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MODULATION OF NON-ADRENERGIC NON-CHOLINERGIC INHIBITORY TRANSMISSION IN RAT DUODENUM: ROLE OF OPIATES AND 5-HYDROXYTRYPTAMINE

A. POSTORINO[1], R. SERIO[1], F. MULÈ[1], E. B. ADAMO[2], G. DI GIOVANNI[1] AND R. MARINI[2]

[1] *Dipartimento di Biologia Cellulare e dello Sviluppo, Laboratorio di Fisiologia Generale, Corso Tukory, 129, 90134 Palermo, Italia*
and [2] *Istituto Pluridisciplinare di Fisiologia umana, Via T. Cannizzaro, 278. 98100 Messina, Italia*

INTRODUCTION

It is known that morphine and opioid drugs affect gastrointestinal motility although data reported in literature show some apparent discrepancies depending on the animal species, the experimental conditions and the opioid agonists used (5, 10). Opioid receptors have been demonstrated throughout the gastrointestinal tract of different species and their presence on neuronal elements of the enteric nervous system suggests a possible role of opiate peptides as neurotransmitters or neuromodulators (9, 12). There is evidence that opioids may interfere with the release of transmitters such as acetylcholine (Ach) and 5-hydroxytryptamine (5-HT) from enteric cholinergic neurons (13, 15, 21) and this finding may explain some of the opioid effects on gastrointestinal motility. In the gut an important nervous pathway in the control of motility is represented by the non-adrenergic non-cholinergic (NANC) nerves. Since our previous research indicated that in the rat duodenum motility is mainly controlled by a NANC inhibitory neuronal system (16, 19), in this study we intended to examine: *i*) whether opioids could interfere with the NANC system in the control of rat duodenal motility, and *ii*) the possible action mechanism involved.

METHODS

Male Wistar rats (250-300 g) were killed by cervical dislocation. A duodenal segment of about 2 cm length was quickly removed and placed in a 5 ml thermostatically controlled organ bath continuously perfused with Krebs solution bubbled with 5% CO_2 in oxygen. The composition of the Krebs solution was the following (mM): NaCl 119, KCl 4.5, $MgSO_4$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1. The experimental set-up used for isometric-isovolumic recordings of duodenal mechanical activity was the same as previously described (19). Intestinal segments were distended with 0.3-0.5 ml physiological solution and pre-loaded with 2 g and a 30 min equilibration period elapsed before starting the experiment. Electrical field stimulation (EFS) was performed by stimuli (0.5 ms, 2-30 pps, supramaximal voltage) in 5s trains applied from a GRASS S88 stimulator via two

platinum ring electrodes (1 cm apart). EFS was performed at intervals of at least 10 min. The preparation was challenged with 1 μM noradrenaline (NA) until a constant response was achieved. Agonists were added to the bath after switching off the perfusion for the testing period (4 min). Antagonists were perfused for at least 15-20 min before agonists were tested. The experimental set-up used allowed us to record the duodenal mechanical activity as changes in both endoluminal pressure and isometric force. All data are given as mean ± SE: n indicates the number of animal preparations. Statistical tests were applied according to the procedure given by Diem (6). Differences were assumed to be significant when tests gave probability levels $<5\%$.

Drugs used were: atropine sulphate, guanethidine sulphate, 5-hydroxytryptamine (5-HT), noradrenaline hydrochloride (NA), tetrodotoxin (TTX), dynorphin 1-13 (DYN); [D-Ala, N-Me-Phe-Gly-ol] enkephalin (DAGO), [D-Pen^{2,5}] enkephalin (DPDPE), naloxone and MR 2266. All drugs were obtained from Sigma except TTX (Calbiochem), guanethidine (generously supplied by Ciba) and MR 2266 (a gift from Boehringer Ingelheim). Stock solutions were prepared in distilled water and kept frozen at -20 C. Fresh drug solutions were made up daily and stored on ice for the duration of the experiment. To reduce adhesion of opioids to the organ bath, walls were siliconized. Care was taken to avoid light exposure of NA and 5-HT.

