

The Antifungal Antibiotic, Clotrimazole, Inhibits Cl⁻ Secretion by Polarized Monolayers of Human Colonic Epithelial Cells

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Abstract

Clotrimazole (CLT) prevents dehydration of the human HbSS red cell through inhibition of Ca⁺⁺-dependent (Gardos) K⁺ channels in vitro (1993. *J. Clin. Invest.* 92:520–526.) and in patients (1996. *J. Clin. Invest.* 97:1227–1234.). Basolateral membrane K⁺ channels of intestinal crypt epithelial cells also participate in secretagogue-stimulated Cl⁻ secretion. We examined the ability of CLT to block intestinal Cl⁻ secretion by inhibition of K⁺ transport. Cl⁻ secretion was measured as short-circuit current (I_{sc}) across monolayers of T84 cells. CLT reversibly inhibited Cl⁻ secretory responses to both cAMP- and Ca²⁺-dependent agonists with IC₅₀ values of ~ 5 μM. Onset of inhibition was more rapid when CLT was applied to the basolateral cell surface. Apical Cl⁻ channel and basolateral NaK2Cl cotransporter activities were unaffected by CLT treatment as assessed by isotopic flux measurement. In contrast, CLT strongly inhibited basolateral ⁸⁶Rb efflux. These data provide evidence that CLT reversibly inhibits Cl⁻ secretion elicited by cAMP-, cGMP-, or Ca²⁺-dependent agonists in T84 cells. CLT acts distal to the generation of cAMP and Ca²⁺ signals, and appears to inhibit basolateral K⁺ channels directly. CLT and related drugs may serve as novel antidiarrheal agents in humans and animals. (*J. Clin. Invest.* 1996. 98:2066–2075.) Key words: clotrimazole • Cl⁻ secretion • T84 cell • K⁺ channels • Ca⁺⁺

Introduction

Intestinal Cl⁻ secretion, the primary transport event of secretory diarrhea (1), requires the coordinated activity of specific membrane ion transporters and channels found in epithelial cells lining crypts of the small and large bowel (2, 3). These so-called “undifferentiated Cl⁻ secreting” cells accumulate Cl⁻ ions above their electrochemical potential through the activities of NaK2Cl cotransporters and Na/K ATPase pumps in the basolateral membrane. Secretion into the intestinal lumen oc-

currs by passive diffusion of Cl⁻ through activated channels in the apical cell membrane (4, 5). Basolateral K⁺ channels recycle transported K⁺ ions out of the cell, and are necessary to maintain the inside-negative electrochemical membrane potential which favors ion secretion (6–10). These membrane proteins function together to secrete Cl⁻. Blockade of any one of these ion transporters or channels will attenuate the Cl⁻ secretory response.

Intestinal Cl⁻ secretion is regulated by agonists which elicit cAMP-, cGMP-, or Ca²⁺-mediated intracellular signals. The activity of apical membrane Cl⁻ channels, including the cystic fibrosis transmembrane regulator (CFTR) (11), depends primarily on ligand-receptor driven cAMP- or cGMP-signaling cascades. These intracellular second messengers also regulate in tandem the activities of basolateral NaK2Cl cotransporters (12–14), and K⁺ channels (6–10, 15–17). Both cyclic nucleotide and Ca²⁺-mediated intracellular signals affect basolateral K⁺ efflux pathways in the intestine, and two pharmacologically distinct K⁺ conductances have been identified (18). One K⁺ efflux pathway displays dependence on intracellular cAMP, and sensitivity to Ba²⁺ salts (8, 19). The other pathway is activated by Ca²⁺ agonists (20, 21), and displays little or no sensitivity to Ba²⁺, tetraethylammonium (TEA), 4-aminopyridine, or apamin (7, 8, 22).

Recently, a Ca²⁺-dependent K⁺ channel in the human intestinal cell line T84 has been biophysically defined (21). Single channel recordings have identified an inwardly rectifying K⁺ conductance of 14–32 pS. Channel activity was dependent on Ca²⁺ and inhibited by charybdotoxin (a component of scorpion venom) and tetraethylammonium (TEA), but not by Ba²⁺, apamin, or 4-aminopyridine. In separate studies using the whole cell perforated patch technique, two distinct K⁺ currents were identified in T84 cells. One K⁺ conductance was sensitive to charybdotoxin and the other was not sensitive to the toxin (23). The molecular identities of these K⁺ channels remain unknown.

Our interest in the use of imidazole antibiotics such as clotrimazole (CLT)¹ to inhibit Cl⁻ secretory responses arises from studies on K⁺ transport in the human red cell (24–27). Recently, CLT was shown to inhibit Ca²⁺-dependent K⁺ channels in rat thymocytes and human erythrocytes (24–28). Ca²⁺-dependent K⁺ channels in the human erythrocyte (also known as the Gardos pathway [29]), and murine erythroleukemia cells (30) display inwardly rectifying unitary conductances of 14–40 pS that are sensitive to blockade by charybdotoxin.

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1. *Abbreviations used in this paper:* CLT, clotrimazole; CRB, carbachol; FSK, forskolin; I_{sc}, short circuit current; VIP, vasoactive intestinal peptide.

These biophysical parameters share some resemblance to those identified for Ca^{2+} -dependent K^+ channels in human intestinal T84 cells, and suggest that CLT may affect K^+ conductances in enterocytes as it does in the red cell.

To test this idea, we utilized the human intestinal cell line, T84. T84 cells form 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 intestinal tissue (3, 31, 32). Our data show that CLT reversibly inhibits both cAMP- and Ca^{2+} -dependent Cl^- secretion, and suggest that CLT acts directly on both Ba^{2+} -sensitive and charybdotoxin-sensitive K^+ efflux pathways. These studies provide evidence that CLT and related compounds may have utility in the treatment of secretory diarrheas of diverse etiology.

Methods

Materials. ^{125}I and ^{86}Rb were obtained from New England Nuclear (Boston, MA) and BaCl_2 from Fisher Scientific Co. (Fair Lawn, NJ). Cholera Toxin was obtained from Calbiochem (San Diego, CA). *Escherichia coli* stable toxin (STa) was obtained from List (Campbell, 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-bisphenyl methanol was kindly provided by Dr. Wolfgang Ritter (Bayer A.G.) HBSS containing (mM) 1.26 CaCl_2 , 0.65 MgSO_4 , 5.37 KCl , 0.441 KH_2PO_4 , 136.9 NaCl , 0.337 Na_2HPO_4 , 5.5 D-glucose and 10 mM Hepes, pH 7.4 was used in all assays except those involving BaCl_2 in which a modified Dulbecco's media containing (mM) 1.0 CaCl_2 , 0.5 MgCl_2 , 145 NaCl , 1 KCl , 5.5 D-glucose, and 10 mM Hepes, pH 7.4 was substituted.

Cell culture. T84 cells obtained from American Type Culture Collection (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- or 5-cm² Transwell inserts (Costar Corp., Cambridge, MA) coated with dilute rat collagen solution as previously described (33, 34). Transepithelial resistances attained stable levels ($> 1,000$ ohms/cm²) 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^- (32). Passages 77 to 91 were used in these studies.

