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CROFELEMER, AN ANTI-SECRETORY ANTIDIARRHEAL PROANTHOCYANIDIN OLIGOMER EXTRACTED FROM *CROTON LECHLERI*, TARGETS TWO DISTINCT INTESTINAL CHLORIDE CHANNELS

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Running title: Crofelemer inhibition of chloride channels

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Non-standard abbreviations: CaCC, calcium-activated chloride channel; CFTR, cystic fibrosis transmembrane conductance regulator; $CFTR_{inh}$-172, thiazolidinone CFTR inhibitor; CPT-cAMP, chlorophenylthio-cAMP; FRT, Fisher rat thyroid; GlyH-101, glycine hydrazide CFTR inhibitor; IBMX, 3-isobutyl-1-methylxanthine

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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 determination of its effect on the major apical membrane transport and signaling processes involved in intestinal fluid transport. Using cell lines and measurement procedures to isolate effects on individual membrane transport proteins, crofelemer at 50 μM had little or no effect on the activity of epithelial Na^+ or K^+ channels, or on cAMP or calcium signaling. Crofelemer inhibited the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel with maximum inhibition of ~60 % and an IC_{50} ~7 μM. 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 thiazolidionone CFTR inhibitors. Crofelemer action resisted washout, with <50 % reversal of CFTR inhibition after 4 hours. Crofelemer was also found to strongly inhibit the intestinal calcium-activated Cl^- channel TMEM16A by a voltage-independent inhibition mechanism with maximum inhibition >90 % and IC_{50} ~6.5 μM. The dual inhibitory action of crofelemer on two structurally unrelated pro-secretory intestinal Cl^- channels may account for its intestinal antisecretory activity.

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INTRODUCTION

Secretory diarrhea remains a global health challenge in developing and developed countries. Intestinal fluid secretion involves Cl^- influx into enterocytes though a $Na^+/K^+/2Cl^-$ symporter on the basolateral membrane, and Cl^- efflux through apical (lumen-facing) Cl^- channels (Barrett and Keely, 2000; Field, 2003; Thiagarajah and Verkman, 2005) (Fig. 1). K^+ channels and a $3Na^+/2K^+$ pump establish the electrochemical driving force for Cl^- secretion. Na^+ and water secretion follow passively in response to active Cl^- secretion. Bacterial enterotoxins produced by *Vibrio cholerae* and *Escherichia coli* elevate cyclic nucleotide concentrations in enterocytes, resulting in Cl^- channel activation and fluid secretion. Na^+ absorption through apical membrane Na^+ channels and electrogenic 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 (ORS) solution. Though ORS 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 pro-secretory membrane transporters mentioned above (reviewed in Field, 2003; Thiagarajah and Verkman, 2005). Our laboratory has focused on targeted inhibitors of the two principal apical membrane Cl^- channels in enterocytes, the cystic fibrosis transmembrane regulator conductance (CFTR), a cAMP-stimulated Cl^- channel, and calcium-activated Cl^- channels (CaCCs). By high-throughput screening and follow-up chemistry, we identified inhibitors of these

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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 thiophenecarboxylate 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, as well as various lung, stomach and other conditions (Ubillas et al., 1994; Jones, 2003; Risco et al., 2003; Rossie 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 therapy of secretory diarrheas associated with acute infections including cholera, as well as 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 Cl^- channels at the luminal membrane of enterocytes.

MATERIALS AND METHODS

Chemicals

Forskolin, apigenin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma. 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) was purchased from Calbiochem. The small-molecule CFTR inhibitors $CFTR_{inh}$-172 and GlyH-101, and the CaCC inhibitor $CaCC_{inh}$-01, were

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synthesized as reported (Ma et al., 2002; Muanprasat et al., 2004; de la Fuente et al., 2008). Crofelemer was provided 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 weight of 2100 daltons, in agreement with a previously reported average molecular weight of 2300 daltons (Ubillas et al., 1994). Fig. 2A shows the putative structure of crofelemer. The material used for the studies here is the same as that used in clinical trials, where it is formulated for oral dosing as modified-release tablets (125 or 250 mg crofelemer per tablet).

Cell culture

Fisher rat thyroid (FRT) cells expressing human CFTR were generated as described (Ma et al., 2002). FRT cells expressing human TMEM16A (cDNA provided by Dr. Luis Galietta, Gaslini Institute, Genoa, Italy) were generated similarly. FRT cells were cultured in F-12 Modified Coon's Medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 350 µg/ml hygromycin and 500 µg/ml geneticin. Primary cultures of human bronchial epithelial cells were maintained at an air-liquid interface as described (Yamaya et al., 1992). T84 cells were cultured in DMEM/Ham's F-12 (1:1) medium

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containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were grown on Snapwell porous filters (Costar 3801) at 37 ^{o}C in 5% 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 ohm·cm). Short-circuit current was measured in Ussing chambers (Vertical diffusion chamber; Costar) 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 KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-HEPES, 10 mM glucose, pH 7.3. Half-Ringer's solution was the same, except that 65 mM NaCl was replaced with Na gluconate, and $CaCl_2$ was increased to 2 mM. The basolateral membrane was permeabilized with 250 µg/ml amphotericin B, as described (Ma et al., 2002). Chambers were bubbled continuously with air. For T84 cells and bronchial epithelial cells, cells were bathed in symmetrical HCO_3^--buffered solution containing (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4), and aerated with 5 % CO_2 at 37 °C. For the measurement of apical K^+ conductance in T84 cells, $NaHCO_3$ and NaCl were replaced with Na gluconate, and Na gluconate in basolateral solution was replaced with K gluconate and bubbled with air. The basolateral membrane was permeabilized with 20 µM amphotericin B. Short-circuit current was measured using a DVC-1000 voltage-clamp apparatus (World Precision Instruments).

Cyclic nucleotide assays

T84 cells were grown in 24-well plates, treated for 45 min with crofelemer, then for 10 min with 0 or 20 µM forskolin, lysed by sonication, centrifuged to remove cell debris, and the

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supernatant was assayed for cAMP according to manufacturer's instructions (Parameter™ cAMP immunoassay kit, R&D Systems).

