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EFFECTS OF ENDORPHINS ON DIFFERENT PARTS OF THE GASTRO-INTESTINAL TRACT OF RAT AND GUINEA-PIG *in vitro*

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1 The spasmogenic and spasmolytic effects of β-lipotropin (LPH) fragments and one analogue were investigated on different parts of the gastro-intestinal tract of guinea-pig and rat *in vitro*.

2 Changes in muscle tone were observed in colon and rectum and to a lesser extent in jejunum and ileum of both species. Rat colon and rectum contracted to the peptides. Guinea-pig colon and rectum relaxed after an initial short-lasting contraction.

3 On the rat rectum (D-ala^2)met-enkephalin, leu-enkephalin, γ-endorphin, α-endorphin and β-LPH 80–91 caused dose-dependent contractions, their ED_{50} values being 0.96×10^{-12} mol, 1.05×10^{-11} mol, 1.22×10^{-11} mol, 1.08×10^{-10} mol, 2.65×10^{-10} mol and 6.5×10^{-9} mol, respectively.

4 Naloxone dose-dependently shifted the dose-response curve of met-enkephalin to the right. Atropine, hexamethonium, burimamide, mepyramine, propranolol and indomethacin did not influence the response to met-enkephalin.

5 In the presence of tetrodotoxin, the ED_{50} for met-enkephalin and the maximal contractor response induced by met-enkephalin, appeared to be increased.

6 The 5-hydroxytryptamine (5-HT) antagonists, methysergide and cyproheptadine, reduced the contractor response in a non-competitive manner. The α-adrenoceptor antagonist phentolamine, in contrast, caused an increase of the maximal response to met-enkephalin of up to 200%. Noradrenergic and tryptaminergic systems, therefore, might be involved in the changes in muscle tone induced by met-enkephalin.

7 These results demonstrate that rectum and colon of guinea-pig and rat are very sensitive to opioid-like peptides.

Introduction

Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris (1975) demonstrated the existence of opiate-like peptides (methionine-enkephalin and leucine-enkephalin) in brain tissue which mimic the influence of morphine on smooth muscle; these effects of the peptides are blocked by opiate antagonists. Recently, the presence of opiate ligands was also shown in several parts of the gastro-intestinal tract of different species (Hughes, Kosterlitz & Smith, 1977). The enkephalins as well as morphine inhibit the electrically-induced contractions of the guinea-pig ileum and the mouse vas deferens (Henderson, Hughes & Kosterlitz, 1972; Hughes *et al.*, 1975). This inhibition has been shown to be a reliable index of the relative potency of various narcotics with respect to analgesia (see review by Kosterlitz & Waterfield, 1975). However, morphine exerts effects widely in the peripheral nervous system. For example, it increases tone and motor activity of the intestine in most mammalian species (Weinstock, 1971). We, therefore, investigated the direct effects of enkephalins and other endorphins on smooth muscle tone of different isolated parts of the gastro-intestinal tract of rat and guinea-pig.

Methods

Experimental designs

Male Wistar rats (CPB, TNO Zeist) weighing between 200 and 300 g and male guinea-pigs (CPB, TNO Zeist) weighing between 250 and 400 g were used for all experiments. Animals were stunned and bled. Segments of gastro-intestinal tract were removed and superfused in a cascade of 4 tissues (Vane, 1964) with Krebs bicarbonate solution at 37°C of the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2 2H_2O$ 2.5, $MgSO_4 7H_2O$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 5.0, gassed with 5% CO_2 and 95% O_2. The flow rate of the Krebs solution was 5 ml/min.

