TRANSLATIONAL PHYSIOLOGY

Diarrhea-associated HIV-1 APIs potentiate muscarinic activation of Cl\(^{-}\) secretion by T84 cells via prolongation of cytosolic Ca\(^{2+}\) signaling

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Rufo, Paul A., Patricia W. Lin, Adriana Andrade, Lianwei Jiang, Lucia Rameh, Charles Flexner, Seth L. Alper, and Wayne I. Lencer. Diarrhea-associated HIV-1 APIs potentiate muscarinic stimulation of Cl\(^{-}\) secretion by T84 cells via prolongation of cytosolic Ca\(^{2+}\) signaling. Am J Physiol Cell Physiol 286: C998–C1008, 2004. First published December 30, 2003; 10.1152/ajpcell.00357.2003.—Aspartyl protease inhibitors (APIs) effectively extend the length and quality of life in human immunodeficiency virus (HIV)-infected patients, but dose-limiting side effects such as lipodystrophy, insulin resistance, and diarrhea have limited their clinical utility. Here, we show that the API nelfinavir induces a secretory form of diarrhea in HIV-infected patients. In vitro studies demonstrate that nelfinavir potentiates muscarinic stimulation of Cl\(^{-}\) secretion by T84 human intestinal cell monolayers through amplification and prolongation of an apical membrane Ca\(^{2+}\)-dependent Cl\(^{-}\) conductance. This stimulated ion secretion is associated with increased magnitude and duration of muscarinally induced intracellular Ca\(^{2+}\) transients via activation of a long-lived, store-operated Ca\(^{2+}\) entry pathway. The enhanced intracellular Ca\(^{2+}\) signal is associated with uncoupling of the Cl\(^{-}\) conductance from downstream intracellular mediators generated normally by muscarinic activation. These data show that APIs modulate Ca\(^{2+}\) signaling in secretory epithelial cells and identify a novel target for treatment of clinically important API side effects.

nelfinavir; clotrimazole; barium

ASPARTYL PROTEASE INHIBITORS (APIs) are important components of most highly active antiretroviral therapy (HAART) regimens used in the treatment of human immunodeficiency virus (HIV)-1 infection. These agents decrease plasma HIV viral load, increase peripheral CD4\(^{+}\) T lymphocyte counts, delay clinical progression, and extend life expectancy (17). However, the clinical utility of these APIs has been limited by serious side effects, which include lipodystrophy, insulin resistance, and diarrhea (14). The mechanistic bases of these dose-limiting side effects of API treatments remain unexplained.

In these studies, we examine the effects of the API nelfinavir on the intestine and on intestinal epithelial cells. Intestinal fluid secretion in the human depends on the closely regulated transport of Cl\(^{-}\) ions by epithelial cells lining the intestinal crypt. Crypt epithelia utilize the basolateral membrane Na\(^{+}\)-K\(^{+}\)-ATPase and Na\(^{+}\)- and K\(^{+}\)-coupled cotransporter NKCC1 to accumulate intracellular Cl\(^{-}\) above its electrochemical equilibrium potential. The regulated opening of apical membrane Cl\(^{-}\) channels in that setting results in a net secretion of Cl\(^{-}\) ions into the intestinal lumen. Coordinated opening of basolateral K\(^{+}\) channels to maintain an inside-negative membrane potential sustains the Cl\(^{-}\) secretory response by enhancing both the electrical gradient favoring electrogenic apical Cl\(^{-}\) exit and the chemical gradient favoring Na\(^{+}\) and K\(^{+}\)-coupled Cl\(^{-}\) uptake by basolateral NKCC1. Water and Na\(^{+}\) are thought to follow Cl\(^{-}\) passively into the intestinal lumen to effect net fluid secretion.

Neural, endocrine, paracrine, and autocrine mechanisms tightly regulate intestinal fluid secretion in the human via agonists that utilize either cyclic nucleotides or Ca\(^{2+}\) as second messengers. Agonists that depend on adenosine 3',5'-cyclic monophosphate (cAMP) to initiate Cl\(^{-}\) secretion activate the apical membrane Cl\(^{-}\) channel CFTR (cystic fibrosis transmembrane receptor) and the basolateral membrane K\(^{+}\) channel KCNJ1/KCNE3 (2, 10, 32, 42). Agonists that utilize Ca\(^{2+}\) as a second messenger activate the apical membrane Ca\(^{2+}\)-activated Cl\(^{-}\) conductance and the basolateral membrane K\(^{+}\) channel IK1 (KCNN4) (22, 24, 25, 47).

Muscarinic innervation of intestinal crypts regulates Cl\(^{-}\) secretion through local release of acetylcholine. The secretory response induced in the crypt epithelial cell requires an elevation of intracellular Ca\(^{2+}\) that initially activates an apical membrane Ca\(^{2+}\)-sensitive Cl\(^{-}\) conductance. However, coordinate generation of inositol 3,4,5,6-tetrakisphosphate (IP\(_{4}\)) and phosphorylation of the MAP kinase intermediates extracellular signal-regulated kinase (ERK) and p38 rapidly downregulate this Ca\(^{2+}\)-sensitive Cl\(^{-}\) conductance to keep muscarinally induced Cl\(^{-}\) secretory responses short-lived (2, 7, 23, 29–31).
In the current study, we have found that the API nelfinavir induces a secretory form of diarrhea in HIV-1-infected patients. In vitro studies demonstrate that nelfinavir potentiates muscarinic stimulation of Cl\(^-\) secretion in the human intestinal cell line T84 through the prolongation of a long-lived, store-operated Ca\(^{2+}\) entry pathway. The resulting prolonged period of increased intracellular Ca\(^{2+}\) correlates with uncoupling of the apical membrane Ca\(^{2+}\)-dependent Cl\(^-\) conductance from effects of the downregulatory signals IP\(_3\), phosphorylated ERK (pERK), and phospho-p38, all present at normal levels. We propose that this prolonged, store-operated Ca\(^{2+}\) influx provokes in intestinal epithelia the enhanced Cl\(^-\) secretion and consequent secretory diarrhea observed clinically in patients treated with APIs.

