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Multiple KCNQ Potassium Channel Subtypes Mediate Basal Anion Secretion from the Human Airway Epithelial Cell Line Calu-3

Shasta L. Moser · Scott A. Harron · Julie Crack · James P. Fawcett · Elizabeth A. Cowley

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Abstract Potassium channels play an important role in providing a driving force for anion secretion from secretory epithelia. To investigate the role of KCNQ K^+ channels in mediating rates of basal anion secretion across the human airway submucosal gland serous cell model, the Calu-3 cell, we examined the expression, localization and function of these channels. In addition to our previous knowledge that Calu-3 cells express KCNQ1, using reverse transcriptase polymerase chain reaction we determined expression of KCNQ3, KCNQ4 and KCNQ5 mRNA transcripts. Immunoblotting detected KCNQ1, KCNQ3 and KCNQ5 proteins, while KCNQ4 protein was not found. Immunolocalization using polarized Calu-3 cell monolayers revealed that KCNQ1 and KCNQ3 were located in or toward the apical membrane of the cells, while KCNQ5 was detected in the apical and lateral membranes. Transepithelial transport studies revealed a small chromanol 293B-sensitive current at the apical membrane, likely KCNQ1. Application of XE991, an inhibitor of all members of the KCNQ channel family, inhibited the basal short-circuit current when applied to both sides of the cells to a greater extent than 293B, with the largest inhibition seen upon apical application. This result was confirmed using linopiridine, a less potent analogue of XE991, and suggests that functional KCNQ3 and KCNQ5, in addition to KCNQ1, are present at the apical aspect of these cells. These results demonstrate the role of a number of KCNQ channel members in controlling basal anion secretion across Calu-3 cells, while also demonstrating the importance of apically located K^+ channels in mediating anion secretion in the airway epithelium.

Keywords Potassium channel · KCNQ · Airway epithelia · Anion secretion · 293B · XE991

S. L. Moser · S. A. Harron · J. Crack · E. A. Cowley (✉)
Department of Physiology and Biophysics, Dalhousie University, B3H 1X5 Halifax, Nova Scotia, Canada
e-mail: elizabeth.cowley@dal.ca

J. P. Fawcett
Department of Pharmacology, Dalhousie University, B3H 1X5 Halifax, Nova Scotia, Canada

Introduction

Epithelial K^+ channels contribute to a variety of cellular functions including the absorption and secretion of fluids and electrolytes across the epithelial barrier (Warth, 2003). The rate of transepithelial anion secretion is known to be critically dependent upon the activity of K^+ channels since opening K^+ channels hyperpolarizes the cell, thus increasing the driving force for anion efflux (Smith & Frizzell, 1984). Until recently, it was assumed that the K^+ channels involved in mediating anion secretion in the airway were located in the basolateral membrane (Smith & Frizzell, 1984; McCann & Welsh, 1990; Devor et al., 1996). However, two small-conductance Ca^{2+}-activated K^+ (SK) channels have been detected in the human bronchial epithelial cell line 16HBE14o- and localized to both the apical and basolateral membrane (Bernard et al., 2003). Additionally, a number of two-pore domain K^+ (K_2P) channels have been found to be expressed both apically and basolaterally in the human airway epithelial cell line Calu-3 (Davis & Cowley, 2006). Finally, there is electrophysiological evidence that an inwardly rectifying K^+ channel is located in the apical membrane of Calu-3 cells (Wu et al., 2004).

Calu-3 cells are widely used as a model of airway submucosal gland serous cells (Shen et al., 1994; Haws

et al., 1994; Moon et al., 1997; Davis & Cowley, 2006). Serous cells have been implicated in the pathogenesis of cystic fibrosis (CF) lung disease (Pilewski & Frizzell, 1999) since they express high levels of the CF transmembrane conductance regulator (CFTR) Cl^- channel, mutations in which result in CF (Engelhardt et al., 1992). Calu-3 cells exhibit basal anion secretion, mediated exclusively via CFTR (Haws et al., 1994), which can be significantly increased by elevation of intracellular Ca^{2+} or cyclic adenosine monophosphate (cAMP) (Shen et al., 1994; Moon et al., 1997; Roy et al., 2006). This basal anion secretion is inhibited by a number of K^+ channel blockers (Cowley & Linsdell, 2002). Conversely, because activation of K^+ channels is strongly prosecretory in Calu-3 cells (Devor et al., 1999; Davis & Cowley, 2006), they represent a potential therapeutic target for manipulation in CF when anion secretion is abnormal. We previously reported that Calu-3 cells express mRNA transcripts for the intermediate-conductance Ca^{2+}-activated channel hIK (KCNN4), as well as the voltage-gated KCNQ1 and two of its accessory subunits, KCNE2 and KCNE3. We concluded that while KCNN4 contributed to the Ca^{2+}-activated anion secretion seen in Calu-3 cells, their basal and cAMP-activated secretion was likely dependent on one or more KCNQ1-containing channel complexes (Cowley & Linsdell, 2002). Subsequently, we reported that basal anion secretion in these cells is also affected by the activity of K_2P channels (Davis & Cowley, 2006).

The KCNQ (Kv 7) family is comprised of a group of five voltage-gated K^+ channels (KCNQ1–5 or Kv 7.1–7.5) ascribed diverse physiological functions (Robbins, 2001). The KCNQ members are six-transmembrane domain–spanning proteins and channels are formed by the assembly of four of the same KCNQ α-subunits or coassembly of different KCNQ proteins (Cooper & Jan, 2003; Schroeder et al., 2000a, 2000b). The properties of KCNQ1, KCNQ2, KCNQ3 and KCNQ4 channels are altered by coassembly with various smaller accessory β-subunits from the KCNE family, termed KCNE1–5 (Robbins, 2001; Strutz-Seebohm et al., 2006). While KCNQ1 has been described in numerous epithelia, expression of KCNQ2–5 is more normally associated with the central nervous system (CNS). However, since recent studies have described expression of members of the KCNQ family in a variety of tissues, these channels may have wider physiological roles than have been previously thought (Lan et al., 2005; Kharkovets et al., 2006). The aims of the present study were to investigate the contribution of KCNQ1 to the rate of basal anion secretion in Calu-3 cells. While confirming a role for KCNQ1, we additionally identify other members of the KCNQ channel family in Calu-3 cells, which likely play an even more important role than KCNQ1 in mediating basal anion secretion. Furthermore, our results localize a number of KCNQ channel types to the apical membrane of Calu-3 cells.

