

**CO-RELEASE OF ENKEPHALINS AND PRECURSORS WITH
CATECHOLAMINES FROM THE PERFUSED CAT ADRENAL GLAND
IN SITU**

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SUMMARY

1. We have compared the nature of the enkephalin-like material derived from proenkephalin present in the intact cat adrenal gland with the material co-released with catecholamines from the perfused adrenal in response to splanchnic nerve stimulation and to perfusions with solutions containing acetylcholine (ACh) or high potassium chloride (KCl).

2. In cat adrenals most of the enkephalin-like material was in the form of large enkephalin-containing peptides. Free (met)enkephalin immunoreactivity represented only 25% of the total (met)enkephalin immunoreactivity as determined by enzymatic digestion of large enkephalin-containing fragments.

3. Electrical stimulation (15 Hz) of the splanchnic nerve or perfusion of the gland with ACh (0.1 mM) or KCl (50 mM), applied for 10 min, induced an immediate release of free (met)enkephalin immunoreactivity, (met)enkephalyl-arg-phe immunoreactivity, and of large (met)enkephalin-containing peptides. The release by all three modes of stimulation followed a pattern that paralleled the output of catecholamines. A rapid fatigue of all secretory processes developed during the stimulation periods, similar to that observed for catecholamines. During splanchnic nerve stimulation, each nanomole of catecholamine output was accompanied by the output of 0.4 pmol free (met)enkephalin immunoreactivity, of 1.1 pmol total (met)enkephalin immunoreactivity and of 0.1 pmol (met)enkephalyl-arg-phe immunoreactivity.

4. Analysis of the perfusate by high-pressure liquid chromatography revealed that (met)enkephalin, (met)enkephalyl-arg-phe and (met)enkephalyl-arg-gly-leu were released in molar ratios of 4 to 1 to 1 which is similar to the ratio found in the precursor, proenkephalin.

5. The ratio of total (met)enkephalin immunoreactivity to free (met)enkephalin immunoreactivity in the perfusate was the same (approximately 2.7) during two successive periods of splanchnic nerve stimulation separated by 10 min. When release was evoked by increasing the K⁺ concentration to 50 mM-KCl, this ratio was increased more than twofold compared with that obtained by electrical stimulation of the splanchnic nerve.

6. Analysis of the perfusate by gel filtration showed that, during splanchnic nerve stimulation, 47% of the total (met)enkephalin immunoreactivity eluted in fractions

containing fragments of low molecular weight. When KCl was used as stimulus only 12% of total (met)enkephalin immunoreactivity eluted in these fractions.

7. The results indicate that the nature of the released peptides depends on the type of stimulus used to evoke release. It appears that under physiological conditions most of the (met)enkephalin immunoreactivity-containing materials that are released, are final products obtained by complete processing of proenkephalin. When the stimulus is due to the presence of ACh or KCl in the perfusion fluid, partially processed peptides are also released.

INTRODUCTION

Recent studies on enkephalin biosynthesis have shown that (met)enkephalin and (leu)enkephalin are derived from a common, large precursor, proenkephalin, that contains four copies of (met)enkephalin and one of (leu)enkephalin (for review see Rossier, 1982; Chrétien, Boileau, Lazure & Seidah, 1983). In addition, this precursor contains at least two carboxy-extended enkephalins, the octapeptide (met)enkephalyl-arg-gly-leu and the heptapeptide (met)enkephalyl-arg-phe (Rossier, Audigier, Ling, Cros & Udenfriend, 1980; Kilpatrick, Jones, Kojima & Udenfriend, 1981; Ikeda, Nakao, Yoshimasa, Yanaihara, Numa & Imura, 1982).

Several studies have indicated that enkephalin immunoreactive products or opioid peptide-like products are co-released with catecholamines from the adrenal medulla *in vitro* (Kilpatrick, Lewis, Stein & Udenfriend, 1980; Stine, Yang & Costa, 1980; Livett, Dean, Whelan, Udenfriend & Rossier, 1981; Rossier, Dean, Livett & Udenfriend, 1981; Corder, Mason, Perrett, Lowry, Clement-Jones, Linton, Besser & Rees, 1982) and *in vivo* (Hexum, Hanbauer, Govoni, Yang & Costa, 1980; Govoni, Hanbauer, Hexum, Yang, Kelly & Costa, 1981). Furthermore, it was recently shown (Livett, Day, Elde & Howe, 1982; Peltö-Huikko, Salminen & Hernoven, 1982) that in the adrenal medulla, enkephalin immunoreactivity is present in adrenergic chromaffin cells but not in noradrenergic cells. We have recently confirmed the results of these immunohistochemical studies by subcellular fractionation of bovine adrenal medulla, in which we found that enkephalin-like material is associated mainly with adrenergic chromaffin granules (Roisin, Artola, Henry & Rossier, 1983).

In the present study we have characterized the nature of the fragments of proenkephalin released by electrical stimulation of the splanchnic nerve. We have found that (met)enkephalin, (met)enkephalyl-arg-gly-leu and (met)enkephalyl-arg-phe are released in a molar ratio similar to the ratio found in the precursor, proenkephalin. It was shown that in addition to free enkephalins, large fragments of the precursor are released, particularly when more powerful stimuli are used, such as perfusion with 50 mM-KCl or with 0.1 mM-acetylcholine chloride (ACh).

