SPI-0211 activates T84 cell chloride transport and recombinant human CIC-2 chloride currents

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SPI-0211 (lubiprostone) is a gastrointestine-targeted bicyclic fatty acid that enhances intestinal fluid secretion and has been shown to be safe and highly effective in treating constipation in clinical trials (18, 19). The gastrointestinal effects of SPI-0211 appear to be mediated by increased Cl− secretion, because the Cl− concentration in secreted intestinal fluid increases after SPI-0211 administration (41). The mechanism by which SPI-0211 enhances Cl− secretion is not known, but an increased Cl− concentration could result from enhanced Cl− channel currents in gastrointestinal epithelial membranes.

In epithelial cells, it is established that Cl− movement across the basolateral membrane occurs via the Na+-K+-2Cl− co-transporter and that Cl− movement across the apical (luminal) membrane occurs via specific Cl− channels. Ca2+-activated K+ channels in the basolateral membrane help to set the driving force for Cl− movement across the cell and tight junctions between cells simultaneously provide transepithelial resistance to the movement of other ions and molecules. Figure 1 schematically illustrates possible Cl− transport mechanisms for epithelial cells in which both cystic fibrosis transmembrane regulator (CFTR) and CIC-2 Cl− channels are present in the apical membrane.

CIC-2 is a member of the CIC Cl− channel family, which consists of nine members that are widely distributed in nature (6, 20, 27, 40, 42). CIC-2 is found in the intestine (1, 5, 15, 23, 28), gastric parietal cells (26, 34), liver (3, 6, 8, 33, 35), retina (12), parotid acinar cells (30), cardiac tissue (4), and neuronal cells (36). CFTR is also a Cl− channel, and mutations in it lead to cystic fibrosis. CFTR is also widely distributed in the tissues (14, 21) and is found in the apical membrane of epithelial cells (31), including the intestine (5, 15, 29) and T84 intestinal cell lines (39). Intestinal epithelial cells are also known to contain Ca2+-activated Cl− channels (2).

Several studies have demonstrated the presence of CIC-2 in intestinal tissues. However, there may be species differences in the development of CIC-2 expression, function, and localization. For example, recent studies have demonstrated that the CIC-2 protein is present in the murine intestine, albeit not in the apical membrane but at the tight junction complexes (16). In adult rabbit stomach, CIC-2 mRNA is present in parietal cells and CIC-2 protein is present in the apical membrane of the parietal cell (34). Furthermore, immunostaining indicates that CIC-2 protein is present in the apical membrane of human intestine (23). In the present study, adult rabbit was used to study the relative abundance and distribution of CIC-2 mRNA in the intestine.

The presence of CIC-2 in the apical intestinal membrane suggests a possible functional role for CIC-2 in apical Cl− transport, although this remains to be fully elucidated. With the exception of Cl− transport studies in guinea pig intestine (5) and murine intestine (16), studies of CIC-2 in the intact intestines of other species have not been found in the literature. CIC-2-mediated Cl− transport has been studied in two human intestinal cell lines: Caco-2 (28) and T84 (1). Caco-2 cells...
contain CIC-2 protein, which contributes to Cl⁻ secretion, but the distribution of the protein in Caco-2 cells is at the tight junction (28). Reports of patch-clamp studies of CIC-2 in T84 cells suggest a role in Cl⁻ transport (1); however, immunostaining suggested that only a small subset of cells in the cultures contained CIC-2 when grown on glass coverslips (23). In the present study, the abundance and distribution of CIC-2 in T84 cells grown on permeable supports were investigated to determine whether T84 cells could be used to study the effects of SPI-0211 on CIC-2- and CFTR-mediated Cl⁻ transport.

CFTR is activated by PKA and inhibited by arachidonic acid (22), whereas CIC-2 activation can occur via PKA or arachidonic acid and other lipids in a PKA-independent manner (8, 38). Effects of SPI-0211 were studied in nontransfected human embryonic kidney (HEK)-293 cells and in HEK-293 cells stably transfected with human CIC-2 or human CFTR. To differentiate the effects on CFTR and CIC-2, experiments were performed in the presence and absence of myristoylated protein kinase inhibitor (mPKI), a specific PKA inhibitor.

This study shows that SPI-0211 is a potent PKA-independent activator of human CIC-2 (not CFTR), suggesting that CIC-2 may be a target for the beneficial effects of SPI-0211 (18, 19). These findings also suggest an important physiological role for CIC-2 in intestinal fluid transport.

MATERIALS AND METHODS

Materials. SPI-0211 was obtained from Suncam Pharmaceuticals (Sanda, Japan) as frozen aliquots of either 100 μM or 2 mM solutions in 100% DMSO. DMSO, also obtained as frozen aliquots from Suncam Pharmaceuticals, was used to dilute the SPI-0211 and when testing for vehicle effects. AlexaFluor 488-labeled goat-anti-chicken antibody and AlexaFluor 488-labeled phalloidin were obtained from Molecular Probes (Eugene, OR). MEM, Lipofectamine, hygromycin, pCEP vector, G-418, pcDNA3.1, DMEM/Ham’s F-12 medium, and the SuperScript preamplification system and random primers DNA labeling kit were purchased from Invitrogen (Carlsbad, CA). HEK-293 cells were obtained from the American Type Culture Collection (Manassas, VA). Forskolin and mPKI were obtained from Calbiochem-Novabiochem (San Diego, CA), isobutylmethyl xanthine (IBMX); a phosphodiesterase inhibitor) and nystatin were purchased from Sigma-Aldrich (St. Louis, MO), and arachidonic acid was obtained from Avanti Polar Lipids (Alabaster, AL). 1-Ethyl-2-benzimidazolone-1 (EBIO) was purchased from Tocris Cookson (Ellisville, MO). Oligotex Direct mRNA columns were obtained from Qagen (Valencia, CA), and Takara Ex-Tag DNA polymerase was purchased from Fisher Scientific. Borsilicate glass (no. 7052) was obtained from Garner Glass (Claremont, CA). Chicken anti-CIC-2 antibody (3) was a kind gift from Dr. Carol J. Blaisdell (Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD).

