Expression of the chloride channel CLC-K in human airway epithelial cells

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Abstract: Airway submucosal gland function is severely disrupted in cystic fibrosis (CF), as a result of genetic mutation of the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane Cl^- channel. To identify other Cl^- channel types that could potentially substitute for lost CFTR function in these cells, we investigated the functional and molecular expression of Cl^- channels in Calu-3 cells, a human cell line model of the submucosal gland serous cell. Whole cell patch clamp recording from these cells identified outwardly rectified, pH- and calcium-sensitive Cl^- currents that resemble those previously ascribed to ClC-K type chloride channels. Using reverse transcription – polymerase chain reaction, we identified expression of mRNA for ClC-2, ClC-3, ClC-4, ClC-5, ClC-6, ClC-7, ClC-Ka, and ClC-Kb, as well as the common ClC-K channel β subunit barttin. Western blotting confirmed that Calu-3 cells express both ClC-K and barttin protein. Thus, Calu-3 cells express multiple members of the ClC family of Cl^- channels that, if also expressed in native submucosal gland serous cells within the CF lung, could perhaps act to partially substitute lost CFTR function. Furthermore, this work represents the first evidence for functional ClC-K chloride channel expression within the lung.

Key words: chloride channel, epithelial transport, airway, cystic fibrosis.

Résumé : La fonction de la glande sous-muqueuse des voies aériennes est fortement perturbée dans la fibrose kystique du pancréas (FK), en raison d'une mutation génétique du régulateur de conductance transmembranaire (CFTR), un canal Cl^- de la membrane apicale. Pour identifier d'autres types de canaux Cl^- susceptibles de se substituer à la fonction perdue du CFTR dans ces cellules, nous avons examiné l'expression fonctionnelle et moléculaire des canaux Cl^- dans les cellules Calu-3, un modèle de lignée cellulaire humaine de la cellule séreuse de la glande sous-muqueuse. Un enregistrement patch clamp en configuration cellule entière a identifié des courants Cl^- à rectification sortante, sensibles au calcium et au pH, qui ressemblent à ceux déjà attribués aux canaux de type ClC-K. La technique RT–PCR a permis d'identifier l'expression de l'ARNm pour ClC-2, ClC-3, ClC-4, ClC-5, ClC-6, ClC-7, ClC-8, ClC-Ka et ClC-Kb, ainsi que la sous-unité β commune aux canaux ClC-K, barttine. Le transfert de type Western a confirmé que les cellules Calu-3 expriment tant les canaux ClC-K que la protéine barttine. Ainsi, les cellules Calu-3 expriment de multiples membres de la famille ClC des canaux Cl^- qui, s'il sont aussi exprimés dans les cellules séreuses sous-muqueuses natives du poumon FK, pourraient peut-être agir aussi comme substitut partiel de la fonction CFTR perdue. De plus, ce travail représente le premier témoignage de l'expression de canaux chlorure ClC-K fonctionnels dans le poumon.

Mots clés : canal chlorure, transport épithélial, voie aérienne, fibrose kystique du pancréas.

[Traduit par la Rédaction]

Introduction

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel found in the apical membrane of many different epithelial cell types (Pilewski and Frizzell 1999; Sheppard and Welsh 1999). Dysfunction of CFTR in CF epithelia results in pleiotropic symptoms of pancreatic, gastrointestinal, and reproductive disease (Pilewski and Frizzell 1999). However, most of the morbidity and mortality currently associated with CF is the result of lung disease (Pilewski and Frizzell 1999; Gibson et al. 2003). Within the lung, CFTR is expressed predominantly in submucosal glands (Jiang and Engelhardt 1998), where it is thought to underlie transepithelial anion secretion, which is the driving force for glandular fluid secretion (Pilewski and Frizzell 1999; Verkman et al. 2003; Ballard and Inglis 2004). Consistent with the high level of CFTR expression in submucosal gland serous cells, it has been proposed that these cells represent the primary site of CF lung pathology (Pilewski and Frizzell 1999; Verkman et al. 2003). Serous cells have been termed the primary defensive cell of the airway mucosa (Basbaum et al. 1990; Pilewski and Frizzell 1999), and their dysfunction may contribute not only to CF, but also to other inflammatory lung diseases, such as asthma and chronic bronchitis (Finkbeiner 1999).

It has long been suggested that activation of alternate, non-CFTR Cl^- channels could, at least in part, overcome

Received 25 May 2005. Published on the NRC Research Press Web site at http://cjpp.nrc.ca on 25 January 2006.

