Calcium Dependence of Serotonin-induced Changes in Rabbit Ileal Electrolyte Transport

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ABSTRACT These studies describe the calcium dependence of the serotonin-induced changes in active electrolyte transport in rabbit ileum in vitro. In the presence of a standard calcium concentration (1.2 mM) in the serosal bathing fluid, serosal serotonin caused a transient increase in short-circuit current and a prolonged decrease in net Na and Cl fluxes. Removing calcium from the serosal (no calcium plus 1 mM EGTA) but not the mucosal bathing fluid inhibited the serotoin-induced increase in ileal short-circuit current, and also completely blocked the serotonin effects on net Na and net Cl fluxes. This inhibition was rapidly reversed by readding calcium. Removing serosal calcium did not inhibit all active electrolyte transport processes, as the effect of a maximum concentration of theophylline (10 mM) was not altered. Similarly, *d,l*-verapamil, a calcium channel blocker, inhibited the serotonin-induced changes in short-circuit current and in net Na and net Cl fluxes, but did not alter the theophylline effects. In contrast, *d*-verapamil, a stereoisomer which does not block calcium channels, did not inhibit the serotonin-induced changes.

The calcium dependence of these serotonin effects was associated with increased uptake of ^{45}Ca into rabbit ileum, including increased ^{45}Ca uptake from the serosal surface. Serotonin also increased the rate of ^{45}Ca efflux from rabbit ileum into a calcium-free solution, compatible with serotonin increasing the ileal plasma membrane permeability to calcium.

It is postulated that serotonin affects active intestinal electrolyte transport by a mechanism dependent on serosal but not mucosal calcium that involves an increase in the intestinal plasma membrane permeability to calcium, and perhaps an increase in intracellular calcium.

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INTRODUCTION

The intracellular mediators of active intestinal electrolyte secretion identified to date include the adenylate cyclase-3',5'-cyclic adenosine monophosphate (cAMP) system, the guanylate cyclase-3',5'-cyclic guanosine monophosphate (cGMP) system, and calcium (1–5). Although the adenylate cyclase-cAMP system was the first identified mediator of active electrolyte secretion, it is still not known how often this system is involved in causing intestinal secretion in vivo. Although intestinal secretion induced by cholera toxin, the prostaglandins, and heat-labile *Escherichia coli* enterotoxin appears to be causally related to increased mucosal adenylate cyclase activity (4), the circumstance for other secretagogues appears less clear. For instance, *Shigella dysenteriae I* enterotoxin-induced secretion in rabbit ileum was associated with an increase in adenylate cyclase activity; however, this increase was only detectable several hours after onset of electrolyte secretion (6). While differences in the sensitivity of detecting changes in active electrolyte transport and in stimulation of the adenylate cyclase-cAMP system could explain the differences, it is just as likely that this intestinal electrolyte secretion is caused by another intracellular mediator. Dihydroxy bile salts and several laxatives that cause intestinal secretion stimulate the intestinal mucosal adenylate cyclase-cAMP system, but also alter many other factors that have been shown to change electrolyte transport (7). In these circumstances, it is unclear which of the multiple factors is responsible for causing the changes in electrolyte transport.

The role of the guanylate cyclase-cGMP system in mediating active electrolyte secretion remains only partially described. Heat-stable *E. coli* enterotoxin-induced secretion in rabbit ileum is associated with an increase in ileal guanylate cyclase activity and in cGMP content. However, although heat stable *E. coli* enterotoxin increases cGMP content throughout the small and large intestine of the rabbit, it does not appear to alter electrolyte transport, as judged by a change in potential difference, in the duodenum and

distal colon (8, 9). This suggests that there is not a one-to-one correlation between changes in cGMP content and changes in intestinal electrogenic electrolyte transport.

That calcium plays a role as an intracellular mediator of active electrolyte secretion was only recently recognized. The calcium ionophore A23187 causes active electrolyte secretion in rabbit ileum and colon without altering ileal mucosal cAMP content (10, 11). Whether calcium represents a common intracellular mediator of intestinal electrolyte secretion is unclear, although Field (10) suggested that calcium might be involved in the effect of carbachol and serotonin on rabbit ileum, inasmuch as there was partial abolition of the increase in short-circuit current induced by these agents when calcium was removed from the serosal bathing solution.

We have previously demonstrated that serotonin alters active electrolyte transport in rabbit ileum and that this ileal secretion is not associated with a change in rabbit ileal adenylate cyclase or cyclic nucleotide phosphodiesterase activities (12, 13). The purpose of this study was to determine whether the serotonin-induced changes in electrolyte transport in rabbit ileum were calcium dependent and to determine the nature of this calcium dependence.

METHODS

Male New Zealand albino rabbits weighing 2–2½ kg were maintained on a standard rabbit chow diet with free access to water. The animals were anesthetized with sodium pentobarbital. The distal ileum was removed and epithelial sheets prepared for study as previously described (12).

In vitro transmural electrolyte fluxes. The methods used to determine transmural ileal electrolyte transport have been previously described (12, 14). In brief, stripped ileal mucosa was mounted as a flat sheet between two lucite modified Ussing chambers having an aperture of 1.13 cm^2, and oxygenated and maintained at 37°C. Transmural potential difference (PD),[1] short-circuit current (Isc), conductance (G), and unidirectional fluxes of Na and Cl were determined. An automatic voltage clamp corrected for fluid resistance between the PD sensing bridges and provided continuous short circuiting of the tissue. Unidirectional fluxes of Na and Cl were measured using ^{22}Na and ^{36}Cl on paired tissue differing in G by <25%. The labeled side samples were obtained before and after the flux determinations; the unlabeled side samples were replaced with an equal volume of unlabeled bathing solution. The residual ion flux (J^R), which represents the net electrogenic ion transport not accounted for by the net Na and Cl fluxes, was calculated by $J^R = Isc - J^{Na}_{net} + J^{Cl}_{net}$. Ion fluxes were generally determined between 20–80 min after isotope addition, though some experiments were continued for 150 min. Whenever a pharmacologic agent was added in the middle of an experiment, at least 10 min was allowed for equilibration before flux studies were begun again. Results of individual experiments were obtained by averaging ion fluxes with two to four separate 20-min flux periods being used in each part of an experiment. A negative sign before a net ion flux indicates net secretion; a positive sign, net absorption.

Usually six to eight pieces of ileum from a single animal were studied simultaneously. In all experiments involving the effects of serotonin as modified by a drug, serotonin and the drug were tested simultaneously to allow for internal controls. A typical experiment would consist of untreated control, serotonin alone, drug alone, and serotonin plus drug. In all experiments involving the effect of serotonin as modified by a change in the composition of either the mucosal or the serosal bathing solution, the Ringer's-HCO_3 solution and altered bathing solutions were also studied simultaneously. A typical experiment would consist of untreated control in Ringer's-HCO_3, serotonin in Ringer's-HCO_3, untreated control in altered bathing solution, and serotonin in altered bathing solution.