RESULTS

Rat isolated duodenal segments showed spontaneous mechanical activity which persisted to pretreatment with TTX or atropine and guanethidine (16). EFS, both in the absence and in the presence of atropine (1 μM) and guanethidine (1 μM) invariably induced a TTX (1 μM)-sensitive response of duodenal muscle. This response, clearly due to the intramural NANC nerve activation, was detectable, in our experimental set-up, as a fall both in endoluminal pressure and isometric tension and it was followed by a rebound contraction (inner inserts of Fig. 1). Since no difference was observed in the response obtained in the presence or in the absence of atropine and guanethidine, these drugs were added to the perfusing solution at the concentration of 1 μM at the beginning of each experiment. The relationships between the stimulus frequency and the amplitude of fall in endoluminal pressure and in isometric tension in response to EFS are shown in Fig. 1. The amplitude of the relaxant response was frequency-dependent reaching the maximum at 30 pps (14.4 ± 0.6 cm H_2O ($n=12$) and 3.85 ± 0.3 g; $n=12$). So, we chose to test opiates on the maximal NANC relaxation induced by EFS with a train of 30 pps.

Opioid peptides tested were DAGO, DPDPE and DYN agonists to μ, δ and k receptors, respectively. These drugs failed to change spontaneous activity and basal tone during the 4 min testing period and the relaxation to 1μM NA was not affected by opioids even at the highest concentrations used. Opioids caused a dose-dependent increase in the amplitude of the response to EFS with a threshold concentration of about 0.001 nM. The maximum effect was reached at a concentration of 1μM for all of the three agonists (data are reported in Table 1). Fig. 2 shows the dose-response curves to each tested opioid for pressure and tension, respectively. The statistical analysis of the calculated median effective dose (ED_{50})

Table 1. — *Effects induced in rat duodenum by maximal dose of opioid peptides on pressure and tension responses to EFS performed at 30 pps.*
All data are expressed as mean values ± S. E. from 5 different animal preparations. * significantly different from control value.

	Pressure (cm H_2O)	Tension (g)
Control	14.35 ± 0.3	3.82 ± 0.2
DAGO (1 μM)	20.73 ± 0.5*	5.63 ± 0.1*
Control	14.34 ± 0.1	3.84 ± 0.5
DPDPE (1 μM)	20.02 ± 0.1*	5.49 ± 0.1*
Control	14.30 ± 0.2	3.84 ± 0.2
DYN (1 μM)	20.35 ± 0.9*	4.60 ± 0.1*

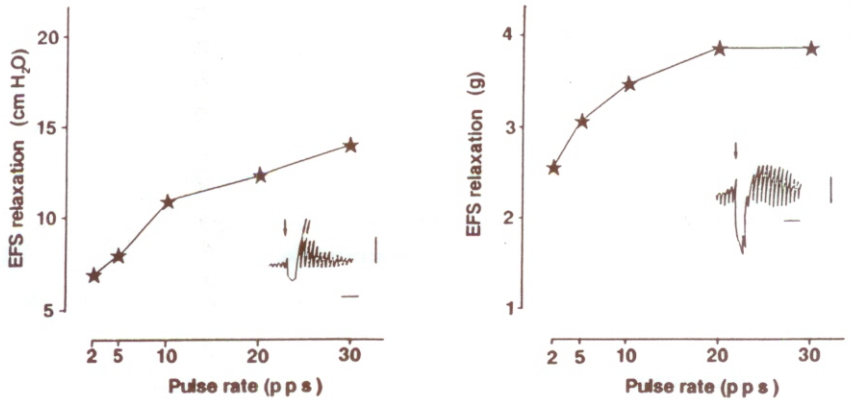

Fig. 1 – *Relationship between stimulus frequency and amplitude of EFS evoked fall both in endoluminal pressure (left panel) and in isometric tension (right panel).*

Note that maximal amplitude of the response was reached at 30 pps (14.4 ± 0.6 cm H_20; 3.8 ± 0.3 g; n = 12). Vertical bars: 15 cm H_20 and 2 g. Horizontal bar: 1 min.

values for each response curve pointed out that no significant difference was found when ED_{50} values for pressure and tension curves to DAGO or DPDPE or DYN were compared (Fig. 3). Moreover, a rank order of potency for the different opioids tested was found being DPDPE > DAGO > DYN. Naloxone, an opioid antagonist which preferentially acts at μ receptors but is also antagonist at δ and k receptors, perfused at 0.1 μM concentration, did not change the spontaneous mechanical activity and it failed to modify NA-and EFS-induced relaxation. Moreover, naloxone pretreatment antagonized the effects induced by the opioid peptides. It shifted to the right the concentration-response curves to each opioid peptide, significantly enhancing the ED_{50} values (Figs. 2 and 3). DYN induced effects on EFS response were tested also after pretreatment with MR 2266 which is considered a specific k receptor antagonist (2). As shown in Fig. 4, the dose-response curve to DYN was antagonized by 1 μM MR 2266 and the calculated ED_{50} values were significanty modified (from $5.1 \pm 0.9 \times 10^{-9}$M to $1.1 \pm 0.1 \times 10^{-8}$M and from

