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Research Article

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The Antifungal Antibiotic, Clotrimazole, Inhibits Chloride Secretion by Human Intestinal T84 Cells via Blockade of Distinct Basolateral K^+ Conductances

Demonstration of Efficacy in Intact Rabbit Colon and in an In Vivo Mouse Model of Cholera

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Abstract

The antifungal antibiotic clotrimazole (CLT) blocks directly and with high potency the Ca^{2+}-activated K^+ channels of human erythrocytes, erythroleukemia cells, and ferret vascular smooth muscle cells. We recently reported that CLT inhibits Cl^- secretion in human intestinal T84 cells, likely by affecting K^+ transport (Rufo, P.A., L. Jiang, S.J. Moe, C. Brugnara, S.L. Alper, and W.I. Lencer. 1996. *J. Clin. Invest.* 98:2066–2075). To determine if CLT had direct effects on K^+ conductances in T84 cells, we selectively permeabilized apical membranes of confluent T84 cell monolayers using the ionophore amphotericin B. This technique permits direct measurement of basolateral K^+ transport. We found that CLT and a stable des-imidazolyl derivative inhibited directly two pharmacologically distinct basolateral membrane K^+conductances, but had no effect on apical membrane Cl^- conductances. The effects of CLT on Cl^- secretion were also examined in intact tissue. CLT inhibited forskolin-induced Cl^- secretion in rabbit colonic mucosal sheets mounted in Ussing chambers by 91%. CLT also inhibited cholera toxin–induced intestinal Cl^- secretion in intact mice by 94%. These data provide direct evidence that CLT blocks Cl^- secretion in intestinal T84 cells by inhibition of basolateral K^+ conductances, and show that CLT inhibits salt and water secretion from intact tissue in vitro and in vivo. The results further support the suggestion that CLT and its metabolites may show clinical efficacy in the treatment of secretory diarrheas of diverse etiologies. (*J. Clin. Invest.* 1997. 100:3111–3120.) Key words: K^+ channels • amphotericin B • clotrimazole • Cl^- secretion • secretory diarrhea

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Introduction

Activated Cl^- secretion from the intestinal crypt is thought to play a major role in secretory diarrhea of most etiologies (1). Epithelial cells lining the crypt lumen express the full complement of channels, cotransporters, and pumps required for the active transport and secretion of Cl^- (2). Activation of Cl^- channels resident in the apical membrane of crypt epithelial cells leads to Cl^- efflux into the crypt lumen. This electrogenic anion efflux hyperpolarizes the crypt, thereby driving Na^+ and water across the epithelial tight junctions to produce salt (NaCl) and water secretion.

Generation of the electrochemical driving force required for Cl^- secretion by crypt epithelial cells depends on their ability to accumulate intracellular Cl^- ions greater than the predicted electrochemical equilibrium concentrations. Available data indicate that Cl^- enters the cell across the basolateral membrane through the activity of Na-K-2Cl cotransporters (3) driven by a strong inwardly directed electrochemical Na^+ gradient established by the basolaterally located Na^+-K^+-ATPase pump. To maintain membrane potential at rest and during Cl^- secretory responses, both Na^+ and K^+ must be recycled out of the cell through the basolateral membrane. The Na^+-K^+-ATPase pump serves to recycle Na^+, while basolateral membrane K^+ channels recycle transported K^+. As in all secretory epithelia, the channels and transporters of the crypt epithelial cell segregated in basolateral and apical membranes operate in concert to achieve vectorial ion transport. Inhibition of any one of these transport pathways attenuates transepithelial Cl^- transport and thereby inhibits the secretory response.

The human intestinal T84 cell line forms confluent monolayers of well-differentiated columnar epithelia that exhibit high transepithelial resistances, polarized apical and basolateral membranes, and cAMP- and Ca^{2+}-regulated Cl^- secretory pathways analogous to those found in native intestine (2, 4–6). Both cyclic nucleotide- and Ca^{2+}-mediated intracellular signals regulate basolateral K^+ conductances in T84 cells. Two pharmacologically distinct K^+ conductances have been identified (7–9). One K^+ conductance required for Cl^- secretion is activated by intracellular cAMP (10), and is sensitive to Ba^{2+} salts (8, 11). The other is activated by Ca^{2+} agonists (12, 13) and displays sensitivity to charybdotoxin (8) but minimal or no sensitivity to Ba^{2+}, tetraethylammonium (TEA), 4-aminopyridine, or apamin (11, 14, 15).

We have reported that the imidazole antibiotic, clotrimazole (CLT),[1] and its metabolites, block conductive K^+ transport in human (16, 17) and mouse erythrocytes (17), and inhibit K^+ channels in murine erythroleukemia (18) and ferret

vascular smooth muscle cells (19). Moreover, the erythroid effects were achievable by systemic administration of these drugs at very high doses in mice and high doses in humans without significant toxicity (17, 20, 21). On the basis of these results, we reasoned that CLT might inhibit Cl^- secretion in the human intestine by inhibiting basolateral conductive K^+ pathways.

We tested this hypothesis using the human intestinal T84 cell line (8). The results of these studies demonstrated that CLT reversibly inhibits at micromolar concentrations transepithelial Cl^- secretion stimulated in T84 cells by either cAMP- or Ca^{2++}-dependent agonists (8). CLT acted at distal steps in the cAMP- and Ca^{2+}-dependent signaling cascades, strongly inhibiting agonist-stimulated basolateral K^+ (^{86}Rb) efflux while exerting no effect on basolateral membrane Na-K-2Cl cotransport or on apical membrane Cl^- transport. These data suggested that CLT may inhibit Cl^- secretion in the intestine via inhibition of basolateral K^+ channels.

Two aims were addressed in the current study. First, we tested the hypothesis that CLT may inhibit directly basolateral K^+ conductances. To do so, we utilized the ionophore amphotericin B to selectively permeabilize apical membranes of confluent T84 monolayers. T84 monolayers so permeabilized, maintain integrity of intercellular junctions and contralateral basolateral membranes, and thus sustain high transepithelial resistance (22–24). This preparation allowed selective assessment of basolateral K^+ conductances using standard transepithelial electrophysiological techniques. Second, as CLT may represent a class of compounds with potential clinical utility, we examined the ability of CLT to inhibit indices of intestinal salt and water secretion by intact colon and in intact animals.

Our data show that CLT and its stable des-imidazolyl metabolite each inhibited basolateral membrane K^+ conductances stimulated by either cAMP- or Ca^{2+}-dependent signaling pathways. CLT had no effect on cAMP-dependent Cl^- conductances of the apical membrane. Exposure of intact rabbit colon to CLT led to inhibition of transepithelial short circuit current (Isc). Oral administration of CLT to mice inhibited intestinal fluid secretion in response to subsequent challenge with cholera toxin. These studies show that CLT inhibits directly basolateral membrane K^+ conductances and thus identify the mechanism by which CLT inhibits intestinal Cl^- secretion. Moreover, as CLT inhibits fluid secretion from native intestine, CLT and related compounds may have utility in the treatment of secretory diarrheas of diverse etiology.

