Endocrine, *vol. 8, no. 3, 231–240, June 1998* 0969–711X/98/8:231–240/$10.50

Elevated Proenkephalin-Derived Peptide Levels in ACTH-Producing Adenomas

Nucleus and Cytoplasm Localization

Osvaldo Vindrola,[1] Alberto Chervin,[2] Marcelo Vitale,[2] Alejandra N. Mella,[2] Raquel Aloyz,[3] and Armando Basso[2]

[1]*Instituto de Fisiologia, Laboratorio de Bioquimica, Universidad Autonoma de Puebla. Mexico;* [2]*Hospital Santa Lucia. Departamento de Neurocirugia y Endocrinologia. Buenos Aires Argentina; and* [3]*Brain Tumor Research Center. Montreal Neurological Institute. Montreal. Quebec. Canada*

The biosynthesis of met-enkephalin in human pituitary and human pituitary adenomas is still not well known. In this work, we studied the processing of proenkephalin-derived peptides in postmortem human pituitary (PMHP), ACTH-producing adenomas (ACTH-PA), nonfunctioning adenomas (NFA), and GH-producing adenomas (GH-PA). ACTH-PA contained at least 10 times more proenkephalin-derived peptides than PMHP, NFA, and GH-PA. Proenkephalin processing was different in the four tested tissues. In ACTH-PA, proenkephalin was processed to high-, intermediate-, and low-mol-wt products. The highest met-enkephalin-containing peptides levels corresponded to intermediate and low-mol-wt materials, although met-enkephalin-Arg-Phe and synenkephalin immunoreactivity appeared only in high-mol-wt peptides. In PMHP and NFA, met-enkephalin-Arg-Phe immunoreactivity was detected in intermediate- and low-mol-wt materials, and it was absent in GH-PA. Immunoblotting of ACTH-PA showed that met-enkephalin-Arg-Phe immunoreactivity corresponded to peptides of 44, 32–30, 27, and 17 kDa. The 32–30 and 17-kDa molecules were localized in the nuclear fraction where they were extracted after enzymatic digestion with DNase I. Plasmatic met-enkephalin levels did not increase in patients with Cushing´s disease, suggesting that the pentapeptide stored in ACTH-PA was not released to the general circulation. In conclusion, we demonstrated that only ACTH-PA contained high levels of proenkephalin peptides, which were stored in cytoplasm organelles and in the nucleus, probably bound to chromatin. These results suggest an adenoma-specific physiological role of proenkephalin products.

Key Words: Proenkephalin; met-enkephalin; nuclei; pituitary; adenomas; Cushing's disease.

Abbreviations: ACTH-PA, ACTH-producing adenoma; NFA, nonfunctioning adenoma; GH-PA, GH-producing adenoma; PMHP, postmortem human pituitary; IR, immunoreactivity; SYN, synenkephalin.

Introduction

Proenkephalin contains four copies of met-enkephalin and one copy of leu-enkephalin, met-enkephalin-Arg-Gly-Leu, and met-enkephalin-Arg-Phe *(1–6)*. Furthermore, proenkephalin presents at the N-terminus one copy of synenkephalin, a nonopioid peptide, which corresponds to the sequence 1–70 *(1–6)*. Leu-enkephalin is also present in prodynorphin molecule *(1,3,6)*, and met-enkephalin appears in the proopiomelanocortin sequence *(1,3,6)*, whereas synenkephalin is only present in the proenkephalin sequence *(6)*. Strict correlation was always observed between the presence of synenkephalin-containing peptides and the expression of proenkephalin *(6–10)*.

In humans, met-enkephalin was detected in high concentrations in the adrenal medulla, pituitary, spinal cord, brain, sympathetic ganglia, and peripheral neurons *(11)*. In most mammalian species, the posterior pituitary contained high concentrations of preproenkephalin mRNA and met-enkephalin, but little or no pentapeptide was detected in the anterior pituitary *(12–14)*. Nevertheless, high levels of met-enkephalin with no met-enkephalin-Arg-Gly-Leu or met-enkephalin-Arg-Phe were observed in the human anterior pituitary, and most of this immunoreactivity was localized in a large subpopulation of thyrotrophs *(15)*. Immuno-

Received July 7, 1997; Revised February 17, 1998; Accepted February 17, 1998.

Author to whom all correspondence and reprint requests should be addressed. Dr. Osvaldo Vindrola, Professor, Instituto de Fisiologia, Laboratorio de Bioquimica, Universidad Autonoma de Puebla, Apartado postal 406, 72001 Puebla, Mexico. E-mail: ovindrol@siu.cen.buap.mx

Table 1
Total and Free IR-met-enkephalin in PMHP[a]

Patient	Age	Sex	IR-met-enkephalin	
			Total	Free
1	37	F	32.6	12.2
2	42	F	37.6	14.6
3	39	F	39.8	13.8
4	35	M	31.0	12.4
5	39	M	32.0	12.0
Mean			34.6 ± 3.86	13 ± 1.14

[a]The results are expressed in pmol/mg protein and represent the mean ± SD.

Table 2
Total and Free IR-met-enkephalin in ACTH-PA[a]

Patient	Age	Sex	IR-met-enkephalin	
			Total	Free
1	37	F	320.4	149.0
2	49	F	236.1	132.3
3	30	F	359.0	250.0
4	32	F	31.9	15.0
5	37	M	297.0	231.0

[a]The results are expressed in pmol/mg protein.

cytochemical and biochemical studies have demonstrated the presence of met-enkephalin in postmortem human pituitary, nonfunctioning adenomas, and ACTH-producing adenomas *(16–18)*. Furthermore, these adenomas and GH-producing adenomas expressed the prohormone-converting enzymes PC1 and PC2 mRNAs *(19)*. Although these works have demonstrated the presence of met-enkephalin and the expression of prohormone-converting enzymes in pituitary adenomas, the posttranslational processing of proenkephalin has not been characterized.

In the present work, we studied the production and post-translational processing of proenkephalin-derived peptides in postmortem human pituitary (PMHP), ACTH-producing adenomas (ACTH-PA), GH-producing adenomas (GH-PA), and nonfunctioning adenomas (NFA). In addition, the nuclear localization of proenkephalin peptides was analyzed in some ACTH-PA. Plasma levels of met-enkephalin were assayed to ascertain whether peptide tissue changes were associated with their release to the general circulation.

Results

To find the ratio between met-enkephalin-containing precursors and low-mol-wt products, tissue extracts were assayed before (free) and after (total) enzymatic digestion with trypsin and carboxypeptidase B. Total and free immunoreactive (IR) met-enkephalin were detected in PMHP (Table 1), in ACTH-PA (Table 2), in NFA (Table 3), and in GH-PA (Table 4). In PMHP, free IR-met-enkephalin represented about 37% of total IR-met-enkephalin, indicating the prevalence of precursor molecules.

Table 3
Total and Free IR-met-enkephalin in Nonfunctioning Adenomas[a]

Patient	Age	Sex	IR-met-enkephalin	
			Total	Free
1	26	F	7.21	1.93
2	59	F	9.3	5.60
3	65	M	11.7	7.50
4	70	M	13.2	2.97
5	60	M	12.3	2.60
6	42	F	5.24	1.40
7	38	M	ND	ND
8	45	M	ND	ND
9	28	F	11.4	8.21
10	40	M	18.6	5.20
11	28	M	ND	ND

[a]The results are expressed in pmol/mg protein. ND, non-detectable.