Methods

Cell Culture

Calu-3 cells (American Type Culture Collection, Rockville, MD) were cultured in 1:1 Dulbecco's modified Eagle's medium:Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 5% 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen, Burlington, Canada). 16HBE14o- human bronchial epithelial cells and CFBE41o- cystic fibrosis human bronchial epithelial cells (both provided by Dr. Dieter Gruenert, California Pacific Medical Center, San Francisco, CA) were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 5% 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen). Stock flasks of 16HBE14o- and CFBE41o- cells were coated with fibronectin (Biosource, Camarillo, CA) at least 3 h prior to use, and the solution was removed immediately before use. Cells were incubated at 37°C in humidified 5% CO_2/95% air. For RNA and protein extraction, cells were cultured on 100-mm-diameter Falcon culture dishes (Becton Dickinson, Franklin Lanes, NJ). For immunohistochemistry and short-circuit current (I_{sc}) recordings, Calu-3 cells were plated on Snapwell inserts (Corning Costar, Cambridge, MA) and maintained at an air–liquid interface with medium present only on the basolateral side, as previously described (Cowley & Linsdell, 2002).

Measurement of Transepithelial I_{sc}

Monolayers of Calu-3 cells were mounted in an Ussing chamber (World Precision Instruments, Sarasota, FL), and the transepithelial potential was clamped to zero using a DVC-1000 voltage-clamp apparatus (World Precision Instruments). The transepithelial I_{sc} was recorded using Ag-AgCl electrodes in agar bridges and reflects the net movement of ions across the epithelial monolayer. Data were collected using an MP 100 data acquisition system and Acq*Knowledge* computer software, version 3.7.3 (both from Biopac Systems, Goleta, CA). Apical and basolateral solutions were maintained at 37°C by heated water jackets and separately perfused and oxygenated with a 95% O_2:5% CO_2 mixture. Bath solutions were (in 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 (basolateral) or mannitol (apical) (pH 7.4) at 37°C when gassed with 95% O_2:5% CO_2.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from Calu-3, 16HBE14o- and CFBE41o- cells using TRIzol reagent (Invitrogen). RNA was DNase-treated with RQ1 RNase-free DNase (Promega, Madison, WI), and 2 μg DNase-treated RNA was then reverse-transcribed to produce cDNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) in the presence of 5 mM dNTP (Invitrogen Life Technologies) and 1 μM oligo dT (Amersham Pharmacia, Baie D'Urfe, Canada).

Polymerase chain reaction (PCR) was performed for KCNQ1–5 and the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) using the primers and conditions described in Table 1. Primers for KCNQ1 were obtained from Currid et al. (2003), while those for KCNQ2–5 were designed in our laboratory using the Invitrogen Oligo Design Programme (www.invitrogen.com). All primers were purchased from Invitrogen and used at 10 μM. PCR experiments were performed in the presence of 25 mM $MgCl_2$, 10x Taq buffer with KCl, 5 mM dNTP and 2.5 U Taq polymerase (all from MBI Fermentas, Burlington, Canada) in a total reaction volume of 25 μl.

PCR products were visualized on a 1.5% agarose gel containing 250 μg^{-1} ethidium bromide alongside a 100-bp ladder (MBI Fermentas). To confirm the identity of amplified PCR fragments, the product was isolated from the gel using the QIAquick gel extraction kit (Qiagen, Mississauga, Canada) and sequenced at a commercial sequencing facility (DalGEN Microbial Genomics Centre, Halifax, Canada). Each PCR was performed at least three times on different passages of cells.

Western Blotting

Calu-3 and 16HBE14o-cells were removed with a cell scraper and spun down, and the pellet was resuspended in a lysis buffer containing 10% sodium dodecyl sulfate (SDS) and 15 mg/ml dithiothreitol (DTT) in the presence of Complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Total protein lysate was run on a 7.5% polyacrylamide gel and transferred to nitrocellulose membrane (Pall Corporation, Pensacola, FL). For Calu-3 cells, immunoblotting was performed using either a rabbit polyclonal antibody raised against residues 585–604 of the C terminus of human KCNQ1 (sc-20816; Santa Cruz Biotechnology, Santa Cruz, CA; used at 1:1,000 dilution or 0.6 μg/ml), a rabbit polyclonal antibody raised against residues S617–Q676 of human KCNQ3 (5597; Chemicon International, Temecula, CA; used at 1:200 dilution or 1.6 μg/ml), a goat polyclonal antibody raised against residues near the N terminus of human KCNQ4 (sc-9385; Santa Cruz Biotechnology; used between 1:100 and 1:500 dilution), or a rabbit polyclonal antibody against residues M1–R88 of human KCNQ5 (9792; Chemicon International; 1:500 dilution). For 16HBE14o- cells, the antibodies described above were used in addition to a rabbit polyclonal raised against amino acid residues 578–593 of rat KCNQ2 (Alomone Labs, Jerusalem, Israel; 1:200 dilution or 1.4 μg/ml).

After incubation with the primary antibody, the membrane was incubated with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody in 5% nonfat milk at 1:50,000 dilution (all Jackson ImmunoResearch, West Grove, PA). Proteins were detected using the ECL Plus kit (Amersham Pharmacia) following the manufacturer's instructions.

Immunocytochemistry and Microscopy

Calu-3 cells grown on Snapwell filters were fixed in 4% paraformaldehyde (Sigma Aldrich, Oakville, Canada) for 10 min, followed by permeabilization with 0.3% Triton X-100 (Sigma Aldrich) for 15 min. Nonspecific binding was prevented with a 1-h incubation with either 10% goat or 10% rabbit serum as appropriate (Invitrogen Life Technologies). To confirm that Calu-3 cells were polarized under our cell

