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E B Chang, ... , N S Wang, M Field

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### Research Article

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# Secretagogue-induced Changes in Membrane Calcium Permeability in Chicken and Chinchilla Ileal Mucosa

## Selective Inhibition by Loperamide

Eugene B. Chang, David R. Brown,\* Ning Sheng Wang, and Michael Field

Department of Medicine, Gastrointestinal Unit, Columbia University, New York, 10032; and \*Department of Veterinary Biology, University of Minnesota, St. Paul, Minnesota 55108

### Abstract

Substance P (SP), neurotensin (NT), bombesin (BB), serotonin (5HT), and carbamylcholine (CCH) transiently increase electrogenic anion secretion in chinchilla and chicken ileum. SP and CCH also transiently inhibit amiloride-sensitive Na/H exchange in isolated chicken enterocytes. Loperamide (LP) inhibits the short-circuit current responses caused by SP, NT, and BB, but not those caused by CCH, 5HT, Ca ionophore, or cyclic nucleotides. Similarly, LP inhibits the effects of SP, but not those of CCH, on Na/H exchange.

LP inhibition of the SP effects was further studied in isolated chicken enterocytes. CCH and SP transiently increased cytosolic Ca activity by 20–50 nmol/liter, but only the response to SP was inhibited by LP ( $10^{-5}$  M) and by the absence of extracellular Ca. We conclude (a) SP and CCH effects on intestinal electrolyte transport are mediated by increasing enterocyte Ca activity and (b) LP specifically inhibits peptide hormone-activated Ca entry by an opiate receptor-independent mechanism.

### Introduction

Loperamide is a commonly used antidiarrheal drug which is structurally related to the opiate analgesic meperidine. It binds to opiate receptors present in the intestinal longitudinal muscle and myenteric plexus and alters gut motor activity, an action that is only partially reversed by opiate antagonists such as naloxone (1–4). Although the antidiarrheal activity of loperamide stems in large part from its effects on intestinal motor function under conditions of normal absorption (5), a portion of these therapeutic actions may be attributed to its ability to alter ion and fluid transport across the intestinal mucosa under secretory conditions (6). For example, the drug has been reported to attenuate or reverse intestinal secretion induced by cholera toxin (7–9), *Escherichia coli* enterotoxins (10, 11), prostaglandin E<sub>2</sub> (6, 8, 10, 12–15), bile salts and fatty acids (16, 17), or vasoactive intestinal polypeptide (15); it has little or no effect on basal ion fluxes. There are conflicting reports as to whether opiate antagonists decrease these antisecretory effects (8, 10, 13, 15). Loperamide does not affect secretagogue-induced increases in intracellular cyclic AMP, one recognized mediator of secretory processes in the mucosa (7, 8, 14, 18). However, it has been thought

that the drug may affect electrolyte secretion activated by calcium-dependent processes (19, 20). Furthermore, loperamide has been found to bind to calcium channels in rat brain membranes and to block calcium-induced contractions of the guinea pig ileal smooth muscle (21).

We have investigated, through measurements of short-circuit current ( $I_{sc}$ ), <sup>22</sup>Na influx, and intracellular free Ca (Ca<sub>i</sub>) and pH (pH<sub>i</sub>), the cellular mechanisms underlying the antisecretory actions of loperamide in intact chinchilla and chicken ileum and in enterocytes isolated from chicken ileum. Initial studies of  $I_{sc}$  responses to secretagogues and loperamide were performed in chinchilla ileum because of its exceptional responsiveness to these agents. However, subsequent experiments were performed in the equally responsive chicken ileum, because isolated enterocytes could be easily obtained and maintained with excellent viabilities for 2–3 h. These were essential criteria for measurements of intracellular free Ca and pH<sub>i</sub>. We report here that loperamide selectively inhibits the actions of those gut peptides and neurotransmitters that directly activate plasma membrane Ca permeability.

### Methods

**Materials.** Loperamide HCl was provided by Janssen Pharmaceuticals Ltd. (Beerse, Belgium). Etorphine, morphine sulfate, and D600 (methoxyverapamil) were gifts from Richard J. Miller (Dept. of Pharmacological and Physiological Sciences, University of Chicago) and diprenorphine was a gift of Dr. David U'Pritchard (Dept. of Pharmacology, Northwestern University). Quin-2 acetoxyethyl-tetraester (quin-2/AM) was obtained from Calbiochem-Behring (San Diego, CA). [<sup>3</sup>H]quin-2/AM (10.6 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). [<sup>14</sup>C]-methyl α-D-glucopyranoside (275 mCi/mmol) was obtained from New England Nuclear (Boston, MA). 5,6-Carboxyfluorescein diacetate (CF) was obtained from Molecular Probes, (Junction City, OR). All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO). Stock concentrations of etorphine, diprenorphine, prostaglandin E<sub>1</sub>, D600, and loperamide were dissolved in 50% ethanol. A23187, CF, and quin-2/AM were dissolved in dimethylsulfoxide (DMSO).

