A synthetic prostone activates apical chloride channels in A6 epithelial cells

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Departments of1Physiology and 2Pediatrics and 3The Center for Cell and Molecular Signaling, Emory University School of Medicine, Atlanta, Georgia; and 4Sucampo Pharmaceuticals, Inc., Bethesda, Maryland

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Bao HF, Liu L, Self J, Duke BJ, Ueno R, Eaton DC. A synthetic prostone activates apical chloride channels in A6 epithelial cells. Am J Physiol Gastrointest Liver Physiol 295: 234–251, 2008. First published May 29, 2008; doi:10.1152/ajpgi.00366.2007.—The bicyclic fatty acid lubiprostone (formerly known as SPI-0211) activates two types of anion channels in A6 cells. Both channel types are rarely, if ever, observed in untreated cells. The first channel type was activated at low concentrations of lubiprostone (<100 nM) in >80% of cell-attached patches and had a unit conductance of ~3–4 pS. The second channel type required higher concentrations (>100 nM) of lubiprostone to activate, was observed in ~30% of patches, and had a unit conductance of 8–9 pS. The properties of the first type of channel were consistent with CIC-2 and the second with CFTR. CIC-2’s unit current strongly inwardly rectified that could be best fit by models of the channel with multiple energy barrier and multiple anion binding sites in the conductance pore. The open probability and mean open time of CIC-2 was voltage dependent, decreasing dramatically as the patches were depolarized. The order of anion selectivity for CIC-2 was Cl > Br > NO3 > I > SCN, where SCN is thiocyanate. CIC-2 was a “double-barreled” channel favoring even numbers of levels over odd numbers as if the channel protein had two conductance pathways that opened independently of one another. The channel could be, at least, partially blocked by glibenclamide. The properties of the channel in A6 cells were indistinguishable from CIC-2 channels stably transfected in HEK293 cells. CFTR in the patches had a selectivity of Cl > Br >> NO3 ≈ SCN ≈ I. It outwardly rectified as expected for a single-site anion channel. Because of its properties, CIC-2 is uniquely suitable to promote anion secretion with little anion reabsorption. CFTR, on the other hand, could promote either reabsorption or secretion depending on the anion driving forces.

CIC-2; CFTR; single-channel recording; lubiprostone; SPI-0211

EPITHELIAL TISSUES ARE DESIGNED to form a barrier between two body compartments and to facilitate transport from one compartment to the other, thereby, regulating the composition of the compartments. In particular, most epithelia regulate the salt and water balance of their luminal compartment. Regulation of salt balance involves active transport of sodium (Na+) and chloride (Cl–), but, in eukaryotic organisms, water movement across epithelia is driven osmotically. That is, water movement depends on prior transport of osmotic equivalents, usually salt. Therefore, to regulate water balance requires the ability to regulate both salt reabsorption and salt secretion. An enormous amount of research (some performed by us) has examined the reabsorptive phase of the balance process, particularly that which involves reabsorption of Na+ through apical epithelial Na channels (ENaC) (8, 37, 57, 74, 82, 99).

But even for reabsorption, Na+ uptake is only part of the salt reabsorption equation: there must also be a pathway for the movement of anions. In the past, the Cl– channel protein, cystic fibrosis transmembrane conductance regulator (CFTR), which is defective in patients with cystic fibrosis, has been suggested as the pathway for anion movement in many epithelia. CFTR can certainly act as an entry pathway for Cl– entry into epithelial cells in parallel with Na+ entry through ENaC; however, fluid balance requires appropriate balance between salt reabsorption and salt secretion, but regulation of secretion has not been studied as extensively as reabsorption.

In salt-transporting epithelial cells, Cl– movement across the basolateral membrane occurs via an energy-dependent Na+/K+-2Cl– cotransporter and possibly Cl– channels (like the Cl– channel that leads to defective reabsorption in one form of Bartter’s Syndrome). Cl– movement across the apical (luminal) membrane occurs via specific Cl– channels. CFTR may be involved in secretion since when CFTR is defective there appears to be excess reabsorption in the lung and intestine. CFTR is widely distributed in tissues (43, 69) and is found in the apical membrane of epithelial cells (96), including the intestine (17, 49, 93), T84 intestinal cell lines (113), and lung (12). Nonetheless, in tissues that also transport Na+, CFTR is not particularly well suited to promote secretion (19), and, except for pathophysiological circumstances, other chloride channels may be more important for stimulated secretion. In particular, the Cl– channel, CIC-2, has been suggested as a route for chloride movement across the luminal membrane of several different epithelia. CIC-2 is a member of the CIC Cl– channel family, which consists of at least nine members that are widely distributed in nature (27, 65, 84, 111, 115). CIC-2 is found in the intestine (3, 17, 51, 72, 86), gastric parietal cells (77, 103), liver (98), lung (9, 27, 33, 101, 104), retina (38), and parotid acinar cells (95). However, the properties of CIC-2 appear different depending on the conditions and tissue in which the channels are examined. In the present study, we examined CIC-2 at a single-channel level in the A6 epithelial cell model and in HEK293 cells and examined the effect of a pharmacological agent, lubiprostone (which has been reported to open Cl– channels), on anion channels in A6 cells. Some investigators have suggested that the lubiprostone-mediated Cl– flux is mediated by the CIC-2 Cl– channel (15, 31). CIC-2-mediated Cl– transport has been studied in two human intestinal cell lines: Caco-2 (86) and T84 (3). Caco-2 cells contain CIC-2 protein, which contributes to Cl– secretion (86). Patch-clamp studies of CIC-2 in T84 cells also suggest a role in Cl– transport (3); however, immunostaining suggested that only a small subset of cells in the cultures contained CIC-2 when grown on glass coverslips (31, 72). Therefore, a second objective of this study was to determine whether one of the...
anion channels activated by lubiprostone in A6 cells is CIC-2 by examining the biophysical properties of the channels at a single-channel level.

This study shows that lubiprostone even at low doses is a potent activator of CIC-2, but at much higher doses, it also opens CFTR (to a lesser extent). These findings suggest a possible physiological role for CIC-2 in epithelial fluid transport.