Table 1 PCR primer sequences and conditions

Primer name	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)	PCR conditions	Product size (bp)
KCNQ1[a]	CTTTGCCATCTCTTCTTTG	AGTGTTGGGCTCTTCCTTAC	95°C 30 s, 58°C 60 s, 72°C 60 s, 35 cycles	411
KCNQ2	GCAAGCTGCAGAATTTCCTC	AGTACTCCACGCCAAACACC	95°C 30 s, 54°C 30 s, 72°C 60 s, 40 cycles	201
KCNQ3	AAGCATGATGGGGAAGTTTG	GGGCTGACTTTGTCAATGGT	95°C 30 s, 60°C 30 s, 72°C 60 s, 40 cycles	279
KCNQ4	TGAAGGACGTCATTGAGCAG	AGTCCCTCAGTCCATGTTGG	95°C 30 s, 60°C 30 s, 72°C 60 s, 40 cycles	422
KCNQ5	CGCTTTCGTTTTTCTCCTTG	CGAGCAAACCTCAGTCTTCC	95°C 30 s, 56°C 30 s, 72°C 60 s, 40 cycles	207
HPRT[b]	GCCAGACTTTGTTGGATTTG	CTCTCATCTTAGGCTTTGTATTTTG	95°C 30 s, 60°C 30 s, 72°C 60 s, 40 cycles	141

[a] Currid et al. (2003)

[b] Erovic et al. (2003)

culture conditions, initial experiments were performed using either a mouse monoclonal antibody against residues 1048–1247 of human ZO-1 (Zonula occludens-1; 610966; BD Bioscience, Franklin Lakes, NJ; used at 1:200 dilution or 0.5 μg/ml) or a mouse monoclonal against rabbit Na,K-ATPase (clone C464.6; Upstate Biotechnology, Lake Placid, NY; 1:500 dilution or 1 μg/ml) as markers of tight junctions and the basolateral membrane, respectively. Then, costaining experiments were performed in which cells were incubated overnight in the presence of two primary and two secondary antibodies at the same time, namely, anti-ZO-1 with either anti-KCNQ1, -KCNQ3, -KCNQ4 or 0KCNQ5 primary antibody (as described above). Secondary antibodies were prepared by diluting Alexo Fluor 488 or 633 goat immunoglobulin G (Molecular Probes, Burlington, Canada) in phosphate-buffered saline containing 2% goat serum, and cells were incubated for 1 h. To mount the monolayer of cells, the entire membrane filter was cut from its base using a razor blade, mounted on a silinated slide and coverslipped using fluorescence mounting medium (DakoCytomation, Carpinteria, CA).

The contribution of the background signal was determined in experiments omitting the primary antibody. Cells were examined using a META LSM 510 laser scanning confocal microscope (Carl Zeiss, North York, Canada) at the Cellular Microscope and Digital Imaging Facility at Dalhousie University. To determine the location of proteins, Z-sectioning was performed by obtaining 1-μm horizontal optical sections of the cells from focal points predetermined by the user. In all cases, images were obtained at x25 magnification with a pinhole diameter of 0.67 μm at a wavelength of 488 nm. Detector gain was set at 505 and final images were obtained by averaging seven images with a scan speed of 15.73 s. Sections were then rotated using Zeiss LSM 5 Image Brower software, version 3,2,0,115 (Carl Zeiss) to produce *x*-axis images.

Chemicals

Stock solutions of 100 mM chromanol 293B (Tocris, Ellisville, MO) and 100 mM forskolin (Sigma Aldrich) were dissolved in dimethylsulfoxide (DMSO) so that the final bath concentration was $\leq$0.01%. Application of DMSO alone had no effect on the monolayers (results not shown). Stock solutions of 100 mM XE991 dihydrochloride and 100 mM linopiridine (both Tocris) were dissolved in the appropriate buffer.

Statistics

All data are presented as mean ± standard error of the mean. To determine 50% inhibitory concentration (IC_{50}) values, the Hill equation was utilized as follows: Percentage inhibition = (maximum inhibition $[B]^n$)/$\{(IC)^n + [B]^n\}$, where n is the Hill coefficient or slope factor, $[B]$ is the concentration of drug and IC is IC_{50}.

Results

KCNQ channels play a role in controlling the basal anion secretion from Calu-3 cells: The basal, unstimulated I_{sc} in Calu-3 cells has previously been determined to be almost exclusively accounted for by anion secretion via CFTR (Devor et al., 1999; Singh et al., 1997). Application of a number of K^+ channel inhibitors is known to reduce this basal I_{sc} (Cowley & Linsdell, 2002; Davis & Cowley, 2006). Therefore, in this study, we initially wished to investigate the potential contribution of KCNQ1 to the basal I_{sc}.

The mean basal I_{sc} for Calu-3 cells monolayers was 26.04 ± 1.09 μA/cm^2 (range 6.38–54.65 , $n = 62$), which is consistent with previous reports (Shen et al., 1994; Cowley & Linsdell, 2002). However, due to the wide range in basal I_{sc} values, we normalized the data by calculating the percentage change in the I_{sc} after drug application.

Initially, to examine whether KCNQ1 plays a role in mediating basal anion secretion in Calu-3 cells, we utilized the KCNQ1-specific blocker chromanol 293B (Lohrmann et al., 1995; Bleich et al., 1997). Apical application of 100 μM 293B reduced the basal I_{sc} by 4.41 ± 0.39 μA/cm^2 or 13.48 ± 1.12% ($n = 12$, Fig. 1a, b). However, there was virtually no effect when 293B was applied to the basolateral aspect of the monolayer (decrease of 0.62 ± 0.60 μA/cm^2 or 1.89 ± 1.10%, $n = 3$; Fig. 1a, b). When increasing concentrations of 293B were applied to the apical membrane, the IC_{50} was calculated to be 12.41 μM with a maximum inhibition of approximately 14% ($n = 5$, Fig. 1c).

To further investigate the contribution of KCNQ1 to basal secretion, we additionally utilized XE991, an agent that inhibits all members of the KCNQ channel family (Zaczek et al., 1998; Wang et al., 2000). Basolateral application of XE991 (100 μM) inhibited the I_{sc} by 4.57 ± 0.38 μA/cm^2 or 19.40 ± 1.69% ($n = 9$, Fig. 2a, f). Inhibition by XE991 was concentration-dependent, with slight recovery of the current occurring after applications of 300 and 1,000 μM (Fig. 2b). Fitting this curve to the Hill equation gave an IC_{50} for basolaterally applied XE991 equal to 97.7 μM and a maximum inhibition of 34.5% ($n = 4$).