Patch-clamp analysis of CFTR and TMEM16A Cl^- channel function

Whole-cell recordings were made on FRT cells stably expressing CFTR or TMEM16A. The pipette solution for CFTR contained (in mM) 140 N-methyl D-glucamine chloride (NMDG-Cl), 5 EGTA, 1 $MgCl_2$, 1 Tris-ATP and 10 HEPES (pH 7.2). The pipette solution for TMEM16A contained (in mM) 130 CsCl, 0.5 EGTA, 1 $MgCl_2$, 1 Tris-ATP and 10 HEPES (pH 7.2). The bath solution contained (in mM) 140 NMDG-Cl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4). All measurements were done at room temperature (22–25 °C). Pipettes were pulled from borosilicate glass and had resistances of 3–5 Mohm after fire polishing. Seal resistances were between 3 and 10 Gohm. After establishing the whole-cell configuration, CFTR was activated by forskolin and IBMX, and TMEM16A 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 mV 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.

Single-channel analysis of CFTR was done in the cell-attached configuration using fire-polished pipettes with a resistance of 6–10 Mohm. The pipette solution contained (in mM) 140 NMDG-Cl, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES (pH 7.4), and the KCl bath solution contained (in mM) 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES (pH 7.4). Recordings were performed at room temperature using an Axopatch-200B (Axon Instruments). Voltage and

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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 (Axon Instruments).

Calcium signaling measurements

Measurements of $[Ca^{2+}]_i$ 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 (Molecular Probes). Fura-2 loaded T84 cells were mounted in a perfusion chamber on the stage of an inverted fluorescence microscope. The cells were superfused with (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose and 10 HEPES (pH 7.4). Fura-2 fluorescence was recorded at excitation wavelengths of 340 nm 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^- secretion by T84 human intestinal epithelial cells

To test whether crofelemer reduces intestinal cell Cl^- secretion, short-circuit current was measured in T84 cells in symmetrical physiological solutions (without plasma membrane permeabilization). Fig. 2B shows crofelemer concentration-dependent inhibition of the increase in short-circuit current produced by the cAMP agonist forskolin (top) and the calcium agonists ATP (middle) and thapsigargin (bottom). Measurements with the calcium agonists were done in the presence of $CFTR_{inh}$-172 to inhibit CFTR. Whereas crofelemer inhibition of forskolin-induced current, which is mainly CFTR-dependent, was slow, weak and partial, inhibition of ATP and thapsigargin-induced current was nearly complete at 10 μM crofelemer. This inhibition of current

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induced by calcium agonists suggests that crofelemer inhibits both CFTR and CaCC channels, with apparently much stronger inhibition of the latter. Further measurements were done using transfected cell systems to study crofelemer effects on CFTR and CaCCs in isolation.

Though crofelemer is membrane impermeable because of its size and polarity, and thus should access only the luminal surface of intestinal epithelial cells, we investigated possible effects of basolateral application of crofelemer on apical membrane CFTR and CaCC. Studies were done in T84 cells in which 50 μM crofelemer was added to the basolateral bathing solution for 10 min prior to measurement of short-circuit current. Interestingly, though crofelemer did not affect the initial currents induced by the CFTR and CaCC agonists forskolin and ATP, respectively, crofelemer slowly reduced Cl^- secretion over many minutes (Fig. 2C). Though probably not relevant to its antidiarrheal mechanisms, crofelemer may act at the basolateral surface of T84 cells to inhibit one or more transporters involved in transepithelial Cl^- secretion.

Crofelemer is a partial antagonist of CFTR Cl^- conductance

CFTR Cl^- current was measured in CFTR-expressing FRT cells in which the basolateral membrane was permeabilized by amphotericin B and a transepithelial Cl^- gradient was applied. FRT cells were used here because they lack intrinsic chloride channel activity, form tight junctions, grow quickly, and are readily transfected with chloride channel cDNAs and reporter indicators (Verkman and Galietta, 2009). Under these conditions the measured current provides a direct quantitative measure of CFTR Cl^- conductance. Fig. 3A shows apical membrane current measurements in which CFTR Cl^- conductance was stimulated by CPT-cAMP, which was followed by addition of different concentrations of crofelemer in the apical bathing solution. Increasing concentrations of crofelemer produced notably more rapid, though partial inhibition of

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CFTR Cl^- current. Addition of crofelemer to the basolateral bathing solution did not inhibit current (not shown). As summarized in Fig. 3B (open circles) the apparent IC_{50} (giving 50 % inhibition of Cl^- current) for crofelemer was ~7 μM, and the maximal inhibition was ~60 %. Similar results were obtained when the apical and basolateral bathing solutions were switched (high Cl^- in apical solution) (Fig. 3B, filled circles), indicating that crofelemer inhibition of CFTR does not depend on Cl^- concentration. In contrast to the partial inhibition by crofelemer, maximal CFTR inhibition by $CFTR_{inh}$-172 or GlyH-101 is approximately 100 % (see below).

Measurements were done to investigate whether the crofelemer inhibition potency depends on the CFTR activation mechanism. Fig. 4A shows similar responses to 50, 200 and 500 μM crofelemer using agonists that activate CFTR directly (apigenin) or through cAMP-dependent CFTR phosphorylation (forskolin). The reversibility of crofelemer inhibition of CFTR was investigated, since washout during secretory diarrhea is a concern with use of a non-absorbable antisecretory agent. Fig. 4B shows apical current measurements in which CFTR Cl^- current was stimulated by CPT-cAMP and then inhibited by different concentrations of crofelemer. Following extensive washing, residual CFTR inhibition was determined from the current after re-stimulation by CPT-cAMP. In control studies in the absence of crofelemer, washout (of CPT-cAMP) followed by re-stimulation produced a similar current to that seen in the initial stimulation. However, following 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 (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 pre-added crofelemer. Fig. 4C (left) shows

