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### Research Article

The presence of enkephalins in the intestine and the use of opiates to treat diarrheal diseases suggests that enkephalins may affect intestinal ion transport. Using isolated rabbit ileal mucosa, we found that leucine enkephalin, methionine enkephalin, and D Ala2-methionine enkephalin amide (D Ala2-Met E) decreased the short circuit current (Isc) and potential difference although the effect of D Ala2-Met E was more pronounced and prolonged. D Ala2-Met E increased net sodium (+1.27  $\pm$  0.5  $\mu$  eq/cm<sup>2</sup>h), and chloride absorption (+2.33  $\pm$  0.4), and increased tissue conductance by 37%. Although the effect of enkaphalin on ion transport is opposite that of cyclic AMP, D-Ala2-Met had no effect on basal or vasoactive intestinal polypeptide-stimulated cyclic AMP levels. The effect of D-Ala2-Met E on Isc was blocked by naloxone, suggesting the involvement of specific opiate receptors. Tetrodotoxin completely blocked the decrease in Isc induced by D-Ala2-Met E but not by epinephrine, inferring that enkephalins are preganglionic neurotransmitters. The effect of D-Ala2-Met E on Isc was not blocked by phentolamine, haloperidol, or pretreatment of animals with 6-hydroxydopamine, suggesting that enkephalin does not affect the Isc by stimulating the release of alpha-adrenergic or dopaminergic agonists. D-Ala2-Met E also decreased the Isc in the presence of carbachol and bethanechol, indicating that enkephalin does not inhibit the release of acetylcholine. Further, up to 10  $\mu$  M atropine [...]

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# Effect of D-Alanine Methionine Enkephalin Amide on Ion Transport in Rabbit Ileum

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**ABSTRACT** The presence of enkephalins in the intestine and the use of opiates to treat diarrheal diseases suggests that enkephalins may affect intestinal ion transport. Using isolated rabbit ileal mucosa, we found that leucine enkephalin, methionine enkephalin, and D-Ala<sub>2</sub>-methionine enkephalin amide (D-Ala<sub>2</sub>-Met E) decreased the short circuit current ( $I_{sc}$ ) and potential difference although the effect of D-Ala<sub>2</sub>-Met E was more pronounced and prolonged. D-Ala<sub>2</sub>-Met E increased net sodium ( $+1.27 \pm 0.5 \mu\text{eq}/\text{cm}^2\text{h}$ ), and chloride absorption ( $+2.33 \pm 0.4$ ), and increased tissue conductance by 37%. Although the effect of enkephalin on ion transport is opposite that of cyclic AMP, D-Ala<sub>2</sub>-Met E had no effect on basal or vasoactive intestinal polypeptide-stimulated cyclic AMP levels. The effect of D-Ala<sub>2</sub>-Met E on  $I_{sc}$  was blocked by naloxone, suggesting the involvement of specific opiate receptors. Tetrodotoxin completely blocked the decrease in  $I_{sc}$  induced by D-Ala<sub>2</sub>-Met E but not by epinephrine, inferring that enkephalins are preganglionic neurotransmitters. The effect of D-Ala<sub>2</sub>-Met E on  $I_{sc}$  was not blocked by phentolamine, haloperidol, or pretreatment of animals with 6-hydroxydopamine, suggesting that enkephalin does not affect the  $I_{sc}$  by stimulating the release of  $\alpha$ -adrenergic or dopaminergic agonists. D-Ala<sub>2</sub>-Met E also decreased the  $I_{sc}$  in the presence of carbachol and bethanechol, indicating that enkephalin does not inhibit the release of acetylcholine. Further, up to 10  $\mu\text{M}$  atropine had no effect on the  $I_{sc}$ . These studies demonstrate that enkephalins stimulate intestinal ion transport and may do so by stimulating (or inhibiting) the release of a nonadrenergic, noncholinergic neurotransmitter.

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## INTRODUCTION

In 1975, Hughes et al. (1) isolated and identified two pentapeptides from the brain that bind specifically with opiate receptors and are presumed to be natural agonists for these receptor sites. These pentapeptides, the enkephalins, differ only in their NH<sub>2</sub>-terminal amino acid, one being leucine, the other, methionine. Leucine enkephalin and methionine enkephalin have subsequently been detected in peripheral tissue. In 1976, Smith et al. (2) detected enkephalins in rabbit and guinea pig ileum. More recent study has shown that endogenous opiates appear to be released from a guinea pig ileal muscle preparation in response to electrical field stimulation (3) or prolonged distension (4). Methionine enkephalin has been found throughout the gastrointestinal tract of humans; the largest quantities being present in the antrum of the stomach (5). Most studies of the effect of enkephalins in the intestine have been directed at smooth muscle function, and these peptides, like classic opiates, inhibit smooth muscle contractility (1, 3, 4, 6). The presence of enkephalin in the intestine, however, suggests that enkephalins may affect other functions such as ion transport. To separate the effect of enkephalins on motility from those on ion transport, we used an in vitro technique in which the serosal and muscular layers were removed from the intestine, leaving only the mucosa and submucosa. In this study, we demonstrate that the enkephalins increase Na and Cl absorption in the rabbit ileum.

## METHODS

Male New Zealand rabbits weighing 2–3 kg were killed with intravenous pentobarbital or an air bolus and ileal tissue rapidly removed to a chilled (4°C) Ringer solution. The tissue was rinsed with iced Ringer solution, drawn over a pipette, and the serosal and muscle layers stripped from the mucosal tissue as described previously (7). Segments of the epithelial tissue were pinned between Lucite half-chambers and the chambers attached to mucosal and serosal reservoirs. These reservoirs contained 8–10 ml of Ringer solution (pH, 7.4)

of the following composition: Na, 140; Cl, 119.8; K, 5.2;  $\text{HCO}_3$ , 25; Mg, 1.2; Ca, 1.2;  $\text{H}_2\text{PO}_4$ , 0.4; and  $\text{HPO}_4$ , 2.4 mM, respectively. Mucosal solutions contained 10 mM mannitol; serosal solutions, 10 mM glucose. Na-free Ringer solution was prepared by equimolar replacement of Na in Ringer solution with choline chloride, and  $\text{HCO}_3$ -free or Cl-free Ringer solution by equimolar replacement with isethionate.

