Br. J. Pharmac (1987), **92**, 291–298

[Met^5]enkephalin acts via δ-opioid receptors to inhibit pelvic nerve-evoked contractions of cat distal colon

Charles Kennedy & [1] Jacob Krier

Department of Physiology, Michigan State University, East Lansing, Michigan 48824–1101, U.S.A.

1 The effects of opioids on the sacral parasympathetic outflow to cat distal colon were studied *in vitro* using muscle strips orientated in the axis of the longitudinal muscle layer, with pelvic nerves attached. Electrical stimulation of the pelvic nerves evoked contractions that were blocked by atropine (1×10^{-6} M) and tetrodotoxin (3×10^{-7} M).

2 [D-Pen^2, D-Pen^5]enkephalin and [Met^5]- and [Leu^5]enkephalin caused concentration-dependent, reversible inhibition of pelvic nerve-evoked contractions, with IC_{50} values of 8.3×10^{-10} M, 2.2×10^{-9} M and 2.1×10^{-9} M respectively.

3 Morphine (1×10^{-7}–1×10^{-5} M) and [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (1×10^{-8}–1×10^{-6} M) and U-50,488H (1×10^{-8}–1×10^{-6} M) were much less potent as inhibitors than [Met^5]- or [Leu^5]enkephalin.

4 Naloxone (1×10^{-7} M), an antagonist at each of the three opioid receptor types, antagonized the effects of both [Met^5]enkephalin and morphine. However, ICI 174,864, a specific δ-opioid receptor antagonist, antagonised the effects of [Met^5]enkephalin only.

5 The inhibitory actions of [Met^5]enkephalin were inversely related to frequency of pelvic nerve stimulation. Also, [Met^5]enkephalin at a concentration (3×10^{-9} M) which produced a large inhibition of neurogenic contractions, had no effect on contractions to exogenous acetylcholine. These results suggest a prejunctional site for inhibitory opioid receptors.

6 In summary, prejunctional inhibitory δ-opioid receptors are present on the sacral parasympathetic outflow to cat distal colon; κ- and/or μ-opioid receptors may also be present, but appear to be of lesser importance.

Introduction

Immunohistochemical techniques have shown that enkephalin-like substances are present in intrinsic neurones of the myenteric plexus throughout the gastrointestinal tract of many species, including man (Elde *et al.*, 1976; Polak *et al.*, 1977; Alumets *et al.*, 1978; Larsson & Stengaard-Pedersen, 1982). At this site, it has been proposed that enkephalins may be neurotransmitters or neuromodulators acting upon cholinergic neurones to inhibit release of acetylcholine (ACh) (see Furness & Costa, 1982). For example, the evoked release of endogenous enkephalin-like compounds has been demonstrated from guinea-pig ileum (Schulz *et al.*, 1977; Clark & Smith, 1983), a tissue in which enkephalins inhibit neurogenic cholinergic twitch contractions (Waterfield *et al.*, 1977). Electrophysiological recordings indicate that this may be due to a naloxone-sensitive hyperpolarization of enteric neurones (North, 1986) and reduction in evoked excitatory postsynaptic potential amplitude (Cherubini *et al.*, 1985).

The actions of enkephalins on the colon have been relatively little studied. The pelvic nerves supply the sacral parasympathetic motor innervation to this organ (Langley & Anderson, 1895; de Groat & Krier, 1976; 1978). Enkephalin-like immunoreactivity has been shown within both sacral preganglionic neurones (Glazer & Basbaum, 1980) and neurones of the colonic myenteric plexus (see above). Furthermore, enkephalins inhibit pelvic nerve-evoked excitatory postsynaptic potentials in parasympathetic colonic neurones (Kennedy & Krier, 1987a,b) and excitatory junction potentials in this tissue (Blanquet *et al.*, 1982). Thus, endogenous enkephalins may be important modulators of motor activity of the colon.

The aim of the present study was to determine more precisely the effects of opioids on pelvic nerve-evoked contractions of cat distal colon longitudinal muscle *in vitro* and the opioid receptor type(s) involved (Martin

[1] Correspondence.

et al., 1976; Lord *et al.*, 1977). The latter were characterized by use of analogues that have been proposed to be selective for opioid receptors in other tissues. Parts of this study have been published previously (Kennedy & Krier, 1986).

Methods

Cats of either sex (1.0–2.5 kg) were anaesthetized with sodium pentobarbitone (30–35 mg kg^{-1}, i.p.) and exsanguinated. A segment of distal colon 4–5 cm long immediately proximal to the rectum and with a 2–3 cm length of both pelvic nerves attached, was excised. The preparation was cut along the antimesenteric border and the mucosal layer was surgically separated from the external muscle layers. A portion of circular and longitudinal muscle 10–20 mm in the axis of the longitudinal muscle layer and 3–5 mm wide, with pelvic nerve attached, was isolated. This tissue was mounted vertically in the longitudinal axis under isometric conditions in a 90 ml organ bath and bathed in modified Krebs solution at 37°C, bubbled with 95% O_2 and 5% CO_2, of the following composition (mM); Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 134, HCO_3^- 15.5, $H_2PO_4^-$ 1.2 and glucose 11.5. Circular muscle mechanical activity in separate preparations, was studied; these preparations consisted of strips 10–20 mm in the axis of the circular muscle layer and 3–5 mm in the axis of the longitudinal muscle layer in the absence of pelvic nerves. They were mounted vertically in the circular axis. All preparations were equilibrated for 60 min under an optimal resting tension equivalent to a load of 2 g. This optimal tension was determined by repeatedly stretching the muscle strips and adding ACh (1×10^{-4} M) between stretches. When contractions to ACh ceased to increase with increasing stretch, that tension was considered optimal.