Apical application of 100 μM XE991 inhibited the basal I_{sc} by 11.41 ± 0.79 μA/cm^2 or 45.09 ± 1.38% ($n = 18$, Fig. 2d, f). Since the maximal inhibitory effect of apically applied XE991 on the basal I_{sc} was so much larger than that of 293B, this suggests that additional KCNQ channels are

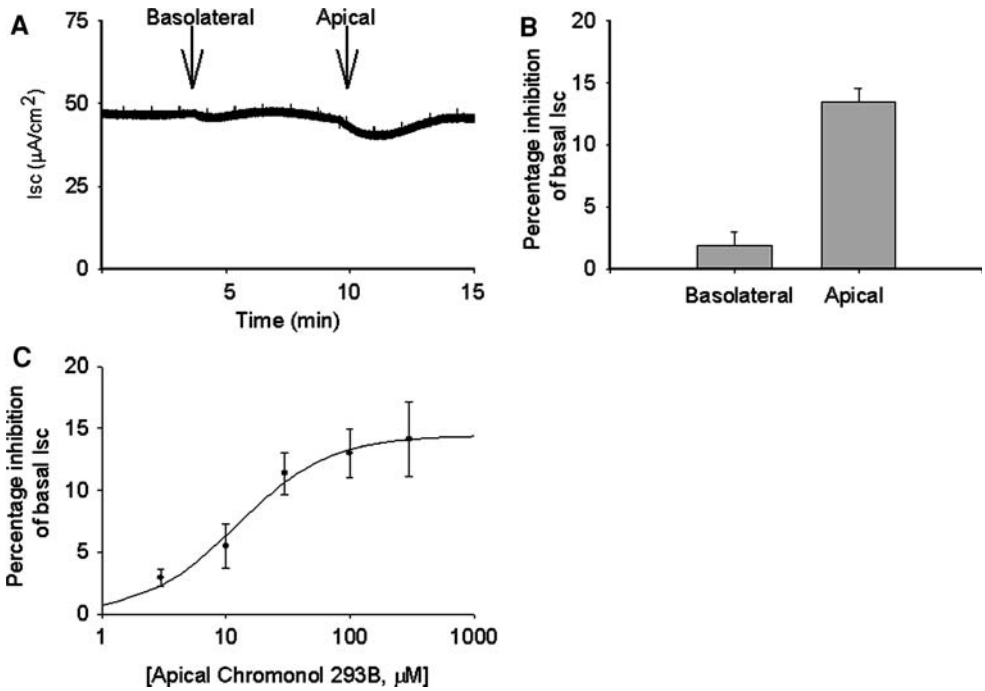

Fig. 1 The basal I_{sc} in Calu-3 cells is inhibited by apical application of a KCNQ1 inhibitor. Apical application of chromanol 293B (100 μM) inhibited the basal I_{sc}, while basolateral application showed virtually no inhibition (**a, b**). When 293B was applied to the apical membrane in a concentration-dependent manner, the IC_{50} was found to be 12.84 μM, with a slope of 1.17 (**c**)

present on the apical membrane of Calu-3 cells and that their activity contributes to the basal I_{sc}. To further investigate this possibility, 293B (100 μM) was applied to the apical aspect of the cells subsequent to inhibition by XE991 (100 μM). Here, 293B application had no further effect on I_{sc} ($n = 3$, Fig. 2e), suggesting that XE991 was already fully inhibiting KCNQ1. When increasing concentrations of XE991 were applied to the apical membrane, increasing inhibition was observed for concentrations up to 300 μM XE991 (Fig. 2g), when a paradoxical stimulation of I_{sc} was observed (increase $= 34.54 \pm 11.23$ μA/cm^2 or $254.90 \pm 54.67\%$, $n = 4$; Fig. 2g). Attempts to calculate an IC_{50} for apical application of XE991 using the Hill equation were therefore inappropriate, due to the apparent dual mechanism of action of XE9991. However, 200 μM XE991 caused inhibition of $52.94 \pm 5.65\%$ ($n = 4$, Fig. 2h).

To confirm the role of KCNQ family members in mediating the basal anion secretion from Calu-3 cells, the effects of linopiridine, described as a less potent analogue of XE991 (Nickolson et al., 1990; Zaczek et al., 1998), were also investigated. Apical application of linopiridine (100 μM) inhibited the basal I_{sc} by 3.52 ± 0.38 μA/cm^2 or $11.20 \pm 0.87\%$ ($n = 6$, Fig. 3a, b), which was often followed by a slight apparent recovery in the I_{sc} (Fig. 3a), while basolateral application inhibited the I_{sc} by 2.24 ± 0.44 μA/cm^2 or $6.01 \pm 1.25\%$ ($n = 3$, Fig. 3a, b). Figure 3c shows apical application of increasing doses of linopiridine, which resulted in increasing inhibition of the I_{sc}, typically followed by a recovery toward baseline. However, application of a large dose of this agent (1,000 μM) produced an initially rapid inhibition followed by a large transient increase in I_{sc} (39.05 ± 3.85 μA/cm^2 or $255.80 \pm 80.21\%$, Fig. 3c). Thus, it appears that while one or more type of channel is inhibited by linopiridine, at higher doses this inhibitory effect appears to be masked by a simultaneous stimulatory effect, as was the case with XE991.

Airway Epithelial Cell Lines Express KCNQ mRNA

The results of the above series of I_{sc} measurements, especially with regard to XE991, suggest that a number of KCNQ family members are present in Calu-3 cells, at both the apical and basolateral membrane. Therefore, we used reverse transcriptase (RT) PCR to investigate possible expression of KCNQ channel mRNA. KCNQ1 mRNA was previously detected in Calu-3 cells (Cowley & Linsdell, 2002). In the present study, RT-PCR on total RNA extracted from Calu-3 cells additionally detected amplicons of the appropriate size for KCNQ3, KCNQ4 and KCNQ5 (Fig. 4a). Despite repeated attempts, we were unable to detect a band for KCNQ2 in Calu-3 cells, although a positive result was obtained when we used human testis cDNA as a positive control (results not shown), confirming the specificity of our primers. PCR for the housekeeping gene HPRT was performed to confirm the integrity of the cDNA (results not shown). Fragments were not detected when water was substituted for the cDNA template. Positive PCR fragments were sequenced, and a comparison of all sequenced products with the published sequences for KCNQ3–5 (National Center for