**METHODS**

*Clinical studies.* Eight HIV-infected subjects (ages 21–54) with diarrhea (stool output ≥300 g/24 h) for longer than 1 mo while on nelfinavir-containing regimens were admitted for 48 h and received a controlled diet (~4,200 calories/day; 50% carbohydrates, 12% protein, and 38% fat). Subjects remained on their prescribed daily dose of nelfinavir (5 subjects received 1,250 mg twice daily, and 3 subjects received 750 mg 3 times daily). Antidiarrheal medications were discontinued 3 days before and during hospitalization. Subjects receiving APIs other than nelfinavir or with any condition known to cause diarrhea were excluded from study participation. Stool output was collected in preweighed containers and stored at 4°C. Specimens were weighed, homogenized, and centrifuged for 10 min at 2,000 rpm. Fecal supernatants were analyzed for [Na\(^+\)] and [K\(^+\)] by ion-selective electrodes (Hitachi 917; Boehringer Mannheim), and pH and osmotic content were measured. Fecal osmolar gap was defined as the difference between predicted ([Na\(^+\) + K\(^+\)] × 2) and estimated fecal osmolality (290 mosmol/kg H\(_2\)O) (15). Osmotic diarrhea was defined as a fecal osmolar gap of >60 mosmol/kg H\(_2\)O with stool [Cl\(^-\)] ≤ 15 meq/l and [Na\(^+\)] ≤ 30 meq/l and a stool pH ≥ 6.0. Secretory diarrhea was defined as a fecal osmolar gap of ≥60 mosmol/kg H\(_2\)O with stool [Cl\(^-\)] < 15 meq/l and [Na\(^+\)] > 30 meq/l and a stool pH < 6.0. Secretory diarrhea was defined as a fecal osmolar gap of >60 mosmol/kg H\(_2\)O with stool [Cl\(^-\)] ≥ 15 meq/l and [Na\(^+\)] ≥ 30 meq/l and a stool pH ≥ 6.0. Secretory diarrhea was defined as a fecal osmolar gap of >60 mosmol/kg H\(_2\)O with stool [Cl\(^-\)] ≥ 15 meq/l and [Na\(^+\)] ≥ 30 meq/l and a stool pH ≥ 6.0.

*Materials.* Nelfinavir (Agouron Pharmaceuticals, La Jolla, CA), saquinavir (Roche Pharmaceuticals, Nutley, NJ), indinavir (Merck, West Point, PA), and ritonavir (Abbott Laboratories, North Chicago, IL) were used without excipients as kindly provided by the manufacturers. Stock solutions (20 mM) were stored at 4°C in equal parts of ethanol and DMSO. Cells were pretreated for 30 min with nelfinavir (or other API) unless otherwise stated. Anti-pERK (New England Biolabs, Beverly, MA) and anti-phospho-p38 antibodies (Cell Signaling, Beverly, MA) were used at 1:1,000 dilution. \[^{3}H\]inositol was obtained from PerkinElmer (Boston, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**Short-circuit measurement in intact monolayers.** Short-circuit current (I\(_{sc}\)) and transepithelial resistance were measured in confluent T84 cell monolayers grown on 0.33-cm\(^2\) inserts of high-Cl\(^-\) or normal I\(_{sc}\) as the sole permeant ions (Table 1). Basolateral membrane K\(^{+}\) conductances, measured as short-circuit current (I\(_{blk}\)), were measured in cells permeabilized apically with 20 μM amphotericin B, in the absence of asymmetrical buffers that imposed a basolaterally directed sevenfold K\(^+\) gradient (apical solution 4, basal solution 5; see Table 1) as previously described (41). Transmembrane potential was clamped at 0 mV, and I\(_{blk}\) was measured before and after stimulation with CCh. Apical Cl\(^-\) conductances, measured as short-circuit current (I\(_{apCl}\)), were measured in cells permeabilized basolaterally with 100 μM amphotericin B, in the presence of symmetric high-Cl\(^-\) buffer (solution 1) with transmembrane potential clamped at +10 mV (apical) as previously described (34). I\(_{apCl}\) was measured before and after thapsigargin stimulation. Anion selectivity was measured in asymmetrical nelfinavir-containing buffers that imposed an apically directed ~20-fold gradient of either I\(^-\) (basal solution 6, apical solution 7) or Cl\(^-\) (basal solution 2, apical solution 3) as the sole permeant ions. Transepithelial currents were measured during 1-s voltage clamp periods ranging from ~80 to +80 mV and normalized to baseline I\(_{sc}\) at rest as described (34). Baseline current-voltage (I-V) curves obtained in the absence of agonist were subtracted from those measured after agonist treatment to calculate agonist-induced currents.