Abstracts of some of the findings of this present paper have been published (Chaminade, Foutz & Rossier, 1983; Rossier, Chaminade, Foutz, Patey & Chabrier, 1983; Rossier, Liston, Patey, Chaminade, Foutz, Cupo, Giraud, Roisin, Henry, Verbanck & Vanderhaeghen, 1983).

METHODS

Perfusion and stimulation of the adrenal gland

Nine cats of either sex, weighing 2.1–4.9 kg, were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.). Supplementary doses of anaesthetic were given when necessary through a catheter in the saphenous vein. In three cats both adrenal glands were removed and frozen for analysis of their contents of catecholamines and enkephalin-like material. In six cats a tracheal cannula was inserted for artificial ventilation with oxygen-enriched air. A slight hypocapnia was maintained in order to suppress spontaneous respiratory movements (Beckman LB2 CO₂ analyser). The abdomen was opened by a mid-line incision and the entire gastrointestinal tract removed. The right adrenal gland was excised and frozen immediately; the left gland was perfused *in situ* by inserting a catheter into the abdominal aorta below the renal artery (Douglas & Rubin, 1961). The aorta was tied above the coeliac axis and all other vessels were ligated except those directly supplying the adrenal gland. The perfusate was collected through a catheter inserted into the adreno-lumbar vein arising from the adrenal gland. This vein was then tied off close to the vena cava. The gland was perfused at room temperature at a constant pressure of about 70 mmHg. The perfusion was with a bicarbonate Krebs–Ringer solution saturated with 5% CO₂ and 95% O₂ (pH 7.4) that had the following composition (mM): NaCl, 120; KCl, 5; CaCl₂, 2.6; MgSO₄, 0.67; KH₂PO₄, 1.2; NaHCO₃, 27.5; glucose 5.9. The splanchnic nerves were then exposed through an opening of the thorax and placed, above the diaphragm, on silver hook electrodes which were immersed in a pool of mineral oil. Artificial ventilation was then stopped and the carotid arteries were cut. The perfusate was collected in tubes at 2.5 min intervals and kept on ice. When 50 mM-KCl was present in the perfusate fluid, the intervals were 1.25 min. After a 20 min period for collection of perfusate to obtain a base line, there were two periods of electrical stimulation of the splanchnic nerves (15 Hz, 15 V, 1 ms square pulses) for 10 min; these were separated by a further period for base-line collection. After a third period for base-line collection, fluid containing 0.1 mM-ACh was perfused for 10 min. There was a fourth period for base-line collection followed by a perfusion with fluid containing 50 mM-KCl but only 75 mM-NaCl.

Catecholamine assay

Total catecholamines were assayed by means of a spectrofluorimeter for basic fluorescence (excitation wave-length of 280 nm, emission wave-length of 330 nm; Anderson & Young, 1981). Adrenaline bitartrate (Sigma, St Louis) was used as standard. Reverse-phase, high-pressure liquid chromatography (h.p.l.c.) of the fluorescent material verified that the fluorescence in the perfusate was due to noradrenaline and adrenaline; no other fluorescent substances were detectable.

Radioimmunoassay

(Met)enkephalin and (met)enkephalyl-arg-phe were assayed by specific radioimmunoassays (r.i.a.) described in detail by Patey, Cupo, Giraud, Chaminade & Rossier (1983). The threshold of sensitivity for (met)enkephalin was 0.7 fmol per tube with a cross-reactivity of 10% towards (leu)enkephalin, of 4% towards (met)enkephalyl-arg-phe and 5% toward (met)enkephalyl-arg-gly-leu. The IC₅₀ (50% of maximum binding) for (met)enkephalin was 10 fmol per tube. The threshold of sensitivity for (met)enkephalyl-arg-phe was 45 fmol per tube with cross-reactivities of 1% for both (met)enkephalin and (leu)enkephalin and less than 0.1% for (met)enkephalyl-arg-gly-leu. The IC₅₀ for (met)enkephalyl-arg-phe was 80 fmol per tube.

Total (met)enkephalin

In order to assay all the (met)enkephalin sequences present in fragments of proenkephalin, the samples were subjected to sequential enzymatic treatments with trypsin and carboxypeptidase B as described previously (Liston, Vanderhaeghen & Rossier, 1983). Samples were diluted tenfold with a solution containing 50 mM-Tris HCl and 2 mM-CaCl₂ (pH 8.4) and transferred to a heating bath of 95 °C for 10 min to inactivate any proteolytic activity. Trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (TPCK trypsin, Worthington, Freehold, NJ, U.S.A.) was added to give a final concentration of 20 µg/ml. The samples were incubated at 37 °C for 2 h and the trypsin was then inactivated by incubation for 10 min at 95 °C. Thereafter, carboxypeptidase B (Boehringer, Mannheim, F.R.G.) was added to give a concentration of 0.1 µg/ml and the mixture

was incubated at 37 °C for 1 h. After this last incubation the enzyme was again heat inactivated before assaying by radioimmunoassay for (met)enkephalin. This treatment converted (met)enkephalyl-arg-phe and (met)enkephalyl-arg-gly-leu into (met)enkephalin.