If one of the sets of experiments on tissue from a given animal proved unsuccessful, the entire experiment was discarded. In each series of experiments, the effect of serotonin was determined with respect to control tissue from the same animal. This accounts for the effect of serotonin on net Na and Cl fluxes being slightly different when reported in different sections of the results.

Unless specified, the bathing solution consisted of Ringer's-HCO_3 composed (in mM) of Na, 140; K, 5.2; Ca, 1.2; Mg, 1.2; Cl, 119.8; HCO_3, 25; HPO_4, 2.4; and H_2PO_4, 0.4; pH was 7.4 after bubbling with 95% O_2–5% CO_2. 10 mM glucose was added to the serosal and 10 mM mannitol to the mucosal bathing fluids at the time of mounting the tissue.

In experiments evaluating the dependence of the serotonin effect on calcium in the bathing solution, calcium sulfate was used to alter the concentration of calcium and the zero calcium solution contained 1 mM EGTA. In all studies, magnesium was present in the usual concentration, and calcium was not removed from mucosal and serosal bathing solutions simultaneously. Mannitol was used to maintain bathing solutions at equal osmolarities and the pH of all solutions was 7.4.

Various concentrations of serotonin creatinine sulfate (Sigma Chemical Co., St. Louis, Mo.) were added as a solution to the bathing fluid, usually at the same time as the addition of the isotope, and were compared with an equal volume of Ringer's-HCO_3; the latter is referred to throughout as the untreated control. We previously demonstrated that creatinine sulfate did not affect electrolyte transport in ileum when the same wide range of concentrations as serotonin was studied (12), and creatinine sulfate was not used in these experiments.

In vitro ^{45}Ca uptake. ^{45}Ca uptake was determined into epithelial sheets of ileum of ~50 mg wet wt, maintained at 37°C in a shaking water bath, and oxygenated by bubbling with 95% O_2–5% CO_2. After preincubation for 30 min in Ringer's-HCO_3 (~2 ml/50 mg of tissue), ^{45}Ca (New England Nuclear, Boston, Mass.) uptake was determined in the presence of [^3H]polyethylene glycol (PEG), 900 mol wt (New England Nuclear), and unlabeled PEG of similar molecular weight (Sigma Chemical Co.), 0.5 g/liter, as an extracellular space marker. In preliminary experiments, the time to establish an extracellular marker steady state was determined and the extracellular space marker was present in the uptake experiments for that number of minutes preceding addition of ^{45}Ca. In determining the effect of serotonin on ileal ^{45}Ca uptake from the mucosal and serosal surfaces simultaneously, it was initially demonstrated that the rate of ^{45}Ca uptake became constant ~30–45 min after addition of ^{45}Ca to ileum and remained constant for at least 105 min after addition of ^{45}Ca. Consequently, 60 min after ^{45}Ca addition, and after

[1] *Abbreviations used in this paper*: ERC, efflux rate coefficient; G, conductance; Isc, short-circuit current; J^R, residual ion flux; PD, potential difference; PEG, polyethylene glycol.

making several determinations of the initial rate of ^{45}Ca uptake in untreated control tissue, the remaining tissue was divided into three flasks containing Ringer's-HCO_3 alone and with serotonin or creatinine sulfate. ^{45}Ca uptake was determined for ~ the next 45 min.

Similar ^{45}Ca uptake experiments were performed from only the serosal surface of stripped ileal mucosa. These experiments were performed in the open-circuited state in tissue mounted in Ussing chambers bathed with Ringer's-HCO_3 on both the mucosal and serosal surfaces. After 30 min of preincubation, serosal ^{45}Ca uptake was measured again using [^3H]-PEG, 900 mol wt, as a serosal extracellular space marker. In these experiments, the rate of ^{45}Ca uptake was measured between 1–15 min after addition of ^{45}Ca in the presence and absence of serosal serotonin (2.6 μM).

Following exposure to ^{45}Ca, the tissue was washed three times in iced isotonic mannitol, blotted lightly, weighed, placed into 1 ml Protosol (New England Nuclear), and incubated overnight at 55°C to dissolve the tissue; then ^{45}Ca and ^3H were determined by liquid scintillation spectrometry using the method of external standards. Calcium uptake was calculated as previously described (15), subtracting the ^{45}Ca in the extracellular fluid from the total tissue ^{45}Ca to give the intracellular ^{45}Ca. Uptake values of ^{45}Ca were calculated by dividing the intracellular ^{45}Ca by the external fluid specific activity and expressing the ^{45}Ca uptake in nanomoles calcium per milligram wet weight per minute. The time of uptake was considered to be the time from addition of the tissue to a solution containing ^{45}Ca until the tissue was placed in iced mannitol.

In vitro ^{45}Ca efflux. The rate of ^{45}Ca efflux from ileal epithelial sheets of ~100 mg wet wt was determined as previously described (11, 16). The ileal tissue was incubated for 60 min in Ringer's-HCO_3 (~2 ml/100 mg tissue) containing ^{45}Ca, 50 μCi/ml, in a shaking water bath at 37°C and oxygenated by bubbling with 95% O_2–5% CO_2. Following this labeling period, the majority of the extracellular ^{45}Ca was removed by rinsing the tissue three times in isotonic iced mannitol and placing each piece of ileum separately for 15 min in an oxygenated flask containing a similar volume of Ringer's-HCO_3 at 37°C. Efflux of ^{45}Ca was then measured from each piece of tissue over eight 10-min periods by placing each separately into an oxygenated flask in a shaking water bath at 37°C containing 2 ml of one of three solutions without ^{45}Ca. After each 10-min period, each piece of tissue was transferred to another similar flask. For the first three 10-min periods, all tissue was in Ringer's-HCO_3, but then for the subsequent five 10-min periods one-third of the tissue was exposed to Ringer's-HCO_3, one-third to a similar solution containing serotonin (0.26 mM), and one-third to a solution containing theophylline (10 mM). In parallel experiments, ileal ^{45}Ca efflux was similarly measured into Ringer's-HCO_3 with the calcium omitted (EGTA not added) and with the calcium increased to 10 mM; all solutions were pH 7.4.

After measuring the efflux rate for 80 min, each piece of tissue was blotted lightly, weighed, placed into 1 ml Protosol and incubated overnight at 55°C. ^{45}Ca was determined by liquid scintillation spectrometry. In addition, radioactivity was determined in tissue handled identically up to the initial efflux measurement, but in which the calcium efflux rate was not measured. ^{45}Ca in the external fluid was also similarly determined. The calcium efflux rate coefficient (ERC) was calculated as previously described (16), using the formula: $ERC = \Delta C/C_m\Delta t \times 100\%$, where ΔC is the radioactivity lost from the tissue during the time interval Δt, and C_m is the mean radioactivity left in the tissue during the interval of the efflux measurement; C_m is calculated by a weighted average of the tissue radioactivity using the values at the beginning and end of the overall efflux measurement. The calcium ERC of the untreated control tissue was taken as 100%, and the changes induced by serotonin or theophylline into solutions of the same calcium concentrations were expressed as percent of the simultaneously studied untreated control tissue.

