Calcium Modulation of the Effects of Serotonin, Carbachol, and Histamine on Rabbit Ileal Ion Transport

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(Submitted November 18, 1993; sent for revision February 1; accepted February 18, 1994)

In mammalian intestine, a number of secretagogues have been shown to work through either cyclic nucleotide or calcium mediated pathways to elicit ion secretion. Because excessive intestinal electrolyte and fluid secretion is central to the pathogenesis of a variety of diarrheal disorders, understanding of these processes is essential to the development of future clinical treatments.

In the current study, the effects of serotonin (5HT), histamine, and carbachol on intestinal ion transport were examined in in vitro preparations of rabbit ileum. All three agonists induced a rapid and transient increase short-circuit current (ΔIsc) across ileal mucosa. Inhibition of the ΔIsc response of all three agents in chloride-free solution or in the presence of bumetanide confirmed that chloride is the main electrolyte involved in electrogenic ion secretion. Pretreatment of tissue with tetrodotoxin or atropine did not effect secretagogue-mediated electrolyte secretion. While tachyphylaxis of ΔIsc response was shown to develop after repeated exposure of a secretagogue to tissue, ΔIsc responses after sequential addition of different agonists indicate that cross-tachyphylaxis between agents did not occur.

Serotonin, histamine, and carbachol have previously been reported to mediate electrolyte secretion through calcium-dependent pathways. To access the role of extracellular calcium in regulating ion secretion, the effect of verapamil on each agent was tested; verapamil decreased 5HT-induced ΔIsc by 65.2% and histamine response by 33.5%, but had no effect on carbachol-elicted chloride secretion. An additive secretory effect was found upon simultaneous exposure of 5HT and carbachol to the system; no other combination of agents produced a significant additive effect. Findings from this study support previous work which has suggested that multiple calcium pathways may be involved in mediating chloride secretion in mammalian intestine.

In mammalian intestine, the balance between active secretory and active absorptive processes determines net ionic transport. Regulation of active ion secretion has been extensively studied in mammalian intestine. Chloride secretion appears to be the major process, occurring in the crypts of the jejunum, ileum, and descending colon [1, 2]. The cellular mechanism for intestinal chloride secretion has been studied most extensively in the colon [3–6]. The basolateral Na+-K+-ATPase creates an electrochemical gradient

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Abbreviations used: ΔIsc, change in short circuit current; 5HT, 5 hydroxytryptamine; CCH, carbachol; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide; TMB-8, 3,4,5 trimethoxy benzoate-8.
focusing the movement of sodium into intestinal cells. Chloride entry across the basolateral membrane is coupled to sodium and potassium movement (Na⁺−K⁺−2Cl⁻ co-transport). The chloride ions exit from the cell into the intestinal lumen across the chloride permeable apical membrane. It is currently accepted that apical membrane chloride permeability is the rate limiting step for intestinal chloride secretion [7].

Since water follows ion movement across osmotic gradients, the elucidation of these processes is essential to the delineation of the pathophysiology of a variety of diarrheal diseases. A number of secretagogues, including serotonin (5HT)⁴, vasoactive intestinal peptide (VIP), cholera toxin, and heat-labile E. Coli enterotoxin cause severe secretory diarrhea by stimulating electrogenic chloride secretion [8-14]. The secretory effects of VIP [15, 16], cholera toxin [17], and heat-labile E. Coli enterotoxin [14] have been shown to be mediated by changes in intracellular levels of cyclic AMP. In contrast, experimental evidence suggests that serotonin regulates electrolyte secretion in mammalian intestine through calcium-dependent mechanisms.

Initial evidence that calcium might function as a second messenger in intestinal electrolyte transport was derived from observations in rabbit ileum and descending colon that calcium ionophore A23187 increased chloride secretion without affecting cyclic AMP levels [19, 20]. Further support for the role of calcium has come from the identification of a number of neurohumoral agents normally present in the intestinal mucosa that alter ion transport by mechanisms that appear to involve changes in intracellular calcium levels. Serotonin, carbachol [21, 22], histamine [23, 24], substance P [25, 26], neurotensin [25, 26], and bradykinin [27] are among the growing number of agents believed to mediate their secretory effects through calcium-dependent processes.

