J. Pharm. Pharmacol. 1983, 35: 823–825
Communicated June 24, 1983

© 1983 J. Pharm. Pharmacol.

[D-Ala[2],D-Met[5]NH$_2$]-Enkephalin inhibits acetylcholine release from the submucosal plexus of rat colon

TIMOTHY S. GAGINELLA*, ZE-AI C. WU, *The Ohio State University, College of Pharmacy and Department of Pharmacology, Hoffmann-La Roche Inc.*

Opiate-induced constipation has been traditionally attributed to stimulation of intestinal circular smooth muscle. However, the concept that morphine and other opiates act purely by affecting intestinal motility has been questioned (Powell 1981). Indeed, morphine and the synthetic enkephalin analogues, [D-Ala[2],D-Met[5]NH$_2$]-enkephalin, [D-Ala[2],D-Leu[5]]-enkephalin, and Me-Tyr-D-Met-Gly-Phe-Pro-NH$_2$, decrease the potential difference and short circuit current in ileal mucosa (Dobbins et al 1980; McKay et al 1981; Miller et al 1981). These in-vitro studies and those of Coupar (1978) and Beubler & Lembeck (1979) in-vivo suggest that the antidiarrhoeal effect of opiates involves enhanced absorption of electrolytes and water by the mucosa.

Opiates may enhance electrolyte absorption by two principal mechanisms: by direct interaction with epithelial cell membrane receptors and by inhibition of acetylcholine (which causes electrolyte secretion) release from mucosal/submucosal nerves. We have tested the latter possibility, by using a muscle-stripped preparation to study the effects of opiates at the mucosal level on evoked release of [^{3}H]acetylcholine (ACh).

Materials and methods

The methods used to study [^{3}H]ACh release from the colonic mucosal preparation have been previously reported (Wu et al 1982). Briefly, pieces (ca 1 mm wide) of rat colonic mucosa were incubated for 1 h with [^{3}H]choline (0·2 μM, specific activity 80 Ci mmol^{-1}) in 2 ml of oxygenated (95% O$_2$–5% CO$_2$) Krebs buffer (containing 50 μM physostigmine) at 37 °C in a Dubnoff metabolic incubator. At the end of this period, tissue was collected on a Whatman GF/B glass fiber filter under vacuum, followed by 2 washes (5 ml) with ice-cold Krebs buffer. The tissue was placed into incubation baskets fabricated from sections of plastic culture tubes (Falcon, 13 mm diameter) with a piece of nylon mesh (mesh size 0·13 mm^2) as the bottom. After washing (37 °C, 1 h) release was measured over 90 min. The buffer used during the wash and the release periods contained 10 μM hemicholinium-3 and 50 μM physostigmine.

The tissue was depolarized twice with buffer containing 50 mM K$^+$. Morphine or [D-Ala[2],D-Met[5]NH$_2$]-enkephalin (ENK) were added to the buffer 20 min before and during the second K$^+$ depolarization. Ethylketocyclazocine was added 20 min before the second K$^+$ stimulation and remained present to the end of the experiment. Samples (2 ml) of the incubation medium were counted for total ^{3}H. In other experiments the remaining 3 ml was used to verify by column chromatography that released ^{3}H represented authentic ACh (Wu et al 1982). At the end of the experiment, tissue was solubilized and counted in a Beckman LS-345 liquid scintillation counter; counting efficiency was approximately 38%.

The stimulated release was calculated as the increased release over baseline and expressed as S_1 and S_2 for the first and second K$^+$-stimulated release phases, respectively. The ratio S_2/S_1 was computed for each experiment in order to compare the inhibitory effects of the opiates.

Source of drugs. Choline chloride [methyl-^{3}H] was purchased from New England Nuclear Corp., Boston, MA; [D-Ala[2],D-Met[5]NH$_2$]-enkephalin was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Morphine sulphate and naloxone were obtained from Merck Chemical Co. and Endo Laboratories, respectively. Ethylketocyclazocine methanesulfonate was a gift from Dr M. Feigenson of Sterling-Wingthrop Research Institute, Rensselaer, NY. Hemicholinium-3 and physostigmine salicylate were purchased and from Sigma Chemical Co., St Louis, MO.