**Solutions employed.** In *in vitro* experiments with chinchilla intestine, the bathing solution contained in millimoles per liter: Na, 144; K, 5; Ca, 1.25; Mg, 1.1; Cl, 125; HCO<sub>3</sub>, 25; H<sub>2</sub>PO<sub>4</sub>, 0.3; HPO<sub>4</sub>, 1.65, and was bubbled with 5% CO<sub>2</sub> in O<sub>2</sub>. In *in vitro* experiments with chicken intestine, the bathing solution contained in millimoles per liter: Na, 137; K, 6; Cl, 145; Mg, 0.9; SO<sub>4</sub>, 0.4; H<sub>2</sub>PO<sub>4</sub>, 0.4; Ca, 1; Hepes, 20 (pH 7.4); fructose, 10, and was bubbled with 100% O<sub>2</sub> (modified Hanks' buffered saline or HBSS). The medium used for isolation of chicken enterocytes contained in millimoles per liter: Na, 100; K, 6; Tris base, 55 (pH 7.4);

Address reprint requests to Dr. Chang.

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1. **Abbreviations used in this paper:** BB, bombesin; CF, 5,6-carboxyfluorescein diacetate; CCH, carbamylcholine; DADLE, D-alanine<sup>2</sup>,D-leucine<sup>3</sup>-enkephalin; 5HT, serotonin; IP<sub>3</sub>, inositol 1,4,5 trisphosphate;  $I_{sc}$ , short-circuit current; NT, neurotensin; quin-2/AM, quin-2 acetoxy-methyl-tetraester; SP, substance P.

Cl, 160; Mg, 1; Ca, 1; HPO<sub>4</sub>, 3; fructose, 10, and was bubbled with 100% O<sub>2</sub> (isolation buffer). It also contained 0.1% BSA, 0.01% trypsin inhibitor, and 0.15% hyaluronidase.

**Ion transport studies.** Intestinal segments of 2–3 cm in length from 500–750-g chinchillas and 10–12-wk-old white leghorn chickens were stripped of their serosa and underlying longitudinal muscle layer and incubated at 37°C in Ussing chambers as previously described (22). D-glucose (20 mmol/liter) was added to the serosal side and an equimolar amount of mannitol was added to the mucosal side. Transmural potential difference, resistance, and short-circuit current ( $I_{sc}$ ) were determined as previously described (22). Secretagogue effects were determined by measuring the difference between the maximal increases in  $I_{sc}$  and prestimulated  $I_{sc}$  values. The effects of loperamide, D-600, etorphine, morphine, trifluoperazine (TFP), and D-alanine<sup>2</sup>,D-leucine<sup>5</sup>-enkephalin (DADLE) on secretagogue-induced  $I_{sc}$  changes were determined by addition of these compounds to the serosal reservoir 5–10 min before addition of secretagogue. To quantitate the potency of loperamide's inhibitory effects, the ID<sub>50</sub> for each series of tissues was determined by approximation of the concentration of loperamide producing a 50% inhibition of the secretagogue response.

**Isolation of chicken enterocytes.** Epithelial cells from the distal half of chicken small intestine were isolated using a modification of the method described by Kimmich (23). Flat sheets of intestine of 3–5 cm in length were incubated in isolation buffer at 39°C for 45 min in a shaking waterbath. The resulting cell slurry was then filtered through nylon stocking material to remove mucus and intact tissue. This cell filtrate was washed twice with HBSS and the final pellet was resuspended in Ca-free HBSS to a cell concentration between 10 and 15 million cells/ml.

**Quin-2 loading of isolated chicken enterocytes.** Isolated enterocytes were initially incubated with quin-2/AM (60 μmol/liter) in calcium-free HBSS for 10 min at 3°C. The final DMSO concentration was <0.1%. Omission of Ca minimized the activity of any carboxyl-esterases released from damaged cells. After 10 min, extracellular Ca was restored to 1 mmol/liter and the incubation was continued for an additional 20 min. Quin-2 loading was estimated to be between 1 and 2 mmol/liter cell volume using <sup>3</sup>[H]quin-2/AM. After loading, the cell suspension was diluted fivefold with cold HBSS and centrifuged at 200 g for 1 min. The pellet was gently resuspended in HBSS and kept at ice temperature. Periodically an aliquot would be removed and diluted twofold with HBSS and centrifuged at 200 g for 1 min. This pellet was then resuspended in HBSS to yield 1–3 million cells/ml and transferred to a cuvette.

**Fluorescence measurements.** Measurements were made with a Farrand Mark I spectrofluorometer (Farrand Optical Co., Valhalla, NY) with a modified thermostated cuvette chamber which had a motor-driven rotary mixing unit that maintained a homogeneous cell suspension. All measurements were made at 39°C in 1-cm<sup>2</sup> quartz cuvettes. Drugs and hormones were introduced into the cuvette with a 10-μl Hamilton syringe introduced through a guide mounted in the cuvette chamber lid. Standard monochromator settings were 339 nm excitation with 5-nm slits; 490 nm emission with 10-nm slits. In every instance an excitation spectrum was generated to confirm that quin-2 was in its free acid form (peak at 339 nm) and that the cells were sufficiently loaded. Determinations of intracellular free Ca ([Ca]<sub>i</sub>) were made according to the method of Tsien et al. (24) with the exception that  $F_{max}$  was determined after addition of 10 mmol/liter digitonin to disrupt the cells.  $F_{min}$  was obtained by setting [Ca]<sub>i</sub> in 1 nM by adding 2 mM EGTA and enough Tris base to increase pH to >8.3. [Ca]<sub>i</sub> was calculated using the equation  $[Ca]_i = 115 \text{ nM} \times (F - F_{min}) / (F_{max} - F)$ ; see Tsien et al. (24). Minimal autofluorescence of loperamide (at 1 μmol/liter) was noted; this was subtracted from fluorescence measurements in the presence of quin-2. No quenching of quin-2 fluorescence was noted by any of the compounds tested.