Intracellular regulation of intestinal ion and fluid transport by calcium-mediated agonists has previously been studied in a number of mammalian systems. The actions of serotonin, histamine, and carbachol have been shown to be independent of intracellular cyclic nucleotide messenger pathways; electrolyte transport induced by each of these agents was not associated with changes in cyclic AMP levels [10, 19, 22, 24, 28]. Serotonin-induced chloride secretion appears to be dependent on the presence of extracellular calcium; in rabbit ileum, serotonin-elicted electrolyte transport has been associated with increased uptake of calcium into mucosal cells, suggesting that serotonin's major mechanism of action involves enhanced ileal plasma membrane permeability [18]. A recent study suggests that carbachol and histamine may mediate intestinal secretion through a separate calcium pathway - induction of phosphatidylinositol hydrolysis and subsequent release of calcium from intracellular stores [29, 30].

In the present study, we examined the secretory effects of these three agonists using freshly isolated mucosa from rabbit ileum. While the use of cultured colonic epithelial tumor cell lines permits detailed study of receptor and signal transduction mechanisms in a simplified system that is devoid of neural elements and peptide hormones, complex interrelated regulatory responses to multiple secretagogues are best studied using intact physiological systems. Because the composition, organization, and physical properties of the extracellular matrix appear to play critical roles in modulating cell phenotype and function, two-dimensional cultured cell layers may not be ideal systems in which to study these effects [21, 31-33]. In contrast, while investigations in freshly isolated ileal mucosa
do not precisely localize the site of agonist action, they more closely mimic intact gastrointestinal systems, and thus, may serve as better models for human disease. While prior studies in Tma colonic tumor cell lines have reported additive secretory effects between a variety of intestinal secretagogues [29, 34–36], no experiment to date has examined the combination of agents proposed to act through separate calcium pathways. The goal of the current study was to evaluate further the individual and additive effects that three different putative calcium agonists (serotonin, histamine, and carbachol) exert on rabbit ileal ion transport.

**MATERIALS AND METHODS**

**Materials.** Serotonin (5-hydroxytryptamine creatinine sulfate), carbachol, histamine, bumetanide, diphenhydramine, cimetidine, atropine sulfate, tetrodotoxin, and ketamine were purchased from Sigma Chemical (St. Louis, MO). Verapamil was purchased from Searle Pharmaceuticals (Chicago, IL).

**ΔIsc measurements in ileal mucosa.** New Zealand White rabbits weighing 3–4 kg were maintained on standard rabbit chow and water *ad lib.* After anesthesia with 10 mg/kg of intravenous ketamine, segments of distal ileum were removed, opened along the mesenteric border, and rinsed in Krebs Ringers-bicarbonate solution (glucose free). The serosa and muscularis propria were bluntly stripped from the specimen leaving intact mucosa, submucosa, and muscularis mucosa. The stripped mucosal tissues were mounted as a flat sheet across lucite Ussing Chambers with a surface area of 1.13 cm². Equal volumes of glucose-free Krebs Ringers-bicarbonate solution (10 mL) were placed on the serosal and the mucosal sides of the chambers. The bathing solution had the following composition (mM): Na⁺ 141; K⁺ 5; Ca²⁺ 1.2; Mg²⁺ 1.2; Cl⁻ 122; HCO₃⁻ 25; HPO₄²⁻ 1.4; H₂PO₄⁻ 0.6. A chloride-free solution was produced by replacement of Cl⁻ ions with an equal concentration of glutamate. For all solutions, the pH was maintained at 7.4. The bathing solutions were circulated and oxygenated with a gas mixture of 95% O₂ and 5% CO₂. Using the water-jacketed system described by Schultz and Zalusky [37], the temperature of the system was maintained at 38°C.

Potential differences across the mucosal membrane were measured by voltage electrodes immersed in the bathing solution on either side of the membrane. Electrodes (Ag/AgCl) connected to a voltage clamp device (World Precision Instruments DVC 1000) were used to set the transepithelial potential difference at zero. This current, designated the short circuit current (ΔIsc), is a direct measure of net electrogenic ion transport across the membrane [38]. Conductance, an indicator of tissue viability, was determined by dividing ΔIsc by the spontaneous potential difference. In this study, to control for differences in tissue viability, conductances of paired tissues were within ± 20% of each other.