Measurement of electrogenic Cl^- secretion (Short-circuit current [I_{sc}]). Confluent monolayers were transferred to HBSS at 37°C. Serosal and mucosal reservoirs were interfaced with calomel and Ag-AgCl electrodes via 5% agar bridges made with Ringer's buffer. Transepithelial resistance was measured during 25 or 50 μA current pulses using a dual voltage clamp device (University of Iowa, Iowa City, IA). I_{sc} was calculated using Ohm's law as previously described (33, 34). Previous studies have shown that secretagogue-activated changes in I_{sc} represent almost entirely apical Cl^- secretion (12).

^{86}Rb and ^{125}I efflux studies. Confluent monolayers on 5-cm² Transwell inserts were used 10–14 d after plating. ^{125}I and ^{86}Rb flux were measured as indicators of apical Cl^- channel and basolateral K^+ channel activities as previously described (35). Monolayers were preincubated at 37°C apically and basolaterally with 4 $\mu\text{Ci/ml}$ ^{125}I or ^{86}Rb in HBSS for 90 min, with 33 μM CLT absent or present during the final 30 min of this 90 min preincubation period. Loading of ^{86}Rb was nearly identical in monolayers treated or not treated with CLT. Loading of ^{125}I into T84 monolayers pretreated with CLT appeared to be increased slightly (34 \pm 11%) over nontreated controls. However, this

difference was not statistically significant. After washing twice in fresh HBSS, 0.5-ml aliquots were obtained every 2 min from the apical reservoir for ^{125}I efflux studies and replaced with fresh HBSS. For ^{86}Rb efflux studies, 1-ml samples were obtained every 2 min from the basolateral reservoir and replaced with fresh HBSS. Four baseline samples were obtained before agonist treatment with vasoactive intestinal peptide (VIP, 5 nM) or thapsigargin (5 μM). After 30 min, cell monolayers were rinsed, cut from their plastic inserts, and residual cell-associated radioactivities were determined. Monolayers were maintained at 37°C in room air throughout the study. ^{125}I and ^{86}Rb activities were determined by gamma or scintillation counting, and normalized to percent total uptake as previously described (35).

^{86}Rb uptake studies. Confluent monolayers on 5-cm² Transwell inserts were preincubated for 30 min at 37°C in HBSS or HBSS containing CLT (33 μM). Subsets of both control and CLT treated monolayers were then treated or not treated with bumetanide (10 μM , for 12 min). All monolayers were then exposed to VIP (5 nM) basolaterally and shifted to HBSS containing 1 $\mu\text{Ci/ml}$ ^{86}Rb for 3 min at 37°C. ^{86}Rb uptake was terminated by washing the inserts in an ice-cold solution containing 100 mM MgCl_2 , and 10 mM Tris-Cl, pH 7.4. Monolayers were cut from their inserts, placed into scintillation vials, and counted using standard methods as above.

Fluorescence ratio imaging of intracellular free Ca^{2+} ion concentration. T84 cells cultured at subconfluent density on collagen-coated 5-cm² coverslips were incubated in growth media containing 2 μM Fura-2-acetoxymethyl ester (Fura-2 AM; Molecular Probes, Eugene, OR) for 30 min at 37°C in humidified 5% CO_2 , then washed and mounted in a modified Leiden chamber in which the coverslip constituted the bottom. 1 ml of HBSS was added to the coverslip chamber on the stage of an inverted microscope (IMT-2; Olympus Corp., Lake Success, NY) equipped with a Dage-MTI CCD7 series video camera (Dage-MTI, Michigan City, IN).

The intracellular concentration of free calcium ($[\text{Ca}^{2+}]_i$) was measured in room air at room temperature by Fura-2 fluorescence ratio imaging using an Image-1 digital imaging system (Universal Imaging, West Chester, PA) equipped with GenIISys image intensifier (Dage-

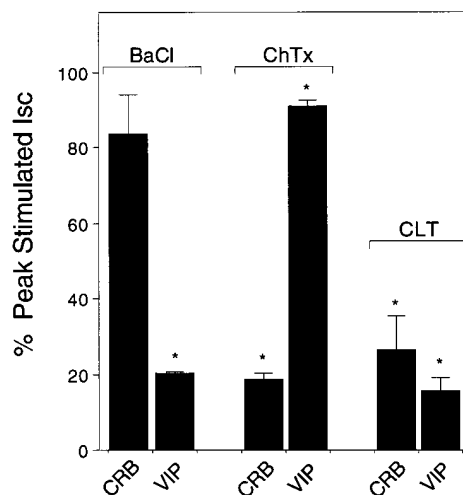


Figure 1. Effect of CLT on the Cl^- secretory response (I_{sc}) in T84 cells. Monolayers were pretreated with or without BaCl_2 (3 mM, $n = 7$), charybdotoxin (100 μM , $n = 4$), or clotrimazole (33 μM , $n = 7$) for 30 min at 37°C, then stimulated basolaterally with VIP (5 μM) or carbachol (CRB, 100 μM). Peak I_{sc} values ($\mu\text{A}/\text{cm}^2$, mean \pm SEM) in the absence of inhibitors were 83 \pm 3 (VIP) and 50 \pm 8 (CRB). CLT inhibited the Cl^- secretory response to both agonists. *Significant differences between treated and untreated control monolayers as determined by ANOVA, $P = 0.005$ – 0.0001 .

MTI), a Pinnacle REO-650 optical disk drive (Pinnacle Micro, Inc., Irvine, CA), color video monitor and printer, as described previously (36). Isolated cells and cells at the periphery of cell clusters accumulated Fura-2 to higher levels than cells in the center of clusters. Therefore, intracellular free $[Ca^{2+}]_i$ was monitored in the cells at the edges of clusters. All individual cells and cell clusters demonstrating Fura-2 uptake were included in data analysis. Exposure of cells to 0.25% Pluronic F-127 (Molecular Probes) during Fura-2 loading did not increase Fura-2 accumulation in cells in the center or at the edges of the cell clusters.

Fura-2 fluorescence images were monitored at an emission wavelength of 510 nm, with alternating excitation wavelengths of 340 and 380 nm. Images and ratio data sets were collected at intervals ranging between 6 and 120 s, and stored to optical disk for later image replay and data processing. In vitro calibration of the Fura-2 free acid fluorescence ratio to the concentration of free Ca^{2+} was performed in vitro by the method of Grynkewicz et al. (36), using a value for the K_d of Ca^{2+} binding to Fura-2 of 224 nM. R_{min} was determined at 10 nM free Ca^{2+} , and R_{max} was determined at 40 μM free Ca^{2+} . In situ calibration of Fura-2 fluorescence ratio in T84 cells was performed

with 2 μM of the nonfluorescent Ca^{2+} ionophore, 4-Br-A23187 (Molecular Probes), in the presence of EGTA/ Ca^{2+} buffers of varying free Ca^{2+} concentration. Multipoint in situ calibration could not be completed, due to loss of T84 cell adhesion to the glass coverslips during the period of cell equilibration in ionophore/ Ca^{2+} /EGTA solutions of low free $[Ca^{2+}]_i$. Consequently, reported measurements of $[Ca^{2+}]_i$ in T84 cells are based on in vitro calibration of the Fura-2 fluorescence ratio. However, in situ fluorescence ratio calibration was successfully performed at nominal $[Ca^{2+}]_i$ values of 100 and 200 nM. The ratios measured in situ at these two concentrations did not differ significantly from those determined by the in vitro calibration procedure at the same values of free $[Ca^{2+}]_i$ ($P > 0.2$).