Agents used in these studies included *d,l*-verapamil and *d*-verapamil (Knoll Pharmaceutical Co., Whippany, N. J., kindly supplied by Mr. A. Graham, Manager of Medical Affairs); theophylline (Eastman Kodak Co., Rochester, N. Y.); and EGTA (Sigma Chemical Co.).

Ileal mucosa was sampled for histology. Tissue was oriented with the villi up, placed in 10% formalin, stained with hematoxylin and eosin, coded, and examined using previously published criteria (17) to determine whether the tissue was histologically normal. All data reported were from histologically normal ileum.

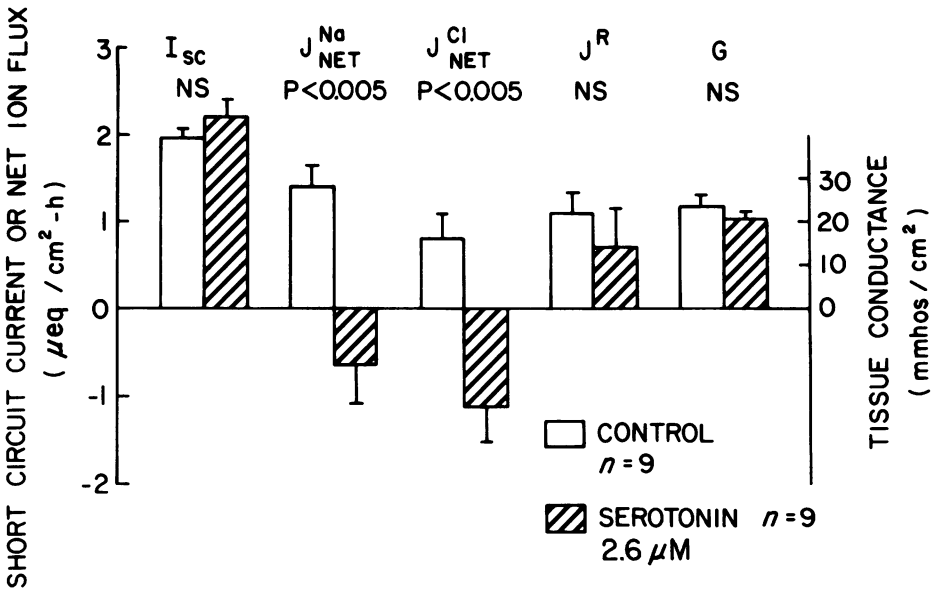
Statistical analyses were performed by *t* test for paired and unpaired data and were two-tailed; linear regression analyses were performed by the method of least squares. All results were expressed as mean±SE.

RESULTS

As demonstrated previously (12), serotonin addition to the serosal but not to the mucosal surface of the ileum caused a short-lived increase in short-circuit current that peaked within 1 min of addition, approached base line within 2 min, and reached base line within 10 min of addition. The explanation for the serotonin-induced peak in short-circuit current is not understood, and there is no evidence that it is related to the effect of serotonin on transmural electrolyte transport. Serotonin also caused a prolonged effect on electrolyte transport, as demonstrated in Fig. 1, which was constant 20–150 min after serotonin addition when compared with simultaneously studied control tissue. This consisted of a decrease in the mucosal-to-serosal movement of Na and Cl and a slight but significant increase in serosal-to-mucosal Cl movement. This resulted in a decrease in net Na transport to approximately zero and in net Cl secretion. During the period of flux determinations, there was no change in the Isc, PD, G, or J^R. Both Isc and electrolyte flux changes were dose dependent with threshold at 26 nM, half-maximal effect at 0.35–0.41 μM, and a maximum effect at 26 μM (12).

Calcium dependence of serotonin effect on active ileal electrolyte transport. To determine the calcium dependence of the serotonin-induced changes in ileal electrolyte transport, the effect of serotonin (2.6 μM) on electrolyte transport (Table I) was determined in the presence of normal calcium and with a calcium-free (no calcium plus 1.0 mM EGTA) bathing solution on either the serosal or mucosal surface.

When serotonin was added to the serosal surface of ileal mucosa exposed for 30 min to a calcium-free serosal bathing solution, the maximum serotonin-induced increase in Isc was 56% of that which occurred when a normal calcium-containing bathing solution



	Na		Cl	
	ms	sm	ms	sm
CONTROL	10.60	9.38	7.42	6.62
SEROTONIN	9.06	9.70	6.13	7.25
	P<0.001	NS	<0.005	<0.02

FIGURE 1 Effect of serosal serotonin (2.6 μM) on rabbit ileal electrolyte transport 20–150 min after serotonin addition. *n* refers to number of animals studied. ms refers to mucosal-to-serosal and sm refers to serosal-to-mucosal unidirectional ion fluxes. *P* values represent comparisons of untreated control and serotonin-treated tissue from the same animals (paired *t* test).

was present for the same period of time (14.9±3.0 vs. 26.8±3.2 μA/cm² in calcium-free and normal calcium serosal bathing solutions, respectively). Exposure of the mucosal surface to the calcium-free bathing solution for a similar period of time did not significantly alter the Isc response (21.1±2.4 μA/cm²). Removing serosal calcium for as short a period of time as 1 min before serotonin addition caused a significant decrease (20.0±2.1 μA/cm²; 25%) in the maximum serotonin-induced increase in Isc.

Removing serosal calcium significantly increased the tissue conductance (18.4±1.3 vs. 34.7±2.8 mmho/cm², $P < 0.001$). The conductance was constant during the 80-min period of flux determinations and up to 150 min. The absence of serosal calcium prevented the serotonin-induced changes in net Na and Cl transport, and also prevented the serotonin-induced decrease in the mucosal-to-serosal movement of both Na and Cl, but did not prevent the serotonin-induced increase in serosal-to-mucosal Cl flux. Both in the presence and absence of serosal calcium, serotonin did not alter Isc, PD, G, or J^R during the period of flux determinations.