The electrical potential difference (PD)<sup>1</sup> across the mucosa was measured by calomel half-cell electrodes in 3 M KCl and monitored with a potentiometer. The spontaneous tissue PD was short-circuited by an automatic voltage clamp via Ag/AgCl<sub>2</sub> electrodes throughout the experiments, except for periods of 5–10 s every 5 min when the spontaneous PD was recorded. Conductance was calculated from the spontaneous PD and the short circuit current ( $I_{sc}$ ) according to Ohm's law, except when tissue PD was <0.8 mV. When this occurred, the tissue was clamped at -2.0 mV and the current required to produce this PD was used to calculate conductance.

Ion flux experiments were performed using <sup>22</sup>Na and <sup>24</sup>Na to measure bidirectional sodium fluxes in single tissues and <sup>36</sup>Cl to measure oppositely directed chloride fluxes on matched tissue pairs, as described previously (8). In addition, some studies were done using <sup>24</sup>Na and <sup>36</sup>Cl oppositely directed on matched tissue pairs from the same animal. Tissue pairs were discarded if conductance differed by >30%. Results with both methods were similar, and thus combined for this presentation. In ion flux experiments, an initial base-line 20-min flux period was collected beginning 50 min after mounting the tissue and addition of isotopes to the reservoir. After completion of this flux period, enkephalin was added only to the serosal reservoir, and 10 min later, a second 20-min flux period was sampled. Three tissue pairs were excluded from the analysis of <sup>36</sup>Cl flux because the  $I_{sc}$  decreased <15  $\mu\text{A}/\text{cm}^2$  after the addition of D-Ala<sub>2</sub>-methionine enkephalin amide (D-Ala<sub>2</sub>-Met E).

In experiments to determine cyclic AMP levels, rabbit ileum was removed and stripped of its serosal and muscular layers as described above. The mucosa was cut into small pieces weighing 30–50 mg and incubated in a 37°C Ringer solution for 20 min. D-Ala<sub>2</sub>Met E was then added and the incubation continued for another 5–20 min. The tissue was continuously oxygenated with 95% O<sub>2</sub>–5% CO<sub>2</sub> during the incubation. At the end of the incubation, the tissue was homogenized in an ether:ethanol (1:1) solution. Cyclic AMP was measured by the method of Brown et al. (9) and protein content was assayed by the method of Lowry et al. (10). Results are expressed as picomoles cyclic AMP per milligram protein.

Enkephalins were obtained from Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.; naloxone hydrochloride from Endo Laboratories, Garden City, N. Y.; tetrodotoxin from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; phentolamine mesylate as Regitine from Ciba-Geigy Corp., Pharmaceuticals Div., Summit, N. J.; phenoxybenzamine hydrochloride from Smith Kline and French Laboratories, Philadelphia, Pa.; epinephrine and 6-hydroxydopamine from Sigma Chemical Co., St. Louis, Mo.; and tyramine from City Chemical Corp., New York.

Results are expressed as mean  $\pm$  SEM. Statistical analysis was performed using the paired and unpaired Student's *t* test (11).

<sup>1</sup> Abbreviations used in this paper: D-Ala<sub>2</sub>-Met E, D-Ala<sub>2</sub>-methionine enkephalin amide;  $I_{sc}$ , short circuit current; M, mucosal; PD, electrical potential difference; S, serosal; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide.

## RESULTS

**Effect on PD,  $I_{sc}$ , Na, and Cl flux.** The effect of the naturally occurring peptides, leucine enkephalin and methionine enkephalin, and a synthetic analog of methionine enkephalin, D-Ala<sub>2</sub>-Met E on  $I_{sc}$  and PD was determined in the initial studies. There was considerable interanimal variation in magnitude of electrical response to the enkephalins. Leucine enkephalin and methionine enkephalin produced similar decreases in  $I_{sc}$ ; however, their effect was short lived and the  $I_{sc}$  quickly reverted to control levels (Fig. 1). In contrast, D-Ala<sub>2</sub>-Met E caused a more pronounced decrease in  $I_{sc}$  that did not quickly revert toward basal levels. The decrease in the  $I_{sc}$  induced by D-Ala<sub>2</sub>-Met E was frequently of sufficient magnitude to cause a reversal in the direction of the  $I_{sc}$  (Fig. 2). The PD also decreased after the addition of D-Ala<sub>2</sub>-Met E and, like the  $I_{sc}$ , frequently reversed with the mucosal side of the tissue becoming positively charged (Fig. 2). Tissue conductance increased by ~37%.

Despite its marked effect on PD and  $I_{sc}$ , 5  $\mu\text{M}$  D-Ala<sub>2</sub>-Met E did not reduce the change in PD or  $I_{sc}$  produced by either 2 mM theophylline (Fig. 2), an agent which stimulates electrogenic chloride secretion, or 10 mM glucose. The addition of 10 mM glucose

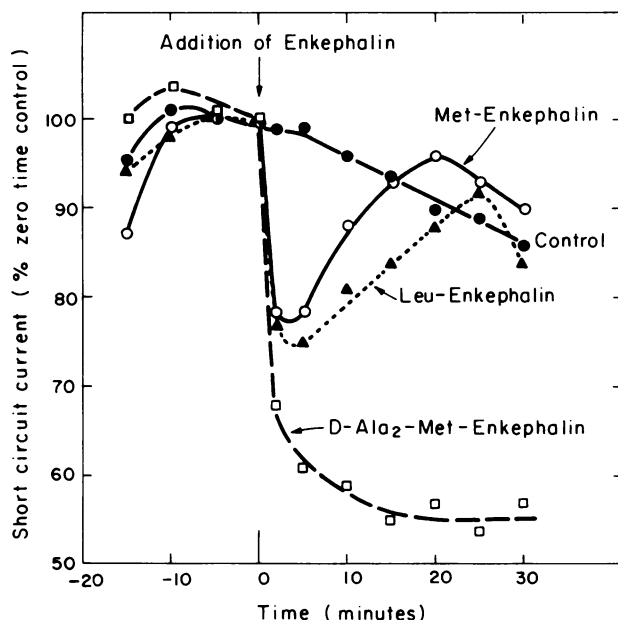


FIGURE 1 The effect of methionine enkephalin (○), leucine enkephalin (▲), D-Ala<sub>2</sub>-Met E (□), and no addition (●), on  $I_{sc}$  in the rabbit ileum. 10  $\mu\text{M}$  of the enkephalins were added to the serosal bathing solution at zero time (40 min after mounting the tissue). The  $I_{sc}$  is expressed as the percentage of the zero time  $I_{sc}$  (100%) for each group of tissues. For each group the value represents the mean of five tissues from five animals.