Bipolar platinum electrodes were placed on the central end of the pelvic nerve. Intramural nerves were stimulated via two platinum plate electrodes 8 mm apart and either side of the tissue. Rectangular pulses (120 mA, 0.5 ms duration, 2 Hz) were applied for 10 s at 1 min intervals from a Grass S48 stimulator and a current amplifier. These parameters could evoke reproducible contractions of a consistent magnitude over the duration of the experiment, which were not obscured by spontaneous mechanical activity. In one series of experiments, tissues were stimulated at 2, 4 and 8 Hz in order to study the frequency-dependence of opioid inhibition.

Drugs were added to the bathing solution as single doses. Evoked contractions obtained in the presence of opioids were expressed as a percentage of 3–6 contractions obtained in their absence and immediately prior to their addition. In each experiment, in which contractions were evoked by either pelvic nerve or electrical field stimulation, [Met^5]enkephalin (3×10^{-9} M, pelvic nerve; 1×10^{-7} M, electrical field stimulation) was added repeatedly at intervals of 20 min for 1 h until a consistent inhibition was produced. Tissues in which such a reproducible inhibition could not be produced were discarded.

Naloxone and ICI 174,864 were added to the bathing solution 5–10 min before addition of agonists. Antagonistic actions of these two drugs were measured in different tissues from control responses since only one log concentration-response curve could be obtained from a tissue. This was due to both the long time required to produce a full curve (a 20 min dose cycle was needed to avoid desensitization) and also to the appearance in the latter part of an experiment of further spontaneous mechanical activity, which obscured neurogenic contractions. Thus, the equilibrium constant (K_e) with confidence limits could not be determined accurately for these antagonists. However, approximate K_e values could be

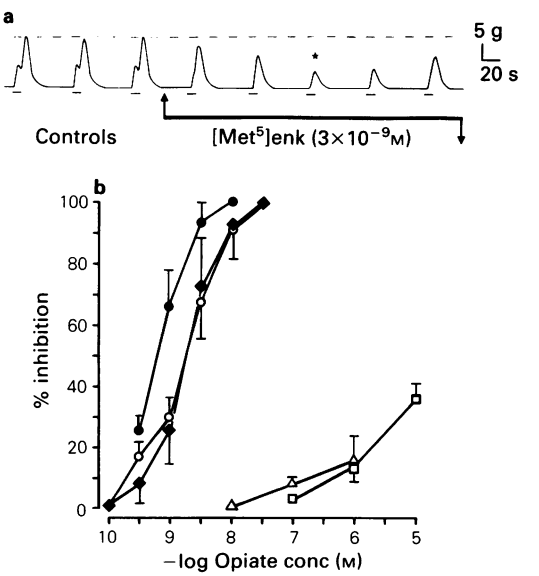

Figure 1 Cat distal colon longitudinal muscle strip *in vitro*: inhibition of pelvic nerve-induced contractions (2 Hz, 120 mA, 0.5 ms pulse duration, for 10 s, at 1 min intervals) (a) by [Met^5]enkephalin (Met-enk) (3×10^{-9} M): maximum inhibition indicated by asterisk broken horizontal line indicates level of control contractions. (b) By [D-Pen^2, D-Pen^5]enkephlain (3×10^{-10}–1×10^{-8} M) (●) ($n = 4$), [Met^5]enkephalin (1×10^{-10}–3×10^{-8} M) (○) ($n = 6$), [Leu^5]enkephalin (1×10^{-10}–3×10^{-8} M) (◆) ($n = 4$), morphine (1×10^{-7}–1×10^{-5} M) (□) ($n = 6$) and U-50,488H (1×10^{-7}–1×10^{-6} M) (△) ($n = 4$). Vertical lines: s.e.mean.

obtained by inserting IC_{50} or IC_{25} values into the following equation: K_e = [antagonist]/(DR − 1) (Kosterlitz & Watt, 1968). Thus, K_e is the concentration of antagonist which requires a doubling of agonist concentration in order to produce the same size of response.

All responses have been expressed as the mean ± s.e.mean, and the geometric mean and its 95% confidence intervals (95% confidence limits) calculated for EC_{50}, IC_{50} and IC_{25} values (Fleming *et al.*, 1972). Results were analysed using Student's unpaired *t* test, except where the frequency-dependence of the action [Met⁵]enkephalin was studied, when Student's paired *t* test was used. A probability of less than 0.05 was considered significant.

Drugs used were, acetylcholine chloride, atropine sulphate, hexamethonium bromide, [Leu⁵]enkephalin acetate, [Met⁵]enkephalin acetate, naloxone hydrochloride, tetrodotoxin (Sigma Chemical Company); morphine sulphate (Eli Lilly and Company); U-50,488H (*trans* 3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl]-benzene acetamide methane sulphonate) (Upjohn Company); [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin and [D-Pen², D-Pen⁵]enkephalin (Peninsula Laboratories); ICI 174,864 (Allyl₂-Tyr-Aib-Aib-Phe-Leu-OH) (Cambridge Research Biochemicals Ltd.) and sodium pentobarbitone (Abbot Laboratories). Drugs were dissolved in distilled water or Krebs solution.