Gel filtration

Samples were fractionated on a column of Sephadex G100 (90 × 1.6 cm) equilibrated in 1 M-acetic acid. The column was eluted at a flow rate of 8 ml/h at room temperature. Fractions of 5 ml were collected and lyophilized; they were reconstituted with 1 ml 50 mM-Tris HCl, pH 8.4, containing 2 mM-CaCl₂.

Analysis of enkephalin congeners by high-performance liquid chromatography

Samples corresponding to fractions 30–37 of the gel filtration column (Fig. 4) were pooled and applied to an Altex (Palo-Alto) reverse phase Ultrasphere-OCTYL column (25 × 0.46 cm, 5 μm particle size). The column was pre-equilibrated with 0.25 M-triethylammonium formate, pH 3.1, and eluted with a discontinuous gradient from 0 to 50% acetonitrile in buffer with a flow rate of 1 ml/min. Synthetic peptides were used as calibration standards (see Giraud, Castanas, Patey, Oliver & Rossier, 1983, for details). Fractions of 1 ml were evaporated in a Speed-Vac concentrator (Savant Instrument, Hicksville, NY) and resuspended in 50 mM-Tris HCl buffer, pH 8.4, containing 2 mM-CaCl₂. Free (met)enkephalin immunoreactivity was determined in an aliquot of each of the fractions. Other aliquots were subjected to sequential enzymatic treatment with trypsin and carboxypeptidase in order to liberate (met)enkephalin from any larger peptides, to determine the free and the generated (met)enkephalin immunoreactivities.

Preparation of tissue homogenates

Whole adrenal glands were weighed and homogenized with a Polytron (Kinetika, Luzern, Switzerland) in 2.5 ml 1 M-acetic acid at 0 °C. Homogenates were centrifuged at 50 000 *g* for two hours. The supernatant was lyophilized and reconstituted in 50 mM-Tris HCl buffer of pH 8.4 containing 2 mM-CaCl₂. After another centrifugation, supernatants were kept frozen until they were assayed.

RESULTS

Characterization of the enkephalin-like materials in the cat adrenal medulla

The adrenal glands from three cats were removed without perfusion and frozen before homogenization in 1 M-acetic acid. After centrifugation, the supernatants were analysed for total catecholamine, free (met)enkephalin and (met)enkephalyl-arg-phe. No difference was found between contents of the right and left adrenal glands of the same animal. Free (met)enkephalin immunoreactivity is approximately one-quarter of total (met)enkephalin immunoreactivity (Table 1).

This finding indicates that most of the (met)enkephalin sequences are in the form of larger molecules; this hypothesis was confirmed by gel filtration of the adrenals of three cats (Fig. 1). In the absence of any enzymatic treatment, (met)enkephalin immunoreactivity is mainly associated with a peak eluting in fractions 30–37 just before the total volume of the G100 column, corresponding to the elution profile of synthetic (met)enkephalin, whereas other peaks are found having retention times of molecules larger than (met)enkephalin (M_r 20 000–50 000). Enkephalin sequences present in these large peptides were assayed after sequential enzymatic treatment with trypsin and carboxypeptidase B, to liberate free (met)enkephalin (Liston *et al.* 1983); after such enzymatic treatment, the main peaks of (met)enkephalin immunoreactivity correspond to the elution times of the larger peptide molecules.

On the other hand, total (met)enkephalin immunoreactivity includes free (met)enkephalin due to degradation of large molecules and the low molecular weight

TABLE 1. Total catecholamines and enkephalin-like immunoreactive materials in cat, control and non-perfused adrenal glands

Weight	0.22 ± 0.06 g
Total CA	184 ± 42 nmol
(Met)immunoreactivity	68.5 ± 18 pmol
Total (met)immunoreactivity	254 ± 82 pmol
Total (met)immunoreactivity/(met)immunoreactivity	3.70
Heptapeptide immunoreactivity	23.2 ± 3.6 pmol
CA/(met)immunoreactivity	2.69 × 10 ³
CA/total (met)immunoreactivity	0.72 × 10 ³
CA/heptapeptide immunoreactivity	7.93 × 10 ³

Adrenals ($n = 6$) from three cats were homogenized as described in Methods. Results (weight or content per gland) are mean ± s.e. of the mean. CA, catecholamines; (met), free (met)enkephalin; total (met), total (met)enkephalin; heptapeptide = (met)enkephalyl-arg⁶-phe⁷.

