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Characterization of basolateral K^+ channels underlying anion secretion in the human airway cell line Calu-3

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Transepithelial anion secretion in many tissues depends upon the activity of basolateral channels. Using monolayers of the Calu-3 cell line, a human submucosal serous cell model mounted in an Ussing chamber apparatus, we investigated the nature of the K^+ channels involved in basal, cAMP- and Ca^{2+}-stimulated anion secretion, as reflected by the transepithelial short circuit current (I_{sc}). The non-specific K^+ channel inhibitor Ba^{2+} inhibited the basal I_{sc} by either 77 or 16 % when applied directly to the basolateral or apical membranes, respectively, indicating that a basolateral K^+ conductance is required for maintenance of basal anion secretion. Using the K^+ channel blockers clofilium and clotrimazole, we found basal I_{sc} to be sensitive to clofilium, with a small clotrimazole-sensitive component. By stimulating the cAMP and Ca^{2+} pathways, we determined that cAMP-stimulated anion secretion was almost entirely abolished by clofilium, but insensitive to clotrimazole. In contrast, the Ca^{2+}-stimulated response was sensitive to both clofilium and clotrimazole. Thus, pharmacologically distinct basolateral K^+ channels are differentially involved in the control of anion secretion under different conditions. Isolation of the basolateral K^+ conductance in permeabilized monolayers revealed a small basal and forskolin-stimulated I_{sc}. Finally, using the reverse transcriptase-polymerase chain reaction, we found that Calu-3 cells express the K^+ channel genes *KCNN4* and *KCNQ1* and the subunits *KCNE2* and *KCNE3.* We conclude that while KCNN4 contributes to Ca^{2+}-activated anion secretion by Calu-3 cells, basal and cAMP-activated secretion are more critically dependent on other K^+ channel types, possibly involving one or more class of KCNQ1-containing channel complexes.

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Human airway serous cells, found within submucosal glands, are of fundamental importance in maintaining a sterile environment within the lung. In addition to secreting a variety of antimicrobial factors (Basbaum *et al.* 1990), they contribute to the quantity and composition of the airway surface liquid, both of which have been implicated in primary host defence mechanisms (Smith *et al.* 1996; Wine, 1999; Boucher, 1999). Serous cells are also the principal pulmonary site of expression of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Engelhardt *et al.* 1992; Jacquot *et al.* 1993), mutations in which cause cystic fibrosis (CF; Riordan *et al.* 1989). CFTR is a cAMP-dependent channel located in the apical membrane of a variety of epithelial cell types where it acts as the predominant Cl^- conductance (Pilewski & Frizzell, 1999). Precisely how absence of functional CFTR in the airway leads to the devastating pulmonary pathology associated with CF remains unclear. However, the high degree of expression of CFTR in the serous cell makes it likely that the function of these cells is among the most affected in CF. Therefore, understanding fluid and electrolyte transport in serous cells provides important information as to the role of CFTR in normal airway physiology and may lead to meaningful insights into the pathogenesis of CF lung disease.

The Calu-3 cell line, originally derived from a lung adeno-carcinoma (Shen *et al.* 1994), has been widely used as a model of the serous cell (Haws *et al.* 1994; Moon *et al.* 1997; Lee *et al* 1998; Devor *et al.* 1999; Pilewski & Frizzell, 1999). Calu-3 cells express high levels of CFTR (Shen *et al.* 1994), form polarized monolayers with a transepithelial resistance of approximately 100 Ω cm^2 (Shen *et al.* 1994), express markers of serous cell function such as lysozyme, lactoferrin and secretory leukocyte protease inhibitor (Finkbeiner *et al.* 1993) and demonstrate active trans-epithelial anion secretion in response to a number of pharmacological stimuli, including those which raise intracellular cAMP or Ca^{2+} concentrations (Shen *et al.* 1994; Moon *et al.* 1997). While it appears that the anion secreted may be either Cl^- (Shen *et al* 1994; Singh *et al.* 1997; Devor *et al.* 1999) or HCO_3^- (Illek *et al.* 1997; Lee *et al.* 1998; Devor *et al.* 1999) depending upon conditions, in both cases the final step appears to be electrodiffusional

Table 1. Primer sequences, expected size of the RT-PCR product, and PCR conditions for *KCNE2, KCNE3, KCNQ1, KCNN4* and *CFTR*

Primer		Expected size (bp)	Conditions (denaturation; annealing; extension)
KCNE2	5´-TCTTCCGAAGGATTTTTATTACTTAT-3´ 5´-ACCATCCATGAGAACATTGGT-3´	304	95 °C/1 min; 58 °C/1 min; 72 °C/2 min; 30 cycles
KCNE3	5´-TACCAATGGAACGGAGACCT-3´ 5´-GGGTCACTACGCTTGTCCAC-3´	264	95 °C/1 min; 58 °C/1 min; 72 °C/2 min; 30 cycles
KCNN4 *	5´-CGGCGTCCTGCTCAACG-3´ 5´-CACCAGCAGGGCTGTGCAG-3´	337	95 °C/1 min; 62 °C/30 s; 72 °C/90 s; 30 cycles
KCNQ1 †	5´-TTCTGGATGGAGATCGTG-3´ 5´-GCCTTCCGGATGTAGATC-3´	738	95 °C/30 s; 63 °C/1 min; 72 °C/1 min; 35 cycles
CFTR	5´-CAAGGAGGAACGCTCTATCG-3´ 5´-ACGCCTGTAACAACTCCCAG-3´	326	95 °C/1 min; 58 °C/1 min; 72 °C/2 min; 30 cycles

* Warth *et al.* (1999); † Mall *et al.* (2000).

anion exit across the apical membrane via CFTR (Illek *et al.* 1997; Lee *et al.* 1998; Devor *et al.* 1999).

A number of studies have demonstrated that the rate of transepithelial anion secretion is dependent upon the activity of basolateral K^+ channels, since exit of K^+ across the basolateral membrane hyperpolarizes the cell, increasing the electrochemical driving force for anion efflux through open apical membrane channels (Smith & Frizzell, 1984; McCann & Welsh, 1990; Devor *et al.* 1996). A similar mechanism has been proposed in Calu-3 cells (Moon *et al.* 1997; Devor *et al.* 1999); however, despite this potentially important role, little is known about the nature of these basolateral K^+ channels or their role in controlling transepithelial anion secretion. We therefore wished to investigate how different basolateral K^+ channels may influence the cAMP- and Ca^{2+}-mediated changes in transepithelial ion transport observed in Calu-3 cells. We investigated the effects of known epithelial K^+ channel inhibitors on the I_{sc} recorded across monolayers of Calu-3 cells under both basal and stimulated conditions. Furthermore, using RT-PCR we have identified a number of K^+ channel genes and modifying channel subunits which may be integrally involved in anion secretion across these epithelial cells.

