J. Membrane Biol. 176, 217–221 (2000)
DOI: 10.1007/s00232001091

The Journal of
Membrane
Biology

Evidence that Basolateral But Not Apical Membrane Localization of Outwardly Rectifying Depolarization-Induced Cl^- Channel in Airway Epithelia

T.-H. Hwang, H.-J. Lee, N.-K. Lee, Y.C. Choi
Department of Pharmacology & Science Institute of Medicine, Dong-A Medical College, Pusan, South Korea 602-103

Received: 7 October 1999/Revised: 24 April 2000

Abstract. The rat primary cultured-airway monolayer had been an excellent model for deciphering the ion channel after nystatin permeabilization of its basolateral or apical membrane (Hwang et al., 1996). After apical membrane permeabilization of rat primary cultured-airway monolayer, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)-sensitive outwardly rectifying depolarization-induced Cl^- (BORDIC) currents were observed across the basolateral membrane in symmetrical NMG-Cl solution in this study. No significant Cl^- current induced by the application of voltage clamping was observed across the apical membrane in symmetrical NMG-Cl solution after basolateral membrane permeabilization. The halide permeability sequence for BORDIC current was $Br^- \fallingdotseq I^- > Cl^-$. BORDIC current was not affected by basolaterally applied bumetanide (0.5 mM). Basolateral DIDS (0.2 mM) but not apical DIDS inhibited CFTR mediated short-circuit current (I_{sc}) in an intact monolayer of rat airway epithelia, a T84 human colonal epithelial cell line, and a Calu-3 human airway epithelial cell line. This is the first report showing that depolarization induced Cl^- current is present on the basolateral membrane of airway epithelia.

Key words: Airway epithelia — Basolateral membrane — Chloride current

Introduction

Cystic fibrosis (CF) is a genetic disease characterized by a reduction of Cl^- conductance in many epithelia (Riordan et al., 1989; Anderson et al., 1991). Kinetically distinct macroscopic Cl^- currents, recorded either with conventional voltage clamp or whole-cell patch clamp techniques, have been identified in polarized cells including airway epithelia.

Outwardly rectifying depolarization induced Cl^- channel (ORDIC) as well as the cystic fibrosis transmembrane conductance regulator (CFTR) which is directly coded by the CF gene have been associated with low Cl^- conduction of CF (Tabcharani & Hanrahan, 1991; Egan et al., 1992; Anderson, 1992). Outwardly rectifying chloride channels (ORCC) which are distinguished from ORDIC also have been reported to be important for CFTR function (Schwiebert et al., 1995). Ca^{2+}-activated chloride channel (CaCC) and volume-regulated chloride channel (VRCC), both of which are not directly involved in CFTR function have been reported to be important for maintaining of physiological function in a variety epithelia (Valverde et al., 1995). However, the genes of the ORDIC, ORCC, CaCC, and VRCC channels have not yet been identified, and information on membrane localization of those have been lacking while the genetic identification and the apical membrane localization of CFTR has been well documented.

In this study, we cultured rat airway epithelial cells on permeable supports and used nystatin to permeabilize the apical or basolateral membrane. We have shown previously that inwardly rectifying K^+ current (IRK) and slowly activated outwardly rectified K^+ currents (I_{sk}) under a transepithelial voltage clamp is present on the basolateral membrane of rat airway epithelia (Hwang et al., 1996), a finding which was confirmed for murine airway epithelia (MacVinsh et al., 1998). Using rat airway epithelia we show here an outwardly rectifying depolarization induced chloride (BORDIC) current is present on the basolateral membrane of rat airway epithelial monolayers bathed symmetrical bicarbonate-free, K-free and NMG-Cl Ringer's solutions. However, BORDIC currents were not present in the apical membrane, while CFTR currents and CaCC currents were recorded across the apical but not the basolateral membranes.