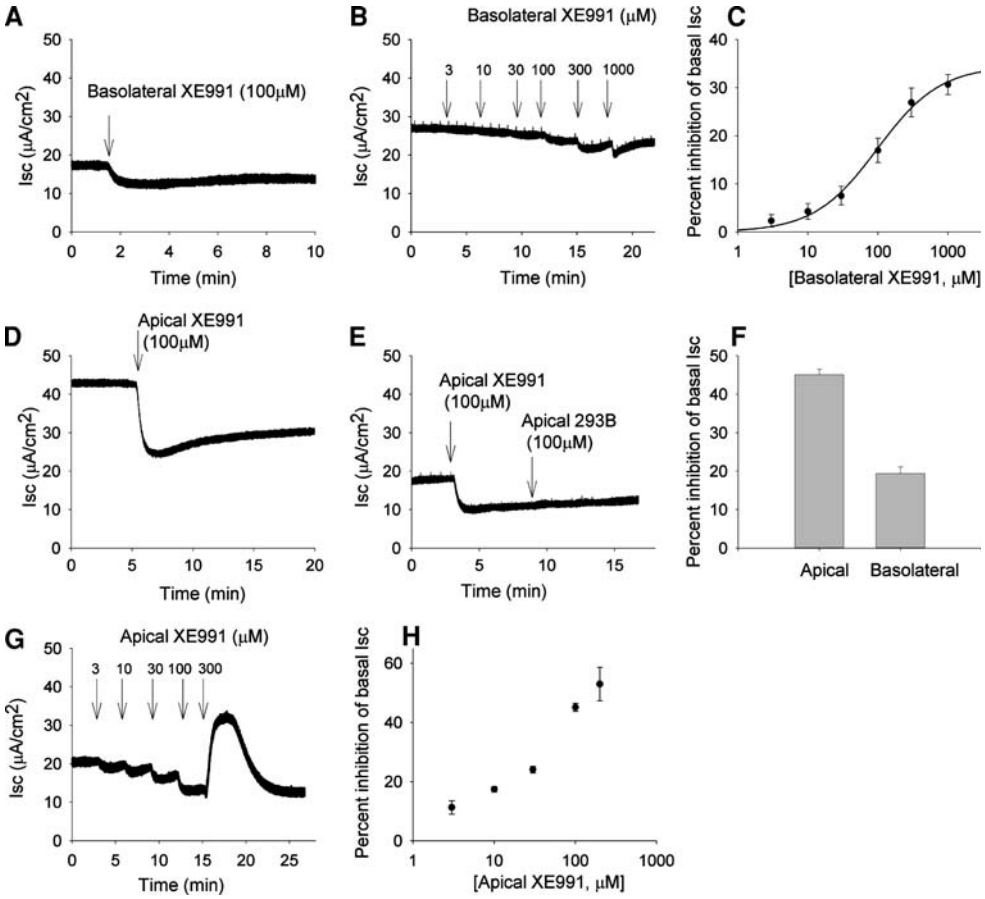

Fig. 2 XE991 affects the basal I_{sc} of Calu-3 cells. Basolateral application of the KCNQ channel inhibitor XE991 (100 μM) inhibited the basal I_{sc} (**a**). Increasing concentrations of XE991 applied basolaterally caused increasing inhibition (**b**), while analysis determined an IC_{50} of 97.7 μM with a Hill slope of 0.96 (**c**). Apical application of XE991 (100 μM) inhibited the basal I_{sc} to a greater extent than basolateral application (**d, f**), while apical application of 293B following XE991 had no further effect on the I_{sc} (**e**). Finally, when XE991 was applied to the apical membrane in a concentration-dependent manner, stimulation of the I_{sc} was observed at 300 μM (**g**). However, analysis of the data between 3 and 200 μM revealed a maximum inhibitory concentration of apical XE991 of approximately 200 μM (**h**)

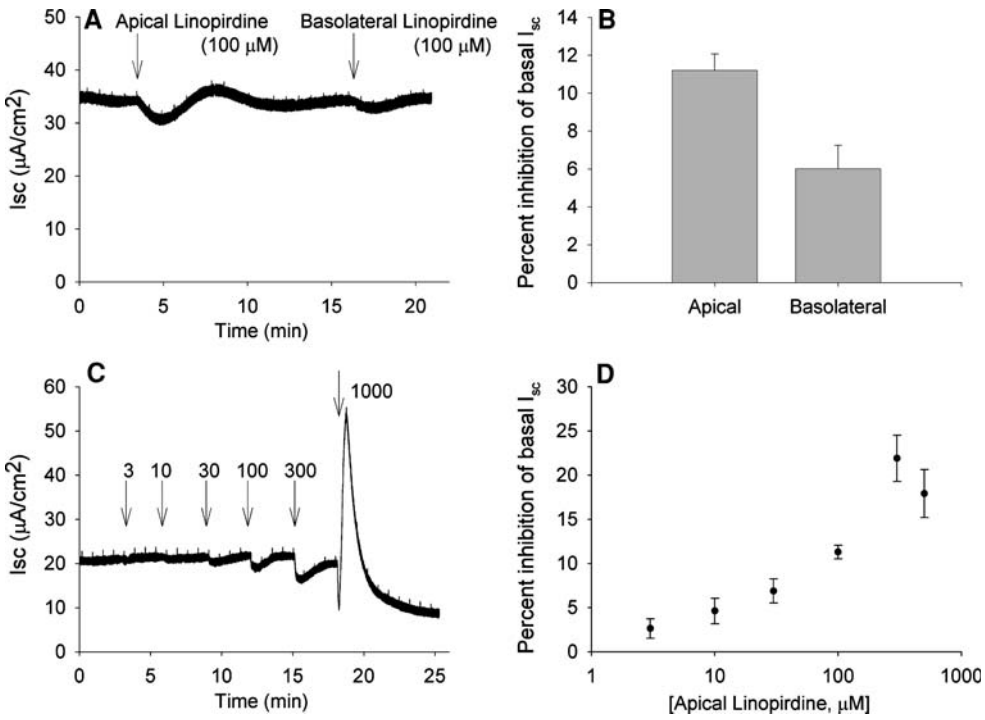

Fig. 3 The basal I_{sc} in Calu-3 cells is both inhibited and stimulated by linopiridine. Apical application of linopiridine (100 μM) inhibited the basal I_{sc}, while application to the basolateral membrane inhibited to a lesser extent (**a, b**). When 100 μM linopirdine was applied to the apical membrane, brief inhibition followed by stimulation of the I_{sc} was observed (**c**). The maximum inhibitory effect observed was approximately 22% (**d**)

Biotechnology Information, Bethesda, MD) confirmed the product identity.