METHODS

Culture of Calu-3 cells

Cells were purchased from the American Type Culture Collection (Rockville, MD, USA) at passage 18. All experiments were performed on cells following an additional 5–12 passages (i.e. final passage number between 23 and 30). They were maintained in 1:1 Dulbecco's modified Eagle's medium–Ham's F-12 nutrient mixture supplemented with 10 % fetal bovine serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} fungizone (all from Gibco BRL, Burlington, ON, Canada) and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. For RNA extraction, cells were grown to confluence in 100 mm diameter Falcon tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA). For measurements of transepithelial short circuit current (I_{sc}), cells were plated onto 12 mm diameter (1.1 cm^2 surface area) Snapwell inserts (Corning Costar, Cambridge, MA, USA). Initially cells were grown submerged in culture medium. However after 3 days of culture, cells were grown at an air–liquid interface, i.e. medium was present only on the basolateral side of the inserts, as previously described (Singh *et al.* 1997; Moon *et al.* 1997). Culture medium was changed every 48 h, and the cells formed a confluent monolayer that held back fluid. Experiments were performed 10–26 days after establishment of the air–liquid interface.

Measurement of transepithelial short-circuit current (I_{sc})

Calu-3 cells grown on Snapwell inserts were mounted in an Ussing chamber (World Precision Instruments (WPI), Sarasota, FL, USA), and the transepithelial potential difference clamped to zero using DVC-1000 voltage-clamp apparatus (WPI). The I_{sc} was recorded using Ag–AgCl electrodes in 3 M KCl agar bridges. Data was sampled at 10 Hz and recorded to computer disk using custom-written software (courtesy of Dr A. S. French). Transepithelial resistance was calculated using Ohm's law by determining the change in current arising from applying 0.4 mV pulses every 50 s. Apical and basolateral solutions were maintained at 37 °C by heated water jackets, and were separately perfused and oxygenated with a 95 % O_2 : 5 % CO_2 mixture. The bath solutions were (mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose or mannitol held at pH 7.4 at 37 °C, when gassed with 5 % CO_2. Glucose was present in the basolateral bath solution, but was substituted with mannitol in the apical solution to eliminate the activity of the Na^+–glucose transporter (Singh *et al.* 1997). Monolayers were equilibrated in these buffers for 30 min before the experiment commenced.

Permeabilized monolayers

To investigate the activity of basolateral K^+ channels in isolation, the apical membrane was permeabilized by the addition of 10 μM of the pore-forming antibiotic amphotericin B, (Sigma, Oakville, ON, Canada) in Cl^--free buffers. The following bath solutions were used. Apical (mM): 145 potassium gluconate, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 magnesuim gluconate, 4 calcium gluconate, 10 glucose, 10 Hepes. Basolateral (mM): 145 sodium gluconate, 3.3 NaH_2PO_4, 0.8 Na_2HPO_4, 1.2 magnesuim gluconate, 4 calcium gluconate, 10 glucose, 10 Hepes. Solutions were adjusted to pH 7.4 at 37 °C. By permeabilizing the apical membrane in the presence of these buffers, a mucosal to serosal K^+ gradient is established and the I_{sc} reflects K^+ movement through basolateral K^+ channels down a concentration gradient.

RNA extraction

Total RNA was extracted from Calu-3 cells using TRIzol reagent (Gibco). RNA was then DNase treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and the product run on a 1 % agarose gel to check integrity. Two micrograms of DNase-treated RNA was then reverse transcribed using M-MLV reverse transcriptase (Gibco) in the presence of 5 mM dNTP and 1 μM oligo dT (Amersham Pharmacia, Baie d'Urfe, PQ, Canada) to produce cDNA.

Polymerase chain reaction

After reverse transcription, polymerase chain reaction (PCR) was performed to amplify DNA fragments using the primers and conditions described in Table 1. The primers and conditions used for *KCNN4* and *KCNQ1* have been described previously (Warth *et al.* 1999; Mall *et al.* 2000). Primers for *KCNE1, KCNE2, KCNE3, KCNH2* and *CFTR* were designed using the published sequences for these genes available from the National Center for Biotechnology Information (NCBI). All custom primers were obtained from Gibco. PCR was performed using primer pairs at 10 μM with 2.5 units *Taq* polymerase (MBI Fermentas, Burlington, ON, Canada), 25 mM $MgCl_2$ and 5 mM dNTP in a total reaction volume of 25 μl. PCR products were visualized by loading a 8 μl sample on a 1.5 % agarose gel containing 250 $\mu g\ l^{-1}$ ethidium bromide, alongside a 100 bp DNA ladder (Gibco).

DNA sequencing

To confirm the identity of the amplified PCR fragments, these products were isolated from the gel using the QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada). DNA was then ligated into the pGEM vector (Promega, Madison, WI, USA), propagated in *E. coli* strain JM109 and sequenced using the Sequenase DNA sequencing kit (USB, Cleveland, OH, USA).

Chemicals

Forskolin, clotrimazole, amphotericin B, 1-ethyl-2-benzimidazolinone (1-EBIO), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-bromo-cAMP) and thapsigargin were obtained from Sigma Aldrich (Oakville, ON, Canada). Clofilium was purchased from RBI (Natick, MA, USA). Stock solutions were made up in either DMSO or ethanol (clotrimazole) so that the final bath concentration of DMSO was ≤ 0.1 %. Application of DMSO or ethanol alone had no effect upon the monolayers.

Statistics

All data are presented as means ± standard error of the mean. Differences between groups were tested for statistical significance using Student's *t* test, with significance determined as $P < 0.05$.

RESULTS

Effect of Ba^{2+} ions on anion secretion in Calu-3 monolayers

The basal I_{sc} in intact monolayers of Calu-3 cells was $30.2 \pm 1.6\ \mu A\ cm^{-2}$ (range, 11.8–65.4 $\mu A\ cm^{-2}$) with an initial resistance of $164.7 \pm 7.4\ \Omega\ cm^2$ ($n = 41$). Basal I_{sc} in these cells has previously been demonstrated to be almost exclusively accounted for by anion secretion (Singh *et al.*