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Changes in the tone of the intestinal segments were recorded with auxotonic levers by means of Harvard transducers (type 368) connected to a Watanabe pen recorder. The initial load on the tissues was 2 g. After being set up, the tissues were allowed to stabilize for 45 min before the experiment was started. Drugs were applied to the tissues, dissolved in a maximum volume of 30 μl of 0.9% w/v NaCl solution (saline). Equal volumes of saline alone were used as control. In each experiment a dose-response curve for methionine-enkephalin (met-enkephalin) was constructed to serve as control. The contractor responses to the other peptides are always expressed as percentages of the met-enkephalin control response. Additions of drugs were made at intervals of 2 to 3 min. Infusions of solutions of antagonists over the tissues were given at a rate of 0.1 ml/min. Dose-response curves in the presence of the antagonists were performed 30 min after starting infusions. Concentrations of the antagonists used (see below) rendered the tissues insensitive to the corresponding agonists (Gilmore, Vane & Wyllie, 1968; Vane, 1971; Eckenfels & Vane, 1972; Gillan & Pollock, 1976; Bérubé, Marceau, Drouin, Rioux & Regoli, 1978).

Drugs

The peptides used were leucine-enkephalin (leu-enkephalin), methione-enkephalin (met-enkephalin; LPH 61–65), (D-ala^2)met-enkephalin, α-endorphin (LPH 61–76), γ-endorphin (LPH 61–77) and LPH 80–91. The LPH fragments and analogue were synthesized and kindly donated by the Scientific Development Group of Organon International B.V., Oss, The Netherlands (Dr H.M. Greven). The highly purified synthetic peptides were stored as the dry form in bottles under dry conditions at room temperature. Solutions of the peptides in saline were prepared each day and kept on ice. The amount of the added endorphins is given as mol, except for the first experiment. The concentration of infused antagonists is given as mol/l (M).

Other drugs used were: phentolamine methane sulphonate (Regitine, CIBA-Geigy), propranolol hydrochloride (ICI), atropine (OPG), methysergide hydrogen-maleinate (Sandoz, AG), cyproheptadine hydrochloride (Merck, Sharp and Dohme), mepyramine chloride (Specia), burimamide (gift from Dr J. Black), indomethacin (gift from Merck, Sharp and Dohme), hexamethonium chloride (OPG), naloxone hydrochloride (generously supplied by Endo Laboratories, Inc.), tetrodotoxin (Calbiochem), acetylcholine chloride (OPG), histamine acid phosphate (BDH-Chemicals), levarterenol bitartrate (noradrenaline) (OPG), isoprenaline sulphate (OPG), 5-hydroxytryptamine creatinine sulphate (Fluka, AG). All drugs were obtained as pure powders except for phentolamine which was obtained as a solution in ampoules.

Statistical analysis

Calculations of the mean effective dose (ED_{50}; i.e. the dose required to produce a contractor response equal to 50% of the maximum response induced by the peptides) and their 95% confidence limits were calculated using linear regression according to the method of least squares. The ED_{50} of met-enkephalin after various pretreatments was considered significantly different from the control ED_{50} of met-enkephalin if it lay outside the 95% confidence limits of the latter and also the control ED_{50} lay outside the 95% confidence limits of the experimental ED_{50}. Parallel line assays were carried out according to de Jonge (1971).

Maximal contractions induced by the peptides are expressed as the mean ± standard error of the mean (s.e. mean). Student's *t* test was used to determine the significance of the difference between the different groups. Results are regarded as significant when $P < 0.05$. pA_2 values were calculated according to the method of Arunlakshana & Schild (1959). Antagonism was regarded as competitive when the slope of the Schild plot was unity.

Results

Effects on different parts of the gastro-intestinal tract

In order to investigate the effects of various endorphins on the muscle tone of different parts of the gastro-intestinal tract of guinea-pig and rat, 10 ng and 1 μg (D-ala^2) met-enkephalin, met-enkephalin, leu-enkephalin, γ-endorphin, α-endorphin and LPH 80–91 were added to the superfusion fluid of the isolated stomach, duodenum, jejunum, ileum, colon and rectum of both species. No changes in basal tone were detectable in rat and guinea-pig stomach; however, from duodenum to rectum changes in tone were observed (Table 1). Intestinal tissue from the rat contracted to the endorphins, while relaxations were induced in the isolated tissues from the guinea-pig. In guinea-pig colon and rectum relaxation was preceded by an initial short-lasting contraction (Figure 1). Interestingly, contractor and relaxant properties increased from duodenum to rectum. In fact, profound activity was observed in rectum and colon of both species. Under the present conditions only these tissues showed spontaneous activity. Of the endorphins tested, (D-ala^2) met-enkephalin, met-enkephalin and leu-enkephalin appeared to be most potent. For further experiments, the rat rectum was selected because of the amplitude of the response to enkephalins.