**Experimental protocol.** Tetrodotoxin (3 x 10⁻⁷ M) and verapamil (5 x 10⁻⁵ M) were added to the mucosal and serosal reservoirs 10 min before addition of the experimental agents. The serosal sides of the chambers were preincubated for 10 min with bumetanide (5 x 10⁻⁴ M), atropine (10⁻⁴ M), cimetidine (10⁻³ M), and diphenhydramine (10⁻⁴ M). In each of the experimental conditions, serotonin (10⁻⁴ M or 10⁻⁵ M), histamine
(10⁻³ M or 10⁻⁴ M), and carbachol (10⁻⁴ M or 10⁻⁵ M) were added to the serosal side of the mounted tissue and ΔIₑₛ response was measured. When agonists were added sequentially, the maximum ΔIₑₛ response to the first agent was measured and short circuit current was then allowed to return to baseline before the addition of the second secretagogue. In studies investigating additive properties, ΔIₑₛ was measured following simultaneous addition of two different agonists to the serosal chamber. Because absolute ΔIₑₛ response varied between different groups of rabbits, studies for each experimental condition were performed within the same group of animals. Ketamine was delivered in double deionized H₂O, bumetanide in dimethyl sulfoxide buffer, and cimetidine in 40% ethyl alcohol solution. All other drugs and agents were administered in glucose-free Krebs Ringers-bicarbonate solution.

Statistics. Data analysis was performed using both paired and unpaired t-tests. Time course results are expressed as mean ± SEM. All other results are expressed as mean (%) ± standard error of the difference of the means (%). p values < 0.05 were considered statistically significant.

RESULTS

Time course. The time courses of ΔIₑₛ for 5HT (10⁻⁴ M), carbachol (10⁻⁵ M) and histamine (10⁻³ M) were determined for a 10 min period following addition of secretagogue. The three agents produced similar time courses: a transient increase in ΔIₑₛ with a rapid return to levels near baseline (Figure 1). The average times to reach maximum ΔIₑₛ were as follows: 5HT (1.11 ± 0.18 min), histamine (0.68 ± 0.04 min) and carbachol (0.93 ± 0.19 min). Return time to ΔIₑₛ less than 25% of maximum value was 10.43 ± 3.51 min for 5HT, 5.71 ± 0.26 min for carbachol, and 6.57 ± 1.53 min for histamine. These time courses were very different from cyclic AMP-mediated responses to VIP; VIP causes a similar initial rise in ΔIₑₛ, but remains elevated at a plateau for greater than 70 min [31, 34].

**Figure 1.** Time courses for each agonist. ΔIₑₛ responses to 5HT (10⁻⁴ M), histamine (HIST; 10⁻³ M), and carbachol (CCH; 10⁻⁵ M) were measured at 1 min intervals (n = 7 for each agonist). ΔIₑₛ responses are represented as mean ± SEM at each time point. For all studies, n = number of tissue strips taken from multiple animals.
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Chloride-free solution. In order to identify the ion responsible for the $\Delta I_{sc}$, measurements were performed in chloride-free medium (Figures 2, 3, and 4). When glutamate was substituted for chloride ions in the bathing solution, the effects of all three agonists on $\Delta I_{sc}$ were significantly reduced. In chloride-free solution, $\Delta I_{sc}$ responses to 5HT ($10^{-5}$ M) and carbachol ($10^{-5}$ M) were inhibited by 71.1% ($p < 0.01$) and 80.4% ($p < 0.001$), respectively. In chloride-free medium, histamine ($10^{-3}$ M) induced $\Delta I_{sc}$ was reduced by

![Figure 2](image-url)  
Figure 2. $\Delta I_{sc}$ responses to 5HT under a variety of experimental conditions. Tissues were pretreated with bumetanide (Bumex; $5 \times 10^{-4}$ M, $n = 7$) or atropine ($10^{-4}$ M, $n = 6$) in serosal bathing solution; or Cl−-free Ringers (n = 6), verapamil (Verap; $5 \times 10^{-5}$ M, n = 7), or tetrodotoxin (TTX; $3 \times 10^{-7}$ M, n = 6) in mucosal and serosal bathing solutions for 10 min prior to addition of 5HT ($10^{-4}$ M except for Cl−-free studies, which used $10^{-5}$ M 5HT). Results are graphed as % inhibition of strips taken from multiple animals.

![Figure 3](image-url)  
Figure 3. $\Delta I_{sc}$ responses to carbachol under a variety of experimental conditions. Tissues were pretreated with bumetanide ($5 \times 10^{-4}$ M, n = 6) or atropine ($10^{-4}$ M, n = 8) in serosal bathing solution; or Cl−-free Ringers (n = 9), verapamil ($5 \times 10^{-5}$ M, n = 9), or tetrodotoxin ($3 \times 10^{-7}$ M, n = 8) in mucosal and serosal bathing solutions for 10 min prior to addition of carbachol to the serosal reservoir. For the Cl−-free, bumetanide, and verapamil studies carbachol ($10^{-5}$ M) was used. Carbachol ($10^{-4}$ M) was added to tissues pretreated with TTX or atropine. Results are graphed as % inhibition of $\Delta I_{sc}$ for each experimental condition. Results are expressed as mean (%) ± SE of the difference of the means (%). *** $p < 0.001$, different than CCH alone.
82.9% (p < 0.001).