When calcium was added back to the serosal bathing solution, the serotonin effect reappeared. After determining the serotonin effect for an initial period of 80 min in the calcium-free solution, the serosal bathing fluid was washed out and replaced with Ringer's-HCO_3, containing 1.2 mM calcium plus the same concentration of serotonin (2.6 μM) and the fluxes determined for another 60 min. The serotonin effects were determined by comparison with electrolyte transport in untreated control tissue exposed to the same serosal bathing solutions as the serotonin-exposed tissue and studied over a similar time period. The serotonin-induced changes in net Na and Cl fluxes in the presence of normal serosal calcium were the same whether the tissue had been continually exposed to 1.2 mM serosal calcium (Table I) or had initially been incubated in the calcium-free bathing solution (ΔJ^{Na}_{net} – 1.39±0.51 μeq/cm²-h; ΔJ^{Cl}_{net} – 1.66±0.74).

To determine whether there was a dose dependence of the serotonin-induced changes in net Na and Cl fluxes on the serosal calcium concentration, serosal Ringer's-HCO_3 was substituted not only with a calcium-free solution, but also with varying concentrations of calcium between 0 and 3 mM. As demonstrated in Fig. 2, serosal calcium concentrations of 0, 0.3, and 0.6 mM inhibited the serotonin-induced changes in

TABLE I

Effect of Serosal Calcium on Serotonin (2.6 μM)-induced Changes in Rabbit Ileal Electrolyte Transport

Bathing fluid [Calcium]												
Mucosal	Serosal	No.	ΔIsc	ΔPD	ΔG	ΔJ^{Na}_{ms}	ΔJ^{Na}_{sm}	ΔJ^{Na}_{net}	ΔJ^{Cl}_{ms}	ΔJ^{Cl}_{sm}	ΔJ^{Cl}_{net}	ΔJ^{R*}
			uA/cm²	*mV*	*mmho/cm²*				*μeq/cm²-h*			
1.2 mM	1.2 mM	15	6.8	−1.6	−2.7	−0.96	0.14	−1.10	−1.14	0.76	−1.90	−0.59
			±	±	±	±	±	±	±	±	±	±
			4.8	0.8	2.2	0.41	0.49	0.40	0.51	0.37	0.23	0.61
	P‡		NS	NS	NS	<0.02	NS	<0.01	<0.02	<0.05	<0.01	NS
1.2 mM	0 + 1 mM EGTA	9	−6.8	−1.3	2.0	−0.51	−0.40	0.11	0.83	0.91	−0.08	−0.44
			±	±	±	±	±	±	±	±	±	±
			5.1	0.4	4.1	0.65	0.41	0.88	0.51	0.44	0.36	0.59
	P‡		NS	NS	NS	NS	NS	NS	NS	<0.05	NS	NS
	P§		NS	NS	NS	NS	NS	NS	<0.05	NS	<0.05	NS
0 + 1 mM EGTA	1.2 mM	6	−4.8	−2.2	1.7	−1.74	0.26	−2.00	−2.07	0.73	−2.80	−0.94
			±	±	±	±	±	±	±	±	±	±
			2.6	1.4	2.3	0.71	0.41	0.77	0.96	0.32	0.84	0.74
	P‡		NS	NS	NS	<0.05	NS	<0.01	<0.02	<0.05	<0.01	NS
	P§		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data were obtained by comparing the effect of serotonin in Ringer's-HCO_3 20–80 min after serotonin addition with simultaneously run, untreated control tissue in Ringer's-HCO_3, and the effect of serotonin in the calcium-free (no calcium plus 1 mM EGTA) bathing solution on the serosal or the mucosal surfaces 20–80 min after serotonin addition to simultaneously run untreated control tissue in the comparable calcium-free bathing solution. In these experiments the tissue was incubated for 30 min in the no calcium-containing solution to allow the conductance to stabilize. When analyzed by each 20-min period, there was no significant effect of time on any parameter.

* $J^R = Isc - J^{Na}_{net} + J^{Cl}_{net}$.

‡ *P* values represent comparisons of serotonin and untreated control tissue from the same animals having the same calcium bathing solution on either the mucosal or serosal surface (paired *t* test).

§ *P* values represent comparisons of serotonin-induced changes (serotonin minus untreated control) in ileal electrolyte transport in the presence and absence of calcium in the bathing solution in tissue from the same animal (unpaired *t* test).

net Na and Cl fluxes. Serotonin did not significantly alter net Na or Cl fluxes at a serosal calcium concentration < 0.6 mM, and a serosal calcium concentration of 0.9 mM was needed for the full serotonin effect. Increasing the serosal calcium concentration above 0.9 mM did not further alter the serotonin effects.

In contrast to the dependence of the serotonin-induced changes in ileal electrolyte transport on the serosal calcium concentration, a calcium-free (no calcium plus 1 mM EGTA) solution on the mucosal surface did not alter any parameter of the serotonin (2.6 μM) effects (Table I).

That removing serosal calcium did not inhibit all active electrolyte transport processes was determined by the use of the cyclic nucleotide phosphodiesterase inhibitor theophylline at a concentration (10 mM) that causes a maximum secretory effect on active ileal electrolyte transport. Theophylline caused similar effects on electrolyte transport in the presence of standard Ringer's-HCO_3 (ΔJ^{Na}_{net} − 1.58±0.25 μeq/cm²-h; ΔJ^{Cl}_{net} − 2.05±0.41; ΔIsc = 75.4±6.4 μA/cm²) and when a calcium-free (no calcium plus 1.0 mM EGTA) serosal solution was present (ΔJ^{Na}_{net} − 1.54 ±0.35 μeq/cm²-h, ΔJ^{Cl}_{net} − 2.23±0.30, ΔIsc = 64.6 ±6.3 μA/cm²).

Effect of verapamil on serotonin-induced changes in ileal electrolyte transport. To get further evidence that removing serosal calcium was not acting by damaging the ileal mucosa, and to verify the calcium dependence of the serotonin-induced changes in Isc and net Na and Cl fluxes, use was made of racemic verapamil. Verapamil was shown previously to block calcium channels in several tissues including cardiac muscle (18–20). In these experiments, *d,l*-verapamil (0.1 mM) was added to the mucosal, serosal, or mucosal plus serosal surfaces following stabilization of the ileal tissue in Ringer's -HCO_3. The effect of serotonin on Isc was determined 5 min after adding verapamil. The conductance in untreated control or serotonin-exposed tissue was similarly changing in the verapamil-exposed tissue. Furthermore, the stabilization of ileal conductance in the presence of verapamil took ~10 min longer than stabilization in Ringer's-HCO_3 alone. Consequently, in the experiments determining the effects of verapamil on serotonin-induced changes in electrolyte fluxes, it was necessary to