METHODS

Cell culture. A highly transporting clone, 2F3, of the Xenopus laevis distal nephron epithelial cell line (A6) was a gift from Dr. Thomas Kleyman and was maintained by standard tissue culture techniques as previously described (6) with a culture medium consisting of a 50% (vol/vol) mix of DMEM and Ham's F-12 medium adjusted to amphibian tonicity plus 0.6% penicillin-1% streptomycin, 5% (vol/vol) fetal bovine serum, 1 mM aldosterone, and 25 mM NaHCO3. For patch-clamp experiments, A6 cells were plated at confluent density on permeable, glutaraldehyde-fixed, collagen-coated Millipore-CM filters (Millipore, Billerica, MA) attached to the bottoms of small Lucite rings (53). This side preparation allowed patch pipette access to the apical membrane and physical separation of the apical and basolateral bath compartments (5, 6, 21, 22, 53, 59, 60, 105–107, 120). Just prior to recording, monolayers were washed with a solution containing 96 mM NaCl, 3.4 mM KCl, 0.8 mM CaCl2, 0.8 mM MgCl2, 10 mM HEPES, or 10 mM Tris, adjusted to pH 7.4 with HCl or NaOH.

HEK293 stably transfected with human CIC-2 were a generous gift of John Cuppoletti and Danuta Malinowska. Untransfected HEK-293 cells were obtained from American Type Culture Collection (Bethesda, MD). Cells were grown under conditions described previously (31).

Lubiprostone was dissolved in spectroscopic grade DMSO to prepare a 1 mM stock solution that was kept at 4°C until diluted so that drug could be added to the apical bathing solution at various drug concentrations with a final DMSO concentration of 0.1% vol/vol. All cells were treated. Patch recording sometimes commenced prior to drug addition and continued after addition. In these cases, DMSO was added as a control for subsequent drug addition. Otherwise, cells were pretreated for 5 to 15 min with appropriate concentrations of drug before recording.

Transepithelial electrical measurements. The transepithelial voltage (Vte) and resistance (Rte) across cell monolayers were measured with an epithelial volt-ohmmeter (World Precision Instruments, Sarasota, FL). The transepithelial current (It) was calculated according to Ohm’s law It = Vte/Rte and normalized by the surface area of the insert. Amiloride (10 μM) was added to the apical side of the inserts at the end of each experiment, and amiloride-sensitive sodium current was calculated as the difference between total current and amiloride-insensitive current.

Patch-clamp recording and analysis. A6 distal nephron cells were visualized with Hoffman modulation optics (Modulation Optics, Greenvile, NY) mounted on a Nikon Diaphot-TMD inverted microscope. Unless otherwise noted, patch pipette and extracellular bath solutions consisted of a physiological amphibian saline containing (in mM): 95 NaCl, 3.4 KCl, 0.8 CaCl2, 0.8 MgCl2, and 10 HEPES or 10 Tris. The solutions were titrated with 0.1 N NaOH or HCl to a pH of 7.3–7.4. Experiments were performed at room temperature. Unitary channel events were measured using an Axopatch 1-D (Axon Instruments, Sunnyvale, CA). Data were acquired using a 902LPF 8-pole Bessel filter (Frequency Devices, Haverhill, MA), TL-2 acquisition hardware and Axotape software (Axon Instruments). By convention, outward flux of Cl−, an anion, produces inward current (pipette to cell) and is represented as downward transitions in single-channel records (inward flux produces outward current-upward deflections).

The total number of functional channels (N) in the patch was determined by observing the number of transitions in the single-channel recordings. As a measure of channel activity, the number of channels open was also calculated. With an estimate of N and a measure of NPo, we also were able to estimate open probability, Po.

Cell-attached patches were used for all experiments. The voltage conventions for patch potentials in epithelial monolayers require special consideration. For high-resistance epithelial monolayers, with the electrical ground in the basolateral compartment, the potential to which channels in the patch membrane are exposed are not simple displacements from the apical membrane potential, but rather, the potential of the pipette is the sum of the applied potential from the patch-clamp amplifier and the transepithelial potential (see Fig. 1). Under typical recording conditions the transepithelial potential depends on the tightness of the monolayer. In the presence of lubiprostone, the potential can be as high as −80 mV (apical potential with respect to the basolateral solution at ground), and, therefore, the potentials in the current-voltage relationships can be offset to the right by as much as 80 mV. The offset will vary depending on the transepithelial voltage, which in turn depends on the transepithelial current and the transepithelial resistance (the primary determinant of which is the shunt or edge resistance).

Data analysis. We used modified constant-field approaches first described by Goldman (48) and later by Hodgkin and Katz (56) to describe the permeability of anions through CIC-2 and CFTR channels. In general, their approach gave the ionic flux (as current) and ion permeability across a cell membrane as described by the following equation:

\[ I = P_e^{-1} \frac{[S]_e - [S]_o}{1 - \exp(-\frac{E_F}{RT})} \]

where I is the current and Pe is the permeability of ion S, [S]e and [S]o are the internal and external concentrations of ion S, z is the valence, and E is the potential difference across the cell membrane, and R, T, and F are constants.

For channels that rectify in a manner opposite that predicted by the above equation, more complicated models that are based on Eyring rate theory are necessary to fit the data. We used three different models: a single-barrier model (109), a two-barrier, one-well model (79), or a three-barrier, two-well model (7, 14, 54). We fit the data to these models using a Marquardt-Levenberg minimization algorithm as provided by the software companion to (97). To avoid local minima, we fit each model 200 different times with randomly selected initial parameters and determined the least square minimum.

Regardless of which method was used model the current, selectivity was determined by the Goldman voltage equation with known concentrations of ions on each side of the membrane and with the reversal potential, Erev, given by

\[ E_{rev} = \frac{RT}{F} \ln \left( \frac{P_e[X]}{P_o[Y]} \right) \]

where P[e] and P[o] and [X] and [Y] are the permeabilities and concentrations of X and Y, respectively.