Bumetanide. Bumetanide, an analogue of furosemide, acts by interfering with the Na⁺-K⁺-2Cl⁻ co-transporter on the basolateral membrane of the cell. To block chloride entry into the cell, the serosal surfaces of the tissues were preincubated with bumetanide (5 x 10⁻⁴ M, Figures 2, 3, and 4). Bumetanide significantly inhibited the increase in short circuit current caused by all three agonists. In tissue pretreated with bumetanide, the SHT (10⁻⁴ M) response was reduced by 54.5% (p < 0.01), while carbachol (10⁻⁵ M) response fell by 67.4% (p < 0.001). Bumetanide caused 47.3% inhibition of histamine (10⁻³ M) induced ΔIₑₑ (p < 0.001).

Verapamil. In order to assess the role of calcium channels in generating the ΔIₑₑ, verapamil (5 x 10⁻⁵ M) was added to the serosal and mucosal reservoirs (Figures 2, 3, and 4). Previous investigations in our laboratory have shown that at concentrations of 5 x 10⁻⁵ M, verapamil significantly inhibits SHT-evoked chloride secretion [39]. In the current study, ΔIₑₑ response to SHT was reduced by 65.2% in tissue pretreated with verapamil (p < 0.001). Verapamil inhibited the histamine (10⁻³ M) response by 33.5% (p < 0.05). However, verapamil had no effect on carbachol (10⁻⁵ M) induced ion transport (5.6% inhibition).

Histamine antagonists. The effects of histamine antagonists on histamine-stimulated ion transport were investigated using diphenhydramine and cimetidine (Figure 4). Pretreatment of the tissue with the H₂-receptor antagonist cimetidine (10⁻³ M) did not effect histamine (10⁻³ M) induced increase in ΔIₑₑ (6.9% inhibition). Conversely, addition of the H₁-receptor blocker diphenhydramine (10⁻⁴ M) to the serosal reservoir totally abolished the ΔIₑₑ response to histamine (99.1% inhibition, p < 0.001). These findings are
consistent with previous studies in rabbit colon [23].

Tetrodotoxin. To determine if the effects of the three agents were indirectly mediated through agonist-stimulated release of an endogenous neurotransmitter, studies were performed in tissues pretreated on both the serosal and mucosal sides with tetrodotoxin (TTX; 3 x 10^-7 M, Figures 2, 3, and 4). In previous studies of guinea pig ileum, similar doses of TTX (10^-7 M) completely abolished increases in \( \Delta I_{sc} \) evoked by both electrical field stimulation and scorpion venom, a substance known to cause neurotransmitter release by depolarization of enteric neurons [40]. The \( \Delta I_{sc} \) responses to 5HT (10^-4 M) and histamine (10^-3 M) were not affected by TTX pretreatment (7.4% and 11.7% reductions in \( \Delta I_{sc} \)). Tetrodotoxin also did not significantly affect carbachol (10^-5 M)-induced ion transport (19.4% inhibition). These results support the conclusion that the \( \Delta I_{sc} \) noted in response to stimulation by the three different agents is independent of a common neural pathway.

Atropine. To determine if the agonist evoked \( \Delta I_{sc} \) responses are autonomically mediated, experiments were performed in tissue serosally preincubated with the muscarinic antagonist atropine (10^-4 M, Figures 2, 3, and 4). In previous studies in guinea pig ileum, atropine (10^-6 M) has been shown to partially reduce serotonin-mediated \( \Delta I_{sc} \) [41]. To ensure that this partial effect was not due to incomplete blockade of muscarinic receptors, a supersaturating dose of atropine (10^-4 M) was used in the current investigation. As expected, atropine totally abolished the \( \Delta I_{sc} \) response to 10^-4 M carbachol (98.3% inhibition, p < 0.001). However, atropine did not effect either the 5HT (10^-4 M) or histamine (10^-3 M) induced \( \Delta I_{sc} \) responses (2.4% increase and 5.3% reduction in \( \Delta I_{sc} \), respectively). Thus, serotonin- and histamine-stimulated electrogenic ion transport is mediated through a mechanism independent of an acetylcholine-dependent autonomic pathway.

