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CONTRACTILE EFFECT OF MORPHINE AND RELATED OPIOID ALKALOIDS, β-ENDORPHIN AND METHIONINE ENKEPHALIN ON THE ISOLATED COLON FROM LONG EVANS RATS

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1 Morphine and related synthetic surrogates as well as β-endorphin and methionine enkephalin caused a contractile response of the longitudinal musculature of the terminal colon of Long Evans rats.

2 The muscular contraction caused by the narcotic analgesics exhibited stereospecificity, with levorphanol being about 50 times more potent than dextrorphan and (−)-methadone 4 times more potent than (+)-methadone. In addition, the rank order in potency of a homologous series of N-alkyl substituted norketobemidones demonstrated that the activity of these compounds in eliciting contractile responses corresponded to that for analgesic efficacy in the rat and also correlated to the ability of these derivatives to inhibit the muscular twitch evoked by electrical stimulation of the guinea-pig ileum.

3 Naloxone blocked the contractile response of the opiates following competitive kinetics; the naloxone pA_2 values for morphine, etorphine, levorphanol and methadone were very close, in spite of the marked differences in potency of these agents.

4 The contractile effect of morphine on the rat colon was abolished by incubation of the tissues with tetrodotoxin 2.0×10^{-7} M or by decreasing the external Ca^{2+} level 100 fold. Increasing the external Ca^{2+} concentration caused an apparent non-competitive antagonism of the response to morphine.

5 Pretreatment of the tissues with hexamethonium 8.3×10^{-5} M caused a modest antagonism of the morphine effect while atropine 5.8×10^{-7} M did not significantly modify the morphine contractile effect. In contrast, methysergide 10^{-5} M caused a 10 fold increase in the morphine EC_{50}.

6 Colons from rats rendered tolerant-dependent on morphine were markedly less sensitive to the contractile effects of morphine than those from placebo-treated controls. Tolerance to morphine was also accompanied by an increased sensitivity to the contractile effects of 5-hydroxytryptamine (5-HT).

7 A marked increase in the spontaneous muscular activity of segments of the terminal colon of rats chronically treated with morphine was found to occur upon removal of the residual morphine in the tissues by repetitive washings. The spontaneous activity was arrested by applications of morphine, suggesting that physical dependence can be demonstrated *in vitro* in this particular preparation.

8 It is concluded that the opiate-induced contractile response is mediated via stereospecific, naloxone-sensitive, opiate receptors and that the muscular response involves the activation of a 5-HT neurone in the nerve terminals of the colon.

Introduction

Morphine produces profound effects on gastrointestinal motility that include changes in peristaltic activity, intraluminal pressure and sphincter tone in a variety of animal species. Evidence has accumulated recently to indicate that the effects of opiates on the gastrointestinal tract are complex and apparently involve both a local and a central site of action. Morphine and its surrogates, including the opioid-peptides, depress the muscular contraction of the coaxially stimulated ileum of the guinea-pig (Paton, 1957; Kosterlitz & Watt, 1968; Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975; Waterfield, Smockum, Hughes, Kosterlitz & Henderson, 1977; Huidobro-Toro, Foree & Way, 1978). In the duodenum and large intestine of man and a variety of experimental animals, morphine decreases the propulsive peristaltic waves, increases the intraluminal pressure and exerts a spasmogenic effect on the internal and external anal sphinters (Plant & Miller, 1926; Forster, 1938; Kaymacalan & Temelli, 1964;

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Burks & Long, 1967; Pruitt, Grubb, Jaquette & Burks, 1974; Grubb & Burks, 1975; Huidobro-Toro & Way, 1976; Gillan & Pollock, 1976; 1980; Huidobro-Toro, Harris, Loh & Way, 1978). Recently, Parolaro, Sala & Gori (1977), Stewart, Weisbrodt & Burks (1977; 1978) and Burks (1980), demonstrated that the intracerebroventricular injection of nanomoles of morphine and related opiates including the endogenous opioid-like peptides to rats, delayed in a dose-dependent fashion the intestinal transit. Both the peripheral and central effects of the opiates on intestinal motility are apparently mediated via opiate receptors, since the actions of the narcotic analgesic drugs on intestinal motility and peristalsis are blocked by naloxone. In addition, after chronic opiate exposure tolerance develops to these morphine effects on intestinal motility (Fenessey, Heimans & Rand, 1969; Pruitt *et al.*, 1974; Huidobro-Toro & Way, 1976; Weisbrodt, Badial-Aceves, Dubrick, Burks & Castro, 1977; Parolaro *et al.*, 1977; Manara, Bianchi, Ferretti, Monferini, Strada & Tavani, 1980), further reinforcing the notion that the gastrointestinal effects of narcotic analgesic drugs are mediated via true opiate receptors.

It is apparent that in the gastrointestinal tract the opiate receptors are localized in the neuronal elements of the enteric nervous system (Pert & Snyder, 1973; Creese & Snyder, 1975). Recent studies using immunofluorescent techniques and tissue extraction procedures have established conclusively the existence of enkephalins within the intrinsic innervation of the intestines of man and a variety of experimental animals (Elde, Hokfelt, Johansson & Terenius, 1976; Polak, Bloom & Sullivan, 1977; Hughes, Kosterlitz & Smith, 1977; Furness & Costa, 1980; Furness, Costa, Franco & Llewellyn-Smith, 1980; Schultzberg, Hokfelt, Nilsson, Terenius, Rehfeld, Brown, Elde, Goldstein & Said, 1980). It is most likely that the local effects of morphine on intestinal motility are mediated via the activation of the endogenous opioid receptors contained in the nervous plexus of the intestinal tract. Concerning the mechanism of action of the opiates in the gut, this appears to vary with the site and from tissue to tissue and from species to species since in some segments of the gut, morphine causes inhibition of neurotransmitter release, while in others it causes release of neurotransmitters. The two best model systems available are the guinea-pig isolated ileum preparation (Kosterlitz & Waterfield, 1975) and the *in vivo* segmental small intestine of the dog. In the first tissue, it is well established that morphine as well as the opioid-like peptides inhibit the release of acetylcholine via the activation of presynaptic opiate receptors, causing relaxation of the gut musculature (Paton, 1957; Schaumann, 1957; Kosterlitz & Watt, 1968; Waterfield, Smockum, Hughes, Kosterlitz & Henderson, 1977; Huidobro-Toro, Hu & Way, 1981). In the *in vivo* small intestine system, acute morphine causes the release of 5-HT from the enteric nervous system supplying the intestine in the dog and rat, thus causing a contractile response (Burks & Long, 1967; Burks, 1973; 1976). It is evident that further research is needed before a definitive picture of the neurotransmitters involved in the action of morphine and specifically the endorphins on gut motility becomes clear.