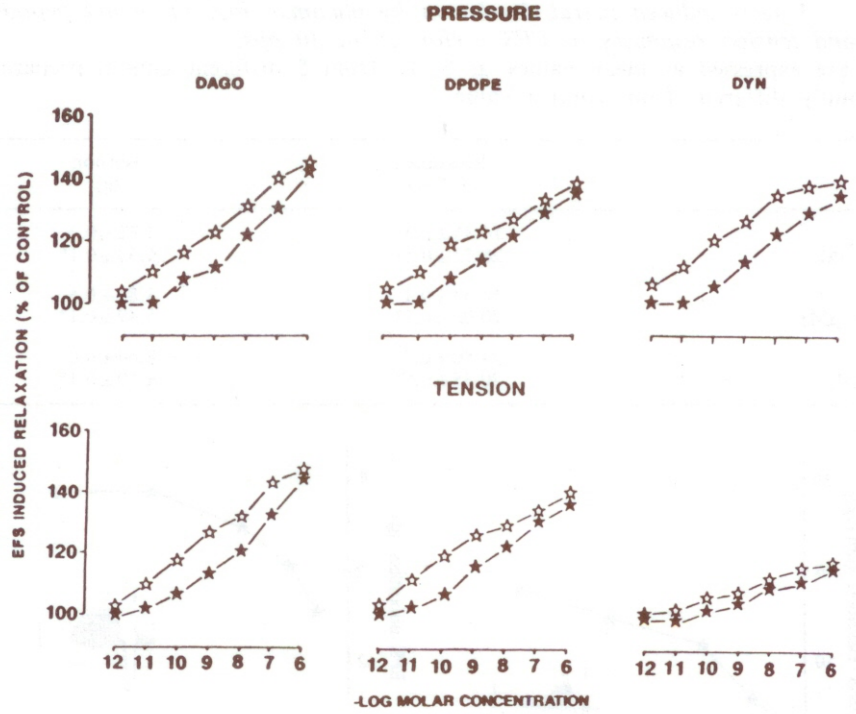

Fig. 2 – *Dose response curves to the tested opioids alone (empty symbols) and after 0.1* μM naloxone pretreatment (full symbols) for pressure and tension.

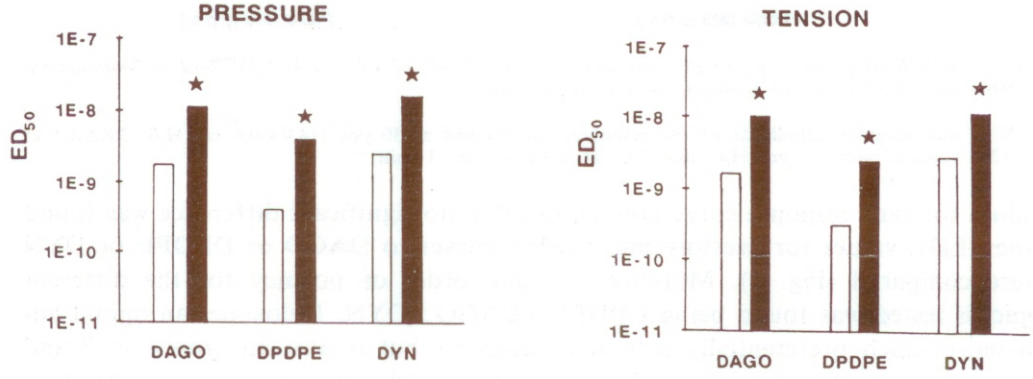

Fig. 3 – *Calculated ED_{50} values for opioid-induced effects on EFS relaxatory response for pressure and tension before (empty columns) and after naloxone pretreatment (full columns).*

Asterisks indicate a significant difference.

$2.2 \pm 0.4 \times 10^{-9}$M to $1.4 \pm 0.4 \times 10^{-8}$M for pressure and tension respectively).