Table 4
Total and Free IR-met-enkephalin in GH-PA[a]

Patient	Age	Sex	IR-met-enkephalin	
			Total	Free
1	62	F	22.0	4.40
2	44	M	4.3	1.66
3	44	F	7.11	5.33
4	48	F	21.2	4.43
5	42	F	44.0	13.0

[a]The results are expressed in pmol/mg protein.

Total and free IR-met-enkephalin increased 8–15 times in ACTH-PA (Tables 1 and 2). In these adenomas, 60–80% of the cells showed positive immunostaining for ACTH. In one of five Cushing's patients (patient 4), the tissue tested corresponded to a second pituitary surgery, and only 30% of adenoma cells were immunopositive for ACTH. Coincidentally total and free IR-met-enkephalin levels in this adenoma did not differ from PMHP values.

In 8 of 11 NFA, total and free IR-met-enkephalin decreased about 70%, and in the remaining NFA, total and free IR-met-enkephalin were not detected (Table 3). In these adenomas, there was no correlation between proenkephalin derived peptides levels and positive immunoreactivity for a particular hormone (Tables 3 and 5).

A significant decrease (56%) in total and free IR-met-enkephalin content was observed in four out of five GH-PA, but no changes appeared in the other adenoma (Table 4). Forty to 70% of GH-PA showed positive immunostaining for GH (Table 6).

Table 5
Clinical Laboratory and Immunostaining Data from Patients with Pituitary Nonfunctioning Adenomas[a]

			Serum levels						Immunostaining				
Patient	Age	Sex	LH	FSH	PRL	T4	E2	T0	LH	FSH	PRL	ACTH	TSH
1	26	F	9.1	2.8	***51***	8.6	72	—	ND	ND	ND	ND	ND
2	59	F	2.2	32.0	***22***	7.8	18	—	ND	ND	ND	ND	ND
3	65	M	10.0	4.4	3	4.8	—	0.2	5%	10%	ND	30%	ND
4	70	M	22.0	20.0	11	7.6	—	1.8	10%	15%	ND	ND	ND
5	60	M	6.0	9.0	18	9.8	—	3.8	70%	60%	10%	ND	10%
6	42	F	8.0	6.0	19	6.1	86	—	10%	40%	5%	ND	5%
7	38	M	3.9	4.5	13	7.9	—	2.6	75%	ND	10%	10%	ND
8	45	M	5.4	2.8	***28***	5.7	—	9.4	5%	ND	5%	5%	ND
9	28	F	17.0	10.0	***86***	9.6	65	—	ND	5%	5%	10%	ND
10	40	M	12.0	2.7	20	8.9	—	0.2	ND	80%	ND	ND	ND
11	28	M	1.9	1.5	15	6.0	—	0.4	30%	40%	ND	ND	ND

[a]T0, testosterone. E2, estradiol. ND, nondetectable. %, Percentage of positive cells in macroadenoma tissue. LH (mUI/mL), FSH (mUI/mL), PRL (ng/mL), T4 (gamma's %), E2 (pg/mL), T0 (ng/mL). Serum levels differing from normal values are marked in bold and italic.

Table 6
Clinical Laboratory and Immunostaining Data from Patients with GH-PA[a]

			Serum levels			
Patients	Age	Sex	GH, ng/mL	IGFI	PRL, ng/mL	Immunostaining
1	62	F	9.6	14.3	15.6	GH 40%
2	44	M	24.8	12.9	7.3	GH 60%
3	44	F	16.8	10.1	12.5	GH 50%
4	48	F	31.5	7.55	15.0	GH 70%
5	42	F	12.7	6.4	10.8	GH 40%
Normal values			0-5	(*)	M:3-16 F:3-20	

[a]%, Percentage of positive cells in macroadenoma tissue. (*) According to Furlanetto et al. *(53)*.

Table 7
Clinical Laboratory and Immunostaining Data from Patients with ACTH-PA

Patient	Age	Sex	Cortisol/serum, µg/dL 8 AM	Cortisol/urinary, µg/24 h	ACTH/serum, µg/mL 8 AM	Immunostaining
1	37	F	36.7	628	89	80%[a]
2	49	F	19.0	354	28	80%[a]
3	30	F	41.0	900	36	80%[a]
4	32	F	32.0	186	40	30%[a]
5	37	M	46.0	827	59	60%[a]
Normal values			7–25	10–100	0–54	

[a]% of ACTH-positive cells in microadenoma tissue.

These results showed that total IR-met-enkephalin content was different in PMHP, ACTH-PA, NFA, and GH-PA. To discover the posttranslational processing of proenkephalin in these tissues, the extracts were applied to gel-filtration chromatography, and fractions were assayed with met-enkephalin, synenkephalin, and met-enkephalin-Arg-Phe radioimmunoassay.

Peptides of high, intermediate, and low-molecular weight displaying IR-met-enkephalin were observed in PMHP (Fig. 1A). Free IR-met-enkephalin appeared in peptides smaller than 6.5 kDa (3.5, 2.0, and 0.5 kDa), and the highest concentrations corresponded to materials eluted in the position of met-enkephalin standard (Fig. 1A). IR-synenkephalin was observed in peptides of 8.5 kDa and

