

# Crofelemer, an Antisecretory Antidiarrheal Proanthocyanidin Oligomer Extracted from *Croton lechleri*, Targets Two Distinct Intestinal Chloride Channels

Lukmanee Tradtrantip, Wan Namkung, and A. S. Verkman

Departments of Medicine and Physiology, University of California, San Francisco, California

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## ABSTRACT

Crofelemer, a purified proanthocyanidin oligomer extracted from the bark latex of *Croton lechleri*, is in clinical trials for secretory diarrheas of various etiologies. We investigated the antisecretory mechanism of crofelemer by determining its effect on the major apical membrane transport and signaling processes involved in intestinal fluid transport. Using cell lines and measurement procedures to isolate the effects on individual membrane transport proteins, crofelemer at 50  $\mu\text{M}$  had little or no effect on the activity of epithelial  $\text{Na}^+$  or  $\text{K}^+$  channels or on cAMP or calcium signaling. Crofelemer inhibited the cystic fibrosis transmembrane regulator (CFTR)  $\text{Cl}^-$  channel with maximum inhibition of  $\sim 60\%$  and an  $\text{IC}_{50} \sim 7 \mu\text{M}$ . Crofelemer

action at an extracellular site on CFTR produced voltage-independent block with stabilization of the channel closed state. Crofelemer did not affect the potency of glycine hydrazide or thiazolidinone CFTR inhibitors. Crofelemer action resisted washout, with  $< 50\%$  reversal of CFTR inhibition after 4 h. Crofelemer was also found to strongly inhibit the intestinal calcium-activated  $\text{Cl}^-$  channel TMEM16A by a voltage-independent inhibition mechanism with maximum inhibition  $> 90\%$  and  $\text{IC}_{50} \sim 6.5 \mu\text{M}$ . The dual inhibitory action of crofelemer on two structurally unrelated prosecretory intestinal  $\text{Cl}^-$  channels may account for its intestinal antisecretory activity.

Secretory diarrhea remains a global health challenge in developing and developed countries. Intestinal fluid secretion involves  $\text{Cl}^-$  influx into enterocytes through an  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symporter on the basolateral membrane and  $\text{Cl}^-$  efflux through apical (lumen-facing)  $\text{Cl}^-$  channels (Barrett and Keely, 2000; Field, 2003; Thiagarajah and Verkman, 2005) (Fig. 1).  $\text{K}^+$  channels and a  $3\text{Na}^+/2\text{K}^+$  pump establish the electrochemical driving force for  $\text{Cl}^-$  secretion.  $\text{Na}^+$  and water secretion follow passively in response to active  $\text{Cl}^-$  secretion. Bacterial enterotoxins produced by *Vibrio cholerae* and *Escherichia coli* elevate cyclic nucleotide concentrations in

enterocytes, resulting in  $\text{Cl}^-$  channel activation and fluid secretion.  $\text{Na}^+$  absorption through apical membrane  $\text{Na}^+$  channels and electrogenic  $\text{Na}^+$ -coupled symporters oppose net fluid secretion. The rate of net intestinal fluid secretion, and hence the severity of secretory diarrhea, is expected to be sensitive to modulators of these transporting systems and to upstream cyclic nucleotide or calcium signaling pathways.

Treatment of secretory diarrheas in developing countries is primarily supportive, involving replacement of intestinal fluid losses using oral rehydration salt solution. Although oral rehydration salt has greatly improved clinical outcome in cholera and other diarrheas, there remains significant mortality from infectious diarrheas, with recurrent major outbreaks. Potential targets for diarrhea therapy to further reduce morbidity and mortality include the bacteria itself (vaccines, antimicrobials), elaborated enterotoxins and their cellular uptake, cellular cyclic nucleotide signaling, and the prosecretory membrane transporters mentioned above (Field, 2003; Thiagarajah and Verkman, 2005). Our laboratory has focused on targeted inhibitors of the two principal apical membrane  $\text{Cl}^-$  chan-

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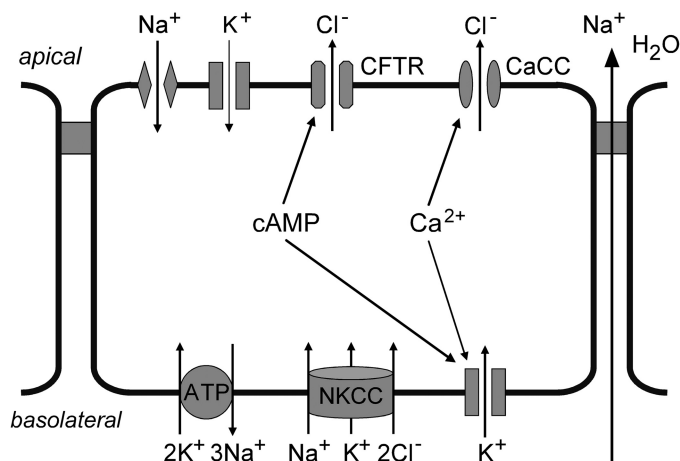
**ABBREVIATIONS:** CFTR, cystic fibrosis transmembrane conductance regulator; CaCC, calcium-activated chloride channel; CFTR<sub>inh</sub>-172, thiazolidinone cystic fibrosis transmembrane conductance regulator inhibitor; CPT-cAMP, chlorophenylthio-cAMP; FRT, Fisher rat thyroid; GlyH-101, glycine hydrazide cystic fibrosis transmembrane conductance regulator inhibitor; IBMX, 3-isobutyl-1-methylxanthine; NMDG-Cl, N-methyl-D-glucamine chloride.

nels in enterocytes: the cystic fibrosis transmembrane regulator conductance (CFTR), a cAMP-stimulated  $\text{Cl}^-$  channel; and calcium-activated  $\text{Cl}^-$  channels (CaCCs). By high-throughput screening and follow-up chemistry, we identified inhibitors of these  $\text{Cl}^-$  channels, including nanomolar-potency thiazolidinone (Ma et al., 2002), glycine hydrazide (Muanprasat et al., 2004) and pyrimido-pyrrolo-quinoxalinedione (Tradtrantip et al., 2009a) CFTR inhibitors, and 3-acyl-2-aminothiophene CaCC inhibitors (De La Fuente et al., 2008). We have also identified thiophene carboxylate activators of phosphodiesterases that reduce cyclic nucleotide concentrations and toxin-induced intestinal fluid secretion (Tradtrantip et al., 2009b).