Correspondence to: T.-H. Hwang

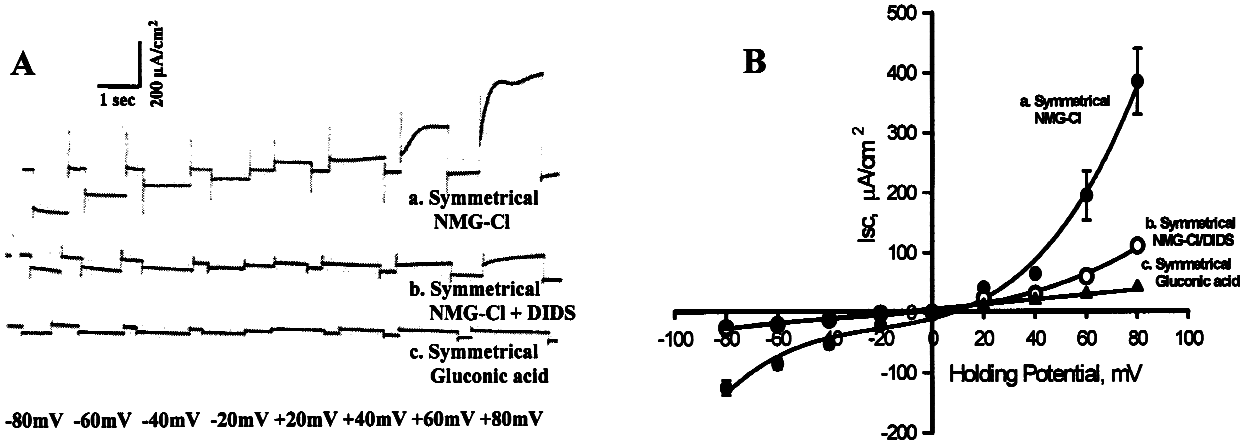

Fig. 1. Outwardly rectifying depolarization induced Cl^- current across the basolateral membrane (BORDIC) in rat airway epithelial monolayer after permeabilization of apical membrane. (*A*) Representative current traces at different clamped voltage applied after nystatin (180 μg/ml) permeabilization of the apical membrane. Currents were elicited by 1 sec clamping pulses from a holding voltage of 0 mV; pulses were from −80 to +80 mV. Symmetrical bicarbonate-free, K^+-free and NMG-Cl Ringer's solution (a, b) or bicarbonate-free, K^+-free and Cl^--free Na-gluconate Ringer's solution (c) was used for Cl^- current recording. To observe DIDS effect, 1 mM DIDS were added into basolateral bath (b). (*B*) Corresponding *I–V* plots of BORDIC current ($n = 12$ each).

Materials and Methods

Cell Culture

T84 cell line and Calu-3 cell line [American Type Culture Collection (ATCC), Rockville, MD] were grown as previously (Hwang et al., 1994). The isolation and culture of rat tracheal epithelia was as described previously (Hwang et al., 1996; Hwang, Schwiebert & Guggino, 1996). Briefly, freshly excised tracheas were incubated at 4°C for 18–24 hours in a CaMg-free, serum-free modified Eagle's minimum essential medium (MEM) containing 0.1% protease XIV (Sigma), and 50,000 U/ml penicillin and 50,000 μg/ml streptomycin (Gibco-BRL solution of both antibiotics). The epithelial cells were removed from the airways by scraping the epithelial surface with a sterile cell scraper, and cells were recovered by centrifugation. The cells were then washed 3× with fresh MEM medium. After the final wash, the cells were resuspended in LHC-8 medium (Biofluids), and final cell suspension (about 10^6 cells) was added to each permeable filter support (12 mm SNAPWELL, Costar, Cambridge, MA). The cells were incubated in an atmosphere of 5% CO_2-95% air at 37°C in the LHC-8 medium containing 5% fetal bovine serum (Gibco-BRL), 50,000 U/ml penicillin and 50,000 μg/ml streptomycin. Cells were cultured for 6–10 days before insertion into a modified, circulating Ussing chamber system constructed to accept SNAPWELL filters (World Precision Instrument, Sarasota, FL).

Measurement of I_{sc}

Transepithelial electrophysiologic measurements were performed in a modified Ussing chamber constructed to accept a SNAPWELL filter. I_{sc} was measured with a DVC-1000 voltage/current clamp (World Precision Instrument) with a voltage clamp mode. The bath solution in intact monolayers of T84 cells, Calu-3 cells, or rat airway epithelial cells was a nominally bicarbonate-free Ringer's solution that was composed of (in mM): 140 NaCl; 2.3 K_2HPO_4; 0.4 KH_2PO_4; 1.2 $CaCl_2$; 1.2 $MgCl_2$; 10 HEPES; and 10 glucose (pH 7.4).