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 doi: 10.1139/y05-112

the deficiency in transepithelial Cl^- permeability, and thus provide a therapeutic benefit in CF (Cuppoletti et al. 2000; Fuller et al. 2001; Wills and Fong 2001). However, to effectively substitute lost CFTR function, alternate Cl^- channels would presumably need to be expressed in those cells in which CFTR normally plays an important role. We have used electrophysiological and molecular techniques to investigate the expression of members of the ClC family of Cl^- channels in the Calu-3 cell line, which, although derived from a human pulmonary adenocarcinoma (Shen et al. 1994), represent a good and widely used model of the human submucosal gland serous cell (Pilewski and Frizzell 1999; Cowley and Linsdell 2002*b*; Hug et al. 2003). While we provide molecular evidence that several ClC family members are expressed in Calu-3 cells, functional evidence suggests a particular role of ClC-K Cl^- channels in membrane Cl^- conductance.

Materials and methods

Experiments were carried out on the Calu-3 human airway epithelial cell line grown in culture as described previously (Cowley and Linsdell 2002*a*). For patch clamp recordings, cells were plated onto glass cover slips coated with 10 μg/mL type I collagen (Sigma–Aldrich, Oakville, Ont.) and used 1–4 days after seeding.

Electrophysiological recording

Currents were recorded using conventional whole-cell patch clamp recording, filtered at 1 kHz using an 8-pole Bessel filter (Warner Instruments, Hamden, Conn.), and digitized at 2 kHz using an Axopatch 200B amplifier (Axon Instruments, Union City, Calif.). Whole-cell currents were recorded during voltage steps between –100 and +80 mV from a holding potential of –40 mV. The intracellular (pipette) solution contained the following: 130 mol CsCl/L, 1 mol NaCl/L, 2 mol $CaCl_2$/L, 1 mol $MgCl_2$/L, 1 mol EGTA/L, 1 mol MgATP/L, and 10 mol *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES)/L, pH 7.4. For experiments in which the extracellular pH was altered, the extracellular (bath) solution contained the following: 140 mol NaCl/L, 4 mol CsCl/L, 2 mol $MgCl_2$/L, 2 mol $CaCl_2$/L, and 10 mol/L pH buffer (2-morpholinoethanesulfonate (MES) for pH 6.4, TES for pH 7.4, or 2-(cyclohexylamino)ethanesulfonate (CHES) for pH 9.4). Where the extracellular concentration of Ca^{2+} was altered, the bath solution contained the following: 140 mol NaCl/L, 4 mol CsCl/L, 2 mol $MgCl_2$/L, 10 mol CHES/L, and 1, 2, or 5 mmol/L $CaCl_2$ (pH 9.4). Pipette resistance was ~5 MΩ using these solutions. Recordings were made at room temperature.

RNA expression

RNA extraction, reverse transcription, polymerase chain reaction (PCR), and DNA sequencing were all carried out according to Cowley and Linsdell (2002*a*). Custom primer sequences and PCR conditions used are described in Table 1. PCR products were visualized by electrophoresis on a 1.5% agarose gel containing 300 μg/L ethidium bromide (Invitrogen, Burlington, Ont.) alongside a 100 bp DNA ladder (Invitrogen).

Protein expression

Cells were removed with a cell scraper, spun down, and the pellet was resuspended in a lysis buffer (60 mmol/L Tris–HCl, pH 6.8) containing 10% sodium dodecyl sulfate and 15 mg/mL dithiothreitol. Complete protease inhibitor (Roche Applied Science, Indianapolis, Ind.) was added and the mixture was sonicated. Total protein (30 μg) was run on a 7.5% polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.). Following blocking in 5% skim milk powder in Tris-buffered saline – Tween (25 mmol/L Tris base, 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.05% Tween-20), immunoblotting was performed using rabbit anti-ClCK (Alomone Laboratories, Jerusalem, Israel) and rabbit anti-barttin (Alpha Diagnostic International, San Antonio, Tex.) primary antibodies, both used following a 1:200 dilution in a mixture of 5% skim milk powder and TBS–Tween, followed by incubation with horseradish peroxidase conjugated goat anti-rabbit IgG (Jackson Immuno, Mississauga, Ont.) at a 1:20 000 – 1:50 000 dilution. Detection was carried out using the ECL Plus chemiluminescence kit according to the manufacturer's instructions (Amersham Pharmacia, Baie d'Urfe, Que.).