Methods

Materials. $BaCl_2$ was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Cholera toxin was obtained from Calbiochem (San Diego, CA). Charybdotoxin was obtained from Peptides International (Louisville, KY). CLT and all other reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. 2-chlorophenyl-*bis*-phenyl methanol (MET II) was kindly provided by Dr. George Krol (Bayer Pharmaceuticals, West Haven, CT). Solutions were prepared as indicated in Table I.

Table I. Buffer Solutions Used for Experiments on Selectively Permeabilized T84 Cell Monolayers

Solutions	1	2	3	4	5	6	7
	HBSS	K (high)	K (low)	Na (high)	Na (low)	Cl (high)	Ringers lactate
NaCl	136.9	—	—	—	—	—	122.0
KCl	5.36	—	—	—	—	—	5.0
N-Meth-Glucamine	—	—	136.9	—	136.9	—	—
K-Gluconate	—	142.5	5.4	—	—	—	—
Na-Gluconate	—	—	—	142.5	5.4	—	—
Choline Cl	—	—	—	—	—	142.3	—
$CaCl_2$	1.25	1.25	1.25	1.25	1.25	1.25	2.0
$MgSO_4$	0.40	0.40	0.40	0.40	0.40	0.40	1.3
KH_2PO_4	—	0.43	0.43	0.43	0.43	0.43	—
Na_2HPO_4	0.35	0.35	0.35	0.35	0.35	0.35	
Hepes	10	10	10	10	10	10	—
Bicarbonate	—	—	—	—	—	—	25.0
D-Glucose	5.6	5.6	5.6	5.6	5.6	5.6	20

Concentrations are reported in millimeters. Final pH of solutions 1–6 was 7.4 in room air. Final pH of solution 7 in 95% O_2/5% O_2 was 7.4.

Cell culture. T84 cells obtained from ATCC (Rockville, MD) were cultured and passaged in equal parts of DME, (1 gram/liter D-glucose) and Ham's F-12 Nutrient mixture, supplemented with 5% newborn calf serum, 15 mM Hepes, 14 mM $NaHCO_3$, 40 mg/liter penicillin, 8 mg/liter ampicillin, and 0.90 mg/liter streptomycin. Cells were seeded at confluent density onto 0.33-cm^2 Transwell inserts (Costar, Cambridge, MA) coated with dilute rat collagen solution as previously described (25, 26). Transepithelial resistances attained stable levels (> 1,000 ohms/cm^2) after 7 d. The development of high transepithelial resistances correlates with the formation of confluent monolayers with well-developed tight junctions as assessed by morphological analysis, and with the ability of monolayers to secrete Cl^- (6). Cells from passages 77 to 91 were used in these studies.

Selective membrane permeabilization and measurement of K^+ transport. Basolateral membrane potassium conductance was measured using the technique developed by Dawson and coworkers (27). Solutions used in all experiments are summarized in Table I. A mucosa to serosa potassium gradient was established across the monolayer using solutions containing K^+ as the major charge-carrying ion (see Table I, *solutions 2 and 3*). Solutions contained 137–143 mM of either the chloride or gluconate salts of Na^+, K^+, *N*-methyl-D-glucamine, or choline, as indicated. Unless otherwise noted, all solutions also contained 5.4 mM appropriate K^+ salt, 1.25 mM $CaCl_2$, 0.40 mM $MgSO_4$, 0.43 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 5.6 mM glucose, and 10 mM Hepes, at a final pH of 7.4 in room air. Apical membranes of confluent monolayers were selectively permeabilized by addition of amphotericin B (20 μM) to apical reservoirs. Intercellular tight junctions and contralateral cell membranes remained intact and impermeant to small solutes in these preparations as evidenced by maintenance of high transepithelial resistance. The initial selective permeabilization of apical membranes was detected as small but stable increases in short circuit current (ΔIsc, 9±2.2 μA/cm^2, $n = 8$, from baseline Isc of 6.3 μA/cm^2) that represent loss of apical membrane resistance after formation of amphotericin B pores. In all cases, transepithelial conductances remained low after apical membrane permeabilization (0.62±.06 mS/cm^2 units, $n = 8$) demonstrating that intercellular tight junctions and contralateral basolateral membranes remained intact. When apical membranes are selectively permeabilized in buffers containing K^+ as the major charge carrying ion, transepithelial short-circuit current (Isc) or conductance (G) becomes a measure of the limit-

1. *Abbreviations used in this paper:* CLT, clotrimazole; G, basolateral K conductance; Isc, short circuit current; MET II, 2-chlorophenyl-*bis*-phenyl methanol.

ing rate of electrogenic transfer of potassium across the basolateral membrane and tight junction combined. Conversely, selective permeabilization of basolateral membranes (by addition of 100 μM amphotericin B to basolateral instead of apical reservoirs) yields measures of ion transport across apical membranes and tight junctions combined (see measurement of Cl^- conductance below). To activate cAMP- and Ca^{2+}-regulated conductances in the permeabilized preparations, forskolin (10 μM), thapsigargin (5 μM), or carbachol (100 μM) were applied to basolateral reservoirs.

Isc and G were measured using calomel electrodes connected via 3 M KCl agar bridges to a dual epithelial voltage clamp (University of Iowa, Iowa City, IA), as described (8, 23). For measurement of current–voltage relationships, currents were elicited in asymmetrical K^+ gluconate solutions (apical solution 2, basolateral solution 3; Table I) by imposition of 1-s test potentials between -80 and +80 in 10-mV increments. These methods have been utilized previously to examine both Cl^- and K^+ transport in T84 cells (10) and HT29-Cl.16E cells (23).

Measurement of Cl^- conductance of the apical plasma membrane. To examine apical membrane Cl^- conductances in T84 cells, cell monolayers were exposed to identical apical and basolateral buffer solutions in which Cl^- was the major charge-carrying ion (Table I). Basolateral membranes were permeabilized by addition of 100 μM amphotericin B to the serosal reservoir as described above. Currents were elicited in symmetrical 142 mM choline chloride buffers (solution 6, Table I) by 1-s test potentials applied in 10-mV increments from -80 to +80 mV, and current–voltage relationships were constructed.

Ussing chamber studies using rabbit colonic mucosa. Four adult male (2.5 kg), New Zealand rabbits (Charles River Laboratories, Cambridge, MA) were killed by an intravenous injection of pentobarbital (0.5 ml/kg). A 15-cm length of distal colon was removed and opened longitudinally. External muscle layers were removed by blunt dissection and colonic mucosal preparations were mounted in an Ussing chamber (DCTSYS; Precision Instrument Design, CA); 1.03 cm^2 surface area (28–30) and incubated in solution 7 (Table I) with and without CLT (30 μM) at 37°C in the presence of 95% O_2/5% CO_2. The fluid volume on each side of the mucosa was 7 ml.

