Tetraethylammonium facilitates the stimulation-evoked loss of the enkephalins from the myenteric plexus of guinea-pig ileum

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The effects of electrical field stimulation on the contents of [Met]enkephalin and [Leu]enkephalin were determined in myenteric plexus–longitudinal muscle preparations of the guinea-pig small intestine. Cycloheximide (0.1 mM) was present in all experiments to prevent de novo biosynthesis. The two enkephalins were separated by high-performance liquid chromatography and assayed on the mouse vas deferens. Stimulation with submaximal pulses (50 mA, 0.5 ms) at a frequency of 10 Hz caused maximal losses of about 35% of [Met]enkephalin and [Leu]enkephalin after 3 h (108,000 pulses). The plot of log (enkephalin content) against number of pulses was steeper during the first 30 min than during the later periods. Tetraethylammonium bromide (TEA, 10 mM) increased the [Met]enkephalin and [Leu]enkephalin contents of the non-stimulated preparations by about 50%. When the preparations were stimulated in the presence of TEA at 50 mA and 1 Hz, the plots of loss of enkephalins against number of pulses were linear until the maximum of about 50% was reached. Compared with stimulation in the absence of TEA, the rate constant was 8 times greater for [Leu]enkephalin and 20 times greater for [Met]enkephalin. The absolute losses per pulse were about 13 times greater for [Leu]enkephalin and 27 times greater for [Met]enkephalin than in the absence of TEA. In the presence of bacitracin and a mixture of dipeptides, the enzymatic degradation of the enkephalins was sufficiently suppressed to cause an overflow of 30–60% of the enkephalins lost from their stores into the perifusing Krebs solution. Until it is possible to determine the preformed precursors, which are present in large quantities, the kinetic relationship between these precursors and the enkephalins cannot be investigated. A similar dilemma exists for the relationship between "released" enkephalins and the losses from their stores.

Introduction

The myenteric plexus–longitudinal muscle preparation of the guinea-pig ileum is rich in enkephalins. The content of [Met]enkephalin (about 400 pmol per gram of tissue) is similar to, and that of [Leu]enkephalin (about 150 pmol/g) is about three times larger than, that of the hypothalamus (Hughes et al. 1977). The plexus contains many fibres with enkephalin-like immunoreactivity (Elde et al. 1976; Linnoila et al. 1978; Jessen et al. 1980); the two enkephalins have been reported
to be present in separate fibres, those with [Met]enkephalin-like immunoreactivity being more numerous than those with [Leu]enkephalin-like immunoreactivity (Larsson et al. 1979).

The anatomical arrangements of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine would make it a suitable model for the investigation of the release of the enkephalins due to electrical stimulation. However, their rapid degradation by exopeptidases and endopeptidases (Hambrook et al. 1976; Malfroy et al. 1979) have so far made it impossible to obtain consistent results. For this reason, an attempt was made (McKnight et al. 1978; Corbett et al. 1980b) to obtain a measure of the release of the enkephalins by determining their loss from the stores caused by electrical stimulation in the presence of cycloheximide, an inhibitor of protein synthesis. Since the loss of the enkephalins was only 4–5 fmol per gram of tissue at stimulation frequencies of either 1 or 10 Hz, the duration of stimulation in these experiments had to be 0.5–4 h to produce a decrease in the enkephalin stores that could be measured with confidence.

In the present series, the experimental procedure was modified in two respects. First, to reduce the length of the stimulation period, tetraethylammonium was added to the bath fluid. This agent has been shown to cause an increase in the evoked transmitter release at the skeletal neuromuscular junction (Koketsu 1958; Katz & Miledi 1979) and at nerve-smooth muscle junctions (Kirpekar et al. 1972; Stjärne 1973; Holman & Suprenant 1980). Secondly, the changes in the stores of [Met]enkephalin and [Leu]enkephalin were measured separately by the use of high-performance liquid chromatography (h.p.l.c.). Some of the preliminary observations have been published (Corbett et al. 1980a). Finally, the advent of potent inhibitors of the peptidases responsible for enkephalin degradation has made it possible to include a few preliminary experiments demonstrating overflow of the two enkephalins into the perfusing medium.