**Table 1. Composition of solutions used for electrophysiology studies**

<table>
<thead>
<tr>
<th>Compound, mM</th>
<th>High Cl(^-)</th>
<th>Normal Cl(^-)</th>
<th>Low Cl(^-)</th>
<th>High K(^+)</th>
<th>Low K(^+)</th>
<th>Normal I(^-)</th>
<th>Low I(^-)</th>
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<td>0.7</td>
<td>0.7</td>
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<td>20</td>
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<tr>
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<td>Ca(^{2+})</td>
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<td>1.25</td>
<td>1.25</td>
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<td>7.9</td>
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<tr>
<td>Mg(^{2+})</td>
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<td>0.4</td>
<td>0.4</td>
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<tr>
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<td>7.9</td>
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<td>137</td>
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<td>I(^-)</td>
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<td>137</td>
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<tr>
<td>t-Glucose</td>
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<td>Total, mM</td>
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<td>305</td>
<td>305</td>
<td>299</td>
<td>299</td>
<td>305</td>
<td>305</td>
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NMDG, N-methyl-d-glucamine.
**Immunoblots of pERK.** T84 cell monolayers (grown on collagen-coated 5-cm² filters) were preincubated in the presence or absence of nelfinavir (0.4 to 40 µM) at 37°C for 30 min. Five minutes after the subsequent addition of CCh, cells were transferred to ice-cold PBS. Total cell lysates were prepared by scraping cells into lysis buffer (1 mM NaF, 1 mM sodium vanadate, 1% Triton X-100, one protease inhibitor MiniTab with EDTA; Hoffman-La Roche, Nutley, NJ) and then clarified by centrifugation. Lysates were analyzed for pERK by SDS-PAGE and immunoblot. Equal protein loads were confirmed by Ponceau stain.

**Immunoblots of phospho-p38.** T84 cell monolayers (grown on collagen-coated 5-cm² filters) were preincubated in the presence or absence of 30 µM nelfinavir for 30 min at 37°C. After exposure to CCh (100 µM) for the indicated intervals (between 0 and 15 min), monolayers were transferred into ice-cold lysis buffer (1 mM NaF, 1 mM sodium vanadate, 1% Triton X-100, one protease inhibitor MiniTab with EDTA; Hoffman-La Roche). Cell lysates were clarified by centrifugation, and phosphorylated p38 was assayed by immunoblot.

**IP₃ measurements.** T84 cell monolayers (grown on collagen-coated 45-cm² filters) were labeled for 24 h in inositol-free DMEM containing 5% fetal calf serum and 5 µCi/ml [³H]inositol. To ensure that cells were studied at steady state, we treated monolayers in the presence or absence of nelfinavir (40 µM) during the final 2.5 h of labeling with [³H]inositol. Three minutes after addition of CCh, inserts were transferred into ice-cold PBS, lysed in 10% trichloroacetic acid by repeated freeze-thaw cycles, and clarified by centrifugation. Total cell lipids were then extracted in H₂O-saturated ether, dried overnight, and analyzed by HPLC as previously described (39).

**Intracellular Ca²⁺ measurements.** T84 cells cultured at subconfluent density on collagen-coated 5-cm² coverslips were incubated at 37°C in growth medium containing 2 µM fura 2-AM (Molecular Probes, Eugene, OR) for 30 min, washed, and mounted in a modified Leiden chamber. T84 clusters containing >20 fura 2-stained cells at the cluster periphery were selected, and the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was measured in all stained cells within a single cluster by fura 2 fluorescence ratio imaging at 20°C in room air, as described previously (41).

**Statistical methods.** Unless otherwise indicated, data were tested for statistical significance by ANOVA (StatView; SAS, Cary, NC). *P* < 0.05 was chosen to denote statistical significance.

**RESULTS**

**Nelfinavir induces secretory diarrhea through selective potentiation of signaling by muscarinic and other Ca²⁺-dependent agonists.** Stool samples collected from eight HIV-infected individuals on chronic nelfinavir therapy exhibited fecal electrolyte concentrations ([Na⁺] and [Cl⁻]) consistent with a secretory process. Stool pH and the fecal supernatant osmotic gap were consistent with a secretory process in seven subjects (Table 2). These data show that HIV-1-infected patients on chronic nelfinavir therapy have a secretory diarrhea.

To examine the cellular mechanisms underlying this secretory defect, we utilized the human intestinal T84 cell line. T84 cells model regulated Cl⁻ secretion in the human intestine (1). Exposure of resting T84 cells to nelfinavir (30 µM) produced no detectable effect on Cl⁻ secretion (*Iₑc*) or transepithelial resistance. Nelfinavir-pretreated monolayers subsequently exposed to CCh showed a three- to fourfold increase in peak Cl⁻ secretory response (Fig. 1A) and a prolonged duration of Cl⁻ secretion (30 min after CCh exposure, *Iₑc* was 3.5 ± 0.3 µA/cm² in control and 9.1 ± 0.83 µA/cm² in nelfinavir-pretreated monolayers, means ± SE, *n* = 7 experiments; *P* ≤ 0.001). Nelfinavir pretreatment did not alter the decrease in transepithelial resistance observed after subsequent treatment with CCh (598 ± 74 and 723 ± 133 Ω·cm², means ± SE, in control and nelfinavir-treated T84 cell monolayers, respectively). Nelfinavir had no effect on Cl⁻ secretion elicited by the Camp-dependent agonists vasoactive intestinal peptide (VIP; 5 nM) (Fig. 1, B and E) or adenosine (10 µM; see below).

**Stool Output, Osmotic Gap, Cl⁻, and K⁺ in HIV-infected patients treated with nelfinavir.**

<table>
<thead>
<tr>
<th>Subject No. (Sex)</th>
<th>Stool Output, g/day</th>
<th>Osmotic Gap, mosmol/kgH₂O</th>
<th>Stool pH</th>
<th>Na⁺, meq/l</th>
<th>Cl⁻, meq/l</th>
<th>K⁺, meq/l</th>
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<tbody>
<tr>
<td>1 (M)</td>
<td>408</td>
<td>−12</td>
<td>6.0</td>
<td>121</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>2 (M)</td>
<td>273</td>
<td>−38</td>
<td>6.0</td>
<td>84</td>
<td>49</td>
<td>79.9</td>
</tr>
<tr>
<td>3 (M)</td>
<td>302</td>
<td>16</td>
<td>7.8</td>
<td>68</td>
<td>41</td>
<td>69</td>
</tr>
<tr>
<td>4 (F)</td>
<td>368</td>
<td>62</td>
<td>7.0</td>
<td>79</td>
<td>39</td>
<td>34.8</td>
</tr>
<tr>
<td>5 (F)</td>
<td>332</td>
<td>32</td>
<td>5.0</td>
<td>73</td>
<td>48</td>
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<td>6 (F)</td>
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<td>6.5</td>
<td>98</td>
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<tr>
<td>7 (M)</td>
<td>256</td>
<td>−36</td>
<td>8.0</td>
<td>116</td>
<td>75</td>
<td>47.1</td>
</tr>
<tr>
<td>8 (M)</td>
<td>3,940</td>
<td>−4</td>
<td>7.5</td>
<td>111</td>
<td>84</td>
<td>36.1</td>
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</table>