**Measurements of intracellular pH.** Isolated enterocytes were suspended in HBSS to a cell concentration of ~15–20 million cells per ml and incubated with CF (20 μM) for 15 min in a waterbath shaker at 37°C. After loading, the suspension volume was diluted twofold with prewarmed buffer. For each pH determination, aliquots of 4–6 ml were washed twice with HBSS and resuspended with the appropriate study buffer to a final volume of 2 ml.

Fluorescence measurements were begun after a 5-min preincubation period. Determination of intracellular pH was made by taking the ratio of fluorescence intensities measured at 530 nm using excitation wavelengths of 490 and 465 nm, respectively. Autofluorescence determined from isolated cells without CF was subtracted in each case. All agents studied exhibited no autofluorescence. Intracellular pH was determined by comparing the corrected ratio measurements to a calibration curve of CF ratio fluorescence in cells suspended in buffer containing in millimoles/liter: K, 130; Cl, 130; Hepes, 20; nigericin, 10<sup>-2</sup>; valinomycin, 4 × 10<sup>-3</sup>. Nigericin and valinomycin were used to collapse any existing H<sup>+</sup> or K<sup>+</sup> gradients, respectively. Under these conditions, intracellular pH can be adjusted by titrating extracellular pH using KOH. Measurements from each cell suspension were made at pH 6.6, 6.8, 7.0, 7.2, and 7.5 after washing two times with pH-adjusted buffer and allowing 5 min for equilibration of intra- and extracellular pH.

**Sodium influx measurements.** Na<sup>+</sup> influx in isolated enterocytes was performed using a modification of the technique described by Kimmich (25). Isolated cells were resuspended in buffer containing in millimoles per liter: Na, 70; N'-methyl,D-glucamine (NMDG), 55; K, 5; Ca, 1.25; Mg, 1.1; Cl, 125; H<sub>2</sub>PO<sub>4</sub>, 0.3; HPO<sub>4</sub>, 1.65; Hepes, 20 (pH 7.4). In all cases, cells were used within 30 min after isolation.

Uptake of <sup>22</sup>Na was initiated by transfer of 1 ml of cell-free suspension buffer containing 0.25 μCi/ml <sup>22</sup>Na to 3 ml of cell suspension (final cell concentration ~5–7 million cells/ml). Aliquots (300 μl) were taken at 0 and 45 s. These were immediately diluted in 1.2 ml of ice-cold sodium-free stop solution containing in millimoles per liter: NMDG, 110; K, 5; Ca, 1.25; Mg, 1.1; Cl, 115; H<sub>2</sub>PO<sub>4</sub>, 0.3; HPO<sub>4</sub>, 1.65; Hepes, 20 (pH 7.4). Samples were rapidly washed twice in Na-free buffer and pellets counted by gamma-ray spectrometry. Pellets were then solubilized with 400 μl of NaOH (1.0 N) and protein content was determined by the method of Lowry in comparison with BSA standards of known concentration. Results were expressed in counts per minute of <sup>22</sup>Na per milligram of protein.

**Determination of cell viability.** Cell viability after quin-2 loading was routinely measured by determination of <sup>14</sup>[C]methyl alpha-glucopyranoside uptakes by isolated enterocytes according to the method of Kimmich (25). Cellular sugar uptake was determined in the presence and absence of phloridzin (200 μmol/liter). Batches of cells with less than 40-fold alpha-glucopyranoside accumulation were rejected.

## Results

**Effects on anion secretion.** Addition of substance P (SP), neurotensin (NT), or bombesin (BB) to the serosal side of chinchilla or chicken ileum produces a transient (3–10 min) increase in  $I_{sc}$  (see Fig. 1). As shown previously for guinea pig ileum (26, 27), this increase in  $I_{sc}$  is due to Cl and/or HCO<sub>3</sub> secretion. Replacement of Cl and HCO<sub>3</sub> with Hepes and gluconate eliminated the  $I_{sc}$  responses to these peptides in both chicken and chinchilla ileum. Furthermore, subsequent addition of SP (10<sup>-5</sup> M), carbamylcholine (CCH) (10<sup>-4</sup> M), or NT (10<sup>-5</sup> M) to tissues maximally stimulated with 8-bromo-cAMP (10<sup>-4</sup> M) failed to produce any further changes in  $I_{sc}$ ; the latter effect being shown to be due to active Cl secretion (18). Direct measurements of transmural isotope fluxes in both tissues were not possible because of the transient nature of these secretagogue responses and because of the large unidirectional passive ion flux movements.