Results

Since control release varies in these type of experiments (ranging from 0·55 ± 0·07 to 0·72 ± 0·10), it was necessary to pair drug effects with control values obtained with tissue from the same animal.

ENK inhibited [^{3}H]acetylcholine release in the rat colonic mucosal preparation. Over the concentration range we tested (4·4 × 10^{-8} M to 4·4 × 10^{-5} M) 4·4 × 10^{-7} M was the lowest concentration that inhibited ACh release. This concentration of ENK significantly ($P <$ 0·005) inhibited (by 32%) K$^+$-evoked [^{3}H]ACh release. The effect was blocked by 10^{-4} M naloxone (Table 1). This concentration of naloxone alone had no effect on K$^+$-evoked ACh release. A lower concentration of naloxone (10^{-5} M) inhibited ENK-induced ACh release, but not significantly ($P >$ 0·05). Morphine, over the concentration range of 10^{-6} to 10^{-3} M, had no significant effect on K$^+$-evoked [^{3}H]ACh release. Ethylketocylazocine at concentrations ranging from

* Correspondence.

Table 1. Effect of [D-Ala^2,D-Met^{5}NH$_2$]-enkephalin (ENK) on K^+-evoked [^{3}H]ACh release from nerves innervating rat colonic mucosa/submucosa.

	[^{3}H]ACh release (S_2/S_1)[a] (n = 6)	P value[b]
Control	0·65 ± 0·06	—
ENK (4·4 × 10^{-7} M)	0·45 ± 0·05	<0·005
ENK + naloxone (1 × 10^{-4} M	0·66 ± 0·07	>0·05

[a] Tissues were depolarized twice by K^+ (50 mM); the first depolarization is designated as S_1 and the second depolarization as S_2. ENK was given 20 min before second depolarization. The ratio (S_2/S_1) of the [^{3}H]ACh released over baseline was calculated to evaluate the effect of the ENK.
[b] Compared with control values. Significance was evaluated by the paired Student's *t*-test.

Table 2. Lack of effect of morphine and ethylketocyclazocine on K^+ (50 mM)-evoked [^{3}H]ACh release.

Treatment	[^{3}H]ACh release (S_2/S_1)[a]
Control[b]	0·55 ± 0·07 (7)
Morphine [M][c]	
10^{-3}	0·47 ± 0·04 (3)
10^{-4}	0·46 ± 0·07 (6)
10^{-5}	0·46 ± 0·08 (3)
10^{-6}	0·53 ± 0·07 (6)
Control	0·72 ± 0·10 (4)
Ethylketocyclazocine [M][c]	
10^{-4}	0·53 ± 0·09 (4)
10^{-5}	0·70 ± 0·11 (4)
10^{-6}	0·72 ± 0·01 (4)
10^{-7}	0·68 ± 0·26 (4)

[a] Values are means ± s.e. Numbers in parentheses are the number of experiments performed.
[b] Not significantly different from control for ethylketocyclazocine.
[c] Morphine was present 20 min before and during second K^+ stimulation, ethylketocyclazocine was present from 20 min before second K^+ stimulation to the end of the experiment. All of the values were not significantly (P > 0·05) different from their respective controls.

10^{-7} to 10^{-4} M also had no significant effect. However, at the highest concentration of ethylketocyclazocine tested (10^{-4} M) a slight (statistically insignificant) inhibition of evoked [^{3}H]ACh release was observed (Table 2).