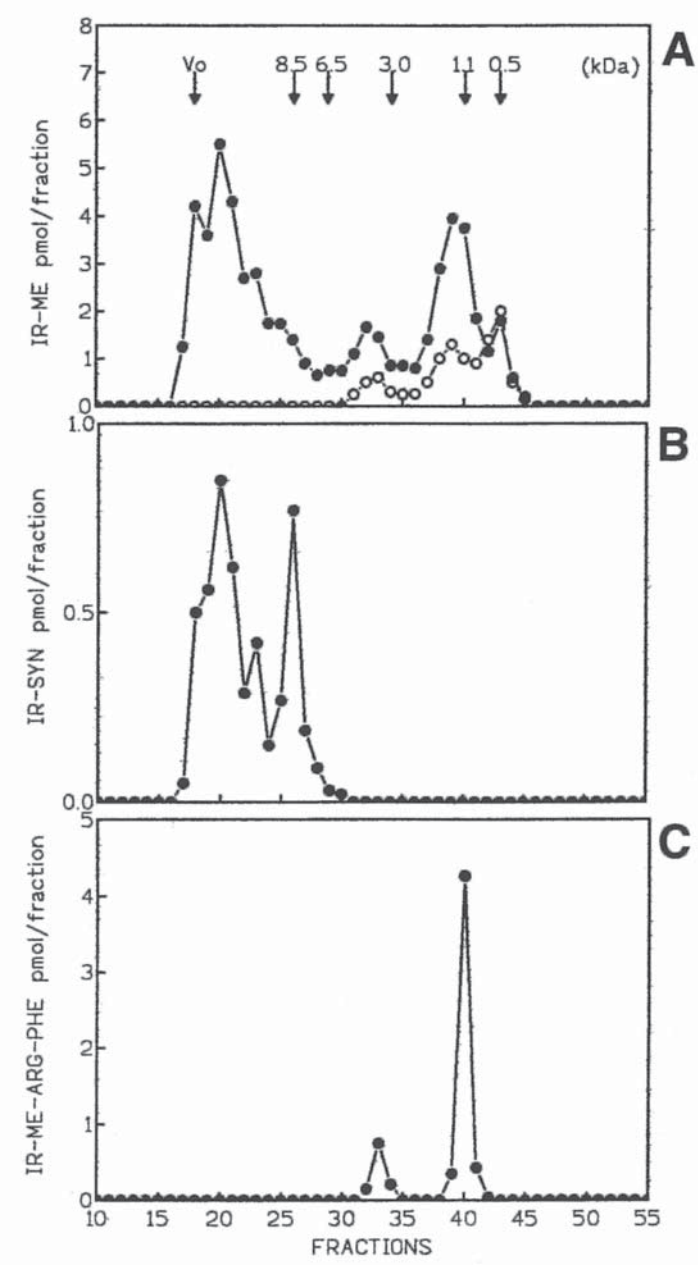

Fig. 1. Sephadex G-50 chromatography of met-enkephalin-containing peptides from 1 *M* acetic acid (pH 1.9 with HCl) extract of PMHP. The 50,000*g* supernatant of the homogenate was applied to a 60 × 1.0 cm column, and 2-mL fractions were collected. Aliquots of each fraction were lyophilized and resuspended in 50 m*M* Tris-HCl buffer, pH 8.4, and 2 m*M* $CaCl_2$. Met-enkephalin (ME) **(A)** and synenkephalin (SYN) **(B)** were assayed before (○) and after (●) enzymatic digestion with trypsin and carboxypeptidase B. ME-Arg-Phe **(C)** was tested without enzymatic digestion. The elution positions of calibration markers were as follow: Vo, hemocyanin; 8.5 kDa, bovine proenkephalin 1-77; 6.5 kDa, aprotinin; 3.0 kDa, amidorphin; 1.1 kDa, [Tyr^{63}] (syn 63-70) synenkephalin; 0.5 kDa, enkephalin.

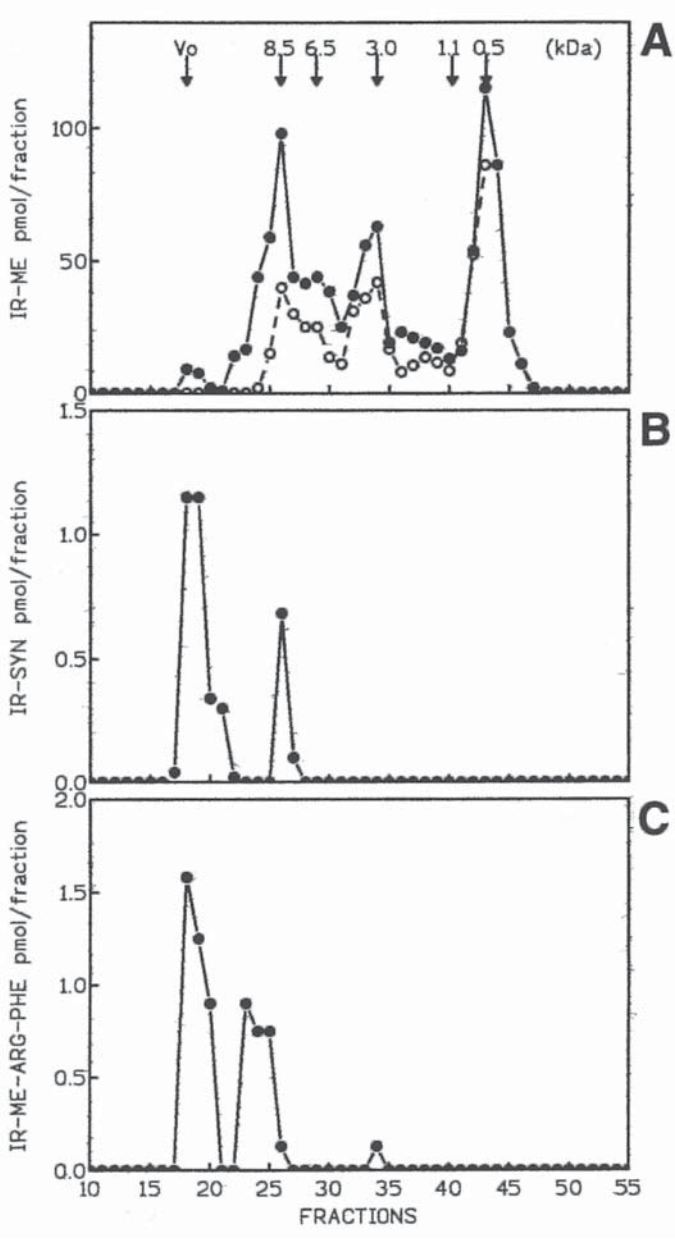

Fig. 2. Met-enkephalin **(A)**, and synenkephalin **(B)**, and met-enkephalin-Arg-Phe-containing peptides **(C)** in ACTH-PA (patient 1). Tissue extract was processed as described in Fig. 1. The elution positions of calibration markers were as described in Fig. 1. Peptides were assayed by RIA before and after enzymatic digestion, as described in Fig. 1.

larger than 8.5 kDa (Fig. 1B). IR-met-enkephalin to IR-synenkephalin ratios were between 8 and 6 in peptides larger than 8.5 kDa (Fig. 1A,B). These ratios corresponded to that predicted for proenkephalin-derived peptides. Free IR-synenkephalin was not detected in PMHP (data not shown). IR-met-enkephalin-Arg-Phe appeared in low-mol-wt peptides eluting in the position of 3.0 and 1.1 kDa standards (Fig. 1C), which correspond to the elution position of peptide B and met-enkephalin-Arg-Phe (data not shown).

IR-met-enkephalin in ACTH-PA (patient 1) appeared in peptides of intermediate and low molecular weight (Fig. 2A). The highest concentrations of IR-met-enkephalin occurred in peptides eluted in the position of met-enkephalin standard. Free IR-met-enkephalin fundamentally eluted in four peaks: two of them occurred between 8.5 and 6.5 kDa stan-

dards, one appeared in the position of 3.5 kDa standard, and one in a major peak of 0.5 kDa. IR-synenkephalin was only observed in peptides of 8.5 kDa or larger than 8.5 kDa (Fig. 2B). IR-met-enkephalin to IR-synenkephalin ratios in materials larger than 8.5 kDa were about 8, according to that predicted for proenkephalin-derived peptides. Free IR-synenkephalin was not detected in ACTH-PA (data not shown). IR-met-enkephalin-Arg-Phe appeared principally in peptides larger than 8.5 kDa (Fig. 2C).

Immunoblotting of ACTH-PA extract, using met-enkephalin-Arg-Phe antibody, showed the presence of 44-, 32–30, 27, and 17 kDa peptides (Fig. 3A). Forty-four- and 27-kDa peptides were localized in postnuclear supernatant; 32- to 30-kDa peptides appeared in postnuclear supernatant and nuclear fraction, whereas 17-kDa molecule was observed only in nuclear fraction. Thirty-two- to 30- and 17-kDa peptides were extracted from nuclear fraction after DNase I treatment (Fig. 3A). PC1 enzyme appeared as a 66-kDa protein, which was exclusively detected in post-nuclear supernatant (Fig. 3B).