Statistics. IC_{50} 's for inhibition of Cl^- secretion were derived from nonlinear fits of dose response data ($n = 7$ and 9 independent experiments for VIP and carbachol, respectively) to Michaelis-Menten kinetics using Deltagraph Pro 3.5 (Deltapoint, Monterey, CA). Origins were constrained to 100% maximal response in the absence of CLT. Tests of significance where indicated were based on ANOVA using Statview 512+ (BrainPower, Calabasas, CA).

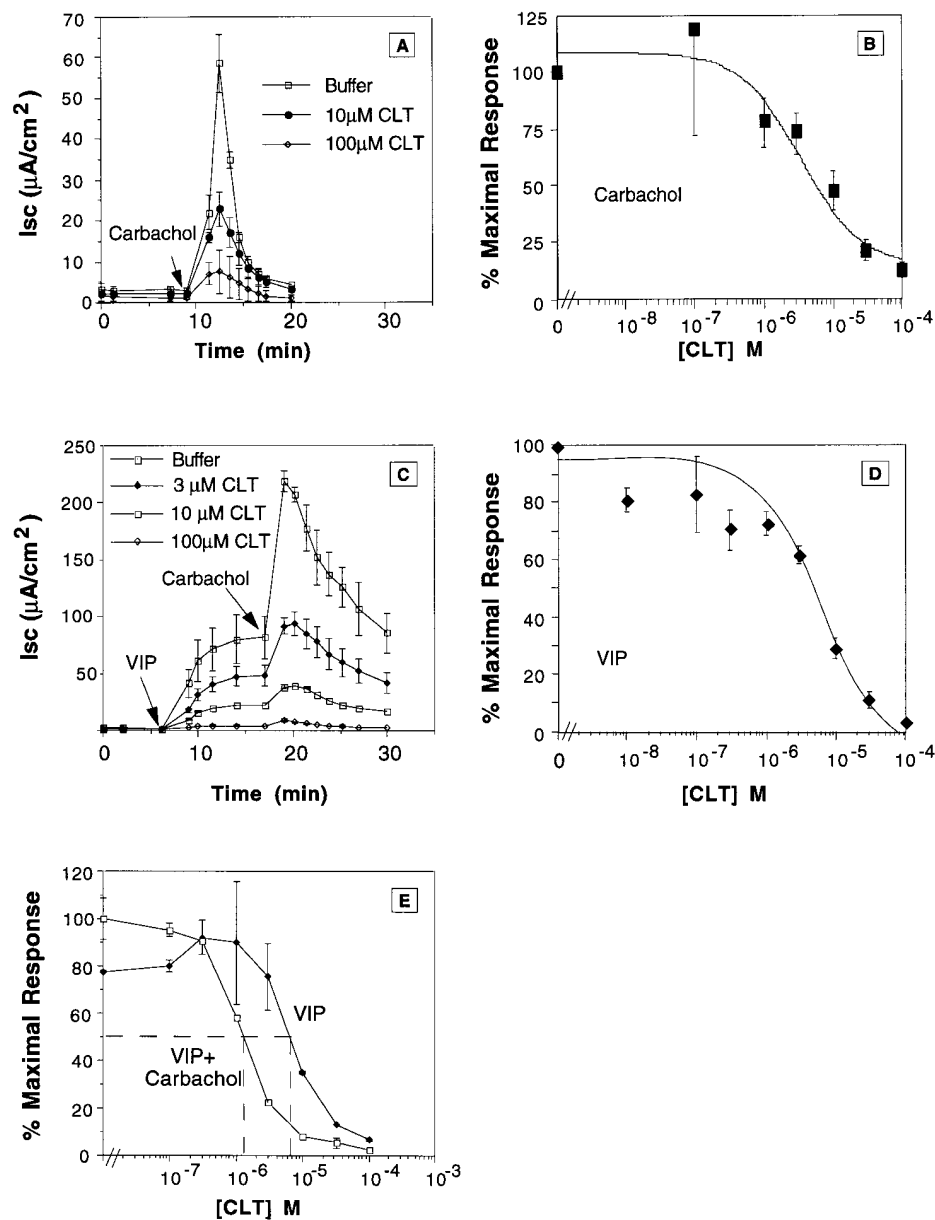


Figure 2. Dose dependence of CLT inhibition. (A) Time course of a representative experiment illustrating the Cl^- secretory response (I_{sc}) elicited by carbachol (100 μM) in the absence or presence of graded doses of CLT. (C) Time course of a representative experiment illustrating the Cl^- secretory response (I_{sc}) elicited by VIP (5 nM) in the absence or presence of graded doses of CLT. (B and D) Dose dependency of CLT action on Cl^- secretory responses elicited by carbachol and VIP, respectively. The I_{sc} values in B and D were normalized to the peak response taken at 1.4 min after addition of carbachol or at 11 min after addition of VIP. IC_{50} 's were derived from nonlinear fits of seven (VIP) or nine (carbachol) independent experiments to Michaelis-Menten kinetics as described in Methods. (E) Representative experiment showing the dose dependency of CLT action on Cl^- secretion elicited by VIP alone or VIP + carbachol. The mean IC_{50} values observed for inhibition of Cl^- secretion after addition of VIP or VIP + carbachol from seven experiments were significantly different (ANOVA $P = 0.007$, $n = 7$).

Results

Clotrimazole reversibly inhibits Cl⁻ secretion elicited by Ca²⁺- or cAMP-dependent agonists. Previous studies have shown that Cl⁻ secretion in T84 cells depends on pharmacologically distinct K⁺ efflux pathways. One pathway participates in the secretory response to cAMP-dependent agonists and displays sensitivity to Ba²⁺ salts (15, 16). The other mediates the response to Ca²⁺-dependent agonists, and is Ba²⁺-insensitive. In agreement with these studies, we found that Ba²⁺ (3 mM) inhibited by 80±0.4% (mean±SEM, *n* = 3) the peak short-circuit current elicited by the cAMP-dependent agonist vasoactive intestinal peptide (VIP, 5 nM), but minimally inhibited (by 16.5±10.7%, mean±SEM, *n* = 4) the Cl⁻ secretory response elicited by the Ca²⁺-dependent agonist carbachol (100 μM) (Fig. 1, bars on left). Charybdotoxin (100 nM) inhibited 81±1% (mean±SEM, *n* = 4) of the Cl⁻ secretory response elicited by carbachol, but only minimally attenuated (by 10±2%, mean±SEM, *n* = 4) Cl⁻ secretion elicited by treatment with the cAMP agonist, VIP (Fig. 1, middle bars). Unlike either Ba²⁺ or charybdotoxin, preincubation with the imidazole antibiotic clotrimazole (CLT, 33 μM) strongly inhibited the Cl⁻ secretory responses elicited by both carbachol and VIP, reducing peak I_{sc}'s by 84±3.5% and 74±8.9% respectively (mean±SEM, *n* = 4) (Fig. 1, bars on right).