Here, we investigate the antisecretory mechanism of crofelemer, a purified proanthocyanidin oligomer extracted from the blood-red bark latex of the South American medicinal plant *Croton lechleri* (dragon's blood). The sap of *C. lechleri* has been used in South American countries like Ecuador and Peru for many years to treat diarrheas, including dysentery and cholera, and various lung, stomach, and other conditions (Ubillas et al., 1994; Jones, 2003; Risco et al., 2003; Rossi et al., 2003). Additional pharmacological studies have shown that crofelemer reduced fluid secretion in cell culture and mouse models (Gabriel et al., 1999). Crofelemer is currently in clinical trials for the treatment of secretory diarrheas associated with acute infections including cholera, chronic diarrhea associated with HIV/AIDS, and diarrhea-predominant irritable bowel syndrome (Holodniy et al., 1999; DiCesare et al., 2002; Mangel and Chaturvedi, 2008). Here, we report that the antisecretory mechanism-of-action of crofelemer involves inhibition of both CFTR and CaCC  $\text{Cl}^-$  channels at the luminal membrane of enterocytes.

## Materials and Methods

**Chemicals.** Forskolin, apigenin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). 8-(4-Chlorophenylthio)-cAMP (CPT-cAMP) was purchased from Calbiochem (San Diego, CA). The small-molecule CFTR inhibitors CFTR<sub>inh</sub>-172 and GlyH-101, and the CaCC inhibitor CaCC<sub>inh</sub>-01, were synthesized as reported previously (Ma et al., 2002; Muanprasat et al., 2004; De La Fuente et al., 2008). Crofelemer was provided



**Fig. 1.** Cellular mechanisms of intestinal fluid secretion by enterocytes, showing chloride secretion through apical membrane chloride channels. See the Introduction for a further explanation.

by Napo Pharmaceuticals Inc. (South San Francisco, CA). Crofelemer was prepared by extraction from the bark latex of *C. lechleri*. After chilling the bark latex to induce a phase separation, the solid residues were discarded, and the supernatant was extracted with butanol. The crofelemer-containing aqueous phase was filtered by tangential flow and subjected to low-pressure liquid chromatography on an ion-exchange column. The crofelemer-enriched fraction was purified on a Sephadex column, and crofelemer was eluted using a mobile phase of aqueous acetone. Crofelemer was then dried under vacuum. Crofelemer is a proanthocyanidin oligomer with an average molecular mass of 2100 Da, in agreement with a previously reported average molecular mass of 2300 Da (Ubillas et al., 1994). Figure 2A shows the putative structure of crofelemer. The material used for the studies here is the same as that used in clinical trials, in which it is formulated for oral dosing as modified-release tablets (125 or 250 mg of crofelemer per tablet).

**Cell Culture.** Fisher rat thyroid (FRT) cells expressing human CFTR were generated as described previously (Ma et al., 2002). FRT cells expressing human TMEM16A (cDNA provided by Dr. Luis Galletta, Gaslini Institute, Genoa, Italy) were generated similarly. FRT cells were cultured in F-12 Modified Coon's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 350  $\mu\text{g}/\text{ml}$  hygromycin, and 500  $\mu\text{g}/\text{ml}$  G418 (Geneticin). Primary cultures of human bronchial epithelial cells were maintained at an air-liquid interface as described previously (Yamaya et al., 1992). T84 cells were cultured in DMEM/Ham's F-12 (1:1) medium containing 10% FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were grown on Snapwell porous filters (Corning Life Sciences, Lowell, MA) at 37°C in 5%  $\text{CO}_2/95\%$  air.

**Short-Circuit Current Measurements.** FRT cells (stably expressing CFTR or TMEM16A) were cultured on Snapwell filters until confluence (transepithelial resistance  $>500 \Omega \cdot \text{cm}$ ). Short-circuit current was measured in Ussing chambers (Vertical diffusion chamber; Corning Life Sciences) with Ringer's solution bathing the basolateral surface and half-Ringer's bathing the apical surface. Ringer's solution contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 10 mM sodium-HEPES, and 10 mM glucose, pH 7.3. Half-Ringer's solution was the same, except that 65 mM NaCl was replaced with sodium gluconate, and  $\text{CaCl}_2$  was increased to 2 mM. The basolateral membrane was permeabilized with 250  $\mu\text{g}/\text{ml}$  amphotericin B, as described previously (Ma et al., 2002). Chambers were bubbled continuously with air. For T84 cells and bronchial epithelial cells, cells were bathed in symmetrical  $\text{HCO}_3^-$ -buffered solution containing 120 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 10 mM D-glucose, 5 mM HEPES, and 25 mM  $\text{NaHCO}_3$ , pH 7.4, and aerated with 5%  $\text{CO}_2$  at 37°C. For the measurement of apical  $\text{K}^+$  conductance in T84 cells,  $\text{NaHCO}_3$  and NaCl were replaced with sodium gluconate, and sodium gluconate in basolateral solution was replaced with potassium gluconate and bubbled with air. The basolateral membrane was permeabilized with 20  $\mu\text{M}$  amphotericin B. Short-circuit current was measured using a DVC-1000 voltage-clamp apparatus (World Precision Instruments, Inc., Sarasota, FL).

**Cyclic Nucleotide Assays.** T84 cells were grown in 24-well plates, treated for 45 min with crofelemer and then for 10 min with 0 or 20  $\mu\text{M}$  forskolin, lysed by sonication, and centrifuged to remove cell debris, and the supernatant was assayed for cAMP according to manufacturer's instructions (Parameter cAMP immunoassay kit; R&D Systems, Minneapolis, MN).