The three peptides appear to stimulate secretion by increasing Ca entry into enterocytes because omission of Ca from the serosal bathing medium reduced their effects on  $I_{sc}$  by >70%. In chinchilla ileum, for example, bombesin (10<sup>-5</sup> M)-stimulated increases in  $I_{sc}$  were reduced from 15 ± 3.0 μA/cm<sup>2</sup> in the presence of serosal Ca ( $n = 6$ , 1.0 mM Ca) to 0.4 ± 0.2 μA/cm<sup>2</sup> in its absence ( $n = 6$ , 0.5 mM EGTA present). SP-stimulated  $I_{sc}$  responses in chicken ileum were likewise reduced from 43 ± 8 μA/

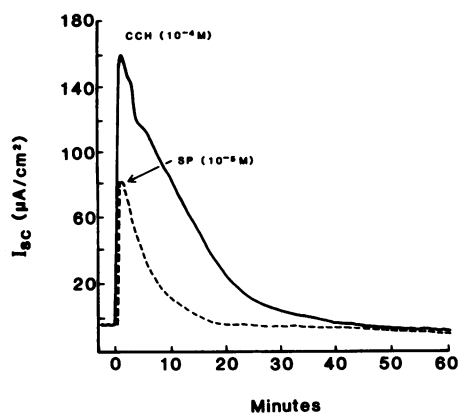


Figure 1. Representative tracings of short-circuit current ( $I_{sc}$ ) responses to substance P (SP) and carbachol (CCH) in chinchilla ileum.

$cm^2$  ( $n = 7$ ) to  $11 \pm 3 \mu A/cm^2$  ( $n = 6$ ) when Ca was removed from the serosal bathing medium.

Loperamide (1–10  $\mu M$  added to the serosal bathing medium) did not alter basal  $I_{sc}$  in either chinchilla or chicken ileum. As shown in Table I, loperamide did, however, inhibit the increases in  $I_{sc}$  produced by SP, NT, and BB. The concentrations of LP producing 50% inhibition of the  $I_{sc}$  responses to SP, NT, and BB in chinchilla ileum were  $2.1 \pm 0.8 \times 10^{-7} M$  ( $n = 7$ ),  $8.0 \pm 1.2 \times 10^{-7} M$  ( $n = 5$ ), and  $1.0 \pm 0.5 \times 10^{-7} M$  ( $n = 5$ ), respectively. Inhibition of these secretory responses by loperamide appears to be noncompetitive ( $ED_{50}$  unchanged) as shown in Fig. 2 A for the dose-related inhibitory effects of loperamide on substance

Table I. Loperamide Inhibition of Secretagogue-induced Increases in Short-Circuit Current Changes ( $\Delta I_{sc}$ ) in Ileum

Secretagogue	Concentration	n	$\Delta I_{sc}$	
			Control	+Loperamide ( $10^{-3} M$ )
	M		$\mu A/cm^2$	$\mu A/cm^2$
<b>Chinchilla</b>				
Substance P	$10^{-6}$	7	$67 \pm 16$	$7 \pm 3^*$
Neurotensin	$10^{-6}$	7	$23 \pm 6$	$2 \pm 1^*$
Bombesin	$10^{-6}$	9	$13 \pm 5$	$1 \pm 1^*$
Carbamylcholine	$10^{-4}$	8	$103 \pm 17$	$126 \pm 13$
Serotonin	$10^{-5}$	5	$62 \pm 18$	$88 \pm 23$
A23187	$10^{-6}$	7	$83 \pm 15$	$70 \pm 9$
Vasoactive intestinal peptide	$10^{-7}$	7	$103 \pm 8$	$109 \pm 7$
$PGE_1$	$10^{-6}$	6	$52 \pm 7$	$62 \pm 9$
Bradykinin	$10^{-6}$	5	$77 \pm 12$	$88 \pm 21$
Theophylline	$10^{-4}$	7	$41 \pm 6$	$39 \pm 6$
8-Bromo-cAMP	$10^{-4}$	7	$52 \pm 9$	$41 \pm 8$
8-Bromo-cGMP	$10^{-4}$	7	$15 \pm 5$	$11 \pm 3$
<b>Chicken</b>				
Substance P	$10^{-5}$	9	$36 \pm 9$	$15 \pm 7^*$
Bombesin	$10^{-6}$	8	$19 \pm 5$	$6 \pm 3^*$
Carbamylcholine	$10^{-4}$	8	$39 \pm 6$	$43 \pm 6$
Serotonin	$10^{-5}$	6	$30 \pm 8$	$30 \pm 5$

Values are means  $\pm$  SEM for ( $n$ ) animals.

\*  $P < 0.05$  by paired  $t$  test determinations.

P-stimulated  $I_{sc}$ . As shown in Fig. 3, loperamide's inhibitory effects were not modified by the presence of the potent opiate antagonist diprenorphine. The potent synthetic opiate etorphine was also found to inhibit the effects of these peptides on  $I_{sc}$  and its inhibitory action was also not modified by diprenorphine (data not shown). These findings suggest that the specific antisecretory effects of loperamide and etorphine are not mediated by opiate receptors. In support of this conclusion is the observation that the  $I_{sc}$  response to these three peptides was not inhibited by either the  $\mu$ -opiate receptor agonist morphine (10  $\mu M$ ) or the delta-receptor selective enkephalin DADLE (10  $\mu M$ ). Loperamide, in contrast to its effect on SP-, NT-, and BB-induced secretory responses, did not inhibit the  $I_{sc}$  responses to CCH (see Fig. 2 B) or serotonin (5HT) (see Table I).