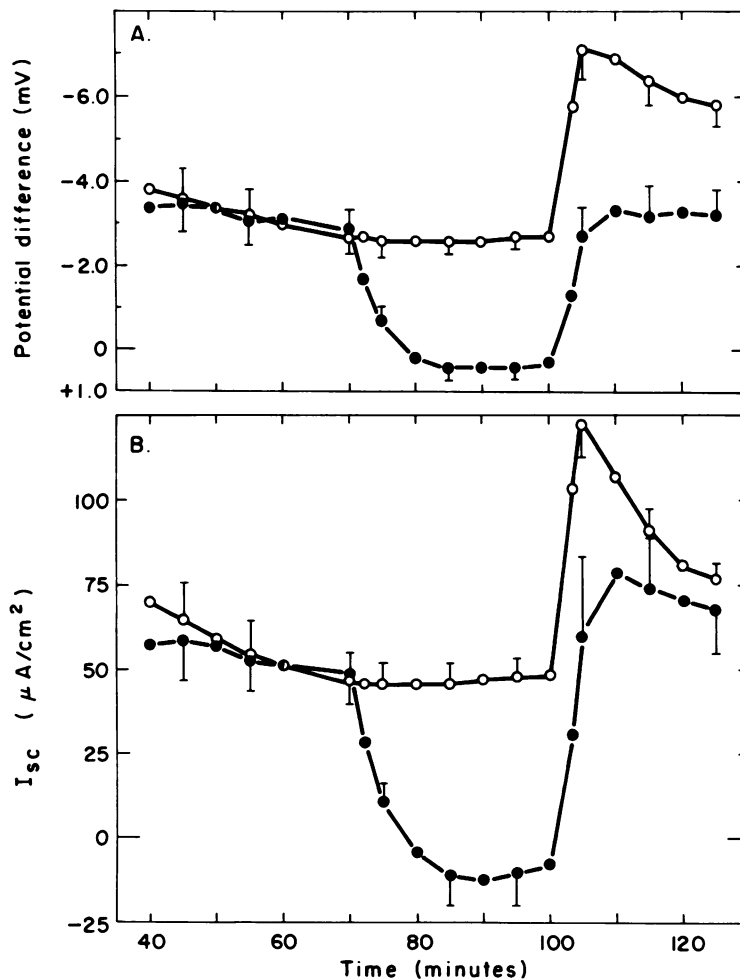


FIGURE 2 The effect of  $1 \mu\text{M}$  D-Ala<sub>2</sub>-Met E and PD and I<sub>sc</sub>. The time shown represents the length of time after mounting the tissue in the Ussing chamber. D-Ala<sub>2</sub>-Met E (●) was added to the serosal reservoir at 70 min and 2 mM theophylline was added to the serosal reservoir at 100 min to both control (○) and enkephalin-treated tissues. Results are expressed as mean  $\pm$  SEM of seven tissues from four animals.

increased PD and I<sub>sc</sub> by an average of  $2.3 \pm 0.1$  mV and  $87 \pm 9 \mu\text{A}/\text{cm}^2$  in enkephalin-treated tissues and  $2.0 \pm 0.2$  mV and  $60 \pm 6 \mu\text{A}/\text{cm}^2$  in control tissues ( $n = 4$ ). Fig. 3 depicts a dose-response curve to D-Ala<sub>2</sub>-Met E. A significant decrease in I<sub>sc</sub> was noted at 50 nM, ( $P < 0.001$ ), and a maximal response was observed at 5  $\mu\text{M}$ .

The effect of naloxone, a specific opiate antagonist, on the D-Ala<sub>2</sub>-Met E-induced decrease in I<sub>sc</sub> is illustrated in Fig. 4. Increasing concentrations of naloxone progressively inhibited the decrease in I<sub>sc</sub>. These concentrations of naloxone usually had no effect or produced a slight increase in the I<sub>sc</sub>, ( $\Delta\text{I}_{\text{sc}}$  after adding 1  $\mu\text{M}$  naloxone was  $+8 \pm 1 \mu\text{A}/\text{cm}^2$ ,  $n = 56$ ) however, occasionally (12% of tissues) naloxone would dramatically increase the I<sub>sc</sub> ( $>20 \mu\text{A}/\text{cm}^2$ ). Concentra-

tions of naloxone greater than 0.1 mM decreased the I<sub>sc</sub>.

Ion replacement experiments were performed to determine the ionic requirements for the decrease in the I<sub>sc</sub> (Table I). Because of interanimal variation in response to enkephalin, tissues from the same animal served as controls. Removal of Na reduced base-line PD and I<sub>sc</sub> to near zero and subsequent addition of D-Ala<sub>2</sub>-Met E produced no additional decrement in PD and I<sub>sc</sub>. Removal of  $\text{HCO}_3^-$  or  $\text{Cl}^-$  reduced the decrease in I<sub>sc</sub> produced by 5  $\mu\text{M}$  D-Ala<sub>2</sub>-Met E by 68 and 66%, respectively.