**Patch-Clamp Analysis of CFTR and TMEM16A  $\text{Cl}^-$  Channel Function.** Whole-cell recordings were made on FRT cells stably expressing CFTR or TMEM16A. The pipette solution for CFTR contained 140 mM *N*-methyl-D-glucamine chloride (NMDG-Cl), 5 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM Tris-ATP, and 10 mM HEPES, pH 7.2. The pipette solution for TMEM16A contained 130 mM CsCl, 0.5 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM Tris-ATP, and 10 mM HEPES, pH 7.2. The bath solution contained 140 mM NMDG-Cl, 1 mM  $\text{CaCl}_2$ , 1 mM

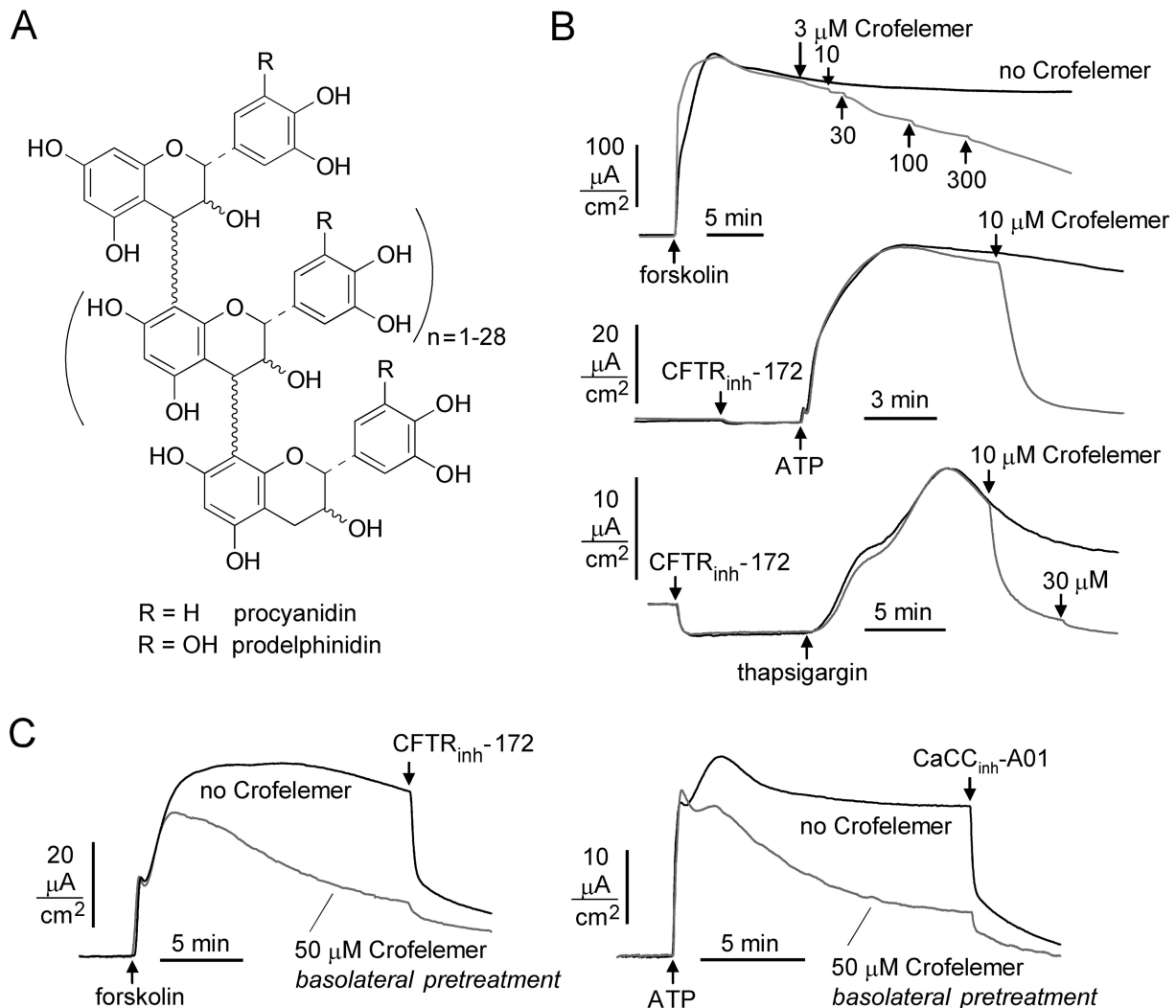
MgCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES, pH 7.4. All measurements were done at room temperature (22–25°C). Pipettes were pulled from borosilicate glass and had resistances of 3 to 5 MΩ after fire-polishing. Seal resistances were between 3 and 10 GΩ. After establishing the whole-cell configuration, CFTR was activated by forskolin and IBMX, and TMEM16A was activated by ATP. Whole-cell currents were elicited by applying hyperpolarizing and depolarizing voltage pulses from a holding potential of 0 mV to potentials between -100 and +100 mV in steps of 20 mV. The current output was filtered at 5 kHz. Currents were digitized and analyzed using an AxoScope 10.0 system and a Digidata 1440A AC/DC converter (Molecular Devices, Sunnyvale, CA).

Single-channel analysis of CFTR was done in the cell-attached configuration using fire-polished pipettes with a resistance of 6 to 10 MΩ. The pipette solution contained 140 mM NMDG-Cl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, and 10 mM HEPES, pH 7.4, and the KCl bath solution contained 140 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, and 10 mM HEPES, pH 7.4. Recordings were performed at room temperature using an Axopatch-200B (Molecular Devices). Voltage and current data were low-pass-filtered at 1 kHz and stored for later analysis. Single-channel data were digitally filtered at 25 Hz and analyzed using Clampfit 10.0 software (Molecular Devices).

**Calcium Signaling Measurements.** Measurements of [Ca<sup>2+</sup>]<sub>i</sub> in confluent monolayers of T84 cells were done by loading cells with fura-2 by 30-min incubation at 37°C with 2 μM fura-2-AM (Invitrogen, Carlsbad, CA). Fura-2-loaded T84 cells were mounted in a perfusion chamber on the stage of an inverted fluorescence microscope. The cells were superfused with 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM D-glucose, and 10 mM HEPES, pH 7.4. Fura-2 fluorescence was recorded at excitation wavelengths of 340 and 380 nm, and the results were expressed as a 340/380 fluorescence ratio. After obtaining baseline measurements, 100 μM ATP was added in the perfusate. Measurements were made in the absence and presence of 50 μM crofelemer.

## Results

**Crofelemer Inhibits Cl<sup>-</sup> Secretion by T84 Human Intestinal Epithelial Cells.** To test whether crofelemer reduces intestinal cell Cl<sup>-</sup> secretion, short-circuit current was measured in T84 cells in symmetrical physiological solutions (without plasma membrane permeabilization). Figure 2B shows crofelemer concentration-dependent inhibition



**Fig. 2.** Crofelemer reduces Cl<sup>-</sup> secretion in T84 human intestinal cells in response to cAMP and calcium-elevating agonists. A, chemical structure of crofelemer (see *Materials and Methods* for explanation) (Ubillas et al., 1994). B, short-circuit current in T84 cells after activation of Cl<sup>-</sup> secretion by forskolin (10 μM), ATP (100 μM), or thapsigargin (1 μM). Indicated concentrations of crofelemer were added to the luminal bathing solution. Where indicated, cells were pretreated with 20 μM CFTR<sub>inh</sub>-172 to inhibit CFTR Cl<sup>-</sup> current. C, short-circuit current measurements showing CFTR (left)- and CaCC (right)-dependent Cl<sup>-</sup> current in the presence of crofelemer (50 μM) added to the basolateral bathing solution 10 min before measurements. CFTR was inhibited by pretreatment with CFTR<sub>inh</sub>-172 (20 μM) for measurement of ATP-induced CaCC activation.