Western blot analysis. Membrane-enriched fractions of proteins were obtained from 2F3 cells by using a lysis buffer (2 mM EGTA; 50 mM Tris-base; 320 mM sucrose, and 2.3% SDS). The protein solutions were titrated with 0.1 N NaOH or HCl to a pH of 4–15% Tris/HCl, loaded in Fig. 2A–C, and subjected to electrophoresis in a 4–15% Tris·HCl polyacrylamide gel, and electroblotted to a 0.45-μm nitrocellulose membrane via the Trans-Blot SD Semi-Dry Blotting System (Bio-Rad, Hercules, CA). Protein expressions were visualized using the Bio-Rad ChemiDoc XRS+ (Hercules, CA) and quantified using the ImageJ image analysis software (National Institutes of Health, Bethesda, MD).
Electrophoretic system with a voltage of 15 V for 45 min in Towbin transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, pH 8.3). The proteins on the membrane were blocked with 5% nonfat dry milk in Tween 20 PBS for 2 h at room temperature. As a control, the primary antibody was preabsorbed with the antigenic peptide at a concentration of 2 μg/ml overnight at 4°C. The membrane was incubated in the solutions either with the primary (CIC-2 or CFTR) antibody at a final concentration of 0.37 μg/ml overnight at 4°C and then was extensively washed with PBS and incubated with the secondary antibody conjugated with horseradish peroxidase at a concentration of 1:3,000 for 1 h at room temperature. Finally, the membrane was washed with PBS and tested by chemiluminescence.

RESULTS

Measurement of intracellular cAMP. Confluent 2F3 cells grown on permeable supports were treated according to the following conditions for 15 min: no treatment control; 20 μM forskolin-positive control; or 10 nM, 50 nM, 100 nM, or 1 μM RU-0211. Cells were lysed with 0.1 N HCl, scraped, and sonicated. cAMP levels were measured by using the acetylated version of the colorimetric cAMP Direct Immunoassay Kit (Calbiochem online catalog no. 116811). A polyclonal antibody to the acetylated version of the colorimetric cAMP Direct Immunoassay Kit (Calbiochem online catalog no. 116811). A polyclonal antibody to cAMP was added to the standards and samples. After a 2-h incubation, plates were washed and substrate was added to produce yellow color change. Color density was read on a microplate reader at 405 nm, with concentration of cAMP inversely proportional to color intensity. The standard curve was used to quantify the concentration of cAMP in each condition. Results were normalized to the amount of protein by conducting a BCA assay and presented as picomoles cAMP per gram protein. Error in the measurement were derived from the standard error of the standard curve.

Chemicals. Amiloride, diphenylcarboxylic acid, and glibenclamide were purchased from Calbiochem or Sigma Chemical (St. Louis, MO). Lubiprostone was obtained from Sucampo Pharmaceuticals (Sanda, Japan) as frozen aliquots of 2 mM solutions in 100% DMSO. DMSO, also obtained as frozen aliquots from Sucampo Pharmaceuticals, was used to dilute the lubiprostone and when testing for vehicle effects.

Statistics. Data are reported as mean values ± SE. In general, cell-attached patch experiments were conducted in a paired fashion: data from each patch membrane served as the control for an experimental manipulation. In this case, the average change in Pn for a group of patches, compared before and after an experimental treatment, was analyzed by a paired t-test or ANOVA for multiple comparisons. For unpaired experiments, traditional t-test or ANOVA was used when the data appeared to be normally distributed; otherwise an appropriate nonparametric test was used. Results were considered significant if P < 0.05. Statistical analysis was performed with SigmaPlot and SigmaStat software (Jandel Scientific, San Rafael, CA).

RESULTS

Lubiprostone increases transepithelial current and voltage in A6 cells. We first tested the ability of lubiprostone to alter transepithelial electrical parameters. Adding even low concentrations of lubiprostone significantly increased transepithelial voltage and current (Fig. 2). Lubiprostone did not produce a significant difference in amiloride-sensitive voltage and current (presumably sodium transport) although there might have been a small increase in current. On the other hand, lubiprostone caused a significant increase in amiloride-insensitive voltage and current. The increase in current produced by even the lowest concentrations of lubiprostone is particularly striking. Lubiprostone (10 nM) significantly decreases the transepithelial resistance from 2.41 ± 0.127 Ω cm² to 1.44 ± 0.0397 Ω cm² (mean ± SE; n = 12). Addition of higher concentrations of lubiprostone further decrease resistance until at 1 μM the resistance is 1.08 ± 0.0462 Ω cm² (mean ± SE; n = 6). The lubiprostone-mediated decrease in resistance is not amiloride sensitive.
Lubiprostone activates Cl⁻ channels in A6 cells. We formed cell-attached patches on A6 cells with either a pipette solution containing 96 mM NaCl plus 3 μM amiloride or saline in which all the Na⁺ had been replaced with N-methyl-D-glucamine (neither saline contained K⁺). This meant that single-channel currents could not be due to ENaC in the first case, or any cation channel in the second case. Under these conditions, we generally saw little, if any, single-channel activity (Figs. 3A and 4A). The all-points amplitude histograms for the entire recording period below the records confirm that the patches reside predominantly in a zero-current state with no channel activity (Fig. 3, B and C and Fig. 4, B and C). After a control recording period, we slowly added 100 nM lubiprostone to the apical bath and after 5–15 min we observed channel activity in over 80% of the patches we examined. The channels were of two different types. The first type of channel, which we suggest is ClC-2 (Fig. 3D), had inward current at zero pipette potential (because of the large lubiprostone-mediated increase in the transepithelial voltage) and increasingly larger inward currents and increasing open probability at all more negative potentials.

Fig. 2. Lubiprostone increases transepithelial current and voltage in A6 cells. A: effect of different concentrations of lubiprostone on total, amiloride-sensitive, and amiloride-insensitive transepithelial voltage. B: effect on transepithelial current. Adding even low concentrations of lubiprostone significantly increased transepithelial voltage and current. Lubiprostone did not produce a significant difference in amiloride-sensitive voltage and current (presumably sodium transport), although there might have been a small increase in current. On the other hand, lubiprostone caused a significant increase in amiloride-insensitive voltage and current. The increase in current produced by even the lowest concentrations of lubiprostone is particularly striking.