Calculated osmotic gap is defined as 290 mosmol/kgH₂O ([fecal [Na⁺] + [fecal [K⁺]]) × 2. HIV, human immunodeficiency virus; M, male; F, female.
described (41). After achievement of steady state, CCh was added and basolateral K⁺/H₄⁺ conductance was measured as the short-circuit current $I_{sc}$. These apically permeabilized monolayers exhibited similar basolateral K⁺/H₄⁺ conductances after muscarinic stimulation in the presence or absence of nelfinavir (Fig. 3, A and B). Thus, nelfinavir has no effect on basolateral K⁺/H₄⁺ conductance activated by CCh stimulation (believed to be mediated by the Ca²⁺/H₄⁺-dependent K⁺/H₄⁺ channel IK₁/KCNN4) (12). Nonetheless, Cl⁻ secretion elicited by nelfinavir requires basolateral K⁺/H₄⁺ conductance and is blocked fully by high concentrations of the K⁺/H₄⁺ channel inhibitor clotrimazole (Fig. 4).

Nelfinavir could also act by increasing the apical membrane Ca²⁺/H₄⁺-dependent Cl⁻ conductance or possibly (though less described (41).

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Fig. 1. Nelfinavir acts synergistically with muscarinic agonists to potentiate Cl⁻ secretion. A: time course of 100 μM carbachol (CCh)-induced short-circuit current ($I_{sc}$) in intact T84 cell monolayers pretreated for 90 min with (○) or without (□) nelfinavir (30 μM). Data are representative of 6 independent experiments. B: time course of 5 nM vasoactive intestinal peptide (VIP)-induced $I_{sc}$ in monolayers pretreated with (●) or without (□) nelfinavir. Data are representative of 3 independent experiments. C: nelfinavir concentration-response curve for monolayers stimulated with CCh (○) and VIP (●). Data are means ± SE; n = 3 experiments. D: time course of $I_{sc}$ induced by thapsigargin in cells pretreated with (●) or without (□) nelfinavir. E: agonist-induced $I_{sc}$ in T84 monolayers pretreated with (light shaded bars) or without (dark shaded bars) nelfinavir. Data are means ± SE; n = 3 experiments for each agonist. *P < 0.05. TDC, tauro-deoxycholate; Thaps, thapsigargin.

Fig. 2. Human immunodeficiency virus (HIV)-1 protease inhibitors potentiate Cl⁻ secretion elicited by the muscarinic agonist CCh. Time courses of CCh-induced $I_{sc}$ are shown in intact T84 monolayers pretreated (●) or untreated (○) with 100 μM saquinavir (A) or indinavir (B). Data for both inhibitors are representative of 2 independent experiments.

Fig. 3. Nelfinavir does not inhibit basolateral K⁺ conductance in T84 cells. A: time course of CCh-induced basolateral membrane K⁺ conductance [$I_{bK}$] in apically permeabilized T84 monolayers pretreated with (●) or without (○) nelfinavir. B: peak increase in $I_{bK}$ above baseline [$\Delta I_{bK}$]. Data are means ± SE; n = 3 experiments. NS, nonsignificant.
Ca$^{2+}$ of the muscarinic agonist CCh in studies assessing apical secretion to inhibition by dithiothreitol (DTT) in monolayers pretreated with (Nelf), or forskolin. Data are means; $n = 2$ experiments. CLT, clotrimazole.

likely, in view of Fig. 1B) through activation of the apical cAMP-dependent CFTR. We therefore permeabilized selectively the basolateral membrane of T84 cell monolayers with amphotericin B. Cells were then studied in symmetrical solutions containing Cl$^-$ as the only permeant ion, and apical Cl$^-$ conductance, measured as $I_{ap(Cl)}$, was measured as previously described (41). Basolateral permeabilization precludes the use of the muscarinic agonist CCh in studies assessing apical Ca$^{2+}$-activated Cl$^-$ conductances. Thus we used the endoplasmonic Ca$^{2+}$-ATPase pump inhibitor thapsigargin in these experiments. These studies showed that thapsigargin-induced $I_{ap(Cl)}$ in basolaterally permeabilized T84 cell monolayers was significantly higher in cells pretreated with nelfinavir than in control cells (Fig. 5, A and B).

To characterize further this nelfinavir-enhanced apical Cl$^-$ conductance, we compared apical Cl$^-$ and I$^-$ conductances of nelfinavir-pretreated, basolaterally permeabilized T84 cells before and after stimulation with thapsigargin or with the cAMP agonist forskolin. Thapsigargin-stimulated apical membrane current measured across nelfinavir-pretreated T84 monolayers in asymmetric I$^-$ solutions [$I_{ap(I)}$] was greater than thapsigargin-stimulated apical membrane current measured in asymmetric Cl$^-$ solutions [$I_{ap(Cl)}$]. This result was consistent with activation of an apical membrane Ca$^{2+}$-gated Cl$^-$ conductance (although the reversal potential of the thapsigargin-stimulated currents suggested either substantial gluconate permeability of the anion conductance or substantial contribution of stimulated cation conductance) (Fig. 5C). In contrast, forskolin stimulated $I_{ap(Cl)}$ to a greater degree than $I_{ap(I)}$ in nelfinavir-pretreated monolayers, consistent with activation of CFTR (Fig. 5D). The nelfinavir-potentiated CCh-induced apical anion conductance also displayed sensitivity to inhibition by dithiothreitol (DTT; 2 mM), in contrast to the forskolin-induced conductance (Fig. 5E). Thus the nelfinavir-enhanced apical membrane Cl$^-$ conductance resembles the Ca$^{2+}$-activated Cl$^-$ conductance CaCC, rather than the cAMP-gated CFTR (18).