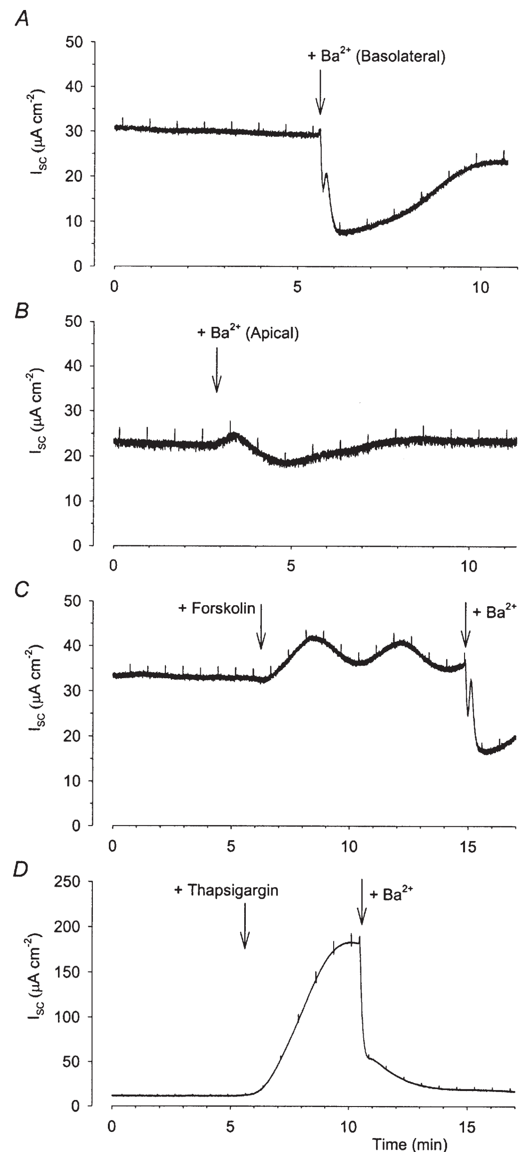

Figure 1. Inhibition of short circuit current (I_{sc}) across Calu-3 monolayers by Ba^{2+}

Basal I_{sc} in Calu-3 monolayers was inhibited by the addition of 5 mM Ba^{2+}, a non-specific inhibitor of K^+ channels, to both the basolateral (*A*) and apical (*B*) membranes. Forskolin- and thapsigargin-stimulated I_{sc} were also inhibited by basolateral application of Ba^{2+} (*C* and *D*). The transient inhibition frequently seen following the addition of Ba^{2+} was due to precipitation of Ba^{2+} from solution as previously reported (Moon *et al.* 1997).

1997). Basal I_{sc} was decreased by the addition of the non-specific K^+ channel inhibitor Ba^{2+} confirming the importance of K^+ channels in transepithelial anion secretion. Furthermore, Ba^{2+} was more effective when applied to the basolateral, rather than the apical, side of the monolayer. When 5 mM Ba^{2+} was added to the basolateral membrane, basal I_{sc} was inhibited by 77.1 ± 3.1 % ($n = 5$, Fig. 1*A*) whereas addition to the apical membrane inhibited only 15.7 ± 2.2 % ($n = 6$, Fig. 1*B*) of the I_{sc}.

Since basolateral K^+ channels appear to play a more significant role than apical channels in the maintenance of basal I_{sc} in Calu-3 cells, we further examined the role of basolateral K^+ channels in cAMP- and Ca^{2+}-stimulated anion secretion. The addition of 2 μM of the cAMP activator forskolin to both apical and basolateral sides of the monolayer resulted in an increased I_{sc} (mean increase 23.2 ± 2.6 μA cm^{-2}; range, 10.7–37.9 μA cm^{-2}; $n = 16$, Fig. 1*C*), as previously reported (Shen *et al.* 1994). Further additions of forskolin had no effect upon the I_{sc} recorded (results not shown). The addition of 300 nM thapsigargin to both sides of the monolayer resulted in a much larger increase in I_{sc} (129.9 ± 20.8 ; range, 86.6–197.2 μA cm^{-2}; $n = 7$, Fig. 1*D*), again confirming previous work (Moon *et al.* 1997). Thapsigargin produces a sustained increase in the intracellular Ca^{2+} concentration by inhibiting the sarcoplasmic reticulum Ca^{2+}-ATPase. The increased I_{sc} recorded from Calu-3 cells upon addition of forskolin and thapsigargin has previously been demonstrated to reflect increased anion secretion (Shen *et al.* 1997; Moon *et al.*

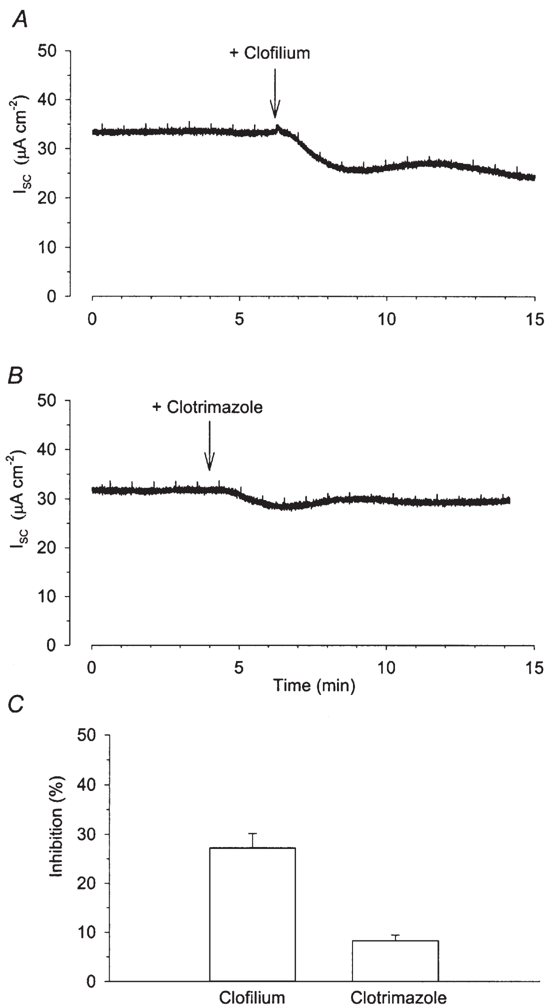

Figure 2. Inhibition of the basal I_{sc} in Calu-3 cells by clofilium and clotrimazole

Addition of 100 μM clofilium to the basolateral membrane of Calu-3 cells reduced the basal I_{sc} (*A*, $n = 6$). Addition of 30 μM clotrimazole to the basolateral membrane resulted in a much smaller decrease in basal I_{sc} (*B*, $n = 8$). *C* shows the percentage of basal I_{sc} inhibited by these agents.