Table 1 Effect of different sequences of β-lipotropin (LPH) and (D-ala^2) met-enkephalin on the muscle tone of different parts of the gastro-intestinal tract of rat and guinea-pig *in vitro*

		Stomach		*Duodenum*		*Jejunum*		*Ileum*		*Colon*		*Rectum*	
Drug	*Dose*	*rat*	*guinea-pig*	*rat*	*guinea-pig*	*rat*	*guinea-pig*	*rat*	*guinea-pig*	*rat*	*guinea-pig*	*rat*	*guinea-pig*
(D-Ala^2)met-	10 ng	0	0	+	−	++	−	++	−	+++	+++	+++++++	++
enkephalin	1 μg	0	0	++	−	++	−	+++	−−	++++	−−−	+++++++++	−−−−−
Met-enkephalin	10 ng	0	0	+	−	+	−	+	−	+++	+−−	+++	−−
	1 μg	0	0	+	−	++	−	++	−	++++	++−−−	+++++++++	+−−−
Leu-enkephalin	10 ng	0	0	0	−	+	0	0	−	++	−	+++	−−
	1 μg	0	0	+	−	++	−	+++	−	++++	++−−−−	+++++++	+−−−
α-Endorphin	10 ng	0	0	0	0	0	0	0	0	+	0	+	0
	1 μg	0	0	0	−	+	−	++	−	++	++−	++	++−−
γ-Endorphin	10 ng	0	0	0	0	+	0	0	0	+	0	+	0
	1 μg	0	0	+	−	++	−	+	−	+++	++	++	++−
LPH 80–91	10 ng	0	0	0	0	0	0	0	0	0	0	0	0
	1 μg	0	0	0	0	0	0	0	0	++	++	++	++−

Amplification of the response was identical in all experiments (50 mV/cm).
Symbols: + contraction up to 10 mm; ++ contraction up to 20 mm etc.; − relaxation up to 10 mm; −− relaxation up to 20 mm etc.: +− contraction followed by relaxation; −+ relaxation followed by contraction; 0 no change in muscle tone.

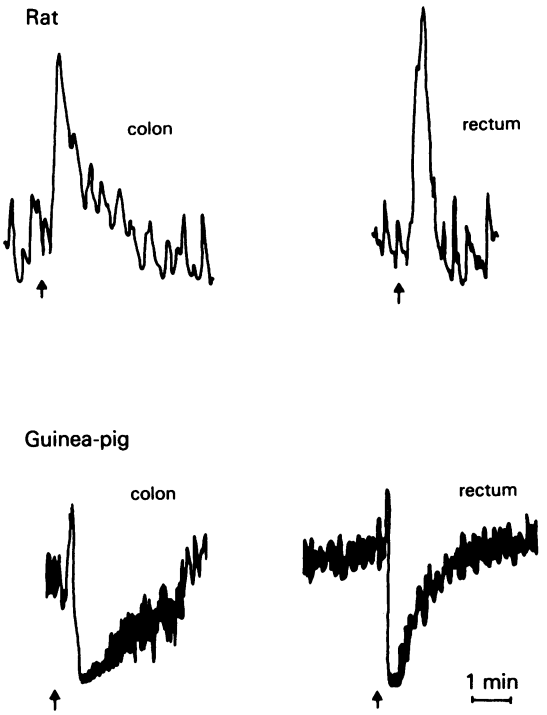

Figure 1 Recording of responses to 1 μg met-enkephalin on rat colon and rectum and guinea-pig colon and rectum *in vitro*.