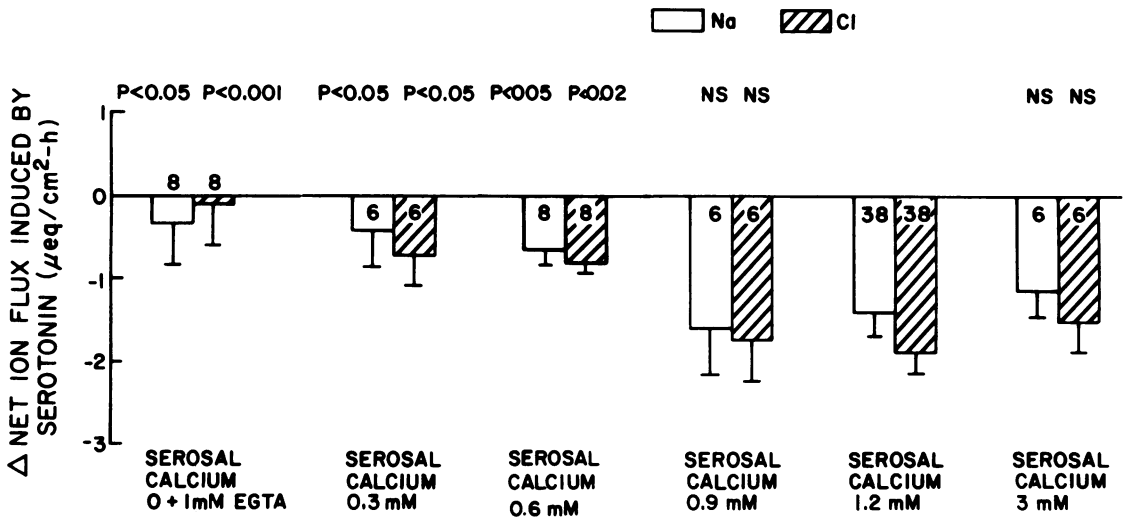


FIGURE 2 Dependence of serotonin (2.6 μM)-induced changes in net Na and Cl fluxes on the serosal calcium concentration. ΔNet Na and Δnet Cl fluxes (serotonin-treated minus untreated control) refer to simultaneously studied ileal tissue having a similar calcium concentration. Numbers in bars represent number of animals studied. *P* values represent comparisons of Δnet Na and Δnet Cl fluxes induced by serotonin in the presence of standard Ringer's-HCO_3 and in the presence of the altered serosal calcium concentration (unpaired *t* test).

initially mount the tissue in bathing solution containing verapamil.

Addition of *d,l*-verapamil (0.1 mM) to the mucosal plus serosal surfaces and to the serosal surface alone for 5 min before serotonin addition significantly decreased the serotonin-induced increase in Isc, although mucosal verapamil had no significant effect (maximum serotonin-induced increase in Isc [μA/cm²]: control, no verapamil, 23.0±2.4; verapamil, mucosal plus serosal surfaces, 7.3±0.8, $P < 0.01$; verapamil, serosal surface only, 7.0±1.9, $P < 0.01$; verapamil, mucosal surface only 23.2±2.4, NS; all comparisons with control).

The presence of *d,l*-verapamil on the mucosal plus serosal surfaces or on the serosal surface alone also inhibited the serotonin-induced changes in net Na and Cl fluxes (Table II). In contrast, in the presence of mucosal *d,l*-verapamil, serotonin still caused significant decreases in net Na and Cl fluxes.

To further document the calcium dependence of the serotonin effects, use was made of *d*-verapamil, an optical isomer of verapamil, which does not appear to alter "calcium channels" (19, 20). In contrast to the effect of *d,l*-verapamil, the presence of *d*-verapamil (0.1 mM) on the serosal surface did not alter the (2.6 μM) serotonin-induced changes in net Na or Cl fluxes (ΔJ^{Na}_{net} − 1.11±0.28 vs. −1.28 + 0.24 μeq/cm²-h; ΔJ^{Cl}_{net} −1.24±0.27 vs. −1.42±0.31 μeq/cm²-h in the absence and presence of serosal *d*-verapamil, respectively.

That the *d,l*-verapamil inhibition of serotonin-induced secretion did not represent a general suppression of active electrolyte secretion was demonstrated again by the use of theophylline. As demonstrated in Table III, the effect of theophylline (10 mM) on net Na and Cl fluxes, and the theophylline-induced increase in Isc were the same whether *d,l*-verapamil (0.1 mM) was present or absent from the mucosal plus serosal surfaces at the time theophylline was added to the serosal surface.

Effect of serotonin on in vitro ileal ^{45}Ca uptake and efflux. Intestinal ^{45}Ca uptake into epithelial sheets of ileum was determined subtracting the ^{45}Ca in the extracellular space determined by use of [^{3}H]PEG, 900 mol wt, as an extracellular space marker. When both mucosal and serosal surfaces were exposed to PEG simultaneously, there was a rapid PEG uptake, which reached a relatively constant value of ~30% of tissue wet wt by 15 min and remained at that value for ~105 min. When PEG was only exposed to the ileal serosal surface, an almost constant value for PEG uptake of ~10% of tissue wet wt was reached by 5 min and the rate was relatively constant for the 30 min studied. If ileal mucosa was incubated for >105 min, occasionally the extracellular space became very large. This was interpreted as loss of tissue integrity.

In untreated control tissue, the rate of ileal ^{45}Ca uptake was measured 1, 5, 15, 30, 45, 60, 65, 75, 85, and 95 min after addition of ^{45}Ca (Fig. 3). ^{45}Ca uptake was ~10% of the maximum after 1 min of exposure to ^{45}Ca, ~50% after 5 min, became constant 30–45 min after ^{45}Ca addition, and remained constant until at least 105 min. This suggests that by 30–45 min, the ^{45}Ca had reached near equilibration with the rapidly exchangeable

TABLE II

Effect of d,l-Verapamil on Δnet Na and Δnet Cl Fluxes Induced by Serotonin (2.6 μM)

	No. of animals	ΔNa	P*	ΔCl	P*
		μeq/cm²-h		μeq/cm²-h	
Control No verapamil	18	−1.38 ± 0.21		−1.61 ± 0.34	
Verapamil Mucosal plus serosal surfaces	12	0.28 ± 0.36	<0.01	−0.05 ± 0.41	<0.01
Verapamil Serosal surface only	8	0.29 ± 0.45	<0.02	−0.42 ± 0.57	<0.05
Verapamil Mucosal surface only	8	−0.87 ± 0.41	NS	−0.96 ± 0.47	NS

Tissue stabilized either in Ringer's-HCO_3 or Ringer's-HCO_3 plus verapamil (0.1 mM) before serotonin addition to serosal surface. Δnet Na and Δnet Cl refer to the effect of serotonin (serotonin minus untreated control) measured 20–80 min after addition of serotonin with both serotonin and untreated control tissue having similar exposure to verapamil. In the verapamil studies, 0.1 mM verapamil was added at the time of tissue mounting ~30 min before addition of serotonin. When analyzed by each 20-min period, there was no significant effect of time on any parameter.