At the present time, very little is known about the *in vitro* effects of morphine and related narcotic analgesics in the colon musculature. In a previous communication we described how morphine causes an excitatory effect in the terminal colon of the rat, an effect that could be blocked by naloxone (Huidobro-Toro & Way, 1976). The present investigation was undertaken in an effort to clarify three aspects of the excitatory effect of morphine and the endorphins on the colon. We were interested in extending our previous observations to that of several narcotic analgesic drugs, including the opioid-like peptides, and in comparing quantitatively the effect of these agents with that of morphine. In addition we wished to discover whether tolerance and physical dependence develops to this excitatory response to morphine *in vitro*. Finally, we wanted to explore the neurochemical basis of this excitatory action of morphine. The present paper summarizes our observations and the pharmacology of this opiate action on the terminal colon of the rat is discussed.

Methods

Adult Long Evans male rats (250–300 g), purchased from Simonsen (Gilroy, CA) were used. Rats were maintained on purina chow and tap water. The animal room was kept at 23–24°C with a 12 h light-dark cycle. Animals were deprived of food overnight before they were killed.

Preparation of the colon for measurement of contractile activity

Rats were killed by a blow on the neck, and the abdomen was opened. A 2–3 cm strip of the terminal portion of the colon was dissected and placed in a 50 ml capacity double jacketed organ bath chamber containing a modified Ringer solution (Kaymacalan & Temelli, 1964) maintained at 26°C, and gassed with a mixture of 95% O_2, 5% CO_2. This temperature was used to avoid excessive spontaneous activity. The composition of the Ringer solution was (g/l): NaCl 9, KCl 0.42, $CaCl_2$ 0.06, $NaHCO_3$ 0.5 and glucose 0.5. Each colon strip was connected to a Grass FT 03 force displacement transducer that was

coupled to a Grass polygraph to record isometric contractions. The preparations were loaded initially with 2 g tension which gradually dropped to about 1 g after the tissue equilibration period. During this interval, the preparations were washed with the Ringer solution every 15 min.

Quantification of drug effects

Drugs were applied regularly to the chambers containing the intestinal strips at 6 min intervals and washed 3 to 4 times in between drug additions with 200 ml Ringer solution. To test for the viability of each preparation, the colon strips were challenged with acetylcholine 5×10^{-8} M. If a prompt contractile response of about 2 to 3 g tension was achieved, the tissue was considered suitable for opiate testing. Routinely, a dose-effect curve for each narcotic analgesic was determined by adding increasing concentrations of each agonist until a maximum response was attained. Results are expressed as a percentage of the maximal (100%) response. The concentration of a drug to cause half maximal muscular contraction (EC_{50}) was interpolated from each individual log dose-response curve as detailed previously by Huidobro-Toro, Harris, Loh & Way, 1978. Drug concentrations are expressed as the final molar concentration of the base. Each strip was used for the bioassay of a single drug; 6 to 8 different preparations were mounted for the study of each opiate.

Antagonism of the opiate responses by naloxone

To ascertain whether the muscular contractions caused by morphine and related narcotic analgesics were mediated via a specific opiate receptor mechanism, naloxone was used as a selective opiate antagonist. To quantitate the antagonism of the opiate response different concentrations of the antagonist were added routinely 2 min before the addition of the agonist tested. After peak agonist contraction ensued, the tissue was extensively washed 3–4 times with at least 200 ml of Ringer solution. Fifteen to 20 min were allowed between each application of naloxone. Dose-response curves for each agonist were obtained in the absence of naloxone and in the presence of 3 concentrations of the opiate antagonist. Schild plots were derived in order to obtain the opiate-naloxone pA_2 values. Analysis of the Schild plots provided the pA_2 value; the slope and the dissociation constant (K_B) were derived from the Schild plots according to Arunlakshana & Schild (1959).

Effect of tolerance development

Rats were rendered tolerant to and dependent on morphine by the subcutaneous implantation of 75 mg morphine pellets (Way, Loh & Shen, 1969). Rats were lightly anaesthetized with ether and pellets were implanted on the back over a period of 72 h as follows: one on the first day, one or two on the second, and three on the third day. During each successive evening an increasing additional dose of morphine sulphate (30, 60, 120 mg/kg respectively) was administered intraperitoneally. On the fourth day, the animals were killed and the sensitivity of the colon to morphine and other agonists was determined by obtaining dose-response curves to the contractile effects of morphine, 5-HT and acetylcholine. Control animals were implanted with placebo pellets and injected with saline (0.9% w/v NaCl solution) but otherwise were treated identically.

To ascertain whether the sensitivity to morphine could be regained after discontinuance of morphine, the morphine pellets were removed from an additional group of implanted rats 72 h after the first morphine pellet implant. The rats were killed a week later and the morphine EC_{50} was determined. In all cases, paired colon segments from morphine- and placebo-treated rats were simultaneously prepared for the performance of dose-response curves.

Effects of tetrodotoxin (TTX), hexamethonium, and Ca^{2+} concentration

To study the influence of neuronal elements on the morphine contractile response, dose-effect curves for morphine, acetylcholine and nicotine were determined before and after the application of TTX 2.0×10^{-7} M or hexamethonium 8.3×10^{-5} M to different colonic preparations. Similarly, dose-response curves to morphine, acetylcholine, and nicotine were determined in Ringer containing 5.4, 0.54 or 0.0054 mM Ca^{2+}. Control experiments included substitution of sucrose to adjust osmotically for the removal of Ca^{2+}. The EC_{50} of morphine, acetylcholine and nicotine determined in the same preparation were compared before and the application of TTX, hexamethonium or the alterations in the external calcium concentration.

Effect of atropine or methysergide

To evaluate the involvement of the cholinergic or the 5-hydroxytryptaminergic system of the gut in the morphine contractile response, dose-response curves for morphine, acetylcholine and 5-HT performed in the absence and presence of atropine 5.8×10^{-7} M or methysergide 1.2×10^{-5} M. The EC_{50} of each agonist was determined in the same preparation before and after the application of each antagonist. Eight different colon preparations were mounted to study the

effect of atropine or methysergide on the contractile responses to morphine. Acetylcholine and 5-HT were always used as the controls to establish the effectiveness of the atropine or methysergide blockade.