Rabbit tissues. Rabbits were anesthetized with sodium pentobarbital according to an Institutional Animal Care and Use Committee-approved animal protocol. Tissues were removed and rapidly frozen in liquid N₂ for mRNA extraction or fixed in 4% paraformaldehyde and embedded in paraffin for in situ hybridization.

Detection of CIC-2 by RT-PCR. Detection of mRNA was performed essentially as previously described (34, 35). Briefly, mRNA was prepared from rapidly frozen rabbit brain, heart, skeletal muscle, large intestine, small intestine, gastric mucosa, and lung using Oligoex Direct mRNA columns. The mRNA (2 μg) from each tissue was pretreated with DNase I and then first-strand cDNA was synthesized using the SuperScript preamplification system. The cDNA was amplified using Ex-Tag DNA polymerase and sequence-specific primers for CIC-2 and β-actin. PCR conditions for amplification of a 468-bp cDNA fragment of the CIC-2 COOH-terminal cytoplasmic domain consisted of 35 cycles of denaturing for 45 s at 94°C, annealing for 45 s at 61°C, and elongating for 2 min at 72°C. To compare the relative levels of CIC-2 mRNA across tissues, a 669-bp cDNA fragment of human β-actin was amplified. PCR conditions for β-actin consisted of 35 cycles of denaturing for 45 s at 94°C, annealing for 45 s at 57°C, and elongating for 2 min at 72°C.

Northern blot analysis. Northern blot analysis was performed as previously described (34, 35). The mRNA was extracted from rapidly frozen adult rabbit tissues (kidney, bladder, small intestine, and large intestine). The mRNA (2–5 μg) from various rabbit tissues was resolved in 1.2% agarose-formaldehyde gel and transferred to a nylon membrane. Rabbit CIC-2 cDNA was excised from pcDNAIII, gel purified, 32P labeled using a random primers DNA labeling kit, and used to screen the blot under medium stringency. A 1.8-kb fragment of human β-actin was amplified. PCR conditions for β-actin consisted of 35 cycles of denaturing for 45 s at 94°C, annealing for 45 s at 57°C, and elongating for 2 min at 72°C.

In situ hybridization. In situ hybridization was performed as previously described (34, 43). Sections were loaded onto saline slides, pretreated with protease K, and acetylated immediately before use (43). A 261-bp cDNA fragment of the CIC-2 COOH-terminal domain was used as a template for the preparation of antisense and sense 35S-labeled cRNA fragments. Hybridization was performed as described previously (34, 43) at 55°C, followed by high-stringency washes. Autoradiography was performed for 10–42 days, and results were analyzed with a Nikon microscope and a dark-field filter. Tissue sections were then stained with hematoxylin and eosin and viewed under bright field.

Cell culture. T84 cells obtained from Dr. Jeffrey B. Matthews (Department of Surgery, University of Cincinnati, Cincinnati, OH) (37) were grown in DMEM/Ham’s F-12 medium with 6% heat-inactivated FBS, 15 mM HEPES, 14.3 mM NaHCO₃, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. T84 cells were grown to confluence, either on 0.3-cm² BioCoat collagen-coated permeable supports (Discovery Labware, Bedford, MA) or on 1.13-cm² collagen-coated Snapwell permeable supports (Corning, Corning, NY). CIC-2-transfected HEK-293 cells were prepared as described by Tewari et al. (38). HEK-293 cells were transfected with His- and T7-tagged human CFTR.
ClC-2 cDNA in pcDNA3.1 using Lipofectamine according to the manufacturer’s instructions. Cells were selected with 300 μg/ml G418. HEK-293 cells were also stably transfected using Lipofectamine with wild-type, expressible human CFTR cDNA in pCEP vector obtained from Dr. Mitch Drumm (Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, OH) (25). Stable transfectants were selected using hygromycin (100 μg/ml). Both ClC-2-transfected and CFTR-transfected HEK-293 cells were grown in MEM supplemented with 5% heat-inactivated horse serum, 0.1 mM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

**Immunofocal microscopy.** Confluent T84 cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 20 min, and washed three times in PBS. Cells were blocked with 1% BSA (RIA grade), 0.05% Tween 20, and 10% goat serum in PBS for 30 min; incubated with chicken anti-CIC-2 antibody (1:300) for 1 h (3) at 22°C; and washed three times for 5 min each with PBS containing 0.05% Tween 20. Cells were then stained for 1 h at 22°C with AlexaFluor 546-labeled goat anti-chicken antibody (1:1,000 dilution), with CIC-2 staining red and AlexaFluor 488-labeled phaloidin (20 U/ml) with F-actin staining green. After three washes as described above, the cells were mounted with GelMount (Biomeda, Foster City, CA) and examined using an LSM 510 Zeiss confocal microscope with argon (488 nm excitation) and HeNe (546 nm excitation) lasers, an Axioplan upright microscope, and a 63× oil objective. X-z and x-y scans were obtained. LSM 510 software was used to process the digitized images obtained.
from Physiologic Instruments (San Diego, CA), was used for normalized to the resistance of the monolayer was monitored with an EVOM epithelial voltohmmeter. Transepithelial resistance of T84 cells was increased from 0.5 to 2 mM to account for chelation of CaCl2 was increased from 0.5 to 2 mM to account for chelation of NaCl and CaCl2 was increased to 4 mM to account for the chelation of Ca2+ by gluconate. In some experiments, an apical-to-basolateral Cl− gradient was imposed. Under these conditions, the Cl− conductance across the apical membrane is directly proportional to the Ie (24). To examine effects on the basolateral membrane, the apical membrane was permeabilized with nystatin and a basolateral-to-apical Cl− gradient was imposed. In preliminary studies, Ie was measured in T84 cells treated with test drug and a final DMSO concentration of 1%. Under these conditions, it was determined that DMSO caused damage to the permeable monolayer support filters. Therefore, final DMSO concentrations were adjusted to 0.1% in subsequent experiments.