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concentration-inhibition studies of CFTR inhibition by $CFTR_{inh}$-172 and GlyH-101. Maximal inhibition ~100 %, with IC_{50} values of ~1 and ~8 μM, respectively. Fig. 4C (right) shows similar concentration-inhibition measurements, in which 50 μM crofelemer was added initially to inhibit CFTR Cl^- current by ~50 %. Despite the partial antagonist mechanism of crofelemer, $CFTR_{inh}$-172 and GlyH-101 were able to inhibit CFTR by nearly 100%. The similar IC_{50} values for $CFTR_{inh}$-172 and GlyH-101 in the absence and presence of crofelemer suggests non-overlapping CFTR inhibition sites for crofelemer and $CFTR_{inh}$-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 μM forskolin produced a membrane current of 179 ± 18 pA/pF (n = 3) at +100 mV (total membrane capacitance 15.8 ± 4 pF). Crofelemer at 50 μM gave ~60 % inhibition of CFTR Cl^- current. Fig. 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, indicting 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 pipette solution (Fig. 5B). Addition of 10 μM forskolin and 100 μM IBMX to the bath resulted in CFTR channel opening. CFTR unitary conductance was 7.2 ± 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 (Po) from 0.71 ± 0.04 (n = 4) to 0.40 ± 0.04 (n = 3). Mean channel open time was not significantly changed, but mean channel closed time was significantly

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increased (Fig. 5B, bottom), indicating that crofelmer stabilizes the channel closed state. These results suggest that crofelemer inhibits CFTR by an altered channel gating mechanism.

Crofelemer is a strong inhibitor of the CaCC TMEM16A

The data in Fig. 2B suggested 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 addition of 1 μM ionomycin to stimulate TMEM16A Cl^- current. Measurements were made in the presence of a transepithelial Cl^- gradient, so that current is a direct, quantitative measure of TMEM16A Cl^- conductance. Fig. 6A shows crofelemer concentration-dependent inhibition of TMEM16A Cl^- current, which was nearly complete at high concentrations of crofelemer. Fig. 6B shows an IC_{50} for crofelemer inhibition of TMEM16A of ~6.5 μM.

Whole-cell membrane current was measured in TMEM16A-expressing FRT cells (Fig. 6C). Stimulation by 100 μM ATP produced a membrane current of 56 ± 13 pA/pF (n = 3) at +100 mV. Pretreatment with 10 μM crofelemer inhibited ATP-induced TMEM16A Cl^- current by 58 % (24 ± 6 pA/pF, n = 3). Fig. 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 (not shown), as seen from the similar shapes of the current-voltage relationships without versus with crofelemer, indicating a voltage-independent block

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mechanism. These results define a second, distinct luminal membrane Cl^- channel target of crofelemer.

Crofelemer has little effect on apical cation channels and cAMP/calcium signaling

The apical membrane of enterocytes also contains Na^+ and K^+ channels, which are potential additional targets of crofelemer. To investigate whether crofelemer alters the activity of the epithelial cell 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 following amiloride provides a quantitative measure of ENaC activity (Yamaya et al., 1994). Fig. 7A shows that pre-treatment of the cell culture with 50 μM crofelemer produced a small, ~20 % inhibition of ENaC activity. Human bronchial epithelial cells also express TMEM16A and have robust CaCC activity. Crofelemer pre-treatment produced a >90 % reduction in short-circuit current following 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 K^+ channels by crofelemer was tested in human bronchial epithelial cells in which the basolateral membrane was permeabilized with amphotericin B 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 following addition of $BaCl_2$, a nonspecific inhibitor of K^+ channels. Fig. 7B shows that pre-treatment with 50 μM crofelemer produced a small, ~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. Fig. 7C shows

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that crofelemer at 50 μM had no significant effect on basal or forskolin-stimulated cAMP concentrations in T84 cells. Fig. 7D shows that crofelemer did not alter basal cytoplasmic calcium concentration, nor did it affect the elevation in calcium concentration following ATP treatment in T84 cells.

DISCUSSION

Crofelemer is a polyphenolic molecule isolated from the latex of the plant species *Croton 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 weight of 2100 daltons. Crofelemer has been characterized by ^{1}H-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→C-6 and/or C-4→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 following 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

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(CFTR) and calcium-stimulated (CaCC) Cl^- channels, with little effect on cation channels or cAMP/calcium signaling. Notably, 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, 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. The reason(s) are not known 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 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 hydrazide class produced a voltage-dependent block, with inward rectification of residual CFTR Cl^- current,

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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 Figs. 4C is consistent with crofelemer action at site different from that of GlyH-101, which occludes the CFTR pore. The larger molecular size of crofelemer compared to 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 Cl^- gradient. However, in the prior studies inhibition of CaCCs was not recognized nor were measurements done in a defined system where 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 Cl^- and fluid secretion in secretory diarrheas caused by certain drugs, including some antiretrovirals and chemotherapeutics, and some viruses (Morris et al. 1999, Barrett, 2000; Kidd and Thorn, 2000; Takahashi et al. 2000; Gyömörey et al. 2001; Rufo et al. 2004; Thiagarajah and Verkman, 2005; Schultheiss et al. 2005, 2006; Farthing, 2006; Lorrot and Vasseur, 2007). In addition to their expression in intestinal epithelial cells, CaCCs are broadly expressed in many cell types where 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 Galietta, 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 Cl^- currents in TMEM16A-transfected cells are similar in electrophysiological characteristics with native CaCCs,

and reduction in CaCC Cl^- current following 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 likely accounts, at least in part, for its inhibition of Cl^- current in T84 cells 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 appears to involve two distinct Cl^- channel targets on the luminal membrane of epithelial cells lining the intestine. We did not investigate the possibility that crofelemer formed in the intestine might have additional cellular effects on enterocytes. The dual inhibition of CFTR and CaCC Cl^- channels by crofelemer provides insights into the understanding of its therapeutic effects in the treatment of secretory diarrheal disorders of various etiologies (DiCesare et al, 2002; Holodniy et al. 1999; Mangel and Chaturvedi, 2008), which share the common feature of excessive Cl^- secretion.

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FOOTNOTES

[*] Authors contributed equally

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FIGURE LEGENDS

Figure 1. Cellular mechanisms of intestinal fluid secretion by enterocytes, showing chloride secretion through apical membrane chloride channels. See text for further explanation.

Figure 2. Crofelemer reduces Cl^- secretion in T84 human intestinal cells in response to cAMP and calcium-elevating agonists. A. Chemical structure of crofelemer (see text for explanation) (from Ubillas et al., 1994). B. Short-circuit current in T84 cells following activation of Cl^- 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 pre-treated with 20 μM $CFTR_{inh}$-172 to inhibit CFTR Cl^- current. C. Short-circuit current measurements showing CFTR (left)- and CaCC (right)-dependent Cl^- current in the presence of crofelemer (50 μM) added to the basolateral bathing solution 10 min prior to measurements. CFTR was inhibited by pretreatment with $CFTR_{inh}$-172 (20 μM) for measurement of ATP-induced CaCC activation.