Results of ion flux studies are summarized in Table II. In control tissues (no additions between periods 1 and 2), there were no significant changes in net or unidirectional Na and Cl fluxes. In the experimental tis-

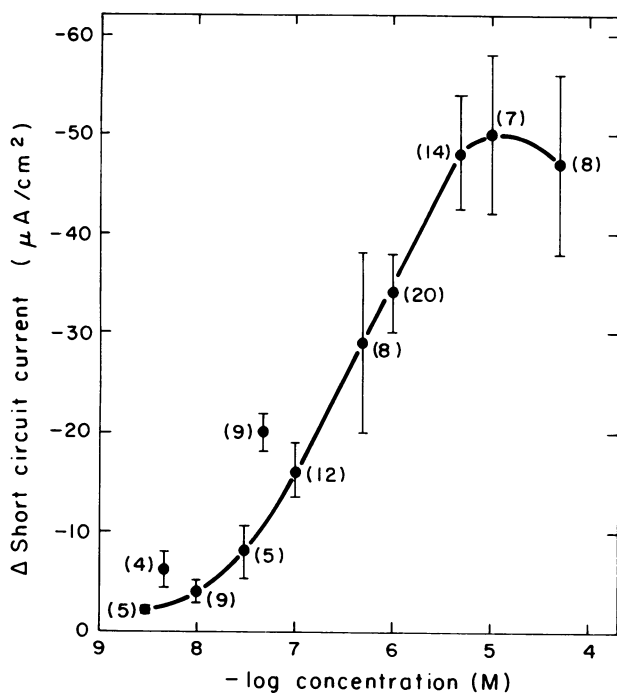


FIGURE 3 The effect of increasing concentrations of D-Ala<sub>2</sub>-Met E on  $I_{sc}$  in the rabbit ileum. D-Ala<sub>2</sub>-Met E was added to the serosal bathing solution 50 min after mounting the tissue. The maximum decrease in  $I_{sc}$  that occurred in the 20-min interval after the addition of D-Ala<sub>2</sub>-Met E was used for calculation. The numbers in parentheses are the number of tissues studied at each concentration.

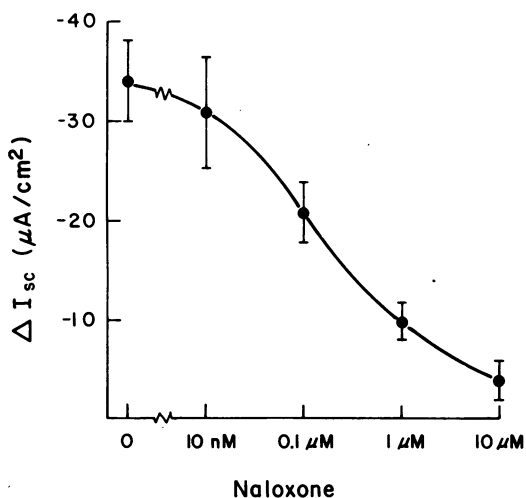


FIGURE 4 The effect of naloxone on D-Ala<sub>2</sub>-Met E induced decrease in  $I_{sc}$ . After the tissues were mounted and the  $I_{sc}$  had stabilized ( $\sim 50$  min) naloxone was added to the serosal bathing solution in the concentrations shown. 15 min later,  $1 \mu\text{M}$  D-Ala<sub>2</sub>-Met E was added to the serosal bath. The maximal decrease in  $I_{sc}$  that occurred during the next 15 min was recorded. Each point represents the mean  $\pm$  SE of at least 10 tissues.

TABLE I  
Effect of Ion Replacement on D-Ala<sub>2</sub>-Met E  
Induced Decrease in PD and  $I_{sc}$

	$\Delta\text{PD}$	$\Delta I_{sc}$	$n$
	mV	$\mu\text{A per cm}^2$	—
Control	$-1.45 \pm 0.13$	$-32.3 \pm 7.6$	6
Na-free Ringer	$-0.03 \pm 0.04$	$-0.7 \pm 1.8$	6
<i>P</i>	$<0.001$	$<0.001$	
Control	$-2.2 \pm 0.1$	$-54.2 \pm 7.4$	4
HCO <sub>3</sub> -free Ringer	$-0.8 \pm 0.2$	$-17.2 \pm 3.9$	6
<i>P</i>	$<0.001$	$<0.01$	
Control	$-3.2 \pm 0.4$	$-67.1 \pm 8.4$	6
Cl-free Ringer	$-1.3 \pm 0.1$	$-23.0 \pm 3.3$	5
<i>P</i>	$<0.01$	$<0.01$	

$5 \mu\text{M}$  D-Ala<sub>2</sub>-Met E was used in the Na-free Ringer experiments and  $1 \mu\text{M}$  D-Ala<sub>2</sub>-Met E in the anion replacement experiments. Na-free Ringer solution was prepared by equimolar replacement of Na in Ringer solution with choline and HCO<sub>3</sub>-free and Cl-free Ringer solution was prepared by equimolar replacement of these anions with isethenate. The maximum decrease in the PD and  $I_{sc}$  in the 20-min period following the addition of D-Ala<sub>2</sub>-Met E is recorded.  $n$  = number of tissues.  $P$  was determined using the unpaired  $t$  test.

sues,  $5 \mu\text{M}$  D-Ala<sub>2</sub>-Met E added between periods 1 and 2 produced a significant increase in net Na ( $+1.3 \pm 0.5 \mu\text{eq/cm}^2 \text{ h}$ ) and net Cl ( $+2.3 \pm 0.4 \mu\text{eq/cm}^2 \text{ h}$ ) absorption. The increases in net Na and Cl absorption were due to increases in the mucosal to serosal (M to S) movement of these ions and to a decrease in the serosal to mucosal (S to M) movements of Cl. The decrease in S to M Cl flux may be more important than appears when one considers that tissue conductance increased by 37%. (The increase in S to M Na flux may be due solely to the increase in tissue conductance.) Residual flux ( $J^R$ ) (which probably represents HCO<sub>3</sub> secretion) fell and reversed in enkephalin-treated tissues while remaining unchanged in controls.