Patch-clamp measurement of whole cell Cl− currents. Patch-clamp and analytical methods were described previously (8, 13, 17, 38). Currents were elicited by voltage-clamp pulses (1,500 ms duration) between +40 and −140 mV in 20-mV increments from a beginning holding potential of −30 mV. Currents were averaged over a 50-ms time course starting at 50 ms and ending at 100 ms. The external solution was normal Tyrode solution containing (in mM) 135 NaCl, 1.8 CaCl2, 1 MgCl2, 5.4 KCl, 10 glucose, and 10 HEPES (pH 7.4 or as indicated). The pipette solution contained (in mM) 130 CsCl, 1 MgCl2, 5 EGTA, 10 HEPES (pH 7.4), and 1 ATP-Mg2+ (pH 7.4). When ATP-Mg2+ (1:1 dilution) was made, 2 mM Cl− was added. These solutions would have a theoretical reversal potential of approximately −3 mV using activity coefficients of pure NaCl and pure CsCl in the bath and pipette solutions. Pipettes were prepared from borosilicate glass and pulled by a two-stage Narashige puller to produce 1- to 1.5-MΩ resistance. Data were acquired with an Axopatch CV-4 headstage, a Digidata 1200 digitizer, and an Axopatch 1D amplifier. Data were analyzed using pClamp 6.04 (Axon Instruments, Union City, CA), Lotus 1-2-3 (IBM, White Plains, NY), and Origin software (OriginLab, Northampton, MA). Initially, 100 µM SPI-0211 was diluted in DMSO to deliver 1 µM SPI-0211 and a final DMSO

Short-circuit current measurements. Two setups were used. Initially, a Plexiglas chamber was used for short-circuit current (Isc) measurements across confluent T84 cell monolayers grown on 0.3-cm² permeable support filters (World Precision Instruments, Sarasota, FL). Electrical measurements were obtained with a 742C voltage-clamp device (Department of Biomedical Engineering, University of Iowa, Iowa City, IA). The temperature was held constant at 37 °C by circulating heated water through the water jacket of the chamber. The output of the amplifier was plotted on an analog chart recorder. Background current was subtracted from Ie after the addition of compound, and Ie was then normalized to the filter area (0.3 cm²). With the use of this Ie setup, Ie measurements in T84 cells were performed as described by Loffing et al. (24). The basolateral membrane solution contained (in mM) 116 NaCl, 24 NaHCO3, 3 KCl, 2 MgCl2, 0.5 CaCl2, 3.6 sodium-HEPES, 4.4 hydrogen-HEPES (pH 7.4), and 10 glucose. The apical membrane bath solution was identical, with the exceptions that the Cl− concentration was reduced by substituting sodium gluconate for NaCl and CaCl2 was added from 0.5 to 2 mM to account for the chelation of Ca2+ by gluconate and glucose was omitted. Both solutions were bubbled with 95% O2-5% CO2, which also served to help mix the solutions. In all cases in which this setup was used, the basolateral membrane was permeabilized with 200 µg/ml nystatin.

Subsequently, the EasyMount Ussing chamber system (6 chambers) with VCCMC6 multichannel current-voltage (I-V) clamps, purchased from Physiologic Instruments (San Diego, CA), was used for Isc measurements across confluent T84 cell monolayers. Transepithelial resistance of T84 cells was measured with an EVOM epithelial voltohmmeter (World Precision Instruments). Cells were used when the transepithelial resistance of the monolayer was >1,200 Ω. For Isc measurements, special sliders (Physiologic Instruments) were used for Snapwell cell culture inserts (1.13 cm²). The solutions were continuously gassed with 95% O2-5% CO2, as well as passed stirring, and the temperature was held constant at 37 °C with a heating block. The clamps were connected to Acquire and Analyse software (Physiologic Instruments) for automatic data collection from all six of the Ussing chambers. Ag/AgCl reference electrodes were used for measuring transepithelial voltage and passing current. Isc measurements were performed as described by DeVor et al. (10). For measurements in intact T84 cells, symmetrical bath solutions were used. Basolateral and apical membrane bath solutions contained (in mM) 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 MgCl2, and 1.2 CaCl2 (pH 7.4). The basolateral solution had 10 mM glucose. To ensure the absence of any Na+–glucose cotransport, the apical solution contained 10 mM mannitol (7). To examine effects on the apical membrane alone, the basolateral membrane was permeabilized with 200 µg/ml nystatin and a basolateral-to-apical Cl− gradient was imposed by using an apical membrane bath solution that was identical, except that the Cl− concentration was reduced by substituting sodium gluconate for NaCl and CaCl2 was added from 0.5 to 2 mM to account for the chelation of Ca2+ by gluconate. In some experiments, an apical-to-basolateral Cl− gradient was imposed. Under these conditions, the Cl− conductance across the apical membrane is directly proportional to the Isc (24). To examine effects on the basolateral membrane, the apical membrane was permeabilized with nystatin and a basolateral-to-apical Cl− gradient was imposed. In preliminary studies, Isc was measured in T84 cells treated with test drug and a final DMSO concentration of 1%. Under these conditions, it was determined that DMSO caused damage to the permeable monolayer support filters. Therefore, final DMSO concentrations were adjusted to 0.1% in subsequent experiments.