Figure 3. Crofelemer inhibition of CFTR Cl^- conductance. A. Apical membrane current in CFTR-expressing FRT cells following permeabilization with amphotericin B and in the presence of a transepithelial Cl^- gradient (apical $[Cl^-]$ 75 mM, basolateral $[Cl^-]$ 150 mM). CFTR Cl^- conductance was activated by 100 μM CPT-cAMP followed by addition of indicated concentrations of crofelemer to the luminal solution. B. Crofelemer concentration-inhibition of CFTR Cl^- current measured at 20 min after crofelemer application (S.E. n=3-5). Data shown for experiments as in A (open circles) and with reversed Cl^- gradient (apical $[Cl^-]$ 150 mM, basolateral $[Cl^-]$ 75 mM) (filled circles).

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Figure 4. Characterization of crofelemer inhibition of CFTR Cl^- conductance. A. Crofelemer inhibition of CFTR following 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 readded. C. Investigation of possible synergy/competition of crofelemer with small-molecule CFTR inhibitors. (left) Apical membrane current following CFTR activation by CPT-cAMP and inhibition by $CFTR_{inh}$-172 or GlyH-101. (right) Crofelemer (50 μM) was added to inhibit CFTR Cl^- current by ~50-60 %, followed by indicated concentrations of $CFTR_{inh}$-172 or GlyH-101.

Figure 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 ±100 mV in steps of 20 mV in the absence and presence of 50 μM crofelemer. CFTR was stimulated by forskolin. (right) Current/voltage (I/V) plot of mean currents at the middle of each voltage pulse from experiments as in A (S.E., n=3). Fitted IC_{50} 6.5 μM. B. (left) Single-channel recordings were done in the cell-attached configuration. CFTR was activated by 10 μM forskolin and 100 μM IBMX. Pipette potential was + 80 mV. (right) Summary of crofelemer effect on CFTR channel open probability (Po), mean open time and mean closed time (S.E., n=3-4, * $P < 0.05$). o, open channel state; c, closed channel state.

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Figure 6. Crofelemer inhibition of calcium-activated Cl^- channels. A. Apical membrane current in TMEM16A-expressing FRT cells in the presence of a transepithelial Cl^- gradient (apical [Cl^-] 70 mM, basolateral [Cl^-] 140 mM). B. Crofelemer concentration-dependence of TMEM16A Cl^- current inhibition. C. Whole-cell TMEM16A current recorded at a holding potential at 0 mV, and pulsing to voltages between ±100 mV in steps of 20 mV in the absence and presence of 10 μM crofelemer. TMEM16A was stimulated by 100 μM ATP. D. Current/voltage (I/V) plot of mean currents (at the middle of each voltage pulse). Mean currents were normalized as current densities (pA/pF).

Figure 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 vs. with pre-treatment with 50 μM crofelemer in the luminal solution. Where indicated amiloride (10 μM) and UTP (100 μM) were added. (right) Summary of differences in short-circuit current following amiloride and UTP additions (S.E., n=3, * $P < 0.05$). B. Apical membrane K^+ current in human bronchial epithelial cells following basolateral membrane permeabilization with 20 μM amphotericin B and in the presence of a K^+ gradient (apical [K^+] 5 mM, basolateral [K^+] 150 mM). C. Cyclic AMP levels in T84 cell homogenates under basal conditions and at 10 min after treatment with 20 μM forskolin. Differences +/- crofelemer not significant. D. Calcium signaling measured by fura-2 fluorescence in T84 cells under basal conditions and following ATP (100 μM). Where indicated cells were pre-treated with 50 μM crofelemer. Inset summarizes the peak ATP increase in fura-2 fluorescence ratio (S.E., n=4). Difference not significant.