Potential difference (PD) and Isc were monitored and registered at 10-min intervals as previously described (28–31). Apical (luminal) and basolateral (serosal) buffer solutions were interfaced via Ag-AgCl electrodes (Voltage/Current Clamp, model VCC600; Physiologic Instruments, Inc., San Diego, CA) and Ringer/agar bridges to a voltage clamp device (model DVC-1000; Voltage/Current Clamp, World Precision Instruments, Inc., Sarasota, FL). After stabilization of baseline PD and Isc, the colon preparations were incubated in the presence or absence of serosal CLT (30 μM) for 30 min, and then stimulated by the addition of forskolin (10 μM) or carbachol (100 μM) to the serosal reservoir.

Mouse model of secretory diarrhea. Female Balb/C mice aged 6–8 wk (Charles River Laboratories) were gavage fed either CLT (150 mg/kg per d administered in two divided doses, dissolved in peanut oil at 20 mg/ml) or vehicle control over 7 d. On day 7, mice were challenged by gavage with PBS containing 25 μg purified cholera toxin (Calbiochem) in the absence or presence of 30 μM clotrimazole, or with PBS alone. 5 h later, the animals were killed by CO_2 anesthesia and cervical dislocation. The animals were rapidly weighed, the peritoneal cavities were exposed, and ligatures tied at the proximal duodenum and distal rectum. Fluid secretion caused by oral administration of cholera toxin was readily apparent as fluid distended loops of bowel. To quantify intestinal fluid secretion, the entire intestine was dissected free of mesentery, removed as a single unit, and weighed. Ratios of the intestinal weight to total body weight were compared among treatment groups. The entire procedure took < 2 min.

Statistics. Tests of significance were based on ANOVA using Statview 512+ (BrainPower, Calabasas, CA). Data represent mean± SEM (when $n \geq 3$). In the one instance in which $n = 2$, the data are presented as a mean value with the associated error bar representing 50% of the range of the two data points (see Figure 8, *column 2*).

Results

Direct assay of basolateral K^+ conductance in selectively permeabilized T84 cell monolayers

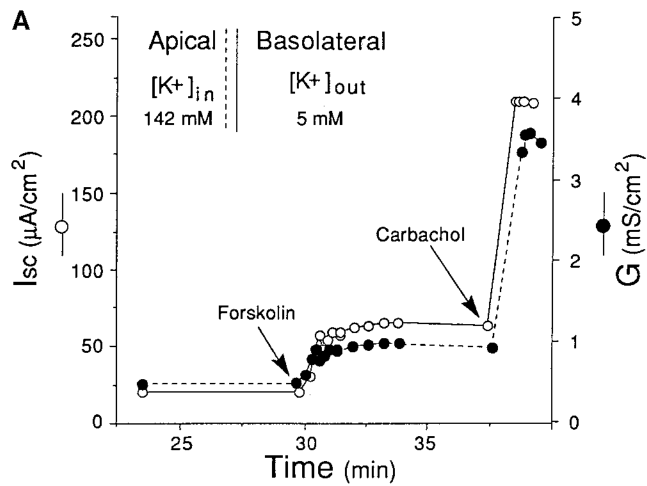
Basolaterally directed K^+ conductances were examined in T84 monolayers permeabilized apically by pretreatment with amphotericin B. Apical and basolateral buffers contained K^+ as the major charge carrying permeant ion. All studies were performed with a 137-mM basolaterally directed K^+ gradient (apical solution 2, basolateral solution 3; Table I). Fig. 1 *A* shows conductive K^+ transport in apically permeabilized monolayers before and after the ordered additions of the cAMP-dependent agonist forskolin and the Ca^{2+}-dependent agonist carbachol.

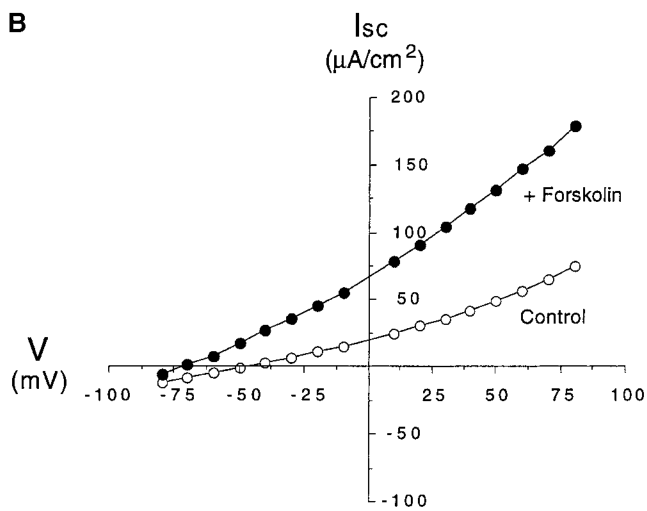
Treatment of apically permeabilized monolayers with 10 μM forskolin (Fig. 1 *A*, *arrow*) led to a brisk increase in Isc with parallel increase in G. The subsequent addition of carbachol (100 μM) led to a further increase in Isc and G. As previously described in intact T84 monolayers, the forskolin-induced changes in Isc were sustained, whereas the effect of carbachol was short-lived (8, 10, 14, 32). Both Isc and G returned to baseline values within 5 min after addition of carbachol. Under these defined conditions, changes in Isc and G represent either agonist-induced activation of K^+ conductive membrane channels, or loosening of intracellular tight junctions, or both.

Fig. 1 *B* and *C* show current/voltage (*Isc*/*V*) relations for apically permeabilized monolayers before and after agonist stimulation. Thapsigargin was used in place of carbachol as a Ca^{2+}-dependent agonist in these studies because the K^+ transients elicited by thapsigargin achieved steady-state conductances of much longer duration (8). In the presence of basolaterally directed K^+ gradients (apical solution 2, basolateral solution 3; Table I), both forskolin (Fig. 1 *B*) and thapsigargin (Fig. 1 *C*) each activated outward currents (mucosal to serosal) that displayed mild outward rectification at positive transepithelial voltages and shifted apparent reversal potentials to more negative values consistent with activation of basolateral membrane K^+ conductances, or after loosening of intercellular tight junctions, or both.

To show that the agonist-induced changes in transepithelial Isc and G represent primarily activation of basolateral membrane K^+ conductances rather than opening of intercellular tight junctions, we replaced K^+ with Na^+ as the major charge carrying permeant ion (apical solution 4, basolateral solution 5; Table I). Passive transport across intercellular tight junctions does not display high selectivity between these cations (33). Thus, if agonist-induced changes in Isc were due to loosening of intercellular junctions, enhancement of transepithelial Isc and G should be equally apparent in buffers containing Na^+ as the major charge carrying permeant ion. In these K^+-free conditions however, neither forskolin nor thapsigargin elicited detectable increases in Isc or G (Fig. 2). Moreover, apparent reversal potentials did not change. These data indicate that the agonist-induced increases in cation conductances were specific to K^+ transport and likely due to activation of basolateral membrane K^+ channels.