To investigate whether KCNQ channel mRNA expression was exclusive to the secretory Calu-3 cells or whether expression of these channels may be more widespread in airway epithelia, we additionally investigated expression in the human bronchial airway epithelial cell line 16HBE14o-, which represents an example of normal bronchial epithelia derived by cell transformation rather than from an adenocarcinoma. When RT-PCR was performed using the same KCNQ primers, amplicons were detected for KCNQ1, KCNQ2, KCNQ4 and KCNQ5 in 16HBE14o- cells (Fig. 4b) and the identities were confirmed with sequencing. Despite repeated attempts using different PCR conditions, there was no band detected for KCNQ3. Similar results were obtained with the CFBE41o- cells (results not shown). Thus, there appears to be more extensive, and differential, expression of KCNQ channel members in human airway epithelia than was previously appreciated.

Calu-3 Cells Express KCNQ Proteins

To examine the protein expression of the KCNQ channels identified above in Calu-3 cells, Western blotting was performed. Using a polyclonal rabbit anti-human KCNQ1 antibody, a protein of approximately 70 kDa was detected (Fig. 4c), which is consistent with KCNQ1 protein expression in these cells. A 95-kDa band, corresponding to the predicted 96-kDa size for KCNQ3 (Yus-Nájera et al., 2003), was detected using an anti-KCNQ3 antibody; however, an unidentified 45-kDa protein was also detected (Fig. 4c). Since we had detected KCNQ4 mRNA, we investigated the corresponding protein expression using an anti-human KCNQ4 antibody; however, no protein was detected despite increasing protein and antibody concentrations (results not shown). Finally, an anti-human KCNQ5 antibody detected proteins of approximately 115 and 50 kDa, with an additional faint band at approximately 95 kDa (Fig. 4c). The predicted molecular weight of KCNQ5 ranges 99–102 kDa (Schroeder et al., 2000a; Lerche et al., 2000). Unfortunately, peptides corresponding to the epitope sequences of the KCNQ antibodies of interest were not commercially available and could not be used to confirm specificity. Therefore, control experiments consisted of those in which the primary antibody was omitted (Fig. 4c).

To further investigate the expression of KCNQ family members in normal airway epithelial cells, Western blotting was also performed on 16HBE14o- cells (Fig. 4d). While KCNQ2 and KCNQ5 proteins were detected, we were unable to detect any KCNQ1 protein in 16HBE14o- cells despite repeated attempts. Since an appropriate blocking peptide designed to block protein-antigen complex formation was available for use with the KCNQ2 antibody, in this case the control experiment consisted of an immunoblot performed in the presence of an excess of this blocking peptide.

KCNQ Localization in Calu-3 Cell Monolayers

To determine KCNQ protein localization in Calu-3 cells, costaining experiments were performed using an anti-ZO-1 antibody and the same anti-KCNQ antibodies that were

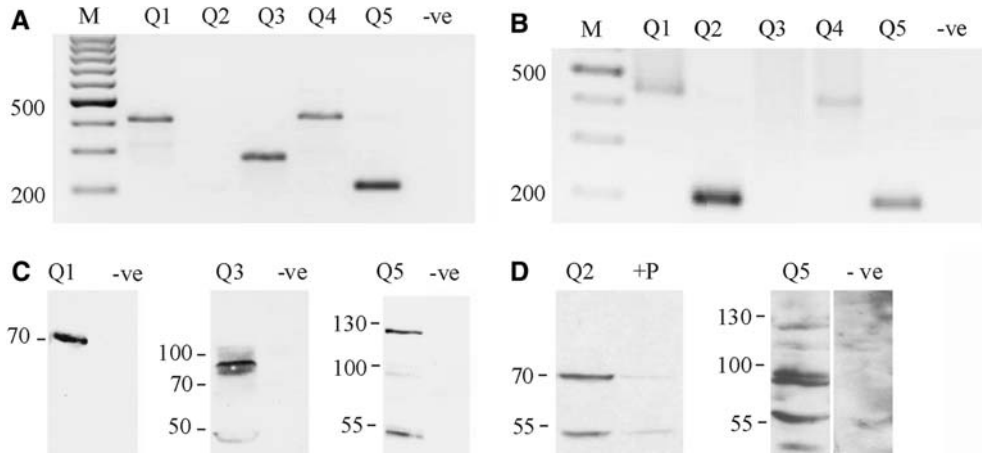

Fig. 4 Airway epithelial cell lines express mRNA and protein for KCNQ channels. Using RT-PCR, transcripts were detected for KCNQ1, KCNQ3, KCNQ4 and KCNQ5 in Calu-3 cells (**a**) and KCNQ1, KCNQ2, KCNQ4 and KCNQ5 in 16HBE14o- cells (**b**). M, 100-bp marker. In the negative control (-ve), water was substituted for template cDNA. All fragments were of the expected size and sequenced to confirm identity. Immunoblotting of protein from Calu-3 cell lysate confirmed expression of KCNQ1, KCNQ3 and KCNQ5 proteins (**c**), while only KCNQ2 and KCNQ5 proteins could be detected in 16HBE14o- cells (**d**). Proteins were not detected when the primary antibody was omitted (-ve) or when run in the presence of an appropriate blocking peptide (+P, available for KCNQ2 only). All experiments were performed in triplicate