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Fig. 3. Immunoconfocal microscopy of ClC-2 Cl− channels in the human T84 gastrointestinal epithelial cell line. Confluent T84 cells grown on permeable supports were fixed and permeabilized. Cells were stained with chicken anti-CIC-2 antibody and AlexaFluor 546-labeled goat anti-chicken antibody, which stains CIC-2 red, and with AlexaFluor 488-labeled phalloidin, which stains F-actin green. A: x-z scan through the cells (arrow in B) from the basolateral side (top) to the basolateral side (bottom, medium and support side). C: anti-CIC-2 antibody omitted. In A, B, and C, top images show CIC-2 staining, middle images show F-actin staining, and bottom images show overlays of CIC-2 and F-actin staining. CIC-2 colocalizes with actin at the apical surface (yellow) of the T84 cells.
RESULTS

Identification, distribution, and localization of CIC-2 mRNA and protein in the rabbit gastrointestinal tract and in human T84 cells. To determine whether CIC-2 was present in adult rabbit tissues, RT-PCR was used. A 459-bp fragment of CIC-2 and a 669-bp fragment of β-actin were amplified. β-Actin was used to control for the amount of cDNA used. As shown in Fig. 2A, CIC-2 mRNA was present in both the large and small intestines as well as in gastric mucosa, brain, heart, skeletal muscle, and lung. Northern blotting and in situ hybridization were performed to determine the transcript sizes, verify the RT-PCR results, and localize the mRNA. As shown in Fig. 2B, the major species detected in the small intestine and large intestine by Northern blotting is a 3.4-kb mRNA transcript. In Fig. 2C, in situ hybridization shows CIC-2 mRNA in the epithelial cells lining both the small and large intestine. No specific labeling was seen with the sense control riboprobe. Thus CIC-2 mRNA is present in the adult rabbit intestine. T84 cells, a human intestinal cell line, were then screened for the presence and localization of CIC-2 protein by immunofluorescence microscopy, and the results are shown in Fig. 3. In confluent monolayers of T84 cells grown on permeable supports, CIC-2 Cl⁻ channel protein was stained red and F-actin, staining green, outlines the cells. Fig. 3A shows the x-z scan obtained

concentration of 1%. In later experiments, 2 nM SPI-0211 was diluted in DMSO to deliver 1 nM-10 μM SPI-0211 and a final DMSO concentration of 0.1%; the specific conditions are indicated for individual experiments. To determine maximal Cl⁻ currents in transfected HEK-293 cells, either 5 μM forskolin (a PKA activator) plus 20 μM IBMX or 1 μM arachidonic acid was added at the end of experiments. Forskolin/IBMX stimulates CIC-2 via a PKA-dependent mechanism, while stimulation of CIC-2 by arachidonic acid is PKA independent (8, 38). Each of these treatments increased the final DMSO concentration by no more than 0.1%. In some studies evaluating the role of PKA in CIC-2 activation, 0.4 μM mPKI was added to inhibit PKA before activation with test agents.

Statistical significance. The statistical significance of the difference between two means was determined using Student’s t-test. Origin 5.0 software was used to fit curves and linear plots.

Fig. 5. Effect of varying SPI-0211 concentration on Iₑₑ or ΔIₑₑ (B) in nystatin-permeabilized T84 cells. A: effect of 20 nM and 250 nM SPI-0211 (added to both sides) on Iₑₑ measured in T84 cells with the basolateral membrane permeabilized with nystatin (200 μg/ml) and a basolateral-to-apical Cl⁻ gradient imposed. DMSO controls were subtracted. Means ± SE (n = 9) are plotted. *P < 0.001 apical vs. basolateral Iₑₑ.

Fig. 4. Effect of 1-ethyl-2-benzimidazolinone (1-EBIO) and SPI-0211 (lubiprostone) on short-circuit current (Iₑₑ) in intact T84 cells and effect of SPI-0211 on Iₑₑ in nystatin-permeabilized T84 cells. A: Iₑₑ of intact T84 cells was allowed to equilibrate and then was measured after addition of 300 μM 1-EBIO. When the maximum Iₑₑ response was reached, 1 μM SPI-0211 was added. Iₑₑ was normalized to area. Both compounds were added to both sides. DMSO controls are also shown. Data are means ± SE. B, C, DMSO (n = 6) with apical-to-basolateral Cl⁻ gradient; ●, 1-EBIO (n = 11), followed by DMSO (n = 3); ●, 1-EBIO (n = 11), followed by SPI-0211 (n = 8). *P < 0.001 vs. DMSO. #P < 0.01 vs. DMSO. B: Iₑₑ was allowed to equilibrate, and then nystatin (200 μg/ml) was added to the basolateral membrane. A Cl⁻ gradient was then imposed either apically to basolaterally or vice versa. After 10-min equilibration with nystatin, 1 μM SPI-0211 was added to both sides. When the response to SPI-0211 reached its maximum, 300 μM 1-EBIO was added to both sides. DMSO (0.1%) controls are also shown. Data are means ± SE. ●, DMSO (n = 8); ○, DMSO (n = 6) with apical-to-basolateral Cl⁻ gradient; ●, SPI-0211 (n = 7); □, DMSO (n = 5) with basolateral-to-apical Cl⁻ gradient. C: bar graph comparing the effect of SPI-0211 on Iₑₑ across apical membrane (A) (closed bar, basolateral membrane permeabilized with nystatin) and basolateral membrane (BL) (open bar, apical membrane permeabilized with nystatin) of T84 cells with a basolateral-to-apical Cl⁻ gradient imposed. DMSO controls were subtracted. Means ± SE (n = 9) are plotted. *P < 0.001 apical vs. basolateral Iₑₑ.
**Table 1. Kinetic analysis of ΔI<sub>sc</sub> measured across monolayers of T84 cells in response to varying concentrations of SPI-0211**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>ΔI&lt;sub&gt;sc max&lt;/sub&gt;, μA/cm²</th>
<th>y²</th>
<th>R</th>
<th>P value</th>
<th>Hill coefficient</th>
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<tr>
<td>Hyperbolic plot</td>
<td>18.24±3.72 (11)</td>
<td>77.82±5.04 (11)</td>
<td>37.39</td>
<td>0.982±0.001 (10)</td>
<td>&lt;0.005</td>
<td>1.14±0.08 (8)</td>
</tr>
<tr>
<td>Lineweaver-Burk plot</td>
<td>18.82±3.55 (3)</td>
<td>75.88±6.91 (3)</td>
<td></td>
<td>0.985±0.026 (8)</td>
<td>0.0001</td>
<td></td>
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<tr>
<td>Hill plot</td>
<td></td>
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Modified Michaelis-Menten hyperbolic fit used the equation \( \Delta I_{sc} = \frac{\text{I}_{sc} \text{max}}{\text{EC}_{50} + \text{[SPI-0211]}} \), where \( \Delta I_{sc \text{ max}} \) is predicted maximum change in \( I_{sc} \) and EC<sub>50</sub> is SPI-0211 concentration ([SPI-0211]) required for half-maximal response. The analysis is constrained to 0 because change in \( I_{sc} \) was measured. Results are means ± SE (no. of data points). R is correlation coefficient.