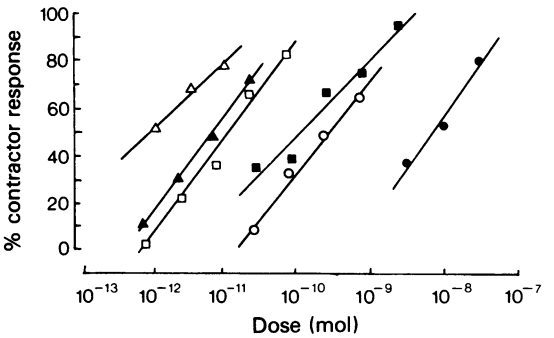

Figure 2 Log concentration-response relation of LPH fragments and analogue on the contractor response of the rat rectum *in vitro*. Contractor responses are expressed as percentage of the met-enkephalin control response. (△) (D-ala^2)met-enkephalin; (▲) met-enkephalin; (□) leu-enkephalin; (■) γ-endorphin; (○) α-endorphin; (●) LPH 80-91. Data shown are means of dose-response curves on 5 to 8 different tissues.

Dose-response effects on the rat rectum

Dose-response curves were obtained for (D-ala^2)met-enkephalin, met-enkephalin, leu-enkephalin, γ-endorphin α-endorphin and LPH 80-91. Met-enkephalin was used as an internal standard for each of the LPH fragments. Regression lines were calculated for the straight part of the curves and accordingly compared to those calculated for met-enkephalin (Figure 2, Table 2). Regression lines of the different endorphins appeared to be parallel to that of met-enkephalin (Figure 2). Maximal contractions of the various entities were not different from those obtained with met-enkephalin. These contractor responses do not reflect the maximal contractor potency of the tissue since we found that acetylcholine can induce much stronger contractions. Mol for mol, (D-ala^2) met-enkephalin was the most potent endorphin. This peptide was approximately 11 times more potent than met-enkephalin. Chain elongation of LPH 61-65 (met-enkephalin) up to 76 or 77 caused a profound decrease in potency. The terminal part of the LPH molecule (LPH 80–91) showed only minor activity being approximately 1300 times less potent than met-enkephalin.

Table 2 Relative agonist potencies of different sequences of β-lipotropin (LPH) and (D-ala^2)met-enkephalin on the rat rectum *in vitro* as compared to met-enkephalin

Drug	n	ED_{50} (*95% confidence limits*) (*mol* × 10^{-11})	ED_{50} *ratio*	% *maximum contraction met-enkephalin*
Met-enkephalin	7	1.05 (0.76–1.40)		97 ± 5
(D-Ala^2)met-enkephalin		0.096 (0.038–0.172)	10.94	83 ± 6
Met-enkephalin	8	0.74 (0.50–1.18)		100 ± 0
Leu-enkephalin		1.22 (0.90–1.79)	0.61	114 ± 12
Met-enkephalin	8	0.82 (0.47–1.33)		99 ± 2
γ-Endorphin		10.8 (4.80–19.00)	0.076	110 ± 7
Met-enkephalin	8	1.45 (0.78–2.30)		96 ± 1
α-Endorphin		26.50 (18.50–44.00)	0.055	77 ± 13
Met-enkephalin	8	0.050 (0.26–0.95)		91 ± 5
LPH 80–91	5	650 (142–5800)	0.0008	82 ± 11

Antagonism by naloxone

The opiate antagonist, naloxone, caused a shift of the dose-response curve of met-enkephalin to the right. ED_{50} values for met-enkephalin, in the presence of various doses of naloxone were calculated from the regression lines (Table 3). From these values the ratio between equiactive doses of met-enkephalin without and in the presence of increasing concentrations of naloxone could be calculated and, accordingly, log (dose ratio −1) was plotted against the negative logarithm of mol/l of naloxone. The slope of this plot (−1.05; see Table 3 and Fig. 3) did not deviate significantly from the theoretical slope of −1 for competitive antagonism. The pA_2 value is represented by the point of intersection of the regression line and abscissa and was found to be 7.34 with confidence limits from 7.24 to 7.50. Interestingly, the infusion of naloxone itself caused a slight increase in spontaneous activity of the rectum when doses of 10^{-6} M or higher were used.