Results

Opioid-induced inhibition of pelvic nerve-evoked contractions

Effects of [Met⁵]- and [Leu⁵]enkephalin Electrical stimulation of the pelvic nerve (2 Hz, 120 mA, 0.5 ms pulse duration, for 10 s at 1 min intervals) produced contractions with a latency of onset of 2–4 s, which were 44 ± 2% (n = 30) of the maximum contraction to exogenous ACh (1×10^{-4} M), and which returned to baseline within 30–45 s. These contractions were abolished by cutting the nerve at a site between the muscle strip and the electrodes (n = 6), by tetrodotoxin (3×10^{-7} M) (n = 6) or by atropine (1×10^{-6} M) (n = 19), indicating that the responses were neurogenic and mediated by ACh.

[Met⁵]enkephalin (1×10^{-10}–3×10^{-8} M) caused concentration-dependent inhibition of pelvic nerve-evoked contractions (Figure 1a,b) with an IC_{50} of 2.2×10^{-9} M (95% confidence limits, 9.4×10^{-10}–5.8×10^{-9} M). The time course of a typical inhibitory response in one preparation is shown in Figure 1a. Onset of inhibition was rapid and maximal depression was seen within 3–6 min. Partial recovery of evoked contractions was evident in the continued presence of [Met⁵]enkephalin. Total recovery was quickly seen following washout. [Leu⁵]-enkephalin (1×10^{-10}–3×10^{-8} M) was equipotent with [Met⁵]enkephalin and had a similar time course of action (Figure 1b) ($IC_{50} = 2.1 \times 10^{-9}$ M, 95% confidence limits, 5.9×10^{-10}–7.1×10^{-9} M).

Effects of morphine Morphine (1×10^{-7}–1×10^{-5} M) was a much less potent inhibitor than [Met⁵]enkephalin (Figure 1b). The time course of inhibition to morphine was similar to that of [Met⁵]enkephalin but morphine did not consistently produce 50% inhibition. Therefore, IC_{25} values were calculated ([Met⁵]enkephalin $IC_{25} = 9.4 \times 10^{-10}$ M, 95% confidence limits, 3.6×10^{-10}–2.5×10^{-9} M: morphine $IC_{25} = 3.7 \times 10^{-6}$ M, 95% confidence limits, 1.2×10^{-6}–1.2×10^{-5} M). These show a [Met⁵]enkephalin: morphine potency ratio of approximately 4,000:1 at this level. This large difference in potency is not due to an acquired insensitivity to opioids of the tissues on which morphine was tested, since a high sensitivity to [Met⁵]enkephalin was seen following discontinued exposure to morphine in these tissues.

At 1×10^{-4} M, morphine potentiated neurogenic responses (37 ± 6% increase: n = 6). This developed over 4–6 min and was maintained above control values until washout.

Effects of δ, κ and μ-opioid receptor-selective agonists [D-Pen², D-Pen⁵]enkephalin (3×10^{-10}–1×10^{-8} M), a proposed δ-opioid receptor-selective agonist (Mosberg *et al.*, 1983; Corbett *et al.*, 1984), was almost three times as potent as [Met⁵]enkephalin in inhibiting pelvic nerve-evoked contractions (Figure 1b) (IC_{50}) = 8.3×10^{-10} M, 95% confidence limits, 3.8×10^{-10}–1.8×10^{-9} M). However, U-50,488H (1×10^{-8}–1×10^{-6} M), a proposed κ-opioid receptor-selective agonist (Piercey *et al.*, 1982), was much less potent, inhibiting evoked contractions with a similar potency to morphine (Figure 1b). U-50,488H did not consistently produce 25% inhibition: at 1×10^{-5} M, it potentiated evoked contractions (14 ± 8% increase; n = 4) in a similar manner to morphine. [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (1×10^{-9}–1×10^{-8} M), a proposed μ-opioid receptor-selective agonist (Handa *et al.*, 1981; Cotton *et al.*, 1984; Corbett *et al.*, 1984), had no effect on evoked contractions. Higher concentrations (1×10^{-7} and 1×10^{-6} M) potentiated these contractions (8 ± 1% increase: n = 4 and 12 ± 2% increase: n = 4 respectively). Thus, δ-opioid receptor agonists were potent inhibitors of neurogenic contractions, whereas κ- or μ-opioid agonists were not.

Effect of naloxone on the actions of opioids

Naloxone (1×10^{-7} M) had no effect on colonic spontaneous contractions and basal tone, nor on the

magnitude of pelvic nerve-evoked contractions. However, it did produce a significant ($P < 0.05$) parallel shift to the right of the log concentration-response curve to [Met^5]enkephalin (1×10^{-9}–1×10^{-7} M) (Figure 2a) ($IC_{50} = 1.0 \times 10^{-8}$ M, 95% confidence limits, 5.3×10^{-9}–2.0×10^{-8} M). This gives an approximate K_e value for naloxone of 2.8×10^{-8} M (see Methods). The log concentration-response curve for morphine (1×10^{-6}–1×10^{-4} M) was also shifted to the right by naloxone (1×10^{-7} M) (Figure 2a). However, since 25% inhibition was not always obtained, a potency ratio and approximate K_e value could not be obtained. There was a significant difference between the action of each concentration of morphine tested in the presence and the absence of naloxone (1×10^{-7} M) ($P < 0.05$). Furthermore, the potentiating action of morphine (1×10^{-4} M) was also antagonized. Thus, the inhibitory actions of [Met^5]enkephalin and the inhibitory and excitatory actions of morphine are mediated via opioid receptors.