Results

Whole-cell patch clamp recording revealed the presence of a rapidly activating, outwardly rectified Cl^- conductance in Calu-3 cells (Fig. 1A). The amplitude of this current was reversibly enhanced by increasing extracellular pH (Fig. 1A and 1B), and also by increasing extracellular Ca^{2+} concentration (Fig. 1C). This whole-cell current was confirmed as a Cl^- current by the dependence of the current reversal potential on the estimated Cl^- equilibrium potential (data not shown).

To investigate the molecular identity of the chloride channel underlying this current, we performed reverse transcription–PCR (RT–PCR) on total RNA extracted from Calu-3 cells. PCR reactions with specific primer pairs (see Table 1) revealed the expression of the Cl^- channel genes *ClC-2*, *ClC-3*, *ClC-4*, *ClC-5*, *ClC-6*, and *ClC-7* (Fig. 2). In contrast, multiple attempts to identify *ClC-1* expression were unsuccessful; this channel has previously been described as being expressed exclusively in skeletal muscle cells (Jentsch et al. 2002). Because of their high sequence homology, we were unable to design PCR primers that would selectively amplify *ClC-Ka* or *ClC-Kb*. However, a primer pair that matched both *ClC-Ka* and *ClC-Kb* did yield a PCR product (Fig. 2) that, upon DNA sequencing, was found to contain a mixture of 2 different cDNAs that matched the published sequences of *ClC-Ka* and *ClC-Kb*. Barttin, the common β subunit for both ClC-Ka and ClC-Kb, was also positively identified by RT–PCR (Fig. 2). For all RT–PCR experiments, DNA was recovered from the gel and sequenced; comparison with published sequences (National Center for Biotechnology Information) confirmed that Calu-3 cells express mRNA for ClC-2, ClC-3, ClC-4, ClC-5, ClC-6, ClC-7, ClC-Ka, ClC-Kb, and barttin.

The voltage dependence, time course, as well as pH and Ca^{2+} sensitivity of the whole-cell current shown in Fig. 1, are reminiscent of ClC-K channel activity (Estévez et al. 2001; Waldegger et al. 2002; Lourdel et al. 2003; Jeck et al. 2004). Since ClC-Ka, ClC-Kb, and barttin mRNA are all

Table 1. Primer sequences and PCR conditions used to detect chloride channel cDNA.

Primer	Sequence*	Expected product size (bp)	PCR conditions (denaturation; annealing; extension)
ClC-2	5′-GGAGTGGTGCTGAAAGAATACC-3′ 5′-TCGATGCTGAAGAGGACGC-3′	270	94 °C/30 s; 53 °C/30 s; 72 °C/30 s; 40 cycles
ClC-3	5′-GGGCACTGGCCGGATTAATAGACA-3′ 5′-TGCACCAAAAGCTACAGAAACCC-3′	600	94 °C/30 s; 63 °C/60 s; 72 °C/60 s; 34 cycles
ClC-4	5′-TCCCAATGAAAGATCATGCA-3′ 5′-ATACGCCTTGAGCAGAAGGA-3′	280	94 °C/30 s; 53 °C/30 s; 72 °C/30 s; 40 cycles
ClC-5	5′-GTTCTGGATGTAAAGCGGGA-3′ 5′-TTTTCAGTGATTGCATATTGGC-3′	310	94 °C/30 s; 53 °C/30 s; 72 °C/30 s; 40 cycles
ClC-6	5′-GCCTTTTTGTGCCTTCTCTG-3′ 5′-GCTGGATGGGTAGGACTTCA-3′	592	94 °C/30 s; 55 °C/30 s; 72 °C/60 s; 35 cycles
ClC-7	5′-CTTCATTGACATCGTGGTGG-3′ 5′-AATTCAGGGTGAACGTGGAG-3′	592	94 °C/30 s; 55 °C/30 s; 72 °C/60 s; 35 cycles
ClC-K	5′-GGACGTTCCCTTCGACCT-3′ 5′-CCCCTGCCAGAGCATACC-3′	592	94 °C/30 s; 55 °C/30 s; 72 °C/60 s; 35 cycles
Barttin	5′-GGATCCATGGCTGACGAGAAGACCTTC-3′ 5′-GAATTCTCAGCCTTGGGTGTCAGGCTC-3′	974	95 °C/30 s; 68 °C/180 s; 35 cycles