Fig. 4. Nelfinavir-induced Cl$^-$ secretion is inhibited by the K$^+$ channel blocker clotrimazole. CCh-induced Cl$^-$ secretion in control and nelfinavir-pretreated intact cell monolayers in the absence (solid bars) or presence (shaded bars) of 30 µM clotrimazole. Data are means; $n = 2$ experiments. CLT, clotrimazole.

Fig. 5. Nelfinavir increases T84 transepithelial Cl$^-$ secretion by potentiation of a Ca$^{2+}$-dependent apical Cl$^-$ conductance. A: time course of 5 µM thapsigargin-induced apical membrane current [$I_{ap(Cl)}$] in basolaterally permeabilized T84 monolayers pretreated with (C) or without (●) nelfinavir and studied in Cl$^-$ solutions. B: increase in $I_{ap(Cl)}$ measured 15 min after exposure to 5 µM thapsigargin [$\Delta I_{ap(Cl)}$]. Data are means ± SE; $n = 7$ experiments. $P < 0.05$. The voltage dependence of 5 µM thapsigargin-induced (C) or 10 µM forskolin-induced (D) apical membrane currents [$I_{ap(anion)}$] in basolaterally permeabilized T84 monolayers were studied as described in METHODS in media containing either I$^-$ (●) or Cl$^-$ (○) as sole permeant anions. Data are means ± SE; $n = 3–7$ experiments. E: sensitivity of Cl$^-$ secretion to inhibition by dithiothreitol (DTT) in monolayers treated with CCh alone, CCh in the presence of nelfinavir (Nelf), or forskolin. Data are means ± SE; $n = 3–7$ experiments.
The transient elevation of $I_{sc}$ that typifies muscarinically induced $\text{Cl}^-$ secretion in T84 cells is generally attributed to rapid downregulation of apical membrane $\text{Cl}^-$ channels by the parallel synthesis of IP$_4$ and the phosphorylation of the MAP kinase intermediates ERK and p38 (29–31). These downregulators of apical $\text{Ca}^{2+}$-dependent $\text{Cl}^-$ conductance render it refractory to further stimulation by $\text{Ca}^{2+}$-dependent agonists for up to 30 min after withdrawal of muscarinic activation (26).

Downregulation of basolateral membrane $\text{K}^+$ channels by transactivation of the EGF receptor also follows muscarinic activation in T84 cells and also contributes to the transient nature of CCh-elevated $I_{sc}$. However, basolateral $\text{K}^+$ conductance is unaltered in neflavinir-pretreated cells as shown in Fig. 3, A and B. We therefore tested whether neflavinir potentiation of apical $\text{Cl}^-$ conductance might be explained by inhibition of the muscarinic activation of IP$_4$ synthesis or by inhibition of phosphorylation of ERK and p38.

We first tested whether neflavinir had any effect on the refractory period to $\text{Ca}^{2+}$-dependent agonists observed after muscarinic activation. Control monolayers stimulated with CCh exhibited a typical increase in $I_{sc}$ followed by a rapid return to baseline. There followed a period refractory to subsequent treatment with thapsigargin (Fig. 6A). In contrast, neflavinir-pretreated monolayers failed to exhibit such a refractory period after muscarinic stimulation (Fig. 6A). The neflavinir-pretreated cells displayed normal sensitivity to thapsigargin exposure only 15 min after the initial exposure to CCh. This response resembles that of cells exposed to thapsigargin without pretreatment with neflavinir or CCh (Fig. 6A). The mean results from three independent studies (Fig. 6B) show that neflavinir abrogates the refractory period seen normally in T84 cells after stimulation with CCh. Despite abolition of the post-CCh-refractory period of T84 cells by neflavinir pretreatment, neflavinir had no detectable effect on CCh-induced $I_{sc}$ from downregulation by phosphorylated ERK (pERK), phospho-p38, and inositol 3,4,5,6-tetrakisphosphate (IP$_4$). A: time course of $I_{sc}$ in intact T84 monolayers treated without CCh (●) or with CCh in the presence (●) or absence (○) of neflavinir. Thapsigargin was applied to all monolayers at 45 min. B: peak $\Delta I_{sc}$ induced by thapsigargin. Data are means ± SE; n = 3 experiments. C: Western blot of total cell extracts for pERK prepared from intact T84 cell monolayers pretreated with or without the indicated concentrations of neflavinir and subsequently exposed or not exposed to 100 $\mu$M CCh for 5 min. Data are representative of 2 independent experiments. D: Western blot of total cell extracts for phospho-p38 prepared from intact T84 cell monolayers pretreated with or without the indicated concentrations of neflavinir and subsequently exposed or not exposed to 100 $\mu$M CCh for 5 min. Data are representative of 2 independent experiments. E–H: HPLC analyses of [3H]IP$_4$ in total cell extracts prepared from control (E and G) or neflavinir-pretreated monolayers (F and H) and subsequently exposed with (+Neflavinir) neflavinir. The gel was divided and vertically offset for visual clarity. E–H: HPLC analyses of [3H]IP$_4$ in total cell extracts prepared from control (E and G) or neflavinir-pretreated monolayers (F and H) and subsequently exposed (G and H) or not to CCh (E and F). IP$_4$ controls were run in parallel (not shown). IP$_4$ levels were similar in control (14,268 dpm) and neflavinir-pretreated (15,191 dpm) T84 cell monolayers. Data are representative of 2 independent experiments.
levels of pERK (Fig. 6C), phospho-p38 (Fig. 6D), or IP$_3$ (Fig. 6, E–H). These data demonstrate that the Ca$^{2+}$-activated Cl$^-$ conductance in nelfinavir-pretreated cells is functionally uncoupled from normal levels of these physiological downregulatory signals.