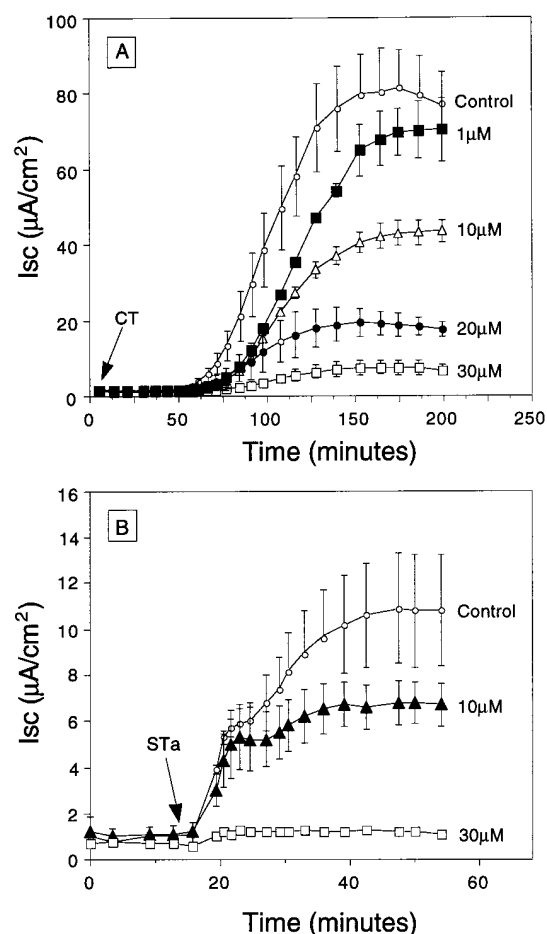


Figure 3. Effect of graded doses of CLT on the secretory response elicited by cholera toxin (A, *n* = 3) or *E. coli* heat stable toxin (StA), (B, *n* = 2).

Fig. 2 shows the dose dependence of inhibition of Cl⁻ secretion by CLT. These inhibitory effects of CLT on Cl⁻ secretion were maximal after 30 min incubation (data not shown). Fig. 2, A and C show the time courses of the Cl⁻ secretory response elicited by carbachol or VIP in the absence or presence of increasing concentrations of CLT. To examine possible effects of CLT on the synergy between cAMP- and Ca²⁺-mediated agonists, monolayers initially stimulated with VIP (Fig. 2 C) were allowed to reach steady-state levels of secretion and then additionally exposed to carbachol (100 μM). CLT was slightly more effective in inhibiting the secretory response to carbachol (Fig. 2 B) than to cAMP (Fig. 2 D) with IC₅₀ values of 3.5±1.0 and 6.1±0.9 μM, respectively (mean±SE, *n* = 9 or 7). This was most clearly seen when the effects of CLT on cAMP- (Fig. 2 E, VIP alone) and Ca²⁺-dependent secretory pathways (Fig. 2 E, VIP + carbachol) were examined on the same monolayers. In low doses (~10⁻⁷ M or less), CLT increased slightly (by 5–10%) the Cl⁻ secretory responses to either agonist, but this finding was variable. CLT inhibited effectively the secretory response to cholera toxin (20 nM, a cAMP-dependent agonist) and *E. coli* heat-stable toxin (100 nM, a cGMP-agonist) (IC₅₀ values of 10 μM and 15 μM, respectively) (Fig. 3). Inhibition of Cl⁻ secretion by CLT (10 μM) was fully reversible (96±2%, *n* = 4) after 60 min recovery in the presence of 0.01 mg/ml bovine serum albumin.

Among the imidazole antibiotics, CLT was the most potent inhibitor of cAMP and Ca²⁺ elicited Cl⁻ secretion (Table I). Ketoconazole, econazole, and miconazole were effective but less potent. Among the drugs tested, only CLT was more potent in blocking carbachol-induced secretion than VIP-induced secretion. Each of the other compounds tested were either more effective inhibitors of the secretory response to cAMP agonists, or had no detectable effect on the secretory response to Ca²⁺ agonists. A stable metabolite of CLT lacking the imidazole ring, 2-chlorophenyl-bis-phenyl methanol, was nearly as effective as CLT (Table I). Metronidazole was not effective in blocking either cAMP- or Ca²⁺-mediated Cl⁻ secretory processes.

Clotrimazole acts at distal steps in the cAMP- and Ca²⁺-dependent signal transduction pathways. To determine the site of CLT action, we examined the effect of CLT pretreatment on monolayers stimulated with agonists that initiate Cl⁻ secretion at sequential steps in the cAMP-signaling cascade (Fig. 4A).

Table I. Pharmacology of Imidazole Derivatives

Agent		VIP	IC ₅₀ (μM) Synergy	Carbachol
Clotrimazole	(<i>n</i> = 7–9)	6.1±0.9	2.7±0.6	3.5±1.0
2-chlorophenyl- bis-phenyl methanol	(<i>n</i> = 2–6)	4.5±1.9	4.8±1.3	23±7.1
Ketoconazole	(<i>n</i> = 3)	42.6±4.6	69±29	ND
Econazole	(<i>n</i> = 4)	37.5±5.0	97.5±10.0	ND
Miconazole	(<i>n</i> = 4)	10±0.0	>>100	ND
Metronidazole	(<i>n</i> = 2)	No effect	No effect	ND
BaCl	(<i>n</i> = 4)	48.8±10	8,300±1,700	7,300±1,800

Synergy represents the increase in short circuit current (I_{sc}) elicited by carbachol (100 μM) applied to monolayers initially stimulated with VIP (5 nM) and allowed to reach steady state levels of secretion.

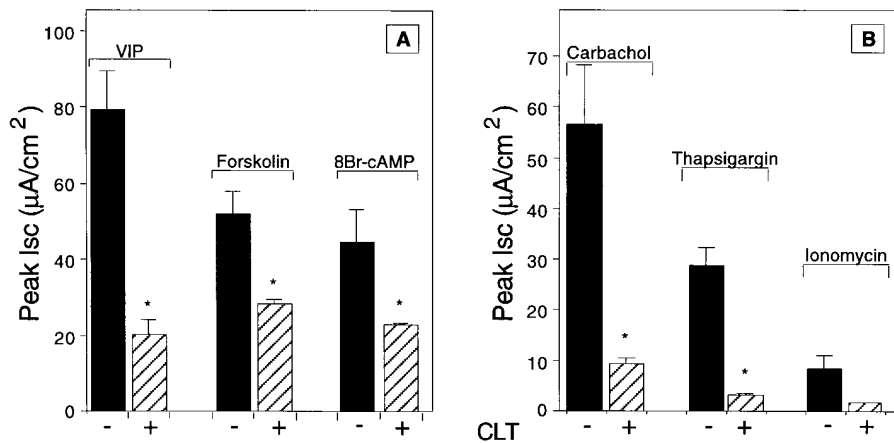


Figure 4. Effect of CLT on peak Cl^- secretory response to cAMP- (A) or Ca^{2+} -dependent agonists (B). T84 monolayers were pretreated for 30 min at 37°C in the absence (solid bars) or presence (hatched bars) of CLT ($10\ \mu\text{M}$ for cAMP agonists, and $33\ \mu\text{M}$ for Ca^{2+} agonists) and stimulated with the cAMP agonists (A) VIP ($5\ \text{nM}$, $n = 10$), forskolin ($10\ \mu\text{M}$, $n = 5$), or 8Br-cAMP ($3\ \text{mM}$, $n = 5$), or with the Ca^{2+} -dependent agonists (B) carbachol ($100\ \mu\text{M}$, $n = 8$), thapsigargin ($5\ \mu\text{M}$, $n = 12$), or ionomycin ($4\ \mu\text{M}$, $n = 5$), (mean \pm SEM), (* ANOVA $P \leq 0.003$). Peak currents were measured at 12 min after stimulation with VIP and after 1.5 min after stimulation with carbachol.