Two pharmacologically distinct K^+ (^{86}Rb) efflux pathways have been identified in intact T84 cells and monolayers (8, 11, 14, 23, 34–37). One pathway participates in the secretory response to cAMP-dependent agonists and is inhibited by Ba^{2+} salts. The second K^+ efflux pathway mediates the response to Ca^{2+}-dependent agonists, and is relatively Ba^{2+}-insensitive but





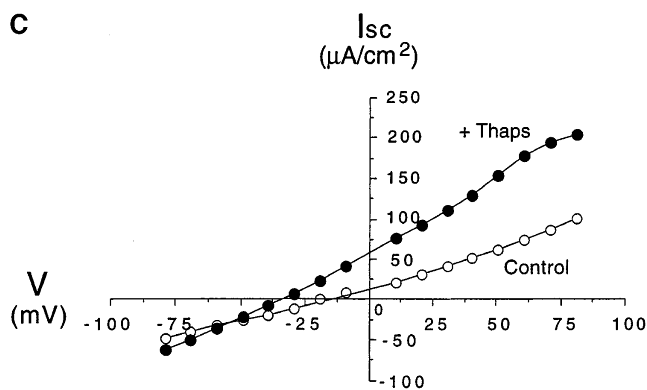


Figure 1. (*A*) Activation of basolateral K^+ conductance by 10 μM forskolin and 100 μM carbachol. A basolaterally directed K^+ gradient was established across the monolayer and the luminal plasma membrane was permeabilized with 20 μM amphotericin B 30 min before the addition of forskolin. Subsequent additions of forskolin and carbachol to the basolateral reservoir are indicated (*arrows*). Both agonists increased Isc (*open circles*, *scale to left*) and G (*filled circles*, *scale to right*). Results are representative of eight experiments. (*B*) Steady-state short circuit current/voltage relationship (*Isc*/*V*) of apically permeabilized monolayers in the presence of a basolaterally directed K^+ gradient in the absence (*open circles*) and presence (*filled circles*) of 10 μM forskolin. Representative of eight independent studies. (*C*) Steady-state Isc/V relationship of apically permeabilized monolayers in the presence of a basolaterally directed K^+ gradient in the absence (*open circles*) and presence (*filled circles*) of 5 μM thapsigargin (*Thaps*). Representative of four independent studies. In all panels, the apical compartment contained solution 2 and the basal compartment contained solution 3 (see Table I).

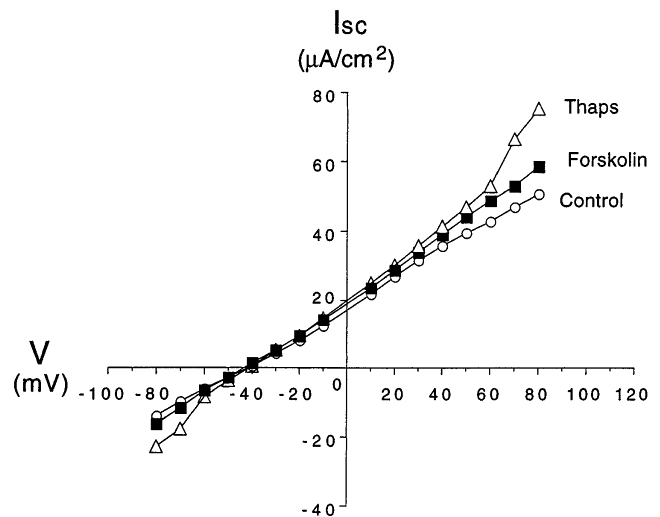


Figure 2. Steady-state current–voltage (*Isc*/*V*) relationship of Na^+ transport in apically permeabilized T84 monolayers. A basolaterally directed Na^+ gradient was imposed (solution 4 apical, solution 5 basolateral, see Table I) before selective apical (luminal) membrane permeabilization with 20 μM amphotericin B. Currents were recorded at the indicated voltage-clamped potentials. The ordinate indicates the Isc measured on the same T84 monolayers before (*control*, *open circles*) and after stimulation with the cAMP-dependent agonist forskolin (*forskolin*, *filled squares*) or the Ca^{2+}-dependent agonist thapsigargin (*Thaps*, *open triangles*). The figure presents one of two independent experiments with nearly identical results.

inhibited by the scorpion venom charybdotoxin (8). Thus, to confirm that the agonist-induced K^+ currents observed in apically permeabilized monolayers likely represent K^+ transport across basolateral membrane K^+ channels, we examined the sensitivity of these currents to Ba^{2+} and charybdotoxin.

Fig. 3 *A* shows the effect of $BaCl_2$ on K^+ currents induced by the ordered additions of forskolin and carbachol in apically permeabilized T84 monolayers. Treatment with $BaCl_2$ (3 mM) inhibited basolateral membrane K^+ conductances induced by the cAMP-dependent agonist forskolin, but had little or no effect on K^+ currents induced by the subsequent addition of the Ca^{2+}-dependent agonist carbachol. In contrast, pretreatment of permeabilized monolayers with charybdotoxin (100 nM, Fig. 3 *B*) had no detectable effect on K^+ transport induced by forskolin but inhibited strongly basolateral membrane K^+ currents induced by carbachol. Thus, in apically permeabilized monolayers the differential sensitivity of basolateral membrane conductive K^+ transport to inhibition by the K^+ channel blockers $BaCl_2$ and charybdotoxin paralleled exactly the selective effect of these inhibitors on K^+ transport in intact monolayers (as deduced from measurements of Isc_{Cl}) (8, 34). Taken together, these data provide strong evidence that transepithelial currents induced by forskolin or carbachol in apically permeabilized monolayers represent K^+ transport through dis-