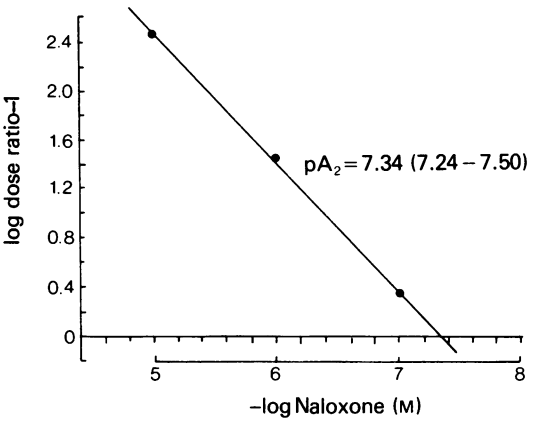

Figure 3 pA_2 estimation for met-enkephalin-naloxone on the rat rectum *in vitro*. Ordinate scale: log (dose ratio −1); Abscissa scale: −log naloxone (M).

Effect of non-opiate receptor antagonists

Subsequently, we tested the effect of several non-opiate receptor inhibitors on the response to met-enkephalin. Muscarinic receptor blockade by atropine (2×10^{-6} M), nicotinic receptor blockade by hexamethonium (2×10^{-6} M), β-adrenoceptor blockade by propranolol (7.7×10^{-6} M) or prostaglandin synthesis inhibition by indomethacin (5.6×10^{-6} M) did not substantially affect the dose-response curve to met-enkephalin (Table 4). A higher concentration of atropine (10^{-5} M) also had no significant effect (ED_{50} ratio met-enkephalin/met-enkephalin + atropine: 0.67; $n = 4$). In the presence of mepyramine, responses to low doses of met-enkephalin were enhanced as shown by the reduced ED_{50} value for met-enkephalin, but the maximum response was unaltered. Profound effects were observed with the α-adrenoceptor blocking agent, phentolamine (Figure 4a). In the presence of phentolamine (2×10^{-6} M) the maximal response to met-enkephalin was doubled. In contrast, the 5-HT antagonists, methysergide and cyproheptadine, reduced the contractor response to met-enkephalin in a dose-dependent manner (Figure 4b and 4c). In addition, phentolamine induced an increase of spontaneous activity of the rectum while the 5-HT antagonists inhibited this activity.

In one other experiment the response to met-enkephalin in the presence of tetrodotoxin (2×10^{-7} and 2×10^{-6} M) was investigated. It appeared that the tissue became more sensitive to met-enkephalin. The ED_{50} value for met-enkephalin was markedly increased in the presence of tetrodotoxin (Table 4). In addition, the maximal contractor response to met-enkephalin was enhanced. Tetrodotoxin induced a small increase of spontaneous activity of the rectum. In order to investigate whether or not the doses of the various non-opiate antagonists that were used were

Table 3 The contractor response to met-enkephalin on the rat rectum *in vitro* in the presence of various concentrations of naloxone

Concentration of naloxone hydrochloride (M)	n	*ED_{50} (95% confidence limits) for met-enkephalin* (mol × 10^{-11})	*Dose-ratio*
None	4	1.36 (1.00–1.90)	
1×10^{-7}		4.50 (2.45–8.75)	3.31
None	7	0.63 (0.38–1.00)	
1×10^{-6}		19.2 (12.0–3.00)	30.47
None	6	1.80 (0.70–4.70)	
1×10^{-5}		525 (130–7000)	291.67