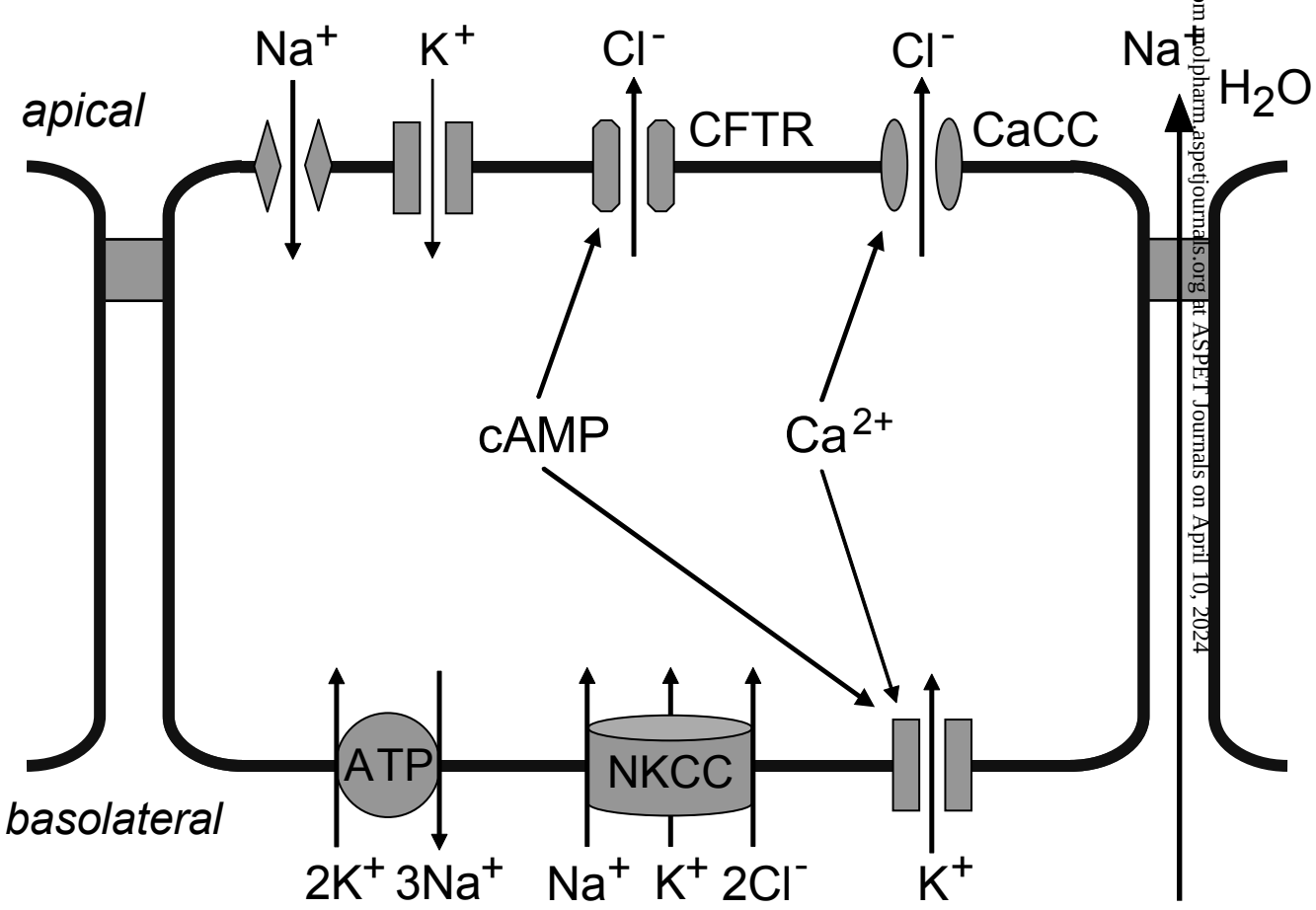

Figure 1

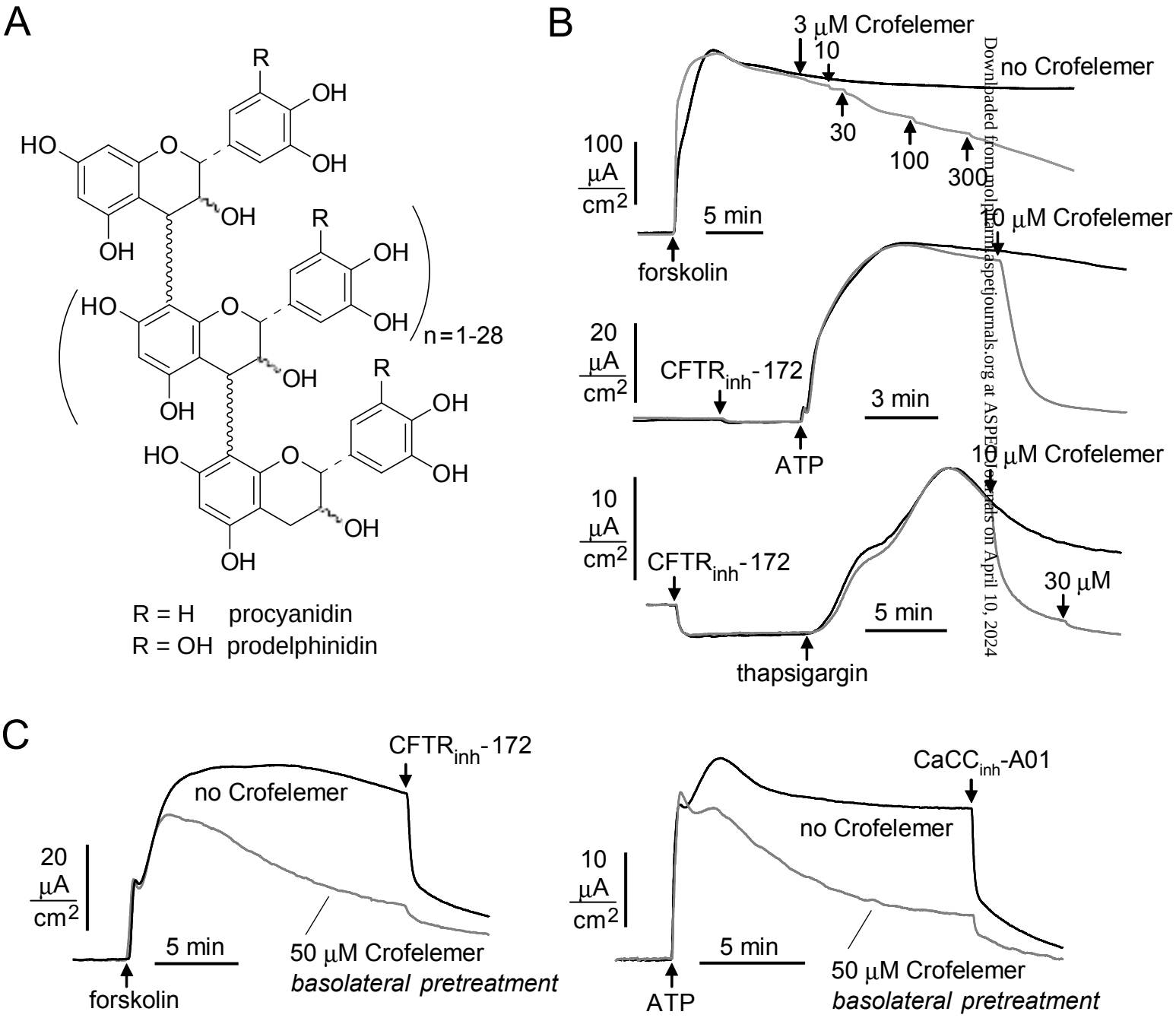

Figure 2