**Nelfinavir potentiates cytosolic [Ca$^{2+}$] signaling.** Because the effects of nelfinavir pretreatment were observed only in cells exposed to Ca$^{2+}$-dependent agonists, we examined the effect of nelfinavir on intracellular Ca$^{2+}$ signaling in fura 2-loaded T84 cells. Nelfinavir itself had no detectable effect on [Ca$^{2+}$], in resting cells (not shown). After muscarinic stimulation, however, Ca$^{2+}$ transients in nelfinavir-pretreated cells were increased in magnitude and duration compared with those observed in cells not exposed to nelfinavir (Fig. 7A). The peak increase in [Ca$^{2+}$], induced by CCh in nelfinavir-pretreated cells was 138 ± 10 nM (n = 9) vs. 56 ± 4 nM (n = 4; means ± SE) in cells unexposed to nelfinavir. In contrast, nelfinavir pretreatment had no detectable effect on intracellular Ca$^{2+}$ transients induced by CCh in a Ca$^{2+}$-free bath (increase in [Ca$^{2+}$]: 43 ± 7 vs. 45 ± 5 nM, respectively, n = 3; mean ± SE) (Fig. 7B). Thus the enhanced [Ca$^{2+}$]$_i$ response induced by CCh in nelfinavir-pretreated cells was entirely dependent on influx of extracellular Ca$^{2+}$. The enhanced muscarinic Cl$^-$ secretion observed in nelfinavir-pretreated monolayers was inhibited by the Ca$^{2+}$-permeable cation channel inhibitor SKF-96365 (50 μM) (Fig. 8, n = 3, P < 0.05). In contrast, the L-type Ca$^{2+}$ channel blockers verapamil (25 μM) and nifedipine (1 μM) were without apparent effect on nelfinavir-induced secretory responses (not shown).

Basolateral addition of Ba$^{2+}$ (3 mM) to T84 monolayers inhibited the enhancement of the muscarine-induced $I_{sc}$ observed in nelfinavir-pretreated monolayers (Fig. 9A), whereas apical Ba$^{2+}$ had no effect (not shown). As expected, basolateral application of Ba$^{2+}$ to untreated monolayers failed to inhibit normal CCh-induced Cl$^-$ secretion (Fig. 9A; summarized in Fig. 9B). Moreover, basolateral Ba$^{2+}$ did not inhibit the CCh-triggered increase in basolateral K$^+$ conductance in apically permeabilized T84 cells pretreated with nelfinavir (Fig. 9, C and D) confirming directly that Ba$^{2+}$ does not affect IK1 in this experimental system. The inhibitory effect of Ba$^{2+}$ is also not due to inhibition of the K$^+$ channel KCNQ1/KCNE3, because the chromotin inhibitor of this channel, 293B, similarly had no effect on the potentiation of the CCh-induced $I_{sc}$ in intact monolayers pretreated with nelfinavir (not shown). Thus the inhibition by Ba$^{2+}$ of the enhanced Ca$^{2+}$ transient in nelfinavir-pretreated T84 cells did not appear secondary to inhibition of either the cAMP-regulated K$^+$ channel KCNQ1/KCNE3 or the Ca$^{2+}$-gated K$^+$ channel IK1.

We then considered the possibility that Ba$^{2+}$ blocks a CCh-activated Ca$^{2+}$ entry pathway in nelfinavir-pretreated cells. As shown in Fig. 7A, CCh induced [Ca$^{2+}$]$_i$ transients in nelfinavir-pretreated, fura 2-loaded T84 cells that exceeded both in magnitude and duration those observed in cells unexposed to nelfinavir. Addition of Ba$^{2+}$ to the bath rapidly reduced [Ca$^{2+}$]$_i$ toward baseline levels (Fig. 7A). Thus Ba$^{2+}$ exposure of nelfinavir-pretreated T84 cells inhibits in parallel the nelfinavir-potentiated $I_{sc}$ across monolayers and the nelfinavir-potentiated [Ca$^{2+}$]$_i$ transient recorded on coverslips, without detectable inhibition of basolateral K$^+$ conductance. This correlation suggested that Ba$^{2+}$ might also inhibit the nelfinavir-associated escape from the refractory period that follows muscarinic activation. Indeed, Ba$^{2+}$ inhibited fully the $I_{sc}$ induced by thapsigargin added soon after muscarinic activation (Fig. 9, E and F). These data suggest that nelfinavir pretreatment potentiates Ca$^{2+}$ entry in CCh-stimulated T84 cell monolayers via a mechanism that can be inhibited by basolateral exposure to Ba$^{2+}$. However, in T84 cells not pretreated with nelfinavir, Ba$^{2+}$ had no effect on CCh-activated $I_{sc}$ or on the CCh-induced [Ca$^{2+}$]$_i$, transient. Thus nelfinavir-elicted, Ba$^{2+}$-sensitive, CCh-activated Ca$^{2+}$ entry is not part of the normal response to muscarinic stimulation in untreated T84 cells.