In NFA extract (patient 10), most of the IR-met-enkephalin was detected in peptides smaller than 3.5 kDa (Fig. 4A). Three peaks of free IR-met-enkephalin of 3.5, 2.0, and 0.5 kDa were observed. IR-synenkephalin appeared in molecules larger than 8.5 kDa (Fig. 4B), where IR-met-enkephalin to IR-synenkephalin ratios were between 8 and 2 (Fig. 4A,B). IR-met-enkephalin-Arg-Phe was observed in low-mol-wt peptides, eluting nearly 3.0- and 1.1-kDa standards (Fig. 4B).

In GH-PA (patient 4), IR-met-enkephalin was mainly detected in high- and intermediate-mol-wt peptides (Fig. 5A). IR-synenkephalin was observed in high-mol-wt peptides (Fig. 5B), where IR-met-enkephalin to IR-synenkephalin ratios were between 7 and 8 (Fig. 5A,B). IR-met-enkephalin-Arg-Phe was not detected in the chromatographic fractions of GH-PA extracts.

Fractions 41–46 from G-50 chromatography of ACTH-PA (Fig. 2A) were pooled and applied to reverse-phase HPLC. Aliquots of HPLC fractions were assayed for IR-met-enkephalin by radioimmunoassay. IR-met-enkephalin was detected in two peaks, corresponding to the position of met-enkephalin and oxidized met-enkephalin standards (Fig. 6).

Plasma IR-met-enkephalin levels in some of the patients with ACTH-PA, NFA, and GH-PA, obtained 15 d before surgery, were compared with normal subjects. IR-met-enkephalin did not change in the plasma of five patients with ACTH-PA or in the three patients with GH-PA (Fig. 7). However, a significant increase of plasma IR-met-enkephalin content was observed in 5 of 11 patients with NFA.

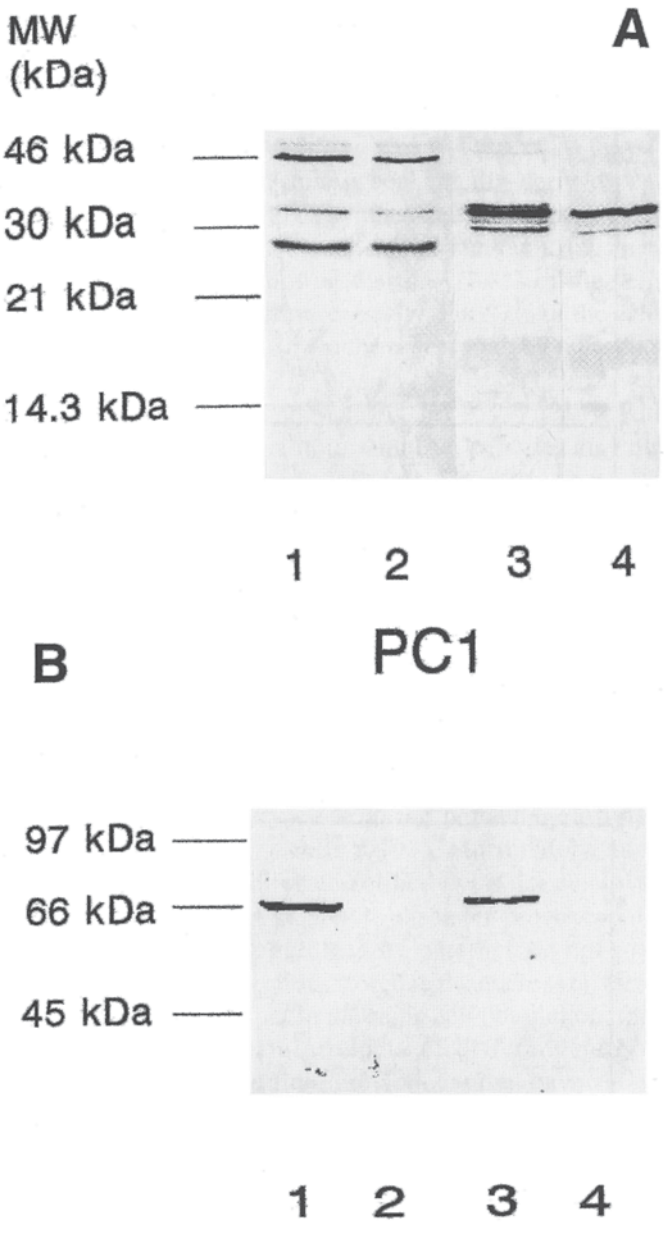

Fig. 3. Analysis of proenkephalin derived peptides **(A)** and PC1 enzyme **(B)** in ACTH-PA by gel electrophoresis and immunoblotting. Samples (about 30 μg protein/lane) of two different ACTH-PAs (patients 6 and 7) were electrophoresed in 15% (proenkephalin peptides) or 8.8% (PC1) SDS-polyacrylamide gels and then electroblotted to a nitrocellulose membrane. Proenkephalin products were detected with met-enkephalin-Arg-Phe directed antibody. **(A)** Lanes 1 and 2 postnuclear supernatant, and lanes 3 and 4, peptides released from nuclear extract after DNase I digestion. **(B)** PC1 protein was detected with NH2-terminus-directed antibody (DAM3): lanes 1 and 3 postnuclear supernatant, and lanes 2 and 4 supernatant after DNase I digestion of nuclear extract.

Discussion

The results of the present work showed that ACTH-PA synthesized at least 10 times more proenkephalin-derived peptides than PMHP, NFA, and GHP. In this adenoma, proenkephalin was processed to high-, intermediate-, and low-mol-wt products. The highest levels of met-enkephalin-containing peptides corresponded to intermediate- and low-mol-wt materials, whereas met-enkephalin-Arg-Phe and