T84 monolayers were preincubated in HBSS in the presence or absence of CLT ($33\ \mu\text{M}$) and then stimulated with either $5\ \mu\text{M}$ VIP (which activates adenylate cyclase through heterotrimeric GTPase-linked cell surface receptors), $10\ \mu\text{M}$ forskolin (which activates adenylate cyclase directly), or $3\ \text{mM}$ 8Br-cAMP (a direct stimulator of protein kinase A). CLT ($10\ \mu\text{M}$) inhibited the secretory response to each of these agonists (Fig. 4 A). These data provide evidence that CLT acts at a step distal to the activation of protein kinase A.

Ca^{2+} -dependent intracellular signaling in T84 and other nonexcitable cells involves recruitment of inositol (1, 4, 5) triphosphate (IP_3)-dependent intracellular Ca^{2+} stores and subsequent activation of plasma membrane Ca^{2+} influx pathways (3, 37–39). Downstream events are mediated by $[\text{Ca}^{2+}]_i$, IP_3 , diacylglycerol, or as yet unidentified diffusible factors (39–41). To examine the site of CLT action along this signaling cascade, T84 monolayers pretreated in the presence or absence of CLT ($33\ \mu\text{M}$) were stimulated with the Ca^{2+} -dependent agonists carbachol ($100\ \mu\text{M}$, which elicits both Ca^{2+} and IP_3 signals), thapsigargin ($5\ \mu\text{M}$, which elevates cytoplasmic Ca^{2+} via inhibition of endoplasmic reticulum Ca^{2+} -ATPase [42–45]), or the Ca^{2+} ionophore ionomycin ($10\ \mu\text{M}$). Fig. 4 B illustrates that CLT ($33\ \mu\text{M}$) inhibited by 80–90% the Cl^- secretory response to each of these reagents. These data suggest that CLT acts at steps in the secretory response distal to the release of intracellular Ca^{2+} stores.

Clotrimazole does not inhibit apical membrane anion conductance or basolateral $\text{NaK}2\text{Cl}$ cotransporters. Inhibition of

electrogenic Cl^- secretion might occur by blockade of apical membrane Cl^- channels, or blockade of basolaterally situated $\text{NaK}2\text{Cl}$ cotransporters, K^+ channels, or NaK ATPase pumps. To determine if CLT affected ion conductance through apical membrane Cl^- channels, we examined the time course of ^{125}I efflux from T84 monolayers pretreated in the presence or absence of CLT (35). Fig. 5 A shows that CLT had little or no effect on the time course of ^{125}I efflux from monolayers treated with VIP. Rate constants for ^{125}I efflux from monolayers treated or not treated with CLT were indistinguishable (0.0637 vs 0.0645% uptake/minute, $n = 2$ independent experiments with 2 independent monolayers in each experiment). CLT had similar lack of effect on ^{125}I efflux stimulated by thapsigargin (data not shown, $n = 2$). These data do not support any effect of CLT on apical Cl^- channels.

We next tested the effect of CLT on basolateral $\text{NaK}2\text{Cl}$ cotransporters, as assessed by bumetanide-sensitive ^{86}Rb uptake (13, 14) (Fig. 5 B). CLT treatment reduced the total amount of ^{86}Rb uptake by $53.6 \pm 5.8\%$ (mean \pm SEM, $n = 6$), but had no effect on the fractional component that was bumetanide-sensitive (88 ± 3.2 vs $75.2 \pm 12.7\%$ total uptake, mean \pm SEM). Taken together, these data suggest that CLT does not affect Cl^- secretion in T84 cells via inhibition of either apical membrane Cl^- channels or basolateral membrane $\text{NaK}2\text{Cl}$ cotransporters.

Clotrimazole inhibits K^+ efflux in T84 cells. We examined whether CLT might inhibit K^+ permeability in T84 cells as it does in the human red cell (26). K^+ channel activity was esti-

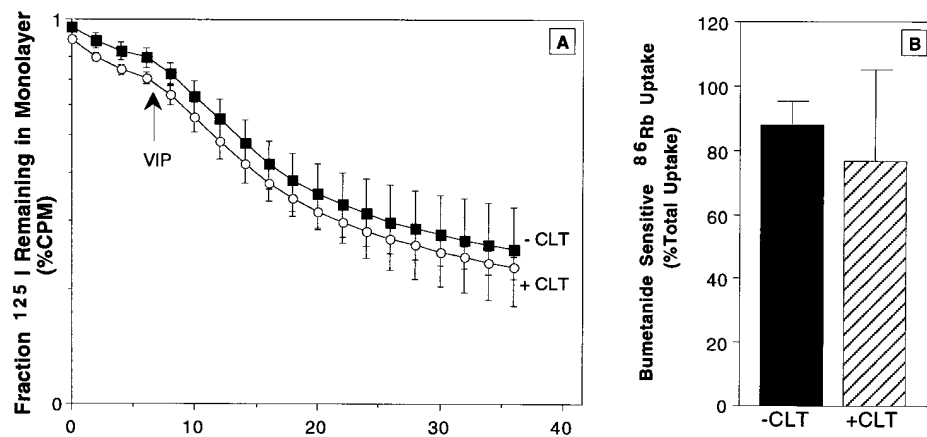


Figure 5. Effect of CLT on apical ^{125}I efflux and basolateral ^{86}Rb uptake by T84 monolayers. (A) T84 monolayers in the absence (closed squares) or presence (open circles) of $33\ \mu\text{M}$ CLT were treated with VIP (arrow). CLT had no effect on basal or VIP stimulated ^{125}I efflux ($n = 4$ individual monolayers). Data are representative of two similar experiments each performed with two independent monolayers in each group. (B) Bumetanide ($10\ \mu\text{M}$) sensitive ^{86}Rb uptake in T84 monolayers pretreated in the absence (solid bar) or presence (hatched bar) of $33\ \mu\text{M}$ CLT, ($n = 6$).