Statistical analysis

The two tail Student's *t* test was used to compare the EC_{50} value obtained for each agonist before and after application of naloxone and other drug antagonists. In some instances (Tables 3 and 4), the 95% confidence limits and the potency ratio were determined according to the method of Litchfield & Wilcoxon (1949). Significance was set at a *P* value less than 0.05.

Drugs

Morphine sulphate, codeine phosphate and nalorphine hydrochloride were purchased from Mallinkrodt Chemical Co. (St. Louis, MO). Levorphanol and dextrorphan tartrate were gifts from Hoffmann La Roche (Nutley, N.J.) and naloxone hydrochloride from Endo Laboratories (Garden City, N.Y.). Etorphine hydrochloride, (−)- and (+)-methadone hydrochloride and the N-alkyl derivatives of norketobemidone were generously provided by Dr E.L. May. Human β-endorphin was a gift from Professor C.H. Li, methionine- and leucine-enkephalin were purchased from Bachem (Torrance, CA). Methysergide was a gift from Sandoz (Basel, Switzerland). L-Pentazocine lactate (30 mg/ml ampoules) and pethidine hydrochloride (50 mg/ml ampoules) were gifts from Sterling Winthrop Laboratories. Tetrodotoxin, atropine sulphate, acetylcholine chloride, carbachol bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Nicotine tartrate was obtained from K & K Laboratories (Plainview, N.Y.). All inorganic salts and reagents used to prepare the Ringer solution were reagent grade obtained from Fisher Scientific Co.

Results

Contractile effects of morphine and its surrogates

Morphine and other agonists produced dose-dependent contractions of the longitudinal muscular layers of the isolated colon of the rat. The muscular effect produced by the application of morphine was not a sustained contraction since the maximal tension was followed by a relaxation to base line tension. It is apparent that morphine causes the initiation of waves of contraction and relaxation. In this study, we considered for quantitative analysis only the initial contractile response, i.e., that corresponding to the contractile phase of the first wave cycle induced by the application of the narcotic analgesics. As an example of the contractile response attained by the application of different concentrations of morphine, see Figure 1. It should be noted that after the application of larger concentrations of morphine and related analogues (about 10–20 μM) the opiates produced vigorous muscular spasms (Figure 1).

Figure 2 shows the dose-effect curves for several of the narcotic agonists tested. Their rank order of potency is consistent with respect to their analgesic efficacy and ability to inhibit the coaxially stimulated guinea-pig ileum. Etorphine with an EC_{50} of 2.8×10^{-10} M, was the most active compound tested. The contractile response caused by the narcotic analgesics was found to be stereo-selective as indicated by the fact that levorphanol was about 42 times more active than dextrorphan and (−)-methadone about 4 times more than its (+)-stereoisomer (Table 1). Nalorphine and pentazocine were 17 and 39 times less potent than morphine respectively. Moreover, when their maximal contractile effectiveness was

Figure 1 Contractile effect of morphine sulphate (MS) on the rat terminal colon. Increasing concentrations of MS were added to a colonic intestinal strip maintained at 26°C in Ringer solution with 95% O_2 and 5% CO_2. Isotonic contractions from the longitudinal muscular layers were recorded isometrically. Additions of MS were made every 6 min and the preparation was washed 3–4 times after drug application. Doses refer to μg of MS added to a 50 ml bath. At the beginning of the experiment, 0.45 μg (5×10^{-8} M) acetylcholine (ACh) was applied to check the suitability of the preparation.

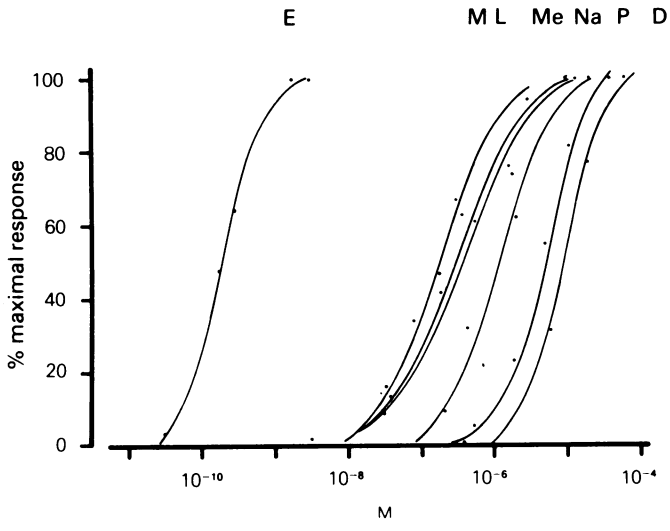

Figure 2 Dose-response curves to the excitatory action induced by various narcotics analgesics in the rat isolated colon. Increasing concentrations of etorphine (E), morphine (M), levorphanol (L), methadone (Me), nalorphine (Na), pentazocine (P), and dextrorphan (D) were applied to separate colon strips. The mean % of the maximal contraction attained by 4–6 colonic preparations is plotted. The abscissa scale represents the – log of the final molar concentration of the narcotics (base) in the bath chamber.

compared to that of morphine, it was consistently found that the maximal response to these two narcotic drugs was less than 100%, indicating some properties of partial agonists. The dose-response curves of all the opiates tested were parallel to that of morphine (Figure 2) indicating that all these compounds probably interact at a common receptor site.

Table 1 Sensitivity of the rat colon to different narcotics and other agonists

Agonist	$\bar{X}$ *EC50 ± s.e.mean* (M)
Morphine	$2.15 \pm 0.2 \times 10^{-7}$
Etorphine	$2.77 \pm 0.9 \times 10^{-10}$
Codeine	$2.49 \pm 0.2 \times 10^{-5}$
Pethidine	$6.42 \pm 0.1 \times 10^{-6}$
Nalorphine	$3.70 \pm 0.7 \times 10^{-6}$
Pentazocine	$8.40 \pm 0.5 \times 10^{-6}$
Levorphanol	$2.60 \pm 0.3 \times 10^{-7}$
Dextrorphan	$1.09 \pm 0.1 \times 10^{-5}$
(−)-Methadone	$5.34 \pm 0.4 \times 10^{-7}$
(+)-Methadone	$2.09 \pm 0.3 \times 10^{-6}$
Methionine enkephalin	$8.50 \pm 1.3 \times 10^{-8}$
β-Endorphin	$3.00 \pm 0.7 \times 10^{-8}$
Carbamylcholine	$6.3 \pm 0.5 \times 10^{-8}$
Acetylcholine	$4.0 \pm 0.4 \times 10^{-8}$
5-Hydroxytryptamine	$5.0 \pm 0.5 \times 10^{-6}$
Histamine	$5.11 \pm 1.3 \times 10^{-5}$

The mean EC_{50} is derived from 6–8 different colonic strip preparations.