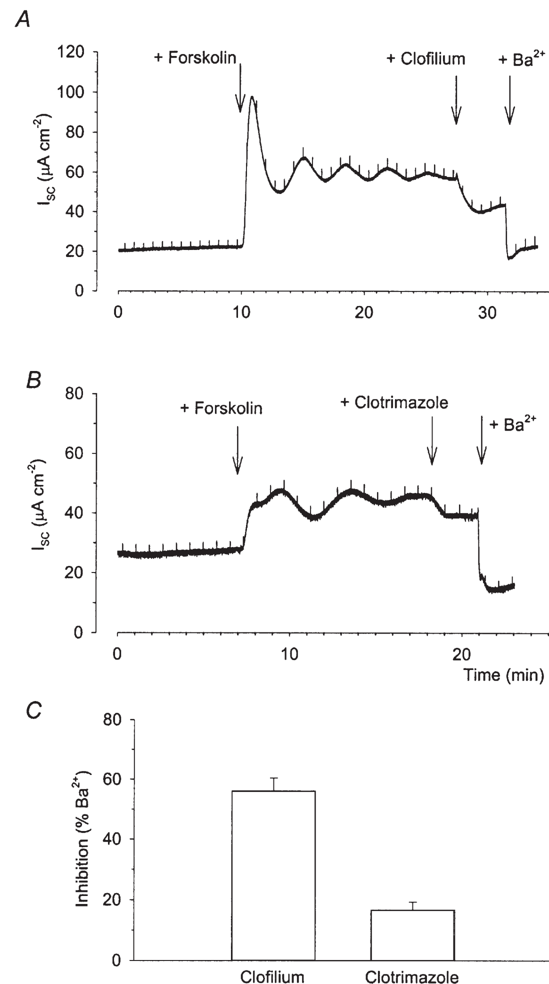

Figure 3. Effects of clofilium and clotrimazole on the cAMP-stimulated increase in I_{sc} in Calu-3 cells

When Calu-3 monolayers were stimulated with 2 μM forskolin applied to both apical and basolateral sides of the membrane, the subsequent addition of 100 μM clofilium to the basolateral membrane reduced the I_{sc} (*A*, $n = 8$). Addition of 30 μM clotrimazole to the basolateral membrane (*B*, $n = 8$) resulted in a much smaller decrease in I_{sc}. At the end of every experiment 5 mM Ba^{2+} was added to the basolateral membrane, which further inhibited the I_{sc}. *C*, reduction in I_{sc} caused by clofilium and clotrimazole expressed as a percentage of the Ba^{2+}-inhibitable I_{sc} (as described in the text).

1997; Devor *et al.* 1999). Addition of Ba^{2+} to the basolateral side of the membrane inhibited both the forskolin- and thapsigargin-stimulated I_{sc}, indicating a role for basolateral K^+ channels in cAMP- and Ca^{2+}-stimulated secretion (Fig. 1*C* and *D*).

Pharmacological characterization of basolateral K^+ channels in intact Calu-3 monolayers

To investigate further the nature of the basolateral K^+ channels responsible for maintenance of anion secretion, we examined the effects of the K^+ channel inhibitors clofilium and clotrimazole. The addition of 100 μM clofilium, described at this concentration as an inhibitor of epithelial basolateral K^+ channels in the rat trachea (Hwang *et al.* 1996), mouse trachea (Lock & Valverde, 2000) and mouse colon and nose (MacVinish *et al.* 1998), to the basolateral membrane significantly reduced the basal I_{sc} (mean decrease of 7.9 ± 1.0 $\mu A\ cm^{-2}$ corresponding to 27.2 ± 2.9 % of the baseline, $n = 6$; Fig. 2*A* and *C*). Addition of 30 μM of the antifungal agent clotrimazole, an inhibitor of Ca^{2+}-activated K^+ channels (Devor *et al.* 1996, 1999), to the basolateral membrane resulted in a much smaller decrease in basal I_{sc} (2.3 ± 0.3 $\mu A\ cm^{-2}$ or 11.3 % of the baseline, $n = 8$; Fig. 2*B* and *C*). A series of preliminary experiments confirmed that these concentrations of clofilium and clotrimazole were maximally effective, and that further addition of the agent produced no further inhibition (results not shown). These results suggest that clofilium-sensitive K^+ channels play a larger role in maintaining basal I_{sc} than clotrimazole-sensitive channels.

cAMP stimulation. To characterize pharmacologically the basolateral K^+ channel types involved in the cAMP secretory response, we next investigated the effects of clofilium and clotrimazole on the forskolin-stimulated I_{sc}. In the first series of experiments (Fig. 3) cells were stimulated with 2 μM forskolin prior to the application of these different K^+ channel blockers. The maximally effective concentration of clofilium (100 μM) caused a mean decrease in the forskolin-stimulated I_{sc} of 21.1 ± 2.3 $\mu A\ cm^{-2}$ ($n = 8$, Fig. 3*A*). In contrast, a maximally effective concentration of clotrimazole (30 μM) produced a much smaller reduction in the forskolin-stimulated I_{sc} of only 3.5 ± 0.7 $\mu A\ cm^{-2}$ ($n = 8$; Fig. 3*B*). As the amplitude of the response to forskolin was variable, 5 mM Ba^{2+} was added to the basolateral side of the monolayer at the end of the experiment and results expressed as the effect on the Ba^{2+}-sensitive I_{sc}. Following forskolin stimulation, clofilium blocked 56.2 ± 4.3 % ($n = 7$) of the Ba^{2+}-sensitive I_{sc}, while clotrimazole blocked only 16.7 ± 2.6 % ($n = 8$; Fig. 3*C*).

In an attempt to dissociate whether the decreases observed in forskolin-stimulated I_{sc} with clofilium and clotrimazole (Fig. 3) were primarily due to inhibition of the response to forskolin or merely reflected a concurrent inhibition of the basal I_{sc} (Fig. 1), a parallel series of experiments was performed in which the inhibitor was added prior to the forskolin stimulus (Fig. 4). When 100 μM clofilium was added to the basolateral membrane of the Calu-3 monolayer, the subsequent response to forskolin was almost completely abolished (Fig. 4*B* and *D*; the mean increase

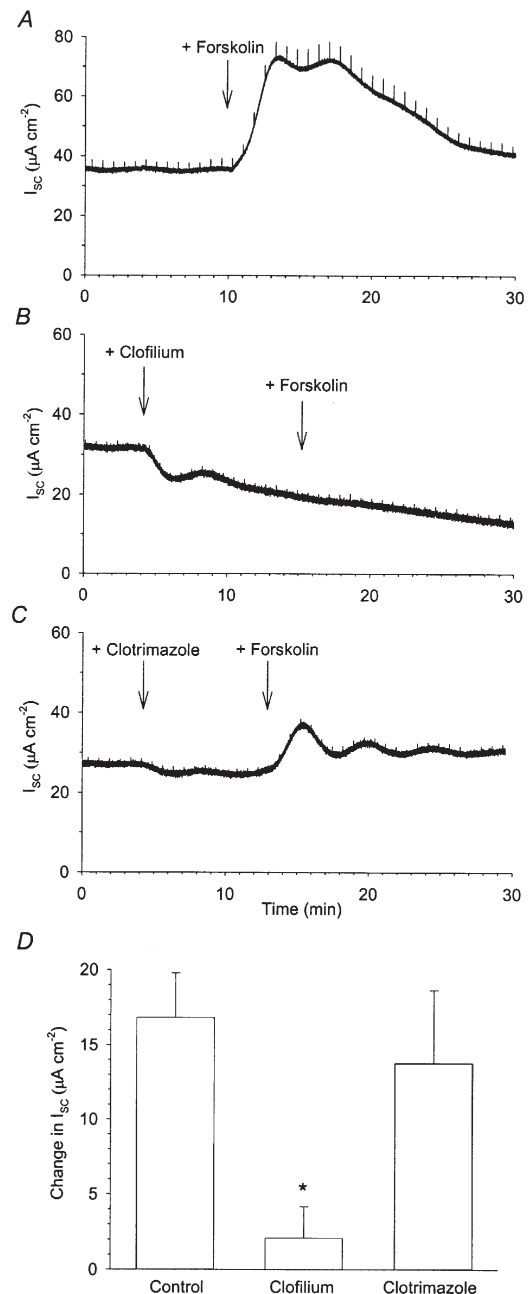

Figure 4. Effect of prior treatment with clofilium or clotrimazole on the cAMP-stimulated increase in I_{sc} in Calu-3 cells