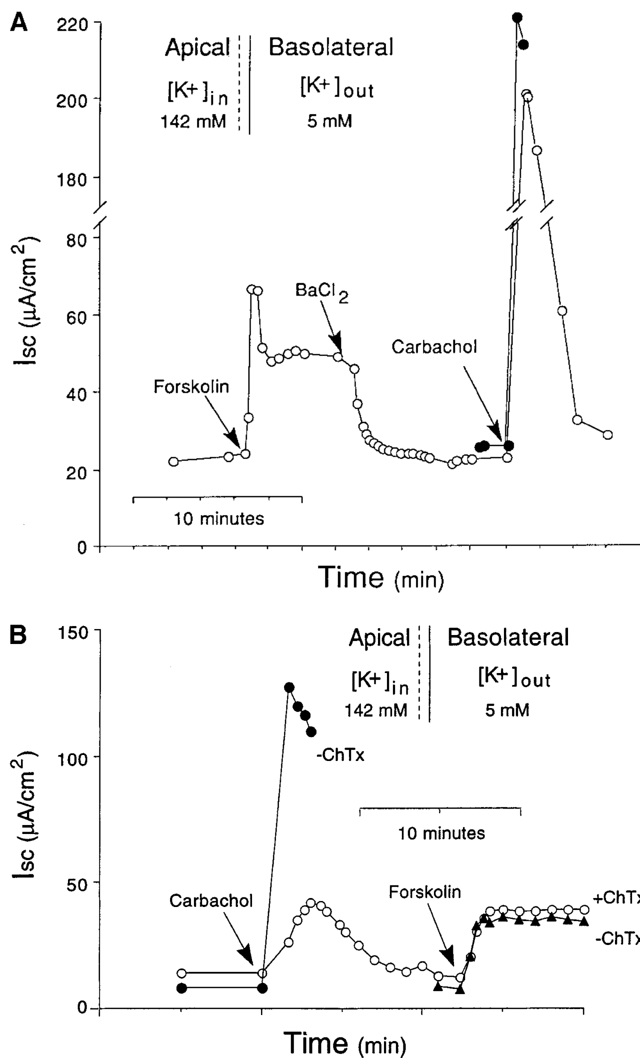


Figure 3. Effect of $BaCl_2$ and charybdotoxin on forskolin- and carbachol-induced K^+ conductances in apically permeabilized monolayers. A basolaterally directed K^+ gradient was established across the monolayers (solution 2 apical, solution 3 basolateral, see Table I), and the apical (luminal) plasma membrane was then permeabilized with 20 μM amphotericin B 30 min before the addition of agonist. Arrows indicate the addition of 10 μM forskolin, 100 μM carbachol, 3 mM $BaCl_2$, or 100 nM charybdotoxin (*ChTx*). (*A*) The time course of the Isc response to the ordered additions of forskolin and carbachol is shown for an apically permeabilized monolayer pretreated with $BaCl_2$ (*open circles*). $BaCl_2$ inhibits forskolin-induced Isc (*open circles*) but has little or no effect on Isc induced by carbachol. Representative of four independent experiments. For comparison, the Isc response elicited by carbachol applied to an apically permeabilized monolayer not treated with $BaCl_2$ (*filled circles*) is superimposed on the time course. Representative of eight independent experiments. (*B*) The time course of the Isc response to the ordered additions of carbachol and forskolin is shown for an apically permeabilized monolayer pretreated with charybdotoxin (*open circles*). Charybdotoxin inhibits the Isc elicited by carbachol, but has no effect on forskolin-induced Isc. Representative of four independent experiments. For comparison, the Isc responses elicited by either carbachol (*filled circles*) or forskolin (*filled triangles*) applied to separate apically permeabilized monolayers not treated with charybdotoxin are superimposed on the time course. Each tracing is representative of eight independent experiments.

tinct basolateral membrane K^+ channels. As such, we term these currents Isc-bl_K.

These data, summarized in Table II, show that Isc-bl_K represents K^+ transport through at least two classes of basolateral membrane K^+ conductances, and agree with previous findings on K^+ transport in human T84, HT29, and tracheal epithelial monolayers (8, 9, 11).

Both clotrimazole and its stable metabolite, 2-chlorophenyl-bis-phenyl methanol inhibit Isc-bl_K stimulated by either cAMP- or Ca^{2+}-dependent agonists

We next tested the hypothesis that CLT inhibition of transepithelial Cl^- transport in T84 cells can be attributed to direct inhibition of basolateral membrane K^+ channels. Fig. 4 *A* shows that CLT significantly inhibited Isc-bl_K after treatment with the cAMP-dependent agonist forskolin (10 μM) and then subsequently with the Ca^{2+}-dependent agonist carbachol (100 μM). Steady-state Isc-bl_K/V relationships after stimulation of monolayers with either forskolin (Fig. 5 *A*) or with thapsigargin (Fig. 5 *B*) show that CLT inhibited strongly both cAMP- and Ca^{2+}-induced K^+ conductances. Nearly identical results were obtained with the des-imidazolyl metabolite of CLT, 2-chlorophenyl-*bis*-phenyl methanol (Fig. 4 *B*). These data, summarized in Table II, show that CLT inhibits both cAMP- and Ca^{2+}-sensitive basolateral membrane K^+ conductances in T84 cells.

Clotrimazole has no effect on apical membrane Cl^- conductance in basolaterally permeabilized monolayers. Our previous studies in intact T84 monolayers showed that CLT had no detectable effect on forskolin- and thapsigargin-activated ^{125}I efflux, suggesting that CLT did not affect apical membrane Cl^- channels (8). To confirm and extend these findings, we examined Cl^- transport in T84 cell monolayers permeabilized basolaterally by amphotericin B. Basolaterally permeabilized monolayers also exhibited low transepithelial conductances (typically 1.25 mS/cm^2 at ±80 mV) indicating that intercellular tight junctions and contralateral apical membranes remained intact. These studies were performed in symmetrical 142-mM Cl^- solutions with Cl^- as the major charge carrying permeant anion (apical solution 6, basal solution 6, Table I). As such, transepithelial currents activated by the cAMP-dependent agonist forskolin and driven by applied transepithelial potentials likely represent Cl^- transport through apical membrane Cl^- channels termed Isc-ap_{Cl} (as defined above for K^+ transport and termed Isc-bl_K).

In basolaterally permeabilized monolayers not treated with CLT, the addition of forskolin (10 μM) to basolateral reservoirs increased Isc-ap_{Cl} 2.9±0.3-fold over baseline (n = 10). The cAMP-dependent increase in Isc-ap_{Cl} presumably represents activation of the cystic fibrosis transmembrane regulator Cl^- channel (CFTR) present in or mobilized to the apical plasma membrane (38–40). However, in contrast to the inhibitory effect of CLT on Isc-bl_K, CLT had no detectable effect on forskolin-stimulated Isc-ap_{Cl}. Steady-state Isc/V relationships for Isc-ap_{Cl} were nearly identical in forskolin-stimulated monolayers assayed in the absence or in the presence of CLT (Fig. 6, representative of three independent experiments). Thus, CLT likely inhibits Cl^- secretion in intact T84 cell monolayers via direct inhibition of basolateral membrane K^+ channels without direct effect on apical membrane Cl^- channels. These data are consistent with our previous results on the effect of CLT on ^{86}Rb and ^{125}I efflux from intact cells (8).

Table II. Effect of K^+ Channel Inhibitors on Basolateral K^+ Conductances in Apically Permeabilized T84 Cell Monolayers

	Control (n = 8)		$BaCl_2$ (3 mM) (n = 4)		ChTx (100 mM) (n = 4)		CLT (30 μM) (n = 7)		MET II (30 μM) (n = 3)	
	Isc	G	Isc	G	Isc	G	Isc	G	Isc	G
	μA/cm²	*mS/cm²*	*μA/cm²*	*mS/cm²*	*μA/cm²*	*mS/cm²*	*μA/cm²*	*mS/cm²*	*μA/cm²*	*mS/cm²*
Baseline	15.3±2.1	0.6±0.1	16.2±2.0	0.79±0.10	13.7±1.5	0.5±0.0	8.7±1.1*	0.6±0.5	11.2±1.2	0.5±0.0
Forskolin (10 μM)	38.9±5.1	0.9±0.1	24.8±2.0*	0.9±0.1	42.4±1.8	0.8±0.0	18.1±1.6*	0.7±0.1	20.0±0.3*	0.7±0.0*
Carbachol (100 μM)	220.6±15.3	4.3±0.4	195.8±5.7	4.0±0.3	67.1±16.0*	2.2±0.4*	29.4±6.2*	1.0±0.1*	63.7±16.3*	2.1±0.6*

Transepithelial currents and conductances measured in apically permeabilized T84 monolayers in the absence or presence of inhibitors. Data presented as mean±SEM. Baseline indicates stable current and conductance measurements after apical permeabilization with amphotericin B. Isc represents basolateral K conductance in μA/cm² (Isc-bl_K) as defined in text. G represents basolateral K conductance in mS/cm². *Significant differences between means in control and treated monolayers at baseline or after forskolin and carbachol treatment, as assessed by ANOVA ($P \leq 0.0001$) with multiple comparisons ($P \leq 0.05$). *CLT*, Clotrimazole; *ChTxn*, Charybdotoxin; *Met II*, 2 chlorophenyl-*bis*-phenyl methanol.