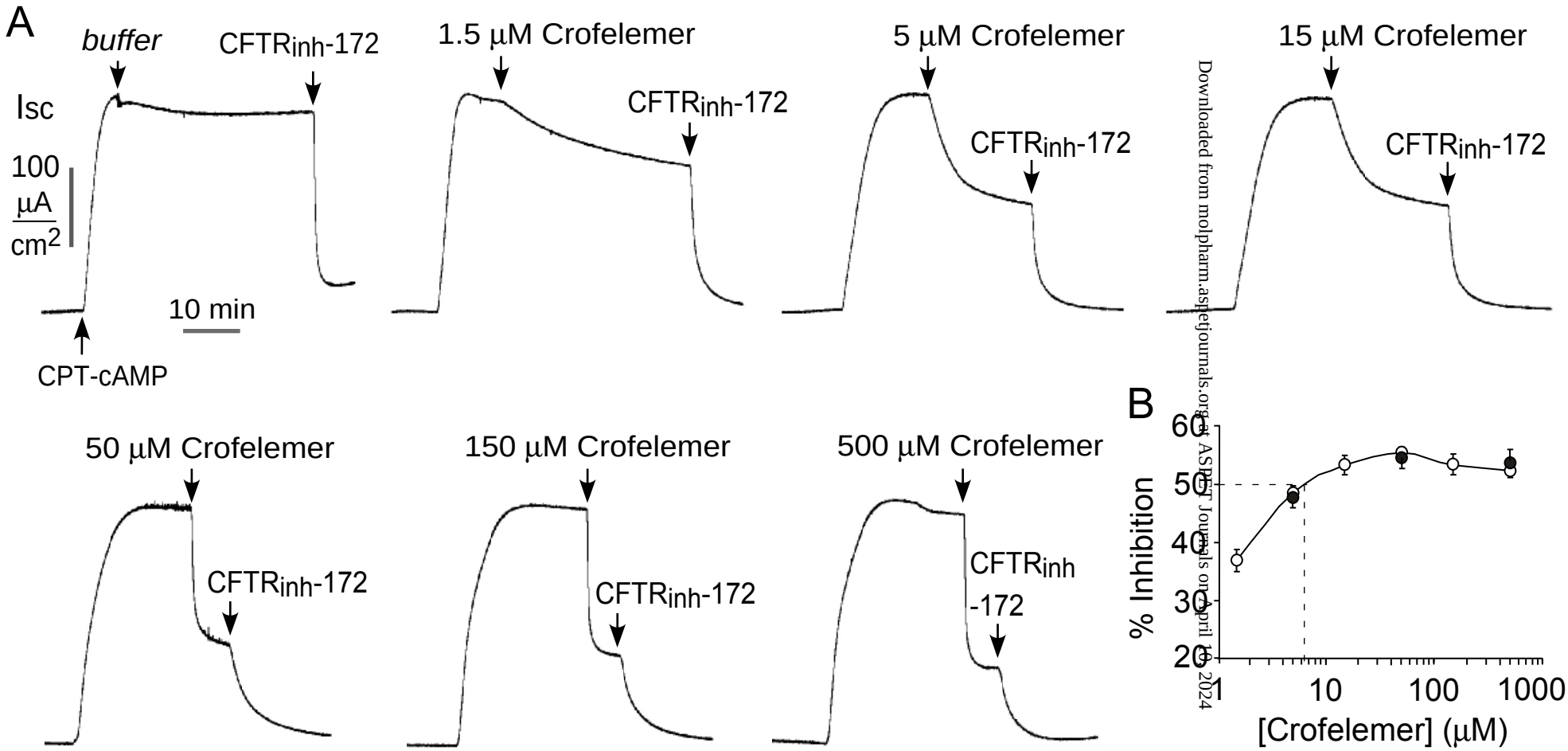

Figure 3

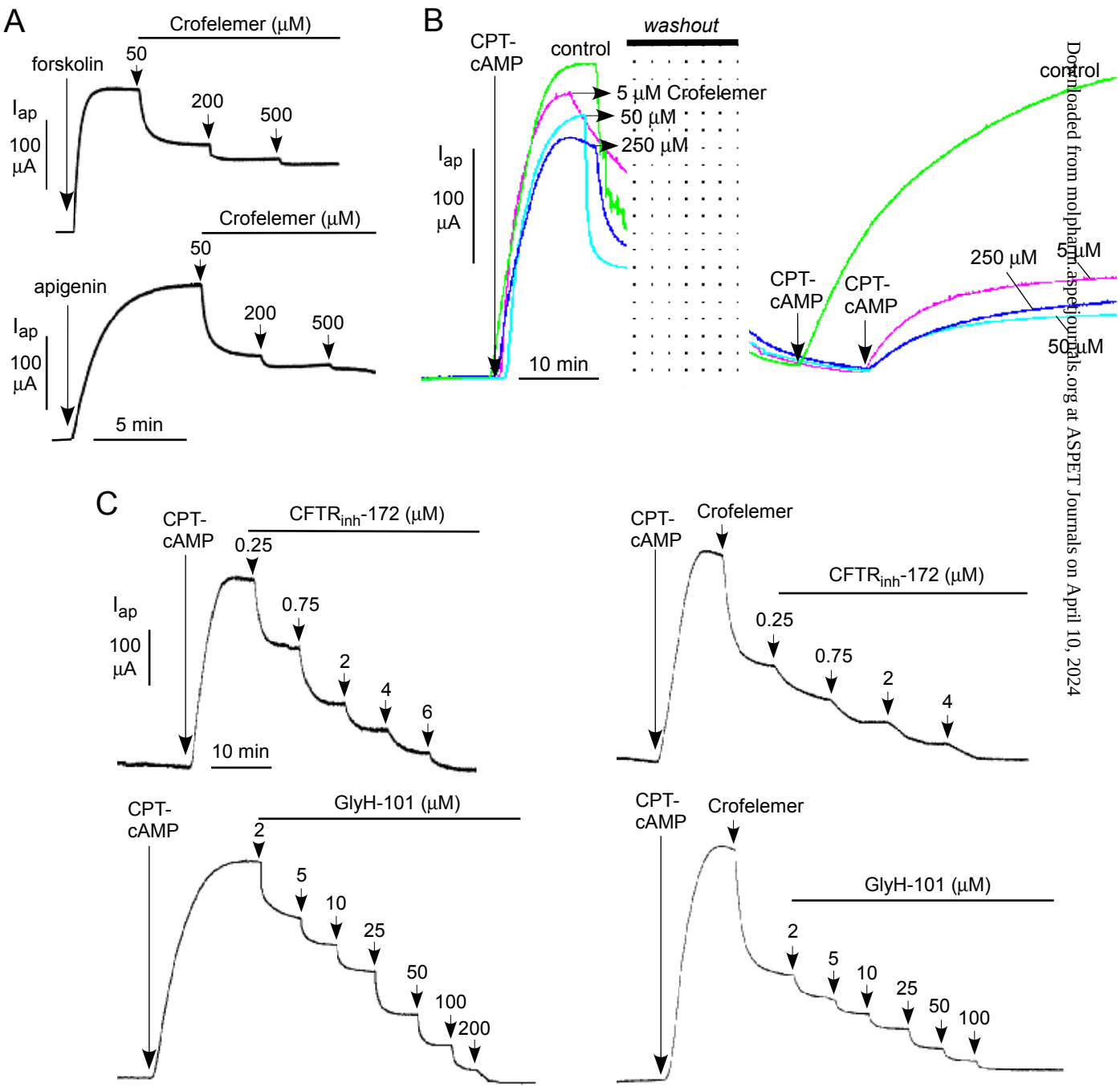

Figure 4