We tested the role of intracellular Ca$^{2+}$ stores in the regulation of the nelfinavir-elicted Ca$^{2+}$ entry pathway. Intracellular Ca$^{2+}$ stores of T84 cells grown on coverslips were depleted by muscarinic stimulation in nominally Ca$^{2+}$-free bath. In the absence of extracellular Ca$^{2+}$, CCh induced small [Ca$^{2+}$]$_i$ transients with...
indistinguishable peak [Ca$^{2+}$]i values 134 ± 58 nM above baseline in nelfinavir-pretreated cells and 108 ± 38 nM in untreated cells (n = 4, mean ± SE) that rapidly returned to baseline levels (Fig. 10, A and B). Readdition of 2.7 mM extracellular Ca$^{2+}$ in the continued presence of CCh rapidly increased [Ca$^{2+}$]i to peak values of 229 ± 31 and 183 ± 27 nM above baseline in nelfinavir-pretreated and untreated cells, respectively (n = 4, means ± SE) (Fig. 10, A and B). However, the rate of the subsequent decline in [Ca$^{2+}$]i in nelfinavir-pretreated cells was much slower than that in untreated cells. [Ca$^{2+}$]i in the absence of nelfinavir fell 78 ± 4% (mean ± SE) from peak values within 8 min after bath Ca$^{2+}$ readdition (Fig. 10, A and C). In contrast, [Ca$^{2+}$]i in nelfinavir-pretreated cells decreased only 30 ± 3% from peak levels during the same period (P < 0.0005; Fig. 10, B and C). Thus influx-dependent elevation of [Ca$^{2+}$]i following bath Ca$^{2+}$ readdition to CCh-stimulated T84 cells was prolonged by nelfinavir pretreatment.

After depletion of intracellular Ca$^{2+}$ stores by thapsigargin exposure of T84 cells in a nominally Ca$^{2+}$-free medium, readdition of bath Ca$^{2+}$ induced an elevation of [Ca$^{2+}$]i, larger than that observed after CCh stimulation, with a peak value of 402 ± 11 nM above baseline (n = 4, mean ± SE; Fig. 10D). This store-depletion-activated Ca$^{2+}$ influx was larger still in nelfinavir-pretreated cells, with a peak value of 816 ± 125 nM above baseline (n = 4, mean ± SE, P < 0.05; Fig. 10E).

Moreover, the rate of subsequent [Ca$^{2+}$]i decline in nelfinavir-pretreated cells was again much slower than that in untreated cells. Whereas 15 min after bath Ca$^{2+}$ readdition, [Ca$^{2+}$]i had declined 52 ± 6% from peak values in untreated cells, this decline was only 26 ± 4% in nelfinavir-pretreated cells (means ± SE, P < 0.0005; Fig. 10F). Thus nelfinavir pretreatment enhanced both the magnitude and duration of thapsigargin-induced Ca$^{2+}$ influx in T84 cells. These data suggest activation by nelfinavir of a store-operated plasmalemmal Ca$^{2+}$ entry pathway.

**DISCUSSION**

These studies provide the first mechanistic insights into the pathophysiology of API-induced diarrhea in HIV-infected patients. Nelfinavir potentiates muscarinic Cl$^{-}$ secretion in intestinal epithelial cells by recruitment of a slowly inactivating basolateral Ca$^{2+}$ entry pathway that can be further activated by depletion of intracellular Ca$^{2+}$ stores and is inhibited by basolateral exposure to Ba$^{2+}$. This additional Ca$^{2+}$ uptake pathway differs from that activated by CCh in the normal muscarinic signal transduction cascade, because Ba$^{2+}$ has no detectable effect on CCh-activated I_{ap}(Cl) in untreated cells. The enhanced [Ca$^{2+}$]i signal induced by nelfinavir may cause or contribute to the uncoupling of a CaCC-like apical Cl$^{-}$-
conductance from normal downregulation by IP₃, pERK, and phospho-p38. The resulting prolonged activation of apical Cl⁻ conductance contributes to (and may suffice to explain) the enhanced Cl⁻ secretory response. This mechanism of action is distinct from that recently proposed for the chemotherapeutic agent flavopiridol, which elicits a secretory response in T84 cells via inhibition of the downregulatory signals affecting the apical Ca²⁺-activated Cl⁻ conductance (27).

On the basis of these results, we propose that nelfinavir acts in vivo directly on the intestinal mucosa to enhance the activity of muscarinic and other Ca²⁺-dependent agonists by recruiting an additional store-operated plasmalemmal Ca²⁺ entry pathway to potentiate an otherwise normal secretory response. Such a mechanism of action on intestinal Cl⁻ secretion should initiate a secretory form of diarrhea, a prediction confirmed by our clinical studies in hospitalized HIV-infected adults. At peak in vivo plasma concentrations of 6 μM for nelfinavir, the Cl⁻ secretory response would be near the ED₅₀ for nelfinavir’s in vitro effect on T84 cells. Thus small differences among individual patient plasma nelfinavir concentrations due to differences in drug metabolism or excretion may have large effects on Cl⁻ secretion and diarrhea severity.