Fig. 3. Lubiprostone activates small-conductance Cl⁻ channels in A6 cells. A: typical single-channel records from a cell-attached patch before application of lubiprostone with no obvious channel activity (patch potentials to the left of the traces are the potential displacement from the apical membrane potential-about −40 mM). The all-points amplitude histograms (0 mV at B; −60 mV at C) confirm that the patch is predominantly in a zero-current state (marked with an arrow to designate the closed state). D: after addition of 100 nM lubiprostone channel activity is easily observed and the all-points histograms (0 mV at E; −60 mV at F) reflect the fact that there are 2 current levels that were not previously visible (marked as open 1 and open 2). In this figure (and all subsequent figures), the arrows mark the level of the closed state.
The second type of channel is the CFTR Cl⁻ channel that has previously been described in A6 cells (71, 80, 81) and that has currents that usually reverse near or slightly positive to zero (no potential applied to the pipette) and characteristic flickery openings at positive pipette potentials (Fig. 4D). The all-points histograms below the records show that both types of channels spend a significant amount of time in one or more open states (Fig. 3, E and F and Fig. 4, E and F).

**CIC-2 and CFTR proteins are detectable by Western blotting in A6 cells.** We used conventional Western blotting methods on lysates of A6 cells and blotted for ClC-2 and CFTR using commercially available antibodies. Figure 5 shows that both proteins are easily detectable in A6 cells. CFTR had been previously reported (71, 80, 81), but ClC-2 has not been shown before in A6 cells although it has been described in several other epithelia (2, 10, 17, 18, 23, 29, 34, 35, 41, 72, 85, 87, 114).

The two channels have different properties. Examination of single-channel records in cell-attached patches after exposure to 100 nM lubiprostone over a large range of voltages shows that the two channel types have distinctive and different biophysical characteristics. The channel we have identified as CIC-2 (Fig. 6A) has long open times at hyperpolarizing potentials, but both the current and the open probability decrease as the membrane is depolarized. At positive membrane potentials channel openings are small and difficult to detect. In contrast, in a lubiprostone-treated cell-attached patch containing CFTR (Fig. 7A), single-channel currents at positive potentials are easily detectable. At more negative potentials, the current becomes smaller until it reverses near zero (in a cell-attached patch, zero refers to no applied potential; that is, the patch membrane is near the transepithelial potential of the monolayer, probably −30 to −40 mV in this case). At potentials more negative than the reversal potential, channel openings are still easy to see, but the openings are now interrupted by fast closures. Such flickery, burstlike events are characteristic of CFTR in A6 cells.

**The rectification of the two channel types is different.** Some of these properties are reflected in the current voltage relationships for the channels (Fig. 6B). The single-channel currents for CIC-2 strongly inwardly rectify. Given that the concentration of Cl⁻ is greater outside the cell (96 mM) than inside (−40 mV in this case), one might expect inward flux of Cl⁻ (outward current) to be greater than outward flux (inward current) when, in fact, it is the reverse. This implies that the permeability for Cl⁻ is smaller for Cl⁻ entry than for Cl⁻ exit. Such a property is consistent with a pore that has one or more barriers to ion movement and possible binding sites within the pore that will produce greater access to the inner mouth of the pore than the outer mouth. Such a pore would be expected to favor outward flux over inward; that is, the flux ratio should vary as some higher power of Cl⁻ concentration and voltage. We fit the current-voltage data to several models that contained one or more energy barriers and energy wells (as described in METHODS). The fit to two of the models is shown in Fig. 8 with best-fit values given in Table 1. As is obvious, the fit to a single energy barrier, although the model can produce strong inward rectification, fits the data very poorly. The fit of either model 2 (2 barriers and 1 well) or model 3 (3 barriers and 2 wells) produces a fit that appears the same. However, model 2 has low-energy barriers and predicts an extremely high intracellular chloride concentration. The low-energy barriers seem in-
consistent with the small unit conductance of ClC-2. Therefore, we tend to favor model 3 or a model with even more barriers and wells.

The reversal potential for ClC-2 in Fig. 6B appears more positive than might be expected for a Cl\(^-\) channel. Of course, it is necessary to remember that channels in cell-attached patches on cells in a tight epithelial monolayer also measure the transepithelial potential (Fig. 1); i.e., the voltages in the current-voltage relationship are the displacement of the patch potential from the transepithelial potential. For example, +20 mV pipette potential is 20 mV more positive than the transepithelial potential. Since lubiprostone activates transepithelial Cl\(^-\) transport, the transepithelial potential can be quite large if the monolayer is high resistance. The transepithelial potential will shift the current-voltage relationship to the right by up to 90 mV (see Fig. 2). This is approximately the deviation of the reversal potential from zero in Fig. 6B. Therefore, the apparent reversal potential will depend on variability in transepithelial potential: if the monolayer is intact the reversal potential may be far to the right; if there is even a slight amount of edge damage the reversal potential will be close to zero. To test this possibility we reasoned that if we found a patch that had both CFTR and ClC-2, they should both reverse at the same potential whether it is very positive or close to zero. Figure 9 shows single-channel records and the current-voltage relationships for just such a patch with the two types of channels reversing at the same positive potential.

The current-voltage relationship for CFTR (Fig. 7B) rectifies in a way expected for a single-site anion channel with more flux from the side that has a higher concentration of Cl\(^-\) (outward current/inward flux is greater than inward current/outward flux). The current through CFTR channels can be fit well with the Goldman-Hodgkin-Katz current equation. The fit provides estimates for CFTR channel permeability (6.74 ± 0.525 × 10\(^{-6}\) cm/s), the transepithelial potential (−41 ± 8.4 mV apical compartment negative), and the intracellular Cl\(^-\) activity (38 ± 8.9. mM consistent with the value of 24 ± 11. mM predicted by model 3 for ClC-2).