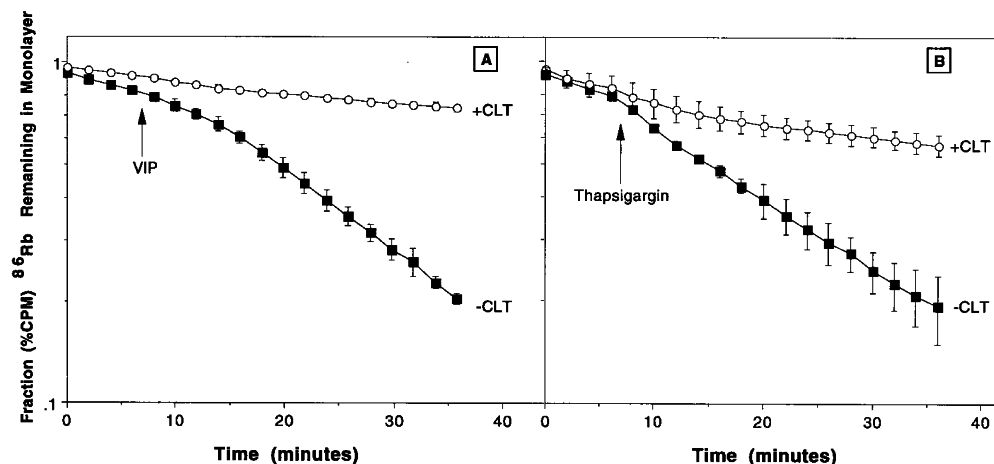


Figure 6. Effect of CLT on basolateral ^{86}Rb efflux. Time course of basolateral ^{86}Rb efflux from T84 monolayers pretreated for 30 min at 37°C in the absence (closed squares) or presence (open circles) of $33\ \mu\text{M}$ CLT. Data represent three similar experiments each performed with two independent monolayers in each group.

ated by measurement of ^{86}Rb efflux (35). Fig. 6 shows that CLT significantly inhibited the rate of ^{86}Rb efflux after treatment with the cAMP agonist VIP ($5\ \mu\text{M}$, Fig. 6 A). The rate constant for VIP-stimulated ^{86}Rb efflux was reduced by 87% in monolayers treated with CLT (0.0062 vs 0.0465% uptake/minute, $n = 3$ independent experiments with 2 independent monolayers in each experiment). CLT inhibited to a similar degree ^{86}Rb efflux from monolayers stimulated with thapsigargin (Fig. 6 B, rate constants 0.011 vs 0.048% uptake/minute, $n = 3$ independent experiments with two independent monolayers in each experiment). These data strongly suggest that CLT can inhibit Cl^- secretion by blockade of K^+ transport through both Ba^{2+} -sensitive and charybdotoxin-sensitive channels.

In further support of this hypothesis, we found that the inhibitory effect of CLT was more rapid in onset when CLT was applied to basolateral than to apical cell surfaces. Fig. 7 shows tests of sidedness of inhibition of VIP-stimulated Cl^- secretion by CLT. As seen in Fig. 7, A and B, most rapid inhibition was achieved by incubation with CLT on both sides of the mono-

layer. However, basolateral application alone was almost as effective. Fig. 7 C shows that the apparent potency of inhibition of CLT at a fixed time point was greater when applied basolaterally than apically. This preferential action of CLT at the basolateral surface of the cell is consistent with the hypothesis that its principal targets are basolateral K^+ channels. It is likely that the attenuated rate of inhibition by apically applied clotrimazole represents diffusion through the monolayer due to the lipid solubility of the compound.

Clotrimazole elicits intracellular Ca^{2+} transients in T84 cells but does not elicit a Cl^- secretory response. Recently, CLT was found to have direct effects on Ca^{2+} signaling in Swiss 3T3 fibroblasts (46). Treatment with CLT ($10\ \mu\text{M}$), elicited a rapid increase in intracellular Ca^{2+} via release from intracellular stores, but the normally ensuing influx of extracellular Ca^{2+} was inhibited. This resulted in the depletion of intracellular Ca^{2+} stores as evidenced by a reduction of cytoplasmic Ca^{2+} activity below baseline, and the attenuation of the $[\text{Ca}^{2+}]_i$ response to subsequent addition of thapsigargin (46). These

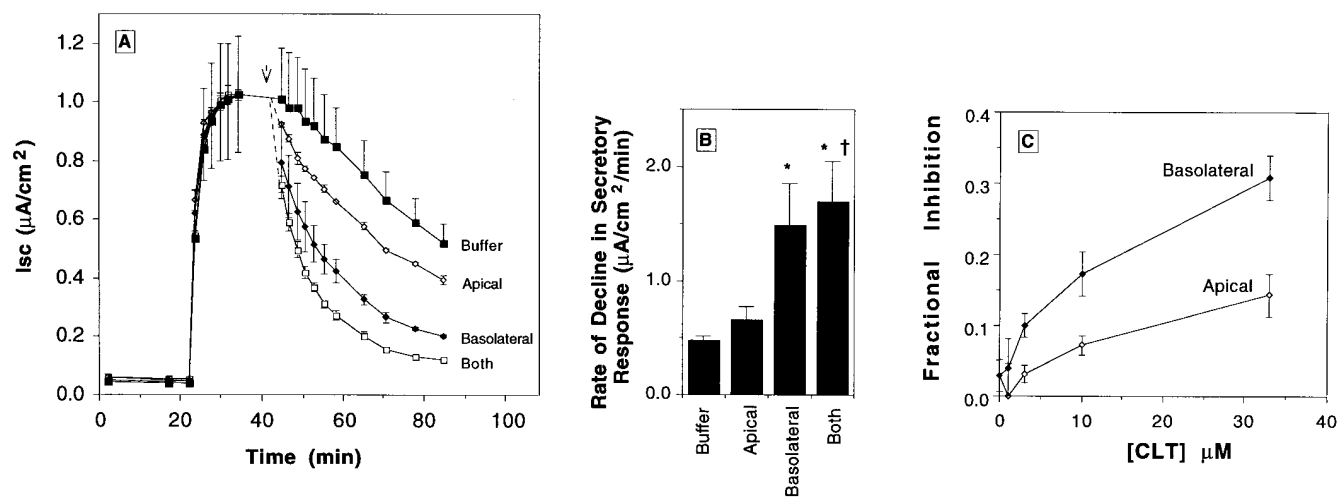


Figure 7. Polarity of inhibition by CLT. (A) T84 monolayers were stimulated with VIP ($5\ \text{nM}$) and subsequently treated (arrow) with buffer (closed squares) or CLT ($33\ \mu\text{M}$) added either to apical (open diamonds), basolateral (closed diamonds), or both cell surfaces (open squares). (A) is representative of five similar experiments using $33\ \mu\text{M}$ CLT. (B) Initial rate of CLT induced secretory inhibition when CLT ($10\ \mu\text{M}$) was applied to apical, basolateral, or both cell surfaces (mean \pm SD, $n = 3$ independent experiments performed with 2 or 3 independent monolayers in each group). * Statistically different from buffer control (ANOVA, $P < 0.05$). † Statistical different from apical treatment with CLT and buffer control (ANOVA, $P < 0.05$). (C) Dose dependence of CLT action added to apical or to basolateral cell surfaces, measured immediately after addition of CLT ($n = 4$).