Measurement of Basolateral or Apical Cl^- Current

Chloride current flowing through the basolateral or apical membrane was measured in a symmetrical bicarbonate-free, K^+-free, and N-methyl-D-glucamine (NMG) Cl^- solution which bathed the apical and basolateral chambers. This solution was made by substituting Na_2HPO_4 and NaH_2PO_4 for K_2HPO_4 and KH_2PO_4, respectively, and NMG-Cl^- for NaCl as a small amount of K^+ records inwardly rectifying K^+ current which masks the outward rectification of Cl^- current. Basolateral chloride current recordings were made in the presence of BAPTA-AM (50 μM), ouabain (0.1 mM), and nifedipine (100 μM) in the basolateral bath to exclude the Ca^{2+}-mediated current and cation current. Chloride free solution was made by substituting Na-gluconate and Na-phosphates for NaCl and K-phosphates respectively. NaCl, NaI or NaBr Ringer's solution instead of NMG-Cl was used for the measurement of anion selectivity as no detectable Na^+ current was recorded across the basolateral membrane. Nystatin (180 μg/ml) was added to the apical or basolateral bath to permeabilize the respective membrane. To prepare a voltage-current curve of channels, currents were elicited by 1 sec test potential from −80 to +80 mV in 20 mV increments in the symmetrical solution.

Results

Figure 1*A* shows representative BORDIC current traces at different clamped voltages applied after nystatin permeabilization of the apical membrane. Immediately after nystatin permeabilization of the apical membrane, the basolateral membrane was clamped to 0 mV, voltages between −80 and +80 mV (holding voltage = 0 mV)

were applied to the basolateral membrane and the resulting currents were recorded. A bicarbonate-free, K^+-free and N-methyl-D-glucamine (NMG) Cl^- bilateral symmetrical solution was used to record Cl^- current. Permeabilization and current recordings were done in the presence of BAPTA-AM (50 μM), ouabain (0.1 mM), and nifedipine (0.1 mM) to exclude the Ca^{2+}-mediated current and cationic current. Figure 1*B* shows corresponding *I–V* plots of the currents shown in Fig. 1*A*. The BORDIC current was strongly inhibited by basolateral 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, 1 mM), and completely diminished in the symmetrical Cl^--free gluconate solution. Basolateral bumetanide (0.5 mM) did not affect BORDIC current at all (*data not shown*). Halide selectivity for the BORDIC current was measured in symmetrical NaCl, NaI, or NaBr Ringer's solution, which solution was substituted for NMG-Cl. As no detectable Na^+ current was observed across the basolateral membrane (*data not shown*), the halide selectivity experiment was done in the Na^+ containing halide instead of NMG-halide Ringer's solution.

Figure 2*A* shows *I–V* plot of anion currents which were recorded by the application of voltages between −80 and +80 mV (holding voltage = 0 mV) in the symmetrical Na^+ containing halide solutions after permeabilization of apical membrane. The representative currents induced by +80 mV clamped voltage were shown in Fig. 2*B*. Figure 2*A* and *B* indicates that halide selectivity for BORDIC current is $Br^- = I^- > Cl^-$. The BORDIC current was recorded reproducibly in most of the rat airway epithelial monolayers we tested ($n > 105$), and specifically across the basolateral membrane. To address the membrane specificity of BORDIC current, we asked whether depolarization activated Cl^- current across the apical membrane is present or not after permeabilization of the basolateral membrane. Figure 3 shows that no detectable current was recorded by the application +80 mV clamped voltage across the apical membrane in the symmetrical bicarbonate-free, K^+ free and NMG-Cl solution that was used for BORDIC current recording. However the application +80 mV clamped voltage from 0 mV holding voltage after stimulation of 8-Br-cAMP (0.1 mM for 10 min) or ionomycin (1 μM for 10 min) induced Cl^- current across the apical membrane.

As BORDIC current was strongly inhibited by the basolateral DIDS, the effect of basolateral or apical DIDS on the cAMP-mediated Cl^- secretion in an intact monolayer was observed. Bicarbonate free normal Ringer's solution was used for this experiment. Figure 4*A* shows that the short circuit current (I_{sc}), increased by 8-Br-cAMP (1 mM), was significantly inhibited by basolateral but not apical DIDS (0.2 mM) in intact monolayer of rat airway epithelia. The same results were observed in an intact monolayer of T84 colonic cell lines and Calu-3 human airway cell lines (Fig. 4*B*).