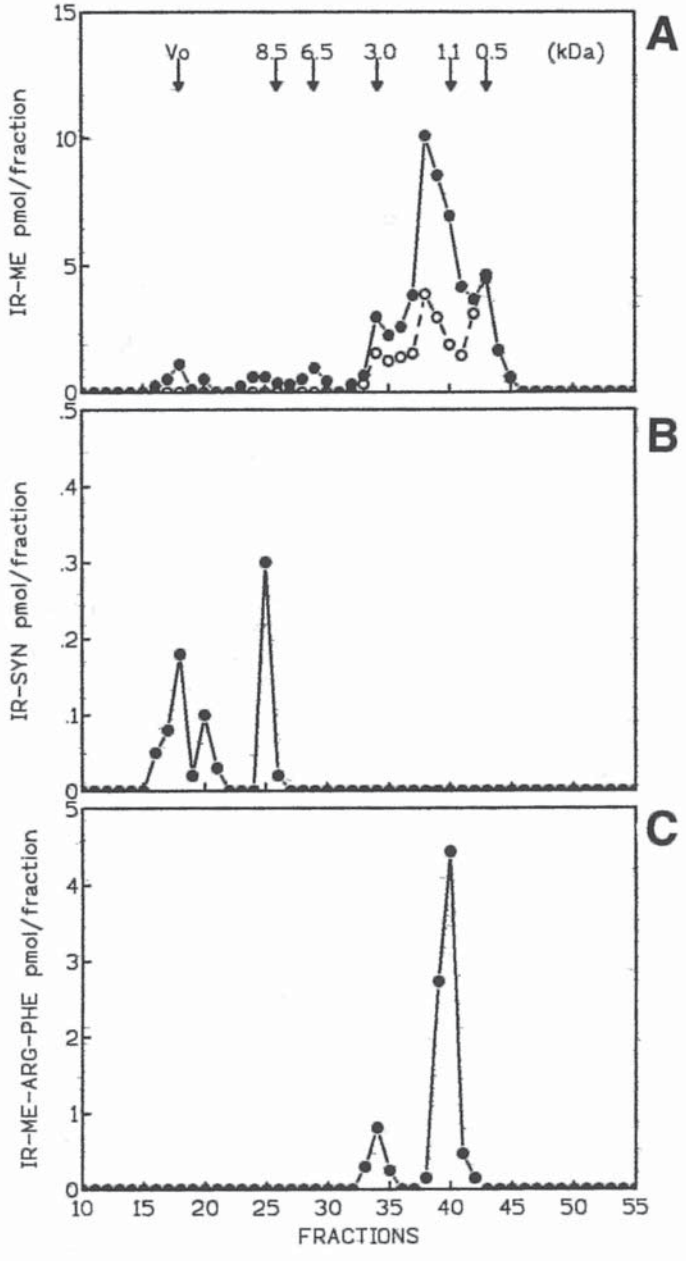

Fig. 4. Met-enkephalin **(A)**, synenkephalin **(B)**, and met-enkephalin-Arg-Phe-containing peptides **(C)** in NFA (patient 10). Tissue extract was processed as described in Fig. 1. The elution positions of calibration markers were as described in Fig. 1. Peptides were assayed by RIA before and after enzymatic digestion, as described in Fig. 1.

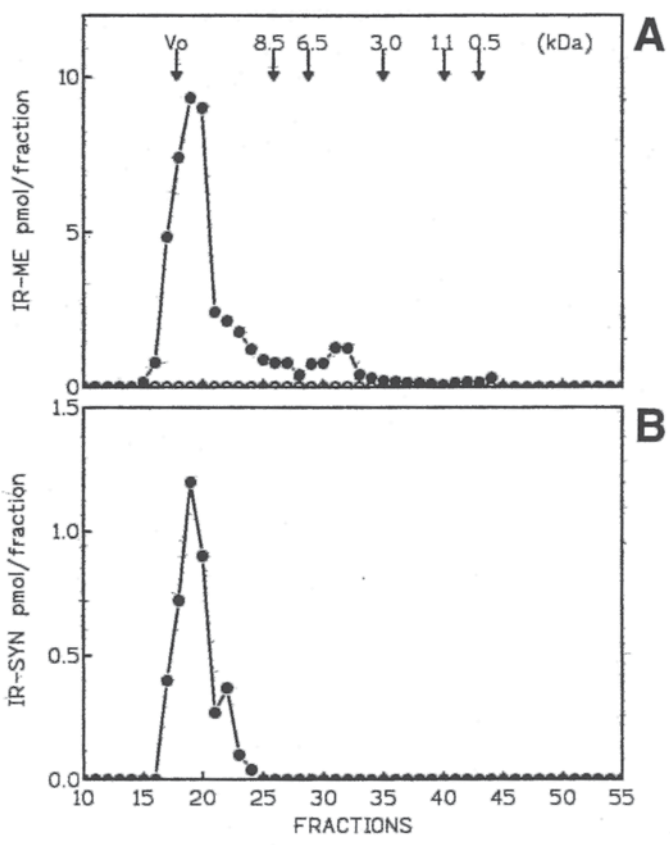

Fig. 5. Met-enkephalin **(A)** and synenkephalin **(B)** in GH-PA (patient 4). Tissue extract was processed as described in Fig. 1. The elution positions of calibration markers were as described in Fig. 1. Peptides were assayed by RIA before and after enzymatic digestion, as described in Fig. 1.

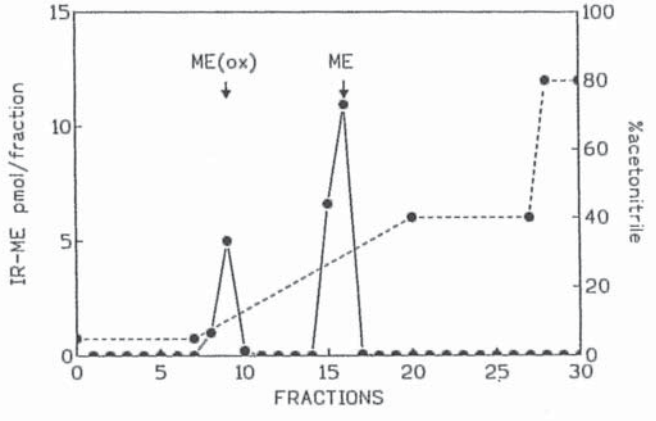

Fig. 6. HPLC of gel-filtration eluates. Aliquots of fractions 41-45 of G-50 Sephadex (Fig. 2) were pooled and evaporated, resuspended in 0.1% TFA, and applied to a reverse-phase HPLC. Elution was performed using an acetonitrile gradient. The flow rate was 0.5 mL/min, and 1-mL fractions were collected, resuspended in RIA buffer, and assayed for met-enkephalin. The arrows indicate the position of met-enkephalin (ME) and oxidized met-enkephalin (ME ox).

synenkephalin immunoreactivity appeared only in high-mol-wt peptides. These results were different from those observed in PMHP and NFA, where heptapeptide immunoreactivity was mainly detected in intermediate- and low-mol-wt materials. Immunoblotting assays of ACTH-PA extracts showed that most of the heptapeptide immunoreactivity was observed in peptides of 44, 32–30, 27, and 17 kDa. The 44- and 27-kDa molecules were mainly localized in postnuclear supernatant, 32–30 kDa peptides appeared in postnuclear supernatant and nuclear fraction, whereas the 17-kDa molecule was only detected in the nuclear fraction. Furthermore, 32–30 and 17-kDa peptides were extracted from the nuclear fraction after DNase I treat-

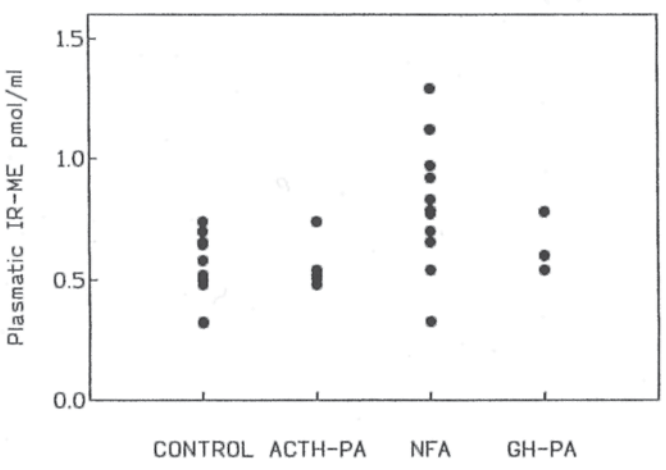

Fig. 7. Plasmatic levels of met-enkephalin in patients with ACTH-PA, NFA, and GH-PA. Two milliliters of plasma were purified through Amberlite XAD-2 column, and ME was assayed by RIA. Controls: 0.546 ± 0.146, ACTH-PA: 0.557 ± 0.105, NFA: 0.810 ± 0.267, GH-PA: 0.640 ± 0.125. Results are means ± SD.

ment. This result suggests that nuclear proenkephalin-derived peptides were bound to chromatin.