Lubiprostone activates ClC-2 at much lower dose than CFTR. We were somewhat surprised that lubiprostone activated CFTR but thought that the activation might be associated only with the higher doses of lubiprostone. Therefore, we examined the lubiprostone dose-response relationship for ClC-2 and CFTR by examining the frequency with which we observed specific channels after treatment with different concentrations of lubiprostone. In Fig. 10, we then plotted the percentage of patches with either ClC-2 or CFTR vs. dose and fitted the data to the following dose equation:

\[
\text{fractional response} = \frac{A_{\text{max}}[\text{dose}]^b}{K_{1/2} + [\text{dose}]^b}
\]

where \(A_{\text{max}}\) is the maximum fraction of patches with channels, \(K_{1/2}\) is the half-activating dose, and \(b\) is the slope of the dose relationship (the Hill coefficient). As is obvious from Fig. 10, much higher doses of lubiprostone are required to activate CFTR. The \(K_{1/2}\) for ClC-2 is 69 ± 18.8 nM whereas for CFTR the \(K_{1/2}\) is 791 ± 273 nM. These \(K_{1/2}\) values are significantly different from one another (\(P < 0.01\)). The maximal responses for ClC-2 and CFTR are 69 ± 7.8 and 61 ± 5.4% of patches with channels, respectively, and the slope coefficient for ClC-2 and CFTR is 1.5 ± 0.37 and 0.84 ± 0.13, respectively (all...
values are means ± SE). These results show that it requires at least 10-fold more lubiprostone to activate CFTR than ClC-2 and is consistent with using lubiprostone as an activator of ClC-2. We were interested in the mechanism by which lubiprostone activated either ClC-2 or CFTR. Traditionally, CFTR is activated by increases in intracellular cAMP. However, an increase in cAMP does not appear to be how lubiprostone increases channel activity (neither ClC-2 or CFTR) since when we measured cAMP (Fig. 11), we found that lubiprostone did not increase cAMP even at high dosages even though the positive control, forskolin, produced a robust cAMP increase.

The open probability of ClC-2 decreases with depolarizing voltages. Figure 4 showed that ClC-2 single-channel currents strongly rectify as the apical membrane was made more positive; however, besides the smaller than expected outward current, the frequency of ClC-2 openings also decreased. That is, the open probability of ClC-2 decreased at positive potentials. Figure 12 shows the voltage sensitivity of the channel.
open probability. The decrease in open probability can be fit by a Boltzmann relationship with half-maximal activation at 170 ± 8.4 mV and the open probability decreasing e-fold for every 43 ± 8.4 mV of depolarization. We were able to determine the open probability by first measuring $N_p O$ and then using the fact that at very hyperpolarized potentials the open probability is large enough that even recording for a relatively short period of time allows an accurate count of the number of channels in the patch.

The decrease in open probability was due to both a voltage-dependent decrease in channel mean open time and an increase in mean closed time. To obtain a better idea of the voltage dependence of channel opening and closing, we took advantage of the fact that the mean duration in either the open or closed state changes according to the following expression:

$$\tau(V) = \tau(0) \exp \left(-\frac{\alpha V F}{R T}\right)$$

where $\tau(V)$ is the mean duration (open or closed time) at a voltage $V$; $\tau(0)$ is the mean duration (open or closed time) of the channel at 0 mV; and $\alpha$ is the voltage dependence of the channel. One way to interpret $\alpha$ is as the fraction of the membrane field detected by charged sites on the channel although for ClC-2 the interpretation may be more complicated (75). The value for $\alpha$ can be obtained by determining the slope of the relationship between log $\tau(V)$ and voltage or by fitting the relationship between $\tau(V)$ and voltage to the expression above. Figure 12, B and C shows the voltage dependence of mean open and closed times for positive potentials. We examined patches that had no more than two channels (at hyperpolarized potentials) and then held the patches at positive potentials since the open probability was so low that we did not observe overlapping open events. Unbiased open time analysis is possible if only one current level is observed in a patch even if the patch contains more than one channel. However, our estimates of closed time are biased if the patches contain more than one channel. However, the voltage dependence of mean closed time is not affected by counting since there is the same bias at all potentials. With no applied potential, the mean open time is 874 ± 22.6 ms and the mean open time decreases with increasing voltage as if the voltage dependence for channel

![Table 1. Best fit parameters for Eyring models of CLC2 current-voltage relationships](image)

<table>
<thead>
<tr>
<th>Model 1 (1 barrier)</th>
<th>Model 2 (2 barriers, 1 well)</th>
<th>Model 3 (3 barriers, 2 wells)</th>
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<tbody>
<tr>
<td>$\Delta G$, KJ/mol</td>
<td>$\delta$</td>
<td>$\Delta G$, KJ/mol</td>
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<tr>
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<td>6.6</td>
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<tr>
<td>Well 2</td>
<td>-0.1</td>
<td>0.18</td>
</tr>
<tr>
<td>Barrier 3</td>
<td>874 ± 22.6 ms</td>
<td>37 mM</td>
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<tr>
<td>[Cl$^-$]</td>
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$\Delta G$, height or depth of the energy barrier or well; $\delta$, electrical distance from the outside of the membrane to the barrier or well.

Fig. 9. The transepithelial potential shifts the single-channel current-voltage relationships to the right. The reversal potential for CIC-2 in Fig. 6B appears more positive than might be expected for a Cl$^-$ channel, but channels in cell-attached patches on cells in a tight epithelial monolayer also measure the transepithelial potential (Fig. 1); i.e., the voltages in the current-voltage relationship are the displacement of the patch potential from the transepithelial potential. Since lubiprostone activates transepithelial Cl$^-$ transport, the transepithelial potential can be quite large if the monolayer is high resistance. The transepithelial potential will shift the current-voltage relationship to the right by up to 90 mV (see Fig. 2). This is approximately the deviation of the reversal potential from zero in Fig. 6B. Therefore, the apparent reversal potential will depend on variability in transepithelial potential: if the monolayer is intact the reversal potential may be far to the right; if there is even a very slight amount of edge damage the reversal potential will be close to zero. To test this possibility we reasoned that if we found a patch that had both CFTR and CIC-2, they should both reverse at the same potential whether it is very positive or close to zero. The current-voltage relationship above shows single-channel records (A) and the current-voltage relationships (B) for just such a patch with the 2 types of channels reversing at the same positive potential.
increases with increasing voltage as if the voltage sensor for the inward flux of Cl\(^-\) membrane potential. Thus the change in the open time reduces the apparent level of ClC-2 activity, respectively, and the slope coefficient for ClC-2 and CFTR is 0.37 and 0.84, respectively (all values are means ± SE).