Furthermore, we evaluated the possible involvement of the 5-hydroxytryptaminergic system in the effects induced by opioids on NANC relaxation of rat duodenum. Since no significant difference was found in the observed effects induced by opioids

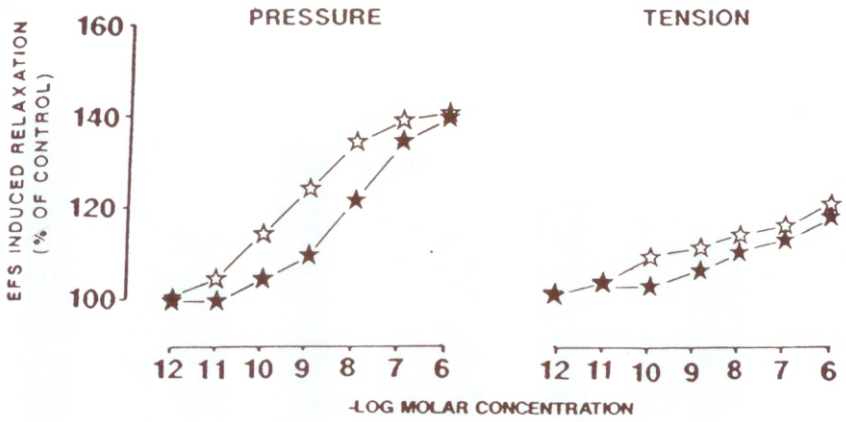

Fig. 4 – *Dose-response curve to DYN before (empty symbols) and after 1μM MR 2266 (full symbols) pretreatment for pressure and tension.*

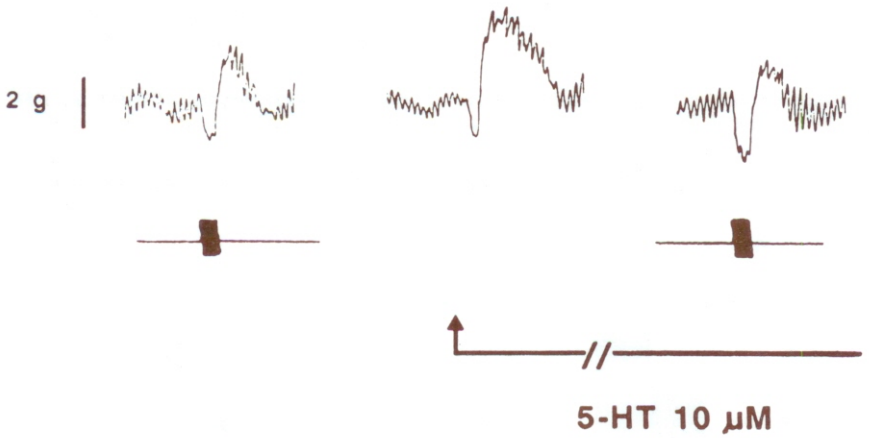

Fig. 5 – *5-HT induced effects on rat duodenal muscle.*

Left trace: control EFS induced relaxation. Central trace: 5-HT, *per se*, evoked relaxation in the presence of atropine and guanethidine. Right trace: 5-HT induced potentiation of response to EFS. Vertical bar: 2 g.

on pressure and tension, only the results about isometric tension will be presented. 5-HT (0.01 μM-10 μM) *per se*, in the presence of atropine (1 μM) and guanethidine (1 μM) caused a dose-dependent, transient relaxation of duodenal muscle which was TTX-sensitive (Fig. 5). Moreover, 5-HT caused an increase in the response to EFS (Fig. 5) which was abolished by the desensitization of 5-HT receptors, achieved by exposing the preparation to high doses of 5-HT (10 μM) continuously for 1 h until duodenum was unresponsive to 5-HT (10 μM) (Fig. 6). Naloxone pretreatment at the 0.1 μM concentration, which antagonized opioid-induced effects on NANC relaxation, failed to modify 5-HT induced effects on NANC response (Fig. 6). Lastly, the effects induced by maximal dose of opioids on EFS-induced relaxation were abolished by 5-HT receptor desensitization (Fig. 7).