Bottger and Spruce *(20)* were the first to describe the presence of proenkephalin-derived molecules in the nucleus of fibroblast and myoblast cell lines. This nuclear proenkephalin corresponded only to 32–30 kDa molecules, which were bound to chromatin *(20)*. On the other hand, proenkephalin products stored in endocrine secretory granules corresponded principally to intermediate- and low-mol-wt materials *(1–6)*. Furthermore, ACTH-PA contained prohormones converting enzymes PC1 and PC2 mRNA *(19)*, and PC1 protein (this article), which was localized exclusively in postnuclear supernatant. These data suggest that the translational products of preproenkephalin mRNA in ACTH-PA may follow two pathways: the secretory pathway and the nucleus. In the secretory pathway met-enkephalin-Arg-Phe may be processed to met-enkephalin, but in the nucleus, this molecule remains intact at the carboxy-terminus of high-mol-wt peptides. Corticotroph tumor cell line (AtT-20), stably transfected with rat preproenkephalin cDNA, produced a 35–32-kDa met-enkephalin-Arg-Phe-containing molecules, peptide B, and the authentic heptapeptide *(21)*. However, the 44-, 27-, and 17-kDa peptides observed in ACTH-PA were not reported in AtT-20 cells or in some other transfected cell lines *(21–23)*. In these studies, transfected cDNA contained only the sequence of exon 2 and 3 of rat preproenkephalin DNA *(21,22)*. The endogenous products detected in ACTH-PA may be produced by alternative transcriptional or post-transcriptional events of proenkephalin DNA as have been described in rat and human tissues *(24)*. When preproenkephalin cDNA lacking signal peptide codifying sequence was transfected to COS cells, the translational products were the 32- to 30-kDa doublet, and they were detected in the nucleus *(20)*. Different endogenous proenkephalin translational products might be produced by different transcriptional start sites in the expression of preproenkephalin RNA or by alternative splicing of preproenkephalin RNA *(24)*.

Nuclear and cytoplasmic proenkephalin-derived peptides in ACTH-PA could be synthesized by the same or by different cells. Immunocytochemical studies using met-enkephalin antibody showed that only 7.8% of the cells displayed positive immunoreactivity, but 87% of the cells contained ACTH *(17,18)*. On the other hand, by reproducing previous data *(25–27)*, plasma met-enkephalin levels did not change in patients with ACTH-PA. These results suggest that proenkephalin low-mol-wt peptides may not be stored in secretory granules of ACTH-producing cells. However, met-enkephalin released to the general circulation could be quickly cleaved by proteases, because it has a very short half-life in plasma *(11)*. On the other hand, proenkephalin peptides may be stored in secretory granules of another cell population that requires stimulation to release them to the general circulation. In human anterior pituitary, met-enkephalin immunoreactivity was localized in a large subpopulation of thyrotroph cells *(15)*. Furthermore, the percentage of thyrotroph cells (7.1%) in ACTH-PA was very close to the percentage of met-enkephalin-containing cells *(17)*. Unfortunately, met-enkephalin-Arg-Phe-directed antibody was not employed in these cytochemical studies to discover proenkephalin nuclear peptides. Regardless of whether or not proenkephalin-derived peptides stored in the nucleus or secretory pathway are present in the same cell population, they may be the translation products of different mRNA. Supporting this suggestion, previous reports have shown that different preproenkephalin mRNAs have been detected in one and/or different brain regions *(24)*, as well as in a mouse T-cell line clone *(24)*.

Compared with ACTH-PA, very low levels of proenkephalin-derived peptides were observed in PMHP, NFA, and GH-PA. In PMHP, proenkephalin was processed to high-, intermediate-, and low-mol-wt met-enkephalin-containing peptides, but in NFA, principally fully processed products were observed. Furthermore, in PMHP and NFA, met-enkephalin-Arg-Phe immunoreactivity appeared mainly in intermediate- and low-mol-wt peptides, as occurred in brain regions and adrenal gland *(1–6)*. The chromatographic profile of free met-enkephalin in PMHP was similar to those reported by Roth et al. *(15)*. NFA showed a significant decrease of proenkephalin-derived peptides' levels that were associated with increased plasma met-enkephalin content in patients with NFA. These results suggest an increased peptide secretion in these adenomas.

GH-PA mainly contained high-mol-wt met-enkephalin- and synenkephalin-containing peptides. However, IR-met-enkephalin Arg-Phe was absent, and met-enkephalin molecule was not detected. Furthermore, plasma met-

enkephalin levels did not change in patients with GH-PA. These results indicate that proenkephalin posttranslational processing was different in the three groups of adenomas, suggesting a differential physiological role.

Several reports showed that secreted met-enkephalin- and met-enkephalin-containing peptides participate in tumoral activity *(28–30)*; in addition, a nuclear δ opioid receptor has been described in a tumoral cell line *(31)*. In most of the cases, met-enkephalin-containing peptides inhibited tumoral activity *(28–30)*. In vivo and in vitro studies have shown that met-enkephalin directly inhibited ACTH release in human corticotroph *(32–34)*. However, this effect was abolished in corticotroph cells of patients with Cushing's disease, suggesting a decrease of opioids receptor levels *(32–34)*. In rat brain, reduction of the number of opioid receptors induced increased expression of the proenkephalin gene *(35)*. We speculate that a decreased number of opioid receptors in corticotroph cells might induce the increase of met-enkephalin-containing peptides' levels in ACTH-PA. Proenkephalin mRNA and proenkephalin-derived peptides are expressed in cells of mesodermal, ectodermal, and endodermal tissues in early stage of embryonic development *(36–40)*. In mesodermal cell lines, proenkephalin immunoreactivity has been detected in the nucleus and associated with chromatin *(20)*. Furthermore, when fibroblasts were transfected with preproenkephalin DNA antisense, the cell proliferation was inhibited *(41)*. Taken together these results suggest that proenkephalin products present in nuclear and secretory pathways may have a physiological role in ACTH-PA.

In conclusion, we demonstrated that only ACTH-PA contained high levels of nuclear and secretory proenkephalin peptides. These results suggest a probable adenoma-specific physiological role of proenkephalin products.

Materials and Methods

In this study, 21 patients (12 females and 9 males) between the ages of 26 and 70 were tested. Five patients had Cushing's disease; 11 patients had NFA, and 5 patients had GH-PA, showing clinical acromegaly.

The adenomas were classified as ACTH-PA, GH-PA, and NFA, based on preoperative clinical and laboratory data, and immunocytochemical staining of tissues removed at surgery *(42–44)*. Tables 5, 6, and 7 show clinical laboratory and immunocytochemical data. PMHP controls were obtained from individuals who died from severe wounds that did not involve the head. Adenomas and control pituitary glands were stored at –70°C until their use.