Discussion

Opiates have been shown to enhance electrolyte absorption across rabbit and guinea-pig ileal mucosa (McKay et al 1981; Miller et al 1981). Enkephalin anlogues reportedly are more potent in this regard than morphine (Dobbins et al 1980). The opiate effects may be direct or may be mediated through modulation of the release of an endogenous substance that itself tends to cause secretion. Functional cholinergic nerve endings are indeed present in the rat colonic mucosa (Wu et al 1982). Moreover, ACh, pilocarpine and bethanechol produce effects functionally opposite to the opiates; they (directly or indirectly) cause intestinal secretion (blood-to-lumen flux) of electrolytes and water (Browning et al 1977; Isaacs et al 1976; Tidball 1961; Hubel 1977). Therefore, since absorptive and secretory fluxes normally occur simultaneously in the intestine (Hendrix & Bayless 1970) antagonism by opiates of blood-to-lumen flux could result in net absorption (lumen-to-blood flux). Thus, reducing the amount of water in the lumen would tend to have an antidiarrhoeal (constipative) effect.

Our results indicate that ENK inhibits the release of [^{3}H]ACh from the intestinal epithelium but that morphine and ethylketocyclazocine have essentially no effect on the release. These findings are in agreement with the results obtained by Kachur & Miller (1982) in functional (ion transport) studies; morphine was ineffective and ethylketocyclazocine was much less potent than [D-Ala^2,D-Leu^5]-enkephalin.

At least four subtypes of opiate receptors (δ, μ, κ, σ) have been postulated. Enkephalins have a higher affinity for δ-type opiate receptors, morphine is relatively more selective as an agonist at μ-type receptors, and ethylketocyclazocine is an agonist primarily of the κ-opiate receptor (Chang & Cuatrecasas 1981). Based upon this classification, it has been suggested (Miller et al 1981; Kachur & Miller 1982) that the effect of enkephalin analogues on ion transport across the intestinal mucosa may be mediated by δ-receptors. The relative order of potency, ENK > ethylketocyclazocine > morphine, on ACh release from colonic mucosa in the present study tends to support the idea that δ-type receptors may be involved in the mucosal response to opiates. A larger series of opiates including the agonists [D-Ala^2,D-Leu^5]-enkephalin and the non-peptide etorphine and the antagonist diprenorphine will be required to more firmly implicate the δ-type receptor in the mechanism for the mucosal response. Furthermore, our finding that a high concentration of naloxone was required to antagonize the effect of ENK on [^{3}H]ACh release is in agreement with the relative selectivity of naloxone, which is less potent as an antagonist at δ than at μ-type opiate receptors.

Endogenous opiate peptides may serve under certain circumstances as physiological modulators of intestinal electrolyte transport, indirectly through inhibition of ACh release from submucosal cholinergic nerves. Hardcastle et al (1982) found that morphine had no effect on basal or prostaglandin-stimulated cyclic AMP production in isolated enterocytes and they suggested that opiates may act on the myenteric nerves to alter secretion. The data of Dobbins et al (1980) support a neurally-mediated effect of opiates as they found that tetrodotoxin completely blocked the response of the mucosa to ENK. These authors felt that acetylcholine was not the mediator of secretion in their ileal preparation because atropine did not produce a response similar to ENK and cholinergic agonists did not antagonize the

effect of ENK. However, Dobbins et al (1982) do not address the issue of whether another mediator, such as the secretagogue vasoactive intestinal polypeptide, (VIP), might be released by ACh. VIP is present in the mucosa/submucosa of the rabbit ileum, where it is released by electrical field stimulation (Gaginella et al 1981), and cholinergic-VIPergic interactions are possible within the neural plexuses of the gut (Gaginella & O'Dorisio 1979).

A direct effect of opiates on the intestinal epithelial cells cannot be completely ignored. However, in separate studies we have been unable to detect specific binding sites (receptors) for opiate ligands on membranes of isolated rat ileal and colonic epithelial cells (Gaginella et al 1983).

This work was supported in part by USPHS Grant AM 21932.