The action of the Ca-channel blocker D600 on SP-stimulated changes in  $I_{sc}$  is strikingly similar to that of loperamide: (Fig. 2 A) and neither inhibits the action of CCH (Fig. 2 B). Increased  $I_{sc}$  stimulated by CCH are inhibited by atropine ( $10^{-5} M$ ), but not by hexamethonium ( $10^{-5}$ ), suggesting that this effect is mediated by muscarinic cholinergic receptors. Furthermore, removal of Ca from the serosal bathing medium (with 0.5 mM EGTA present) reduced  $I_{sc}$  responses to CCH and 5HT by only 30% and 10%, respectively, indicating that their effects are largely independent of extracellular Ca. Because the actions of SP, NT, and BB are dependent on extracellular Ca, loperamide appears to act by blocking a specific membrane receptor-mediated Ca permeability, mimicking the action of D600. Loperamide does

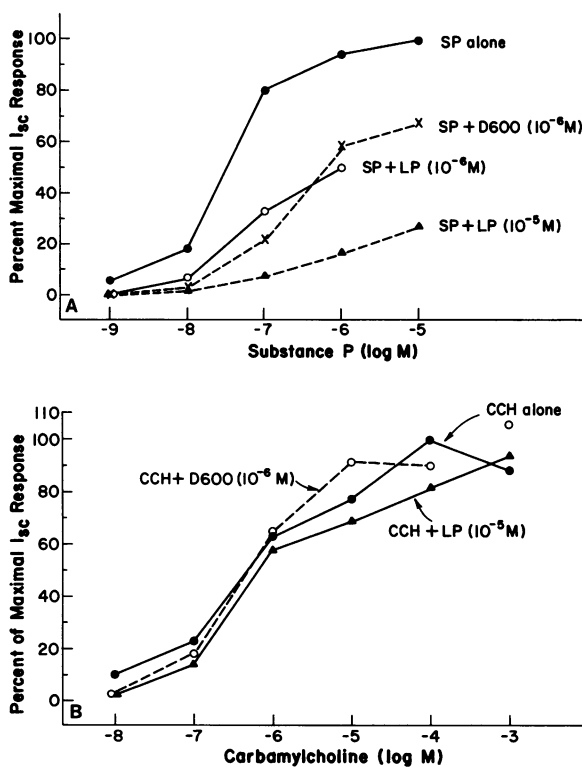


Figure 2. Effects in chinchilla ileum of loperamide (LP) and D600 (methoxyverapamil) on increases in  $I_{sc}$  stimulated by substance P (A) and by carbamylcholine (CCH) (B).  $I_{sc}$  responses were determined by the difference between the peak  $I_{sc}$  response to secretagogues and pre-stimulated  $I_{sc}$  values. For SP and CCH, maximal  $I_{sc}$  responses (100%) were  $70 \pm 10$  and  $115 \pm 15 \mu A/cm^2$ , respectively. Each condition was tested on tissues from five to nine animals.

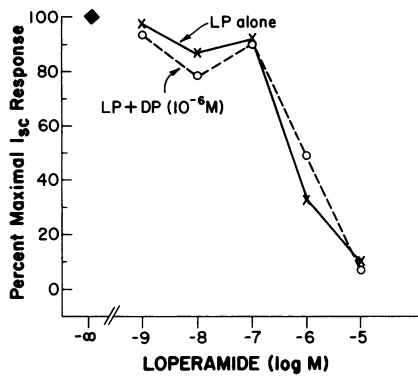


Figure 3. Inhibitory effects of loperamide (LP) alone or LP and the opiate receptor antagonist diprenorphine (DP) on increases in  $I_{sc}$  produced by substance P ( $10^{-6}$  M) in chinchilla ileum. Values are means of experiments on tissues from six animals.

not inhibit the secretory response to the Ca-ionophore A23187 (Table I); this reinforces the notion that loperamide blocks a specific agonist-induced Ca permeability in the basolateral membrane and does not interfere with an intracellular locus of Ca action. It has been suggested that loperamide binds to and inhibits calmodulin (18–20). However, unlike loperamide and D600 which selectively inhibit  $I_{sc}$  responses to SP, the calmodulin inhibitor TFP was found to inhibit the  $I_{sc}$  responses to both SP and carbachol (Table II). Loperamide also does not inhibit the secretory responses to bradykinin, 8-bromo-cAMP, 8-bromo-cGMP, vasoactive intestine peptide, prostaglandin  $E_1$ , or theophylline (Table I).

**Effects on Na/H exchange.** The effects of loperamide on Na uptake by isolated chicken enterocytes are shown in Figs. 4 and 5. Na uptake into the chicken enterocytes, particularly that component regulated by intestinal hormones and neurotransmitters, has been shown to occur predominantly by an amiloride-sensitive Na/H exchange in the apical membrane (28–30). The effects of SP and carbachol on  $^{22}$ Na influx into isolated enterocytes are shown in Fig. 4. Na influx was inhibited by ~50% 1 min after addition of either agent. Pretreatment of the enterocytes with loperamide ( $10^{-5}$  M) blocked the decrease in Na influx caused by SP, but not that caused by carbachol. Loperamide had no effect on basal Na influx. Similarly, when the intracellular

Table II. Effects of Trifluoperazine, D600, and Loperamide on  $I_{sc}$  Response to Substance P and Carbachol in Chinchilla Ileum

n	Inhibitor	$\Delta I_{sc}$ response	
		SP ( $10^{-6}$ M)	CCH ( $10^{-4}$ M)
		$\mu A/cm^2$	$\mu A/cm^2$
4	-TFP	73±22	122±19
	+TFP ( $10^{-4}$ M)	24±8*	56±6*
7	-LP	67±16	103±17
	+LP ( $10^{-5}$ M)	7±3*	126±13
7	-D600	63±11	124±14
	+D600 ( $10^{-5}$ M)	26±5*	112±16

\*  $P < 0.05$  by  $t$  test analysis of paired variates.