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across four cells, indicated by the arrow in Fig. 3B. The ClC-2 protein was localized at or near the apical membrane in the bulk of the T84 cells. The very small amount of punctate staining in the basolateral membranes is likely to be insignificant in relation to the major staining observed at the apical membrane. As shown in Fig. 3C, when the primary antibody to ClC-2 was omitted, staining was virtually eliminated, demonstrating specificity of the antibody. The small amount of very low red staining is likely secondary antibody trapped in tissue spaces.

**I<sub>sc</sub> studies.** The effect of SPI-0211 on transepithelial Cl⁻ transport of T84 cells was then studied. The cells were grown on permeable supports to form a monolayer and mounted in an Ussing chamber, and \( I_{sc} \) was measured and normalized to area. Figure 4A shows the effect on \( I_{sc} \) of 300 μM 1-EBIO followed by 1 μM SPI-0211. Both compounds (both lipophilic) were added to the apical and basolateral sides, and because they were dissolved in DMSO, the appropriate DMSO controls are also shown. 1-EBIO caused a large, sustained increase in \( I_{sc} \) (\( P < 0.001 \) vs. DMSO) as previously reported (9). SPI-0211 caused a further large, significant, and sustained increase in \( I_{sc} \) (\( P < 0.001 \) vs. 1-EBIO + DMSO), while DMSO alone had no effect. The current measured (\( I_{sc} \)) is a measure of transepithelial Cl⁻ transport (9). Thus both 1-EBIO and SPI-0211 caused increased transepithelial Cl⁻ transport. The effect of 1-EBIO on apical Cl⁻ transport is due to the opening of a basolateral Ca<sup>2+</sup>-activated K⁺ channel and activation of CFTR (9, 11). In further experiments, the effect of SPI-0211 added to either the apical or the basolateral membrane bath solutions was examined. Over the time course observed (15 min), SPI-0211 was slightly more effective (\( P < 0.05 \)) when added to the apical side [change in \( I_{sc} (\Delta I_{sc}) = 72.16 ± 4.37 \) μA/cm² (\( n = 6 \))] than it was when added to the basolateral side [\( \Delta I_{sc} = 56.15 ± 4.44 \) μA/cm² (\( n = 6 \))].

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Fig. 6. Effect of 1 μM SPI-0211 on Cl⁻ channel currents in ClC-2-transfected and nontransfected human embryonic kidney (HEK)-293 cells. A: representative whole cell Cl⁻ currents in HEK-293 cells stably transfected with recombinant human ClC-2 Cl⁻ channels without [control (C)] and with 1 μM SPI-0211 treatment and subsequently treated with 5 μM forskolin/20 μM IBMX (F/I) and then with 1 μM arachidonic acid (AA). B: representative whole cell patch-clamp Cl⁻ currents in nontransfected HEK-293 cells before and after treatment with 1 μM SPI-0211. C: current-voltage (I-V) curves for the Cl⁻ currents shown in A normalized to cell capacitance (proportional to cell membrane area) and expressed as pA/pF. Data are means ± SE (\( n = 3 \)) of cells with no treatment (control) and cells subsequently treated with 1 μM SPI-0211 (●), with 5 μM F/I (●), or with 1 μM AA (●). I-V curves for nontransfected cells are not shown. D: summarized data for normalized slope conductance (ns) for data presented in A–C. Data are means ± SE (\( n = 3 \)). * \( P < 0.001 \) vs. control. # \( P < 0.01 \) vs. control.
obtained with a reversed (apical to basolateral) Cl\(^{-}\) gradient, together with results (24). The results are shown in Fig. 4B.

Fig. 7. Effect of myristoylated protein kinase inhibitor (mPKI) on SPI-0211-treated whole cell Cl\(-\) currents in T84 cells. However, because T84 cells contain both ClC-2 and CFTR channels in T84 cells. However, because T84 cells contain both ClC-2 and CFTR channels and perhaps other channels in their apical membranes, more definitive studies were pursued to evaluate the effects of SPI-0211 on human CIC-2 and human CFTR separately using HEK-293 cells stably transfected with their apical membranes, more definitively. Patch-clamp studies (17) were used to study whole cell Cl\(-\) currents (8, 13, 38).