ClC-2 is a “double-barreled” channel. At least two other members of the ClC family of Cl\(^-\) channel proteins form dimers and, thus, have two ion-conductance pathways for each channel protein that open and close independently of one another (92, 123). Despite the independence, the fact that each protein molecule in effect contains two channels means the patches will appear to contain even numbers of channels and patches. Since counting the number of channels in a patch involves counting the number of current levels and since the two halves of the dimers open independently of one another, it might still be possible to observe patches with what appear to be odd numbers of channels. This would occur when the recording period was so short or the open probability was so low that there was a low probability of seeing both conductance pathways open at the same time. We have discussed the problem of determining whether a channel contains two conducting pathways in previous work (68). We recorded from a6 patches containing alternative anions and then comparing in the same batch of cells the reversal potential for the anions. In our experiments, we filled the pipettes with either 96 mM NaCl, NaBr, or NaNO\(_3\) or 115 mM NaI or NaSCN, where SCN is thiocyanate. Figure 14 shows the single-channel currents for inward and outward currents for Cl\(^-\), Br\(^-\), and NO\(_3\). Outward Br\(^-\) current is relatively easy to detect, but outward NO\(_3\) current is difficult to observe. Figure 15 shows current-voltage relationships for the different ions near the reversal potentials. The currents were fit to the Goldman-Hodgkin-Katz current equation assuming one permeable coefficient for outward current (flux of anion from the pipette into the cell) and another for inward current (see METHODS). It is obvious from the reversal potentials that the order of permeability for CIC-2 is Cl\(^-\) > Br\(^-\) > NO\(_3\) > I\(^-\) > SCN\(^-\) and for CFTR is Cl\(^-\) > Br\(^-\) > NO\(_3\) > I\(^-\) = SCN\(^-\). Table 2 gives values for the permeability ratios and estimates of the specific permeabilities for the ions that had significant outward current. The position of NO\(_3\) in the sequence for CIC-2 is ambiguous because little outward current was recorded and, therefore, the reversal potential was poorly determined. This implies that NO\(_3\) could be lower (but not higher) in the sequence of anions. For CFTR, we observed only small outward currents for SCN\(^-\) and no outward current for NO\(_3\), or I\(^-\), even at the most depolarizing potentials (up to +250 mV), implying that the permeability of these latter two ions is <1% of Cl\(^-\) permeability.

Fig. 10. Lubiprostone activates CIC-2 at much lower doses than CFTR. This figure shows the frequency of CIC-2 and CFTR in patches in response to lubiprostone measured as the percentage of patches that contained one channel or the other. The frequency of CIC-2 (●) and CFTR (○) were fitted (solid lines) to the Hill equation (see RESULTS). This figure shows that much higher doses of lubiprostone are required to activate CFTR. The half-activating dose for CIC-2 is 69 ± 18.8 nM whereas for CFTR the dose is 791 ± 273 nM. The maximal response for CIC-2 and CFTR is 69 ± 7.8 and 61 ± 5.4% of patches with channels, respectively, and the slope coefficient for CIC-2 and CFTR is 1.5 ± 0.37 and 0.84 ± 0.13, respectively (all values are means ± SE).

CIC-2 and CFTR are more permeable to Cl\(^-\) than other anions. Part of the biophysical characterization of ion channels is a determination of the selectivity of the channels for different ions. Examination of selectivity of single ion channels in cell-attached patches involves filling the pipettes with salines containing alternative anions and then comparing in the same batch of cells the reversal potential for the anions. In our experiments, we filled the pipettes with either 96 mM NaCl, NaBr, or NaNO\(_3\) or 115 mM NaI or NaSCN, where SCN is thiocyanate. The position of NO\(_3\) in the sequence for CIC-2 is ambiguous because little outward current was recorded and, therefore, the reversal potential was poorly determined. This implies that NO\(_3\) could be lower (but not higher) in the sequence of anions. For CFTR, we observed only small outward currents for SCN\(^-\) and no outward current for NO\(_3\), or I\(^-\), even at the most depolarizing potentials (up to +250 mV), implying that the permeability of these latter two ions is <1% of Cl\(^-\) permeability.

Fig. 11. Lubiprostone does not increase intracellular cAMP. Lubiprostone did not increase cAMP even at high dosages even though the positive control, forskolin, produced a robust cAMP increase.
Thiocyanate and iodide are CIC-2 channel blockers. If inward flux of anions through CIC-2 is independent of outward flux, then application of any anion regardless of its permeability to the external surface of the channel should have no effect on the current mediated by the ion on the internal surface of the channel. In other words, even though I\(^-\) and SCN\(^-\) have a very low permeability (little outward current), their application should not affect efflux of Cl\(^-\) (inward current). Figure 16 shows that this is definitely not the case. Both I\(^-\) and SCN\(^-\) reduce the mean open time of the channel for inward currents when open times are usually relatively long. At \(-60\) mV, the mean open and closed times with external and internal Cl\(^-\) were 211 \(\pm\) 2.94 ms and 549 \(\pm\) 4.47 ms, respectively. At the same potential, the mean open and closed times in the presence of I\(^-\) were 12.2 \(\pm\) 4.24 and 85.4 \(\pm\) 0.464 ms, respectively, and in the presence of SCN\(^-\) were 18.2 \(\pm\) 2.46 and 108 \(\pm\) 0.199 ms, respectively. Such “trans-side” block has been extensively described for multi-ion cation channels (42, 54, 118) and is consistent with either model 2 or model 3 described above.

Lubiprostone activates CIC-2 in stably transfected HEK293 cells. There are few descriptions of the single-channel properties of CIC-2 channels. Only one study in cortical astrocytes (92) provided data that could be compared with ours, and the quantitative and qualitative comparisons supported our conclusion that we were examining CIC-2. However, to further support our conclusion, we examined the properties of the predominant anion channels in HEK293 cells stably transfected with human CIC-2 (31). The patches we formed on HEK cells were not as stable or as high resistance as those on A6 cells; however, the anion channel activated by lubiprostone had channel kinetics (Fig. 17A) and a current-voltage relationship (Fig. 17B) that were essentially indistinguishable from the channels in A6 cells we had identified as CIC-2 channels.