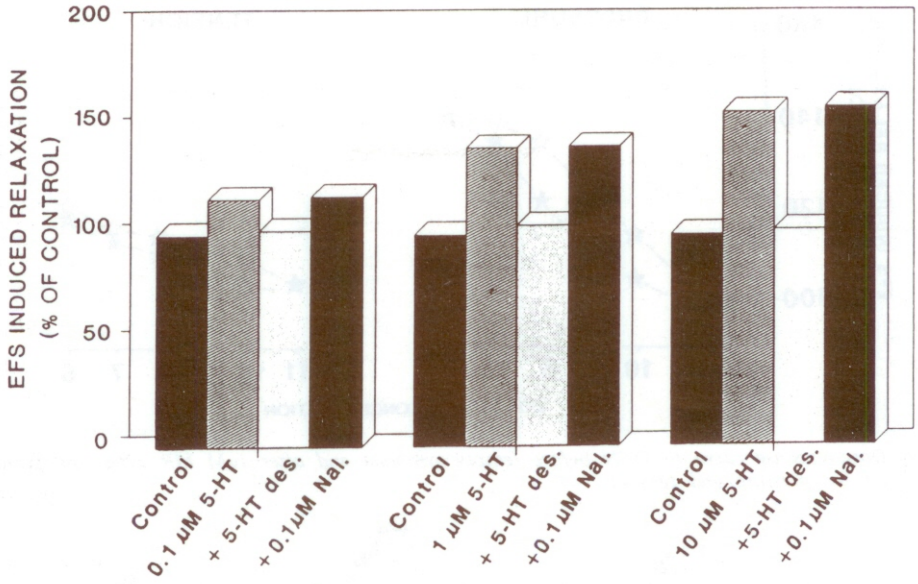

Fig. 6 – *Histogram related to potentiating effects of different concentrations of 5-HT on EFS induced relaxation alone, after 5-HT receptor desensitization and after 0.1 μM naloxone pretreatment.*

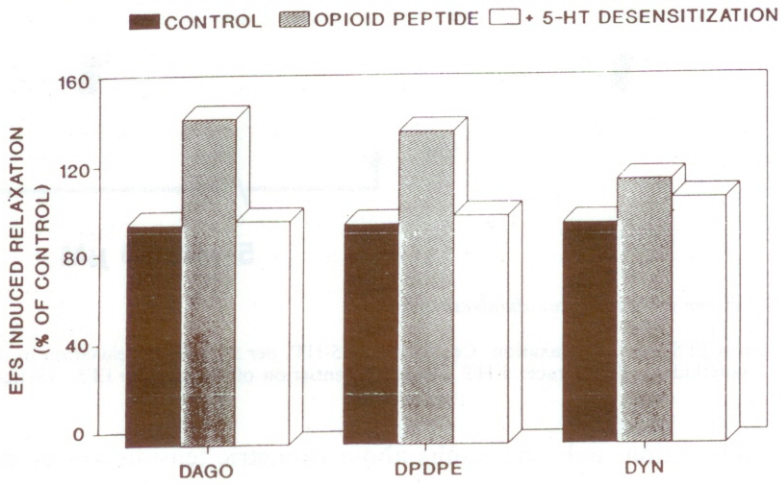

Fig. 7 – *Histogram related to 5-HT receptor desensitization effect on 1μM opioid potentiation of EFS induced relaxation.*

DISCUSSION

Electrical field stimulation (EFS) in rat duodenum constantly results in a transient relaxation followed by a rebound contraction. The frequency of stimulation affects the amplitude of the evoked response, but it fails to discriminate between relaxant and contractor responses. The relaxant response, which persisted unchanged in

the presence of atropine and guanethidine, but was abolished by TTX, is due to NANC intramural nerve activation.

Results from the present study demonstrate that opioids can modulate the NANC inhibitory intramural neurons. In fact, the amplitude of the relaxation in response to EFS was enhanced in the presence of DAGO, DPDPE and DYN. This effect is specifically due to opioid receptor activation since it was competitively antagonized by the pure opioid antagonist naloxone. We could not reproduce direct muscular response to opioids, as described at least for met-enkephalin, in rat duodenum by Furokawa *et al.* (8). One possible explanation for the absence, in our experiments, of a direct effect of opioids on muscular cells might be that the glucose concentration in our Krebs solution (11 mM) is high enough to suppress the direct response to opiates in a similar way to that described by Shook *et al.* (20). The lack of effect of opioids on muscular cell activity and on the response to NA, indicates that, in our experimental conditions, they do not change muscular cell responsiveness acting just at a neuronal level where they increase NANC mediator release.