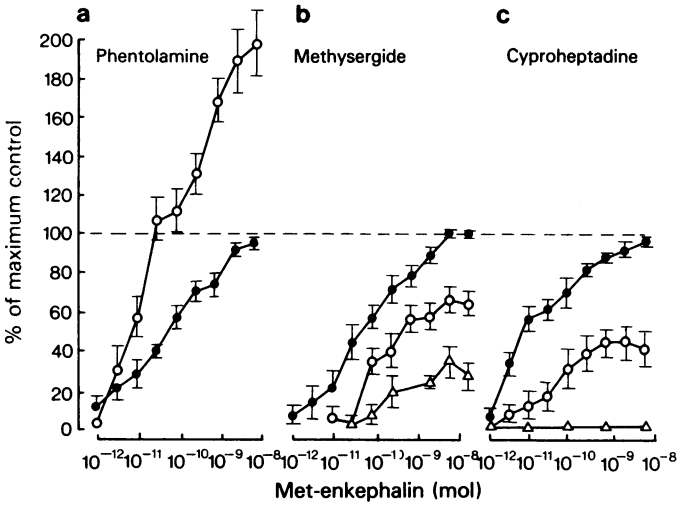

Figure 4 Effects of phentolamine (a), methysergide (b) and cyproheptadine (c) on the contractor response to met-enkephalin of the rat rectum *in vitro*: (●) met-enkephalin; (○) met-enkephalin plus inhibitor (2×10^{-6} M); (△) met-enkephalin plus inhibitor (2×10^{-5} M). Data shown are means of dose-response curves on 4 to 8 different tissues.

able to inhibit the action of the respective agonist, a series of experiments was performed. 5-HT, histamine and acetylcholine caused a dose-dependent contraction of the rat rectum; only acetylcholine-induced contractions exceeded those induced by met-enkephalin. Noradrenaline induced a dose-dependent relaxation and isoprenaline a very small relaxation which was not dose-dependent.

5-HT, histamine and acetylcholine were tested in the absence and in the presence of the various antagonists (same concentrations as used before; see Table 4 and Figure 4). The agonists were added to the rectum till a contraction was observed which was of similar magnitude to that obtained with the ED_{50} dose of met-enkephalin.

It appeared that in the presence of both methyser-

Table 4 The contractor response to met-enkephalin on the rat rectum *in vitro* in the presence of various drugs

Treatment (M)	n	*ED_{50} (95% confidence limits) for met-enkephalin* (mol × 10^{-11})	*% maximum contraction*
None	8	1.25 (0.66–1.95)	95 ± 2
Atropine (2×10^{-6})		3.00 (1.95–5.80)	106 ± 14
None	7	1.50 (1.18–2.10)	100 ± 0
Hexamethonium (2×10^{-6})		1.30 (0.98–1.78)	88 ± 10
None	8	0.67 (0.50–0.85)	95 ± 2
Propranolol (7.7×10^{-6})		0.60 (0.38–0.78)	99 ± 10
None	4	0.84 (0.45–1.53)	93 ± 4
Indomethacin (5.6×10^{-6})		0.92 (0.21–2.18)	95 ± 11
None	7	0.63 (0.47–0.94)	97 ± 3
Mepyramine (2×10^{-6})		0.23* (0.13–0.39)	119 ± 10†
None	6	1.25 (0.98–1.75)	90 ± 4
Burimamide (2×10^{-6})		2.30 (1.60–3.90)	113 ± 11†
None	5	0.87 (0.29–1.20)	94 ± 2
Tetrodotoxin (2×10^{-6})		4.9* (3.1–7.4)	140 ± 15*
Tetrodotoxin (2×10^{-7})		16.1* (5.3–31.1)	152 ± 22*

* Different from control value ($P < 0.05$); † $P < 0.1$.

gide and cyproheptadine more than 1000 times the concentration of 5-HT was needed to obtain a similar response to that obtained in the absence of these antagonists. The corresponding values were 10 in the case of histamine and mepyramine and more than 1000 in the case of acetylcholine and atropine. Noradrenaline was tested in the absence and the presence of phentolamine. To obtain a relaxation that was 50% of the maximal response, 3 times more noradrenaline was needed in the presence of phentolamine.

Discussion

The present results demonstrate that longitudinal smooth muscle of the gastro-intestinal tract of guinea-pig and rat, in particular rectum and colon, are sensitive to opioid-like peptides. The effects may be mediated by opioid-like receptors since naloxone competitively antagonized the response to met-enkephalin. No relationship is present between the observed activity and the endogenous content of opioids in the different parts of the gastro-intestinal tract since, according to Hughes *et al.* (1977), guinea-pig duodenum contains 4 to 5 times more endorphin-like material than colon, while guinea-pig colon was much more sensitive to endorphin than the duodenum.

