In vitro release of [5-methionine]enkephalin and [5-leucine]-enkephalin from the rat globus pallidus*

(opiate peptides/neurotransmitters/brain peptides)

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ABSTRACT Endogenous [5-methionine]enkephalin (Met-enkephalin) and [5-leucine]enkephalin (Leu-enkephalin) are released from perfused slices of rat globus pallidus by increased K⁺ in a Ca²⁺-dependent manner. Tissue perfused for 40 min contained only 26% of the Met-enkephalin and 44% of the Leu-enkephalin found in the freshly dissected tissue. After perfusion, the mean (±SEM) ratio (wt/wt) of Met-enkephalin to Leu-enkephalin was 3.4 ± 0.2 compared with 5.8 ± 0.2 in the fresh tissue. The degradation of trace amounts of synthetic [3H]enkephalins in the perfusing medium during stimulated release seems to reflect the accelerated degradation of enkephalin released from the tissue: 63% of the Met-enkephalin and 23% of the Leu-enkephalin were degraded in a medium containing bacitracin (30 μg/ml). The mean ratio (wt/wt) of the Met-enkephalin to the Leu-enkephalin recovered after release by exposure of slices to 50 mM K⁺ was 2.7 ± 0.3. When perfusates were corrected for degradation, this ratio increased to about 5.5 which is higher than that found in the perfused tissue. The differences in release, tissue loss, and catabolism of the two enkephalins may be reflecting differences in the metabolic systems operating on the pentapeptides, but this interpretation will have to be validated by in vivo release experiments. In any event, these observations strongly suggest that both enkephalins can be considered candidate neurotransmitters in the rat globus pallidus.

The presence of the opioid peptides [5-methionine]enkephalin (Met-enkephalin) and [5-leucine]enkephalin (Leu-enkephalin) (1) in several regions of the central nervous system (2) and the immunohistochemical evidence for their localization in fibers and cell bodies (3) support their consideration as putative central neurotransmitters. The search for a model system for study of the central neurobiology of the enkephalins has led to the striatum (4, 5), and especially the globus pallidus because of this region's high content of enkephalins (ref. 6; unpublished data) and enkephalin-containing fibers. Recent studies in our laboratory (7) have shown that, during in vitro perfusion of slices from rat globus pallidus, enkephalin-like immunoreactive material is released by K⁺ in a Ca²⁺-dependent manner. In the present work we have analyzed the composition of these enkephalin-like substances, both in freshly dissected and perfused tissue and in the released material, as well as some aspects of the metabolic changes of enkephalins during in vitro release experiments. Our results indicate that, although the two enkephalins are released from the globus pallidus, some differences can be found in their catabolism in vitro.

MATERIAL AND METHODS

Biological Samples. Adult male rats (Sprague–Dawley) weighing 150–200 g were decapitated, their brains were re-

moved, and the globus pallidus from each hemisphere was quickly dissected (approximately 15 mg each) from coronal slices (sagittal 1.5–2.25 mm, anterior level 5.0–7.0) according to Konig and Klippel (8). The tissue samples were immediately chopped in two perpendicular directions at 200-μm intervals (Mcllwain tissue chopper, Brinkmann, NY).

Perfusion. Each globus pallidus was suspended in a plastic perfusion chamber as described (7). A slightly modified Krebs–Ringer bicarbonate buffer [127 mM NaCl/3.73 mM KCl/1.8 mM CaCl₂/1.18 mM KH₂PO₄/1.18 mM MgSO₄/20 mM NaHCO₃/D-glucose (2 g/liter)] previously equilibrated with O₂/CO₂ 95:5 (vol/vol), and containing 0.1% bovine serum albumin (Sigma, crystalline) at 30 μg of bacitracin per ml, was pumped (polystatic pumps and Silastic tubing) through the chambers at a flow rate of 250 μl/min. A heating bath kept the temperature inside the chambers at 37°–38°. The perfusate was pumped out of the chambers and fractions were collected every 3 min for 30 min over propylene tubes containing 1 ml of boiling 1 M acetic acid and 20 μl of saturated Na₂EDTA solution. Release of enkephalin was evoked after 24 min by a 6-min exposure to a modified perfusing solution containing 85 mM NaCl/50 mM KCl (7).

Extraction and Processing of Samples. Both the freshly dissected tissue and the perfused tissue were boiled for 15 min in 1.0 M acetic acid solution with 20 μl of saturated Na₂EDTA solution per ml (2 ml per each globus pallidus) and homogenized (9). These tissue extracts as well as the perfuse fractions were centrifuged at 1000 × g for 30 min, frozen overnight, and centrifuged once more. Aliquots of the supernatants were lyophilized.