**Materials and methods**

*(a) Apparatus and preparations*

All experiments were performed on the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine. The experimental procedure was similar to that used in the earlier experiments (Corbett et al. 1980b). The terminal 10 cm of the ileum were discarded and two preparations of 10–12 cm length and 200 mg mass were dissected. The preparations were folded over to give a length of 5–6 cm and suspended in separate organ baths 6 cm long and 0.5 cm wide; the resting tension was 0.5 g (ca. 5 mN). The baths were perfused at a rate of 0.6 ml/min with modified Krebs solution of the following composition (mm): NaCl 118, KCl 4.74, CaCl$_2$ 2.54, KH$_2$PO$_4$ 1.19, MgSO$_4$ 1.2, NaHCO$_3$ 25, glucose 11; it also contained the following L-amino acids at a concentration of 1 µg/ml: Ala, Arg, Asp, Cys, Glu, Gly, His, Leu, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. The bath fluid was at 37 °C and was bubbled with 95% O$_2$ and 5% CO$_2$. For electrical stimulation, the organ baths were fitted with a coiled platinum electrode at the bottom and a platinum foil ring at the top.
(b) Experimental procedure

Before the beginning of the experiment, the preparations were preincubated for 60 min, when they were stimulated to test their viability. For this purpose, rectangular pulses of 0.5 ms duration and supramaximal voltage were applied at 0.1 Hz; the contractions were recorded isometrically and, if the tension did not reach 2 gf (ca. 20 mN), the preparations were discarded. During the actual experiments, the voltage was varied and the current through the bath monitored and adjusted to the desired level. To minimize polarization at the electrodes, the pulses were given biphasic components by means of 2 μF condensers in series with the output of a Grass stimulator capable of delivering currents of up to 120 mA.

After the initial period of preincubation, the preparations were equilibrated for 30 min in the modified Krebs solution containing also cycloheximide (Sigma, 0.1 mM), or tetraethylammonium bromide (TEA, Sigma, 10 mM) in addition to the cycloheximide. One of the preparations served as non-stimulated control while the other was stimulated with different current strengths at frequencies of 0.1, 1 or 10 Hz.

(c) Extraction and estimation of tissue enkephalins

At the end of the experimental period the preparations were weighed, homogenized in 20 ml of ice-cold 0.1 M HCl and centrifuged at 50000 g for 30 min. The enkephalins present in the supernatants were adsorbed onto columns (10 cm × 0.5 cm) of Amberlite XAD-2 resin (B.D.H.) and then eluted with methanol. A portion of this eluate was retained for subsequent estimation of the total enkephalin content by bracket assay on the mouse vas deferens against standard [Met]enkephalin (Hughes et al. 1977).

The major part of the eluate from the XAD-2 column was lyophilized, redissolved in 500 μl of a mixture of 4 volumes of 0.7 M acetic acid and 1 volume of methanol (pH 2.5) and filtered through a cellulose acetate/nitrate filter with a pore size of 0.45 μm (Millipore). The enkephalins in the filtrate were separated by reverse phase h.p.l.c.; they were eluted from a 30 cm × 0.4 cm μBondapak C₁₈ column (Waters) at a rate of 0.8 ml/min by a linear gradient progressing from the solution used for dissolving the XAD-2 elute to 100% methanol in 70 min. The u.v. absorbance of the eluate was continuously monitored at 280 nm. Material eluting with the same retention times as standard [Met]enkephalin and [Leu]enkephalin was lyophilized and bioassayed on the mouse vas deferens against authentic synthetic [Met]enkephalin and [Leu]enkephalin (Hughes et al. 1977). The results of such a separation are shown in figure 1. The more hydrophilic constituents of the eluate from the XAD-2 column are not retained and are eluted in the void volume of the column (ca. 3 ml) whereas the more hydrophobic materials, including the enkephalins, are eluted progressively later.