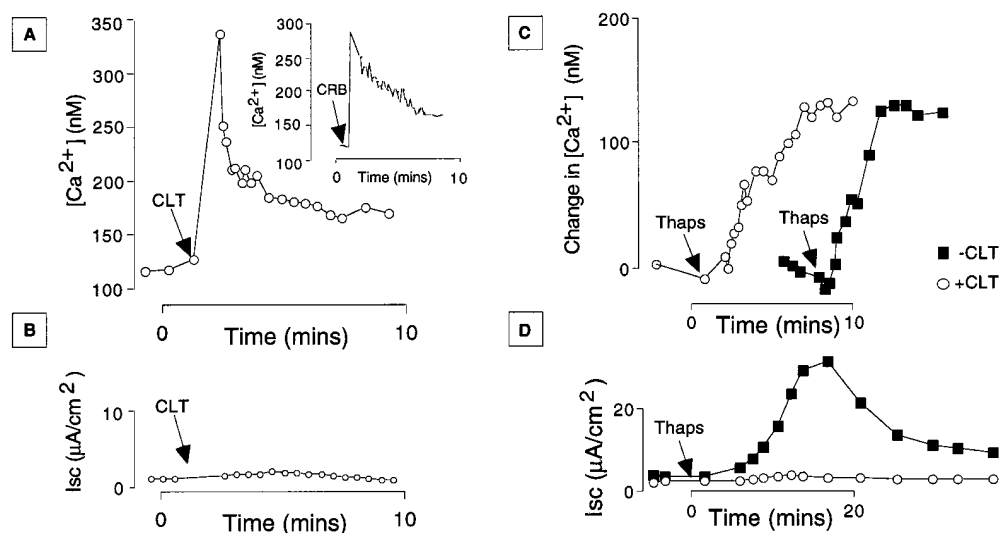


Figure 8. Effect of CLT on intracellular Ca^{2+} signaling in representative individual T84 cell clusters. (A) Time course of $[\text{Ca}^{2+}]_i$ transient elicited by CLT ($10 \mu\text{M}$) in T84 cells grown on glass coverslips and loaded with Fura-2/AM ($n = 32$). Inset shows the time course of Ca^{2+} transients elicited by carbachol ($100 \mu\text{M}$, $n = 13$). (B) Time course of Cl^- secretion (I_{sc}) elicited by CLT in T84 monolayers grown on permeable supports and studied in parallel ($n = 5$). (C) Time course of $[\text{Ca}^{2+}]_i$ transients elicited by thapsigargin in T84 cells grown on glass and pretreated (open circles) or not treated (filled squares) with

CLT for 30 min ($n = 6$). Tracings were offset for visual clarity and denote the change in intracellular $[\text{Ca}^{2+}]_i$ elicited after treatment with thapsigargin. (D) Time course of Cl^- secretion (I_{sc}) elicited by thapsigargin in T84 monolayers grown on permeable supports and pretreated (open circles) or not treated (filled squares) with CLT for 30 min.

studies prompted us to examine the effect of CLT on Ca^{2+} signaling in T84 cells.

Fig. 8 shows that CLT ($10 \mu\text{M}$) elicited a brisk Ca^{2+} transient in T84 cells (Fig. 8, open circles), increasing intracellular Ca^{2+} more than threefold above resting values (125 ± 6 vs peak responses of 353 ± 29 nM, mean \pm SEM, $n = 18$ cell clusters). The initial Ca^{2+} transient elicited by CLT was followed by a significant and prolonged elevation in intracellular Ca^{2+} (168 nM ± 8 , mean \pm SEM, $n = 15$ cell clusters). The elevation in levels of intracellular Ca^{2+} elicited by CLT was similar in magnitude and kinetics to that elicited by the muscarinic agonist carbachol (Fig. 8 insert). CLT did not deplete intracellular Ca^{2+} stores in T84 cells, in so far as subsequent stimulation with thapsigargin (Fig. 8 C) or carbachol (data not shown) produced a Ca^{2+} transient that was only marginally decreased in amplitude from that observed in cells not treated with CLT (CLT pretreated = 96 ± 17 vs thapsigargin = 150 ± 29 nM $[\text{Ca}^{2+}]_i$, mean \pm SEM, $n = 13$ and 11 cell clusters. This small difference approached but did not reach statistical significance, $P = 0.06$, ANOVA). Thus, CLT did not inhibit Cl^- secretion elicited by carbachol or thapsigargin via inhibition of the intracellular Ca^{2+} signal generated by either agonist. These results contrast with the ability of CLT to deplete nearly completely the intracellular Ca^{2+} stores of Swiss 3T3 fibroblasts (46).

Although CLT ($10 \mu\text{M}$) elicited an intracellular Ca^{2+} transient in T84 cells grown on glass (Fig. 8 A), CLT did not elicit a corresponding Cl^- secretory response from T84 monolayers grown on permeable supports and studied in parallel (Fig. 8 B, open circles). CLT clearly retained its bioactivity in this assay, as demonstrated by its inhibition of the Cl^- secretory response elicited by thapsigargin (Fig. 8 D).

Discussion

The results of this study show that the imidazole antibiotic CLT inhibits the Cl^- secretory response of human intestinal T84 cells to both cAMP- and Ca^{2+} -dependent agonists. Inhibition occurs at micromolar concentrations, and is fully revers-

ible. CLT acts at distal steps in cAMP- and Ca^{2+} -dependent signaling cascades, likely by direct inhibition of basolateral K^+ channels. Neither apical Cl^- conductance nor basolateral NaK2Cl cotransport appear to be affected.

Two lines of evidence support the view that CLT directly blocks basolateral K^+ channels. First, two pharmacologically distinct K^+ channels have been identified in intestinal T84 cells (6–9, 15, 16, 47), and CLT appears to inhibit both as evidenced by a nearly fivefold reduction of ^{86}Rb efflux elicited by VIP or by carbachol. The observation that CLT acts at steps distal to the generation of intracellular Ca^{2+} or cAMP, and inhibits the secretory response more efficiently when applied to basolateral cell surfaces is consistent with this proposed mechanism of CLT action. Recently, the 4-sulfonylaminochromanol, 293B, was found to attenuate Cl^- secretory responses in isolated rabbit colon by inhibition of basolateral K^+ channels (48). In contrast to CLT, however, the activity of this compound was restricted to inhibition of cAMP-induced secretion. Secretory responses to Ca^{2+} -agonists were unaffected.

Secondly, CLT competitively displaces ^{125}I -bound charybdotoxin from red cells with ID_{50} equivalent to that for blockade of A23187 activated ^{86}Rb efflux. These data suggest that CLT inhibits K^+ conductance in erythrocytes by binding to the external surface of the Gardos channel (26). Though inhibition by CLT of Cl^- secretion (I_{sc}) in T84 cells is almost 100-fold less potent than inhibition of the Gardos channel, the Ca^{2+} -dependent K^+ channel of the red cell and T84 cells share some biophysical and pharmacological characteristics, including inward rectification, unitary conductance (21, 49), inhibition by charybdotoxin, and insensitivity to Ba^{2+} . CLT has also been shown to inhibit Ca^{2+} -sensitive K^+ conductances in rat thymocytes and Ehrlich ascites tumor cells (27). Based on these data and that from the present study, we propose that CLT may act directly on both charybdotoxin- and Ba^{2+} -sensitive K^+ channels of the human intestinal T84 cell.