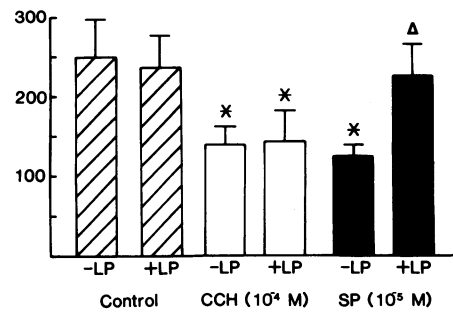


Figure 4.  $^{22}$ Na influx during the first 45 s in isolated chicken enterocytes. Stimulated cells were treated with carbachol (CCH) or substance P (SP) for 1 min prior to initiation of influx measurements. Some groups were pretreated for 5 min with loperamide (+LP,  $10^{-5}$  M) prior to the addition of secretagogue. \*Significant to  $P < 0.05$  by  $t$  test analysis of paired variates compared to controls.  $\Delta$ Significant to  $P < 0.05$  compared to SP effect in the absence of LP.

pH of isolated enterocytes was measured by ratio fluorescence of CF (Fig. 5), transient but significant decreases in ratio fluorescence of  $0.05 \pm 0.01$  and  $0.06 \pm 0.01$  (peak values at 1 min)

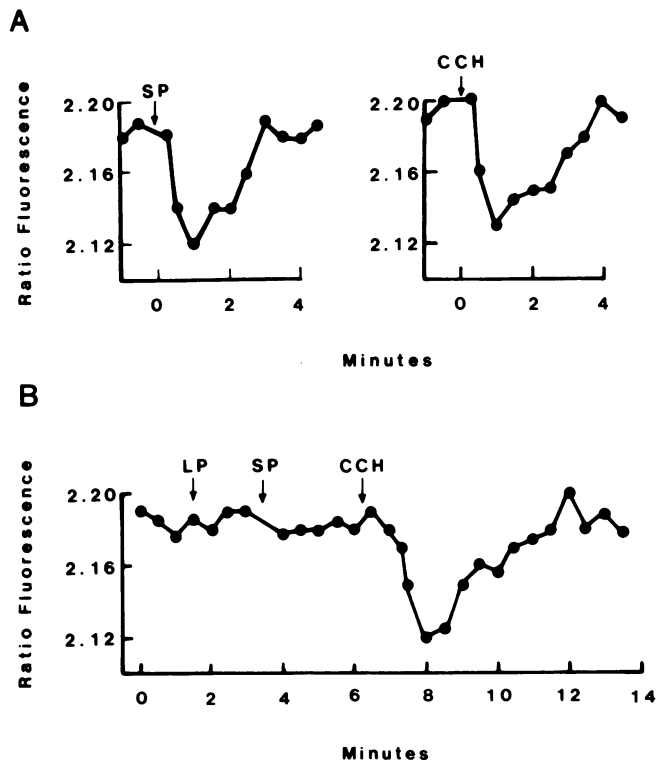


Figure 5. Effects of loperamide (LP) on substance P (SP) and carbachol (CCH)-induced changes in ratio fluorescence measurements from isolated chicken enterocytes loaded with CF. Decreases in ratio values reflect decreases in intracellular pH stimulated by secretagogues. (A) Effects of SP ( $10^{-5}$  M) and CCH ( $10^{-4}$  M) on ratio fluorescence. Each point represents the means of five separate determinations. Both SP and CCH stimulate transient decreases in intracellular pH at 1 min by approximately 0.8–1.0 pH units (see Results). (B) Effects of pretreatment with loperamide ( $10^{-5}$  M) on SP- but not CCH-induced decreases in intracellular pH. LP ( $10^{-5}$  M) by itself did not alter basal fluorescence measurements. Each point represents the means of three separate determinations.

were caused by both SP and carbachol ( $n = 5$  for each), respectively, corresponding to decreases in intracellular pH of  $\sim 0.08$ – $0.1$  pH units (basal intracellular pH  $\sim 6.9$ – $7.0$ ). The  $\text{pH}_i$  responses to both CCH and SP were Na-dependent (i.e., no effect on  $\text{pH}_i$  when Na was replaced with NMDG) and not additive to the acidifying effect of amiloride ( $10^{-5}$ – $3$  M), consistent with an inhibitory effect of these agents on an amiloride-sensitive Na/H exchange. Pretreatment of cells with loperamide ( $10^{-5}$  M) inhibited the effect of SP on  $\text{pH}_i$  but did not affect the  $\text{pH}_i$  response to carbachol (Fig. 5 A and B).

**Effects on intracellular Ca activity.** Basal  $[\text{Ca}]_i$  in isolated chicken enterocytes, as measured with quin-2, was  $148 \pm 7$  nM ( $n = 47$ ) and was not altered by loperamide ( $1$   $\mu\text{M}$ ). Both SP ( $10$   $\mu\text{M}$ ) and CCH ( $100$   $\mu\text{M}$ ) produced short-lived increases in  $[\text{Ca}]_i$  which preceded but were similar in duration to the change in pH caused by these agents (compare CCH responses in Fig. 5 with Fig. 6). As summarized in Table III, loperamide ( $10^{-6}$  M) caused a large inhibition of SP ( $10^{-6}$  M)-induced increases in  $[\text{Ca}]_i$  but did not significantly affect the CCH-induced changes in  $[\text{Ca}]_i$ . CCH-induced  $[\text{Ca}]_i$  changes were blocked, however, by atropine ( $10$   $\mu\text{M}$ ), but not by hexamethonium ( $10^{-5}$  M). Also, in the absence of extracellular Ca, there was not a negligible effect of SP ( $10^{-5}$  M) on  $[\text{Ca}]_i$  (Fig. 6 D), whereas the effect of CCH ( $10^{-4}$  M) on  $[\text{Ca}]_i$  was still present (Fig. 6 C). The predominant effect of Ca removal on the CCH-induced increase in  $[\text{Ca}]_i$  was a more rapid decline in  $[\text{Ca}]_i$  after the peak increase was reached (no significant decrease in peak effect).