Since the amounts of the two enkephalins in the eluate from the XAD-column were less than 100 ng, no peaks of u.v. absorbance could be attributed to them. Therefore, prior to any experimental run, the retention times were established with 2 μg of each of [Met]enkephalin and [Leu]enkephalin (figure 1, lower panel). Under our conditions, the retention time of [Leu]enkephalin was about 5 min longer than that of [Met]enkephalin. At regular intervals, the reliability of the method was
checked by separating a mixture of 50 ng of synthetic [Met]enkephalin and 25 ng of synthetic [Leu]enkephalin by standard runs on the C\textsubscript{18} column. These amounts are of the order found in 200 mg of myenteric plexus–longitudinal muscle. The recovery in the appropriate fraction of [Met]enkephalin was 91 ± 3 \% and that of [Leu]enkephalin 81 ± 4 \% (n = 5).

![Graph](http://rspb.royalsocietypublishing.org/)

**Figure 1.** H.p.l.c. separation of [Met]enkephalin-like and [Leu]enkephalin-like activities on a \( \mu \)Bondapak C\textsubscript{18} reverse phase column: u.v. absorbance profiles at 280 nm (1 cm path length with a 8 \( \mu \)l flow cell) of a typical XAD-2 extract of myenteric plexus–longitudinal muscle preparation of the guinea-pig small intestine; (b) of a mixture of synthetic [Met]enkephalin and [Leu]enkephalin (2 \( \mu \)g of each)). The histograms show [Met]enkephalin-like activity (open columns) and [Leu]enkephalin-like activity (hatched columns), determined by bioassay on the mouse vas deferens and contained in 0.8 ml (1 min) fractions of the eluate whose u.v. absorbance profile is shown in (a). The broken lines show the gradient of the methanol concentration in the eluting solvent.

A measure of the recovery of the enkephalins from the myenteric plexus–longitudinal muscle was obtained in two ways. In the first, the total enkephalin assayed in the lyophilized eluate from the XAD-2 column was compared with the sum of [Met]enkephalin and [Leu]enkephalin in the appropriate fractions after passage through the C\textsubscript{18} column; the two enkephalins assayed separately amounted to 87 ± 1 \% (n = 48) of the total non-separated enkephalins. Recovery of total enkephalins in brain tissue by extraction with 0.1 \( \mathrm{M} \) HCl and subsequent treatment on XAD-2 resin gave in four experiments a recovery of 86 ± 6 \% (Hughes et al. 1977). In three experiments, 50 ng of [Met]enkephalin and 25 ng of [Leu]enkephalin were added to one part of a lyophilized and redissolved eluate from the XAD-2 resin. The assay gave the sum of the added enkephalins and the endogenous [Met]enkephalin and [Leu]enkephalin present in the myenteric plexus. In the remainder of the eluate the endogenous enkephalins were determined. The recovery of added [Met]enkephalin was 82 ± 3 \% and that of [Leu]enkephalin 75 ± 6 \%. The results given in this paper were not corrected for recovery.

In the example of figure 1, the enkephalin-like activities of the tissue extract
shown in the histogram in the lower panel elute with approximately the same retention time as synthetic standard [Met]enkephalin and [Leu]enkephalin, the major part of each peptide being contained in a single fraction of 0.8 ml. No activity was detected in the four fractions intervening between that containing [Met]enkephalin and that containing [Leu]enkephalin or in the four fractions preceding [Met]enkephalin or those following [Leu]enkephalin. Routinely, the fraction corresponding to the median retention time of the standard peptide and the two flanking fractions were collected as a single sample, lyophihzed and assayed.

To ensure further that the opioid activities found in the fractions having the retention times of synthetic [Met]enkephalin and [Leu]enkephalin correspond to the two enkephalins, chromatographic conditions were changed by using an equilibrating solution of pH 5, obtained by the addition of a concentrated ammonia solution to the original acetic acid–methanol mixture of pH 2.5. Although the retention times for the two enkephalins were longer at pH 5 than at pH 2.5, the amounts of [Met]enkephalin and [Leu]enkephalin were the same within the limit of error of the bioassay when the same eluate from the XAD-2 column was used. The opioid activity in the fractions containing [Met]enkephalin and [Leu]enkephalin was completely reversed by naloxone whether the pH of the equilibrating solution was 2.5 or 5.