The homologous series of the N-alkyl substituted norketobemidones, was also studied. The most active compounds in the isolated colon were found to be the N-amyl (C_5) and N-hexyl (C_6) derivatives, which were as potent as morphine. Figure 3 illustrates the EC_{50}s of these compounds arranged by increasing number of carbon atoms in the alkyl chain. Norketobemidone (C_0) was about 10 times less potent than morphine; the contractile activity of these compounds increased about 10 fold from C_1 to C_6. A further increase in the alkyl chain length to C_8 or C_{10} yielded compounds with considerably less activity than that of the parent compound.

The rat colon was also very sensitive to the action of the opioid-like peptides. β-Endorphin had an EC_{50} of 3×10^{-8} M but contractile responses were elicited at a concentration as low as 4.2×10^{-10} M. Methionine-enkephalin was about one-third as active as β-endorphin but approximately 3 times more potent than morphine. β-Endorphin was about 10 times more potent than morphine in producing a contractile response of the rat colon (Table 1).

Both acetylcholine and carbachol produced dose-dependent contractile responses; 5-HT and histamine also produced muscle contractions but their potency was much lower than that of the cholinoceptor agonist drugs. The EC_{50} values of these drugs are indicated in Table 1.

Antagonism by naloxone of responses to the opiates

Naloxone in concentrations up to 2×10^{-4} M did not

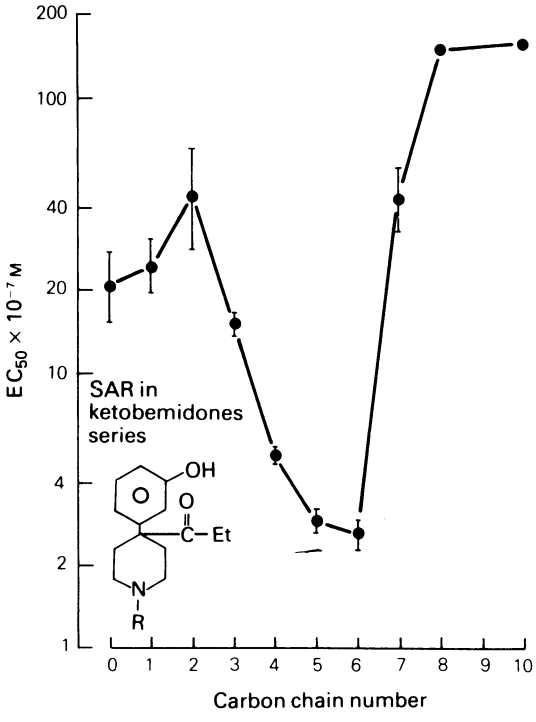

Figure 3 Effect of the substituted N-alkyl derivatives of norketobemidone on the contractile response of the rat colon. The mean EC_{50} of each derivative (C_1–C_{10}) is plotted vs. the number of carbon atoms in the N-alkyl side chain (N–R). At least 4 determinations were performed for each compound excepting the C_8 and C_{10} derivative ($n=2$); vertical lines show s.e.mean. Note the logarithmic scale for the EC_{50} values on the ordinates.

produce a contractile effect but was a potent antagonist of the narcotic responses. Naloxone displaced to the right in a parallel and dose-related fashion the dose-response curves of morphine and other agonists. Schild plot analysis of these data revealed a morphine-naloxone pA_2 value of 7.67 ($n=6$). The pA_2 values obtained for etorphine, levorphanol or (−)-methadone were not statistically different from that of morphine, but were considerably higher than that for the partial agonist pentazocine. The results from this set of experiments are summarized in Table 2. It should be noted that although the EC_{50} of the opiates varied more than a thousand fold, the pA_2 values were not essentially different when compared to that of morphine, suggesting that all these compounds interact with a common opiate receptor system. The slope of the Schild plots is not significantly different from the theoretical value of −1.0 expected for agonists except for pentazocine which had a slope of −1.56. The higher slope value for the pentazocine Schild plots is consistent with its properties of a partial agonist in this tissue. Table 2 shows in addition, the dissociation constant (K_B) derived from the Schild plot for each agonist studied.

Development of tolerance and physical dependence

Considerable tolerance developed to the contractile effect of morphine on the colon after sustained morphine treatment. The morphine dose-response curve was shifted in a parallel fashion to the right in proportion to the number of morphine pellets implanted during the 72 h period. After the implantation of 6 morphine pellets, the narcotic EC_{50} was increased about 90 fold as compared to that of the placebo-treated rats. The morphine EC_{50} in the placebo-treated group was $0.98\ (0.3–2.4) \times 10^{-7}$ M, and that of the morphine-treated group was $9.0\ (3.2–24.8) \times 10^{-6}$ M. Values in parentheses denote the 95% confidence limits, and establish a P value of less than 0.001 denoting the development of tolerance. The results of these experiments are presented as the EC_{50} ratio of placebo- vs. morphine-treated rats in Figure 4. The sensitivity of the colon to acetylcholine was not significantly altered by the morphine treatment, but an increase in sensitivity to 5-HT developed concomitantly to the development of tolerance (Figure 4 and Table 3). After the implantation of 6 morphine pellets, the sensitivity to exogenously applied 5-HT increased approximately 30 fold. The EC_{50} of 5-HT in the placebo-treated group was $3.0\ (1.57–5.70) \times 10^{-6}$ M and that of the morphine-treated group decreased to $1.1\ (0.4–3.20) \times 10^{-7}$ M (P value less than 0.01). Seven

Table 2 Opiate-naloxone interaction in the rat colon: determination of the naloxone pA_2 value

Narcotic	*pA_2*	*Slope*	*K_B (nM)*
Morphine	7.67 ±0.13(6)	−1.19 ±0.12	21.4±2.5
Levorphanol	7.33 ±0.12(6)	−1.30 ±0.10	46.8±6.0
(−)-Methadone	7.82 ±0.23(3)	−1.11 ±0.16	15.1±4.0
Etorphine	8.23 ±0.25(4)	−1.09 ±0.10	6.0±1.5*
Pentazocine	5.43* ±0.10(5)	−1.56* ±0.11	4570±800*