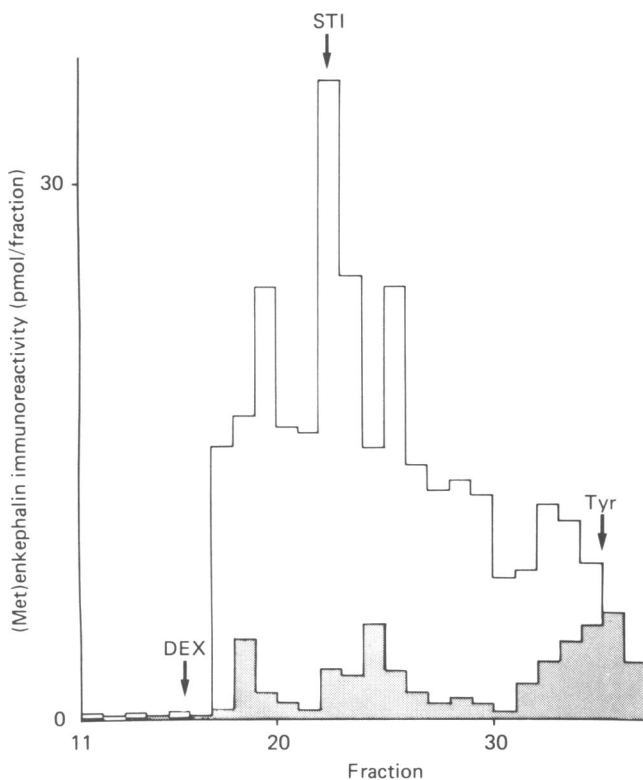


Fig. 1. Free (met)enkephalin immunoreactivity and total (met)enkephalin immunoreactivity in acetic acid (1 M) extracts of three unperfused homogenized cat adrenal glands. The 50000 g supernatant of the homogenate was fractionated on a Sephadex G100 column (90 × 1.6 cm). Fractions were assayed for (met)enkephalin immunoreactivity (pmol/fraction) before (stippled histogram) and after (open histogram) a sequential enzymatic treatment with trypsin and carboxypeptidase B. Calibration markers were: blue dextran (DEX) for the void volume, soybean trypsin inhibitor (STI) for 21000 Da and tyrosine (tyr) for the total volume.

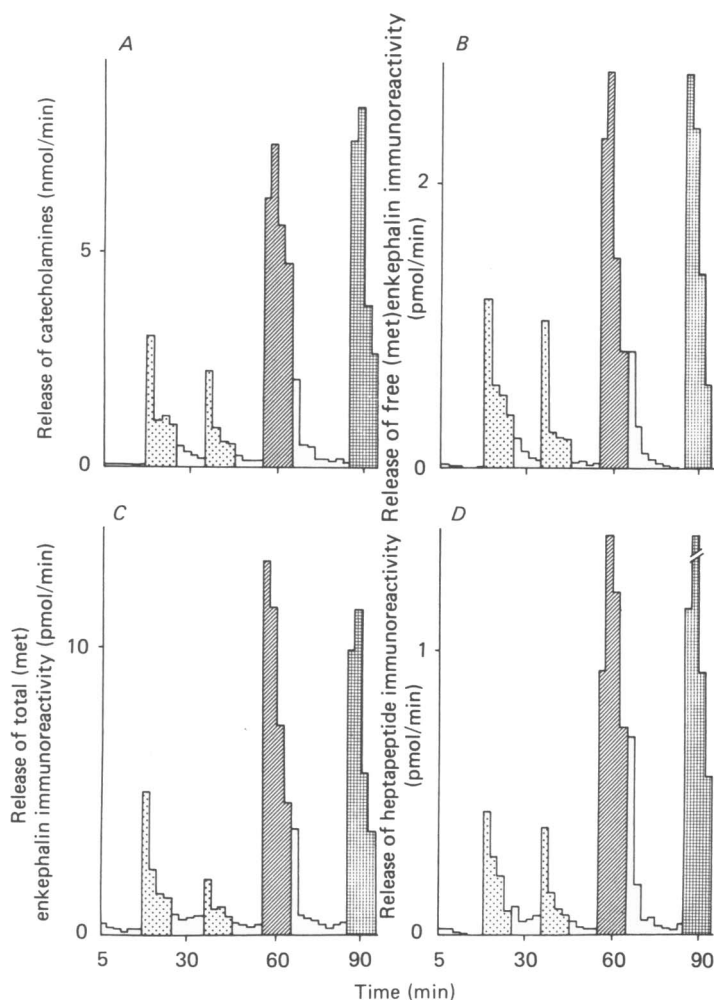


Fig. 2. Release of catecholamines (A), free (B) and total (met)enkephalin immunoreactivity (C) and (met)enkephalyl-arg-phe immunoreactivity (D) from one perfused cat adrenal gland caused by splanchnic nerve stimulation (stippled histogram) or by perfusion with 0.1 mM-ACh (hatched histogram) or with 50 mM-KCl (crossed histogram). After a rest period of 20 min, the splanchnic nerve was stimulated (15 Hz, 15 V, 1 ms pulses) for one 10 min period, and then for a second period after 10 min without stimulation. After another 10 min rest period the gland was perfused with 0.1 mM-ACh in Krebs solution (10 min), and after a 20 min rest period it was perfused with 50 mM-KCl in Krebs solution containing 75 mM-NaCl. Fractions were collected every 2.5 min and the results are expressed in nanomoles of catecholamine or picomoles of immunoreactive peptides released per minute.

peptides eluting just before the total volume of the column. Thus, total (met)enkephalin immunoreactivity is a composite of mainly (met)enkephalin sequences obtained by enzymatic treatment from the precursor and other small enkephalin-containing peptides, such as (met)enkephalyl-arg-phe and (met)-enkephalyl-arg-gly-leu, and, finally, free (met)enkephalin.