Addition of 2 μM forskolin to Calu-3 monolayers produced an increase in I_{sc} (*A*, $n = 16$). When 100 μM clofilium was applied to the basolateral membrane prior to the forskolin stimulus, the increase in I_{sc} was virtually abolished (*B* and *D*). In contrast, the increase in I_{sc} recorded following the application of 2 μM forskolin when 30 μM clotrimazole had been pre-applied to the monolayers was not significantly different from the forskolin-alone response (* significantly different from forskolin stimulated as determined by Student's *t* test; $P < 0.05$).

with forskolin addition was 2.1 ± 2.1 μA cm^{-2}, $n = 3$). In contrast, pretreatment with clotrimazole did not significantly alter the response to forskolin (Fig. 4*C* and *D*; mean increase was 13.7 ± 4.9 μA cm^{-2}, $n = 3$). This suggests that the forskolin-stimulated increase in I_{sc} is more dependent upon the activity of clofilium-sensitive, rather than clotrimazole-sensitive, K^+ channels.

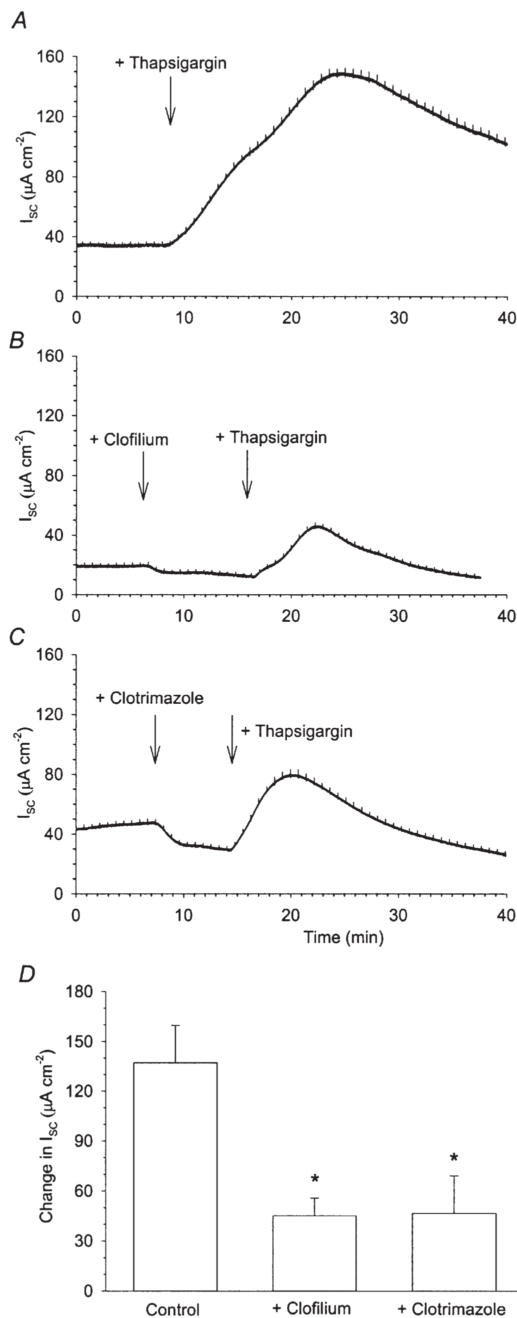

Figure 5. Thapsigargin stimulation of I_{sc} in Calu-3 monolayers

Addition of 300 nM thapsigargin to Calu-3 monolayers produced a large increase in I_{sc} (*A*; $n = 7$). When 100 μM clofilium was applied to the basolateral membrane prior to the thapsigargin stimulus, there was a large increase in I_{sc}, though it was significantly reduced from the thapsigargin-alone response (*B* and *D*). Addition of 30 μM clotrimazole prior to the thapsigargin stimulus also significantly reduced the amplitude of the thapsigargin response (*C* and *D*). * Significantly different from thapsigargin-stimulated I_{sc} as determined by Student's *t* test ($P < 0.05$).

Ca^{2+} stimulation. A corresponding series of experiments was then performed examining the effects of the above K^+ channel blockers on the thapsigargin-stimulated I_{sc}. Figure 5*A* shows a representative example of the large increase in I_{sc} recorded when 300 nM thapsigargin was applied to a monolayer. Unfortunately, due to the transient nature of the response to thapsigargin stimulation, we determined that it was not feasible to quantify the inhibition of this response by subsequent addition of K^+ channel blockers. For this reason, and to avoid ambiguity concerning the effects of blockers on basal *versus* Ca^{2+}-activated I_{sc}, clofilium and clotrimazole were applied prior to the thapsigargin stimulus.

After the addition of clofilium or clotrimazole, (Fig. 5*B* and *C*) thapsigargin still induced a large increase in I_{sc}, though it was significantly smaller than control. Following clofilium pretreatment, the mean increase in I_{sc} was 45.2 ± 10.6 μA cm^{-2} ($n = 3$) while clotrimazole pretreatment reduced the thapsigargin-stimulated increase in I_{sc} to 46.6 ± 10.6 μA cm^{-2} ($n = 3$). These results indicate that both clofilium- and clotrimazole-sensitive channels appear to contribute towards the thapsigargin-stimulated increase in I_{sc}.