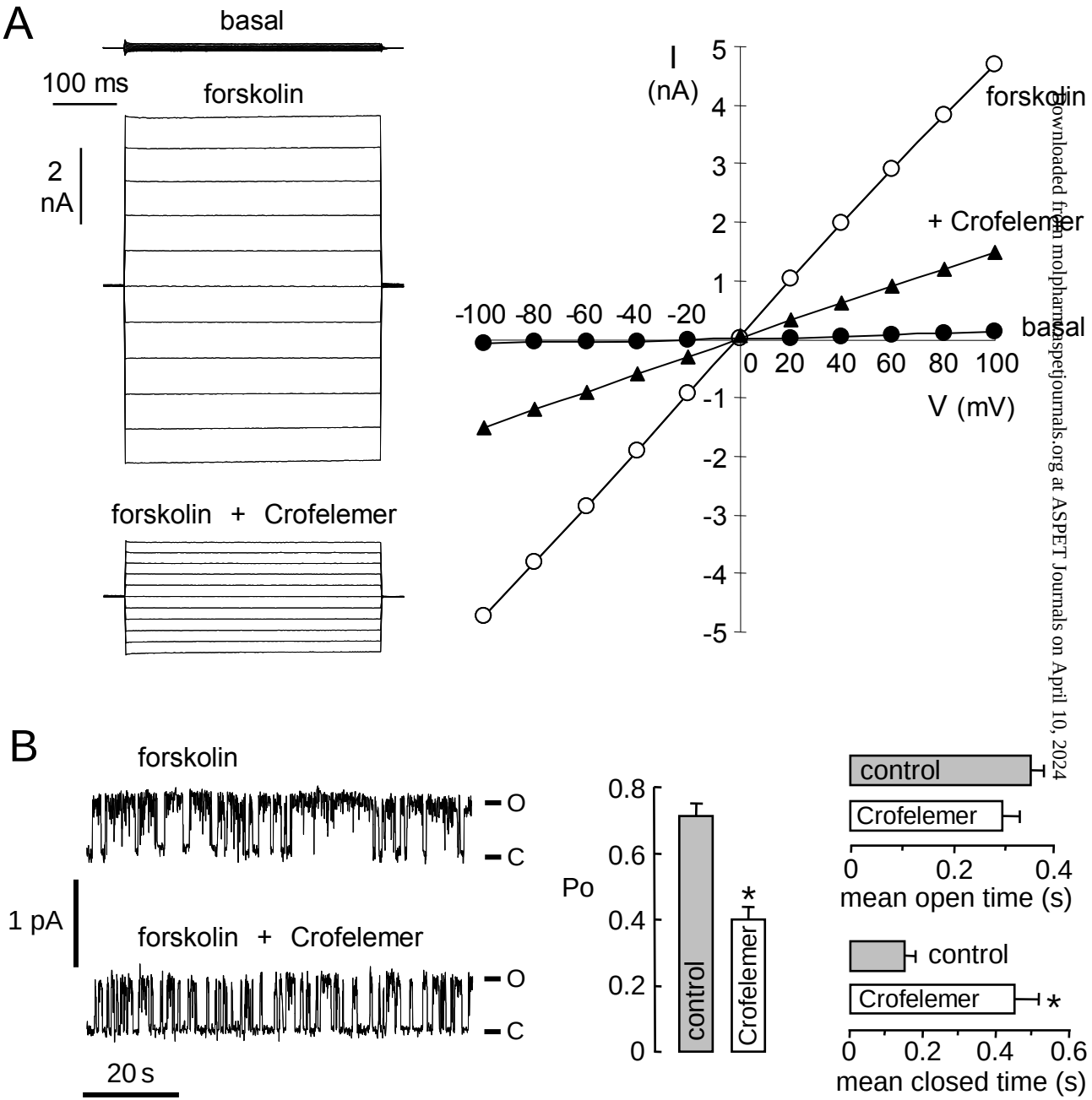

Figure 5

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