Mean values are given ± s.e.mean
*$P<0.05$ as compared to morphine

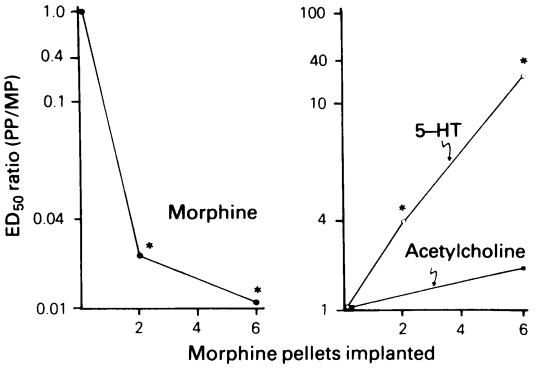

Figure 4 Effect of chronic morphine treatment on the contractile response of the rat colon to morphine, 5-hydroxytryptamine (5-HT) and acetylcholine. Rats were implanted with 2 or 6 morphine pellets (MP) for 72 h. Controls were implanted with placebo pellets (PP). The ratio of the EC_{50} obtained after placebos and morphine pellet treatment of six paired experiments are given (on the ordinate scale) and the number of pellets implanted (on the abscissa scale). The potency ratio was estimated according to Litchfield & Wilcoxon (1949). Note that after chronic morphine treatment there is a reduced sensitivity to morphine but the colon showed an increase in the sensitivity to 5-HT. The acetylcholine potency was not significantly altered by the opiate treatment.

days after the removal of the morphine pellets, the sensitivity to morphine and 5-HT returned to the control-placebo levels, indicating that the process of tolerance is reversible upon the withdrawal of morphine from the system (Table 3).

The colon strips obtained from rats implanted with morphine pellets exhibited more spontaneous activity during the equilibration period before testing for development of tolerance (i.e. before the application of morphine to construct dose-response curves) than those from naive or placebo-pellet implanted animals. The strips of the test group failed to exhibit the typical acute contractile response to a concentration of morphine ordinarily effective in the control group, indicating a condition of tolerance to the excitatory effect of morphine. However, spontaneous activity of the colon from the morphine pellet-implanted animals could be largely reduced by repeated exposure to morphine. These effects are illustrated in Figure 5: the addition of morphine 4×10^{-6} M (M) to a colonic strip from a placebo-implanted rat elicited a burst of contractile responses (Figure 5a, below). However, the same concentration of morphine was ineffective on a strip from a morphine-implanted group despite the fact that the preparation remained responsive to 1.38×10^{-7} M acetylcholine (ACh) (see Figure 5a above). Following exposure to repeated additions of morphine, the colonic strip from the morphine tolerant rat (Figure 5b, above) had essentially no spontaneous activity even though the preparation retained its lack of responsiveness to the application of morphine. It is apparent that the spontaneous activity present in the colon of the tolerant rat, in the absence of additional morphine, is largely abolished by the applications of the opiate. This suggests that during opiate withdrawal, the preparations had a marked muscular activity that was abolished by the addition of morphine at concentrations that did not elicit an excitatory response.

These experiments indicate that tolerance in the colon was paralleled by some degree of 'physical dependence' that could be overcome *in vitro* by further additions of morphine. Naloxone administered to the tolerant-dependent colon did not increase the spontaneous activity during the equilibration period after washing the residual morphine from the colon.

Table 3 Sensitivity of the rat colon to different agonists after the development of tolerance and physical dependence

	EC_{50} (95% confidence limits)		
	Morphine ($\times 10^{-7}$ M)	*Acetylcholine* ($\times 10^{-8}$ M)	*5-Hydroxytryptamine* ($\times 10^{-6}$ M)
Morphine[a]	50.0(24.3–102.5)** (4)		0.8(0.22– 2.88)* (4)
Placebo[b]	0.98(0.24–2.40)(7)	4.0(1.94– 8.4)(12)	3.0(1.57– 5.70) (8)
Morphine pellet removed for 7 days	1.05(0.41–2.66)(4)	3.5(1.55– 7.80) (4)	5.0(1.38–18.10) (4)
Naive-control	2.5 (0.7–4.0)(7)	4.0(1.55–10.28)(12)	5.0(1.91–13.05) (9)

[a]Rats were implanted with pellets of morphine: one at time zero, and a second one 24 h later. In addition rats received daily s.c. injections of morphine at increasing doses detailed in methods. Animals were killed 72 h after the first pellet implant.

[b]Placebo pellets and saline were substituted for morphine.

*$P<0.05$; **$P<0.01$ in relation to the placebo group.

Numbers in parentheses refer to the number of preparations used for each determination.

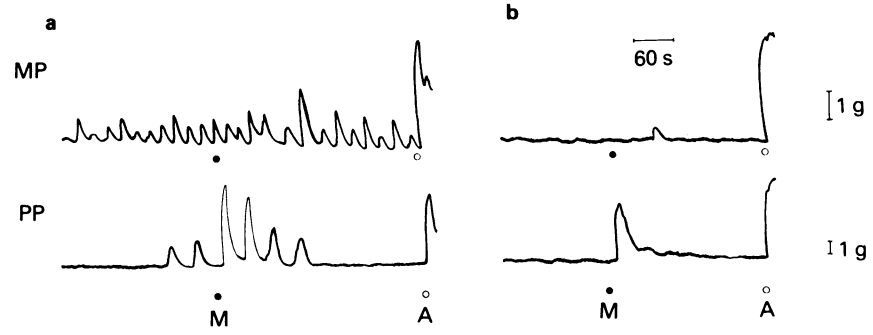

Figure 5 Tracing from the colon of a rat rendered tolerant-dependent on morphine, and its paired control treated with a placebo (PP). The upper record corresponds to a rat implanted with six morphine pellets (MP) as described in methods, and killed 72 h later. Note that the test preparation exhibits considerable spontaneous activity and is not responsive to morphine 4.0×10^{-6} M (M), but reacts normally to acetylcholine 1.38×10^{-7} M (A). During an interval of approximately 50 min (between (a) and (b)), 8 doses of morphine were applied in succession (varying from 0.02 to 6 μg morphine/ml every 6 min). In (b) no spontaneous activity is seen in the gut from the non-tolerant rat. The sensitivity in the lower record is one half of that in the upper record.