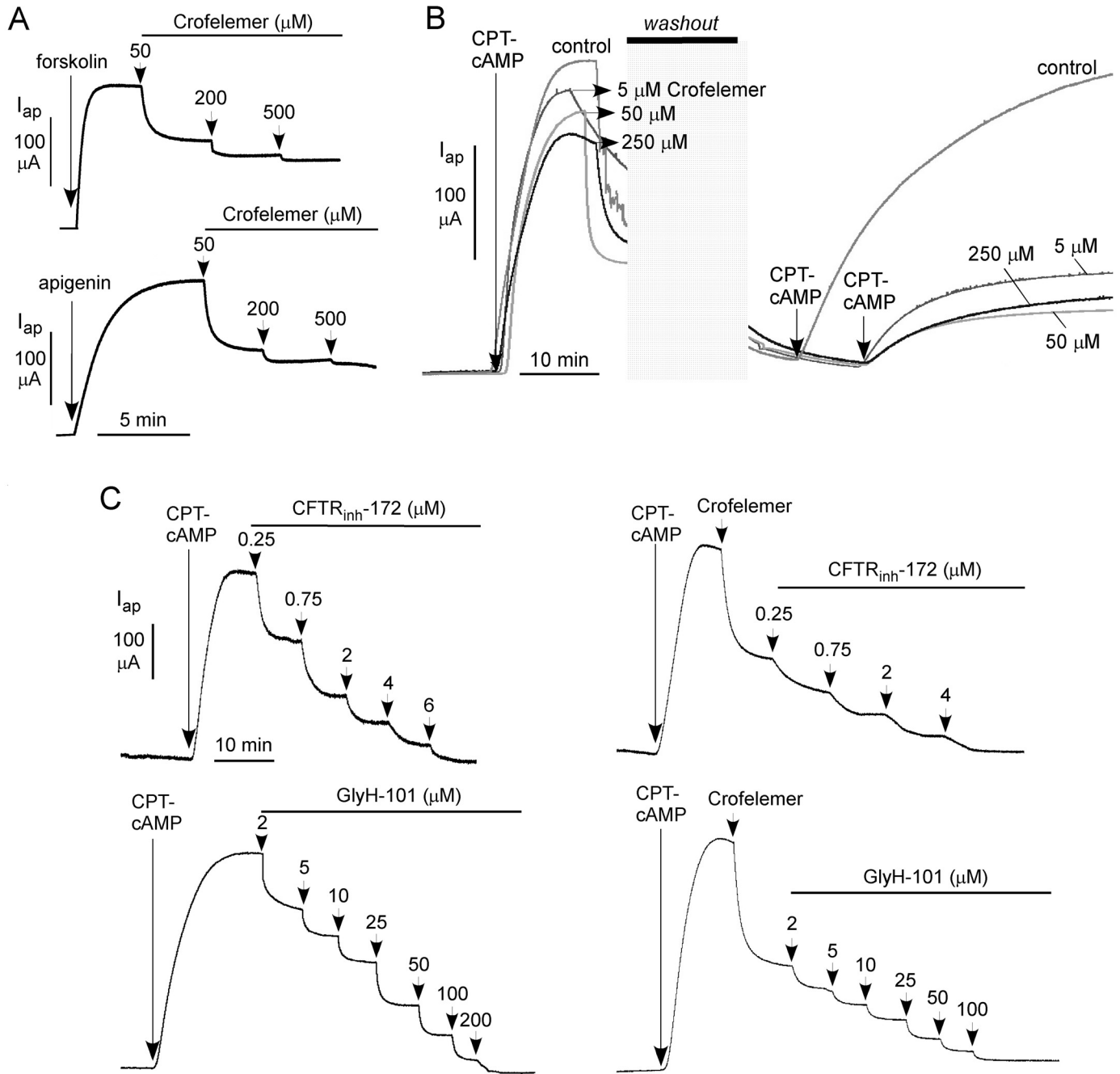




absence of crofelemer, washout (of CPT-cAMP) followed by re-stimulation produced a current similar to that seen in the initial stimulation. However, after inhibition with different concentrations of crofelemer, washout studies showed partial (25–35%) reversal of CFTR inhibition over 30 min. Extended time studies showed <50% reversal of crofelemer inhibition at 4 h (data not shown).

To evaluate the possibility that the site of action of crofelemer on CFTR might overlap with that of the small-molecule thiazolidinone and glycine hydrazide CFTR inhibitors, we compared CFTR inhibition in the absence and presence of preadded crofelemer.

Figure 4C (left) shows concentration-inhibition studies of CFTR inhibition by CFTR<sub>inh</sub>-172 and GlyH-101. Maximal inhibition was ~100%, with IC<sub>50</sub> values of ~1 and ~8 μM, respectively. Figure 4C (right) shows similar concentration-inhibition measurements, in which 50 μM crofelemer was added initially to inhibit CFTR Cl<sup>-</sup> current by ~50%. Despite the partial antagonist mechanism of crofelemer, CFTR<sub>inh</sub>-172 and GlyH-101 were able to inhibit CFTR by nearly 100%. The similar IC<sub>50</sub> values for CFTR<sub>inh</sub>-172 and GlyH-101 in the absence and presence of crofelemer suggests nonoverlapping CFTR inhibition sites for crofelemer and CFTR<sub>inh</sub>-172 or GlyH-101.