(d) Estimation of enkephalins released into the bath fluid

In a few experiments, attempts were made to detect release of enkephalins into the bath fluid during field stimulation in the presence of TEA. The preparations were equilibrated in the modified Krebs solution already described which also contained 10 mM TEA and 0.1 mM cycloheximide. To reduce degradation by peptidases, the following additions were also made: bacitracin (30 μM), Tyr-Gly (0.1 mM), and Leu-Leu, Leu-Gly, Tyr-Tyr and Gly-Phe (all 1 mM). In bovine or rat caudate, bacitracin inhibits about equally well aminopeptidase and the enkephalinase cleaving at Gly–Phe while the mixture Leu-Leu, Leu-Gly, Tyr-Tyr is a potent peptidase inhibitor, being more active against enkephalinase than aminopeptidase (Sullivan et al. 1980). This mixture of dipeptides was successfully used in studies on enkephalin release induced by K+ or veratridine in brain homogenates or striatal slices (Henderson et al. 1978). Tyr-Gly is a potent and Gly-Phe a less active inhibitor of enkephalinase (Fournie-Zaluski et al. 1979; Sullivan et al. 1980).

In these experiments the organ baths (3 ml) were perfused at 1 ml/min and the effluent collected by overflow from a side arm at the top of the bath into plastic centrifuge tubes immersed in ice and containing sufficient 1 M HCl to bring the concentration of HCl in the final volume to 0.1 M together with dithiothreitol (0.65 mM, Sigma) as an antioxidant. Until the adsorption of the enkephalins onto the XAD-2 column the acidified perifusates were kept at 0–4 °C.

(e) Analysis of data

The contents of the tissues were expressed as [Met]enkephalin or [Leu]enkephalin (picomoles per gram (wet mass)) or as [Met]enkephalin equivalents (pmol/g (wet mass)) when total enkephalins were measured.
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To test whether the loss in enkephalins followed first order kinetics, the equation

\[ K = (1/n)(\ln C_n - \ln C_0) \]

was used, where \( K \) is the rate constant, \( n \) the number of electrical pulses applied, \( C_0 \) the enkephalin content of the non-stimulated control preparation and \( C_n \) the enkephalin content of the stimulated preparation after the application of \( n \) pulses. When it was desirable to express the absolute values of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Observations</th>
<th>Total Enkephalin Content</th>
<th>[Met]Enkephalin pmol/g</th>
<th>[Leu]Enkephalin pmol/g</th>
<th>[Met]Enkephalin/ [Leu]Enkephalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, incubation up to 240 min</td>
<td>14</td>
<td>474 ± 24</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cycloheximide (0.1 mM), incubation up to 210 min</td>
<td>18</td>
<td>499 ± 24</td>
<td>252 ± 14</td>
<td>149 ± 11</td>
<td>1.7</td>
</tr>
<tr>
<td>Cycloheximide (0.1 mM) and tetraethylammonium (10 mM), incubation up to 180 min</td>
<td>22</td>
<td>741 ± 37</td>
<td>414 ± 29</td>
<td>207 ± 14</td>
<td>2.0</td>
</tr>
</tbody>
</table>

the contents after different numbers of pulses (figure 3), it was necessary to normalize the \( C_0 \) and \( C_n \) values of each pair to the mean of all \( C_0 \) values of the group of experiments under consideration because there was a rather large variation in the \( C_0 \) values between preparations. Alternatively, when the rate constant was calculated from a relatively small number of values of fractional loss of enkephalin content, the content of the control of each pair of preparations was taken to be 1 and the content of the stimulated preparations expressed as the fraction \( C_n/C_0 \).

**RESULTS**

(a) The effects of cycloheximide and TEA on the enkephalin contents of non-stimulated control preparations

It has been shown previously (Corbett et al. 1980a) that incubation of the myenteric plexus–longitudinal muscle preparations with cycloheximide (0.1 mM) had no effect on the content of total enkephalin. These results have been confirmed in the present series of experiments (table 1).