The absence of inhibition by CLT of ^{125}I efflux from confluent T84 cell monolayers was not expected in view of the predicted depolarization produced by K^+ channel blockade. How-

ever, Venglarik et al. (35) similarly found that the two channel K^+ blockers, Ba^{2+} and charybdotoxin, though effective inhibitors of $I_{sc(Cl^-)}$ and of ^{86}Rb efflux, produced no inhibition of ^{125}I efflux. The reasons for this discrepancy between the effects of K^+ channel blockade on anion secretion measured by short-circuit current and that measured as isotopic efflux remain unclear. However, preliminary experiments confirm in T84 monolayers with selectively permeabilized apical or basolateral membranes that CLT blocks basolateral K^+ conductance and fails to block apical Cl^- conductance (Rufo, P.A. et al., unpublished results).

As in Swiss 3T3 fibroblasts (46), we also find that CLT has direct effect(s) on Ca^{2+} signaling in human intestinal T84 cells. In contrast to 3T3 fibroblasts however, CLT did not appear to deplete intracellular Ca^{2+} stores by inhibition of capacitative plasma membrane Ca^{2+} influx. The prolonged increase in $[Ca^{2+}]_i$ above baseline, and the normal Ca^{2+} response to subsequent treatment with thapsigargin or carbachol indicate that CLT has little or no effect on Ca^{2+} release-activated Ca^{2+} conductances of T84 cells, or that "capacitative Ca^{2+} entry" may be mediated by other pathways in this cell type.

Ca^{2+} transients elicited by CLT did not initiate Cl^- secretion in T84 cells, even though the intracellular Ca^{2+} transients were comparable in magnitude and duration to those elicited by carbachol. The simplest explanation for this observation is that CLT concomitantly inhibits K^+ efflux and thus the Cl^- secretory response. The ability of CLT, an inhibitor of secretagogue-stimulated chloride secretion, to increase intracellular Ca^{2+} directly is reminiscent of the similar property of another chemically unrelated inhibitor of chloride secretion, the chloride channel blocker, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) (50). It is likely that both drugs use distinct mechanisms for elevation of intracellular Ca^{2+} concentration and for blockade of transepithelial chloride secretion.

These data allow for the possibility that CLT may uncouple the Ca^{2+} transient from activation of Cl^- secretion by inhibiting generation or action of a distally acting diffusible intermediate(s) in the Ca^{2+} signaling cascade, as previously hypothesized (41, 51). It is also possible that CLT may promote the generation of other intermediates, such as $Ins(3,4,5,6)P_4$ tetrakisphosphate, which might dissociate intracellular Ca^{2+} transients from Cl^- secretion elicited by carbachol in T84 cells (52). However, these hypotheses do not suffice to explain the blockade of VIP-activated Cl^- secretion.

Among the imidazole antibiotics tested, CLT displayed the greatest potency for inhibition of both the cAMP- and Ca^{2+} -dependent secretory responses. Other imidazole orazole antifungal drugs had little or no inhibitory effect on the response to Ca^{2+} -agonists, indicating that these compounds may distinguish between binding sites on Ba^{2+} -sensitive and charybdotoxin-sensitive channels. For inhibition of Ba^{2+} -sensitive cAMP-dependent secretory responses in T84 cells, we found the rank order of potency: clotrimazole > ketoconazole > econazole > miconazole. In contrast, the rank order potency for inhibition of the Ca^{2+} -sensitive K^+ channel in T84 cells was: clotrimazole > miconazole > econazole > ketoconazole. The nitroimidazole derivative metronidazole had no effect, though metronidazole blocked the Ca^{2+} -sensitive K^+ channel of sickle erythrocytes (26). The differences in rank order of potencies noted for cAMP- and Ca^{2+} -dependent K^+ channels in T84 cells underscore likely differences in primary structure.

In some experiments CLT at low doses ($< 10^{-7}$ M), enhanced slightly the secretory response to both cAMP and Ca^{2+} agonists. Vajanaphanich et al. previously reported stimulation of Cl^- secretion by T84 cells, but at doses up to 1,000-fold higher than the stimulatory doses observed in the present work (53). In contrast to the experiments presented here, these authors observed no inhibition of $I_{sc} Cl^-$ in T84 cells. The variably observed increase in Cl^- secretory responses at very low concentrations of CLT might be explained by the observation that CLT itself elicits an intracellular Ca^{2+} transient. Recent results with other compounds related in structure to CLT have shown inhibition of Cl^- secretion without any observed stimulation at low concentrations (Rufo, P.A. et al., unpublished data).

Clotrimazole is in current therapeutic use as a topical antifungal and is used in oral and vaginal preparations for mucosal candidiasis. Clotrimazole has been tested in humans as a systemic treatment of several conditions including rheumatoid arthritis (54, 55), severe esophageal (56), and systemic chronic mucocutaneous candidiasis (57, 58), and sickle cell disease (24, 26, 60). In the most recent study, oral doses of 20 mg CLT/kg per day over 4–12-wk courses yielded plasma concentrations of 2.8–12.5 μM (CLT and active metabolites) (60) with mild or no toxicity (55, 60). These serum levels approximate or exceed the IC_{50} 's for inhibition of secretagogue-activated Cl^- secretion in T84 cells.

A stable metabolite of CLT lacking the imidazole ring (2-chlorophenyl-*bis*-phenyl methanol) was also found to be effective at inhibiting Cl^- secretory responses in T84 cells with nearly equal potency. These data show that the imidazole ring is not required for inhibitory activity in T84 cells. Adverse effects associated with clotrimazole are dose dependent. Reversible increases in hepatic transaminases and adrenal steroid levels (61) have been reported in humans and animals. These effects are thought to result from interactions between the imidazole ring and cytochrome P-450 enzymes (24, 62, 63). Thus, metabolites of CLT or related compounds having the triphenyl structure but lacking the imidazole ring may display similar or greater efficacy in vivo with less toxicity. Moreover, the ability of compounds like 2-chlorophenyl-*bis*-phenyl methanol to inhibit Cl^- secretion in T84 cells shows that inhibition of P450 enzymes is not required for the inhibition of K^+ channels by CLT.

In summary, these studies show that in the human intestinal cell line T84, the imidazole antibiotic clotrimazole attenuates the Cl^- secretory response elicited by either cAMP- or Ca^{2+} -dependent agonists. Cl^- secretion elicited by *E. coli* heat-labile toxin (assessed using cholera toxin) or heat-stable toxin (STa) was completely inhibited by CLT. These bacterial enterotoxins are primarily responsible for the acute secretory diarrhea encountered by 30–70% of travelers to nonindustrialized regions of the world (64). *E. coli* enterotoxins also cause neonatal diarrhea (scours) of newborn farm animals. CLT appears to dissociate intracellular cAMP and Ca^{2+} signals from Cl^- secretion by inhibiting basolateral membrane K^+ channels. Previous studies with oral clotrimazole have already shown in humans that plasma levels of 3.3 μM or greater are achievable after a single oral dose of 1 gram (24, 60). Such levels would be sufficient for inhibition of the intestinal secretory response as assessed in our in vitro system. Moreover, CLT is metabolized to active compounds of (likely) even lower toxicity. Thus we propose that oral clotrimazole and drugs of related structure may have utility in the treatment of acute and chronic secretory diarrheas of diverse etiologies.

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