Effect of morphine after tetrodotoxin or hexamethonium treatment

Pretreatment of the tissues with TTX produced a complete blockade of the contractile effects of morphine. As can be seen in Figure 6, a significant contractile response could not be obtained with morphine 4.0×10^{-6} M, a concentration 10 times larger than that which consistently produced a maximal response prior to the addition of the neurotoxin. As a control for the effectiveness of the TTX blockade, the toxin antagonized the response to nicotine, but to a lesser extent than that to morphine. The contractile effect produced by acetylcholine was not antagonized by TTX, rather the dose-effect curve to acetylcholine was displaced to the left and a six fold increase in sensitivity to acetylcholine was seen.

Treatment with hexamethonium caused a modest reduction of the morphine effect, the EC_{50} increasing from 1.0 to 2.0×10^{-7} M ($n = 5$). Hexamethonium caused about an 80 fold decrease in the potency of nicotine, increasing the EC_{50} of this ganglionic stimulant from 8.0×10^{-6} M to 6.5×10^{-4} M ($P < 0.01$, $n = 12$). In the same preparations the acetylcholine sensitivity was doubled by hexamethonium; the EC_{50} for acetylcholine decreased from 7.5×10^{-8} M to 3.5×10^{-8} M ($n = 8$).

Effect of Ca^{2+} ions on the contractile effects of morphine

Increasing the Ca^{2+} concentration of the Ringer solution 10 fold antagonized the effect of morphine. Not only was there a significant doubling of the morphine

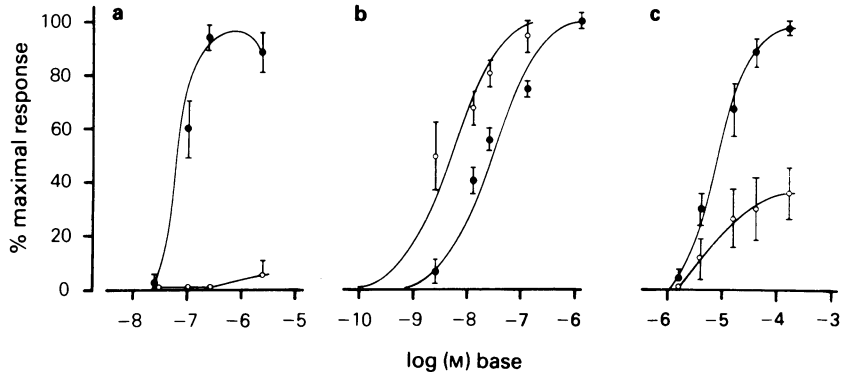

Figure 6 Effect of tetrodotoxin (TTX) on the contractile response of the rat colon to three types of agonists. Dose-effect curves for morphine (a), acetylcholine (b) and nicotine (c) were performed before (●) and after (○) the application of tetrodotoxin (2.0×10^{-7} M). Results are expressed as % of the maximal response obtained before tetrodotoxin. Each point represents the mean of seven experiments performed on different intestinal strips; vertical lines show s.e.mean. $n = 7$ in each case.

Table 4 Effect of Ca^{2+} on the contractile response of the rat colon to morphine, acetylcholine and nicotine

Ca^{2+}	EC_{50} (95% confidence limits) *Morphine* ($\times 10^{-7}$ M)	*Acetylcholine* ($\times 10^{-8}$ M)	*Nicotine* ($\times 10^{-6}$ M)
0.54	6.5(2.70–15.6) (6)	2.0(0.09– 4.40) (8)	9.0(3.75–21.6) (7)
0.0054	NR (6)	8.5(2.42–29.75) (8)	NR (7)
0.54	6.0(1.36–26.34)(13)	4.0(1.26–12.68) (4)	5.0(1.12–22.25) (4)
0.0054 mM + sucrose	NR (3)	15.0(2.25–99.75) (4)	NR (4)
0.54	3.5(1.0 –10.3) (13)	4.2(2.3 –12.1) (17)	10(4.0 –20.8) (11)
5.4	7.0(3.4 –18.7)*(13)	1.8(0.7 – 4.5) (17)	5(1.8 –12.3) (11)

NR = The EC_{50} could not be determined because responses did not reach 50% of the maximal agonist response. *The dose-effect curve was not parallel to the control, and did not reach 100% of the maximal response. Potency ratio indicated a *P* value less than 0.05.

EC_{50} but in addition, the maximal response was not achieved. On the other hand, the sensitivity to acetylcholine was roughly doubled (Table 4).

Decreasing the Ca^{2+} concentration from 0.54 mM to 0.0054 mM caused a complete loss of the contractile effects of morphine and nicotine. Moreover, this manipulation caused a modest four fold decrease in sensitivity to acetylcholine (Table 4).

Effects of atropine and methysergide

Atropine did not block the contractile effect of morphine nor that of 5-HT while producing a 1000 fold blockade of the stimulant effect of acetylcholine. The result of these effects are summarized in Figure 7. In contrast to atropine, methysergide competitively antagonized the contractile effects of morphine and 5-HT. As shown in Figure 8, methysergide displaced the dose-effect curve of 5-HT to the right, shifting the EC_{50} about 12 fold and that of morphine about 18 fold.

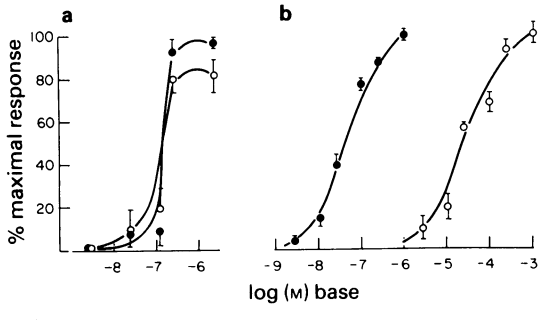

Figure 7 Effect of atropine on the contractile response to morphine (a) and acetylcholine (b) in the rat colon. Dose-response curves were determined before (●) and after (○) the addition of atropine 5.8×10^{-7} M to the Ringer solution. Results are expressed as a % of the maximal contraction attained for each agonist before application of atropine. Symbols represent the mean value and vertical lines the s.e.mean of 12 experiments with acetylcholine and 7 with morphine.