Release of catecholamines and enkephalins

When the left adrenal glands of other cats were perfused with modified Krebs solution *in situ*, electrical stimulation of the splanchnic nerves induced a release of catecholamines (Fig. 2). As previously reported (see review in Douglas, 1975) the amount of catecholamines released during a 10 min electrical stimulation decreased rapidly; during the last 2.5 min of the collection period it was 25–33 % of that released in the first 2.5 min period. A similar decrease was also observed when a much larger release was induced by perfusing the adrenal gland with Krebs solution containing 0.1 mM-ACh or with 50 mM-KCl. With such rather drastic stimuli, the release per minute was about 5 % of the total catecholamine content of the gland during the first 2.5 min of collection and only about 1 % during the fourth period of 2.5 min. The decreases in output were not due to a depletion of catecholamine in the gland, as the release evoked with 50 mM-KCl is of similar order if it follows a perfusion with ACh. When, in these perfusates, free (met)enkephalin immunoreactivity, total (met)enkephalin immunoreactivity and (met)enkephalyl-arg-phe immunoreactivity were assayed, it was found that they were co-released with catecholamines, whether it was induced by electrical stimulation, by ACh or by high KCl concentration (Fig. 2).

Characterization of the released enkephalin-like material

The nature of the (met)enkephalin immunoreactivity and the (met)enkephalyl-arg-phe immunoreactivity which are released into the perfusate during nerve stimulation was examined by h.p.l.c. For this purpose, in another cat, perfusates collected during both nerve stimulation periods were pooled and fractionated on a G100 column (see Fig. 4); there fractions containing substances of low molecular weight (apparent $M_r < 2000$; fractions 30–37) were pooled and analysed by h.p.l.c. (Fig. 3).

In the absence of any enzymatic treatment of the eluate, more than 90 % of the material of low molecular weight, that is (met)enkephalin immunoreactivity, eluted with the same retention time as authentic (met)enkephalin. After enzymatic treatment of the eluant, two additional peaks of (met)enkephalin immunoreactivity were identified, which eluted in the positions of (met)enkephalyl-arg-gly-leu and (met)enkephalyl-arg-phe. A small peak due to immunoreactive material was found which corresponded to the retention time of (leu)enkephalin; this may be due to the cross-reactivity of our antiserum with (leu)enkephalin. The relative proportions found in the three major peaks of (met)enkephalin immunoreactivity (Fig. 3) were 70 % (met)enkephalin, 16 % (met)enkephalyl-arg-gly-leu and 14 % (met)enkephalyl-arg-phe. Thus, in response to splanchnic nerve stimulation, these peptides were released from the adrenal gland in an approximate molar ratio of 4 to 1 to 1, similar to the ratio present in the proenkephalin molecule.

Ratio of amounts of catecholamines to those of enkephalin-like materials in the perfusate

In the series of three cats (one presented in Fig. 2) the released materials were quantified. Animals were submitted to two 10 min periods of splanchnic nerve stimulation, one period of ACh perfusion in two cats and one period of KCl perfusion.