REFERENCES

Beubler, E., Lembeck, F. (1979) Naunyn-Schmiedeberg's Arch. Pharmacol. 300: 113–118
Browning, J. G., Hardcastle, J., Hardcastle, P. T., Sanford, P. A. (1977) J. Physiol. (London) 272: 737–754
Chang, K.-J., Cuatrecasas, P. (1981) Fed. Proc. Fed. Am. Soc. Exp. Biol. 40: 2729–2734
Coupar, J. M. (1978) Br. J. Pharmacol. 63: 57–63
Dobbins, J., Racusen, L., Binder, H. (1980) J. Clin. Invest. 66: 19–28
Gaginella, T. S., Rimele, T. J., Wietecha, M. (1983) J. Physiol. (London) 335: 101–111
Gaginella, T. S., O'Dorisio, T. M., Hubel, K. A. (1981) Regulatory Peptides 2: 165–174
Gaginella, T. S., O'Dorisio, T. M. (1979) in: Binder H. J. (ed.) Mechanisms of Intestinal Secretion, Vasoactive Intestinal Polypeptide: Neuromodulator of Intestinal Secretion? Alan Liss, Inc., New York, NY.
Hardcastle, J., Hardcastle, P. T., Redfern, J. S. (1982) J. Pharm. Pharmacol. 34: 68
Hendrix, T. R., Bayless, T. M. (1970) Ann. Rev. Physiol. 32: 139–164
Hubel, K. A. (1977) Proc. Soc. Exp. Biol. Med. 154: 41–44
Isaacs, P. E. T., Corbett, C. L., Riley, C. L., Hawker, P. C., Turnberg, L. A. (1976) J. Clin. Invest. 58: 535–542
Kachur, J. F., Miller, R. J., Field, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77: 2753–2756
Kachur, J. F., Miller, R. J. (1982) Eur. J. Pharmacol. 81: 177–183
McKay, J. S., Linaker, B. D., Turnberg, L. A. (1981) Gastroenterology 80: 279–284
Miller, R. J., Kachur, J. F., Field, M. (1981) Ann. N.Y. Acad. Sci. 372: 571–593
Powell, D. (1981) Gastroenterology 80: 406–408
Tidball, C. S. (1961) Am. J. Physiol. 200: 309–312
Wu, Z. C., Kisslinger, S. D., Gaginella, T. S. (1982) J. Pharmacol. Exp. Ther. 221: 664–669

J. Pharm. Pharmacol. 1983, 35: 825–827
Communicated April 19, 1983

© 1983 J. Pharm. Pharmacol.

Rapid induction of morphine dependence in the mouse by means of a modified pellet implantation

P. B. St. Leger*, N. A. Armstrong, P. S. J. Spencer, *The Welsh School of Pharmacy, UWIST, Cardiff CF1 3NU, U.K.*

The development of tolerance to and dependence upon the opiates is thought to depend upon the dose and frequency of drug administration. The ability to achieve persistent concentrations of opiates in target tissues during the processes of change and adaptation which underlie tolerance and physical dependence, is thought to be essential. The wide variation in the degree of tolerance to and dependence on opiates in the literature may be explained primarily by the many different methods used and their varying success in ensuring continuing exposure to the opiate during the preparative period. Many laboratory methods have been tested, ranging in complexity from simple but frequent multiple injections of opiate over days or weeks, to the implantation of osmotic mini-pumps (Gianutsos et al 1974; Wei & Loh 1976).

One of the most widely used methods of producing tolerance or dependence (or both) to morphine in laboratory animals is that of implantation of a tablet or pellet. First described by Maggiolo & Huidobro (1961), the pellet has been used extensively by Way et al (e.g. 1968, 1969) who used a pellet formulation described by Gibson & Tingstad (1970). Whilst we found this formulation satisfactory for inducing tolerance and dependence in rats, it was not so in mice. In successive experiments in mice, pellet implantation was followed by death within a few hours in 40 to 85% of animals, suggesting too rapid a release of morphine. Similar problems with different strains of mice have been reported by Brase et al (1977) and have led to the production of alternative 'pellets' (Hui & Roberts 1975). Some investigators have used a silicone-based delivery system, thereby avoiding tableting procedures (Isom et al 1978; Riffee et al 1980).

We describe modifications to the method of formulation equally useful in both mouse and rat studies.

Materials and methods

The following powders were used as received: anhydrous morphine base (Macfarlane Smith Ltd), microcrystalline cellulose (Avicel, Honeywell & Stein, Ltd),

* Correspondence.