Sequential addition of agonists. To determine if tachyphylaxis or cross-tachyphylaxis

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**Figure 6.** Effects of sequential addition of carbachol to the same tissue. ΔIsc responses to carbachol (10⁻⁵ M) were measured alone (n = 19) and following prior exposure to HIST (10⁻⁴ M, n = 9), 5HT (10⁻⁴ M, n = 10), and CA (10⁻⁴ M, n = 11). ΔIsc response to carbachol was partially attenuated by prior exposure to either HIST or 5HT. Tissue previously stimulated by CCH failed to produce a significant ΔIsc response when re-exposed to CCH. * for p < 0.05, *** for p < 0.001, different than CCH alone.

**Figure 7.** Effects of sequential addition of histamine to the same tissue. ΔIsc responses to histamine (10⁻³ M) were measured alone (n = 16) and following prior exposure to CCH (10⁻⁵ M, n = 5), 5HT (10⁻⁴ M, n = 8), and HIST (10⁻³ M, n = 5). Histamine-induced ΔIsc response was partially attenuated by prior exposure to either CCH or 5HT. No additional response was observed when tissue was re-exposed to histamine. *** for p < 0.001, different than HIST alone.

developed, agonists were sequentially to the same tissue and ΔIsc responses were measured (Figures 5, 6, and 7). To ensure that active ion transport due to exposure to the first secretagogue was not still occurring, ΔIsc was allowed to completely return to baseline before addition of the second agent. Measurement of all ΔIsc responses from plateau states allowed us to compare maximum responses for each agonist. A similar pattern in response to sequential addition of agents is seen for each of the three agonists. Ileal tissue fails to produce an increase in ΔIsc when re-exposed to the same agonist. In contrast, tissue that has previously been exposed to one agent still produces a ΔIsc response when
Figure 8. Additive Effects of 5HT, carbachol, and histamine. $\Delta I_{sc}$ responses to individual agonists are compared to $\Delta I_{sc}$ responses to combinations of agonists. (a) $\Delta I_{sc}$ response to simultaneous addition of 5HT ($10^{-4}$ M) and CCH ($10^{-5}$ M) is significantly larger than the response to either agent by itself. ** for $p < 0.01$, different than combined response. (b,c) No significant additive effect is seen between either 5HT ($10^{-4}$ M) and HIST ($10^{-5}$ M) or HIST ($10^{-4}$ M) and CA ($10^{-5}$ M).
exposed to a different agonist. In general, the second response of the tissue to a secretagogue was significantly diminished in magnitude compared to the first response to the same agonist (p < 0.05 or 0.001 for all cases expect 5HT following carbachol). In tissue previously exposed to carbachol, subsequent ΔI_{sc} response to 5HT was not significantly reduced compared to initial 5HT response (Figure 5).

**Additive effects.** Serotonin, histamine, and carbachol were used in combination with one another to determine if their secretory effects were additive (Figure 8). To study additive effects, agents should ideally be added to the system at concentrations eliciting maximum ΔI_{sc} response. In rabbit ileum, previous dose-response studies have shown that administration of 10^{-4} M serotonin produces maximum ΔI_{sc} response [28, 42]. In T84 cells, histamine elicits maximum chloride secretion at doses between 10^{-3} M and 10^{-4} M [24]. Maximum secretory response to direct carbachol stimulation is not as well established. While maximum ΔI_{sc} response of T84 cells to carbachol has been reported to occur at a concentration of 10^{-4} M [29], studies in rabbit ileum have suggested that maximum carbachol doses may stimulate chloride secretion through indirect neural pathways [43]. Because of this, an intermediate dose (10^{-5} M) of carbachol was chosen for this study. In T84 cell dose-response experiments, 10^{-5} M carbachol produced almost 70% of maximum ΔI_{sc} response [21].

In the current study, ΔI_{sc} response to the simultaneous addition of 5HT and carbachol (117.3 ± 12.3 μAmps/cm²) shows that the maximum 5HT (10^{-4} M) response (72.6 ± 4.2, p < 0.005) is significantly increased when used in combination with carbachol (10^{-5} M). Similarly, the additive ΔI_{sc} response of 5HT and carbachol is significantly greater than the response elicited by carbachol (10^{-5} M) alone (117.3 ± 12.3 vs. 75.9 ± 5.7, p < 0.01). Thus, an additive effect appears to exist between 5HT and carbachol.

In contrast, no additive effect was observed when 5HT (10^{-4} M) and histamine (10^{-3} M) are simultaneously added to the system. Serotonin failed to increase the maximum histamine response (5HT ± histamine = 96.6 ± 5.1; 5HT alone = 91.8 ± 6.6; histamine alone = 77.0 ± 13.8). Likewise, no additive effects occurred with the combination of histamine (10^{-4} M) and carbachol (10^{-5} M). Carbachol did not significantly increase the maximum histamine response (histamine + carbachol = 78.9 ± 7.0; histamine alone = 68.7 ± 6.5; carbachol alone = 61.8 ± 7.2).