A change in  $I_{sc}$  indicates a change in electrogenic ion transport. The decrease in  $I_{sc}$  induced by D-Ala<sub>2</sub>-Met E could either be the result of a relative decrease in net cation ( $\text{Na}^+$ ) absorption and/or a relative increase in net anion ( $\text{Cl}^-$  or  $\text{HCO}_3^-$ ) absorption. We therefore correlated changes in  $I_{sc}$  with changes in net ion flux. A linear correlation was found between the change in net Cl absorption and the decrease in  $I_{sc}$  between periods 1 and 2 in the enkephalin-treated animals ( $r = -0.703$ ,  $P < 0.01$ , Fig. 5). No correlation was found between net Na absorption and  $I_{sc}$  ( $r = 0.015$ ).

**Neurotransmitter studies.** Because enkephalins are found in central and peripheral nerve tissue (myenteric plexus) (2), their effect on ion transport may be neurally mediated. That is, the enkephalins may be preganglionic or postganglionic neurotransmitters. If

TABLE II  
Effect of 1  $\mu$ M D-Ala<sub>2</sub>-Met-E on Ion Transport in Rabbit Ileum

		J <sup>Na</sup>				J <sup>Cl</sup>			I <sub>sc</sub>	J <sup>R</sup>	G
		n	M to S	S to M		Net	n	M to S			
Controls											
Period 1	11	9.3±0.6	8.5±0.6	+0.8±0.7	15	7.4±0.6	7.7±0.4	-0.3±0.4	2.0±0.2	0.9±0.8	20.1±1.3
Period 2		10.1±0.6	9.3±0.6	+0.8±0.7		7.1±0.6	7.5±0.4	-0.4±0.5	2.1±0.2	1.0±0.9	21.0±1.4
Δ		+0.8±0.3	+0.8±0.3	-0.01±0.2		-0.3±0.2	-0.2±0.2	-0.1±0.3	+0.1±0.1	+0.1±0.4	+0.1±0.6
P		NS	NS	NS		NS	NS	NS	NS	NS	NS
D-Ala <sub>2</sub> -Met E											
Period 1	13	9.5±0.4	8.9±0.4	+0.6±0.3	14	6.9±0.4	8.0±0.4	-1.1±0.3	1.8±0.1	0.1±0.5	20.6±1.0
Period 2		12.6±0.8	10.7±0.7	1.9±0.6		8.3±0.5	7.0±0.3	1.3±0.5	0.0±0.3	-0.6±0.8	28.3±1.4
Δ		+3.1±0.6	+1.8±0.6	+1.3±0.5		+1.4±0.3	-1.0±0.3	+2.3±0.4	-1.8±0.2	-0.7±0.7	7.7±1.2
P		<0.001	<0.01	<0.05		<0.001	<0.05	<0.001	<0.001	NS	<0.001

Results are expressed as mean±SEM. Sodium flux (J<sup>Na</sup>), chloride flux (J<sup>Cl</sup>), I<sub>sc</sub>, and residual flux (J<sup>R</sup>) are expressed as micro-equivalents per square centimeter per hour, and conductance (G) as millimhos per square centimeter. Period 1 is a 20-min flux period sampled immediately before addition of D-Ala<sub>2</sub>-Met-E to the serosal solution of experimental tissues. Period 2 is a second 20-min flux period sampled following a 10-min equilibration period in experimental tissues. *n* represents tissues or tissue pairs for Na data, and tissue pairs for Cl data. *P* represents differences between period 1 and period 2 in control and in enkephalin-treated tissues, respectively. Three tissue pairs were excluded from analysis of <sup>36</sup>Cl flux because the I<sub>sc</sub> decreased <15  $\mu$ A/cm<sup>2</sup> after the addition of D-Ala<sub>2</sub>-Met E.

enkephalins are preganglionic neurotransmitters, their effect may be blocked by the neurotoxin, tetrodotoxin (TTX). When TTX alone was added to rabbit ileum, the I<sub>sc</sub> and PD decreased, as has been previously described (Table III) (12). The prior addition of TTX did not block a further decrease in I<sub>sc</sub> by epinephrine,

but prevented any further decrease in I<sub>sc</sub> by D-Ala<sub>2</sub>-Met E. Naloxone did not inhibit the decrease in I<sub>sc</sub> produced by TTX. (Data not shown.) These results suggest that D-Ala<sub>2</sub>-Met E may be functioning as a neurotransmitter.

If enkephalin is a preganglionic neurotransmitter, then it could be stimulating adrenergic nerve fibers ( $\alpha$ -adrenergic agonists have effects on ion transport similar to those of enkephalin) (13) or inhibiting cholinergic nerve fibers (cholinergic agonists stimulate net Na and Cl secretion) (14). To test the former possibility, we determined the effect of  $\alpha$ -adrenergic blockade on the enkephalin-induced decrease in I<sub>sc</sub>. Phentolamine alone, like TTX, also decreased the I<sub>sc</sub> (Table IV). However, pretreatment of the tissues with phentolamine inhibited the decrease in I<sub>sc</sub> produced by epinephrine, but not the decrease produced by D-Ala<sub>2</sub>-Met E. Furthermore, phentolamine partially reversed the decrease in I<sub>sc</sub> produced by epinephrine but only augmented the decrease in I<sub>sc</sub> produced by D-Ala<sub>2</sub>-Met E (Fig. 6).

Pretreatment of tissue for 50 min with 10  $\mu$ M phenoxybenzamine, another  $\alpha$ -adrenergic antagonist, significantly inhibited the decrease in I<sub>sc</sub> produced by D-Ala<sub>2</sub>-Met E (from  $-40\pm5$   $\mu$ A/cm<sup>2</sup> to  $-28\pm3$ , *P* = 0.05, *n* = 17). Because of the conflicting results obtained with these two  $\alpha$ -adrenergic antagonists, phentolamine and phenoxybenzamine, animals were pretreated with 6-hydroxydopamine in order to deplete their catecholamine stores. Pretreatment of rabbits with 6-hydroxydopamine 1 d before sacrifice inhibited the decrease in I<sub>sc</sub> induced by tyramine, an adrenergic nerve depolarizer, but had no effect on D-Ala<sub>2</sub>-Met E-induced