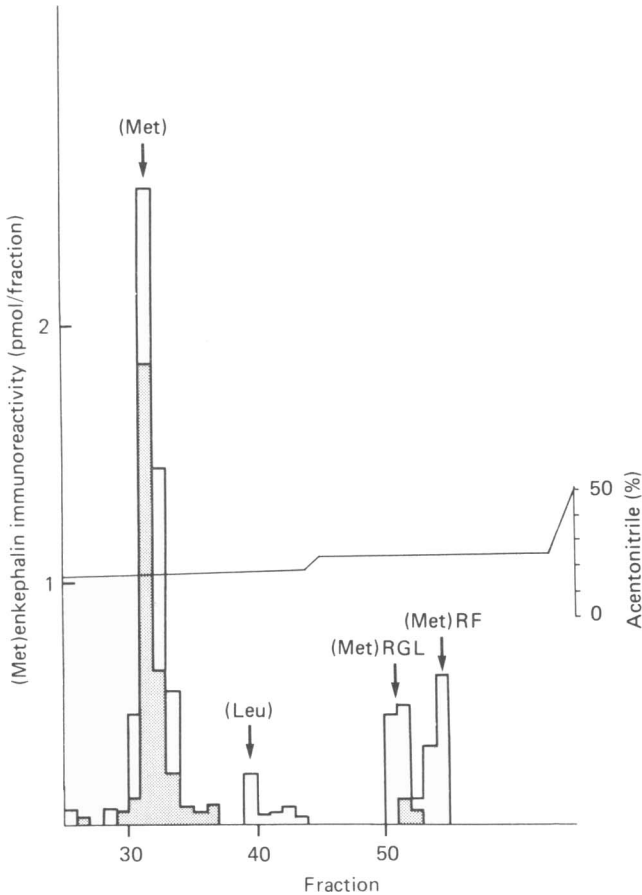


Fig. 3. Identification by h.p.l.c. of the low molecular weight enkephalin-like materials released by nerve stimulation. Pooled fractions obtained during a 10 min stimulation of the splanchnic nerve similar to that shown in Fig. 2 were fractionated on a G100 column. Fractions 30–37 containing eluted material with an apparent $M_r < 3000$ were lyophilized and redissolved in 0.25 M-triethylammonium formate, pH 3.1, and then injected onto the reverse-phase h.p.l.c. column (Altex Ultrasphere-OCTYL, 25×0.46 cm). Elution was with a gradient of increasing concentration of acetonitrile in the same buffer. Fractions were evaporated under reduced pressure and reconstituted in 50 mM-Tris HCl and 2 mM-CaCl₂ buffer of pH 8.4. (Met)enkephalin immunoreactivity was determined before (stippled histogram) and after (open histogram) treatment with trypsin and carboxypeptidase B. Arrows indicate the elution positions of standards, (met)enkephalin (met), (leu)enkephalin (leu), (met)enkephalyl-arg-gly-leu ((met)RGL), and (met)enkephalyl-arg-phe ((met)RF). More than 70% of total (met)enkephalin immunoreactivity eluted between fractions 25 to 60; 12% was eluted in a peak corresponding to (met)enkephalin sulphoxide (fraction 17). The remaining (met)enkephalin immunoreactivity was eluted in several other unidentified peaks.

The ratios of catecholamine to free (met)enkephalin immunoreactivity, catecholamine to total (met)enkephalin immunoreactivity and catecholamine to (met)enkephalyl-arg-phe immunoreactivity were calculated for each 2.5 min collection period (Table 2).

During stimulation of the splanchnic nerve, the ratios are constant in all fractions.

TABLE 2. Ratio of catecholamines to enkephalin-like immunoreactive materials released to the perfusates

Stimulus	2.5 min fractions	CA (nmol/min)	CA/(met) immunoreactivity ($\times 10^3$)	CA/total(met) immunoreactivity ($\times 10^3$)	CA/heptapeptide immunoreactivity ($n = 2, 1$ for ACh; $\times 10^3$)	Total (met) immunoreactivity/(met)immunoreactivity
1st 10 min nerve stimulation	1st	2.17 \pm 0.48	2.46 \pm 0.14	1.02 \pm 0.16	7.14 \pm 0.72	2.57 \pm 0.54
	2nd	1.07 \pm 0.24	2.32 \pm 0.22	0.87 \pm 0.19	6.37 \pm 1.61	3.02 \pm 0.93
	3rd	0.96 \pm 0.16	2.40 \pm 0.08	0.87 \pm 0.16	9.35 \pm 0.81	2.98 \pm 0.57
	4th	0.65 \pm 0.18	2.51 \pm 0.06	1.11 \pm 0.16	8.45 \pm 0.69	2.36 \pm 0.34
	Σ	12.1 \pm 2.5	\bar{M} 2.42 \pm 0.04*	\bar{M} 0.97 \pm 0.06*	\bar{M} 7.83 \pm 0.66	\bar{M} 2.73 \pm 0.16**
2nd 10 min nerve stimulation	1st	1.70 \pm 0.48	2.28 \pm 0.31	0.90 \pm 0.22	14.24 \pm 2.79	2.74 \pm 0.51
	2nd	0.65 \pm 0.15	2.95 \pm 0.37	1.28 \pm 0.42	11.19 \pm 1.08	3.23 \pm 1.46
	3rd	0.53 \pm 0.05	2.71 \pm 0.15	1.29 \pm 0.37	8.90 \pm 2.28	2.61 \pm 0.94
	4th	0.43 \pm 0.12	2.04 \pm 0.86	0.91 \pm 0.41	10.34 \pm 1.98	2.20 \pm 0.71
	Σ	8.3 \pm 1.5	\bar{M} 2.49 \pm 0.20	\bar{M} 1.10 \pm 0.11*	\bar{M} 11.17 \pm 1.13	\bar{M} 2.70 \pm 0.21**
Effect of 0.1 mM-ACh ($n = 2$)	1st	6.67 \pm 0.55	2.25 \pm 0.39	0.63 \pm 0.02	4.73	3.52 \pm 0.54
	2nd	6.30 \pm 1.15	1.97 \pm 0.63	0.43 \pm 0.10	6.53	4.41 \pm 0.46
	3rd	4.48 \pm 1.11	2.51 \pm 1.26	0.39 \pm 0.08	7.77	6.00 \pm 2.00
	4th	2.78 \pm 0.25	2.54 \pm 1.44	0.33 \pm 0.08	6.64	7.04 \pm 2.54
	Σ	50.6 \pm 4.9	\bar{M} 2.32 \pm 0.13	\bar{M} 0.45 \pm 0.07	\bar{M} 6.42 \pm 0.63	\bar{M} 5.24 \pm 0.79
Effect of 50 mM-KCl	1st	10.41 \pm 1.44	3.03 \pm 0.25	0.55 \pm 0.05	9.63 \pm 1.80	5.48 \pm 0.77
	2nd	6.62 \pm 0.99	3.10 \pm 0.21	0.50 \pm 0.01	8.56 \pm 0.93	6.17 \pm 0.41
	3rd	2.95 \pm 0.47	3.60 \pm 0.36	0.56 \pm 0.11	9.58 \pm 2.51	6.41 \pm 2.10
	4th	2.38 \pm 0.26	4.03 \pm 0.29	0.51 \pm 0.05	13.75 \pm 5.45	7.88 \pm 1.80
	Σ	56.3 \pm 1.6	\bar{M} 3.44 \pm 0.23	\bar{M} 0.53 \pm 0.02	\bar{M} 10.38 \pm 1.15	\bar{M} 6.48 \pm 0.50