Discussion

The present results confirm and extend our findings (Huidobro-Toro & Way, 1976; Huidobro-Toro *et al.*, 1978) and those of Gillan & Pollock (1980) indicating that morphine and its surrogates cause a dose-dependent contraction of the terminal colon of the rat. The excitatory responses to morphine were more marked and consistent in the colon derived from Long Evans rats than that from Sprague-Dawley or Wistar rats. However, other investigators (Kaymacalan & Temelli, 1964; F. Huidobro & J. Lewin, personal communication) were successful in obtaining reliable dose-related contractile responses to morphine in colons from Sprague Dawley rats. It is apparent that the Long Evans rats are more sensitive to the morphine effects than other strains of rats. (Recently, we found that the vas deferens of the Long

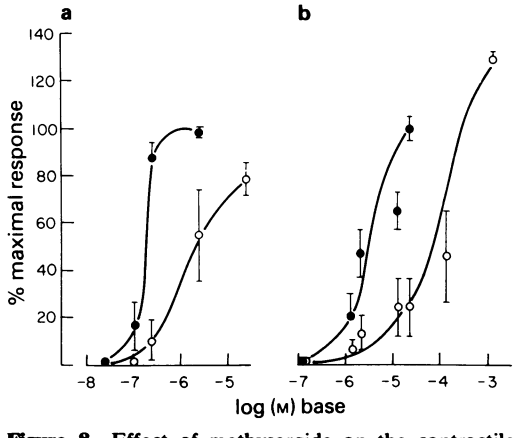

Figure 8 Effect of methysergide on the contractile response to morphine in the rat colon. Dose-response curves for morphine (a) and 5-hydroxytryptamine (b) were constructed before (●) and after (○) the application of methysergide 1.2×10^{-5} M. Symbols represent the mean % of the maximal response and vertical lines the s.e.mean of 6 determinations for morphine and 8 for 5-HT.

Evans rat was the most sensitive duct among that of several rat strains tested in responding to the inhibitory action of β-endorphin in the neuromuscular transmission of this organ; Huidobro-Toro & Way, 1981). In general, concentrations of the opiates in the range of 10 to 1000 nM produced rhythmical contractions of the colon longitudinal musculature. Higher doses of the narcotic analgesics caused a sustained spasm but even this response was easily reversed by washing.

The excitatory effect of morphine in the colonic musculature appears to be selectively mediated via activation of opiate receptors in the neuronal elements of the gut as suggested by several findings. First, the stimulant response to morphine is shared by a variety of narcotic analgesic drugs including the homologous series of the N-alkyl norketobemidones (Oh-ishi & May, 1973). The rank order of potency of the ketobemidones in eliciting this response was similar to that found with other test systems used for measuring agonist efficacy. In brief, the N-amyl derivative was reported to be the most potent and the N-ethyl derivative least potent with respect to analgesic action in rodents (Wilson, Rogers, Pert & Snyder, 1975), in the inhibition of the electrically induced muscular twitches in the guinea-pig ileum (Kosterlitz, Leslie & Waterfield, 1975) and in the binding of these compounds to purified cerebroside sulphate (Loh, Cho, Wu, Harris & Way, 1975). Secondly, the excitatory response is stereospecific as illustrated by the fact that levorphanol is about 50 times more potent than dextrorphan and (−)-methadone more potent than (+)-methadone. Thirdly, the opiate response is antagonized in a competitive fashion by the opiate antagonist naloxone. Fourthly, colons from rats rendered tolerant-dependent on the opiate were found to be markedly resistant to the excitatory action of morphine. In addition, the increased spontaneous activity observed in these tissues upon morphine withdrawal could be interpreted as evidence of opiate 'physical dependence' *in vitro*.

In characterizing the nature of the naloxone blockade of the opiate contractile response in the colon, it was noted that the pA_2 values for morphine, levorphanol, etorphine and methadone were not significantly different from that of morphine, despite the large differences in their agonist potency. The pA_2 value we obtained for the morphine-naloxone interaction in the rat colon is consistent with that found by Takemori, Kupferberg & Miller (1969), Smits & Takemori (1970) and Harris, Loh & Way (1976b) for antinociception in rodents, but slightly lower than that reported for inhibition of electrically induced contractions in the guinea-pig ileum (Kosterlitz & Watt, 1968; Vaught & Takemori, 1978; Huidobro-Toro *et al.*, 1981). Perhaps these differences in pA_2 values could be interpreted as evidence in support of the concept of heterogeneity of opiate receptors in the peripheral system as suggested by Lord, Waterfield, Hughes & Kosterlitz, 1977. Indeed, in the rat colon, β-endorphin is about 10 times more potent than morphine and about 3 times as potent as the enkephalins. These results are in decided contrast to the relative ratio of potency of these agents as compared to that of morphine in the guinea-pig ileum, mouse or rat vas deferens (Lord *et al.*, 1977; Wüster, Schulz & Herz, 1979; Huidobro, Huidobro-Toro & Miranda, 1980; Huidobro-Toro & Way, 1981).

Additional evidence supporting the notion that the excitatory effect of opiates on the rat colon reflects a true agonist response was provided by the fact that this preparation developed tolerance and physical dependence upon the chronic administration of morphine. The colons from rats implanted with morphine pellets were markedly resistant to the stimulatory effect of morphine, although full agonist activity was observed with high concentrations of morphine. These findings could be interpreted as indicating that tolerance develops to this excitatory opiate response. It is of interest that tolerance develops to an excitatory effect of morphine and particularly to this contractile response on the terminal colon in view of the fact that humans do not easily develop tolerance to the constipating action of morphine.

The tolerance exhibited to morphine by the colon was also accompanied apparently by physical dependence, as shown by the increase in spontaneous activity of the isolated colon strips after rinsing off the residual morphine, presumably bound to the tissue. During this washing-equilibrating period, a marked increase in the spontaneous activity of the intestinal tone was observed only in the colons from the morphine-tolerant rats. This colonic hypermotility could possibly indicate signs of opiate withdrawal by the neuronal elements in the colon. The spontaneous activity of the colon musculature upon removal of morphine from the media was very obvious ($P < 0.01$ by the Mann Whitney test) but it largely subsided following the reapplication of morphine to the tissues. Moreover, when the spontaneous activity of the tolerant dependent colonic strips was abolished by the addition of morphine, it could not be exacerbated by the addition of naloxone. It thus appears that there are some elements in common between the effects of morphine in the colon and those observed centrally in that the response elicited at both sites appears to be mediated via selective opiate receptors and that tolerance and physical dependence develop after chronic opiate treatment.