Effect of ICI 174,864 on the actions of opioids

The possibility that [Met^5]enkephalin and morphine were acting via δ-opioid receptors was studied by use of ICI 174,864, a selective antagonist at this receptor (Cotton *et al.*, 1984; Corbett *et al.*, 1984). ICI 174,864 (1×10^{-7} M) caused no change in the magnitude of pelvic nerve-evoked contractions, but did produce a significant ($P < 0.001$) parallel shift to the right of the log concentration-response curve to [Met^5]enkephalin (3×10^{-9}–3×10^{-7} M) (Figure 2b) ($IC_{50} = 3.2 \times 10^{-8}$ M, 95% confidence limits, 1.5×10^{-8}–6.9×10^{-8} M). This gives an approximate K_e value for ICI 174,864 of 7.4×10^{-9} M. In confirmation, it was found that ICI 174,864 (1×10^{-7} M) could block or reverse the inhibitory action of [D-Pen^2, D-Pen^5]enkephalin (3×10^{-10}, 1×10^{-8} M). In contrast, ICI 174,864 (1×10^{-7} M) had no effect on the inhibitory actions of morphine (1×10^{-7}–1×10^{-5} M) (Figure 2b) ($IC_{25} = 2.7 \times 10^{-6}$ M, 95% confidence limits, 1.5×10^{-6}–4.8×10^{-6} M). Likewise, the potentiating action of morphine (1×10^{-4} M) was unaffected ($39 \pm 4\%$ increase: $n = 4$). These results show that the actions of [Met^5]enkephalin, but not those or morphine, may be mediated via δ-opioid receptors.

Studies on the site of action of [Met^5]enkephalin

Preincubation with [Met^5]enkephalin (3×10^{-9} M), a concentration that inhibited pelvic nerve-evoked contractions by $68 \pm 12\%$ ($n = 6$), for 2 min prior to addition of ACh (1×10^{-9}–1×10^{-4} M) had no effect on ACh-evoked contractions (EC_{50} values = control; 2.2×10^{-7} M, 95% confidence limits, 1.1×10^{-7}–4.2×10^{-7} M, $n = 4$: test; 1.8×10^{-7} M, 95% confidence limits, 1.2×10^{-7}–2.8×10^{-7} M, $n = 4$).

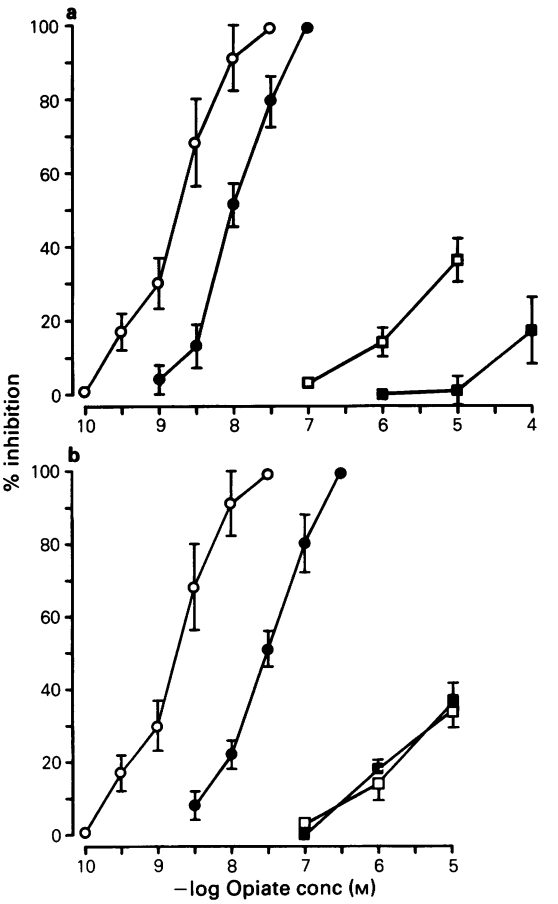

Figure 2 Cat distal colon longitudinal muscle strip *in vitro*: inhibition of pelvic nerve stimulation-induced contractions (2 Hz, 120 mA, 0.5 ms pulse duration, for 10 s, at 1 min intervals) by [Met^5]enkephalin (1×10^{-10}–3×10^{-7} M) (○) and morphine (1×10^{-7}–1×10^{-4} M) (□) (a) in the absence (open symbols) ($n = 6$) and presence (filled symbols) ($n = 4$) of naloxone (1×10^{-7} M), and (b) in the absence (open symbols) ($n = 6$) and presence (filled symbols) ($n = 4$) of ICI 174,864 (10^{-7} M). Vertical lines: s.e.mean.