Adrenal glands of three cats were perfused and stimulated as described in text. Values are means \pm s.e. of the mean of individual values. Σ , total amount of CA released (nmol) in 10 min; \bar{M} mean ratio for four 2.5 min fractions. * $P < 0.05$; ** $P < 0.01$, Students t test for related measures relative to K^+ stimulation. Abbreviations as in Table 1.

There were with each nmol catecholamine 0.41 pmol free (met)enkephalin immunoreactivity, 1.1 pmol total (met)enkephalin immunoreactivity and 0.105 pmol (met)enkephalyl-arg-phe immunoreactivity. These ratios are close to those observed in the extracts of the unstimulated glands (Table 1), where 1 nmol catecholamine corresponds to 0.37 pmol free (met)enkephalin immunoreactivity, 1.38 pmol total (met)enkephalin immunoreactivity and 0.126 pmol (met)enkephalyl-arg-phe immunoreactivity.

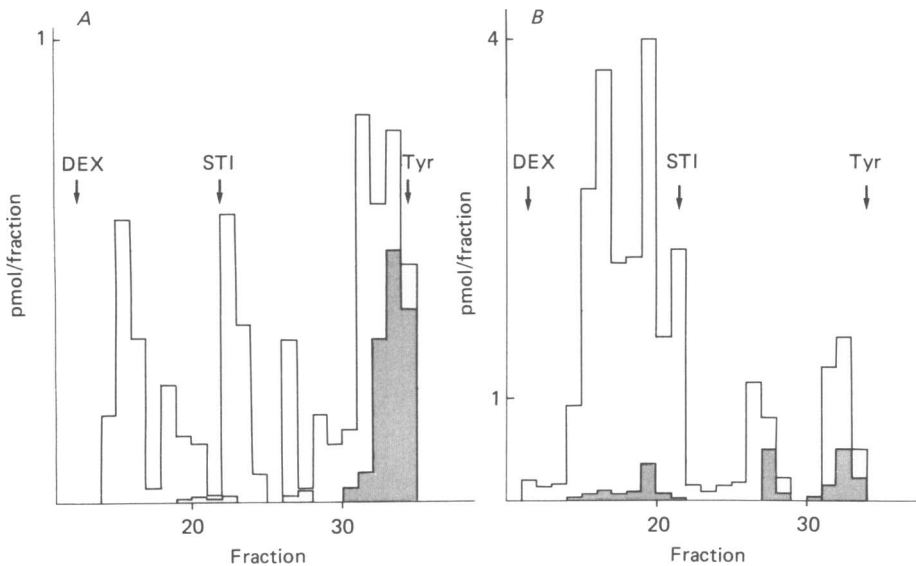


Fig. 4. Gel filtration of perfusates after splanchnic nerve stimulation (A) or perfusion with 50 mM-KCl (B). Same cat as in Fig. 3. Perfusates were applied to a G100 column (90 × 1.6 cm) and eluted in 1 M-acetic acid. Fractions (5 ml) were evaporated and re-suspended in 50 mM-Tris HCl, 2 mM-CaCl₂, buffer of pH 8.4. (Met)enkephalin immunoreactivity was assayed before (stippled histogram) and after (open histogram) digestion with trypsin and carboxypeptidase. Arrows indicate elution peaks of markers as in Fig. 1.

The ratio of catecholamine to total (met)enkephalin immunoreactivity is greater by a factor of two when the splanchnic nerve is stimulated than when 50 mM-KCl is present (Table 2). This observation suggests a relatively greater release of material of high molecular weight during stimulation by excess KCl. The ratio of released catecholamine to (met)enkephalyl-arg-phe was similar with the two types of stimuli (Table 2). The mean ratio of released total (met)enkephalin immunoreactivity to free (met)enkephalin immunoreactivity was not different in response to the two successive periods of splanchnic nerve stimulation but was increased by 240% when the perfusion fluid contained 50 mM-KCl (Table 2).