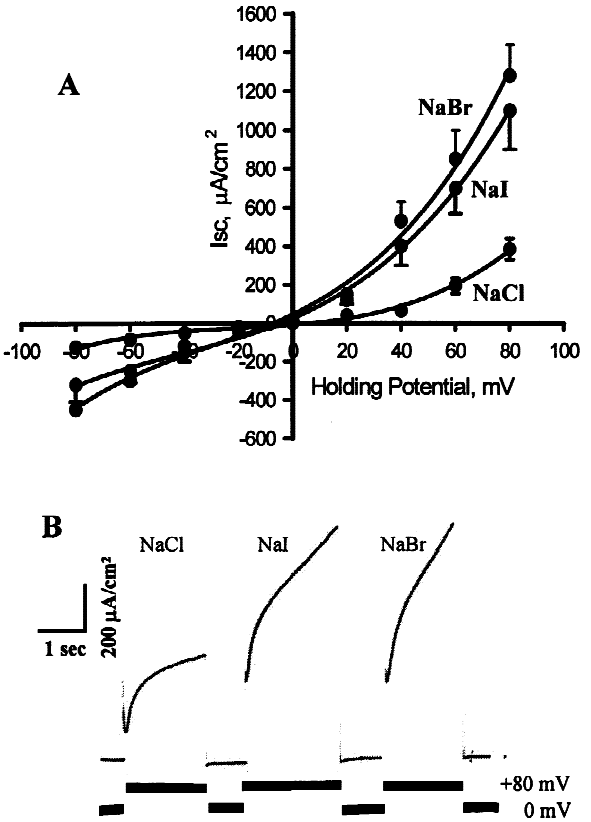

Fig. 2. *I–V* plots of halide permeability for outwardly rectifying depolarization induced Cl^- current across the basolateral membrane (BORDIC) recorded in the symmetrical bicarbonate, K^+ free and NaCl, NaI or NaBr Ringer's solution after permeabilization of apical membrane (*A*). Representative currents trace at +80 mV clamping pulses from a holding voltage of 0 mV applied after nystatin (180 μg/ml) permeabilization of the apical membrane. (*B*).

Discussion

In this study, high resistance rat airway epithelial monolayers were used after apical or basolateral membrane permeabilization with nystatin to functionally isolate the basolateral or apical membrane. The macroscopic currents recorded on the permeabilized rat airway epithelial monolayer under a transepithelial voltage have shown similar biophysical characteristics shown by whole cell or single channel recording in previous experiments (Hwang et al., 1996). Even though a rectified current in symmetrical solution can be recorded on the monolayer of Calu-3 human airway or T84 human colonic epithelial cell lines, it is not easy to quantify the data statistically because transcellular resistance after nystatin treatment

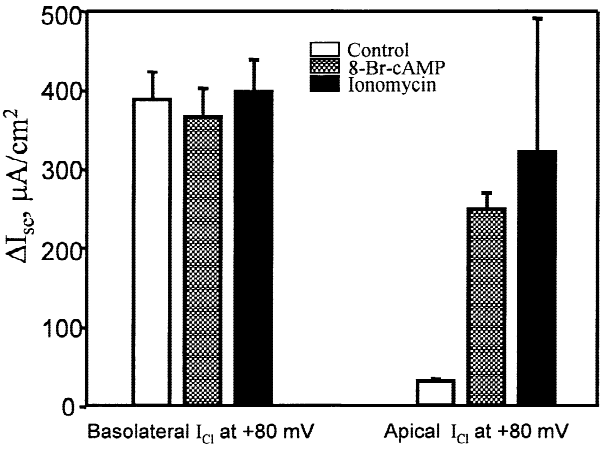

Fig. 3. Comparison of the depolarization induced Cl^- current in the symmetrical bicarbonate-free, K^+-free and NMG-Cl Ringer's solution across the basolateral or apical membrane. The clamped voltage of +80 mV pulses from 0 mV holding potential were applied after permeabilization of apical or basolateral membrane in the absence and presence of 8-Br-cAMP (0.1 mM) or ionomycin (1 μM) ($n = 5$).