*The top and bottom lines represent forward and reverse sequences, respectively.

expressed by Calu-3 cells, we undertook Western blot analysis of Calu-3 cell total lysate using anti-ClC-K and anti-barttin primary antibodies. Antibodies capable of distinguishing between ClC-Ka and ClC-Kb are not currently available. However, a nonspecific anti-ClC-K antibody showed immunoreactivity to 2 bands of approximately 75 kDa (close to the predicted molecular mass of the ClC-K channels) and 50 kDa (Fig. 3A). Anti-barttin antibody was immunoreactive to a protein that comigrated with the 47 kDa molecular mass marker (Fig. 3B), as previously demonstrated for barttin (Estévez et al. 2001). Each of the bands shown in Fig. 3A and 3B were abolished by pre-incubation of the primary antibody with an excess of peptide antigen (not shown).

Discussion

The dominant Cl^- channel underlying anion secretion by submucosal gland serous cells is CFTR (Pilewski and Frizzell 1999; Verkman et al. 2003; Ballard and Inglis 2004), consistent with the greatly disturbed secretory function of this cell type in CF (Pilewski and Frizzell 1999; Verkman et al. 2003). Befitting their use as a model for serous cells, the Calu-3 cell line shows strong expression of CFTR (Shen et al. 1994), which appears to be responsible for the bulk of the apical membrane Cl^- permeability (Haws et al. 1994; Illek et al. 1999). Nevertheless, our present results demonstrate that Calu-3 cells express a plethora of ClC-type Cl^- channels, and that dissociated Calu-3 cells exhibit non-CFTR-mediated membrane Cl^- currents.

Calu-3 cells expressed mRNA for 8 different members of the ClC Cl^- channel family: ClC-2, ClC-3, ClC-4, ClC-5, ClC-6, ClC-7, ClC-Ka, and ClC-Kb, as well as the ClC-K channel β subunit barttin (Fig. 2). Based on sequence homology and functional studies using overexpressed channels, these ClCs are frequently subdivided into 2 groups: ClC-2, ClC-Ka, ClC-Kb, and ClC-1, which are associated with plasma membrane Cl^- currents; and ClC-3, ClC-4, ClC-5, ClC-6, and ClC-7, which may function predominantly in the membranes of intracellular vesicles (Jentsch et al. 2002; Nilius and Droogmans 2003). Nevertheless, it should be pointed out that most of these channels can contribute to plasma membrane Cl^- channels under certain conditions (Jentsch et al. 2002; Nilius and Droogmans 2003), and there is evidence that ClC-3 in particular may contribute to membrane Cl^- currents in some cell types (Huang et al. 2001; Wang et al. 2003; Vessey et al. 2004). However, this evidence has not met with universal acceptance (e.g., see Jentsch et al. 2002).

Of these ClC channel types expressed in Calu-3 cells, the functional properties of the whole-cell Cl^- currents observed in dissociated cells (Fig. 1) most closely resemble the cell-membrane-associated channel types ClC-Ka and ClC-Kb. When overexpressed in mammalian cells (Estévez et al. 2001) or *Xenopus* oocytes (Estévez et al. 2001; Waldegger et al. 2002; Jeck et al. 2004), these channels carried membrane Cl^- currents that were moderately outwardly rectified, and that were enhanced by high extracellular pH and high extracellular Ca^{2+} concentrations. Similar currents have also been observed in rat cochlear cells that endogenously express ClC-K (Ando and Takeuchi 2000), and candidate ClC-K currents, again stimulated by alkaline pH and high extracellular Ca^{2+} concentrations, have been observed at the single-channel level in renal epithelial cells (Sauvé et al. 2000; Lourdel et al. 2003). While the whole-cell currents shown in Fig. 1 do show some resemblance to heterologously expressed ClC-K currents, it is possible that, since these cells express so many different ClC channel types, these currents actually reflect the ensemble activity of several different channel types, among which ClC-K channels play a dominant role. Theoretically, the contribution of ClC-K channels could be further dissected by investigating the ionic selectivity (Waldegger et al. 2002) and blocker sensitivity (Picollo et al. 2004) of the whole-cell current. Indeed, since ClC-Ka and ClC-Kb show some differences in anion selectivity (Waldegger et al. 2002) and blocker sensi-

Fig. 1. Whole-cell chloride currents in Calu-3 cells. (A) Examples of whole-cell currents in a Calu-3 cell at extracellular pH 7.4 (left), and following superfusion with pH 9.4 solution (right). The outward current rectification and rapid activation on depolarization are reminiscent of heterologously expressed ClC-K Cl^- channels. (B) The effect of altering extracellular pH on whole-cell Cl^- current amplitude at a membrane potential of +80 mV. Currents were significantly (*, $P < 0.01$, ANOVA) and reversibly stimulated by alkalization of the extracellular solution. Mean ± SE of data from 5 cells. (C) Whole-cell Cl^- current amplitude at +80 mV was also significantly (*, $P < 0.01$, ANOVA) and reversibly stimulated by increasing extracellular Ca^{2+} concentration.