Effect of 1-EBIO

To investigate the pharmacological specificity of clofilium and clotrimazole, 1-ethyl-2-benzimidazolinone (1-EBIO) was used as a stimulus for anion secretion. This agent has

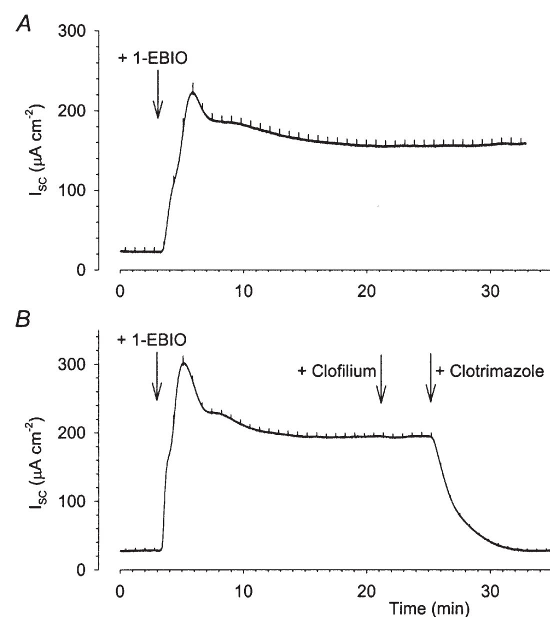

Figure 6. Clotrimazole, but not clofilium, inhibits the 1-EBIO stimulation of I_{sc} in Calu-3 cells

1-EBIO (1 mM) produced a large increase in I_{sc} (*A*, $n = 3$), which was unaffected by the subsequent addition of 100 μM clofilium, but entirely abolished by the addition of 30 μM clotrimazole (*B*).

previously been reported to produce large increases in the I_{sc} when applied to Calu-3 and human bronchial epithelial cells (Devor *et al.* 1999, 2000) by concurrently activating CFTR and basolateral Ca^{2+}-activated K^+ channels. Indeed, when 1 mM 1-EBIO was applied to intact monolayers, it caused a large sustained increase in I_{sc} (190.6 ± 30.1 μA cm^{-2}, $n = 3$; Fig. 6*A*). Application of 100 μM clofilium had no effect upon the response to 1-EBIO, while 30 μM clotrimazole almost completely abolished the secretory response (Fig. 6*B*; $n = 3$). This demonstrates that, at the concentrations used, clofilium has no effect upon the activity of 1-EBIO-sensitive Ca^{2+}-activated K^+ channels, while clotrimazole causes a near-total inhibition of these channels (Devor *et al.* 1999).

Isolation of basolateral K^+ channel currents

To investigate further the nature of the basolateral channels involved in anion secretion from Calu-3 cells, a series of experiments examined the K^+ conductance of the basolateral membrane in isolation. For these experiments, the apical membrane was permeabilized by the addition of 10 μM amphotericin B, in the presence of a mucosal-to-serosal K^+ gradient and monolayers equilibrated for at least 20 min (see Methods). The basal I_{sc} recorded under these conditions which presumably reflects basolateral K^+ current was 5.6 ± 1.5 μA cm^{-2} ($n = 12$). The addition of 2 μM forskolin produced a small increase in the I_{sc} (mean increase 2.5 ± 0.5 μA cm^{-2}; $n = 8$ Fig. 7*A*). The subsequent addition of 100 μM 8-bromo cAMP, to increase the intracellular cAMP levels directly, had no further effect upon the I_{sc}, indicating that the cAMP pathway was maximally stimulated (results not shown). Since the basal and forskolin-stimulated basolateral I_{sc} were so small, it proved impossible to determine the effects of clofilium and clotrimazole on these currents. In contrast, the addition of 1 mM 1-EBIO produced a large increase in I_{sc} (51.4 ± 8.6 μA cm^{-2}, $n = 3$; Fig. 7*B*) which was insensitive to clofilium and almost entirely blocked by 30 μM clotrimazole (Fig. 7*C*).

Molecular characterization of the K^+ channels in Calu-3 cells

By performing RT-PCR on total RNA extracted from cultures of Calu-3 cells, we were able to detect fragments of the K^+ channel genes *KCNN4* and *KCNQ1*, and the K^+ channel subunit genes *KCNE2* and *KCNE3* (Fig. 8). Additionally we performed RT-PCR using primers specific for *CFTR*, known to be abundant in these cells. Fragments were not detected without reverse transcription (results not shown). Despite several attempts at a variety of PCR conditions, we were unable to detect *KCNE1,* or *KCNH2*. When bands were detected using RT-PCR, the DNA was excised, subcloned into the pGEM vector (Promega, Madison, WI, USA) and sequenced. Comparison of the sequenced product with published sequences (National Center for Biotechnology Information) confirmed the expression of *KCNE2, KCNE3, KCNN4* and *KCNQ1* in Calu-3 cells.

DISCUSSION

Submucosal gland serous cells are the predominant site of *CFTR* expression in the human lung (Engelhardt *et al.* 1992; Jacquot *et al.* 1993). The fluid secreted from these cells is integrally involved in the primary host defence mechanisms of the lung, since it contains a host of potent antimicrobial factors (Basbaum *et al.* 1990) and also provides a low viscosity medium for effective mucociliary clearance of debris from the airways (Luk & Dulfano, 1983). It is a logical conclusion that the loss of functional CFTR Cl^- channels which occurs in CF will have a

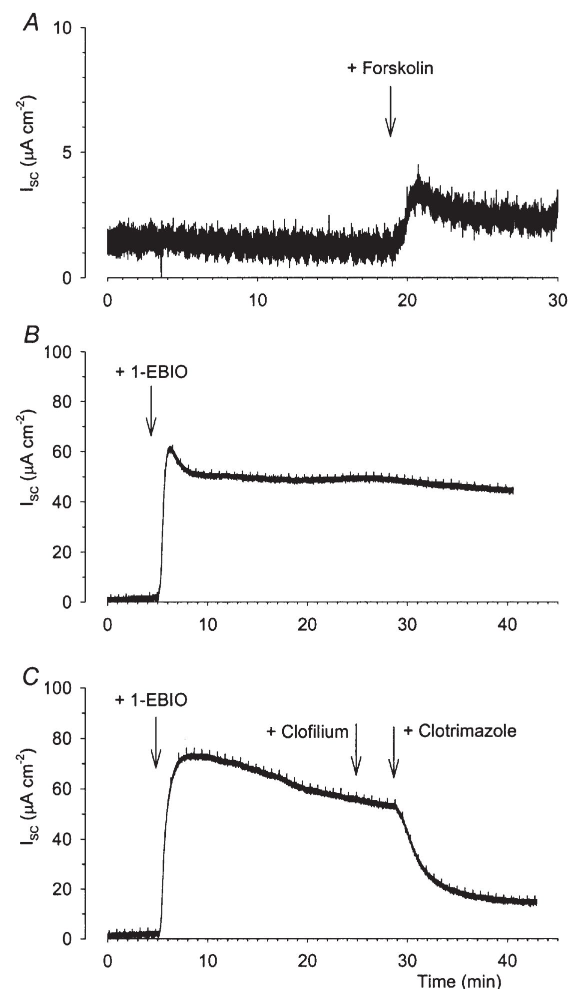

Figure 7. Basolateral K^+ conductances in permeabilized Calu-3 cells

The basolateral K^+ conductance in Calu-3 cells was isolated by permeabilizing the apical membrane. A small basal I_{sc} was recorded under these conditions and the addition of 2 μM forskolin induced a small increase in I_{sc} (*A*, $n = 8$) while the addition of 1 mM 1-EBIO induced a much larger increase in I_{sc} (*B*, $n = 3$). This 1-EBIO-stimulated response in the permeabilized monolayers was unaffected by the addition of 100 μM clofilium, but entirely abolished by the addition of 30 μM clotrimazole (*C*).

profound effect upon the activity of the serous cell. Indeed, it has been proposed that it is the dysfunction of this cell type that is the primary trigger for the pathogenesis of CF lung disease (Jiang *et al.* 1997; Pilewski & Frizzell, 1999). Loss of CFTR from the serous cell could profoundly affect the quantity and/or the composition of glandular secretions, resulting in impaired host defence mechanisms. Knowledge of fluid and electrolyte movement in these cells is therefore important to improve our understanding of normal airway host defence and how this may be impaired in CF. The results of the present study demonstrate the importance of distinct basolateral K^+ channels in controlling anion secretion from a well-characterized model of the serous cell, Calu-3 cells, under different conditions. Furthermore, we identify at the molecular level a number of different K^+ channels and modifying subunits in Calu-3 cells which may account for the differential effects observed.