Other channel blockers. Specific anion channel blockers have been notoriously difficult to find. Several inhibitors of CFTR have been described, but most of these tend to be hydrophobic anions that are likely to interact with many different molecules. We examined two such blockers: diphe-
nylcarboxylic acid and glibenclamide. Both of these agents block CFTR and we confirmed this block in our experiments (data not shown). Unfortunately, they also both block ClC-2 (the effect of glibenclamide on ClC-2 is shown in Fig. 18). Therefore, these blockers do not appear useful for distinguishing the two channel types in whole cell experiments. We further examined the effect of glibenclamide on ClC-2 to determine the nature of the block. Glibenclamide appears to be an open channel blocker since it reduces the mean open time (Fig. 19B) as judged from the open-interval histograms (mean open time at 60 mV before glibenclamide is 2.9 ms, which is reduced to 2.2 ms) and induces a second component in the closed-interval histogram (Fig. 19A). The closed-interval histogram before glibenclamide is best fit (as judged by a $\chi^2$ comparison) by a single exponential distribution with a mean closed time of 3.3 ms, but after glibenclamide, the histogram is best fit by two exponential distributions with mean times of 2.55 and 5.6 ms. The second component (with the shorter mean duration) should represent the mean duration of the glibenclamide-blocked state. Figure 19C shows the open probability vs. time after exposure of the whole cell to glibenclamide. The block is

Fig. 14. ClC-2 and CFTR are more permeable to Cl$^-$ than other anions. In our experiments, we filled the pipettes with either 96 mM NaCl (A), NaBr (B), or NaNO$_3$ (C) (plus amiloride to ensure that there were no cation channels) or 115 mM NaI or NaSCN and recorded single-channel currents for inward and outward currents for Cl$^-$, Br$^-$, and NO$_3^-$. Outward bromide current is relatively easy to detect, but outward NO$_3^-$ current is difficult to observe. In this figure, the arrows mark the level of the closed state.
Table 2. Selectivity of ion channels in A6 cells stimulated by lubiprostone

<table>
<thead>
<tr>
<th>Ion</th>
<th>CIC-2 Channel P_{ion}/P_{Cl}</th>
<th>CFTR Channel P_{ion}/P_{Cl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Br⁻</td>
<td>0.766</td>
<td>0.261</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>0.546</td>
<td>very small</td>
</tr>
<tr>
<td>I⁻</td>
<td>0.00124</td>
<td>very small</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>0.00771</td>
<td>0.0442</td>
</tr>
</tbody>
</table>

Permeability ratios were calculated from the shifts in the reversal potentials in Fig. 9 for different ions in the external (pipette) solution. Permeabilities were calculated from the fits to the current-voltage relationships for inward and outward currents. P_{ion}/P_{Cl} is the ratio of the permeability of the ion in the first column to the chloride permeability. SCN, thiocyanate.

relatively slow (the time constant for block is 466 ± 290 s), presumably reflecting the time necessary for glibenclamide to enter the cell, and, even at long times, the block does not appear to be complete reflecting the fact that glibenclamide is not a particularly effective blocker of CIC-2. Nonetheless, the reduction in P_o is statistically significant (P < 0.001 by Mann-Whitney rank sum test).

The other blocker we examined was cadmium (Cd²⁺) since it has been reported to be a specific blocker of CIC-2 (23, 29, 66, 67, 76, 92, 123). The presumptive mechanism by which Cd²⁺ produces it block is by interaction with a critical cysteine residue in CIC-2. This suggests that Cd²⁺ may not be very specific and since it has previously always been bath applied the route and mechanism of block is not completely clear. In our experiments, we included 1 µM Cd²⁺ in the pipette and did not reduce the incidence of CIC-2 in the patches (~70–80% of patches). If we pretreat the entire monolayer with cadmium for 30 min, we see no CIC-2 channels, or for that matter, any other channels (119). This suggests a generally toxic effect of cadmium due to its sulfhydryl reactivity.

DISCUSSION

The main objectives of this study were to determine clearly which channels were activated by lubiprostone in a model epithelial cell line and to provide a biophysical characterization of these channels at a single-channel level. For comparison, the main results are summarized in Table 3.

Lubiprostone activation of CIC-2 channels. There are surprisingly few examples of single-channel records from CIC-2 channels in native, untransfected cells and only limited examples in any preparations. There has been some disagreement about the properties of the unitary channels underlying CIC-2 (39, 62, 77, 116). At least part of the variability likely has to do with the low conductance of CIC-2 single channels, the disparate conditions under which recordings of CIC-2 activity are made, and the fact that CIC-2 gating is apparently sensitive to changes in intracellular Cl⁻, pH, hormonal agents, voltage protocols, and tonicity (3, 9, 18, 23, 28, 32, 33, 35, 40, 46, 55, 63, 78, 90, 91, 94, 98, 123). Inside-out patch recordings of CIC-2 dimers expressed in *Xenopus* oocytes produces channels with a single-channel conductance of ~3 pS that behaved as a double-barrel-like channel, although the pore stoichiometry of the homomeric channel was not determined (116). CIC-2 expressed in NIH 3T3 cells (13) had similar properties (3–4 pS) to those in oocytes and an inwardly rectifying single-channel conductance. The channels observed in one of the few examples of single-channel recordings from native cells (rat cortical astrocytes) have similar properties to those observed in oocytes and CIC-2-transfected cells (92). These properties are in contrast to a CIC-2 variant from gastric parietal cells, CIC-2G, which when expressed in *Xenopus* oocytes is acti-

![Fig. 16. I⁻ and SCN⁻ block CIC-2 channels. Both I⁻ and SCN⁻ reduce the mean open time of the channel for inward currents when open times are usually relatively long. At −60 mV, the mean open and closed times with external and internal Cl⁻ (top trace) were 211 ± 2.94 ms and 549 ± 4.47 ms, respectively. At the same potential, the mean open and closed times in the presence of I⁻ were 12.2 ± 4.24 ms and 85.4 ± 0.464 ms (bottom trace with expanded section) and in the presence of SCN⁻ were 18.2 ± 2.46 ms and 108 ± 0.199 ms (middle trace with expanded section). Such “trans-side” block has been extensively described for multi-ion cation channels (38, 50, 111). In this figure, the arrows mark the level of the closed state.](http://ajpgi.physiology.org/)
vated by protein kinase A and acid pH with a linear current-voltage curve and a conductance of 29 pS. These properties were measured at high Cl\textsuperscript{−} concentration (800 mM CsCl) (77, 104). The same laboratory has also shown that ClC-2 channels are activated by arachidonic acid and the drug omeprazole in lung and a recombinant expression system (33, 110). The source of this difference in behavior is unclear, but it is interesting to note that in whole cell recordings, even when the steady-state current-voltage relationship rectifies, the current shortly after a voltage step is close to linear (39, 45, 101, 111). Therefore, differences may well have to do with the protocols for recording in different laboratories.