Similarly, two consecutive log concentration-response curves to ACh over the same time course, both in the absence of [Met^5]enkephalin, showed no change in EC_{50} values. Thus, [Met^5]enkephalin does not inhibit the postjunctional action of ACh, suggested that it may be acting at a prejunctional site to inhibit pelvic nerve-evoked contractions. These contractions were $44 \pm 2\%$ ($n = 30$) at 2 Hz, $54 \pm 6\%$ ($n = 5$) at 4 Hz, and $62 \pm 5\%$ ($n = 5$) at 8 Hz, of the maximum contraction evoked by ACh (1×10^{-4} M). [Met^5]enkephalin (3×10^{-9} and 1×10^{-8} M) was significantly

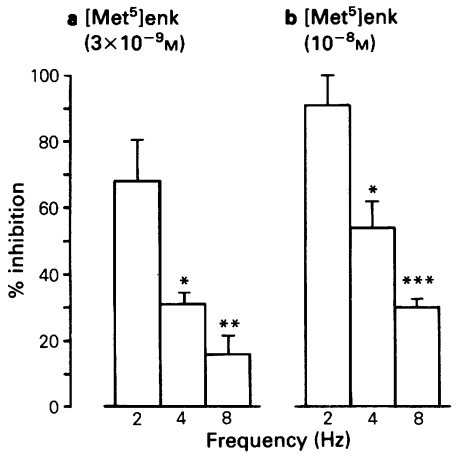

Figure 3 Cat distal colon longitudinal muscle strip *in vitro*: inhibition by [Met⁵]enkephalin ([Met⁵]enk) at (a) 3×10^{-9} M and (b) at 1×10^{-8} M, of pelvic nerve stimulation-induced contractions at 2 Hz ($n = 6$), 4 Hz ($n = 5$) and 8 Hz ($n = 5$) (120 mA, 0.5 ms pulse duration, for 10 s at 1 min intervals). Vertical lines: s.e.mean. Statistical differences are indicated by asterisks ($*P < 0.05$; $**P < 0.01$, $***P < 0.001$).

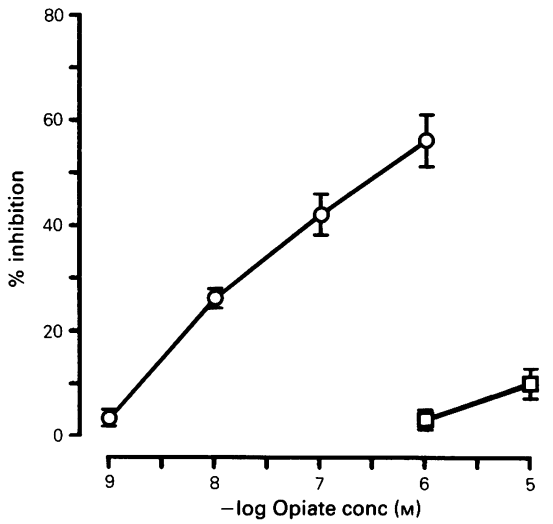

Figure 4 Cat distal colon longitudinal muscle strip *in vitro*: inhibition of contractions evoked by electrical field stimulation (2 Hz, 120 mA, 0.5 ms pulse duration, for 10 s at 1 min intervals) by [Met⁵]enkephalin (1×10^{-9}–1×10^{-6} M) (○) ($n = 6$) and morphine (1×10^{-6}–1×10^{-5} M) (□) ($n = 6$).

more potent in inhibiting contractions evoked at 2 Hz than at 4 and 8 Hz (Figure 3a,b) ($P < 0.05$). Thus, inhibitory potency was inversely related to frequency of stimulation, which is consistent with [Met⁵]enkephalin acting at a prejunctional site to inhibit pelvic nerve-evoked contractions.

Effects of opioids on electrical field stimulation-evoked contractions

Electrical field stimulation (2 Hz, 120 mA, 0.5 ms pulse duration, for 10 s at 1 min intervals) evoked contractions with a similar time course to those evoked by pelvic nerve stimulation but which were slightly larger (53 ± 3% maximum contraction to ACh (1×10^{-4} M): $n = 16$). These contractions were unaffected by hexamethonium (1×10^{-4} M) ($n = 5$) but were rapidly abolished by tetrodotoxin (3×10^{-7} M) ($n = 8$) or atropine (1×10^{-6} M) ($n = 15$), indicating that they were due to stimulation of postganglionic cholinergic neurones.

[Met⁵]enkephalin (1×10^{-9}–1×10^{-6} M) produced a concentration-dependent inhibition of electrical field stimulation-evoked contractions but never abolished them (Figure 4); 50% inhibition was seen at 6.0×10^{-7} M (95% confidence limits, 2.2×10^{-7} M–1.7×10^{-6} M). The time course of inhibition was similar to that for pelvic nerve-evoked contractions (Figure 1a). Higher concentrations of [Met⁵]enkephalin were not used as these appeared to induce prolonged desensitization. [Met⁵]enkephalin was much more potent against pelvic nerve-evoked contractions than against electrical field stimulation-evoked contractions (Figure 4). Since the log concentration-response curves for [Met⁵]enkephalin in Figure 4 are not parallel, a potency ratio was not calculated.

Naloxone (1×10^{-7} M) had no effect on the magnitude of contractions evoked by electrical field stimulation. However, it did produce a parallel shift to the right of the log concentration-response curve to ([Met⁵]enkephalin (3×10^{-9}–1×10^{-6} M). At the 25% inhibition level this represented a 6.2 fold shift ($P < 0.01$), giving an approximate K_e value for naloxone 1.9×10^{-8} M. Thus, the inhibitory effects of [Met⁵]enkephalin are opioid receptor-mediated.