Tissue Extracts

Frozen tissues were resuspended in 10 vol of 1 *M* acetic acid (pH 1.9 with HCl), boiled for 15 min, homogenized with a Polytron, and centrifuged at 50,000*g* for 1 h. The supernatant was stored at –70°C.

Nuclei Isolation

Nuclei were isolated as described previously *(45)*. Immediately after the surgery, the ACTH-PA was immersed in ice-cold 0.32 *M* sucrose. Thirty minutes later, they were homogenized in 10 vol of 0.5 *M* sucrose, 10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 10% glycerol, 1 m*M* EDTA, 1 m*M* dithiotreitol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 1 μg/mL each of aprotinin, leupeptin, and pepstatin A in Dounce homogenizer, with five strokes with loose-fitting pestle and five strokes with light-fitting pestle. The homogenate was centrifuged at 4000*g* for 5 min at 4°C. The supernatant was removed (postnuclear supernatant), and the pellet (nuclei) was processed as described previously by Fey and Penman *(46)*. Briefly, the pellet was digested with RNase-free DNase I (250 μg/mL) in digestion buffer (10 m*M* PIPES pH 6.8/50 m*M* NaCl/300 m*M* sucrose/3 m*M* MgCl/0.5% [v/v] Triton X-100, with PMSF, aprotinin, leupeptin, and pepstatin A) for 20 min at room temperature. Chromatin-associated proteins were released by subsequent 250 m*M* ammonium sulfate extraction and centrifuged at 20,000*g* for 1 h at 4°C. The supernatant was diluted in Laemmli buffer (2X final concentration) and electrophoresed.

Postnuclear supernatant was diluted in acetic acid (final concentration 1 *N*, containing 50 m*M* HCl), boiled for 10 min, homogenized in Polytron, and centrifuged at 20,000*g* for 1 h. The supernatant was concentrated in a 10-kDa cutoff Centricon filter, resuspended in 2X Laemmli buffer, and electrophoresed.

Total and Free Met-enkephalin Assay

Aliquots of tissue extracts were lyophilized and reconstituted in 50 m*M* Tris-HCl buffer, pH 8.4, and 2 m*M* $CaCl_2$. Free and total IR-met-enkephalin and synenkephalin, that is, previous to and after sequential enzymatic digestion with trypsin and carboxypeptidase B, were determined by RIA as previously described *(47,48)*.

Enkephalin, Synenkephalin, and Met-enkephalin-Arg-Phe Assays

Met-enkephalin and synenkephalin were measured by RIA as described previously *(47–49)*. Iodinated met-enkephalin and [Tyr^{63}] (syn 63–70) synenkephalin were used as tracers. We found that met-enkephalin antiserum crossreactivity was 100% with oxidized met-enkephalin, 0.3% with leu-enkephalin, and <0.01% with met-enkephalin-Arg, dynorphin (1–13), and α-, γ-, and β-endorphin. Sensitivity of the assay was 9 fmol; IC_{50} was 130 fmol. The antiserum to [Tyr^{63}] (syn 63-70) synenkephalin showed crossreactivity of 1% with a native 8.6-kDa peptide (proenkephalin 1–77), which, when digested with trypsin, displayed a 20% crossreactivity that reached 100% when digested with trypsin and carboxypeptidase B. No crossreactivity was observed with met-enkephalin, met-enkephalin-Arg, met-enkephalin-Arg-Phe, met-enkephalin-Arg-Gly-Leu,

leu-enkephalin, peptide E and F, dynorphin B, amidorphin, and metorphamide. Sensitivity was 2 fmol; IC_{50} was 50 fmol. ME-Arg-Phe was measured as described previously *(50)*.

Gel Filtration

Samples were applied to a Sephadex G-50 superfine (60 × 1.0 cm) column equilibrated with 1 *M* acetic acid (pH 1.9 with HCl). The elution was performed at a flow rate of 10 mL/h at room temperature; 2-mL fractions were collected, and aliquots were lyophilized and reconstituted in 50 m*M* of Tris-HCl buffer, pH 8.4, and 2 m*M* $CaCl_2$. Eluates were monitored by ultraviolet absorbance at 280 nm. IR-synenkephalin and IR-met-enkephalin were assayed prior to and after enzymatic digestion with trypsin and carboxypeptidase B. IR-met-enkephalin-Arg-Phe was assayed without enzymatic digestion.

HPLC Chromatography

Samples were applied to a reverse-phase column, Ultropac, Lichrosorb RF18, 5 µm, 4 × 250 mm (Waters Corporation, Milford, MA). The column was equilibrated with 10% acetonitrile in TFA 0.1%, and the elution was performed in a discontinuous acetonitrile gradient (10–80%). The flow rate was 0.5 mL/min, and 1-mL fractions were collected. Aliquots were evaporated in a Speed Vac concentrator, and resuspended in the Tris-HCl buffer. All the chromatographic assays were repeated at least three times, using pituitary or tumor extracts from different patients.

Electrophoresis and Immunoblotting

Samples diluted in Laemmli buffer were applied to electrophoresis on 15 and 8.8% acrylamide for proenkephalin peptides and PC1 enzyme, respectively. Blotting protocol was described previously *(51,52)*. Briefly, proteins were transferred to nitrocellulose and blocked with 5% milk in Tris-buffered saline. Blots were then sliced and incubated overnight, at 4°C, with gentle rocking, using specific antibodies diluted in 5% milk. Proenkephalin peptides were identified with met-enkephalin-Arg-Phe directed antibody, diluted 1:400 in 5% milk, whereas PC1 enzyme was tested with NH2-terminus-directed antibody (DAM3) diluted 1:1000. The blots were developed as previously described *(51,52)*.

Plasma Met-enkephalin Assay

Blood was put into polypropylene tubes kept on ice, containing 10 µL/mL of a protease inhibitor cocktail (10,000 IUC/aprotinin, 0.23 m*M* citric acid, and 0.024 m*M* EDTA) and 5 U of heparin. Blood was centrifuged at 4°C, and plasma was immediately stored at –20°C; 2 mL of plasma were diluted to 10 mL with 0.1 *N* HCl and applied to Amberlite XAD-2 column and processed as described previously *(23)*, except that met-enkephalin was eluted with a mixture of 1 *M* acetic acid/acetonitrile (50%/50%). Using this protocol, the recovery of ^{3}H-met-enkephalin (identified by HPLC) added to the blood was between 80 and 90%.

Acknowledgments

We thank Jean Rossier for the donation of SYN and ME-Arg-Phe antibodies; Maria Rosa Padros, Anne Compliment, Isabel Perez Monfort, and Phil Arriaga for the valuable commentaries. This work was supported by grants from CONACYT (Mexico) (O.V. 3898-N9401) and CONICET (Argentina).