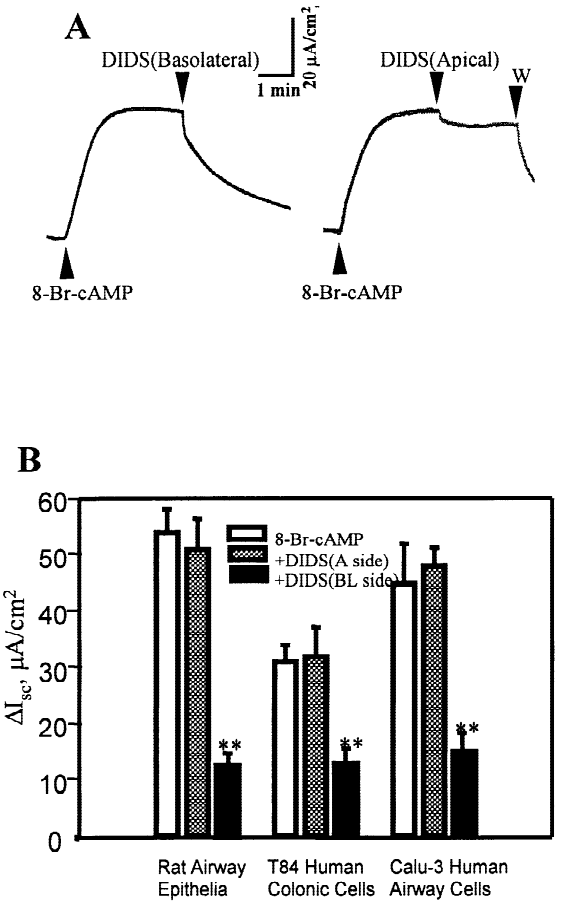

Fig. 4. Effects of basolateral (BL side) or apical (A side) DIDS on cAMP-mediated short circuit current (I_{sc}) in the intact monolayer of rat airway epithelia, T84 human colonic cell lines and Calu-3 human airway cell lines ($n > 8$). (*A*) Representative recordings of DIDS effect during CFTR activation in intact rat airway epithelial monolayer. DIDS (0.2 mM) was added into basolateral or apical bath in the presence of 8-Br-cAMP (0.1 mM) or ionomycin (1 μM). Bicarbonate-free normal Ringer's solution were used. (*B*) A summary of basolateral or apical DIDS effect on cAMP mediated I_{sc}. $^{**}P < 0.01$ compared with values for control current.

is not high enough. Therefore rat airway epithelial monolayer was used for this study unless otherwise indicated.

This study provides evidence that cultured monolayer of rat airway epithelial cells express a basolateral outwardly rectifying chloride conductance (BORDIC). The conductance is DIDS sensitive and not affected by basolaterally applied bumetanide. Cyclic-AMP dependent current activation in the intact monolayer is inhibited by basolateral application of DIDS implying indirectly that the BORDIC conductance contributes to the cAMP-mediated Cl^- secretion.

Bumetanide-sensitive basolateral Na-K-2Cl cotransport have been known for an entry pathway for Cl^- secreted by the CFTR in a variety of epithelia (Welsh & Smith, 1993). However a recent report showed that cAMP increased bumetanide-insensitive Cl^- secretion in jejunum, colon, and airway epithelia isolated from mice lacking the basolateral Na-K-2Cl cotransport, which suggests an alternative Cl^- entry pathway is present on the basolateral membrane (Flagella et al., 1999). The physiologic function of BORDIC current in airway epithelia is not currently proposed. However, we speculate that BORDIC current can be responsible for an alternative pathway for basolateral Cl^- entry based on its activation by depolarization voltage, outward rectification, basolateral DIDS sensitivity on cAMP mediated Cl^- secretion in intact monolayer, and bumetanide insensitivity. As bicarbonate free Ringer's solution was used for this experiment, the possibility of DIDS effect on bicarbonate transport can be excluded. However, it has been reported that DIDS has no effect on cAMP-mediated I_{sc} in primary human tracheal cells (Shen et al., 1995), T84 intestinal crypt cell lines (Brayden et al., 1993), or intact sweat ducts (Morris et al., 1992). The differential results of DIDS on cAMP-mediated I_{sc} in intact rat airway, T84 cells and Calu-3 epithelial monolayer in this study might be due to the basolateral application of DIDS while the effect of apical DIDS has been observed in previous experiments.

The biophysical characteristics and functional significance of outwardly rectifying depolarization induced Cl^- channel (ORDIC) in CFTR expressing epithelial cells have been well reported (Tabcharani & Hanrahan, 1991; Egan et al., 1992). Early studies of ORDIC indicated that it can be activated by cAMP agonists in cell-attached membrane patches or by the catalytic subunit of PKA or PKC in excised, inside-outpatches from airway

or intestinal epithelia (Frizzell, Rechkemmer & Shoemaker, 1986). Moreover, such regulation was reported to be defective in CF patients, which in turn focused a great deal of attention on the channel. The ORDIC channels were suggested to occur on the basolateral membrane, based on the observation that high percentage recording of ORDIC channel activity in nonconfluent cells compared with in confluent cells (Pisam & Ripoche, 1976). After tight junctions are broken, cell polarity is lost and the two membranes mix in nonconfluent cells. However other groups explained that the upregulated activity of ORDIC channels by disruption of monolayer integrity, rather than membrane localization, might be involved in high percentage recording of ORDIC channel activity in nonconfluent cells (Xia, Haws & Wine, 1997). This indicates that no direct evidence regarding the membrane localization of the ORDIC channels has been observed. The apical membrane localization of CFTR and CACC channels recorded in this experiment agree with most prior accounts. Therefore, there is a possibility that the BORDIC current can be ORDIC current. The physiological functions of BORDIC current regarding those of ORDIC and CFTR currents should be illuminated for further study.