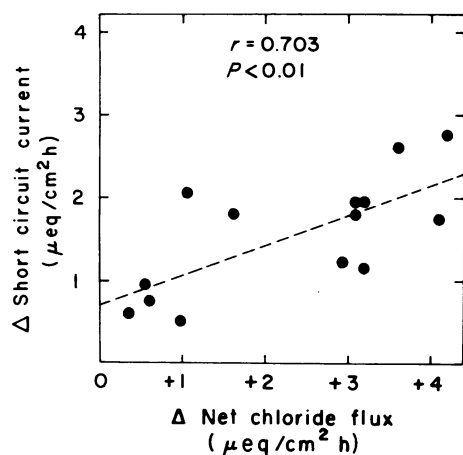


FIGURE 5 The correlation between the change in I<sub>sc</sub> and the change in net sodium flux and chloride flux after the addition of 5  $\mu$ M D-Ala<sub>2</sub>-Met E. The change in I<sub>sc</sub> after the addition of enkephalin is plotted on the ordinate and the simultaneous change in net sodium or chloride flux is plotted on the abscissa. Net sodium and chloride flux were determined as described in Methods (see Table II) by measuring oppositely directed <sup>22</sup>Na and <sup>24</sup>Na movement in a single tissue or oppositely directed <sup>24</sup>Na and <sup>36</sup>Cl movement in a matched tissue pair. The change in I<sub>sc</sub> is that occurring in the single tissue or the mean of the tissue pair.

TABLE III  
Effect of TTX on PD and  $I_{sc}$

Additions	Control tissues			TTX-treated tissues		
	$\Delta PD$	$\Delta I_{sc}$	$n$	$\Delta PD$	$\Delta I_{sc}$	$n$
	mV	$\mu A \text{ per cm}^2$		mV	$\mu A \text{ per cm}^2$	
—	+0.1±0.1	+1±4	14	-1.6±0.2*	-38±5*	19
Epinephrine	-2.2±0.5	-80±18	6	-1.5±0.3†	-51±11†	9
Enkephalin	-1.6±0.3	-47±7	8	-0.0±0.1*	-1±4*	10

1–3 × 0.1  $\mu M$  TTX was added to the serosal reservoir of the “TTX-treated” tissues 35–50 min after mounting the tissue. TTX produced the decrease in PD and  $I_{sc}$  shown on the first line whereas no change in PD or  $I_{sc}$  occurred in control tissues during the same time interval. 15 min after addition of TTX, 50  $\mu M$  epinephrine or 5  $\mu M$  D-Ala<sub>2</sub>-Met E was added to the serosal reservoir of both control and TTX-treated tissues. These concentrations of epinephrine and D-Ala<sub>2</sub>-Met E produce the maximal decrease in  $I_{sc}$  in control tissues. The change in PD and  $I_{sc}$  recorded was the greatest increase or decrease in these parameters in the 20-min period following the addition of epinephrine and enkephalin. 10 mM glucose was present in the mucosal solution of some tissues. Since these tissues responded no differently from tissues without mucosal glucose, the results were combined for analysis. *P* values are compared with control tissues.

\* *P* < 0.001.

† *P* > 0.1.

TABLE IV  
Effect of Pretreatment with Phentolamine and 6-Hydroxydopamine on Enkephalin-induced Decrease in  $I_{sc}$

	Change in $I_{sc}$		<i>P</i>	<i>n</i>
	Control	Treated		
	$\mu A/cm^2$			
Phentolamine*				
Control	+1±3	-35±6	<0.001	11
Epinephrine	-39±8	-8±2	0.01	6
Enkephalin	-37±9	-37±13	NS	4
6-Hydroxydopamine†				
Control	-8±3	-7±3	NS	5
Tyramine	-36±5	-14±2	<0.01	6
Enkephalin	-47±6	-41±6	NS	6

50  $\mu M$  phentolamine was added to the serosal bathing solution of the treated tissue 40–50 min after the tissue was mounted. Phentolamine produced a decrease in the  $I_{sc}$  shown on the first line. 15 min later, 0.1  $\mu M$  epinephrine of 5  $\mu M$  D-Ala<sub>2</sub>-Met E was added to the serosal solution of both control and phentolamine-treated tissues. The greatest change in  $I_{sc}$  over the next 20 min was then recorded. *P* value was determined using the unpaired *t* test. *n* = the number of tissues in each group.

Rabbits were injected with 6-hydroxydopamine (50 mg/kg body wt) intravenously the day before sacrifice. After sacrifice, the tissues were mounted in the Ussing chamber. After the  $I_{sc}$  had stabilized, no additions were made (control), or 5  $\mu M$  D-Ala<sub>2</sub>-Met E or 0.1 mM tyramine was added to the serosal reservoir, and the greatest decrease in  $I_{sc}$  in the 20-min period after the addition of these agents was recorded.

decreases in  $I_{sc}$  (Table IV). Pretreatment of rabbit ileum with 0.1 mM haloperidol, the dopaminergic blocking agent, or 0.1 mM hexamethonium, a nicotinic ganglionic blocker, had no effect on the enkephalin-induced decrease in  $I_{sc}$  (data not shown).

The enkephalins are known to inhibit the release of

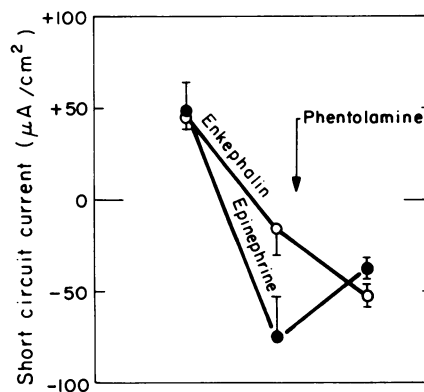


FIGURE 6 The effect of phentolamine on epinephrine- and enkephalin-induced decrease in  $I_{sc}$ . The  $I_{sc}$  just before the addition of D-Ala<sub>2</sub>-Met-E (○) or epinephrine (●), the decrease in  $I_{sc}$  in the 20-min period following the addition of these agents, and the change in  $I_{sc}$  induced by the further addition of phentolamine is shown. Both D-Ala<sub>2</sub>-Met E and epinephrine decreased the  $I_{sc}$ . Phentolamine partially reversed the decrease in  $I_{sc}$  induced by epinephrine but caused a further decrease in  $I_{sc}$  in the enkephalin-treated tissues. The concentrations were (a) D-Ala<sub>2</sub>-Met E, 5  $\mu M$ ; (b) epinephrine, 5–20  $\mu M$ ; and (c) phentolamine, 5–50  $\mu M$ . Six tissues were treated with enkephalin, then phentolamine and five tissues were treated with epinephrine, then phentolamine.