References

1. Akil, H., Watson, S., Young, E., Lewis, M., Khachaturian, H., and Walker, M. (1984). *Ann. Rev. Neurosci.* **7,** 223–255.
2. Comb, M., Seeburg, H. P., Adelman, J., Eiden, L., and Herbert, E. (1982). *Nature* **295,** 663–666.
3. Douglass, J., Civelli, O., and Herbert, E. (1984). *Ann. Rev. Biochem.* **53,** 665–715.
4. Gubler, U., Seeburg, H. P., Hoffman, B. J., Gage, L. P., and Udenfriend, S. (1982). *Nature* **295,** 206–209.
5. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., et al. (1982). *Nature* **295,** 202–206.
6. Rossier, J. (1993). In: *Handbook of Experimental Pharmacology*. Herz, A. (ed.). Berlin: Springer-Verlag.
7. Liston, D., Vanderhaeghen, J. J., and Rossier, J. (1983). *Nature* **302,** 62,63.
8. Liston, D. and Rossier, J. (1984). *Neurosci. Lett.* **48,** 211–216.
9. Rodriguez Vida, M. I., Kleid, M. C., Ase, A., Finkielman, S., Nahmod, V. E., and Vindrola, O. (1994). *Dev. Brain Res.* **77,** 151–156.
10. Song, D. D. and Harlan, R. E. (1993). *Neuroscience* **52,** 883–909.
11. Clemente-Jones, V. and Besser, G. M. (1983). *Br. Med. Bull.* **39,** 95–100.
12. Rossier, J., Battenberg, E., Pittman, Q., Bayon, A., Koda, L., Miller, R. J., et al. (1979). *Nature* **277,** 653–655.
13. Jingami, H., Nakanishi, S., Imura, H., and Numa, S. (1984). *Eur. J. Biochem.* **142,** 441–445.
14. Pittius, C. W., Kley, N., Loeffler, J. P., and Hollt, V. (1985). *EMBO J.* **4,** 1257–1263.
15. Roth, K. A., Lorenz, R. G., Mckeel, D. W., Leykam, J., Barchas, J. D., and Tyler, A. N. (1988). *J. Clin. Invest. Metab.* **66,** 804–810.
16. Desiderio, D. M., Kusmierz, J. J., Zhu, X., Dass, C., Hilton, D., and Roberton, J. T. (1993). *Biol. Mass Spectrom.* **22,** 89–97.
17. Wowra, B. and Peiffer, J. (1984). *Pathol. Res. Pract.* **178,** 349–354.
18. Hossoun, J., Charpin, C., Jaquet, P., Oliver, C., Lissitzky, J. C., Grisoli, F., et al. (1979). *Ann Endocrinol.* **40,** 559,560.
19. Hashimoto, K., Koga, M., Kouhara, H., Arita, N., Hayakawa, T., Kishimoto, T., et al. (1994). *Clin. Endocrinol.* **41,** 185–191.
20. Bottger, A. and Spruce, B. (1995). *J. Cell Biol.* **130,** 1251–1262.
21. Mathis, J. P. and Lindberg, I. (1992). *Endocrinology* **131,** 2287–2296.
22. Thomas, G., Thomas, L., Thorne, B. A., and Herbert, E. (1988). *Biochimie* **70,** 89–87.
23. Comb, M., Liston, D., Martin, M., Rosen, H., and Herbert, E. (1985). *EMBO J.* **4,** 3115–3122.
24. Weisinger, G. (1995). *Biochem J.* **307,** 617–629.
25. Furui, T., Kuwayama, A., and Kageyama, N. (1980). *No To Shinkei* **32,** 305–309.
26. Kimura, T., Ota, K., Matsui, K., Iitake, K., Shoji, M., Inouse, M., et al. (1985). *Endocrinol. Jpn.* **32,** 517–527.
27. Gaillard, R. C., Grossman, A., Smith, R., Rees, L. H., and Besser, G. M. (1981). *Clin. Endocrinol.* **14,** 471–478.
28. Zagon, I. S. and McLaughlin, P. J. (1989). *Brain Res.* **490,** 14–25.
29. Zagon, I. S., McLaughlin, P. J., Goodman, S. R., and Rhodes, R. E. (1987). *J. Natl. Cancer Inst.* **79,** 1059–1065.

30. Zagon, I. S. and Mc Laughlin, P. J. (1988). *Life Sci.* **43,** 1313–1318.
31. Belcheva, M. M., Glucker, S., Chuang, D., Clark, W. G., Jefcoat, L. B., McHale, R. J., et al. (1995). *J. Pharm. Exp. Ther.* **274,** 1513–1523.
32. Lamberts, S. W., Oosterom, R., Bons, E. G., and Uitterlinden, P. (1981). *J. Clin. Endocrinol. Metab.* **53,** 1084–1086.
33. Allolio, B., Winkelmann, W., Hipp, F. X., Kaulen, D., and Mies, R. (1982). *J. Clin. Endocrinol. Metab.* **55,** 1–7.
34. Allolio, B., Deuss, U., Kaulen, D., Leonhanrdt, U., Kallabis, D., Hamel, E., et al. (1986). *J. Clin. Endocrinol. Metab.* **63,** 1427–1431.
35. Morris, B. J., Hollt, V., and Herz, A. (1988). *Neurosci. Lett.* **89,** 80–84.
36. Keshed, E., Polakiewicz, R. D., Itin, A., Ornoy, A., and Rosen, H. (1989). *EMBO J.* **8,** 2917–2923.
37. Kew, D. and Kilpatrick, D. L. (1990). *Mol. Endocrinol.* **4,** 337–340.
38. Polakiewicz, R. D. and Rosen, H. (1990). *Mol. Cell. Biol.* **10,** 736–742.
39. Rosen, H., Polakiewicz, R. D., and Simantov, R. (1990). *Biochem. Biophys. Res. Commun.* **171,** 722–728.
40. Rosen, H., Polakiewicz, R. D., Benzakine, S., and Bar-Shavit, Z. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 3705–3709.
41. Weisinger, G., Zinder, O., and Simantov, R. (1995). *Biochem. Biophys. Res. Commun.* **274,** 530–537.
42. Asa, S. L. and Kovacs, K. (1983). *Clin. Endocrinol. Metab.* **12,** 567–596.
43. Klibanski, A. (1987). *Endocrinol. Metab. Clin. North. Am.* **16,** 793–804.
44. Chang-Moraville, B. M. and Jackson, I. M. (1992). *Endocrinol. Metab. Clin. North Am.* **21,** 649–668.
45. Unlap, T. and Jope, R. S. (1994). *Mol. Brain Res.* **24,** 275–282.
46. Fey, E. G. and Penman S. (1988). *Proc. Natl. Acad. Sci.* **85,** 101–125.
47. Saravia, F., Ase, A., Aloyz, R., Kleid, M. C., Rodriguez Vida, M. I., Nahmod, V. E., et al. (1993). *Endocrinology* **132,** 1431–1437.
48. Vindrola, O., Padros, M. R., Sterin-Prync, A., Ase, A., Finkielman, S., and Nahmod, V. E. (1990). *J. Clin. Invest.* **86,** 531–537.
49. Vindrola, O., Briones, R., Asai, M., and Fernandez Guardiola, A. (1981). *Neurosci. Lett.* **21,** 39–43.
50. Meeters, K. M. and Rossier, J. (1987). *J. Neurochem.* **49,** 721–728.
51. Vindrola, O., Mayer, A. M. S., Citera, G., Spitzer, J. A., and Espinoza L. R. (1994). *Neuropeptides* **27,** 235–244.
52. Vindrola, O. and Lindberg, I. (1992). *Mol. Endocrinol.* **6,** 1088–1094.
53. Furlanetto, R., Underwood, L., and Van Wyk, J. (1977). *J. Clin. Invest.* **60,** 648–653.