The Calu-3 cell line has become a widely used and accepted model of the human serous cell (Haws *et al.* 1994; Shen *et al.* 1994; Singh *et al.* 1997; Devor *et al.* 1999; Pilewski & Frizzell, 1999). Calu-3 cells exhibit electrogenic transepithelial anion (Cl^- and/or HCO_3^-) secretion under basal conditions (Singh *et al.* 1997) which is further stimulated by increases in intracellular cAMP or Ca^{2+} concentrations (Shen *et al.* 1994; Moon *et al.* 1997; Devor *et al.* 1999), consistent with the proposed value of these cells as models of serous cell secretion. Anion secretion occurs via cAMP-activated CFTR Cl^- channels at the apical membrane (Haws *et al.* 1994; Moon *et al.* 1997; Illek *et al.* 1999), but also depends on the activity of K^+ channels. Our finding that the non-specific K^+ channel inhibitor Ba^{2+} produced a much larger decrease in basal I_{sc} when applied to the basolateral versus the apical membrane (Fig. 1) confirms the importance of basolateral K^+ channels in anion secretion. The opening of K^+ channels presumably hyperpolarizes the cell, increasing the electrochemical driving force for anion secretion across the apical membrane. In our model system, whether these channels are located on the apical or basolateral membrane would have little effect on their ability to hyperpolarize the cell; however, our current understanding of transepithelial anion secretion *in vivo* is based upon a model in which K^+ recycling across the basolateral membrane plays a central role in setting the electrochemical driving force for anion secretion (Smith & Frizzell, 1984; McCann & Welsh, 1990; Dawson & Richards, 1990; Cotton, 2000). While our results suggest that Calu-3 cells may possess some apical K^+ channels, the basolateral K^+ channels appear to play a much more important role, consistent with the prevailing model. Furthermore, it appears that pharmacologically distinct basolateral K^+ channels differentially influence anion secretion under different conditions. Thus, the basal I_{sc} is more sensitive to clofilium than clotrimazole (Fig. 2), the cAMP-stimulated I_{sc} is almost completely abolished by clofilium but is clotrimazole insensitive (Fig. 4), while the Ca^{2+}-stimulated I_{sc} is partially blocked by both clofilium and clotrimazole (Fig. 5).

An important requirement for us to conclude that different K^+ channels are responsible for the observed differential effects on basal, cAMP-and Ca^{2+}-stimulated I_{sc} is the pharmacological specificity of clofilium and clotrimazole. The specificity of these blockers at the concentrations used is demonstrated by their effects upon the 1-EBIO-stimulated response, resulting from the activation of basolateral Ca^{2+}-dependent K^+ channels as previously described by Devor *et al* (1999). Thus, the response to 1-EBIO, both in intact (Fig. 6*B*) and permeabilized monolayers (Fig. 7*C*), is clofilium insensitive and almost entirely inhibited by 30 μM clotrimazole.

Despite the large role played by clofilium-sensitive K^+ channels in supporting the basal (Fig. 2) and cAMP-stimulated (Figs 3 and 4) I_{sc}, only small basal and cAMP-

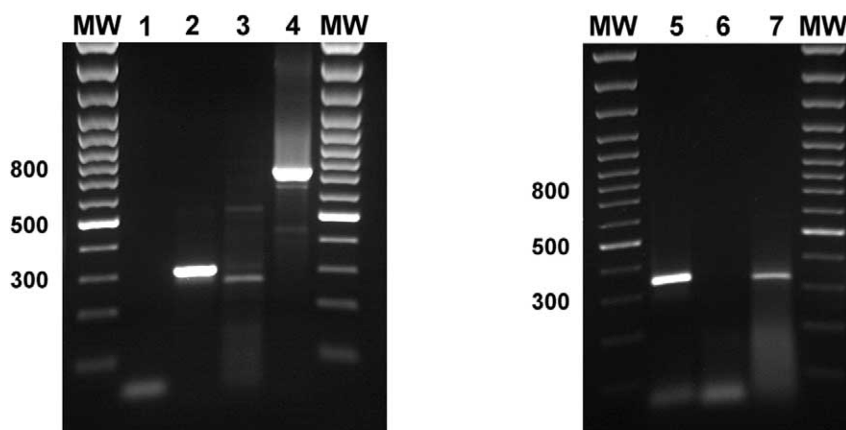

Figure 8. PCR analysis on RNA extracted from Calu-3 cells

Transcripts were detected for *KCNE2* (lane 2), *KCNE3* (lane 3), *KCNQ1* (lane 4), *KCNN4* (lane 5) and *CFTR* (lane 7) but not *KCNE1* (lane 1) or *KCNH2* (lane 6).

activated basolateral K^+ conductances could be identified in permeabilized monolayers (Fig. 7*A*). In contrast, a much larger 1-EBIO-activated K^+ conductance, which was sensitive to clotrimazole but not clofilium, was observed under these conditions (Fig. 7*B*), suggesting that the majority of the basolateral K^+ conductance can be accounted for by Ca^{2+}-activated channels. Due to the small amplitude of the basal and cAMP-stimulated I_{sc}, it was not possible to determine the pharmacological properties of the underlying K^+ channels. However, despite its small size, this conductance would appear to play a pivotal role in transepithelial anion secretion, in particular that stimulated by cAMP. The observation by ourselves and others (Shen *et al.* 1994; Moon *et al.* 1997; Devor *et al.* 1999) that forskolin increases anion secretion in Calu-3 cells could theoretically result either from direct cAMP-stimulation of apical anion channels, or as an indirect consequence of increased K^+ conductance enhancing anion efflux via already open channels. Calu-3 cells possess a significant resting Cl^- conductance, identified as CFTR, which is further increased upon forskolin addition (Shen *et al.* 1994; Haws *et al.* 1994). We here identify that Calu-3 cells possess a significant resting basolateral K^+ conductance that is also increased by forskolin. However, we are unable to determine whether the forskolin-stimulated increase in basolateral K^+ conductance is sufficient or required for the forskolin-stimulated increase in I_{sc}. Nevertheless, our finding that pretreatment with clofilium virtually abolishes the forskolin response in these cells (Fig. 4*B*) suggests that this basolateral K^+ conductance, though small, does play a significant role in the forskolin-stimulated I_{sc}. One interesting possibility is that cAMP causes a parallel increase in the apical anion and basolateral K^+ conductances, maximizing the secretory response.