High-Pressure Liquid Chromatography (HPLC). A high-pressure liquid chromatograph equipped with a Universal In-jector (U6K) and μBondapak CN columns (0.39 X 30 cm) (all from Waters Associates) were used throughout this work. The eluting mixture contained 14% glass-distilled acetonitrile and 86% 0.01 M ammonium acetate (pH adjusted to 4.2 with glacial acetic acid); this eluant was also used to dissolve the samples (100–500 μl). The columns were calibrated with pure synthetic peptides and their retention times were determined by in-line absorption detection at 210 nm. After injection of sample and with a flow rate of 2 ml/min, 400-μl fractions were collected in polypropylene tubes (Gilson Microfractionator) and lyophilized.

Abbreviations: Met-enkephalin, [5-methionine]enkephalin; Leu-enkephalin, [5-leucine]enkephalin; HPLC, high-pressure liquid chromatography.

* This paper is the second part of a study on the release of enkephalins from rat globus pallidus. The first paper is ref. 7.

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Radioimmunoassay. The lyophilisates of tissue extracts, fractions of perfusate, or HPLC fractions were dissolved in small volumes of water, aliquots were taken for scintillation counting when necessary, and the remaining sample was subjected to radioimmunoassay at two dilutions in duplicate. The Leu-enkephalin assay was as described (10). The minimum detectable amount of Leu-enkephalin was 20 pg and the crossreactivity of Leu-enkephalin was 16% (7% in the Gros et al. radioimmunoassay). Both assays were performed with purified 125I-labeled enkephalins.

Chemicals. The purified nonlabeled peptides used as standards in the radioimmunoassays and HPLC were prepared by solid-phase synthesis (12). [tyrosyl-35S]enkephalin (5-L-methionine) (53 Ci/mmol) and [tyrosyl-3,5-3H]enkephalin (5-L-leucine) (25.5 Ci/mmol) were purchased from Amersham (England) and New England Nuclear (Boston, MA), respectively, and purified through HPLC prior to use. L-[2,6-3H]-Tyrosine and carrier-free 125I were obtained from Amersham.

RESULTS

Fig. 1 shows the HPLC fractionation of an extract of freshly dissected globus pallidus tissue. Each enkephalin was identified by its retention time on the basis of synthetic peptides and 3H-labeled internal standards. Endogenous enkephalins were monitored by means of two radioimmunoassays using antisera raised against Met-enkephalin and Leu-enkephalin. The identity of the peaks of immunoreactivity showing the retention times of Met-enkephalin and Leu-enkephalin was further assessed by their relative immunoreactivity with both the Met-enkephalin and Leu-enkephalin antisera, calculated as Met-enkephalin equivalents/Leu-enkephalin equivalents. Immunoreactive components other than Met-enkephalin and Leu-enkephalin showed immunoreactivity ratios from the two radioimmunoassays that were intermediate between those of Leu-enkephalin and Met-enkephalin. These other peaks were also present in chromatograms of perfused tissue extracts (not shown) and in perfusate samples although in different proportions (see Fig. 2c). Such other peaks account for about one-third of the total immunoreactivity with the Leu-enkephalin assay. Dilution curves of these fractions paralleled the standard curves in the assays. Many synthetic enkephalin fragments had retention times in HPLC that cluster around those of these immunoreactive peaks (Fig. 1a). Nevertheless, all these synthetic fragments showed low crossreactivity in the Leu-enkephalin assay (minimum detectable, >10 ng).

In a preliminary attempt to explore the relationship of these nonidentified components to the enkephalins, their molecular weights were estimated by using a Sephadex G-15 column (45 X 0.7 cm) eluted with 50% acetic acid and calibrated with 3H-labeled enkephalins and tyrosine; [3H]tyrosine was also used as an internal standard. The unidentified immunoreactive substances were eluted before tyrosine but no significant amounts of immunoreactivity were eluted before either enkephalin. From these data, it is likely that these components may be degradation products of the enkephalins, which may therefore be highly concentrated both in the fresh and perfused globus pallidus tissue and in the perfusate fractions.

The coupled HPLC/radioimmunoassay analysis of perfused globus pallidus displayed a similar profile to that shown by freshly dissected tissue but had lower amounts of both Met-enkephalin and Leu-enkephalin. The mean ratio of Met-enkephalin to Leu-enkephalin contents also decreased from 5.8 ± 0.2 to 3.4 ± 0.2 (Table 1). Experiments to determine the extent of degradation of each enkephalin during perfusion of tissue slices were performed by adding trace amounts of 3H-labeled Met-enkephalin or Leu-enkephalin in 2 ml of the perfusing medium (Fig. 2a and b). Degradation of [3H]Met-enkephalin in bacitracin-free medium was about 90%; bacitracin...
Table 1. Met-enkephalin and Leu-enkephalin contents in globus pallidus tissue and in material released during K\(^+\) stimulation in vitro

<table>
<thead>
<tr>
<th></th>
<th>Fresh tissue</th>
<th>Perfused tissue*</th>
<th>K(^+)-evoked release(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-enkephalin</td>
<td>54.8 ± 6.2</td>
<td>14.4 ± 1.3</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>9.3 ± 0.7</td>
<td>4.1 ± 0.3</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>Met/Leu enkephalin</td>
<td>5.8 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>2.7 ± 0.3</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM from three or four independent experiments.