Morphine (1×10^{-6}–1×10^{-5} M) had little inhibitory effect on electrical field stimulation-evoked contractions and was much less potent than against pelvic nerve-evoked contractions (Figure 4a). Morphine (1×10^{-4} M) potentiated electrical field stimulation-evoked responses (22 ± 6% increase: $n = 6$), in a manner similar to that described above for pelvic nerve-evoked responses.

Effect of [Met⁵]enkephalin on spontaneous contractions and basal tone of colonic muscle

Opioids had no effect on basal tone of longitudinal muscle strips which were electrically stimulated ($n = 60$) by either pelvic nerves or by electrical field

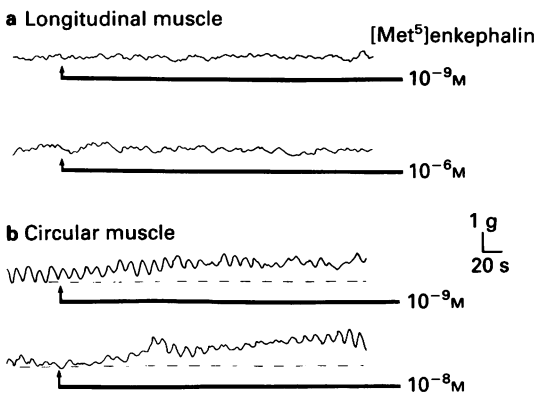

Figure 5 Effect of [Met⁵]enkephalin (1×10^{-9}–1×10^{-6} M) on basal tone and spontaneous contractile activity of (a) longitudinal and (b) circular muscle strips of distal colon *in vitro*, from the same cat. Broken horizontal lines indicate baseline.

stimulation. In addition, [Met⁵]enkephalin (1×10^{-9}–1×10^{-6} M) had no effect on amplitude and frequency of spontaneous contractions, nor did it affect basal tone of longitudinal muscle strips (Figure 5a) ($n = 7$). In contrast, [Met⁵]enkephalin (1×10^{-9}–1×10^{-7} M) increased basal tone and amplitude of spontaneous contractions of circular muscle strips (Figure 5b). Thus [Met⁵]enkephalin contracts circular, but not longitudinal colonic muscle.

Discussion

The results of this study show that [Leu⁵]- and [Met⁵]enkephalin are potent inhibitors of pelvic nerve-evoked contractions of cat distal colon longitudinal muscle *in vitro* and that their effects were mediated by opioid receptors. In contrast, morphine is much less potent. Peripheral opioid receptors have been classified into 3 types, μ, κ and δ, (Martin *et al.*, 1976; Lord *et al.*, 1977). On the basis of this division, several findings in this study indicate that the inhibitory effects of enkephalins are mediated via prejunctional δ-opioid receptors, whereas the inhibitory and excitatory effects of morphine are mediated via μ or κ-opioid receptors or via both subtypes.

First, enkephalins, which are nonselective δ-agonists (Lord *et al.*, 1977), were very potent inhibitors, with an IC_{50} of approximately 2 nM. [D-Pen², D-Pen⁵] enkephalin, a more δ-selective agonist (Mosberg *et al.*, 1983; Corbett *et al.*, 1984; Kosterlitz, 1985), was even more potent. In contrast, morphine, a non-selective μ-receptor agonist (Martin *et al.*, 1976; Lord *et al.*, 1977) and U-50,488H, a selective κ-receptor agonist (Piercey *et al.*, 1982), were both several orders of magnitude less potent. Furthermore, [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin, a μ-selective agonist (Handa *et al.*, 1981; Cotton *et al.*, 1984; Corbett *et al.*, 1984), at all concentrations tested, and morphine and U-50,488H at higher concentrations, caused a small potentiation of neurogenic contractions, rather than inhibition. Thus, only agonists at the δ-opioid receptor were potent inhibitors of pelvic nerve-mediated contractions.

Second, naloxone, an antagonist at each of the three opioid receptor types (Lord *et al.*, 1977), antagonized both [Met⁵]enkephalin and morphine. The K_e value obtained for naloxone against [Met⁵]enkephalin (28 nM) agrees well with those found for naloxone acting at δ-opioid receptors; 21–23 nM (Lord *et al.*, 1977); 23–24 nM (Cotton *et al.*, 1984). K_e values for naloxone acting at μ- and κ-opioid receptors are generally smaller (Lord *et al.*, 1977). A K_e value could not be obtained here for naloxone acting against morphine, though the naloxone-induced shift to the right of the log concentration-response curve for morphine appeared to be greater than that for [Met⁵]enkephalin, suggesting an action at μ- or κ-opioid receptors. Naloxone also antagonized the potentiating action of morphine.