Patch-clamp studies. Patch-clamp studies were performed in both CIC-2-transfected and nontransfected HEK-293 cells after the addition of 1 \(\mu\)M SPI-0211 with a final concentration of 1% DMSO. Current recordings are shown in Fig. 6, A and B; I-V relationships normalized to cell capacitance are shown in Fig. 6C; and the slope conductance is shown in Fig. 6D. Nontransfected HEK-293 cells exhibited minimal endogenous Cl\(-\) channel currents. For these studies, patch-clamp methodologies (17) were used to study whole cell Cl\(-\) currents (8, 13, 38).

To investigate whether the SPI-0211 effect was on an apical Cl\(-\) conductance, the basolateral membrane was permeabilized with nystatin (a polyene antibiotic that allows the movement of ions without the loss of larger molecules, such as ATP, from the cell) and a Cl\(-\) gradient was imposed by reduction of the Cl\(-\) concentration in the solution bathing the apical membranes (24). Under these conditions, the Cl\(-\) conductance across the apical membrane is directly proportional to the Cl\(-\) conductance in CIC-2-transfected HEK-293 cells. The normalized slope conductance (NSpF) is shown. Data are means ± SE (n = 3). A: effect of sequential addition of SPI-0211, F/I, and AA in the absence and presence of mPKI. B: effect of mPKI followed by F/I and then SPI-0211 compared with the effect of mPKI followed by SPI-0211 and then F/I. mPKI, 0.4 \(\mu\)M; SPI-0211, 1 \(\mu\)M; F/I, 5 \(\mu\)M/20 \(\mu\)M; AA, 1 \(\mu\)M. *P < 0.001 with respect to control. #P < 0.05 with respect to control.

Fig. 7. Effect of myristoylated protein kinase inhibitor (mPKI) on SPI-0211-activated Cl\(^{-}\) channel conductance in CIC-2-transfected HEK-293 cells. The normalized slope conductance (NSpF) is shown. Data are means ± SE (n = 3). A: effect of sequential addition of SPI-0211, F/I, and AA in the absence and presence of mPKI. B: effect of mPKI followed by F/I and then SPI-0211 compared with the effect of mPKI followed by SPI-0211 and then F/I. mPKI, 0.4 \(\mu\)M; SPI-0211, 1 \(\mu\)M; F/I, 5 \(\mu\)M/20 \(\mu\)M; AA, 1 \(\mu\)M. *P < 0.001 with respect to control. #P < 0.05 with respect to control.

then added and had no significant effect, indicating that basolateral membrane permeabilization with nystatin was complete. These results suggest that SPI-0211 is activating a Cl\(^{-}\) conductance in the apical membrane of T84 cells. To further rule out basolateral effects, the effect of SPI-0211 on apical and basolateral membrane conductance was examined and compared. I\(_{sc}\) was measured using a basolateral-to-apical Cl\(^{-}\) gradient, with either the basolateral membrane or the apical membrane permeabilized with nystatin. Figure 4C shows that SPI-0211 increased apical I\(_{sc}\) (ΔI\(_{sc}\) = 80.7 ± 6.3 nA/cm\(^2\); n = 9) and had virtually no effect on basolateral I\(_{sc}\) (ΔI\(_{sc}\) = 4.4 ± 2.5 nA/cm\(^2\); n = 9). The effect of varying SPI-0211 concentrations on I\(_{sc}\) after nystatin permeabilization of the basolateral membrane was then examined. Figure 5A shows I\(_{sc}\) traces after addition of 20 and 250 nM SPI-0211, and Fig. 5B shows ΔI\(_{sc}\) normalized to area plotted as a function of SPI-0211 concentration and both data sets (measured on the 2 different I\(_{sc}\) apparatuses) were used. The modified Michaelis-Menten hyperbolic plot was used to fit the data with the equation ΔI\(_{sc}\) = ΔI\(_{sc}\)max [(SPI-0211)/(EC\(_{50}\) + [SPI-0211])]. The analysis is constrained to 0, except for the change in conductance obtained from both hyperbolic and Lineweaver-Burk plots, were similar: EC\(_{50}\) = 18.24 ± 3.72 nM and ΔI\(_{sc}\)max = 77.82 ± 5.04 nA/cm\(^2\); (n = 11) from the hyperbolic plot, and EC\(_{50}\) = 18.82 ± 3.55 nM and ΔI\(_{sc}\)max = 75.88 ± 6.91 nA/cm\(^2\); (n = 3) from the Lineweaver-Burk plot. The hyperbolic plot fit the data very well (P < 0.005): χ\(^2\) = 37.39. The Hill coefficient was 1.14 ± 0.08 (n = 8), indicating not only that the modified Michaelis-Menten hyperbolic plot is the appropriate analysis but also that the simple bimolecular first-order kinetic model is correct. These data indicate that SPI-0211 is a potent activator of apical Cl\(^{-}\) channels in T84 cells. However, because T84 cells contain both CIC-2 and CFTR Cl\(^{-}\) channels and perhaps other channels in their apical membranes, more definitive studies were pursued to evaluate the effects of SPI-0211 on human CIC-2 and human CFTR separately using HEK-293 cells stably transfected with either CIC-2 (8, 38) or CFTR. Nontransfected HEK-293 cells exhibit minimal endogenous Cl\(^{-}\) channel currents. For these studies, patch-clamp methodologies (17) were used to study whole cell Cl\(^{-}\) currents (8, 13, 38).
3) in the presence or absence of 1% DMSO. Arachidonic acid alone (1
M in 1% DMSO) increased ClC-2 channel conductance to 0.694 ± 0.058 nS/pF (n = 3). These results indicate that 1 M SPI-0211 alone fully activated ClC-2 Cl
de channel currents. To examine whether PKA was mediating the activating effect of SPI-0211, the effect of mPKI, the cell-permeant PKA inhibitor, on SPI-0211 activated ClC-2 Cl
de channel currents was investigated. As shown in Fig. 7, mPKI did not inhibit the effect of SPI-0211, which was at a maximum because no further activation with arachidonic acid was observed. However, mPKI did prevent activation by forskolin/
IBMX (Fig. 7B) as previously shown (38). Therefore, SPI-0211 effects on ClC-2 Cl
de channels are not mediated by PKA.