* After 39-min perfusion. The tissue content immediately after release (30 min) was not significantly different.
† Total amount recovered during 6-min exposure to the high K\(^+\) medium containing 30 μg of bacitracin per ml.
‡ Calculated per globus pallidus (both hemispheres).
§ From ratios (wt/wt) calculated in individual experiments.

(30 μg/ml) decreased the degradation to 63%. Degradation of Leu-enkephalin was 62% without bacitracin and 23% with it.

Perfusion of globus pallidus slices with 50 mM K\(^+\) medium containing bacitracin stimulated the release of endogenous Met-enkephalin as well as of Leu-enkephalin (Fig. 2c). Total release over 6 min amounted to about 6% and 8% of the tissue stores, respectively. The Met-enkephalin/Leu-enkephalin ratio (wt/wt) of the recovered enkephalin was 2.7 ± 0.3 (Table 1). The unidentified low molecular weight immunoreactive peaks were also present and showed approximately the same ratios of immunoreactivity between the two radioimmunoassays that were observed in the corresponding peaks of the fresh tissue extract chromatogram (Fig. 1a).

DISCUSSION

Two separate, although probably related, phenomena have been observed in the enkephalin release experiments with perfused slices of rat globus pallidus: an extensive loss of tissue enkephalin and a massive enkephalin degradation in the perfusate. At the end of the perfusion experiments, 74% of the Met-enkephalin and 56% of the Leu-enkephalin originally present in the tissue were lost (Table 1), and the Met-enkephalin/Leu-enkephalin ratio decreased from 5.8 in fresh tissue to 3.4. It has already been reported (7) that the presence of bacitracin in the perfusing medium does not modify the amount of the tissue loss, although it significantly protects the enkephalin released into the perfusate. However, even correcting for degradation, the enkephalin recovered in the perfusate fractions does not account for the total tissue loss (ref. 7; Fig. 2a and b). Therefore, it is possible that, besides leakage of enkephalin from the tissue, significant degradation continues within the slices.

Perfusion of trace amounts of \(^3\)H-labeled enkephalins through slices of globus pallidus showed considerable degradation of Met-enkephalin and Leu-enkephalin, both in the presence of bacitracin (63% and 23%, respectively) and in its absence (91% and 62%, respectively) (Fig. 2a and b). These degradation estimates using \(^3\)H-enkephalin seem to reflect the degradation, in the perfusate, of the endogenous enkephalins. This is supported by the finding (7) that the Leu-enkephalin immunoreactivity recovered in the perfusate after K\(^+\)-evoked release is doubled when bacitracin is present in the perfusing medium. If the amounts of Met-enkephalin and Leu-enkephalin released at high K\(^+\) concentration into the perfusate are corrected for this degradation, the observed Met/Leu enkephalin ratio (Table 1) increases to about 5.5. The latter value is then higher than the ratio, 3.4, found in the perfused tissue. If this calculated ratio indicated preferential release of Met-enkephalin, this could account in part for the greater tissue loss of Met-enkephalin. The ratio of the total tissue loss of Met- and Leu-enkephalin is about 7.7.

In a recent study (13), Met-enkephalin/Leu-enkephalin ratios in whole striatal tissue (rabbit) and in its veratridine-stimulated output were found similar to our values when uncorrected for degradation. Nevertheless, strict comparison of the rabbit values (13) with our results is not possible because, besides the differences in biological samples and releasing agent, different procedures were used to protect, isolate, quantitate, and estimate the recovery and degradation of the enkephalins. The differences in the two enkephalins with respect to their lability in tissue slices, degradation in the perfusate, and in vitro release from the tissue could have functional implications. A faster turnover of Met-enkephalin compared to Leu-enkephalin could be related simply to metabolic independence of the enkephalin systems or to physical compartmentation in cells or subcellular structures.
Because both Met-enkephalin and Leu-enkephalin can be released by K+ in a Ca2+-dependent manner from the globus pallidus, these chemically and pharmacologically similar pentapeptides must be considered as performing interrelated roles, perhaps as neurotransmitters, at least in this constrained area of the nervous system. Although this interrelationship based on co-release may not be generalizable to other brain regions, our data suggest that the coarsely correlated concentrations of Met- and Leu-enkephalin in other brain regions (see ref. 2) may warrant extended neurobiological investigation.

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