless wrong, candidate. In this case, pOMC(65–103) (M_r 3812.9), arising from cleavage at the dibasic sites pOMC(63/64) and pOMC(104/105), seemed a suitable candidate for an ion of mass 3814.9. Re-isolation of fraction 37 for sequence analysis yielded a mixture of four peptides containing the unknown together with N fragment, VP-NP, and Ac-β-endorphin(1–17). Subtractive Edman degradation of the mixture established Xaa-Asn-Asp-Arg-Xaa-Xaa-Xaa-Thr as the partial N-terminal sequence for the unknown. This sequence excluded pOMC(65–103) but was compatible with glycosylated CPP(1–*N*) [Asn-6-Xaa-Thr-8 comprises a typical N-glycosylation site (39)]. Data from Edman degradation of further purified CPP(1–*N*) confirmed this conclusion and tentatively established $N = 19$.

Biological Significance. Full-length CPP has been reported to stimulate the release of prolactin (40). Biological activity of the CPP fragments may also be anticipated because their *in vivo* biosynthesis has recently been shown for the rat (41, 42) [CPP(1–19/20) and CPP(22–37/39)]. By analogy, the bovine counterparts found in this study are likely to be processing products rather than artifacts of the isolation procedure. The processing mechanisms that lead to CPP(1–19) and CPP(22–39) are not known. It is tempting to explain CPP(1–19) via cleavage at the C terminus of Arg-20 followed by carboxypeptidase B action, especially because the rat cleavage site is consistent with the recent proposal (11) that "single" basic cleavage sites are really "double" basic cleavage sites with the two basic amino acids being separated by two, four, or six other amino acids (Fig. 3). However, the corresponding bovine cleavage site does not fulfill the requirement of a second basic amino acid despite the fact that we find the corresponding bovine CPP fragments. The processing of CPP, therefore, must either follow a different mechanism, or the second basic amino acid is not an absolute requirement. AJP is both acidic and contains a high mol % of proline, glutamic and aspartic acid, and threonine. These characteristics have been linked to fast proteolytic degradation *in vivo* (43). Thus the truncated form of AJP [AJP(4–24)] found in this study could be a natural processing product. Finally, the two Lys-γ-MSHs deserve mentioning, because sometimes they are referred to as having no N-terminal lysine. Consistent with earlier reports (28, 31, 44) we observed Lys-γ-MSHs and not γ-MSHs. Unless flanked by proline residues, all basic amino acids are generally removed after cleavage of the neuropeptide precursors at basic sites (12, 45). It remains an unanswered question as to why the Lys-γ-MSHs behave so differently.

CONCLUSION

The more abundant peptides in the pituitary can be efficiently charted by MS, apparently irrespective of peptide class and of posttranslational acetylation and amidation. Neither a RIA, which depends on a specific antigenic determinant, nor a chemical assay, which depends on a specific structural feature, could accomplish this. The charting was not obstructed by breakdown products of structural proteins. Suppression of certain peptides within a mixture was seen, but not to a major extent. In addition to increasing the HPLC resolution, suppression effects due to differences in surface

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                *
....LLDGPSGALLLRLVQLAGAP....   (bovine)

....QLDGPARELLLRLVQLAGTQ....   (rat)
        *   *
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FIG. 3. Comparison of cleavage sites for rat and bovine CPP. CPP(9–28) is shown. Basic sites are indicated by asterisks; conserved residues are underlined.

activity may also be overcome with dynamic FAB (9), in which the target surface is transient in nature.

Qualitative results obtained with PD-MS and FAB-MS were similar, but, as expected, the specificity of detection was higher with the magnetic instrument, whereas sensitivity, which is especially critical for measuring large peptides and charting peptides in small tissue samples, was greater with the time-of-flight instrument. Overall, both MS techniques complemented each other.

At present the dynamic range of our MS charting is limited to one or two orders of magnitude. The abundance of the molecular ions for trace peptides, such as somatostatin or cholecystokinin octapeptide (46), was apparently below detection. We anticipate that specificity and detection levels will improve as other versions of mass spectrometers become available commercially. The specificity of PD-MS can be increased through the use of an electrostatic mirror (47), and the sensitivity of magnetic instruments can be increased through the use of liquid metal ion probes to promote more efficient sample desorption (48) and of array detectors for the simultaneous detection of ions (49). Other developments, such as Fourier-transform MS with secondary ion or PD (50, 51), multiphoton-ionization (52, 53), and matrix-assisted laser desorption (54) may also bring about improvements for the MS charting of peptides.

Although we limited this study to one tissue type, there is no compelling reason why our method should not be more widely applicable. In fact, a recent independent study successfully charted hormones in the catfish pancreas by PD-MS (55). Difficulties, which may arise from increased complexity and lower amounts of peptides in other tissues, should be met by the aforementioned advances emerging in MS. If performed quantitatively, as could be done with mass chromatography by using a coupled HPLC/MS system, MS charting opens the exciting possibility of studying the regulation of whole peptide profiles in discrete tissues.

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