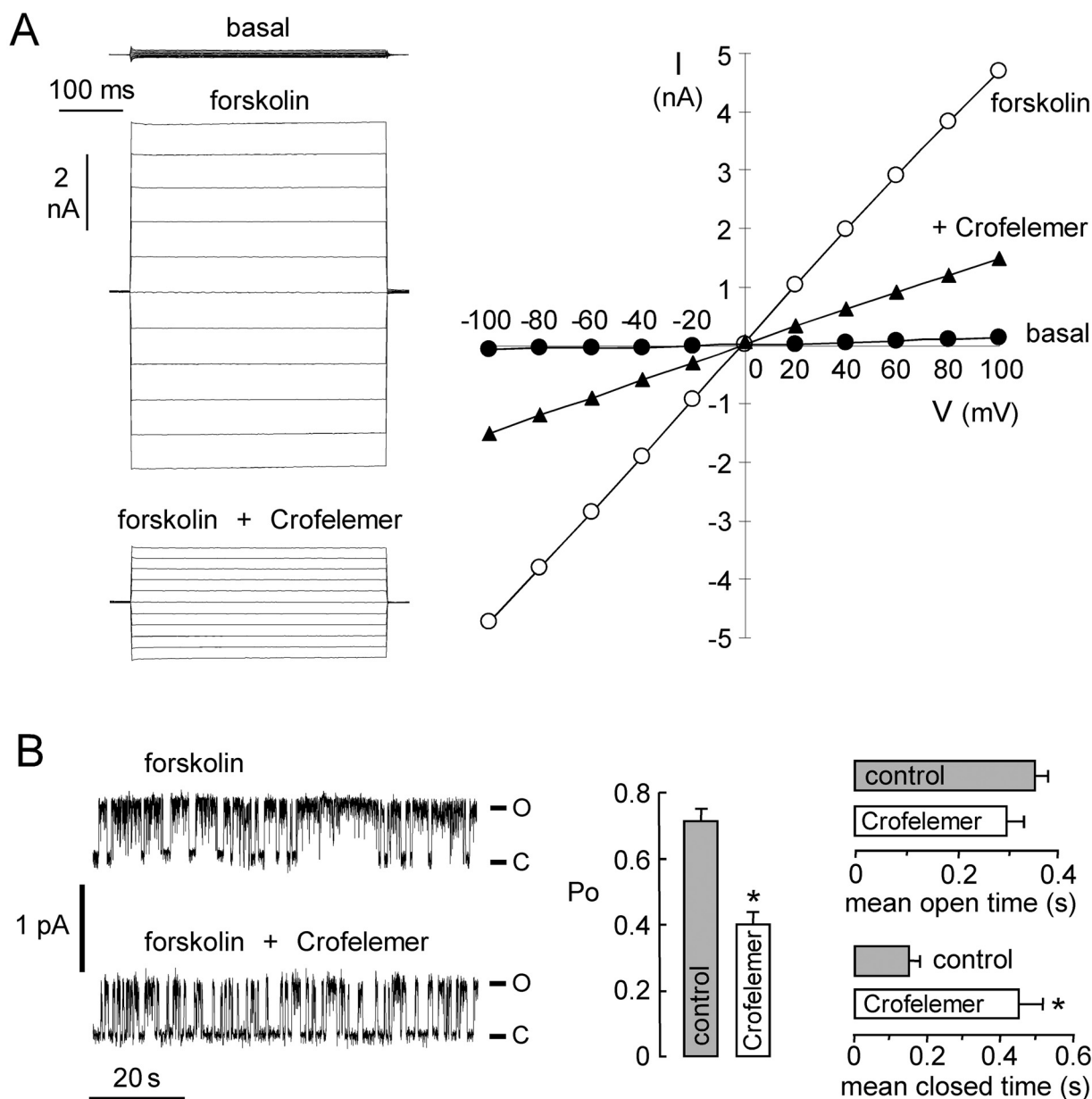


**Fig. 4.** Characterization of crofelemer inhibition of CFTR Cl<sup>-</sup> conductance. A, crofelemer inhibition of CFTR after different agonists including forskolin (20 μM) and apigenin (100 μM). B, slow reversibility of crofelemer inhibition of CFTR. Where indicated, crofelemer was added, the apical solution was washed extensively, and CPT-cAMP was readded. C, investigation of possible synergy/competition of crofelemer with small-molecule CFTR inhibitors. Left, apical membrane current after CFTR activation by CPT-cAMP and inhibition by CFTR<sub>inh</sub>-172 or GlyH-101. Right, crofelemer (50 μM) was added to inhibit CFTR Cl<sup>-</sup> current by ~50 to 60%, followed by indicated concentrations of CFTR<sub>inh</sub>-172 or GlyH-101.

Patch-clamp was done to investigate the molecular mechanism of CFTR inhibition by crofelemer. Whole-cell membrane current was measured in CFTR-expressing FRT cells (Fig. 5A, left). Stimulation by 10  $\mu\text{M}$  forskolin produced a membrane current of  $179 \pm 18$  pA/pF ( $n = 3$ ) at +100 mV (total membrane capacitance,  $15.8 \pm 4$  pF). Crofelemer at 50  $\mu\text{M}$  gave  $\sim 60\%$  inhibition of CFTR  $\text{Cl}^-$  current. Figure 5A (right) shows an approximately linear current-voltage relationship for CFTR, as expected for CFTR. The CFTR current-voltage relationship remained linear after crofelemer addition, indicating a voltage-independent block mechanism, as expected for an uncharged inhibitor.

To assess single-channel CFTR properties, cell-attached patch recordings were done on CFTR-expressing FRT cells in

the absence or presence of crofelemer in the pipette solution (Fig. 5B). The addition of 10  $\mu\text{M}$  forskolin and 100  $\mu\text{M}$  IBMX to the bath resulted in CFTR channel opening. CFTR unitary conductance was  $7.2 \pm 0.1$  pS ( $n = 4$ ), which was not affected by crofelemer. Crofelemer reduced channel activity remarkably, as seen by the less frequent channel openings (Fig. 5B, left). Analysis of single-channel recordings indicated that crofelemer significantly reduced open-channel probability ( $P_o$ ) from  $0.71 \pm 0.04$  ( $n = 4$ ) to  $0.40 \pm 0.04$  ( $n = 3$ ). Mean channel open time was not significantly changed, but mean channel closed time was significantly increased (Fig. 5B, bottom), indicating that crofelemer stabilizes the channel closed state. These results suggest that crofelemer inhibits CFTR by an altered channel-gating mechanism.