## Discussion

The present results indicate that loperamide selectively reverses the effects on intestinal electrolyte transport of the endogenous gut peptides SP, NT, and BB. These peptides stimulate transient increases in transepithelial  $I_{\text{sc}}$ , reflecting stimulation of electrogenic anion secretion. In support of this is the observation that removal of Cl and  $\text{HCO}_3^-$  from the bathing media reduces these responses by over 90% in both chicken and chinchilla ileum.

Table III. Substance P and Carbachol-stimulated Increases in  $[\text{Ca}]_i$  in the Presence and Absence of Loperamide

	-LP	<i>n</i>	$\Delta[\text{Ca}]_i + \text{LP}$ ( $10^{-6}$ M)	<i>n</i>
			<i>nM</i>	
Substance P ( $10^{-6}$ M)	$27 \pm 4$	(6)	$3 \pm 2^*$	(10)
Carbachol ( $10^{-4}$ M)	$44 \pm 9$	(6)	$32 \pm 2$	(5)

Measurements were made in isolated chicken enterocytes loaded with quin-2.

\*  $P < 0.001$  by *t* test analysis of paired variates.

Similar results have been reported in guinea pig and rabbit ilea (26, 27, 31).

In isolated chicken enterocytes, loperamide also reverses the inhibition of Na/H exchange caused by SP, but not that caused by carbachol. These findings are entirely consistent with those observed for  $I_{\text{sc}}$ . Na/H exchange, an electroneutral process not reflected in the  $I_{\text{sc}}$ , appears to be a common mechanism for Na absorption by the small intestine and proximal colon of mammals (29, 31–34). In mammalian small intestine, evidence supports the presence of electroneutral Na/H exchange in villus cells and electrogenic anion secretion in crypt cells (35–37). The small intestine of the chicken appears to be similar. In fact, the presence of an amiloride-sensitive Na/H exchange has been identified in isolated enterocytes, a predominantly villus cell population (28–30).

These selective inhibitory actions of loperamide occur at drug concentrations within a therapeutically relevant range. Furthermore, the actions of loperamide and etorphine, a synthetic opiate, are not blocked by the potent opiate delta- and mu-receptor antagonist diprenorphine, suggesting that their effects are not mediated by enteric opiate receptors. In further support of this was the finding that structurally different opiate agonists such as morphine, a mu-receptor agonist, and DADLE, a specific

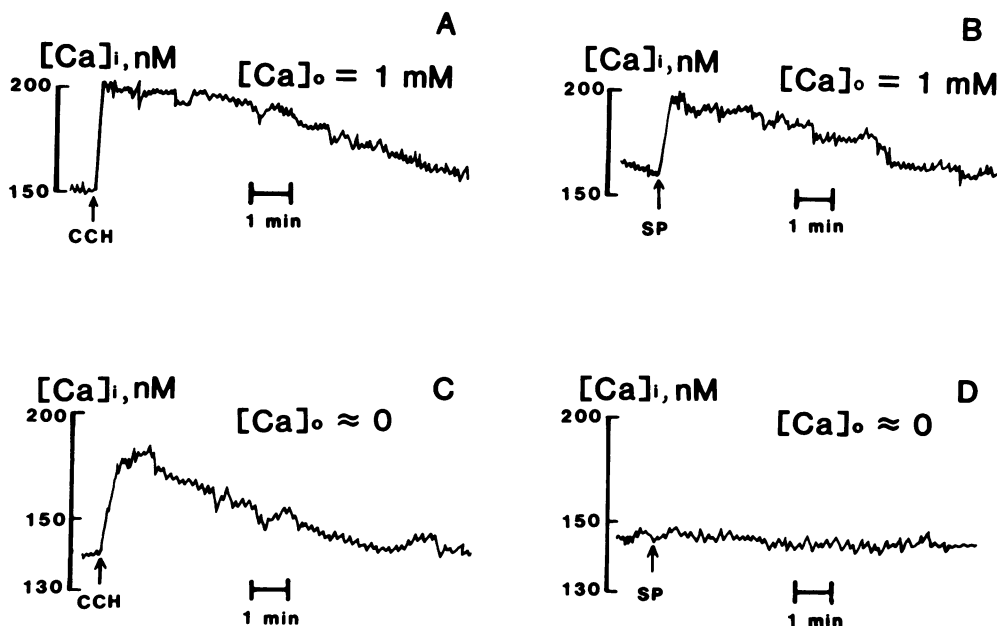


Figure 6. Representative tracings of quin-2 fluorescence from isolated chicken enterocytes. Transient increases in  $[\text{Ca}]_i$  stimulated by carbachol ( $10^{-4}$  M) (A) and substance P ( $10^{-5}$  M) (B) in cells suspended in buffer containing 1.0 mM calcium. Effects of these agents on  $[\text{Ca}]_i$  in cells suspended in Ca-free buffer (0.5 mM EGTA present) are shown in C and D.

delta-receptor agonist, failed to block the effects of SP on  $I_{sc}$ . However, D600, a nonopiate Ca-channel blocker bearing some structural similarities to loperamide and diphenoxylate, had the same selective inhibitory action as did loperamide.