An interesting aspect of this investigation was the examination of the neurochemical basis for the excitatory effect of morphine. The results indicate that the contractile response to morphine is mediated

neurally rather than through the direct activation of the muscle membrane. TTX is a potent and specific blocker of Na^+ channels (Kao, 1966) that impairs axonal conduction without producing blockade of post junctional receptor responses (Ogura, Mori & Watanabe, 1966; Gershon, 1967). If the contractile effect of the opiates on the rat colon were dependent on neuronal component(s), we reasoned that morphine should be completely ineffective in the presence of TTX. This proved to be the case. Although the effect of morphine was completely abolished by TTX the response to acetylcholine, which activates mainly the muscarinic receptor at the postsynaptic muscle membrane was still present. Thus, it would appear that cholinergic neurones are not primarily involved in the contractile response to opiates. In further support of this contention, the ganglionic blocking agent, hexamethonium, in concentrations which increased resistance to nicotine 80 fold, decreased the effect of morphine only two fold.

Further evidence for the neuronal basis of opiate action on the colon is apparent from the Ca^{2+} experiments. Lowering the Ca^{2+} in the Ringer solution 100 fold abolished the responses to morphine and nicotine while the sensitivity to exogenously applied acetylcholine was minimally reduced. These results could be interpreted as meaning that Ca^{2+}, being essential for synaptic transmission (see review by Hubbard, 1973; Rubin, 1974), cannot be reduced below a certain critical level in order to obtain a morphine response. However, Ca^{2+} does not appear to be an essential requirement for the direct, postsynaptic, contractile effect of acetylcholine. It is of interest that increasing the external calcium 10 fold caused an increase in the morphine EC_{50} and reduced the maximal response, indicating an apparent noncompetitive antagonism between the two agonists. The anti-opiate effects of Ca^{2+} have previously been reported *in vitro* (Heimans, 1975; Opmeer & Van Ree, 1979; Hu, Huidobro-Toro & Way, 1980; Huidobro-Toro *et al.*, 1981) and *in vivo* (Kakunaga, Kaneto & Hana, 1966; Harris, Loh & Way, 1975; 1976a).

Concerning the neuronal pathway(s) involved in the contractile effect of opiates, the present data suggest that a 5-hydroxytryptaminergic neurone is most probably linked to the contractile response to morphine in the terminal colon of the rat. In support of this conclusion we showed that methysergide antagonized both the contractile response to morphine and that of exogenously applied 5-HT to approximately the same extent. In contrast, neither atropine nor hexamethonium modified the effect of morphine, indicating that cholinergic neurones are not primarily involved in mediating the response. The present data are entirely consistent with the findings of Burks & Long (1967) and Burks (1973; 1976) who demonstrated that the narcotic analgesic releases 5-HT from the small intestine preparation of the dog or rat, thus explaining the excitatory action of morphine in the small bowel of these species. Further support for a 5-hydroxytryptaminergic component in the local excitatory effect of morphine in the intestine can be derived from the fact that the colons obtained from animals tolerant to morphine had an increased sensitivity to 5-HT coupled with the reduced sensitivity to opiates. The tolerance to morphine exhibited by the colon was accompanied by an increased sensitivity to the effects of exogenously applied 5-HT. The simultaneous development of these two opposite responses during tolerance had been observed earlier in the guinea-pig ileum by Takagi, Takayanagi, Irikura, Nishino, Ichinoseki & Shishide (1965) and Goldstein & Schulz (1973), where narcotics cause an inhibition of the twitch produced by coaxial stimulation of the cholinergic nerves of the ileum. Both effects on the rat colon are reversible since the decreased sensitivity to morphine and increased potency of 5-HT returned to baseline levels after morphine pellet removal. The functional significance of these parallel changes in sensitivity to morphine and 5-HT during development of tolerance are not clear at the present time. Interestingly they occur in two entirely different bioassay preparations in which the opiates produce markedly different neurochemical effects. Because of this argument, it is possible that these dual changes are intrinsically related to tolerance development at least in isolated intestinal preparations. However, since the opiate-induced contraction is apparently mediated at least in part via a 5-hydroxytryptaminergic neurone, this possibility needs further clarification. If opiates release 5-HT in the colon of naive rats and tolerance develops to this action, it might well be that after sustained morphine treatment there is some degree of supersensitivity to this indoleamine due to the lack of release of 5-HT in the continual presence of the opiate. It is also possible to argue that tolerance to the excitatory effect of morphine may develop as a result of a severe depletion of 5-HT from the enteric nerves of the terminal colon. Dependence could be visualized as the recovery phase of the tissue content of 5-HT levels. In support of this postulate, there was an eventual parallel recovery in responsiveness to both morphine and 5-HT to normal baseline levels after eventual elimination of body stores of morphine by removal of the morphine depot.

The question arises as to whether this excitatory action of morphine on the terminal colon of the rat is related to the antidiarrhoeal effect of the opiate. It may be argued that the rhythmic peristalsis of the colon is destroyed by the burst of fast excitatory waves elicited by the opiates. Furthermore, at concentrations of about 10 μM, the opiates and particu-

larly morphine caused the spasmic contracture of the colonic musculature which may well completely stop intestinal transit. It is likely that the local effects of the narcotic analgesics acting on the intrinsic innervation of the gut may explain in part the antidiarrhoeal effect of morphine. However, Stewart *et al.* (1977; 1978) and also Manara *et al.* (1980) have recently shown that morphine and opiate surrogates exert both a central and a peripheral effect on the gastrointestinal tract. Furthermore, Burks (1980) implicated a central component in the mechanism of action of the endogenous opioid-like peptides by demonstrating that β-endorphin administered into the cerebral ventricles of rats delayed in a dose-related fashion the intestinal transit. Thus, it seems possible that the constipation produced by morphine has a local and central site of action.

In conclusion, the isolated terminal colon of the rat offers a new bioassay system to an excitatory action of the opiates. The morphine response is mediated via the activation of opiate receptors probably located in the neuronal elements of the enteric system. The opiate receptors in the colon share common properties with the receptors found in the guinea-pig ileum although the neurochemical correlates of the morphine mechanism(s) of action involved in these two systems are entirely different. It is apparent that in the rat colon the opiates cause excitation via the local release of 5-HT from nerve terminals.

Note added in proof

Nijkamp & Van Ree recently showed that the isolated colon and rectum of the guinea-pig and the rat are sensitive to opioid peptides; the contractile response elicited by these peptides is not cholinergic in nature but antagonized by methysergide or cyproheptadine (Nijkamp, F.P. & Van Ree, J. 1978). Effect of endorphins on different parts of the gastrointestinal tract *in vitro*. In *Characteristics and Functions of Opioids*. ed. Van Ree, J. & Terenins, L. pp.179–180. Amsterdam: Elsevier, North Holland Biomedical Press.

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