On the other hand, the addition of TEA (10 mM) to the Krebs solution containing cycloheximide (0.1 mM) raised the contents of total enkephalin, [Met]enkephalin and [Leu]enkephalin by about 50%. The apparent differences between the increases of [Met]enkephalin and [Leu]enkephalin did not reach significance, as was indicated by only a minor change in the ratio of the two enkephalins from 1.8 to 2.1 (table 1); a ratio of 2.7 was found in untreated preparations (Hughes et al. 1977).

(b) The effect of electrical stimulation on the enkephalin contents

in the absence of TEA

The losses of total enkephalin and of [Met]enkephalin and [Leu]enkephalin due to stimulation at 10 Hz and a current of 50 mA were followed up for 3 h; at the end of this period, during which 108000 pulses had been applied, the contents of
[Met]enkephalin and [Leu]enkephalin had been reduced by 35%. When the contents were plotted logarithmically against the number of pulses, the decrease was shown to be nonlinear (figure 2), the rate of loss of total enkephalins, [Met]enkephalin and [Leu]enkephalin being faster during the first 30 min than during the later periods. When it was assumed that the curve was due to two first

order reactions of different rates, the $K$ values calculated for the losses between 15 and 30 min were $-1.8 \times 10^{-5}$ and $-1.5 \times 10^{-5}$ for [Met]enkephalin and [Leu]enkephalin respectively. The corresponding $K$ values for the losses of [Met]enkephalin and [Leu]enkephalin between 1 and 3 h of stimulation were only $-0.25 \times 10^{-5}$. During the first 30 min period of stimulation, the mean losses per pulse were for [Met]enkephalin 4 fmol per gram of tissue and for [Leu]enkephalin 2 fmol per gram of tissue.

Since prolonged stimulation at 10 Hz was considered to be undesirable, the decreases of the enkephalin contents were determined in three experiments in which the stimulation frequency was 1 Hz and the current 50 mA for 4 h, during which period 14400 pulses were applied. The values for the contents (pmol/g ± s.e.m., $n = 3$) of the non-stimulated and stimulated preparations, respectively, were 517 ± 18 and 436 ± 10 for total enkephalin, 283 ± 11 and 244 ± 12 for [Met]enkephalin and 144 ± 10 and 140 ± 1 for [Leu]enkephalin.

Thus, this experimental approach was not useful. Therefore, it was investigated whether TEA would increase the losses in the enkephalin contents since it is known to raise the release of acetylcholine at the skeletal neuromuscular junction (Katz & Miledi 1979) or noradrenaline at autonomic nerve–smooth muscle junctions (Kirpekar et al. 1972; Stjärne 1973; Holman & Suprenant 1980).
(c) The effects of TEA on the stimulation-induced losses of the enkephalins

When the myenteric plexus–longitudinal muscle preparation was stimulated in the presence of TEA (10 mM) at 1 Hz and 50 mA current strength, the losses in total enkephalin, [Met]enkephalin and [Leu]enkephalin obeyed first-order kinetics (figure 3). The regression lines for $\ln([\text{tissue content}/(\text{pmol/g})]$ on number of stimulating pulses showed the steepest slope for [Met]enkephalin ($K = -36 \times 10^{-5}$) and the smallest slope for [Leu]enkephalin ($K = -12.2 \times 10^{-5}$), with the slope for the total enkephalin being intermediate ($K = -27.5 \times 10^{-5}$). When the average values for the losses occurring per pulse during the first 30 min (1800 pulses) were calculated, they were 110 fmol/g for [Met]enkephalin and 27 fmol/g for [Leu]-enkephalin. The maximum loss of the [Met]enkephalin content of 52% was reached after 30 min while that of [Leu]enkephalin was 35% after 60 min. Thus, for [Met]enkephalin the loss per pulse at 1 Hz in the presence of TEA was about 27 times larger than at 10 Hz in the absence of TEA and the corresponding value for [Leu]enkephalin was 13.