* P values represent comparisons of serotonin-induced Δnet Na and Δnet Cl in the absence and the presence of verapamil in the same animal (paired *t* test).

cytosolic calcium pools. When serotonin (2.6 μM) was added 60 min after ^{45}Ca addition, serotonin induced a rapid increase (Fig. 3) in ^{45}Ca uptake that was significant when first measured 5 min after serotonin addition and was still present 15–35 min after serotonin addition. Because of the large standard errors, the time it took for the maximum effect of serotonin on ^{45}Ca uptake to occur could not be determined. (Calcium uptake after exposure to serotonin in nanomoles per milligram wet weight at 5 min was 1.56±0.37; at 15 min, 1.63±0.15; at 25 min, 2.40±0.76; and at 35 min, 1.79±0.39. Calcium uptake at the same times in control

TABLE III

Effect of d,l-Verapamil on Δnet Na and Δnet Cl Fluxes Induced by Theophylline (10 mM)

	No. of animals	ΔNa	P*	ΔCl	P*	ΔIsc	P*
		μeq/cm²-h		μeq/cm²-h		μA/cm²	
Theophylline	8	−1.58 ± 0.25		−2.05 ± 0.41		75.4 ± 6.4	
Theophylline plus verapamil on mucosal and serosal surfaces	8	−1.91 ± 0.61	NS	−2.86 ± 0.57	NS	79.6 ± 12.0	NS

Tissue stabilized either in Ringer's-HCO_3 or Ringer's-HCO_3 plus verapamil (0.1 mM) before theophylline added to serosal surface. ΔNet Na and Δnet Cl refer to the effect of theophylline (theophylline minus untreated control) measured 20–80 min after addition of theophylline with both theophylline and untreated control tissue having similar exposure to verapamil. ΔIsc refers to the maximum increase in Isc that occurred after theophylline addition. In the verapamil studies, 0.1 mM verapamil was added at the time of tissue mounting ~30 min before addition of theophylline.

* *P* values represent comparisons of Δnet Na, Δnet Cl, and ΔIsc induced by theophylline in the absence and the presence of verapamil in the same animal (paired *t* test).

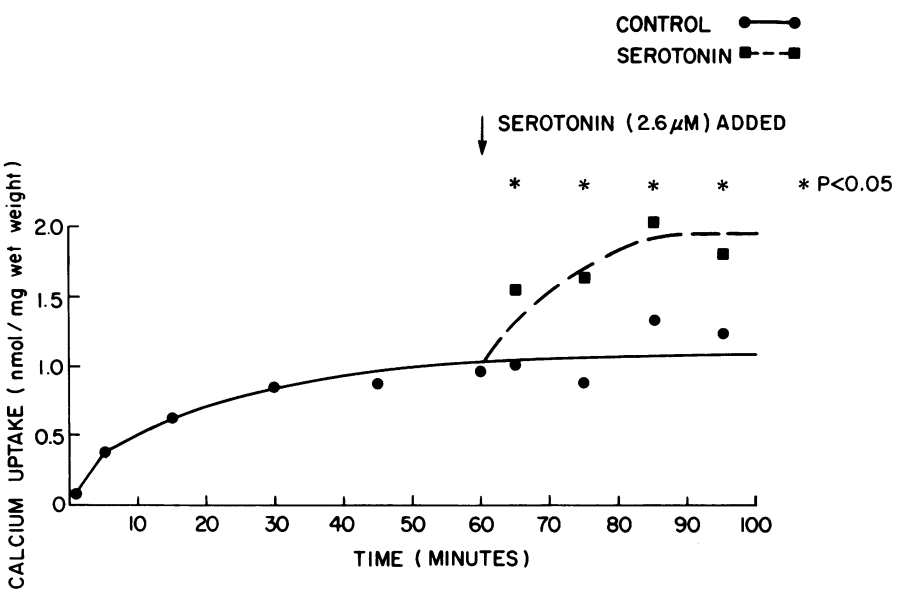


FIGURE 3 Effect of serotonin on in vitro ^{45}Ca uptake into rabbit ileum, measured using [^{3}H]-PEG, 900 mol wt, as an extracellular space marker. Serotonin was added 60 min after ^{45}Ca, after isotopic equilibrium had been reached. *P* values represent comparisons of serotonin exposed and untreated control tissue from the same animal (paired *t* test). Tissue from eight animals was studied.

tissue was, 5 min after serotonin addition, 1.21 ±0.26; after 15 min, 1.11±0.24; after 25 min, 1.07±0.15; and after 35 min, 1.30±0.79; eight animals studied) Because the effect of serotonin on ^{45}Ca uptake was determined at close to isotopic equilibrium, the serotonin-induced increase in ^{45}Ca uptake strongly suggests that serotonin increased the total calcium content of the ileal mucosa. That this effect of serotonin is due to an increase in nonspecific binding of ^{45}Ca is unlikely, because the large increase in ^{45}Ca occurred at a point close to isotopic equilibrium.

The increased calcium content induced by serotonin could have been caused by increased ^{45}Ca uptake or decreased ^{45}Ca efflux. Because we previously demonstrated that serotonin altered active ileal electrolyte transport only from the serosal surface (12), it was determined whether serotonin also altered ^{45}Ca uptake from the serosal surface of the ileum. ^{45}Ca uptake from the serosal bathing surface was determined in stripped ileal mucosa mounted in an Ussing chamber to allow separation of mucosal from serosal bathing fluids and was studied in the open-circuited state with a 5-min preincubation with PEG as an extracellular marker on the serosal surface. As demonstrated in Fig. 4, serotonin significantly increased the ileal uptake of ^{45}Ca from the serosal surface 1, 2, 3, and 5 min after ^{45}Ca addition.

The effect of serotonin on ^{45}Ca efflux from epithelial sheets of rabbit ileum was also determined. When the efflux of ^{45}Ca from ileal epithelial sheets into a no-calcium Ringer's-HCO_3 solution (no EGTA added) was determined (Figs. 5, 6), serotonin (2.6 μM and 0.26 mM) caused an increased rate of ^{45}Ca efflux that became significant after 10 min of exposure to serotonin and continued for the subsequent 40 min of exposure. Serotonin increased the rate of ^{45}Ca efflux only when there was no calcium in the external bathing solution. When calcium was present, serotonin caused a significant decrease of ^{45}Ca efflux, which was slightly but not significantly greater when the external bathing solution had a calcium concentration of 10 mM compared with 1.2 mM. In contrast to the effect of serotonin, theophylline caused an increase in efflux of ^{45}Ca both when there was no calcium in the external bathing solution and when the calcium concentration was 1.2 mM (Fig. 6). Histologic sections of ileal mucosa incubated for comparable periods of time (80 min) in calcium-free and calcium-containing (1.2 mM) solutions were not different by light microscopy and there was no evidence of a significant loss of epithelial cells.