The effect of increasing concentrations of SPI-0211 on Cl
de channel currents in ClC-2-transfected HEK-293 cells was then examined. These studies were designed such that each cell would be treated with progressively increasing SPI-0211 concentrations. Preliminary studies (data not shown) indicated that accumulating DMSO increased EC50 without affecting the maximum velocity. Thus DMSO concentrations were minimized and kept constant by preparing each SPI-0211 concentration to yield a final DMSO concentration of 0.1%. This was accomplished by washing three times with 2 ml of medium before addition of the next higher SPI-0211 concentration. This procedure was used because, as shown in Fig. 8, A–C, SPI-0211 could be washed out, and then the same effect was observed upon reapplying the same concentration. This result also indicates that SPI-0211 effects are reversible. In Fig. 9, the change in ClC-2 Cl
de channel current normalized to capacitance is plotted as a function of SPI-0211 concentration. The modified Michaelis-Menten hyperbolic plot was used to fit the data using the equation 

\[
I = \frac{I_{\text{max}}}{EC_50 + [SPI-0211]}.
\]

Lineweaver-Burk and Hill plots of the data are also plotted as insets in Fig. 9. Table 2 shows that the EC50 and I_{max} maximum predicted change in current (or transport rate) obtained from both hyperbolic and Lineweaver-Burk plots were not significantly different from each other: EC50 = 24.38 ± 5.80 nM and I_{max} = 56.99 ± 3.97 pA/pF (n = 7) from the hyperbolic plot, and EC50 = 17.27 ± 0.53 nM and I_{max} = 50.78 ± 2.66 pA/pF (n = 3) from the Lineweaver-Burk plot. The Hill coefficient was 0.71 ± 0.06 (n = 5). These values are similar to those obtained in the IC_{50} experiments shown in Fig. 6B and Table 1. Based on a Hill coefficient of 1, the modified Michaelis-Menten hyperbolic plot is the appropriate analysis to use, and the simple bimolecular first-order kinetic model is correct.

The above studies with recombinant ClC-2 not only suggested that ClC-2 is a target for SPI-0211 but also strongly
EC 50 is required for half-maximal response. The analysis is constricted to 0 because change in DMSO. Data were cell was then treated with the next higher concentration of SPI-0211 in 0.1% changes of medium were performed to wash away SPI-0211 and DMSO. The insets Origin 5.0 software. Lineweaver-Burk and Hill plots are shown as insets. All parameters calculated are in Table 2.

suggested that the SPI-0211 effects observed with T84 cells could be mediated by effects on CIC-2. However, because T84 cells also contain CFTR, the possibility existed that CFTR might also be a target for SPI-0211. To evaluate this possibility, effects of SPI-0211 on whole cell Cl− currents of HEK-293 cells stably transfected with CFTR rather than CIC-2 were examined. Figure 10 demonstrates that SPI-0211 did not increase the CFTR-mediated Cl− channel current or conductance. In contrast, and as expected, forskolin/IBMX significantly increased CFTR-mediated Cl− channel current and conductance. Therefore, CFTR does not appear to be a target of SPI-0211.

DISCUSSION

The central hypothesis tested in this study was whether an intestinal CIC-2 Cl− channel was responsible for the increased intestinal Cl− observed in SPI-0211 treated animals (41) and for the beneficial effects of SPI-0211 seen in human clinical trials (18, 19). mRNA for CIC-2 was present in the adult rabbit intestine as measured by RT-PCR, Northern blot analysis, and in situ hybridization. The human intestinal T84 cell line was shown to contain CIC-2 uniformly in the apical membrane when grown to confluence on permeable supports. Icsc measurements were used to determine EC50 for SPI-0211 in T84 cells. HEK-293 cells stably expressing human CIC-2 had been used in our laboratory for a number of years (8, 38), and HEK-293 cells stably expressing CFTR were developed for the present study. SPI-0211 did not activate CFTR-mediated Cl− conductance but was shown, using whole cell patch clamp, to activate CIC-2 with an EC50 similar to that observed with activation of Icsc (apical Cl− conductance) in T84 cells. SPI-0211 activation of CIC-2 was shown to be independent of PKA because mPKI had no effect.

T84 cells have been used widely as a model system for the study of intestinal Cl− transport (1, 2, 37, 39). CIC-2 has been studied by patch clamp in T84 cells grown on plastic (1).

![Fig. 9. Effect of varying SPI-0211 concentration on Cl− currents in CIC-2-transfected HEK-293 cells. SPI-0211 concentration is plotted against the change in CIC-2 Cl− channel current, ∆I (pA/pF), measured by patch clamp at −140-mV holding potential, normalized to cell capacitance, and expressed as means ± SE (n = 3). After each addition of SPI-0211 in 0.1% DMSO, 3 changes of medium were performed to wash away SPI-0211 and DMSO. The cell was then treated with the next higher concentration of SPI-0211 in 0.1% DMSO. Data were fit with a modified Michaelis-Menten hyperbolic plot using Origin 5.0 software. Lineweaver-Burk and Hill plots are shown as insets. All parameters calculated are in Table 2.](image1)

![Fig. 10. Effect of SPI-0211 on Cl− channel currents in CFTR-transfected HEK-293 cells. Whole cell Cl− currents were measured in CFTR-transfected HEK-293 cells by patch clamp. A: I-V curves for Cl− currents normalized to cell capacitance: cells with no treatment (control, C), cells subsequently treated with 1 μM SPI-0211 (○), and then with F/I (△). B: summarized data for the normalized slope conductance (nS/pF) of the data presented in A. Data are means ± SE (n = 3). *P < 0.05 vs. control.](image2)