Finally, ICI 174,864, which has a high selectivity for δ-opioid receptors (Cotton *et al.*, 1984; Corbett *et al.*, 1984), antagonized [Met⁵]enkephalin-induced inhibition but had no effect on the actions of morphine. This further indicates that the predominant actions of [Met⁵]enkephalin and morphine were mediated by different receptors. Here, ICI 174,864 caused greater antagonism of [Met⁵]enkephalin than did naloxone. The K_e value for ICI 174,864 (7 nM) was lower than those reported for this compound acting at δ-opioid receptors in the mouse vas deferens *in vitro* (30–31 nM, Cotton *et al.*, 1984; 36 nM, Corbett *et al.*, 1984). The reason for this difference is not known. Thus, the cat distal colon longitudinal muscle-pelvic nerve preparations represents a gastrointestinal tissue where enkephalins act via δ-opioid receptors. This is similar to neurones in guinea-pig ileum submucous plexus (North, 1986) but contrasts with guinea-pig ileum myenteric plexus were both enkephalins and morphine act via μ-opioid receptors (Lord *et al.*, 1977; Corbett *et al.*, 1984).

The results suggest that inhibitory opioid receptors are located prejunctionally, acting to depress evoked ACh release. The inverse relationship between frequency of pelvic nerve stimulation and inhibitory potency of [Met⁵]enkephalin is consistent with such a site and mechanism of action. A postjunctional site of action is unlikely because [Met⁵]enkephalin, at a concentration producing a large inhibition of pelvic nerve-evoked contractions, had no effect on contractions to exogenous ACh. These prejunctional

inhibitory opioid receptors are located at two neuronal sites, namely the myenteric plexus and the extramural colonic ganglia, the latter occurring in variable locations and numbers within colonic branches of the pelvic nerve (Langley & Anderson, 1895; de Groat & Krier, 1976; Krier & Hartman, 1984). Opioid receptors mediating inhibition are present in the myenteric plexus since in the present study, opioids inhibited electrical field stimulation-evoked contractions. Electrophysiological studies indicate that these receptors are also present in colonic ganglia (Kennedy & Krier, 1987a,b).

In this study, naloxone had no effect *per se* on colonic mechanical activity, suggesting that there was no tonic release of enkephalin from myenteric or colonic neurones under these *in vitro* experimental conditions. The possibility cannot be excluded, however, that tonic or intermittent release of enkephalins occurs *in vivo* from sacral preganglionic neurones or colonic myenteric neurones. If enkephalins were released, they might depress cholinergic transmission in efferent sacral parasympathetic pathways which mediate increases in colonic motility during defecation (de Groat & Krier, 1978). In guinea-pig ileum, [Met^5]enkephalin release is intermittent and associated with non-propulsive activity, elevation of intraluminal pressure attenuating this release (Clark & Smith, 1983).

In the present study, [Met^5]enkephalin contracted colonic circular muscle but had no such effect on longitudinal muscle. The evoked contractile activity was similar to that reported in this tissue by Wienbeck & Dunzen (1982), which was naloxone-sensitive but tetrodotoxin- and atropine-resistant. This is consistent with the effects of opioids *in vivo* of increasing intraluminal pressure but decreasing colonic transit (Jaffe & Martin, 1985). Increased intraluminal pressure may be due to contraction of circular muscle whereas decreased colonic transit may be due to prejunctional inhibition of neurogenic contractions.

This work was supported by Research Grant AM 29920 from the National Institutes of Health. J.K. is a recipient of a Research Career Development Award (AM 01273). We thank Dr William H. Percy for advice and criticism, Lynn Sherer for technical assistance and Sharon Shaft for secretarial assistance.

References

ALUMETS, J., HAKANSON, T., SUNDLER, F. & CHANG, K.J. (1978). Leu-enkephalin-like material in nerves and enterochromaffin cells in the gut. *Histochem.*, **56**, 187–196.

BLANQUET, F., BOUVIER, M. & GONELLA, J. (1982). Effects of enkephalins and morphine on spontaneous electrical activity and on junctional potentials elicited by parasympathetic nerve stimulation in cat and rabbit colon. *Br. J. Pharmac.*, **77**, 419–429.

CHERUBINI, E., MORITA, K. & NORTH, R.A. (1985). Opioid inhibition of synaptic transmission in the guinea-pig myenteric plexus. *Br. J. Pharmac.*, **85**, 805–817.

CLARK, S.J. & SMITH, T.W. (1983). The release of met-enkephalin from the guinea-pig ileum at rest and during peristaltic activity. *Life Sci.*, **33** (S1), 465–468.

CORBETT, H.D., GILLAN, M.G.C., KOSTERLITZ, H.W., MCKNIGHT, A.T., PATERSON, S.J. & ROBSON, L.E. (1984). Selectiveness of opioid peptide analogues as agonists and antagonists of the δ-receptor. *Br. J. Pharmac.*, **83**, 271–279.

COTTON, R., GILES, M.G., MILLER, L., SHAW, J.S. & TIMMS, D. (1984). ICI 174864: a highly selective antagonist for the opioid delta-receptor. *Eur. J. Pharmac.*, **97**, 331–332.

DE GROAT, W.C. & KRIER, J. (1976). An electrophysiological study of the sacral parasympathetic pathway to the colon of the cat. *J. Physiol.*, **260**, 425–445.

DE GROAT, W.C. & KRIER, J. (1978). The sacral parasympathetic reflex pathway regulating colon motility and defecation in the cat. *J. Physiol.*, **276**, 481–500.

ELDE, R., HOKFELT, T., JOHANSSON, O. & TERENIUS, L. (1976). Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. *Neurosci.*, **1**, 349–351.

FLEMING, W.W., WESTFALL, D.P., DE LA LANDE, I.S. & JELLETT, L.B. (1972). Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmac. exp. Ther.*, **181**, 339–345.