Clotrimazole inhibits intestinal secretion by native intestine studied in vitro and in vivo

Ussing chamber studies using isolated rabbit colonic mucosa. To test the ability of CLT to block Cl^- secretion in native intestinal tissue, we mounted isolated preparations of rabbit colonic mucosa in Ussing chambers containing modified Ringer's solution (solution 7, Table I) with or without CLT (30 μM). After Isc had stabilized, forskolin (10 μM) was then applied to serosal reservoirs and transepithelial Isc and G were monitored. As shown in Fig. 7, CLT inhibited by 90.6% forskolin-induced changes in Isc.

Murine model of secretory diarrhea. We utilized a murine model of secretory diarrhea to examine the ability of CLT to inhibit toxigenic intestinal fluid secretion in intact animals. Balb/C mice (6–8 wk of age) were gavage fed with CLT (150 mg/kg per d in 12-h divided doses) or with vehicle control for 7 d. Both control and CLT-fed mice were allowed free access to water and chow. Both groups gained weight normally during the 7-d treatment period (4 vs. 8% increase in total body weight in control and CLT treated mice respectively n = 5–10, P = 0.21). After 7 d, the mice were challenged with purified cholera toxin (CT, 25 μg) administered orally by gavage. 5 h after treatment with cholera toxin, the mice were killed and intestinal fluid secretion was measured indirectly as intestinal weight normalized to total body weight as described in Methods. Mice not treated with CLT and challenged or not challenged with CT on day 7 provide two point calibration (Fig. 8, *control vs. CT alone*). Pretreatment with CLT reduced by 94% the increase in normalized intestinal weight induced by cholera toxin (Fig. 8, *CT* + CLT). CLT had no detectable effect on normalized intestinal weight in the absence of cholera toxin (Fig. 8, CLT alone).

Discussion

The results of these studies show that the antifungal clotrimazole inhibits directly two pharmacologically distinct K^+ conductances located on the basolateral membrane of human intestinal T84 cell monolayers. CLT has no detectable effect on apical membrane Cl^- conductance. These data confirm and extend our previous studies on the effects of CLT on K^+ transport in intact T84 cells assessed indirectly as transepithelial Cl^- currents or with unidirectional isotopic efflux studies (8). The results are also consistent with our earlier studies using whole cell and single channel recordings to show a direct effect of CLT and related trityl metabolites on whole cell and single channel K^+ currents in murine erythroleukemia cells (18) and in ferret portal vein smooth muscle cells (19). Taken together, our results are consistent with the hypothesis that blockade of Cl^- secretion by CLT in intestinal T84 cells is secondary to direct blockade of basolateral membrane K^+ channels involved in both cAMP- and Ca^{2+}-regulated Cl^- secretory pathways.

At least two types of K^+ channels are present in T84 cells (12, 13, 15, 41). One channel is activated by cAMP-dependent agonists. This channel may underlie the basolateral membrane K^+ conductance activated by forskolin in apically permeabilized T84 cell monolayers in the current study. A second channel is activated by agents that elevate cytosolic [Ca^{2+}] (12, 40). It is likely that this channel underlies the basolateral membrane K^+ conductance activated by carbachol and by thapsigargin in apically permeabilized T84 monolayers in the current study.

The ability of CLT to inhibit salt and water secretion from native intestinal tissue, as evidenced by blockade of stimulated Isc in rabbit colon in vitro and fluid secretion in mouse intestine in vivo, raises the distinct possibility that CLT and related compounds may display utility in the clinical treatment of secretory diarrheas. High dose CLT has already been administered orally to humans (42–47), and can be therapeutically safe over long term at oral doses of 25 mg/kg per d (21). In support of this view, we found in the current study that CLT had no detectable effect on the growth of mice over a 7-d treatment period. Thus, CLT may inhibit agonist-induced fluid secretion from the mouse intestine (presumably by blockade of basolateral cAMP-dependent K^+ channels) without affecting the net transport and absorption of nutrient substrates. As the bulk of solute absorption in the intestine depends on Na^+-coupled transcellular mechanisms, these data imply that CLT is not likely to depolarize membrane potentials of absorptive intestinal epithelia in vivo. In fact, SADD mice have been treated with CLT (80 mg/kg per d) for 28 d without detectable effects on growth or toxicity (17).