**Fig. 5.** Patch-clamp analysis of crofelemer inhibition of CFTR. A, left, whole-cell CFTR current recorded at a holding potential at 0 mV and pulsing to voltages between  $\pm 100$  mV in steps of 20 mV in the absence and presence of 50  $\mu\text{M}$  crofelemer. CFTR was stimulated by forskolin. Right, I-V plot of mean currents at the middle of each voltage pulse from experiments as in A (S.E.,  $n = 3$ ). Fitted  $\text{IC}_{50} = 6.5$   $\mu\text{M}$ . B, left, single-channel recordings were done in the cell-attached configuration. CFTR was activated by 10  $\mu\text{M}$  forskolin and 100  $\mu\text{M}$  IBMX. Pipette potential was +80 mV. Right, summary of crofelemer effect on CFTR channel  $P_o$ , mean open time, and mean closed time (S.E.,  $n = 3-4$ ; \*,  $P < 0.05$ ). o, open channel state; c, closed channel state.

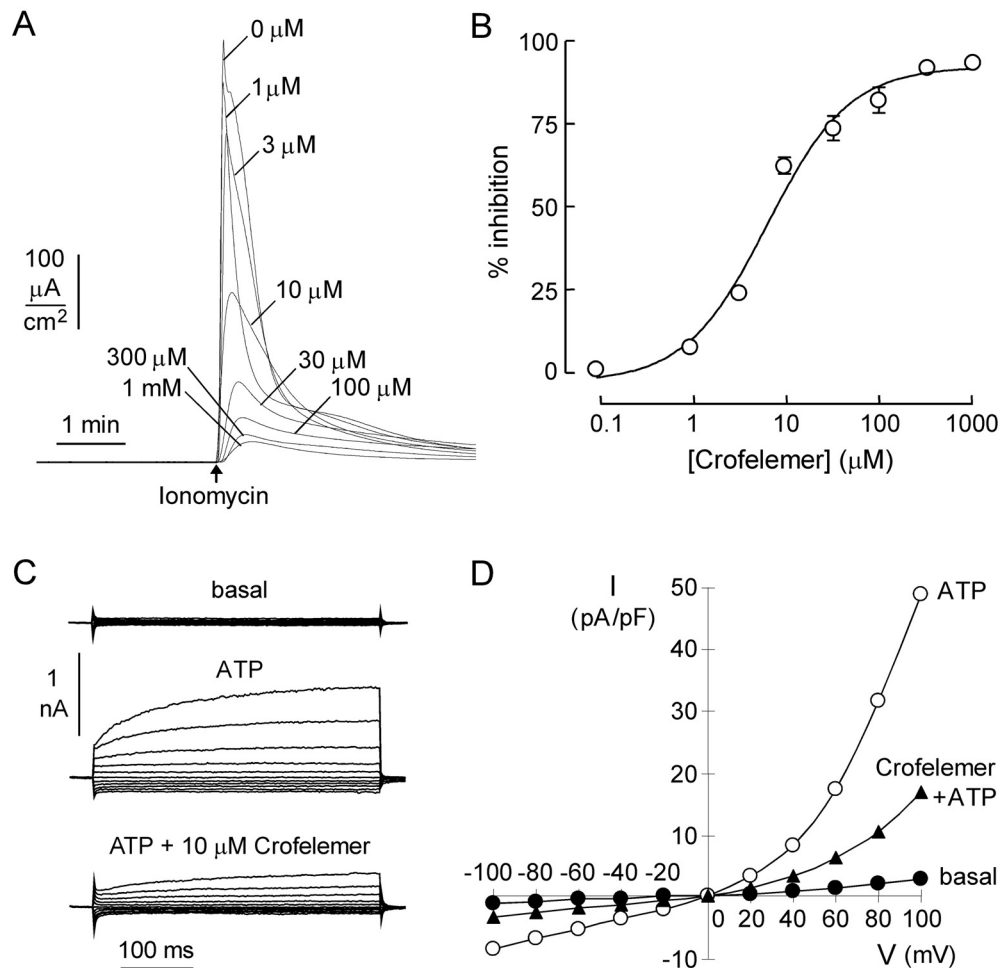
**Crofelemer Is a Strong Inhibitor of the CaCC TMEM16A.** The data in Fig. 2B suggest that crofelemer strongly inhibits CaCC(s) in T84 cells. Recent work has implicated the protein TMEM16A as a CaCC in multiple epithelial cells, including intestinal epithelia, as well as in smooth muscle, nerve, and other cell types (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). To test whether TMEM16A is the CaCC target of crofelemer, FRT epithelial cells stably expressing TMEM16A were pretreated with different concentrations of crofelemer, followed by the addition of 1  $\mu\text{M}$  ionomycin to stimulate TMEM16A  $\text{Cl}^-$  current. Measurements were made in the presence of a transepithelial  $\text{Cl}^-$  gradient, so that current is a direct, quantitative measure of TMEM16A  $\text{Cl}^-$  conductance. Figure 6A shows crofelemer concentration-dependent inhibition of TMEM16A  $\text{Cl}^-$  current, which was nearly complete at high concentrations of crofelemer. Figure 6B shows an  $\text{IC}_{50}$  for crofelemer inhibition of TMEM16A of  $\sim 6.5 \mu\text{M}$ .

Whole-cell membrane current was measured in TMEM16A-expressing FRT cells (Fig. 6C). Stimulation by 100  $\mu\text{M}$  ATP produced a membrane current of  $56 \pm 13 \text{ pA/pF}$  ( $n = 3$ ) at +100 mV. Pretreatment with 10  $\mu\text{M}$  crofelemer inhibited ATP-induced TMEM16A  $\text{Cl}^-$  current by 58% ( $24 \pm 6 \text{ pA/pF}$ ,  $n = 3$ ). Figure 6D shows an outward-rectifying current-voltage relationship for TMEM16A. The TMEM16A current-voltage relationship remained outward-rectifying after crofelemer addition, as expected for an uncharged inhibitor. The

percentage inhibition of TMEM16A by crofelemer was voltage-independent (data not shown), as seen from the similar shapes of the current-voltage relationships without versus with crofelemer, indicating a voltage-independent block mechanism. These results define a second, distinct luminal membrane  $\text{Cl}^-$  channel target of crofelemer.

**Crofelemer Has Little Effect on Apical Cation Channels and cAMP/Calcium Signaling.** The apical membrane of enterocytes also contains  $\text{Na}^+$  and  $\text{K}^+$  channels, which are potential additional targets of crofelemer. To investigate whether crofelemer alters the activity of the epithelial cell  $\text{Na}^+$  channel ENaC, short-circuit current was measured in primary cultures of human bronchial epithelial cells, which robustly express ENaC and in which the change in short-circuit current after amiloride provides a quantitative measure of ENaC activity (Yamaya et al., 1992). Figure 7A shows that pretreatment of the cell culture with 50  $\mu\text{M}$  crofelemer produced a small,  $\sim 20\%$  inhibition of ENaC activity. Human bronchial epithelial cells also express TMEM16A and have robust CaCC activity. Crofelemer pretreatment produced a  $>90\%$  reduction in short-circuit current after the addition of calcium-elevating agonist UTP, in agreement with the conclusions from T84 cells and TMEM16A-transfected FRT cells, above.