It has been reported that μ, δ and k receptors are responsible for the effects induced by opioids at peripheral level (14). The ability of all three tested opioid peptides to increase the NANC response to EFS confirms the presence of μ, and k receptors, although the rank order of potency of opioids we found might indicate a main involvement of δ receptors. DYN, besides being a preferred agonist at k receptors, could act also at μ and δ receptors (2). So, the potentiating effects of DYN do not necessarily indicate that k receptors are present within rat duodenal wall. Results from Mr 2266, a more specific k receptor antagonist (2), allow us to confirm the existence of k receptors involved in the modulation of NANC transmission in rat duodenum.

In rat duodenum, opioids enhance NANC neurotransmitter release and a similar mechanism of action has been proposed to explain inhibitory effects induced by meperidine on opossum LES (18) and by morphine in circular muscle cell of canine jejunum (11). The lack of effect of naloxone on the amplitude of the NANC response to EFS indicates that there is no endogenous tonic release of opiates. The observation that 5HT, in the presence of atropine and guanethidine, elicits a TTX-sensitive relaxation, provides evidence for a stimulant action of this drug on NANC inhibitory neurons. This finding is further confirmed by the increase of the NANC relaxation to EFS induced by 5HT. Relaxant response to 5-HT and the potentiating effect of 5-HT on NANC response fail to occur after 5-HT receptor desensitization indicating that this drug acts on specific 5-HT receptors located on intramural nerves. The presence of 5-HT receptors on NANC intramural inhibitory neurons has been described in the opossum LES (17) and in guinea pig and mouse stomach (1).

Evidence has been reported that intestinal effect of morphine and opiates can be mediated by the release of endogenous 5-HT (3,4) and our results confirm this hypothesis. In fact, in rat duodenum, 5-HT receptor desensitization prevents the potentiating effects of opioids on NANC relaxation evoked by EFS. So, it

is possible to hypothesize that opioids mobilize intestinal 5-HT which in turn stimulates neuronal 5-HT receptors. Our results cannot provide any information about the source of 5-HT.

Although opioids exert an overall constipating action in all mammalian species, the action mechanism (either central or peripheral) may be quite different from one species to another, so it is difficult to compare our results with those obtained in different preparations. The majority of the peripheral actions in gut seem to be a neuromodulation causing an increase or a decrease in neurotransmitter release from enteric neurons (5). A well-documented neuronal effect of opiates is an impairment of cholinergic transmission due to a decrease in Ach release (7, 13). Since in rat duodenum the main nervous control of mechanical activity is represented by NANC inhibitory neurons, it seems likely that opiates act via a modulation of the NANC system. In rat duodenum, in fact, opiates have an inhibitory effect indirectly increasing the release of transmitter/s from inhibitory NANC enteric neurons. The functional link between opioids and NANC neurons is represented by 5-HT release.

SUMMARY

In rat duodenal segments *in vitro*, electrical field stimulation induced a TTX-sensitive relaxation in the presence of atropine and guanethidine. A correlation between the amplitude of the evoked response and stimulus frequency was observed. Opioid peptides DAGO, DPDPE and DYN caused a dose-dependent increase in the amplitude of the response to EFS. Naloxone shifted to the right the dose-response curves for each opioid peptide significantly enhancing the ED_{50} values. The amplitude of the response to EFS was enhanced, dose-dependently, also in the presence of 5-HT. Such an effect induced by 5-HT was prevented by 5-HT receptor desensitization, but persisted unchanged after naloxone pretreatment. Opioids failed to affect the response to EFS after 5-HT receptor desensitization. Results suggest that in rat duodenum opioids modulate NANC inhibitory neurotransmission, indirectly the release of 5-HT.