FURNESS, J.B. & COSTA, M. (1982). Identification of gastrointestinal neurotransmitters. In *Handbook of Experimental Pharmacology: 591 Mediators and Drugs in Gastrointestinal Motility*. ed. Bettaccini, G. pp. 583–662. Berlin, Heidelberg, New York: Springer-Verlag.

GLAZER, E. & BASBAUM, A.I. (1980). Leucine-enkephalin: localization in and axoplasmic transport by sacral parasympathetic preganglionic neurons. *Science*, **208**, 1479–1481.

HANDA, B.K., LANE, A.C., LORD, J.A.H., MORGAN, B.A., RANCE, M.J. & SMITH, C.F.C. (1981). Analogues of $-LPH_{61-64}$ possessing selective agonist activity at mu-opiate receptors. *Eur. J. Pharmac.*, **70**, 531–540.

JAFFE, J.H. & MARTIN, W.R. (1985). Opioid analgesics and antagonists. In *The Pharmacological Basis of Therapeutics*. ed. Gilman, A.G., Goodman, L.S., Rall, T.W. & Murad, F. pp. 491–531. New York, Toronto, London: Macmillan Press.

KENNEDY, C. & KRIER, J. (1986). Methionine-enkephalin acts via opiate delta receptors to potently inhibit pelvic-nerved-evoked contractions of cat distal colon. *Fed Proc.*, **45**, 756.

KENNEDY, C. & KRIER, J. (1987). Delta-opioid receptors mediate inhibition of fast excitatory postsynaptic potentials (e.p.s.ps) in cat parasympathetic colonic ganglia. *Fen. Proc.*, **46**, 1064.

KENNEDY, C. & KRIER, J. (1987b). Delta-opioid receptors mediate inhibition of fast excitatory postsynaptic potentials in cat parasympathetic colonic ganglia. *Br. J.*

Pharmac., (in press).

KOSTERLITZ, H.W. (1985). Opioid peptides and their receptors. *Proc. R. Soc. L.B.*, **225**, 27–40.

KOSTERLITZ, H.W. & WATT, A.J. (1968). Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allyl-noroxymorphone (naloxone). *Br. J. Pharmac.*, **33**, 266–276.

KRIER, J. & HARTMAN, D.A. (1984). Electrical properties and synaptic connections to neurons in parasympathetic colonic ganglia of the cat. *Am. J. Physiol.*, **247**, G52–G61.

LANGLEY, J.N. & ANDERSON, H.K. (1895). On the innervation of the pelvic and adjoining viscera. Part I; The lower portion of the intestine. *J. Physiol.*, 18, **67–105.**

LARSSON, L.I. & STENGAARD-PEDERSEN, K. (1982). Immunocytochemical and ultra-structural differentiation between met-enkephalin, and leu-enkephalin-, and met/leu-enkephalin-immunoreactive neurones of feline gut. *J. Neurosci.*, **2**, 861–878.

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITIZ, H.W. (1977). Endogenous opioid peptides: multiple agonists and receptors. *Nature*, **267**, 495–499.

MARTIN, W.R., EADES, C.G., THOMPSON, J.A., HUPPLER, R.E. & GILBERT, P.E. (1976). The effects of morphine- and nalorphine-like drugs in the non-dependent and morphine-dependent chronic spinal dog. *J. Pharmac. exp. Ther.*, **197**, 517–532.

MOSBERG, H.I., HURST, R. HRUBY, V.J., GEE, K., YAMAMURA, H.I., GALLIGAN, J.J. & BURKS, T.F. (1983). Bis-penicillamine enkephalins possess highly improved specificity towards delta opioid receptors. *Proc. natn. Acad. Sci. U.S.A.*, **80**, 5871–5874.

NORTH, R.A. (1986). Opioid receptor types and membrane ion channels. *Trends Neurosci.*, **9**, 114–17.

PIERCEY, M.F., LAHTI, R.A., SCHROEDER, L.A., EINSPAHR, F.J. & BARSUHN, C. (1982). U-50,488H, a pure Kappa receptor agonist with spinal analgesic loci in the mouse. *Life Sci.*, **31**, 1197–1200.

POLAK, J.M., SULLIVAN, S.N., BLOOM, S.R., FACER, P. & PEARSE, A.G.E. (1977). Enkephalin-like immunoreactivity in the human gastrointestinal tract. *Lancet*, **i**, 972–976.

SHULZ, R., WUSTER, M., SIMANTOL, R., SNYDER, S. & HERZ, A. (1977). Electrically stimulated release of opiate-like material from the myenteric plexus of the guinea-pig ileum. *Eur. J. Pharmac.*, **41**, 367–368.

WATERFIELD, A.A., SMOKCUM, R.W.J., HUGHES, J., KOSTERLITIZ, H.W. & HENDERSON, G. (1977). *In vitro* pharmacology of the opioid peptides, enkephalins and endorphins. *Eur. J. Pharmac.*, 43, 107–116.

WIENBECK, M. & DUNZEN, R.G. (1982). The effects of leucine-enkephalin on the spontaneous motility of the circular muscle of the cat colon. *Gastroenterologie*, **20**, 429–437.

(Received December 12, 1986.
Revised May 5, 1987.
Accepted June 17, 1987.)