In our work, the predominant lubiprostone-induced single Cl\textsuperscript{−} channels had a low unit conductance (3–4 pS), the conductance inwardly rectified, and the open probability was voltage dependent with increased open probability at hyperpo-

Fig. 17. Lubiprostone activates CIC-2 in stably transfected HEK293 cells. We examined the properties of the predominant anion channels in HEK293 cells stably transfected with human CIC-2 (27) (A). The patches we formed on HEK cells were not as stable or as high resistance as those on A6 cells; however, the anion channel activated by lubiprostone had channel kinetics that were essentially indistinguishable from the channels in A6 cells we had identified as CIC-2 channels (Figs. 1 and 4). B: the current-voltage relationship of CIC-2 channels in stably transfected HEK293 cells. The current-voltage relationship of the anion channel activated by lubiprostone was essentially the same as that of the channels in A6 cells we had identified as CIC-2 channels (Fig. 6A). In this figure, the arrows mark the level of the closed state.

Fig. 18. Glibenclamide blocks CIC-2. A: CIC-2 induced by 100 nM lubiprostone before (top) or after glibenclamide (0.1 mM), ordinarily considered a CFTR blocker. The all-point amplitude histograms below show that glibenclamide significantly reduces the open state and increases the occupancy of the closed state (compare histograms in B and C). In this figure, the arrows mark the level of the closed state.
the channels in A6 cells are virtually indistinguishable from those of CIC-2 stably expressed in HEK293, which we and others (31) have shown are activated by lubiprostone. The selectivity sequence for anions of the channels in A6 cells is also the same as that reported by others for CIC-2 (39, 45, 101, 111) and the “double-barreled” nature of the channels is similar to channels we have previously described in rat distal nephron principal cells that are stimulated by PGE2. We therefore conclude that the lubiprostone-induced channels are, indeed, CIC-2. Our results are interesting because, in unstimulated A6 cells, the frequency of observing CIC-2 channels was extremely low (no more than 1 or 2% of successful patches); however, after treatment with lubiprostone virtually every patch (>80%) had activity that appeared to be CIC-2. We verified that the activity was CIC-2 in the patches by examining the current-voltage relationship and voltage sensitivity.

Comparison of lubiprostone-activated channels in A6 cells with channels in other epithelia. We observed that lubiprostone at high concentrations (more than 50 times higher than the concentration necessary to activate CIC-2) activated CFTR. As mentioned above, CFTR channels have been previously reported by us in A6 cells (71). The properties of the CFTR channels activated by lubiprostone are identical to those previously described by us as cAMP-activated channels although we have shown that lubiprostone is not activating CFTR by increasing intracellular cAMP. They also have properties essentially identical to those described for single CFTR channels in many other epithelial preparations (for reviews, see Refs. 36, 52, 61). In T84 cells, another cell line that expresses both CFTR and CIC-2, lubiprostone did not activate CFTR. We do not fully understand why CFTR should be activated in A6 cells but not in T84 or in HEK cells transfected with CFTR and CIC-2. One possibility is that T84 cells (and the transfected HEK cells), unlike A6 cells, are considered exclusively Cl−-secretory cells, whereas A6 cells are a model for more generalized salt transport and salt homeostasis. Therefore, A6 cells require the ability to activate both anion (and cation) secretion and reabsorption. Their more extensive signaling pathways may lead to activation of channels that could promote secretion or reabsorption. Alternatively, we have previously shown that prostaglandins can activate CFTR in A6 cells (68). Lubiprostone is structurally similar to prostaglandin, but does not interact with mammalian prostaglandin receptors; however, prostaglandins are metabolized to prostones. If part of the stimulation of CFTR by PGE2 (11) is not due to the prostaglandins themselves, but rather to their metabolic products,
then the activation of CFTR in A6 cells might not be surprising.

**Blocking CIC-2 channels.** It is unfortunate that there are no effective small-molecule inhibitors of CIC-2 or CFTR. Glenbemclamide has been used as a selective blocker of CFTR to the point of some investigators using the drug as diagnostic for the contribution of CFTR anion flux. However, on the basis of our observation, glenbemclamide is almost as good at blocking CIC-2 as CFTR. Therefore, substantial care must be exercised in the use glenbemclamide, diphenylcarboxylic acid, or other small anionic blockers to determine the contributions of CFTR and CIC-2 to the anion flux in epithelial tissues (83). Cd²⁺ has been used as a blocker of CIC-2, but Cd²⁺ can hardly be considered selective since it blocks many transporters owing to its strong interaction with cysteine residues. In fact, when applied selectively to the isolated apical membrane (inside the patch pipette), Cd²⁺ does not alter the properties of CIC-2 or the frequency with which the channel is observed. Thus any modifiable cysteines that alter CIC-2 activity are not directly accessible from the apical surface of the channel. More promising inhibitors are recently described small peptide toxins derived from scorpion venom that appear to be substantially more selective blockers of CIC-2 (44, 112, 122).

**Implications for epithelial fluid balance.** A major question concerns the physiological role of the two channels we have identified in A6 cells and their role in normal epithelial function. A second question involves the functional implications of lubiprostone activation of the channels. The role of CFTR in epithelial transport has been discussed at great length in the literature. A third question involves the functional implications of lubiprostone activation of the channels. The role of CFTR in epithelial transport has been discussed at great length in the literature.

**Conclusions.** These studies show that lubiprostone is a potent activator of CIC-2 Cl⁻ channels in A6 cells, but, to a much lesser extent, it also activated CFTR. Therefore lubiprostone activation of Cl⁻ transport in the human intestine (15) likely occurs through activation of the CIC-2 Cl⁻ channel. These properties suggest that lubiprostone is an excellent candidate for the clinical treatment of gastrointestinal syndromes involving reduced water content of the intestinal contents. These studies also suggest that, beyond pharmacological activation, CIC-2 Cl⁻ channels may play a physiological role in Cl⁻ transport in the intestine and other epithelial tissues. Further studies are needed to define the specific mechanism by which lubiprostone activates CIC-2 and CFTR Cl⁻ channels.

**ACKNOWLEDGMENTS**

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**REFERENCES**

G250 LUBPROSTONE ACTIVATES Cl− CHANNELS IN A6 CELLS