In other studies, loperamide has been reported to inhibit *in vivo* intestinal fluid accumulation induced by a variety of secretagogues (7–17). On the other hand, the drug had no effect on *in vitro* electrical responses of the rodent intestinal mucosa to prostaglandin  $E_2$  (10, 14) or *E. coli* heat-labile toxin (10), agents that act by increasing intracellular cyclic AMP levels, or to *E. coli* heat-stable toxin (10) which raises cyclic GMP levels in enterocytes (38). We have extended these observations to isolated chinchilla ileal mucosa by demonstrating that loperamide did not alter the  $I_{sc}$  elevations produced by prostaglandin  $E_1$ , bradykinin, vasoactive intestinal peptide, cyclic AMP and cyclic GMP analogues, 5HT, and CCH. In contrast, loperamide pretreatment markedly attenuated or abolished the changes in  $I_{sc}$  responses induced by the secretory gut peptides SP, NT, and BB. Loperamide's inhibitory action, at least for SP, appears to be noncompetitive, indicating that it may be acting at a cellular site separate from the peptide binding sites.

In isolated chicken enterocytes, the increases in free intracellular calcium stimulated by SP were blocked by loperamide, whereas those elicited by carbachol remained intact. Although both agents appear to inhibit Na/H exchange in isolated cells and stimulate  $I_{sc}$  in intact mucosa by increasing  $[Ca]_i$ , the mechanism for increasing intracellular Ca may differ. In support of this are the findings that  $I_{sc}$  and  $[Ca]_i$  responses to SP, unlike those to CCH, or 5HT, have an absolute requirement for extracellular Ca. It is, therefore, likely that loperamide, D600, and etorphine inhibit the effects of certain gut peptides by selectively blocking agonist-activated plasma membrane calcium permeabilities. It is unlikely that LP interferes with an intracellular site for Ca action since it did not inhibit the  $I_{sc}$  responses to the Ca-ionophore A23187. Loperamide inhibition of the Ca-calmodulin complex was also felt to be unlikely because calmodulin-inhibition by TFP appeared to nonselectively inhibit secretion induced by a variety of secretagogues including SP and CCH. Loperamide has recently been found to block Ca channels in brain membranes (2) and may do so also in the gut (21). Conventional Ca channel blocking agents such as verapamil have pro-absorptive actions in the rat intestine (39).

The mechanism by which 5HT and agonists such as carbachol increase  $[Ca]_i$  may involve the activation of membrane phosphatidylinositol metabolism (30, 40, 41). Receptor stimulation of membrane phospholipase C causes the hydrolysis of phosphatidyl 4,5-bisphosphate to inositol 1,4,5 trisphosphate (IP3) and diacylglycerol. IP3 has been shown to increase  $[Ca]_i$  by stimulating the release of Ca from endogenous stores (42, 43). This observation is, therefore, consistent with our findings that agents such as carbachol and 5HT do not have an essential requirement for extracellular Ca for the actions on intestinal electrolyte transport. Loperamide, which seems to act only at the plasma membrane level, does not appear to interfere with the intracellular processes and internal Ca mobilization stimulated by these agents.

The increase in  $[Ca]_i$  stimulated by many agonists suggests that Ca is the intracellular mediator for their actions on electrolyte transport. For example, stimulation of isolated enterocytes by the Ca ionophore A23187 caused a significant and persistent decrease in  $pH_i$  (28), an effect resulting from inhibition of amiloride-sensitive Na/H exchange. Agonist-stimulated increases in

$Ca_i$ , which usually lasts 4–6 min, always precede measurable changes in  $pH_i$  and have a similar duration to the transient acidification effects of these agonists. However, the time course for stimulated changes in  $I_{sc}$  appears to be more prolonged, lasting 3–10 min. The difference may be due to barriers to the diffusion of agonist present in intact intestinal mucosa but not present in isolated cells.

This study provides the first direct evidence that many intestinal secretagogues transiently increase intracellular free calcium, which, as a consequence, stimulates an increase in  $I_{sc}$  and inhibits Na/H exchange. The evidence provided suggests that loperamide, as well as D600 and etorphine, may selectively, block agonist-activated Ca permeability in the plasma membrane of enterocytes such as that seen with SP, NT, and BB. The actions of other secretagogues such as carbachol and serotonin appear to be mediated by mobilization of endogenous calcium, perhaps through the stimulated hydrolysis of phosphatidyl 4,5 bisphosphate and generation of IP3 (30). The antidiarrheal effects of loperamide, D600, and etorphine could, in part, be mediated by their direct selective inhibitory actions on certain intestinal secretagogues. It is also possible that these agents block the action of these and perhaps other peptides on enteric neurons by inhibiting the secondary release of neurotransmitters such as vasoactive intestinal peptide.

Verapamil and other Ca-entry blockers have been used to treat cardiac arrhythmias (44) and esophageal motility disorders (45). Whether loperamide will have similar actions in these or other organ systems remains to be explored.

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