DISCUSSION

We have demonstrated that the serotonin-induced changes in rabbit ileal short-circuit current and in active transport of Na and Cl depend on serosal calcium, inasmuch as removal of serosal calcium or the presence of serosal *d,l*-verapamil, which is known to block "calcium channels" (18–20), inhibited these serotonin-induced effects. In examining possible

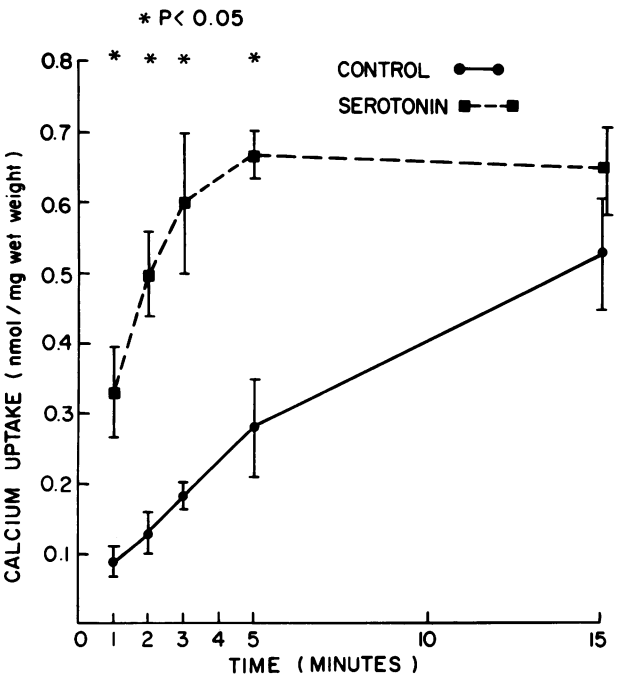


FIGURE 4 Effect of serotonin (2.6 μM) on in vitro ^{45}Ca uptake from the serosal surface alone of rabbit ileal mucosa mounted in Ussing chambers, using serosally placed [^{3}H]-PEG, 900 mol wt, as an extracellular space marker. During these studies the tissue was not short-circuited. Tissue was allowed to equilibrate in Ussing chambers for 25 min, then PEG was allowed to equilibrate with the tissue for 5 min before ^{45}Ca addition, during which time serotonin was also present in the serosal fluid. Extracellular space was not altered by the presence of serotonin. *P* values represent comparisons of serotonin-exposed and untreated control tissue from the same animals (paired *t* test). Tissue from eight animals was studied.

mechanisms of this calcium dependence, we demonstrated that serotonin increased ^{45}Ca uptake into rabbit ileum in vitro studied at close to isotopic equilibrium, which indicated that serotonin caused an increase in ileal calcium content. Consistent with the demonstration that serotonin altered ileal electrolyte transport only when exposed to the serosal surface (an effect dependent on the presence of serosal calcium), serotonin was shown to increase ^{45}Ca uptake from the serosal surface of the ileum. Whether the total explanation for the calcium dependence of the serotonin-induced changes in rabbit ileal electrolyte transport is provided by the increased uptake of ^{45}Ca from the serosal surface, and exactly what this increased calcium is doing at the cellular level, awaits further studies.

The dependence of the ileal effects of serotonin on serosal calcium was determined both by using a calcium-free media which contained 1.0 mM EGTA, and by using *d,l*-verapamil. The reason for using two methods of evaluating the dependence on serosal calcium was because of the possibility that 1.0 mM EGTA might have had an effect independent of lowering the calcium (including a toxic effect), and racemic verapamil has been suggested to be a blocker of sodium as well as calcium channels. That both conditions caused similar changes in the effects of serotonin, that the effect of removing serosal calcium was reversible, that *d*-verapamil, the isomer thought not to block calcium channels, failed to alter the serotonin effects, and that both removing serosal calcium and racemic verapamil did not alter another active intestinal secretory process (theophylline-induced electrolyte secretion) is strong evidence that dependence on serosal calcium has been demonstrated.

In contrast, interpretations of both calcium uptake and calcium efflux experiments are somewhat speculative. The effect of serotonin on intestinal ^{45}Ca uptake performed at close to isotopic equilibrium suggests that serotonin increased the total intestinal calcium content, though the total calcium content was not measured directly. The best explanation for this serotonin-induced increase in both calcium influx and efflux is that serotonin appears to increase the ileal plasma membrane permeability to calcium. There are several possible explanations for the failure of serotonin to increase calcium efflux when calcium was present in the bathing solution. One possibility is that serotonin increased the influx of unlabeled calcium, which then decreased the specific activity of the rapidly exchangeable intracellular calcium pool, and that this decrease then produced a decrease in the measured efflux of ^{45}Ca. Another possibility is that in the absence of extracellular calcium, much of the ^{45}Ca that left the cell was recycled from the unstirred water layer, reducing the efflux rate. In contrast, in the presence of extracellular calcium, the ^{45}Ca that escaped from the tissue was diluted by the unlabeled calcium, preventing the decrease in the ^{45}Ca efflux rate.

The current studies are all consistent with, but do not prove, the hypothesis that the calcium dependence of the serotonin effects on active electrolyte transport is related to the changes in the intracellular, rapidly exchangeable calcium pools. If serotonin increases the ileal plasma membrane permeability to calcium, it would be expected that changes in the bathing solution would cause rapid changes in these intracellular calcium pools. The data consistent with the serotonin effects involving changes in the rapidly exchangeable calcium pools include the following: (*a*) the effect of serotonin on net Na and Cl fluxes was dependent on the calcium concentration bathing the serosal surface in a dose-dependent manner; (*b*) the effect of removing serosal calcium was rapidly reversible; (*c*) removing the serosal calcium for as short a time as 1 min significantly decreased the effect of serotonin on Isc; and (*d*) the greater the time the ileum was exposed to a calcium-free serosal solution, the greater the inhibition