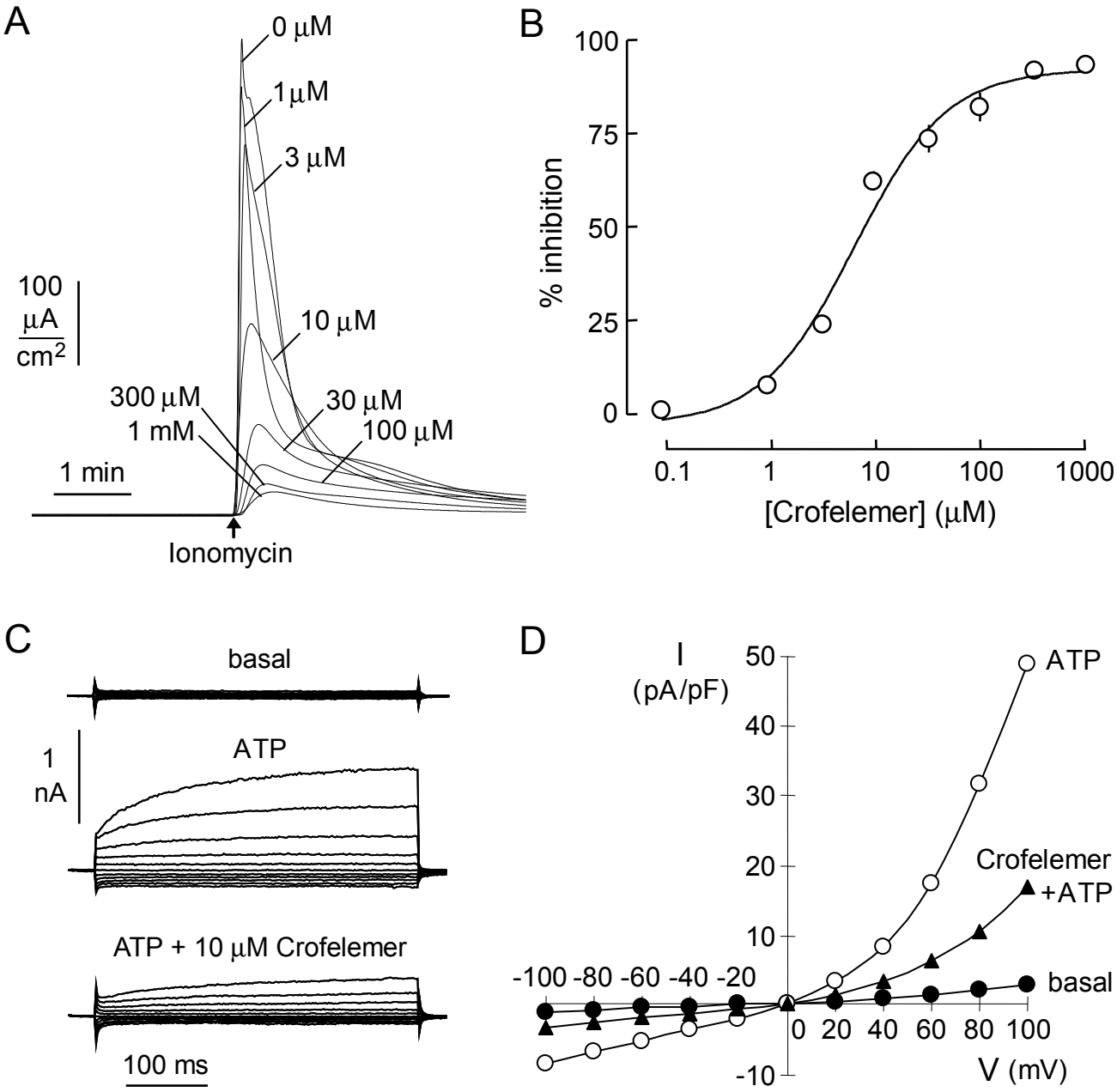

Figure 6

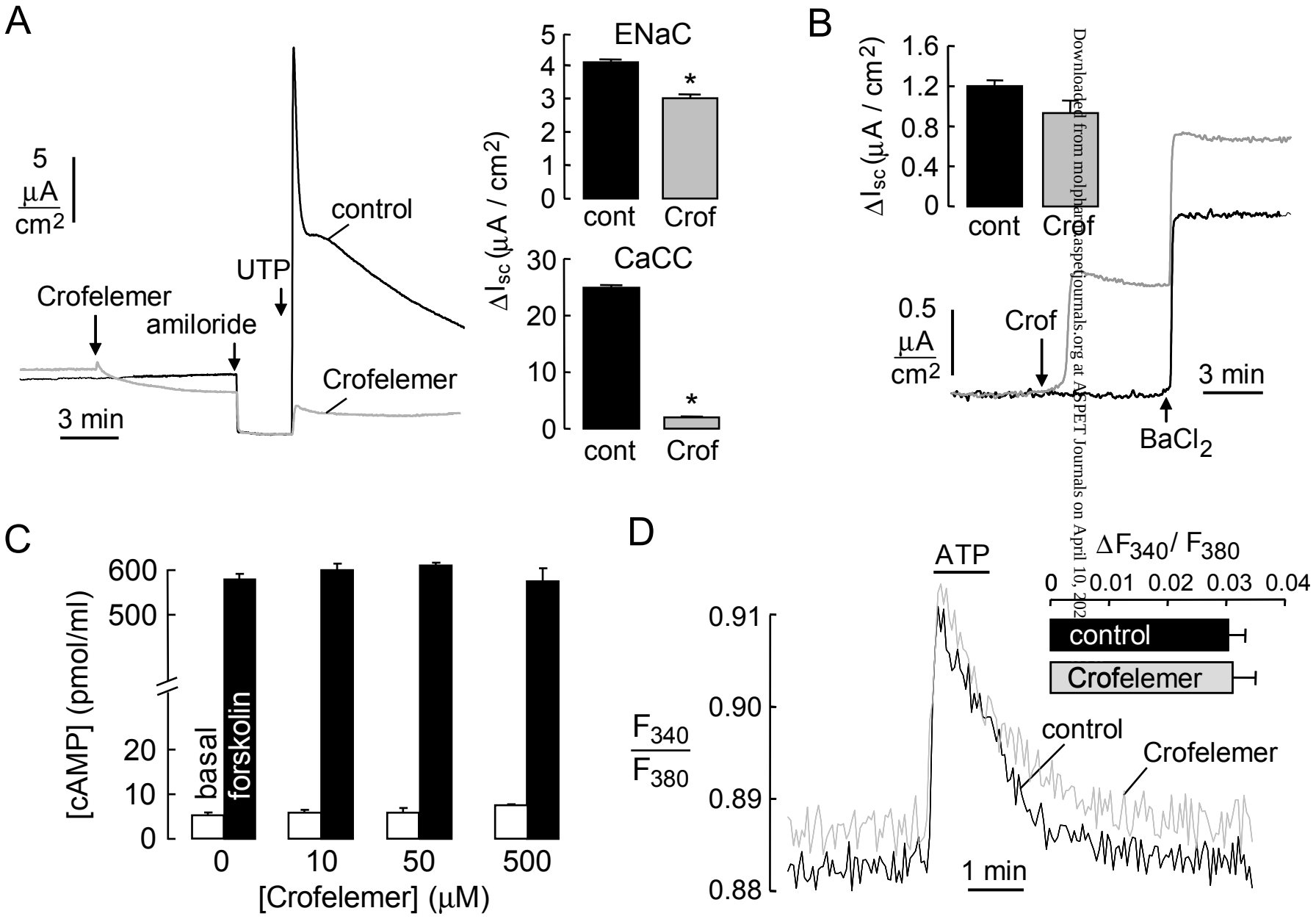

Figure 7