**DISCUSSION**

In this study, we examined the secretory effects of serotonin, carbachol, and histamine in rabbit ileum under a variety of experimental conditions. While previous experiments have investigated additive effects between intestinal secretagogues, most have studied combinations of cAMP-mediated agents and calcium dependent agonists, and nearly all have taken place in T_{84} human colonic cell systems. To our knowledge, no additive studies between putative calcium-mediated secretagogues have previously been reported in rabbit ileum.

Clinically, serotonin appears to be the most important of the calcium-mediated secretagogues. Malignant carcinoid tumors are relatively rare neuroendocrine tumors that typically arise in the gastrointestinal tract and are primarily associated with excessive production and release of serotonin [44]. Several studies suggest that increased serotonin levels
may be responsible for the diarrhea associated with the carcinoid syndrome by inducing a hyper-secretory state in the small intestine [10, 45]. Although the diarrhea caused by Entamoeba histolytica is primarily due to intestinal tissue destruction, recent studies have suggested that serotonin contributes to this disorder by evoking electrolyte and fluid secretion [46, 47].

The study of unidirectional Na\(^+\) and Cl\(^-\) flux measurements in human colonic epithelial cells and rabbit colon and ileum have provided evidence that increases in Δ\(I_{\text{sc}}\) following exposure to 5HT, carbachol, and histamine are due to net chloride secretion [21, 24, 42]. In our studies, inhibition of 5HT, carbachol, and histamine-induced Δ\(I_{\text{sc}}\) in both chloride-free solution and in the presence of bumetanide provide further data to support the proposal that electrogenic chloride secretion is primarily responsible for the observed increases in Δ\(I_{\text{sc}}\). These results are consistent with findings from previous studies. Using rabbit colon, McCabe [23] reported that histamine-elicited increase in Δ\(I_{\text{sc}}\) was reduced by 75% in both chloride-free solution and in tissue pretreated with furosemide. Similarly, Cooke [41] demonstrated that removal of chloride from bathing solution and exposure to furosemide significantly reduced 5HT and carbachol-stimulated Δ\(I_{\text{sc}}\) in guinea pig ileum. In the current study, the incomplete reduction of Δ\(I_{\text{sc}}\) in response to chloride-free conditions and bumetanide may be partially explained by the flux of intracellular chloride or the presence of residual extracellular ions after rinsing of the tissue. Additionally, bumetanide may not be completely effective in inhibiting basolateral Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transport.

Donowitz et al. [42] previously described tachyphylaxis of Δ\(I_{\text{sc}}\) response upon re-exposure of ileal tissue to serotonin. However, when the serosal bathing fluid containing the serotonin was washed out and replaced with an equal volume of Ringers-HCO\(_3\)-solution, allowed to equilibrate, and then re-exposed to serotonin, an increase in Δ\(I_{\text{sc}}\) equal in magnitude to the initial increment occurred. In our study, agonists were added sequentially to the same tissue to determine if tachyphylaxis to the agonists developed. Because removal of serosal bathing fluid which has previously been exposed to a secretagogue appears to offset any tachyphylactic effect [42], no washout was employed between the addition of the first and second agents. For each of the three agonists, tachyphylaxis was observed upon second exposure to the same agent.

In contrast, cross-tachyphylaxis did not develop between the three secretagogues (e.g., tissue previously exposed to 5HT did not lose its ability to respond to histamine). These results indirectly indicate that the actions of one agonist are not mediated through the release of a second agonist. Based on observations that tetrodotoxin and atropine significantly inhibited serotonin-evoked chloride secretion in guinea pig ileum, Cooke et al. [41] had previously suggested that serotonin's effect may be mediated by enteric cholinergic neurons within the submucosal plexus. Our current study in rabbit ileum failed to support these conclusions; instead, our experiments using tetrodotoxin and atropine provide evidence that serotonin, carbachol, and histamine act independently of intermediate neural or acetylcholine-mediated autonomic pathways.