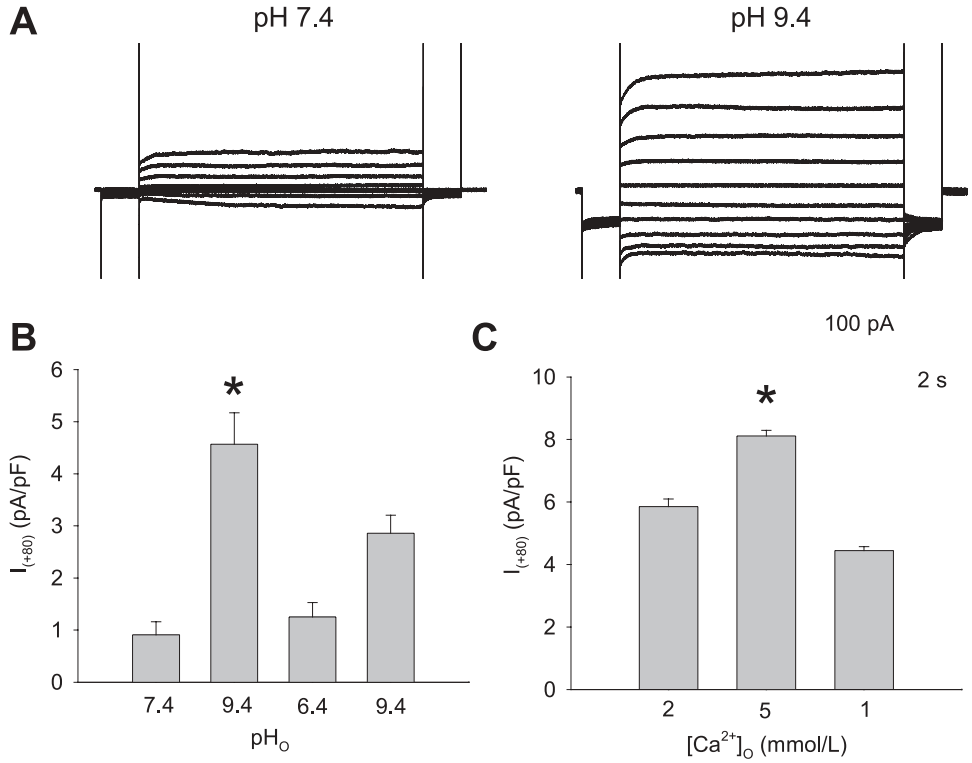
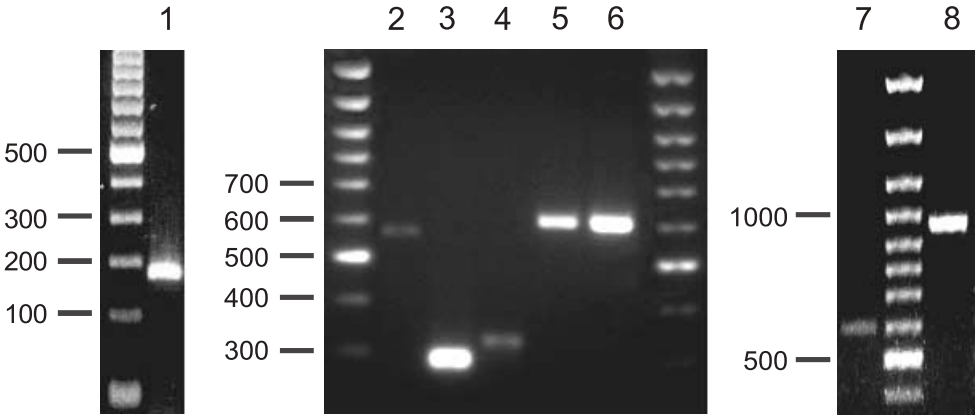


Fig. 2. Calu-3 cells express ClC chloride channel mRNA. Following reverse-transcription – polymerase chain reaction, cDNA transcripts were observed for ClC-2 (lane 1, expected size, 270 bp), ClC-3 (lane 2, 600 bp), ClC-4 (lane 3, 280 bp), ClC-5 (lane 4, 310 bp), ClC-6 (lane 5, 592 bp), ClC-7 (lane 6, 592 bp), ClC-K (lane 7, 592 bp), and barttin (lane 8, 974 bp). The identity of each band was confirmed by DNA sequencing. Unlabelled lanes are molecular weight markers.