This large increase in the losses of [Met]enkephalin and [Leu]enkephalin made it possible to study the effect of stimulation at the low frequency of 0.1 Hz for 2 h

Figure 3. The effects of field stimulation (50 mA, 0.5 ms, 1 Hz) in the presence of cycloheximide (0.1 mM) and TEA (10 mM) on the contents of total enkephalin (■), [Met]enkephalin (●) and [Leu]enkephalin (○) of the myenteric plexus–longitudinal muscle preparations of the guinea-pig small intestine. The regression equations were: for total enkephalin $\ln C_n = -n(27.5 \times 10^{-5}) + 6.606$; for [Met]enkephalin $\ln C_n = -n(36.0 \times 10^{-5}) + 5.982$; and for [Leu]enkephalin $\ln C_n = -n(12.2 \times 10^{-5}) + 5.350$. The observed means of $C_n$ were 753 ± 46 pmol/g for total enkephalin expressed as methionine equivalents, 398 ± 30 pmol/g for [Met]enkephalin and 211 ± 14 pmol/g for [Leu]enkephalin. The sum of [Met]enkephalin and [Leu]enkephalin contents amounted to 612 pmol/g of methionine equivalents, i.e. 82% of total enkephalins. Each point is the mean of at least three observations.
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(720 pulses). As far as [Met]enkephalin was concerned, the loss per pulse (200–240 fmol/g) was about twice as large as that found with stimulation at 1 Hz (table 2). However, there were some anomalies regarding [Leu]enkephalin; the mean contents of the non-stimulated control preparations were just below 60% of the values observed in other groups of experiments. Nevertheless, it may be of significance that with maximal stimulation (100 mA) there was no change in the [Leu]enkephalin content whereas with submaximal stimulation (50 mA) there was an increase in content which, on paired analysis, was significant ($P < 0.025$).

### Table 2. Changes in the [Met]enkephalin and [Leu]enkephalin contents of paired preparations after stimulation at 0.1 Hz for 2 h in the presence of TEA (10 mM)

(The values are the means ± s.e. of three experiments.)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[Met]enkephalin</th>
<th>change per pulse</th>
<th>[Leu]enkephalin</th>
<th>change per pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>441 ± 32</td>
<td>−200</td>
<td>117 ± 10†</td>
<td>+56</td>
</tr>
<tr>
<td>stimulated at 50 mA</td>
<td>297 ± 18</td>
<td></td>
<td>157 ± 9</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>408 ± 29</td>
<td>−240</td>
<td>118 ± 19</td>
<td></td>
</tr>
<tr>
<td>stimulated at 100 mA</td>
<td>235 ± 21</td>
<td></td>
<td>113 ± 8</td>
<td>−7</td>
</tr>
</tbody>
</table>

† With paired analysis, the difference was $40 ± 3$, $P$ being $< 0.025$.

(d) Overflow of [Met]enkephalin and [Leu]enkephalin into the bath fluid

During the last few years, several unsuccessful attempts have been made in this and other laboratories to obtain a consistent overflow of enkephalins into the bath fluid. In recent preliminary experiments, a certain amount of success has been achieved in the following manner: the release of the enkephalins was potentiated by TEA and their degradation was minimized by the addition of bacitracin and of several dipeptides. The details of the procedure are outlined in methods. Experiments with bacitracin or dipeptides alone were unsuccessful.

When in the presence of cycloheximide, bacitracin and the dipeptide mixture the preparations were stimulated at 1 Hz and 50 mA for 30 min, $48 ± 13$ pmol/g of [Met]enkephalin and $16 ± 3$ pmol/g of [Leu]enkephalin ($n = 3$) were found in the bath fluid. In these experiments the ‘recovery’ rate of the lost enkephalins in the bath fluid was low, between 30 and 60%. It may be of significance that, while under identical experimental conditions (1 Hz, 50 mA, 30 min) the ratio of [Met]enkephalin to [Leu]enkephalin was 4 to 5 for loss from the tissue, it was 3 for the enkephalins that appeared in the bath fluid.
DISCUSSION

The experiments described in this paper are an extension of our attempts to investigate the release of the enkephalins evoked by electrical field stimulation of the myenteric plexus–longitudinal muscle preparation of the guinea-pig small intestine (McKnight et al. 1978; Corbett et al. 1980b). Most of the data were obtained from the decrease in the enkephalin content of the preparation when ribosomal synthesis of the precursors was prevented. Their interpretation is limited by two circumstances. First, measurements of the 'release' of the enkephalins into the bathing fluid are still unreliable and, secondly, there is no information as to changes in the stores of the preformed precursors which are large in the myenteric plexus (McKnight et al. 1980) as well as in the striatum (Lewis et al. 1978; Beaumont et al. 1980) and the adrenal medulla (Lewis et al. 1979).