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1997 (F0301). Thanks are due to professor Michael Duffy for proofreading the manuscript.

References

Anderson, M.P. 1992. Chloride channels in the apical membrane of normal and cystic fibrosis airway and intestinal epithelia. *Am. J. Physiol.* **263:**L1–L14

Anderson, M.P., Gregory, R.J., Thompson, S., Souza, D.W., Paul, S., Mulligan, R.C., Smith, A.E., Welsh, M.J. 1991. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* **253:**202–205

Brayden, D.J., Krouse, M.E., Law, T., Wine, J.J. 1993. Stilbenes stimulate T84 Cl^- secretion by elevating Ca^{2+}. *Am. J. Physiol.* **264:** G325–G333

Egan, M., Flotte, T., Afione, S., Solow, R., Zeitlin, P.L., Carter, B.J., Guggino, W.B. 1992. Defective regulation of outwardly rectifying Cl^- channels by protein kinase A corrected by insertion of CFTR. *Nature* **358:**581–584

Flagella, M., Clarke, L.L., Miller, M.L., Erway, L.C., Giannella, R.A., Andringa, A., Gawenis, L.R., Kramer, J., Duffy, J.J., Doetschman, T., Lorenz, J.N., Yamoah, E.N., Cardell, E.L., Shull, G.E. 1999. Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. *J. Biol. Chem.* **274:**26946–26955

Frizzell, R.A., Rechkemmer, G., Shoemaker, R.L. 1986. Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science* **233:**558–560

Hwang, T.H., Jung, J.S., Bae, H.R., Yun, I., Lee, S.H. 1994. Stimulation of Cl^- secretion by $A1F_4^-$ and vanadate in T84 cells. *J. Kor. Med. Sci.* **9:**497–504

Hwang, T.H., Schwiebert, E.M., Guggino, W.B. 1996. Apical and basolateral ATP stimulates tracheal epithelial chloride secretion via multiple purinergic receptors. *Am. J. Physiol.* **270:**C1611–1623

Hwang, T.H., Suh, D.J., Bae, H.R., Lee, S.H., Jung, J.S. 1996. Characterization of K^+ channels in the vasolateral membrane of rat tracheal epithelia. *J. Membrane Biol.* **154:**251–257.

MacVinish, L.J., Hickman, M.E., Mufti, D.A., Durrington, H.J., Cuthbert, A.W. 1998. Importance of basolateral K^+ conductance in maintaining Cl^- secretion in murine nasal and colonic epithelia. *J. Physiol.* **510:**237–247

Morris, A.P., Cunningham, S.A., Benos, D.J., Frizzell, R.A. 1992. Cellular differentiation is required for cAMP but not Ca^{2+}-dependent Cl^- secretion in colonic epithelial cells expressing high levels of cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **267:**5575–5583

Pisam, M., Ripoche, P. 1976. Redistribution of surface macromolecules in dissociated epithelial cells. *J. Cell Biol.* **71:**907–920

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245:**1066–1073

Schwiebert, E.M., Egan, M.E., Twang, T.H., Fulmer, S.B., Allen, S.S., Cutting, G.R., Guggino, W.B. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* **81:**1063–1073

Shen, B.Q., Mrsny, R.J., Finkbeiner, W.E., Widdicombe, J.H. 1995. Role of CFTR in chloride secretion across human tracheal epithelium. *Am. J. Physiol.* **269:**L561–L566

Tabcharani, J.A., Hanrahan, J.W. 1991. On the activation of outwardly rectifying anion channels in excised patches. *Am. J. Physiol.* **261:**G992–G999

Valverde M.A., Hardy, S.P., Sepulveda, F.V. 1995. Chloride channels: a state of flux. *FASEB J.* **9:**509–515

Welsh, M.J., Smith, A.E. 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell* **73:**1251–1254

Xia, Y., Haws, C.M., Wine, J.J. 1997. Disruption of monolayer integrity enables activation of a cystic fibrosis "bypass" channel in human airway epithelia. *Nature Med.* **3:**802–805