Possible inhibition of apical  $\text{K}^+$  channels by crofelemer was tested in human bronchial epithelial cells, in which the basolateral membrane was permeabilized with amphotericin B



**Fig. 6.** Crofelemer inhibition of calcium-activated  $\text{Cl}^-$  channels. A, apical membrane current in TMEM16A-expressing FRT cells in the presence of a transepithelial  $\text{Cl}^-$  gradient (apical  $[\text{Cl}^-]$ , 70 mM; basolateral  $[\text{Cl}^-]$ , 140 mM). B, crofelemer concentration-dependence of TMEM16A  $\text{Cl}^-$  current inhibition. C, whole-cell TMEM16A current recorded at a holding potential of 0 mV and pulsing to voltages between  $\pm 100$  mV in steps of 20 mV in the absence and presence of 10  $\mu\text{M}$  crofelemer. TMEM16A was stimulated by 100  $\mu\text{M}$  ATP. D, I-V plot of mean currents (at the middle of each voltage pulse). Mean currents were normalized as current densities (measured in picoamperes per picofarads).

in the presence of a transepithelial  $K^+$  gradient. Under these conditions, the small measured current is an apical membrane  $K^+$  current. Apical  $K^+$  current was measured after the addition of  $BaCl_2$ , a nonspecific inhibitor of  $K^+$  channels. Figure 7B shows that pretreatment with  $50 \mu M$  crofelemer produced a small,  $\sim 22\%$  inhibition of apical membrane  $K^+$  current.

Last, we tested the possibility that crofelemer action on apical membrane receptor(s) might affect major intracellular signaling pathways, which might secondarily modulate the activities of basolateral membrane transporters to inhibit transcellular  $Cl^-$  secretion indirectly. Figure 7C shows that crofelemer at  $50 \mu M$  had no significant effect on basal or forskolin-stimulated cAMP concentrations in T84 cells. Figure 7D shows that crofelemer did not alter basal cytoplasmic calcium concentration or affect the elevation in calcium concentration after ATP treatment in T84 cells.

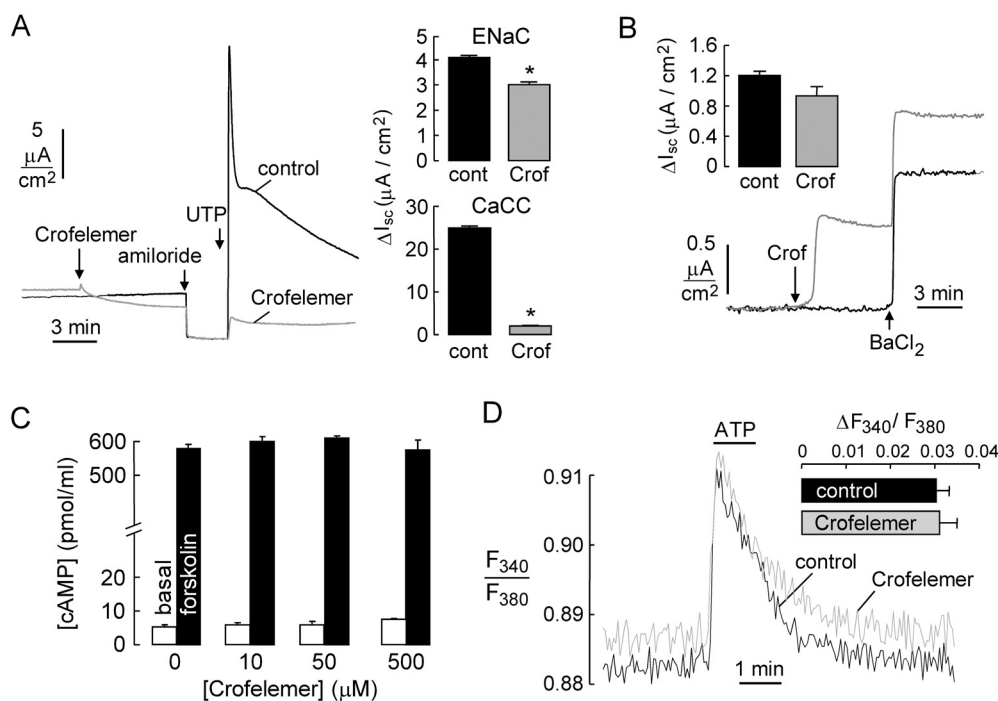
## Discussion

Crofelemer is a polyphenolic molecule isolated from the latex of the plant species *C. lechleri* of the family *Euphorbiaceae*. Crofelemer is an amorphous, dark red-brown powder consisting of an oligomeric proanthocyanidin of varying chain lengths with an average molecular mass of 2100 Da. Crofelemer has been characterized by  $^1H$  NMR,  $^{13}C$  NMR, and mass spectrometry, producing the putative structure shown in Fig. 2A (Ubillas et al., 1994). The polymer chains mostly range from 3 to 30 monomer units linked together in a random sequence through either C-4 $\rightarrow$ C-6 and/or C-4 $\rightarrow$ C-8. The monomeric components are (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin. The relative and absolute configuration of the monomer units was established by optical rotation, circular dichroism, and  $^{13}C$  NMR, and from analysis of monomers after depolymerization. Recently completed clinical studies in adults with acute infectious diarrhea, one with predominantly *E. coli* diarrhea and the other

with *V. cholera*, showed significant clinical benefit of crofelemer in the resolution of the diarrheas (Sharma et al., 2008; Bardhan et al., 2009).