The basis for this difference between the ratios of total (met)enkephalin immunoreactivity to free (met)enkephalin immunoreactivity was determined by an analysis of the perfusate by gel filtration (Fig. 4). The elution profiles of the immunoreactive materials in the perfusates obtained after addition of excess KCl or by splanchnic nerve stimulation differed markedly. Following splanchnic nerve stimulation, 47% of

the total (met)enkephalin immunoreactivity elutes between fraction 30 and the total volume of the column. In response to perfusion with 50 mM-KCl only 12 % of the total (met)enkephalin immunoreactivity is present in these fractions. Thus, the nature of the enkephalin-like material released by the more physiological mechanisms during the period of splanchnic nerve stimulation is different from that released by the depolarization of the chromaffin cells induced by 50 mM-KCl. The more intense stimulus clearly results in a much greater release of high molecular weight enkephalin-containing peptides.

DISCUSSION

We have shown that catecholamines and enkephalin-like immunoreactive materials are released simultaneously from the adrenal gland by splanchnic nerve stimulation or during perfusion with ACh or KCl. The perfusion *in situ* of the cat adrenal gland with a physiological solution has several advantages over other methods. First, release can be obtained by splanchnic nerve stimulation. Models such as adrenal cell cultures or isolated bovine adrenals are restricted to the use of exogenous agents, such as ACh or KCl to induce release from chromaffin cells. *In vivo* cannulation of dog adrenals, which has been used to demonstrate release of enkephalin (Hanbauer, Govoni, Majane, Yang & Costa, 1982), is limited by the rapid degradation of enkephalins since their half-life in rat blood plasma is of the order of 2–3 min (Hambrook, Morgan, Rance & Smith, 1976). Moreover, the assay of enkephalins in blood may necessitate a long extraction procedure, thus giving variable yields from extract to extract. In the present report the more physiological method by stimulating the splanchnic nerves to the adrenal gland was used; furthermore, there is complete removal of blood elements which may interfere with the assays. Therefore, *in situ* perfusion allows accurate measurement of released materials. It is also important to note that the values obtained for the release of catecholamines and enkephalins did not show much variation from cat to cat.

Since the discovery of enkephalin-like material in the adrenal medullary gland, interest has been focused on large enkephalin-containing peptides. The large precursor, proenkephalin, contains four sequences of (met)enkephalin, one of (leu)enkephalin, one of (met)enkephalyl-arg-gly-leu and one of (met)enkephalyl-arg-phe. The complete proenkephalin molecule is not found in large amounts in extracts of chromaffin granules; rather they contain several intermediates in the processing of proenkephalin to the final products (Rossier *et al.* 1983). These intermediates vary in size from 18.2 to 3.2 kDa. Previous studies and the present work have shown that sequential enzymatic digestion of the peptides of the adrenal gland generates up to 80–90 % of the total (met)enkephalin immunoreactivity. It has already been found (Kilpatrick *et al.* 1980; Rossier *et al.* 1981) that the large enkephalin-containing peptides are released together with catecholamines in response to physiological stimuli. The results given in the present paper indicate that such an assumption may be erroneous since we have found that, in response to electrical stimulation of the splanchnic nerves, one-half of the total (met)enkephalin immunoreactivity released comprises free (met)enkephalin, (met)enkephalyl-arg-phe and (met)enkephalyl-arg-gly-leu. A certain amount of enkephalin-containing peptides of large molecular weight was also released. One may even question whether large

enkephalin-containing peptides would have been released if more physiologically relevant conditions of nerve stimulation had been used. Indeed, splanchnic nerve stimulation at 15 Hz is known to provoke massive release of catecholamine from the adrenal gland (Douglas, 1975) and causes an initial loss of 1 % of the catecholamine content of the adrenal gland per minute.

When the gland was perfused with Krebs solution containing ACh or KCl, a proportionally greater amount of enkephalin-containing peptides of larger molecular weight were released. This feature is particularly apparent in Fig. 4, in which, after gel filtration, the contents of perfusates collected during splanchnic nerve stimulation were compared with those obtained during perfusion with 50 mM-KCl. When the perfusion fluid contained 50 mM-KCl, the enkephalin-like material was mainly composed of large enkephalin-containing peptides with an apparent molecular weight greater than 10000. In contrast, during nerve stimulation, most of the enkephalin-like materials had a molecular weight of less than 2000. This difference in the composition of the released material may indicate that in more normal, physiological conditions most of the materials released by the adrenal medulla are the final products obtained after full processing of proenkephalin, namely (met)enkephalin, (leu)enkephalin, (met)enkephalyl-arg-gly-leu and (met)enkephalyl-arg-phe. When high concentrations of depolarizing agents are used, partially processed materials are also released. These findings might be explained by the assumption that the population of chromaffin granules are heterogeneous; granules may be classified into mature granules in which proenkephalin is fully processed and immature granules containing in addition the high molecular weight intermediates of proenkephalin. Physiological stimulation by the splanchnic nerves may preferentially induce the release of the mature granules that contain (met)enkephalin, (met)enkephalyl-arg-gly-leu and (met)enkephalyl-arg-phe in a molar ratio of 4 to 1 to 1. When intense depolarizations are induced by excess K^+ , the pool of mature granules is depleted and immature granules which contain the large enkephalin-containing peptides are released.

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