The opposite responses of the endorphins in the gastro-intestinal tract of guinea-pig and rat may be explained by activation of different types of receptors (Lord, Waterfield, Hughes & Kosterlitz, 1977).

In the rat rectum (D-ala^2)met-enkephalin proved to be the most potent peptide. The observed eleven fold greater potency of this peptide as compared to met-enkephalin may be caused by higher metabolic stability of (D-ala^2)met-enkephalin (Pert, Bowie, Fong & Chang, 1976) as is also assumed for the differences in potency of these peptides with respect to analgesia (Walker, Berntson & Sandman, 1977). Compared to the inhibitory effects of the endorphins in the electrically stimulated guinea-pig ileum, slightly different ratios were found in our test system. (D-Ala^2)met-enkephalin, leu-enkephalin and LPH 61-76 have an equipotent molar ratio to met-enkephalin of respectively 6, 0.33 and 0.44 in inhibiting the electrically stimulated guinea-pig ileum (Waterfield, Smokcum, Hughes, Kosterlitz & Henderson, 1977), while in our system, the respective potencies were 10.9, 0.61 and 0.055.

The value found by us for inhibition of met-enkephalin-induced contractility by naloxone in the rat rectum (7.34) substantially agrees with pA_2 values of 7.27, found for *in vivo* analgesic tests in mice in which morphine was used as agonist (Hayashi & Takemori, 1971), and of 7.14 for *in vivo* inhibition of intestinal motility in rat (Parolaro, Sala & Gori, 1977). Activation of a similar kind of receptor population under these different conditions, therefore, seems likely.

In contrast to the electrically stimulated guinea-pig ileum, the effects of the opioid-like peptides in the rat rectum may not be mediated by acetylcholine since atropine does not inhibit the response to met-enkephalin. A role for 5-HT seems possible since methysergide and cyproheptadine inhibit the contractor response. Although cyproheptadine also has significant anti-histaminic properties (Stone, Wenger, Ludden, Stavorski & Ross, 1961; Trottier & Malone, 1969), this effect does not seem to be responsible since histamine H_1- and H_2-receptor antagonists did not prevent the met-enkephalin response. The observed potentiating effect of α-adrenoceptor blockade points to an inhibitory role for noradrenaline in the met-enkephalin contractor response, since noradrenaline relaxes rat intestinal tissue. The increase in spontaneous activity after even partial α-adrenoceptor blockade, on the other hand, may have caused a facilitation of the contractor response to met-enkephalin.

The effects of met-enkephalin on rat rectum are very similar to those described for morphine on rat colon and rat and dog intestine (Burks, 1973; 1976; Gillan & Pollock, 1976). It was suggested that in dog intestine morphine effects are mediated predominantly by local release of 5-HT. However, in rat colon a different mode of action of morphine was suggested. Our data also do not point to a direct tryptaminergic mediation of the met-enkephalin response since 5-HT antagonists inhibit the effect in a non-competitive manner.

Met-enkephalin could exert its effect on isolated intestinal smooth muscle by a direct action on the smooth muscle fibre (myogenic action) and/or an action on the intramural nerve plexus (neurogenic action). After tetrodotoxin, a specific inhibitor of axonal conduction of nerve action potentials (Kao, 1966), the maximal response to met-enkephalin is potentiated. Removal of the tonic inhibitory nervous influence by tetrodotoxin together with a direct myogenic action of met-enkephalin may explain these phenomena. Noradrenaline may be a likely candidate to mediate this tonic inhibitory influence since after both tetrodotoxin and phentolamine the met-enkephalin response is potentiated and spontaneous activity of the rectum is increased. However, since tetrodotoxin also shifted the met-enkephalin dose-response curve to the right a neurogenic effect of met-enkephalin cannot as yet be excluded.

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