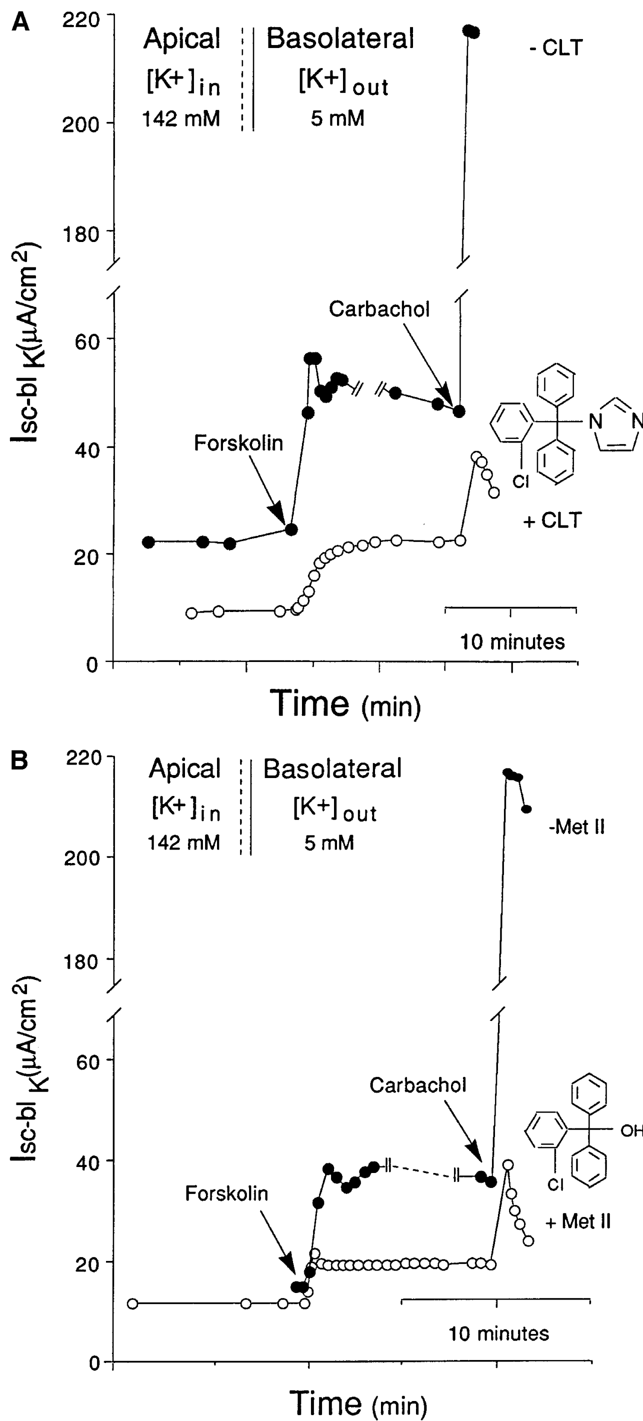


Figure 4. Effect of CLT and MET II on forskolin- and carbachol-induced K^+ currents Isc-bl_K. A basolaterally directed K^+ gradient was established across the monolayer (solution 2 apical, solution 3 basal, see Table I), and the apical plasma membrane was then permeabilized with 20 μM amphotericin B 30 min before the addition of agonist. Arrows indicate the addition of forskolin (10 μM) or carbachol (100 μM). (*A*) The time course of the Isc-bl_K response to the ordered additions of forskolin and carbachol is shown for an apically permeabilized monolayer pretreated with CLT (30 μM, chemical structure shown, *open circles*). CLT inhibited both forskolin- and carbachol-induced Isc-bl_K. Representative of seven independent experiments. For comparison, the Isc responses elicited by forskolin and carbachol applied to a separate apically permeabilized monolayer not treated with CLT is superimposed on the time course (*filled circles*). Representative of eight independent experiments. (*B*) The time course of the Isc-bl_K response to the ordered additions of forskolin and carbachol is shown for an apically permeabilized monolayer pretreated with the stable metabolite of CLT, Met II (30 μM, chemical structure shown, *open circles*). Met II inhibited both forskolin- and carbachol-induced Isc-bl_K. Representative of three independent experiments. For comparison, the Isc responses elicited by forskolin and carbachol applied to a separate apically permeabilized monolayer not treated with Met II is superimposed on the time course (*filled circles*). Representative of eight independent experiments.

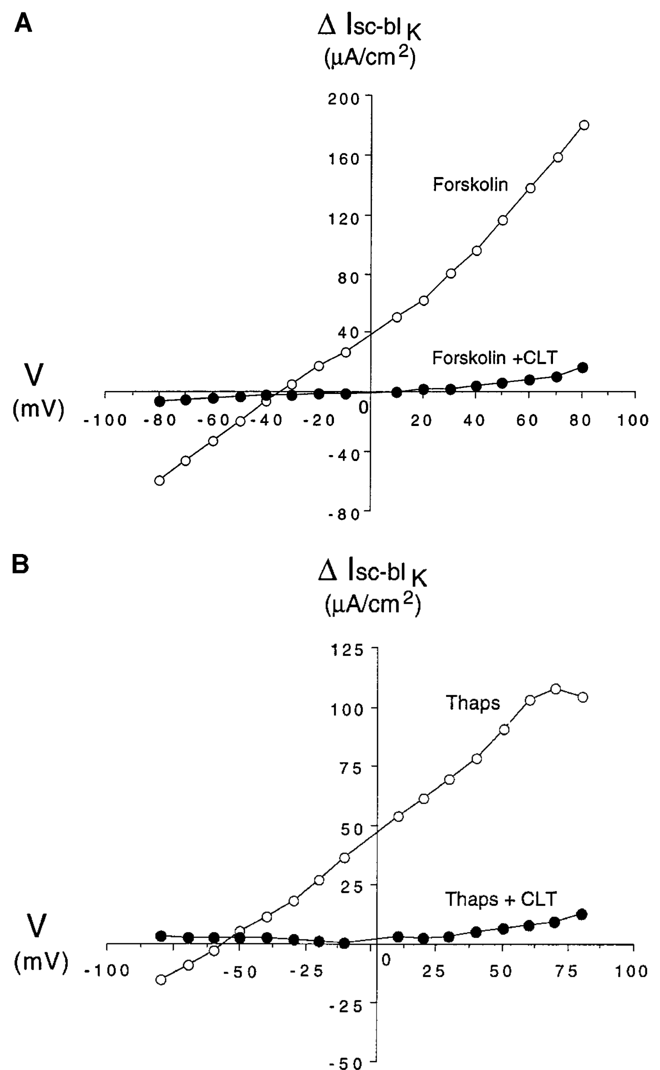


Figure 5. Effect of CLT on steady-state current–voltage relationship of K^+ transport in luminally permeabilized monolayers of T84. A basolaterally directed K^+ gradient was established across the monolayer (solution 2 apical, solution 4 basal, see Table I), which was then apically permeabilized with 20 μM Amphotericin B. Short-circuit currents (*Isc-bl*$_K$) were recorded at the indicated voltage-clamped test potentials. The ordinate indicates the current difference measured on the same T84 monolayer before and 10 min after agonist stimulation (at steady-state Isc). (*A*) Currents (*Isc-bl*$_K$) were elicited by 10 μM forskolin in the absence (*open circles*) or presence (*solid circles*) of 30 μM CLT. (*B*) Currents (Isc-bl_K) were elicited by 5 μM thapsigargin (*Thaps*) in the absence (*open circles*) or presence (*solid circles*) of 30 μM CLT. Each panel is representative of seven independent experiments.

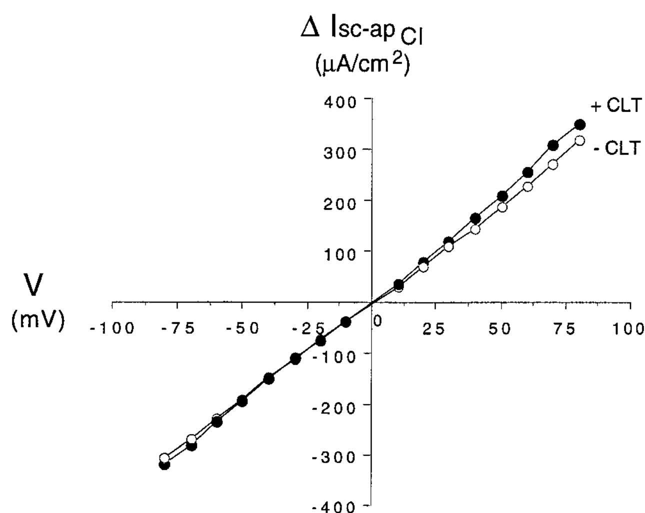


Figure 6. Effect of CLT on steady-state current–voltage relationship for Cl^- transport in basolaterally permeabilized T84 monolayers. A symmetrical high Cl^- solution was established across the monolayer (142 mM choline-chloride; solution 6, see Table I), which was then basolaterally permeabilized with 100 μM amphotericin B. Currents (Isc-ap_{Cl}) were recorded at the indicated voltage-clamped test potentials. The ordinate indicates the change in Isc-ap_{Cl} measured sequentially on the same T84 monolayer at given voltages before and 10 min after stimulation with 10 μM forskolin (at steady-state Isc). Monolayers were studied in the absence (*open circles*) or presence (*solid circles*) of 30 μM CLT. Representative of three independent experiments for each condition.