Previous investigations have shown that the cAMP and intracellular Ca\(^{2+}\) pathways interact to regulate electrogenic ion transport, although the exact nature of this relationship is unclear. Frizzell [20] showed that addition of cAMP to descending rabbit colon
preloaded with $^{45}$Ca$^{2+}$ caused a reversible three-fold increase in Ca$^{2+}$ efflux from the tissue. From these results Frizzell first suggested that $\Delta I_{ac}$ response to cAMP may be partially mediated through Ca$^{2+}$ release from intracellular stores. Similarly, Semrad et al. [48] used Quin-2 fluorescence to demonstrate that cAMP stimulates release of endogenous Ca$^{2+}$ in isolated chicken enterocytes. In addition to cAMP regulating Ca$^{2+}$ levels, recent studies in distal rabbit colon by Calderaro et al. [49] have suggested that intracellular Ca$^{2+}$ levels modulate cAMP-mediated chloride secretion. Compared to experiments performed in 1.2 mM Ca$^{2+}$ Ringers, addition of prostaglandin E$_2$ to tissue incubated in Ca$^{2+}$-free medium was associated with increased cAMP levels, decreased phosphodiesterase activity, increased adenylate cyclase activity, and augmented $\Delta I_{ac}$ response. From these results, Calderaro concluded that intracellular Ca$^{2+}$ decreases the rate of cAMP accumulation, most likely through a direct inhibitory effect of Ca$^{2+}$ on adenylate cyclase activity.

Other studies have investigated the role that calmodulin [50–54] and protein kinase C [51, 55], two different Ca$^{2+}$-dependent regulatory proteins, play in regulating cAMP-induced electrolyte transport. Trifluoperazine and chlorpromazine, neuroleptic agents known to inhibit calmodulin, have been shown to attenuate $\Delta I_{ac}$ response to a variety of cyclic nucleotide-mediated secretagogues (including theophylline, PGE$_2$, VIP, cholera toxin, and 8-Br-cAMP) [52–54]. Recent work in T$_{84}$ cells by Worrall et al. [56] demonstrated that the Ca$^{2+}$-calmodulin-dependent protein kinase II mediates stimulation of apical chloride conductance. Activation of protein kinase C is known to stimulate ion transport in mammalian intestine [57–59]. Studies using phorbol esters (protein kinase C activators) in combination with PGE$_2$ (a cAMP-mediated agonist) suggest that protein kinase C may indirectly modulate PGE$_2$-induced adenylate cyclase activation [55]. Thus, the calcium and cAMP pathways mediating active intestinal electrolyte transport appear to be interrelated in a complex, but poorly understood manner.

Additive effects between small intestinal secretagogues have previously been investigated using human colonic T$_{84}$ epithelial cells [29, 34–36]. These experiments reported that VIP and the calcium ionophore A23187 potentiated the action of one another on chloride secretion [34, 35]. Similarly, combination of carbachol with either prostaglandin E$_1$ or VIP (both cAMP-mediated agents) produced a synergistic secretory response [36]. Based on these studies it was suggested that the basis of the synergism between cAMP and Ca$^{2+}$-mediated Cl$^-$ secretion was due to activation of different sets of Cl$^-$ channels. Cliff [60] performed experiments consistent with this hypothesis, where cAMP and Ca$^{2+}$ were shown to activate Cl$^-$ conductances with different properties. These data suggested that second messengers either activate different Cl$^-$ channels or induce different conductive and kinetic states in the same channel. Dharmasathaphorn [29] demonstrated additive increases for both $\Delta I_{ac}$ response and intracellular Ca$^{2+}$ levels when carbachol and histamine were used in combination, suggesting the existence of multiple calcium-mediated effector mechanisms or the existence of multiple mediators that augment the action of cytosolic Ca$^{2+}$. In the current study, the additive effects between histamine and carbachol were not significant. The disparity in these findings likely reflects model differences in ion transport mechanisms which exist between rabbit ileum and monocultured layers of human T$_{84}$ colon cells.

The dependence of ion transport on extracellular Ca$^{2+}$ in rabbit ileum has previously
been reported for 5HT. Donowitz et al. [18] showed that 5HT-induced $\Delta I_{\text{leak}}$ was abolished in a Ca$^{2+}$-free medium (zero Ca$^{2+}$ and 1 mM EGTA). The size of the $\Delta I_{\text{leak}}$ response was proportional to the amount of extracellular Ca$^{2+}$ and, by use of $^{45}$Ca$^{2+}$, it was apparent that 5HT elicited increased calcium uptake across the serosal surface. In the current study, pretreatment of ileal tissue with verapamil caused a 65% inhibition of 5HT-evoked ion flux (a figure similar to the 68% reduction reported by Donowitz et al.). Evaluated together, the results of these studies support the proposal that 5HT stimulates electrogenic ion transport in rabbit ileum via an extracellular Ca$^{2+}$-dependent mechanism.