The cellular antisecretory targets of crofelemer were investigated, focusing on the principal luminal membrane determinants of intestinal fluid secretion, including ion channels and signaling pathways. We found that crofelemer inhibited apical membrane cAMP-stimulated (CFTR) and calcium-stimulated (CaCC)  $Cl^-$  channels, with little effect on cation channels or cAMP/calcium signaling. It is noteworthy that crofelemer inhibited two distinct  $Cl^-$  channels, which are unrelated in their sequences and putative structures. The dual cellular actions of crofelemer, together with its slow washout, may account for its broad antisecretory activity in diarrheas caused by bacterial enterotoxins, viruses, and other effectors. Inhibition of both CFTR and CaCCs is of particular interest because of cAMP/calcium cross-talk in enterocytes and thus the potential involvement of both types of  $Cl^-$  channels in some diarrheas. Crofelemer action at the basolateral surface of T84 cells also reduced net  $Cl^-$  secretion, perhaps by inhibition of one or more transporters involved in transcellular  $Cl^-$  secretion such as the basolateral  $Na^+/K^+$  pump, NKCC symporter, or  $K^+$  channel(s). Because crofelemer has minimal oral absorption, it is not expected to access the basolateral surface of enterocytes in the intestine, and its action on basolateral transporter(s) is probably not important in its antidiarrheal mechanisms.

Crofelemer acted as a partial antagonist of CFTR  $Cl^-$  conductance, with a concentration-dependent rate of inhibition over several minutes. Washout of the crofelemer was slow, occurring over hours. It is unknown why, unlike thiazolidinone and glycine hydrazide CFTR inhibitors, crofelemer inhibition of CFTR  $Cl^-$  conductance is partial even at very high concentrations. Some possibilities for such partial inhibition include partial external CFTR pore blockade by the large crofelemer molecule and an intrinsically inefficient



**Fig. 7.** Little or no effect of crofelemer on apical membrane cation channels and intracellular cAMP and calcium signaling. A, left, short-circuit current in primary cultures of CFTR-deficient human bronchial epithelial cells without versus with pretreatment with  $50 \mu M$  crofelemer in the luminal solution. Where indicated, amiloride ( $10 \mu M$ ) and UTP ( $100 \mu M$ ) were added. Right, summary of differences in short-circuit current after amiloride and UTP additions (S.E.,  $n = 3$ ; \*,  $P < 0.05$ ). B, apical membrane  $K^+$  current in human bronchial epithelial cells after basolateral membrane permeabilization with  $20 \mu M$  amphotericin B and in the presence of a  $K^+$  gradient (apical  $[K^+]$ ,  $5 \text{ mM}$ ; basolateral  $[K^+]$ ,  $150 \text{ mM}$ ). C, cAMP levels in T84 cell homogenates under basal conditions and at 10 min after treatment with  $20 \mu M$  forskolin. Differences  $\pm$  crofelemer were not significant. D, calcium signaling measured by fura-2 fluorescence in T84 cells under basal conditions and after ATP addition ( $100 \mu M$ ). Inset, peak ATP increase in fura-2 fluorescence ratio (S.E.,  $n = 4$ ). Difference was not significant.



allosteric inhibition mechanism. Patch-clamp analysis indicated that crofelemer action on the extracellular-facing CFTR surface produces voltage-independent channel inhibition with stabilization of the channel closed state and without rapid channel flicker. Therefore, crofelemer inhibition of CFTR is unlikely to involve direct pore occlusion. In contrast, CFTR inhibitors of the glycine hydrazone class produced a voltage-dependent block, with inward rectification of residual CFTR  $\text{Cl}^-$  current, and direct pore occlusion with rapid flicker in membrane current (Muanprasat et al., 2004; Sonawane et al., 2006, 2007, 2008). The independence of crofelemer and GlyH-101 action seen in Fig. 4C is consistent with crofelemer action at a site different from that of GlyH-101, which occludes the CFTR pore. The larger molecular size of crofelemer compared with GlyH-101 is consistent with crofelemer action at a site outside of the CFTR pore. Prior studies (Gabriel et al., 1999) suggested evidence for CFTR inhibition by crofelemer in T84 cells in the presence of a large  $\text{Cl}^-$  gradient. However, in the previous studies, inhibition of CaCCs was not recognized, and measurements were not done in a defined system in which CFTR could be studied in isolation from CaCCs and other ion channels.

We discovered that crofelemer strongly inhibited CaCC(s). There is evidence, albeit indirect, suggesting that CaCCs in intestinal epithelial cells provide an important route for  $\text{Cl}^-$  and fluid secretion in secretory diarrheas caused by certain drugs, including some antiretrovirals and chemotherapeutics, and some viruses (Morris et al., 1999; Barrett and Keely, 2000; Kidd and Thorn, 2000; Takahashi et al., 2000; Gyömörey et al., 2001; Rufo et al., 2004; Schultheiss et al., 2005, 2006; Thiagarajah and Verkman, 2005; Lorrot and Vasseur, 2007). In addition to their expression in intestinal epithelial cells, CaCCs are broadly expressed in many cell types, in which they are involved in different functions, including transepithelial fluid secretion, olfactory and sensory signal transduction, smooth muscle contraction, and cardiac excitation (Hartzell et al., 2005; Verkman and Galiotta, 2009). The molecular identity of CaCCs was unclear until recently, when three independent laboratories reported TMEM16A (anoctamin-1) as a CaCC (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Several lines of evidence supported the conclusion that TMEM16A is a CaCC, including the demonstration that CaCC  $\text{Cl}^-$  currents in TMEM16A-transfected cells are similar in electrophysiological characteristics with native CaCCs and reduction in CaCC  $\text{Cl}^-$  current after RNAi knockdown of TMEM16A. TMEM16A is expressed broadly in epithelial and other cell types in multiple organs, including intestinal epithelium. It is not known at this time whether intestinal epithelial cells express other CaCCs as well. We found here that crofelemer strongly inhibited human TMEM16A, which probably accounts, at least in part, for its inhibition of  $\text{Cl}^-$  current in T84 cells after addition of calcium-elevating agonists. The precise role of TMEM16A in various secretory diarrheas remains to be elucidated.

In conclusion, the cellular antisecretory action of crofelemer seems to involve two distinct  $\text{Cl}^-$  channel targets on the luminal membrane of epithelial cells lining the intestine. We did not investigate the possibility that crofelemer metabolites formed in the intestine might have additional cellular effects on enterocytes. The dual inhibition of CFTR and CaCC  $\text{Cl}^-$  channels by crofelemer provides insights

into the understanding of its therapeutic effects in the treatment of secretory diarrheal disorders of various etiologies (Holodniy et al., 1999; DiCesare et al., 2002; Mangel and Chaturvedi, 2008), which share the common feature of excessive  $\text{Cl}^-$  secretion.

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**Address correspondence to:** Dr. Alan S. Verkman, 1246 Health Sciences East Tower, Box 0521, University of California, San Francisco, San Francisco, CA 94143-0521. E-mail: alan.verkman@ucsf.edu.

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