Other tested APIs also potentiate the muscarinically induced Cl⁻ secretory response in T84 cells. Furthermore, nelfinavir effectively potentiates the Cl⁻ secretory responses elicited by a wide range of Ca²⁺-dependent agonists, including those induced by bile acids often present in the human colon. Thus the secretory diarrhea described in up to 30% of API-treated patients may be the result of enhanced secretion by normal neurocrine and paracrine secretory regulators, triggered by subclinical degrees of bile acid malabsorption as well as other genetic, dietary, or environmental factors.
tion by DTT. Although the molecular identity of the intestinal crypt cell Ca\(^{2+}\)-activated Cl\(^{-}\) conductance remains uncertain, members of the CLCA gene family have been proposed as candidates (3, 19). This apical Ca\(^{2+}\)-activated Cl\(^{-}\) conductance is thus a therapeutic target, but specific inhibitors for it and for the cloned CLCA channels remain unidentified. Chlorotixin, active against the Ca\(^{2+}\)-activated Cl\(^{-}\) conductance of gliomas and astrocytes, appears inactive in T84 cells (33). Our results also show that nelfinavir effectively uncouples the apical Cl\(^{-}\) conductance from downregulation by the intracellular mediators IP3, pERK, and phospho-p38 but has no apparent effect on basolateral K\(^{+}\) conductance after muscarinic activation. Thus nelfinavir may elevate levels of intracellular antagonists of these downregulatory signals to potentiate the physiological agonists of Ca\(^{2+}\)-dependent intestinal Cl\(^{-}\) secretion.

The reversal potential of thapsigargin-activated apical membrane currents in nelfinavir-pretreated cells (Fig. 5C) suggests that nelfinavir may potentiate in parallel an apical membrane Ca\(^{2+}\)-activated cation conductance and an apical Ca\(^{2+}\)-activated Cl\(^{-}\) conductance, as suggested also by our previous studies in the absence of nelfinavir (34). Merlin et al. (34) also showed that gluconate permeability of the thapsigargin-induced T84 cell apical membrane conductance is minimal. Similarly, CCh-induced increase in I\(_{sc}\) across intact, nelfinavir-pretreated T84 cell monolayers is abrogated in nominally Cl\(^{-}\)-free, symmetrical sodium gluconate solutions (not shown). Activation of apical cation currents is consistent with previous reports of nonspecific cation currents in T84 cells (4, 8, 11, 45, 46).

We have previously shown that the imidazole antifungal clotrimazole and its des-imidazolyl metabolite block intestinal Cl\(^{-}\) secretion by inhibition of both the cAMP-activated K\(^{+}\) conductance (likely mediated by KCNQ1/KCNE3) and the Ca\(^{2+}\)-gated K\(^{+}\) conductance likely mediated by IK1 (KCNN4) (41). Blockade of the appropriate K\(^{+}\) channel(s) fully inhibits Cl\(^{-}\) secretion induced by either cAMP or Ca\(^{2+}\)-dependent agonists in vitro and by cAMP-dependent agonists in vivo. Basolateral K\(^{+}\) channel activity is also required for nelfinavir-stimulated Cl\(^{-}\) secretion by T84 cells and is blocked fully by 30 \(\mu M\) clotrimazole, although at this concentration clotrimazole is nonspecific. At the more specific concentration of 1 \(\mu M\), clotrimazole had no effect on CCh-induced Cl\(^{-}\) secretion in cells pretreated with nelfinavir (or (previously reported) without nelfinavir (not shown). Clotrimazole has been administered in humans at doses sufficient to block IK1 with minimal toxicity (5, 6). Thus clotrimazole or other more specific IK1 blockers (16, 44) may be useful for treatment of the secretory diarrhea induced by nelfinavir and other APIs used in the treatment of HIV.

Direct blockade of the nelfinavir-recruited Ca\(^{2+}\) entry pathway, however, might affect more selectively the adverse effects of APIs on regulation of intestinal Cl\(^{-}\) secretion. Such blockade would allow for specific inhibition of API-induced potentiation of Ca\(^{2+}\)-dependent Cl\(^{-}\) secretory responses without affecting the normal muscarine-induced secretory response. Thus molecular identification of this Ca\(^{2+}\) entry pathway might facilitate development of specific inhibitors of this pathway as well as APIs that do not increase its activity.

One family of plasmalemmal Ca\(^{2+}\)-permeable cation channels is the transient receptor potential (TRP) superfamily (9, 36). TRPV6 (ECaC2/CaT1) and TRPV5 (ECaC1/CaT2) are the most extensively studied TRP channels of the intestine. ECaC2/CaT1 has been localized by in situ hybridization to surface enterocytes of the rat (37) but appears to be absent from human colon (21, 38). ECaC1/CaT2 has been immunolocalized to the apical membrane of villous tip enterocytes in rabbit duodenum (20) and in transverse and distal colon of the human (21, 38). In addition, TRPV6 overexpressed in some cultured cells confers increased store-operated cation channel activities. However, the pathway recruited by nelfinavir in T84 cells may represent a basolateral pathway of intestinal crypt cells.

This novel pathway is notable for the ability of Ba\(^{2+}\) to block the nelfinavir-associated enhancement of apical Cl\(^{-}\) secretion as well as for the enhanced magnitude and prolonged duration of muscarinally induced [Ca\(^{2+}\)]\(_{i}\) elevation. In contrast, Ba\(^{2+}\) has no effect on the muscarinically induced I\(_{sc}\) in cells not treated with nelfinavir. Thus the inhibitory effect of Ba\(^{2+}\) on nelfinavir-potentiated Cl\(^{-}\) secretion may represent a Ba\(^{2+}\) block of the nelfinavir-induced Ca\(^{2+}\) entry pathway. It is also possible that Ba\(^{2+}\) may permeate the nelfinavir-induced Ca\(^{2+}\) entry pathway once inside the cell, Ba\(^{2+}\) might then block a nelfinavir-induced, Ca\(^{2+}\)-dependent reversal of the normal inactivation processes for the apical membrane Ca\(^{2+}\)-activated Cl\(^{-}\) conductance. Thus the molecular identity of the nelfinavir target(s) remains to be determined.

The effects of nelfinavir on Ca\(^{2+}\) signaling in intestinal epithelial cells may similarly apply to other cell types affected by API-based therapeutics. If so, altered Ca\(^{2+}\) signaling in adipocytes, myocytes, or hepatocytes may contribute to other API-associated dose-limiting side effects including lipodystrophy and insulin resistance (13, 43).

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