Progress has been made in several directions. A reliable method for the estimation of [Met]enkcephalin and [Leu]enkcephalin after separation by high-performance liquid chromatography has been developed. Further, by inhibiting the degrading enzymes at least partially, [Met]enkcephalin and [Leu]enkcephalin have been found in the bath fluid in quantities corresponding to 30–60% of the enkephalins lost from the stores during field stimulation. However, at present the 'recovery' rates are not consistent enough to be helpful in the interpretation of the mechanisms underlying the losses from the tissue stores.

When the earlier experiments on the loss of enkephalin due to submaximal field stimulation (50 mA) at 10 Hz (Corbett et al. 1980b) were repeated, it could not be confirmed that the plots of log [Met]enkcephalin and of log [Leu]enkcephalin against number of stimuli were linear. Since the period of stimulation lasted for 3 h, it was suspected that impulse transmission was defective. Therefore use was made of the increase in release observed with other transmitters in the presence of TEA (Kirpekar et al. 1972; Stjärne 1973; Katz & Miledi 1979; Holman & Suprenant 1980). It was found that 10 mM TEA increased the rate constant of the loss of [Met]enkcephalin from 1.8 × 10⁻⁵ to 36 × 10⁻⁵ and that of [Leu]enkcephalin from 1.5 × 10⁻⁵ to 12.2 × 10⁻⁵. This increase in rate of loss made possible the use of a stimulation frequency of 1 Hz instead of 10 Hz; the maximal loss of about 50% of the stores was obtained after 30 min of stimulation with [Met]enkcephalin and after 60 min with [Leu]enkcephalin.

An interesting observation was the rise of 40 to 50% in the contents of [Met]enkcephalin and of [Leu]enkcephalin by TEA when the preparations were not stimulated and ribosomal synthesis was prevented by cycloheximide. This finding implies that on stimulation in the presence of TEA the absolute decrease per pulse in the enkephalin stores is about 50% larger than would be expected from the rate constants. Although there is so far no evidence as to the source of the enkephalins added to the stores in the presence of TEA, it may be permissible to assume that they are derived from the precursor stores. In this context, it should be remembered that the non-stimulated myenteric plexus–longitudinal muscle preparation is not absolutely quiescent and at low-grade activity the processing of precursors to the enkephalins may exceed the requirements of the release mechanism. Such an interpretation may also explain the rise in the total enkephalin content found
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previously when the preparations were stimulated at 1 Hz for 2 h in the absence of cycloheximide (McKnight et al. 1978).

The addition of TEA makes possible the measurement of change in the contents of enkephalins at low stimulation frequencies, e.g. 0.1 Hz. In a preliminary experiment it was shown that after 720 pulses in 2 h 50% of the [Met]enkephalin stores were lost. The loss per pulse was about twice as large as that obtained by stimulation at 1 Hz when 1800 pulses in 30 min were required for a similar loss.

In conclusion, we have shown that, in the presence of cycloheximide, TEA causes large increases in the losses of [Met]enkephalin and [Leu]enkephalin when the myenteric plexus–longitudinal muscle preparation of the guinea-pig small intestine is excited by field stimulation. These effects of TEA and the fact that the stimulation-induced loss of enkephalin is tetrodotoxin-sensitive (McKnight et al. 1978) indicate that the loss is neurogenic. As long as it is not possible to correlate the changes in tissue content with changes in the preformed precursor and with changes in the overflow of the enkephalins into the bath fluid, no useful conclusions can be drawn with regard to the observation that [Met]enkephalin appears to be lost more rapidly than [Leu]enkephalin. That the preformed precursors can replenish or increase the enkephalin stores is shown by the rise of the latter in the quiescent preparation after addition of TEA to the bath fluid.

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References


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