acetylcholine from brain and from electrically stimulated guinea pig ileal muscle (15, 16). If enkephalins were inhibiting the release of cholinergic agonists, then cholinergic antagonists should have produced a similar decrease in  $I_{sc}$  as D-Ala<sub>2</sub>-Met E. However, addition of up to 10  $\mu$ M atropine, a muscarinic blocker, to the serosal reservoir had no effect on  $I_{sc}$  (data not shown). Furthermore, addition of 1  $\mu$ M carbachol or 100  $\mu$ M bethanechol to the serosal solution did not inhibit the decrease in  $I_{sc}$  induced by D-Ala<sub>2</sub>-Met E (data not shown). Thus, in the presence of excess cholinergic stimulation, D-Ala<sub>2</sub>-Met E was still effective, further suggesting that it does not work solely by inhibiting the release of cholinergic agonists.

**Effect on cyclic AMP.** 0.1 and 1.0  $\mu$ M D-Ala<sub>2</sub>-Met E had no effect on basal tissue cyclic AMP levels after 5-, 10-, and 15-min incubations (Table V). Furthermore, 5  $\mu$ M D-Ala<sub>2</sub>-Met E did not inhibit the increase in tissue cyclic AMP levels induced by vasoactive intestinal polypeptide (VIP) although it slightly inhibited the increase induced by prostaglandin E<sub>1</sub> and theophylline.

## DISCUSSION

Opiates and related compounds have been used in the treatment of diarrheal diseases for many years. Opiates have long been known to inhibit intestinal motility and slow the transit time of luminal contents. It has been assumed that the antidiarrheal effect of opiates is

the result of this effect on transit time, because increased time of exposure to intestinal or colonic mucosa will result in increased fluid and electrolyte absorption (if net absorption is occurring in the tissue).

Although increased motility may play a role in some diarrheal disease, alterations of intestinal fluid and electrolyte transport processes are generally believed to be important factors in the pathophysiology of most diarrheal states. It is possible, therefore, that the mechanism of action of antidiarrheal agents may be secondary to a direct stimulatory effect on electrolyte absorption, in addition to any action on intestinal motor function. In this regard, we have recently demonstrated that codeine stimulates net Na and Cl absorption in the rabbit ileum (17).

The discovery of endogenous opioid pentapeptides and the demonstration of their presence in the intestine, suggested the possibility that the enkephalins may play a role in the physiologic control of ion transport in the intestine. Enkephalins are known to affect other intestinal functions. Enkephalins stimulate gastric acid secretion and inhibit pancreatic exocrine secretion (18, 19). Methionine enkephalin also augments histamine-induced gastric acid secretion and increases gastric mucosal blood flow (18).

In our initial studies, we found that methionine enkephalin, leucine enkephalin, and D-Ala<sub>2</sub>-Met E, a synthetic analog of the naturally occurring methionine enkephalin, all decreased the  $I_{sc}$  in the rabbit ileum (Fig. 1). However, the effect of methionine and leu-

TABLE V  
Effect of Enkephalin on Mucosal Tissue Cyclic AMP Levels

min .....	Time		
	5	10	15
Effect on basal cyclic AMP:			
Controls	13.5±0.8	10.5±1.5	13.4±0.8
Enkephalin (1 $\mu$ M)	13.1±1.0	13.2±0.8	12.6±1.5
Enkephalin (0.1 $\mu$ M)	12.8±1.1	14.8±1.8	10.6±1.2
	PGE <sub>1</sub> 0.1 mM	VIP 1 $\mu$ M	Theo (2 mM)
Effect on stimulated cyclic AMP:			
Control	34.8±2.2	22.7±1.0	26.2±2.0
Enkephalin (5 $\mu$ M)	27.8±1.3	21.9±1.0	19.6±1.5

Ileum was removed, stripped of its muscular layers, cut into 30–50-mg pieces and preincubated for 20 min at 37°C. 1  $\mu$ M or 0.1  $\mu$ M D-Ala<sub>2</sub>-Met E was added to the incubation flask of some of the tissues and the incubation continued an additional 5, 10, or 15 min. 0.1 mM prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), 1  $\mu$ M VIP or 2 mM theophylline (Theo) alone, or in addition to 5  $\mu$ M D-Ala<sub>2</sub>-Met E, was added to the incubation flask after the 20-min preincubation. The incubation was then continued another 5 min for PGE<sub>1</sub>, 10 min for VIP, and 20 min for theophylline. Results are expressed as picomoles cyclic AMP per milligram protein. Each result is the mean±SEM of 12 tissues, from four animals on basal cyclic AMP and three animals on stimulated cyclic AMP.



cine enkephalin on the  $I_{sc}$  was only transient. This may be because of the fact that the natural analogs are more susceptible to tissue proteases or that the synthetic analog has a greater affinity for the opiate receptor (20). Therefore, D-Ala<sub>2</sub>-Met E was used in subsequent studies. D-Ala<sub>2</sub>-Met E decreased  $I_{sc}$ , increased tissue conductance and increased net Na and Cl absorption. The effect on  $I_{sc}$  was seen with concentrations of enkephalin as low as 50 nM (Fig. 3). This concentration is compatible with a physiologic role for the enkephalins in intestinal ion transport. Furthermore, the observation that the effect of D-Ala<sub>2</sub>-Met E on  $I_{sc}$  was inhibited by naloxone, a specific opiate antagonist (Fig. 4), indicates that the interaction of enkephalins on ion transport requires specific opiate receptors and is further evidence of a potential physiologic role for these compounds.