The differential effects of clofilium and clotrimazole suggests the presence of at least two distinct classes of basolateral K^+ channels controlling anion secretion from Calu-3 cells. Using RT-PCR, we detected expression of the K^+ channel genes *KCNN4* and *KCNQ1* in these cells (Fig. 8). *KCNN4* encodes an intermediate-conductance Ca^{2+}-activated K^+ channel (Ishii *et al.* 1997; Joiner *et al.* 1997; Logsdon *et al.* 1997), activated by 1-EBIO and inhibited by clotrimazole (Jensen *et al.* 1998), and which has previously been suggested to form the basolateral Ca^{2+}-activated K^+ conductance of Calu-3 cells (Devor *et al.* 1999). *KCNQ1* encodes the KvLQT K^+ channel protein, which coassembles with the small accessory subunit minK (encoded by the *KCNE1* gene) to form a slow voltage-gated cardiac K^+ channel, mutated in long QT syndrome type 1 (Suessbrich & Busch 1999; Sanguinetti, 2000). KCNQ1 also coassembles with the related subunits MiRP1 (encoded by the *KCNE2* gene) to form a constitutively open K^+ channel (Tinel *et al.* 2000), and MiRP2 (encoded by *KCNE3*; Schroeder *et al.* 2000). Coassembly of *KCNQ1* and *KCNE3* gene products may form the cAMP-activated basolateral K^+ channel of intestinal epithelial cells (Schroeder *et al.* 2000; Kunzelmann *et al.* 2001). We detected *KCNE2* and *KCNE3*, but not *KCNE1*, in Calu-3 cells (Fig. 8), suggesting the possibility that Calu-3 cells may express different KCNQ1-containing K^+ channel complexes with distinct biophysical properties. *KCNQ1* has previously been suggested to contribute to airway epithelial basolateral K^+ conductance (Mall *et al.* 2000) and forms channels which are sensitive to clofilium (Honoré *et al.* 1991; Suessbrich & Busch, 1999; Warth & Bleich, 2000). *KCNE2* also coassembles with the cardiac K^+ channel HERG (encoded by the gene *KCNH2*) to form a clofilium-sensitive, voltage-gated K^+ channel (Suessbrich *et al.* 1997; Abbott *et al.* 1999). However, we were unable to detect *KCNH2* in Calu-3 cells (Fig. 8). It must be stressed that our inability to detect *KCNE1* or *KCNH2* by RT-PCR, despite multiple attempts under different conditions, does not unequivocally rule out the possibility that these genes are expressed by Calu-3 cells. Furthermore, this study is by no means a comprehensive investigation of K^+ channel expression in this cell line. For example, recent studies have indicated an extensive complement of K^+ channel types and subunits in rat pituitary cells (Wulfsen *et al.* 2000) and it is not unlikely that K^+ channels exist in Calu-3 cells in addition to those identified here. However, our positive identification of *KCNE2, KCNE3, KCNN4* and *KCNQ1*, following isolation and partial sequencing of these gene fragments, demonstrates that these genes are expressed in Calu-3 cells, and that their gene products could potentially contribute to basolateral K^+ conductances involved in the control of anion secretion.

The large clotrimazole and 1-EBIO-sensitive, clofilium-insensitive, K^+ conductance (Fig. 7) we observed is most probably accounted for by KCNN4 (Fig. 8) as previously proposed (Devor *et al.* 1999). This channel appears to play a role in Ca^{2+}-activated secretion (Fig. 5) but does not appear to contribute to the cAMP-activated I_{sc} (Fig. 4). We also demonstrated a second K^+ conductance, carried by a clofilium-sensitive channel, which is involved in basal (Fig. 2) and cAMP-stimulated (Figs 3 and 4) secretion and which interestingly also appears to contribute toward Ca^{2+}-stimulated secretion (Fig. 5). Our finding that Calu-3 cells express *KCNQ1* make this a possible candidate for forming the clofilium-sensitive basolateral K^+ conductance, since *KCNQ1* forms clofilium-sensitive basally active and/or cAMP-stimulated channels (Honoré *et al.* 1991; Suessbrich & Busch, 1999; Warth & Bleich, 2000; Schroeder *et al.* 2000; Tinel *et al.* 2000) Furthermore, we also find that Calu-3 cells express *KCNE2* and *KCNE3*, both of which coassemble with *KCNQ1* and regulate its biophysical properties (Schroeder *et al.* 2000; Tinel *et al.* 2000). Given the essential role of clofilium-sensitive K^+ channels in the cAMP-stimulated I_{sc} in Calu-3 cells (Fig. 5)

it is tempting to suggest that *KCNQ1–KCNE3* may serve a similar role in airway epithelia to that described in colonic epithelia (Schroeder *et al.* 2000), though we have no functional evidence of the role of *KCNE2* or *KCNE3*.

Cultured submucosal gland cells secrete fluid in response to agents which stimulate cAMP- or Ca^{2+} signalling pathways (Yamaya *et al.* 1991). As previously described, we find that Calu-3 cells exhibit both cAMP and Ca^{2+}-dependent increases in I_{sc} (Figs 1, 3–5; Shen *et al.* 1994; Moon *et al.* 1997). The present work emphasizes the pivotal role of basolateral K^+ channels in secretion by Calu-3 cells, and demonstrates clearly that distinct K^+ channel types support secretion under different conditions. Ca^{2+}-stimulated secretion is dependent, not only on the activity of a clotrimazole-sensitive channel, but also on additional K^+ channels sensitive to clofilium. We believe our finding that Calu-3 cells express *KCNN4* make this a strong candidate for a clotrimazole-sensitive channel. Conversely, cAMP-stimulated secretion is almost completely dependent on non-clotrimazole-sensitive channels, possibly formed by one or more KCNQ1-containing channels.

Loss of CFTR-mediated secretion from submucosal gland serous cells probably plays a fundamental role in pathogenesis of CF lung disease. Using a well-defined model of the human serous cell, we demonstrate that pharmacologically differential K^+ conductances underlie basal, cAMP- and Ca^{2+}-stimulated secretion and describe candidate genes that may be responsible for these channels. The co-ordinated activity of both apical CFTR and basolateral K^+ channels is required for normal activity of these cells, thus strategies which aim to circumvent loss of functional CFTR in CF-affected epithelia must also consider the basolateral K^+ conductance.

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