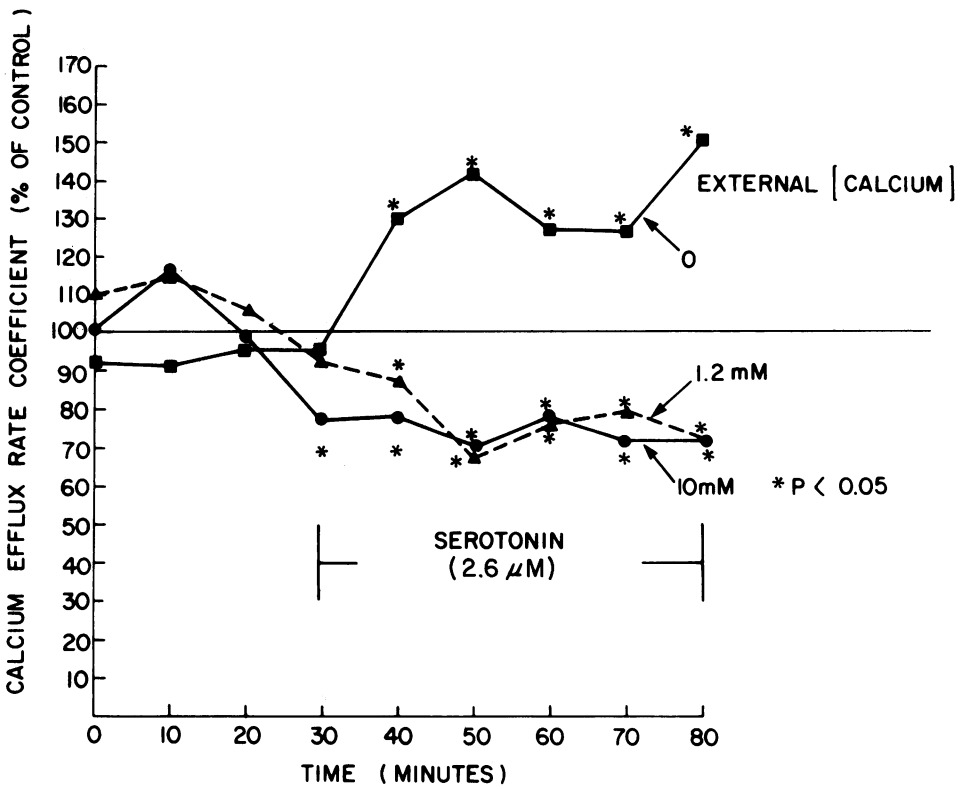


FIGURE 5 In vitro ^{45}Ca efflux from rabbit ileum into solutions having calcium concentrations of 0, 1.2, or 10 mM. Efflux from tissue exposed to serotonin (2.6 μM) was compared with untreated control tissue at each time period and the results presented as percentage of the control tissue. *P* values represent comparisons between serotonin-exposed and untreated control tissue from the same animal (paired *t* test). Tissue from eight animals was studied.

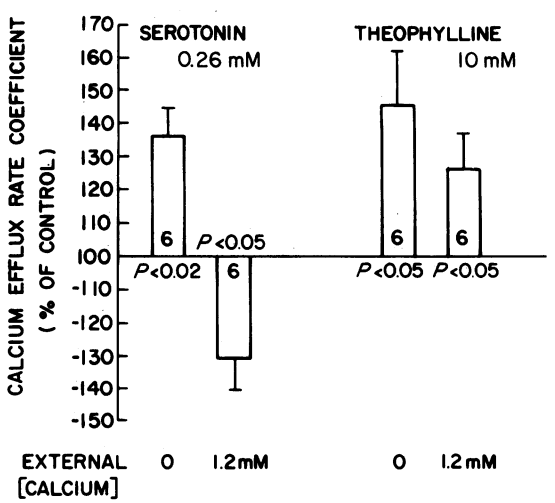


FIGURE 6 Effect of serotonin and theophylline on calcium efflux from rabbit ileum into solutions having calcium concentrations of 0 or 1.2 mM. The concentrations of serotonin and theophylline were those that maximally changed electrolyte transport. The calcium ERC for each of the 10-min periods of exposure to either serotonin or theophylline were determined as a percentage of the control period and the mean value±SE plotted. *P* values represent comparisons between serotonin-exposed or theophylline-exposed and untreated control tissue from the same animal (paired *t* test). Numbers in parentheses represent number of animals studied.

of the serotonin effect on Isc. In addition, although *d,l*-verapamil is thought to block only membrane channels and not to work at an intracellular site, because of the estimated small size of the intracellular, rapidly exchangeable calcium pools, *d,l*-verapamil would also be expected to produce a decrease in intracellular calcium (21). We have previously suggested that the major effect of serotonin on active electrolyte transport is to inhibit the neutral NaCl uptake process at the brush border (12). This, plus the dependence of the serotonin effects on serosal calcium and the apparent serotonin-induced increase in intestinal calcium content, suggests that the neutral NaCl uptake process at the brush border can be inhibited by increasing the intracellular, rapidly exchangeable calcium pools.

Both the calcium ionophore A23187 and serotonin have now been shown to alter active electrolyte transport by a calcium-dependent mechanism. However, serotonin and the calcium ionophore do not act in

an identical manner. In contrast to the dependence of the effect of serotonin on the serosal calcium concentration, after removal of serosal calcium the calcium ionophore A23187 still altered active electrolyte transport in rabbit ileum (10). In addition to inhibiting the neutral NaCl absorptive process, the calcium ionophore increased rabbit ileal Isc and induced electrogenic Cl secretion (10). The calcium ionophore has been suggested to increase intracellular calcium both by increasing the calcium permeability of the plasma membrane and by mobilizing calcium from intracellular stores (22, 23). When the changes caused by ionophore A23187 were compared in the absence and presence of normal serosal calcium, that part of the ionophore effect that disappeared when serosal calcium was removed was just that which serotonin induced: i.e., a decrease in the mucosal-to-serosal movement of Na and Cl and a decrease in net Na movement (10). The explanation for the difference of the serotonin-induced and calcium ionophore-induced changes in active ileal electrolyte transport is not known. Possibilities are that the ionophore causes a quantitatively greater increase in intracellular calcium, that the mobilization of intracellular calcium by the ionophore causes the additional effects by a separate intracellular process, and that the ionophore affects a population of cells which are not altered by serotonin. A further question is whether the calcium dependence of the serotonin-induced changes in ileal electrolyte transport represents the mechanism by which other intestinal secretagogues act, or whether this is unique for serotonin. That the former may be the case is suggested because the carbamylcholine-induced increase in rabbit ileal Isc was also abolished by removing serosal calcium (10). Determining whether intestinal secretagogues still alter intestinal electrolyte transport after removal of serosal calcium or in the presence of serosal *d,l*-verapamil seems to be a satisfactory way to determine whether intestinal secretagogues act similarly to serotonin and are calcium dependent. An alternate but less direct approach would be to determine which secretagogues increase uptake of ^{45}Ca at isotopic equilibrium. In contrast, it would not be acceptable to measure ^{45}Ca efflux to suggest a calcium-dependent mechanism because theophylline, the effect of which on intestinal electrolyte transport is not dependent on serosal calcium (though it may be dependent on intracellular calcium and may involve mobilization of intracellular calcium stores) also increased ^{45}Ca efflux. To date, the mechanism by which many intestinal secretagogues affect intestinal electrolyte transport remains unknown. Extensive studies of their effects on the adenylate cyclase-cAMP system strongly suggest that other intracellular mediators are often involved (24–29). Use of the in vitro techniques outlined in this paper seems to be a promising approach to increased knowledge of the involvement of calcium in active electrolyte transport processes and of the pathophysiology of diarrhea.

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