In contrast, in the current study verapamil failed to influence the $\Delta I_{\text{leak}}$ response to carbachol, suggesting that extracellular calcium does not play a significant role in carbachol mediated ion transport. This is consistent with studies in human colonic T84 epithelial cells where carbachol-induced $\Delta I_{\text{leak}}$ response did not require extracellular Ca$^{2+}$ [22]. In the same study, carbachol significantly increased free cytosolic Ca$^{2+}$ levels (measured using Quin-2 fluorescent spectrofluorometry). Direct measurements of inositol trisphosphate suggest that phospholipid turnover is involved in carbachol's secretory action [30]. Inositol phosphate raises cytosolic Ca$^{2+}$ levels through release of intracellular stores.

Additional support for the proposal that carbachol acts through intracellular Ca$^{2+}$ release was provided by experiments using dantrolene [61] and 3,4,5 trimethoxybenzoate-8 (TMB-8) [62], agents known to prevent mobilization of Ca$^{2+}$ from intracellular stores. Both agents totally prevented carbachol stimulation of electrogenic ion transport, but failed to inhibit the effects of 5HT. The differing responses of 5HT and carbachol to verapamil, dantrolene, and TMB-8 imply that the Ca$^{2+}$ utilized by these two secretagogues may originate from different sources. Calcium mediating the 5HT response appears to be derived mostly from extracellular sources, while the cytosolic Ca$^{2+}$ rise following carbachol exposure presumably reflects mobilization from intracellular stores. These data may explain the additive effect noted in our study between 5HT and carbachol. Adding the two agonists simultaneously may raise intracellular Ca$^{2+}$ levels above those reached by either agent alone. It is possible that this increase in intracellular Ca$^{2+}$ is responsible for the augmented $\Delta I_{\text{leak}}$ response observed after simultaneous exposure to 5HT and carbachol. Additionally, this difference in calcium mobilization may in part explain the observation that $\Delta I_{\text{leak}}$ response to 5HT was unaffected by prior exposure of the tissue to carbachol.

The nature of the Ca$^{2+}$-dependent mechanism through which histamine mediates electrogenic ion transport is less clear. It has been hypothesized that histamine acts through hydrolysis of phosphatidylinositol with a resultant increase in free cytosolic Ca$^{2+}$ in a manner similar to carbachol [24, 29]. In contrast, McCabe [23] reported that histamine-induced $\Delta I_{\text{leak}}$ is almost completely abolished in Ca$^{2+}$-free solution, suggesting that extracellular Ca$^{2+}$ may play a major role in mediating histamine-elicited ion transport. In the present study, verapamil inhibited histamine-stimulated electrolyte transport by 33.5%, demonstrating that the response is only partially dependent on extracellular Ca$^{2+}$. On the other hand, no additive effect was seen when histamine and carbachol were used in combination, raising the possibility that the two agents work through a common Ca$^{2+}$ pathway. These data suggest that intracellular and extracellular Ca$^{2+}$ sources may both play important roles in mediating histamine-induced $\Delta I_{\text{leak}}$ response.
The results of our experiments are not without limitations. Additive effects between agonists are ideally studied with agents at the concentration required for maximum physiological response. The use of sub-maximal carbachol doses could be interpreted as providing data that might not necessarily be comparable. However, suggestion of neuronally mediated carbachol responses at maximum doses would have provided similarly complex interpretation. Additionally, our conclusions about the existence of multiple calcium-mediated pathways are based in part on prior studies performed in calcium-free conditions [18, 22, 23] and with intracellular calcium depleting agents [61, 62]. These experiments were not repeated in our present study. Further studies in both isolated mucosa and cultured epithelial cell lines will be required to elucidate more completely the intracellular mechanisms by which calcium regulates electrogenic ion transport. In particular, the effect of intracellular calcium depleting agents (dantrolene or TMB-8) on histamine-stimulated ion secretion must be examined.

In summary, we have used a rabbit model to investigate the secretory effects of serotonin, carbachol, and histamine. We have confirmed that chloride is the main ion responsible for the current measured across ileal mucosa, and have established information about tachyphylaxis to and cross-tachyphylaxis between agents. Studies with verapamil suggest that each of the agonists depends on the presence of extracellular calcium to mediate chloride secretion to differing degrees. Additive studies were performed between each combination of secretagogues; only the combination of 5HT and carbachol produced a significant additive secretory effect. These findings support previous studies which have suggested that multiple calcium-mediated effector mechanisms may be involved in the regulation of intestinal electrolyte and fluid secretion.

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