The ability of the des-imidazolyl CLT metabolite, MET II, to inhibit K^+ transport confirms our earlier observations that the imidazole ring, though strictly required for inhibition of cytochrome P-450 enzymes, is not required for inhibition of Isc-bl_K in T84 cells. As an inhibitor of cytochrome P-450 enzymes, CLT shows preference for arachidonate epoxygenase (48). Although epoxygenase metabolites have been shown to regulate K^+ channel activity in rat kidney cells (49), the ability of CLT and its metabolites to inhibit K^+ transport in human T84 cells appears not to be mediated through this pathway. This lack of requirement for cytochrome P-450 interaction resembles a similar pharmacology identified for the inhibition by CLT and its analogues of large conductance Ca^{2+}-dependent K^+ channels (K_{Ca}) of ferret portal vein vascular smooth muscle cells (19) and in the intermediate conductance K_{Ca} channels in murine erythroleukemia cells (18). Inhibition of cytochrome P-450 was similarly found not necessary for in vivo inhibition of erythroid Ca^{2+}-activated ^{86}Rb flux in humans (50). These results are of clinical importance. In humans, adverse effects associated with oral delivery of clotrimazole are dose dependent, reversible, and thought to result largely if not entirely from interactions between the imidazole ring and cytochrome P-450 enzymes (43, 50, 51). Thus trityl-based structures related to CLT but lacking the imidazole ring may be found to exhibit a larger in vivo therapeutic window with similar or greater therapeutic potency.

The use of K^+ channel blockers to inhibit epithelial chloride secretion has also been explored by other laboratories (52, 53). Greger and colleagues noted that a group of cromanol compounds selected from agents screened for the ability to block Isc in isolated rabbit colon turned out to be ineffective as Cl^- channel blockers, but effective as blockers of K^+ channel activity elicited by cAMP-dependent agonists. The most potent of these was compound 293B (52, 53). Like 293B, CLT was found to inhibit K^+ conductance activated by cAMP-dependent agonists. CLT differs from 293B, however, in its ability also to block Ca^{2+}-activated K^+ conductances (references 16–18, 54–57, and this study).

In preliminary studies, we have shown that the distinct K^+ conductances activated by cAMP- and Ca^{2+}-dependent secretagogues also display different pharmacological profiles of inhibition by a family of CLT-related compounds that lack the imidazole ring. These compounds are expected to display no P-450 inhibitory activity. Moreover, the pharmacologic profiles of inhibition for both types of K^+ conductance expressed in the human intestinal T84 cell differ in turn from the profile of inhibition for Ca^{2+}-activated K^+ channel of intermediate conductance present in human erythrocytes. We therefore expect that CLT-related compounds capable of greater inhibitory specificity than that exhibited by the parent compound will become available in the near future.

In summary, based on the results of our studies in rabbit colon in vitro, in mouse intestine in vivo, and in human intestinal T84 cells in culture (reference 8, and this study), we propose that the effects of CLT on fluid secretion in intact intestine are mediated by blockade of basolateral K^+ channels expressed in Cl^- secreting cells of the intestinal crypts. As CLT inhibits Cl^- secretion elicited by both cAMP- and Ca^{2+}-dependent ago-

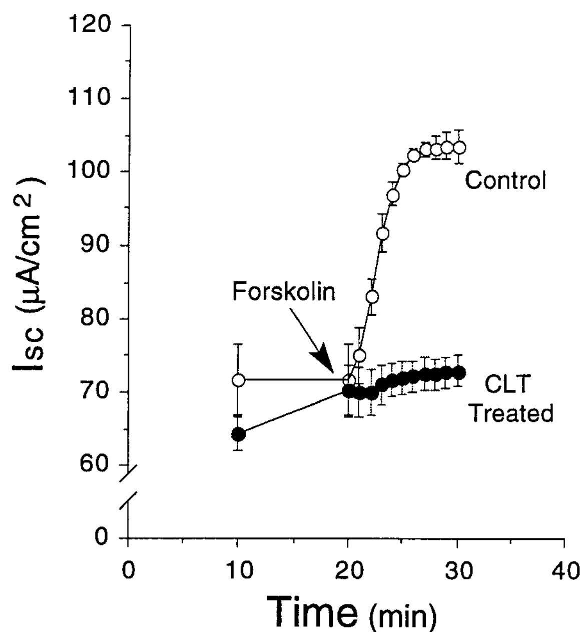


Figure 7. CLT (30 μM) inhibits forskolin-induced changes in short circuit current (*Isc*) in isolated rabbit colon mounted in Ussing chambers. Rabbit colonic mucosal preparations were dissected free of supporting structures and mounted in Ussing chambers. Mucosal and serosal reservoirs contained symmetric buffer solutions of modified lactated Ringers solution (solution 7, see Table I) in the absence (*open circles*) or presence (*filled circles*) of 30 μM CLT. After reaching stable Isc levels, mucosal preparations were stimulated by the addition of forskolin (10 μM) to the serosal reservoir. Representative of four independent experiments, each containing measurements on tissue from four separate animals.

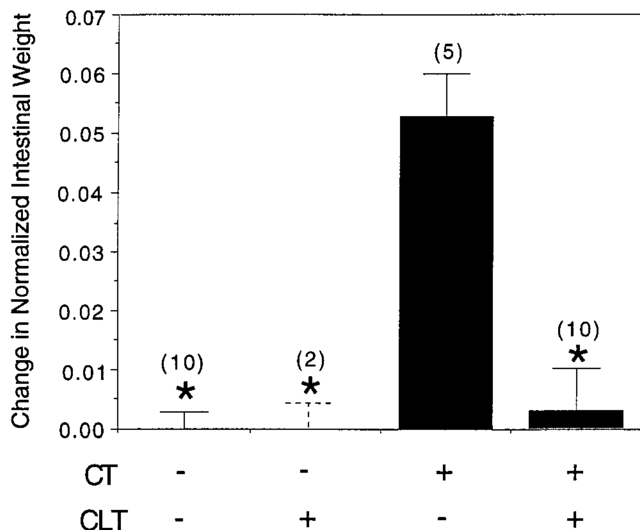


Figure 8. Oral administration of CLT inhibits intestinal fluid secretion in an in vivo mouse model of enterotoxin-induced secretory diarrhea. Animals were gavage fed either CLT (150 mg/kg per d) or vehicle control in two divided doses for 7 d and then challenged orally with cholera toxin (*CT*, 25 μg) or PBS buffer as control. Intestinal fluid secretion was measured gravimetrically and normalized to total animal body weight as described in Methods. The number of animals in each treatment group is shown in parentheses. Two mice treated with CLT were not challenged with CT, and the dashed bar reports average and range of the two animals in this group. Mean intestinal weight in animals not treated with CLT or CT was 1.55±0.11 grams: normalized intestinal weight, calculated by dividing intestinal weight by total body weight, had a mean value of 0.093±0.003 in this group. *Statistically significant differences among test and control groups as assessed by ANOVA ($P < 0.0001$), and multiple comparison procedures set at $P \leq 0.05$.

nists, CLT and related des-imidazolyl compounds may display utility in the treatment of secretory diarrheas of diverse etiologies.

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