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REFERENCES

1. BULBRING, E. and GERSHON, M. D. 5-hydroxytryptamine participation in the vagal inhibitory innervation of the stomach. *J. Physiol., Lond.*, **192**: 832-846, 1968.
2. BURKS, T. F. Actions of drugs on gastrointestinal motility. Pp. 723-744. In: JOHNSON, L. R. (Ed.). *Physiology of the gastrointestinal tract.* Vol. I. New York, Raven Press, 1977.
3. BURKS, T. F. Mediation by 5-hydroxytryptamine in nerve muscle preparation from

the guinea pig small intestine and colon. *J. Pharmacol. Exp. Ther.*, **185**: 530-539, 1973.
4. BURKS, T. F. and LONG, J. P. Release of intestinal 5-hydroxytryptamine by morphine and related agents. *J. Pharmacol Exp. Ther.*, **156**: 267-276, 1967.
5. DANIEL, E. E., COLLINS, S. M., FOX, J. E. T. and HUIZINGA, J. D. Pharmacology of neuroendocrine peptides. Pp. 759-816. In: WOOD, I. D. and SHULTZ, S. G. (Eds.), *Handbook of Physiology*. Sec. 6: *The Gastrointestinal System Motility and Circulation*. Vol. 1. New York, Raven Press, 1989.
6. DIEM, K. *Wissenschaftliche Tabellen Geigy. Teilband Statistik. 8. Auflage*, Basel, Ciba-Geigy, 1980.
7. DUGGAN, A. W. and NORTH, R. A. Electrophysiology of opioids. *Pharmacol. Rev.*, **35**: 219-281, 1984.
8. FUROKAWA, T., NOMOTO, T. and TONOUE, K. Myogenic receptivity for methionine-enkephalin in rat duodenum: comparison with guinea pig duodenum. *Eur. J. Pharmacol.*, **66**: 129-132, 1980.
9. FURNESS, J. B. and COSTA, M. Types of nerves in the enteric nervous system. *Neuroscience*, **5**: 1-20, 1980.
10. FURNESS, J. B. and COSTA, M. Identification of gastrointestinal neurotransmitters. Pp. 383-462 In: BERTACCINI, G. (Ed.) *Mediators and Drugs in Gastrointestinal Motility*. Vol. I. Berlin, Springer Verlag, 1982.
11. GILBERT, R. J., SARNA, S. K. and HARDER, D. R. Effect of morphine on electrophysiological properties of circular and longitudinal muscles. *Amer. J. Physiol.*, **252**: G333-G338, 1987.
12. HUGHES, J., KOSTERLITZ, H. W. and SMITH, T. W. The distribution of methionine-enkephalin and leucine-enkephalin in the brain and peripheral tissue. *Br. J. Pharmacol.*, **61**: 639-647, 1977.
13. KOSTERLITZ, H. W. and WATERFIELD, A. A. In vitro models in the study of structure-activity relationships of narcotic analgesie. *Ann. Rev. Pharmacol.*, **15**: 29-47, 1975.
14. MILLER, R. J. and HIRNING, L. D. Opioid peptides of the gut. Pp. 631-660. In SHULTZ, S. G., WOOD, J. D. and RAUNER, B. B. (Eds.), *The Gastrointestinal System II, Handbook of Physiology*. Sec. 6: *The Gastrointestinal System Motility and Circulation*. Vol. II. New York, Raven Press, 1989.
15. PATON, W. D. M. The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea pig ileum. *Br. J. Pharmacol.*, **12**: 119-127, 1957.
16. POSTORINO, A., MANCINELLI, R., RACANICCHI, C., ADAMO, E. B. and MARINI, R. Spontaneous electromechanical activity in the rat duodenum, in vitro. *Arch. int. Physiol. Biochem.*, **98**: 35-40, 1990.
17. RATTAN, S. and GOYAL, R. K. Evidence of 5-HT participation in vagal inhibitory pathway to opossum LES. *Amer. J. Physiol.*, **234**: E273-E276, 1978.
18. RATTAN, S. and GOYAL, R. K. Identification and localization of opioid receptors in the opossum lower esophageal sphincter. *J. Pharmacol. Exp. Ther.*, **224**: 391-397, 1983.
19. SERIO, R., MULÉ, F., ADAMO, E. B. and POSTORINO, A. Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic, nerves in rat duodenum. *Eur. J. Pharmacol.*, **182**: 487-495, 1990.
20. SHOOK, J. E., KACHUR, J. F., BRASE, D. A. and DEWEY, W. A. Morphine dependence and diabetes. II. Alterations of normorphine potency in the guinea pig ileum and mouse vas deferens and of ileal morphine dependence by changes in glucose concentration. *J. Pharmacol. Exp. Ther.*, **237**: 848-852, 1986.
21. SZERB, J. C. Correlation between acetylcholine release and activity in the guinea pig ileum myenteric plexus: effect of morphine. *Neuroscience*, **7**: 327-340, 1982.