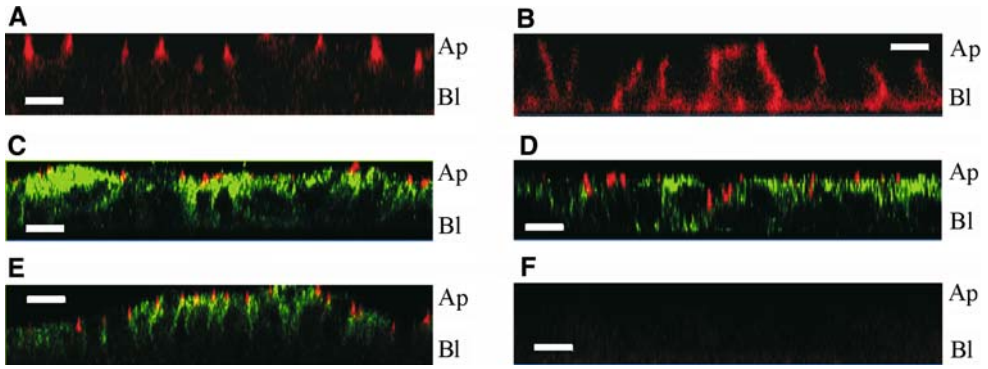

Fig. 5 Immunolocalization of KCNQ channels in polarized Calu-3 cell monolayers. Immunolocalization of ZO-1 (**a**) and Na-K-ATPase (**b**, both *red*) confirm polarization of the Calu-3 cell monolayers. Costaining experiments for KCNQ protein (*green*) with the tight junction marker ZO-1 revealed the majority of positive staining for KCNQ1 at or toward the apical aspect of the cells (**c**). A similar pattern of distribution was observed for KCNQ3 protein, though some basolateral staining is apparent (**d**). Staining for KCNQ5 protein (**e**) was localized to the apical and lateral membranes. All specific staining was abolished when incubation was performed in the absence of the primary antibodies (representative example shown in **f**). No staining was detected for KCNQ4 (not shown). Scale bar = 10 μM

used for Western blotting. Initially, for orientation purposes and to confirm polarization of the Calu-3 monolayers, ZO-1 and the Na-K-ATPase proteins were used as markers for tight junctions and the basolateral membrane, respectively (Fig. 5a, b).

The anti-KCNQ1 antibody detected protein mainly toward the apical membrane, as shown by the close proximity of the KCNQ1 staining and the ZO-1 staining (Fig. 5c). While the bulk of the staining appears at the apical membrane, or immediately subapical, a small amount of KCNQ1 protein does appear in the lateral and basolateral membrane (Fig. 5c). Using the anti-KCNQ3 antibody, KCNQ3 protein was again found at or near the apical membrane, with a very small amount of staining in the basolateral membrane (Fig. 5d). No staining was detected for KCNQ4 when the anti-KCNQ4 antibody was used, even when the antibody dilution was reduced from 1:500 to 1:100 (results not shown), which is consistent with our failure to detect the protein via immunoblotting. Finally, KCNQ5 was located in both the apical and lateral membranes but not in the basal membrane (Fig. 5e). In some areas, KCNQ5 was colocalized with ZO-1, as indicated by the yellow staining. When the primary antibodies were omitted in these experiments as a control, very minimal staining was detected due to background from the secondary antibodies (representative example shown in Fig. 5f).

Discussion

K^+ channels are important in providing an electrochemical gradient for anion secretion in a variety of epithelia (Warth, 2003). Previous work demonstrated expression of the KCNQ1 K^+ channel in the Calu-3 cell line (Cowley & Linsdell, 2002). The aim of the present study was to examine the contribution of KCNQ1 to the resting I_{sc} of Calu-3 cells. However, in addressing this aim, we also identified a number of additional KCNQ K^+ channel members present in Calu-3 cells which appear to play more important roles in maintaining the basal I_{sc}.

KCNQ1 is expressed in many tissues, including the heart and numerous epithelia (Bleich & Warth, 2000). Coassembly of KCNQ1 with KCNE2 and/or KCNE3 has been shown to form basally active, cAMP-stimulated channels (Tinel et al., 2000; Heitzmann et al., 2004; Schroeder et al., 2000a); and since KCNQ1 and both KCNE2 and KCNE3 are expressed in Calu-3 cells, it was proposed that KCNQ1 may coassemble with KCNE2 or KCNE3 in this secretory cell type (Cowley & Linsdell, 2002). Our finding that 16HBE14o- cells express KCNQ1 at the mRNA, but not the protein, level was somewhat surprising. However, while both Bernard et al. (2003) and Mall et al. (2000) described expression of KCNQ1 mRNA in 16HBE14o- cells, neither demonstrated protein expression. Furthermore, Bernard et al. concluded that there was no 293B-sensitive cAMP-stimulated conductance in these cells, suggesting that these cells do not possess functional KCNQ1.The results of the present study indicate a chromonol 293B-sensitive channel at the apical, though not at the basolateral, membrane of Calu-3 cells (Fig. 1a, b). Furthermore, this is consistent with our immunolocalization data, placing KCNQ1 protein predominantly in or toward the apical membrane (Fig. 5c). KCNQ1 protein can be localized either apically or basolaterally depending on the epithelial type. In the apical membrane of gastric parietal cells, KCNQ1 is colocalized with H^+/K^+ATPase, where it serves to secrete K^+ into the lumen for recycling by H^+/K^+ATPase (Grahammer et al., 2001). In the basolateral membrane of nasal epithelial cells (Mall et al.,

2000), colon and small intestine (Schroeder et al., 2000b), KCNQ1 has been shown to be important in maintaining cAMP-stimulated Cl^- secretion across the apical membrane. Furthermore, the sensitivity of KCNQ1 to 293B depends upon its association with KCNE accessory subunits (Tinel et al., 2000; Schroeder et al., 2000b; Heitzmann et al., 2004; Bett et al., 2006). In the present study, apical application of 293B gave an IC_{50} of 12.41 μM for inhibition of the basal I_{sc} in Calu-3 cells, which is consistent with coassembly of KCNQ1 with KCNE2 and/or KCNE3 in the apical membrane of Calu-3 cells.

In order to confirm and extend our studies with 293B, we additionally used XE991, which inhibits all members of the KCNQ channel family (Zaczek et al., 1998; Wang et al., 2000). XE991 and its less potent derivative linopiridine are usually described as cognitive enhancers (Zaczek et al., 1998), due to their ability to increase release of certain neurotransmitters. While a recent report indicates that XE991 is able to inhibit ether-a-go-go channels (ERG) in addition to KCNQ channels (Elmedyb et al., 2007), previous work from our own laboratory failed to detect ERG in Calu-3 cells (Cowley & Linsdell, 2002); therefore, we are confident that we are examining KCNQ-mediated events. Intriguingly, we found that application of XE991 to the apical membrane (up to 200 μM) caused a much larger maximal inhibition of the basal I_{sc} (53%) than did 293B (14%). Basolateral application of XE991 also inhibited the resting I_{sc} to a much greater extent than 293B (approximately 20% inhibition vs. 2%; Figs. 1b and 2f). These results indicate the possible presence of additional KCNQ channels at both the apical and basolateral membranes of Calu-3 cells and prompted us to examine whether these cells express any other KCNQ channels. Using RT-PCR, we were able to detect mRNA expression for KCNQ3, KCNQ4 and KCNQ5 (Fig. 4a), though we were only able to detect protein for KCNQ3 and KCNQ5 (Fig. 4b). Immunolocalization revealed KCNQ3 protein in or toward the apical membrane, while KCNQ5 protein appeared apically and laterally (Fig. 5d, e). Thus, we propose that the large inhibition of basal I_{sc} produced in response to apical application of XE991 (and to a lesser extent linopiridine) is the result of simultaneous inhibition of KCNQ1, KCNQ3 and/or KCNQ5. Indeed, these findings suggest that functional KCNQ3 and/or KCNQ5 channels are expressed apically in Calu-3 cells and are more important than KCNQ1 in controlling basal transepithelial anion secretion. Similarly, we suggest that the inhibition seen with basolateral XE991 is a result of the laterally detected KCNQ5 protein (Fig. 5e).