Table 2. Kinetic analysis of ∆I measured as Cl− channel current by patch clamp of human CIC-2 expressed in HEK-293 cells in response to varying concentrations of SPI-0211

<table>
<thead>
<tr>
<th>Analysis</th>
<th>EC50, nM</th>
<th>ΔI_{max}, pA/pF</th>
<th>χ²</th>
<th>R</th>
<th>P Value</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperbolic plot</td>
<td>24.38 ± 5.80* (7)</td>
<td>56.99 ± 3.97 (7)</td>
<td>15.80</td>
<td>0.996 ± 0.001 (6)</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>Lineweaver-Burk plot</td>
<td>17.27 ± 0.53* (3)</td>
<td>50.78 ± 2.66 (3)</td>
<td>0.990 ± 0.027 (5)</td>
<td>&lt;0.0001</td>
<td>0.71 ± 0.06 (5)</td>
<td></td>
</tr>
</tbody>
</table>

Modified Michaelis-Menten hyperbolic fit used the equation $\Delta I = \Delta I_{max} \cdot [SPI-0211] / [EC_{50} + [SPI-0211]]$, where $\Delta I_{max}$ is predicted maximum change in $I$ and $EC_{50}$ is [SPI-0211] required for half-maximal response. The analysis is constricted to 0 because change in $I$ was measured. Results are means ± SE (no. of data points). $R$ is correlation coefficient. *Not significantly different from each other.
Previous localization studies suggested that CIC-2 was present in the apical membrane of T84 cells grown on glass slides, but only in a small population of such cells (23). However, in the present studies of T84 cells grown to confluence on permeable supports, the CIC-2 Cl\(^-\) channel protein was expressed in the apical membrane and in virtually all cells. Therefore, these T84 cells can be used to study Cl\(^-\) transport by the \(I_{sc}\) technique. \(I_{sc}\) was studied in intact T84 cells and then in cells with a basolateral-to-apical Cl\(^-\) gradient and the basolateral membrane permeabilized by nystatin. Under the latter conditions, Cl\(^-\) conductance across the apical membrane is directly proportional to \(I_{sc}\) (24). As we have shown, transepithelial Cl\(^-\) transport in T84 cells is potently activated by SPI-0211. This effect is due to increased Cl\(^-\) transport across the apical and not the basolateral membrane. CFTR is also present in these cells (39). When correlated with the effect of SPI-0211 on recombinant CIC-2 and CFTR, the activation of Cl\(^-\) transport in T84 cells by SPI-0211 strongly suggests that non-CFTR Cl\(^-\) channels (likely CIC-2) in the apical membrane were responsible. Potent and selective inhibitors of CIC-2 do not exist, leaving open the question whether some non-CFTR Cl\(^-\) channel other than CIC-2 is also a target of SPI-0211 in T84 cells.

Effects of SPI-0211 on CIC-2 and CFTR were tested in HEK-293 cells transfected with recombinant human CIC-2 or CFTR Cl\(^-\) channels using whole cell patch clamp. HEK-293 cells have a low level of endogenous Cl\(^-\) currents (8, 13, 38) and have been used widely to study recombinant Cl\(^-\) channels (8, 13, 38) using patch-clamp techniques (17). In patch-clamp studies, it was demonstrated that SPI-0211 strongly stimulated CIC-2 channel currents but had no effect on CFTR channel currents. There was good agreement between the \(EC_{50}\) in T84 \(I_{sc}\) studies and in CIC-2-expressing HEK-293 patch-clamp studies, providing evidence that CIC-2 may be the target of SPI-0211 in T84 cells. Because CIC-2 is also known to be present in the human small and large intestines (23), these results were consistent with the therapeutic benefits of SPI-0211 being the result of activation of CIC-2-mediated Cl\(^-\) transport in intestinal epithelia.

In most studies, the extent of CIC-2 activation by SPI-0211 in CIC-2-expressing HEK-293 cells was assessed by comparison with other well-established activators of CIC-2, such as forskolin/IBMX or arachidonic acid (8, 38). When cells were treated with forskolin/IBMX and arachidonic acid after treatment with SPI-0211, CIC-2 activation levels were similar to those with SPI-0211 alone, indicating that all of these treatments activate the same protein, namely, CIC-2, and that SPI-0211 resulted in maximum activation of CIC-2. Experiments with mPKI evaluated the role of PKA in mediating CIC-2 activation by SPI-0211. In contrast to forskolin/IBMX, SPI-0211 activation of CIC-2 did not require PKA, as previously also shown to be the case with arachidonic acid (8, 38).

These studies show that SPI-0211 is a potent activator of CIC-2 Cl\(^-\) channels expressed in HEK-293 cells and Cl\(^-\) transport in T84 cells. No effects were observed on CFTR in these systems. Therefore, SPI-0211 activation of Cl\(^-\) transport in the human intestine (41) may occur through activation of the CIC-2 Cl\(^-\) channel. These properties suggest that SPI-0211 is an excellent candidate for the clinical treatment of many gastrointestinal syndromes. These studies also suggest that CIC-2 Cl\(^-\) channels may play a physiological role in Cl\(^-\) transport in the intestine. Further studies are needed to elucidate the mechanism by which SPI-0211 activates CIC-2 Cl\(^-\) channels.

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\(I_{sc}\) measurements using the \(L_{sh}\) chamber from World Precision Instruments were performed by Venkataraman Muthiah-Narakaran.

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DISCLOSURES

J. Cuppoletti, M. L. Patchen, and R. Ueno have financial interests in Sucampo Pharmaceuticals, Inc.

J. Cuppoletti serves as a consultant to, R. Ueno is an owner of, and M. L. Patchen is a former chief executive officer of Sucampo Pharmaceuticals, Inc.

REFERENCES