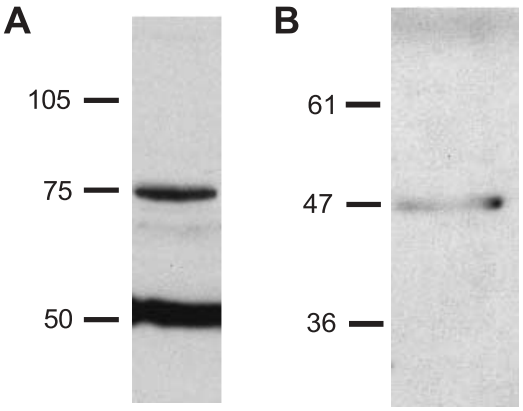


tivity (Picollo et al. 2004), these same kinds of experiments could be used to gain some information on the relative contribution of these 2 channel types to whole-cell Cl^- current.

Both ClC-2 (Gyömörey et al. 2000; Mohammad-Panah et al. 2001; Catalán et al. 2002;) and ClC-4 (Mohammad-Panah et al. 2002) have previously been suggested to contribute to epithelial cell membrane Cl^- conductance. However, the whole-cell Cl^- current shown in Fig. 1 does not share key functional properties associated with these channel types: ClC-2 shows inward rectification and is stimulated by acidic pH (Jentsch et al. 2002), and ClC-4 shows very strong outward rectification (Friedrich et al. 1999).

Previously, ClC-K channels have been identified only in Cl^- absorptive renal epithelial cells (Jentsch et al. 2002; Uchida and Sasaki 2005) and K^+ secretory cells in the inner ear (Estévez et al. 2001; Jentsch et al. 2002). In both of

Fig. 3. Expression of (A) ClC-K and (B) barttin protein in Calu-3 cell extracts by Western blotting. Each of the protein bands shown was eliminated by preincubation of the primary antibody with an excess of peptide antigen (not shown). The predicted molecular masses of ClC-K Cl^- channels and barttin are approximately 75 and 47 kDa, respectively.



these tissues, ClC-K channel expression appears to be restricted to the basolateral membrane (Estévez et al. 2001; Jentsch et al. 2002). While ClC-K channels appear to be present in the membrane of Calu-3 cells, the functional role of these channels in polarized Calu-3 epithelial cells will clearly depend on their subcellular localization. Unfortunately, we have been unable to determine the localization of either ClC-K or barttin in polarized Calu-3 cell monolayers, using either immunofluorescence directed against endogenous proteins, or against overexpressed, epitope-tagged ClC-Kb or barttin proteins, and as a result, we can only speculate as to their role in serous cell physiology. CFTR appears to be the dominant apical membrane Cl^- transport pathway in these cells (Haws et al. 1994; Illek et al. 1999); however, other Cl^- channels, which are also found in the apical membrane, may also contribute to transepithelial anion secretion and could represent bona fide therapeutic targets in the CF airway. Functional evidence also suggests that Calu-3 cells express Cl^- channels in the basolateral membrane (Szkotak et al. 2003); however, the molecular identity of these channels is not known. Basolateral Cl^- channels in Calu-3 cells could play a role in switching between Cl^- and HCO_3^- secretion (Szkotak et al. 2003), or could allow Cl^- absorption under certain conditions (Uyekubo et al. 1998).

This work identifies ClC-K Cl^- channels as being expressed in human airway epithelial cells and demonstrates that they contribute to plasma membrane Cl^- conductance. As far as we know, this represents the first evidence for this channel type in airway cells or in any anion secretory cell type. We suggest that these channels may contribute to the control of anion and fluid secretion by submucosal glands in the lung.

Acknowledgements

This work was supported by the Nova Scotia Health Research Foundation. J.L.M. was supported by scholarship awards from the Natural Sciences and Engineering Research Council of Canada and the Killam Trusts.

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