One unexpected finding during the course of this study was the paradoxical stimulation of anion secretion by large doses of XE991 (and linopiridine) applied to the apical aspect of Calu-3 cells (Figs. 2g and 3c). The reason for the switch from an inhibitory to a secretory effect is not immediately apparent and is presently under investigation in our laboratory. However, theoretical possibilities could include stimulation of an apical Cl^- conductance (most likely CFTR given the dominance of this channel in the apical membrane of Calu-3 cells) or the inhibition of an apical K^+ channel. Whatever the mechanism, our findings indicate that the effect of XE991 on transepithelial transport is more complex than initially anticipated and that care must be taken when using this compound in CFTR-expressing tissues.

KCNQ2 and KCNQ3 coassemble in the CNS to form the M current, a K^+ channel that is important in neuronal excitability in the CNS (Wang et al., 2000). Outside the CNS, KCNQ3 mRNA is expressed in the human cochlea, spleen (Robbins, 2001) and liver (Lan et al., 2005), although the physiological roles of KCNQ2 and KCNQ3 in these tissues have not yet been closely examined. The present study detected KCNQ3, but not KCNQ2, mRNA transcripts in Calu-3 cells, while the human bronchial airway epithelial cell line 16HBE14o- demonstrated a differential pattern of expression, with KCNQ2, but not KCNQ3, mRNA and protein detected. Thus, interestingly, it appears that while KCNQ2 and KCNQ3 are both expressed in airway epithelial cells, they are not coexpressed in the same cell lines. MacVinish et al. (2001) detected mRNA transcripts for KCNQ1 and KCNQ4 in murine lung and nasal epithelium, KCNQ5 in the lung and KCNQ2 in the nasal epithelium, though they suggested this latter finding may be due to contamination from the olfactory bulb. One possibility is that in the airway epithelial cells we have studied KCNQ2 and KCNQ3 may be integral to channels with novel physiological roles in the lung distinct from than that of the M-channel in the CNS. Furthermore, our finding that airway epithelial cells appear to differentially express either KCNQ2 or KCNQ3 may indicate that these channels may be able to substitute for each other while forming functional complexes.

KCNQ3 and KCNQ5 have also been shown to coassemble to form a channel with similar electrophysiological properties as those of the native M-channel (Lerche et al., 2000; Schroeder et al., 2000b). KCNQ5 is often coexpressed with KCNQ3 in many areas of the brain, leading to speculation that in these tissues the M-channel may be formed by KCNQ3 and KCNQ5 rather than only KCNQ2 and KCNQ3 (Lerche et al., 2000; Schroeder et al., 2000a). Our finding that KCNQ3 and KCNQ5 are both localized apically in Calu-3 cells raises the interesting possibility that similar channels may be present in Calu-3 cells. Ideally, we would have liked to perform colocalization experiments to determine the degree to which KCNQ3 and KCNQ5 colocalize, if any, in Calu-3 cells. However, this was not possible in the present

study because the anti-KCNQ3 and anti-KCNQ5 antibodies used were both raised in a rabbit host.

The rate of transepithelial anion secretion is ultimately dependent upon the activity of K^+ channels since K^+ exit hyperpolarizes the cell, increasing the driving force for anion efflux through open anion channels in the apical membrane (Smith & Frizzell, 1984). In the model proposed by Silva et al. (1977), K^+ channels supporting apical anion secretion were thought to be located in the basolateral membrane, which was supported by a great amount of functional evidence (Smith & Frizzell, 1984; McCann & Welsh, 1990; Hwang et al., 1996; MacVinish et al., 1998; Mall et al., 2000; Grahammer et al., 2001; Cowley & Linsdell, 2002). However, Cook & Young (1989) revised this model after determining that epithelial secretion rates would be optimal if 11–25% of K^+ conductance was in the apical membrane. Therefore, our results support this model by providing functional evidence that apically located K^+ channels do indeed mediate epithelial anion secretion. Previous work by ourselves (Davis & Cowley, 2006) and others (Wu et al., 2004) strongly suggests an increasing number of K^+ channel types located at the apical membrane of Calu-3 cells. Localization of the KCNQ channels to the apical membrane could possibly provide an advantage when considering their potential to drive anion secretion. Ion channels are often regulated via interactions with nearby proteins or other molecules (Marx, 2003). KCNQ1 and KCNQ2 are both known to associate with A-kinase anchoring proteins (AKAPs), which facilitate protein kinase A or C modulation of the channels (Marx et al., 2002; Hoshi et al., 2003). It is likely that KCNQ3–5 associate with the same AKAP as KCNQ2 since the AKAP binding region is conserved across KCNQ2–5 (Hoshi et al., 2003). CFTR is linked to an AKAP in Calu-3 cells and forms a signaling complex with the β_2-adrenergic receptor (Naren et al., 2003); therefore, it is possible that CFTR and/or β receptors interact with the KCNQ channels via AKAPs, perhaps resulting in the formation of a coordinated secretory complex.

In summary, our findings are the first to describe expression of KCNQ2–5 in human airway epithelial cell lines and suggest, together with work on the mouse (MacVinish et al., 2001), that these channels are more extensively expressed in airway tissue than previously appreciated. Furthermore, these channels play a role in mediating basal anion secretion and demonstrate the importance of apically located K^+ channels in mediating anion secretion in airway epithelia.

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