The observation that low dose naloxone occasionally caused a dramatic increase in  $I_{sc}$  is intriguing and suggests that naloxone is blocking the effect of an endogenous opiate. Because this was an unusual occurrence, we thought it might be related to separating the muscle layer (and myenteric plexus) from the tissue. Therefore, we determined the effect of naloxone on unstripped preparations of ileum. However, naloxone-induced increase in  $I_{sc}$  was not seen more frequently in the unstripped than the stripped preparation (unpublished observation). Thus, we are not certain of the significance of the occasional increase in  $I_{sc}$  seen with low dose naloxone.

The presence of methionine enkephalin in the endocrine cells of intestinal mucosa and in the myenteric plexus suggests that the enkephalins may either have a paracrine effect or function via neural pathways. Our observation that TTX blocked the decrease in  $I_{sc}$  produced by enkephalin suggests that the effect of enkephalin involves neural pathways. However, TTX alone decreased  $I_{sc}$ , and TTX has been previously shown to stimulate net Na and Cl absorption in the rabbit ileum (13). Therefore, we cannot exclude the possibility that TTX is blocking an effect of enkephalin directly on the enterocyte. TTX does not appear to occupy opiate receptors, however, because its effect on  $I_{sc}$  is not blocked by naloxone. The ability of TTX to block the decrease in  $I_{sc}$  produced by D-Ala<sub>2</sub>-Met E is not nonspecific because it did not inhibit epinephrine-induced decreases in  $I_{sc}$ .

Although the TTX studies indicate that enkephalins may be preganglionic neurotransmitters, we were unable to demonstrate adrenergic or cholinergic involvement, further suggesting that the TTX effect may be directly on the enterocyte. At the present time, therefore, it is unclear whether the effect of enkephalin is mediated via neural pathways. If so, it seems to involve nonadrenergic, noncholinergic nerve fibers. It is

possible that enkephalins are affecting peptidergic nerve fibers. Enkephalins could be inhibiting the release of VIP or stimulating the release of somatostatin (21).

5  $\mu$ M D-Ala<sub>2</sub>-Met E stimulated net Na and Cl absorption and decreased  $I_{sc}$ . The ion responsible for the decrease in the  $I_{sc}$  was not readily apparent since there was no significant change in residual flux and net Cl absorption was not statistically greater than net Na absorption ( $2.33 \pm 0.4 \mu\text{eq/cm}^2$  vs.  $1.27 \pm 0.5 \mu\text{eq/cm}^2$ ). However, there was a strong linear correlation between the decrease in  $I_{sc}$  and the increase in net Cl absorption, suggested that Cl absorption accounted in part for the decrease in the  $I_{sc}$  (Fig. 5).

Any model to explain the effect of enkephalin on ion transport based on flux data alone is, of course, tentative. The data, however, is compatible with two effects based on current concepts in ion transport. D-Ala<sub>2</sub>-Met E may stimulate neutral Na-Cl influx across the brush border of the enterocyte. These ions then move into the lateral intercellular space. Because the tight junction is cation selective, some Na leaks back into the mucosal solution. This would result in an excess of Cl absorption over Na absorption and decrease the  $I_{sc}$ . Neutral Na-Cl uptake has been shown to be present in rabbit ileum and other epithelia (22). In the teleost intestine, coupled NaCl influx across the brush border has been demonstrated but net chloride absorption exceeds net sodium absorption and accounts for the  $I_{sc}$  (23, 24). Field proposes that after sodium and chloride enter the lateral intercellular space, sodium diffuses back into the mucosal solution but chloride does not because the tight junction is cation selective (22–24). Thus, “electrogenic” chloride absorption results because of back leak of sodium across the tight junction. It should be pointed out that the separation of Na and Cl in the lateral intercellular space occurs only under short-circuit conditions.

D-Ala<sub>2</sub>-Met E may also inhibit Cl permeability at the brush border which would result in a decrease in S to M Cl movement, a decrease in the  $I_{sc}$  and an increase in net Cl absorption. Cyclic AMP, which has the opposite effect of D-Ala<sub>2</sub>-Met E in the rabbit ileum, may work primarily by increasing the permeability of the brush border membrane to Cl (22, 25–27). Thus it is not unreasonable to postulate that an agent that stimulates absorption may do so by decreasing Cl permeability at the brush border. Experiments with isolated brush border membrane vesicles are currently in progress to test these hypotheses.

As mentioned above, the effects of enkephalins on ion transport are more or less opposite those of cyclic AMP. Cyclic AMP (a) stimulates electrogenic chloride secretion; (b) inhibits neutral NaCl influx; (c) increases

the permselectivity of the shunt pathway and/or permeability of the brush border to chloride (25–30). Enkephalins could thus: (a) be inhibiting the production of cyclic AMP; (b) blocking the effect of cyclic AMP on ion transport; or (c) working independently of the cyclic nucleotides, but having the opposite effect. We did not find an effect of D-Ala<sub>2</sub>-Met E on basal cyclic AMP levels or on VIP-stimulated cyclic AMP levels although prostaglandin and theophylline-stimulated cyclic AMP levels were slightly depressed (Table V). Therefore, it is unlikely that an enkephalin effect on cyclic AMP metabolism explains its effect on ion transport, especially in the basal state. When theophylline was added to enkephalin-treated tissues (Fig. 2), there was no inhibition of the theophylline-induced increase in the I<sub>sc</sub>; thus, enkephalins do not appear to be blocking the effect of cyclic AMP either. These findings imply that enkephalins affect a similar mechanism as cyclic AMP but in the opposite direction. Cyclic guanosine 5'-monophosphate (GMP) has also been implicated in intestinal secretion and it is possible that enkephalins are affecting the metabolism or blocking the production of this nucleotide (31).

These studies indicate that the enkephalins may play a physiologic role in intestinal ion transport. The enkephalins or other endorphins may ultimately have therapeutic value also. Although diarrhea has been treated for years with opiates, it has been assumed that the antidiarrheal effect of the opiates is due to their recognized effects on motility. These present studies indicate that the opiates may affect ion transport as well.

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