Maxi K$^+$ channel mediates regulatory volume decrease response in a human bronchial epithelial cell line

José M. Fernández-Fernández, José M., Muriel Nobles, Aoife Currid, Esther Vázquez, and Miguel A. Valverde. Maxi K$^+$ channel mediates regulatory volume decrease response in a human bronchial epithelial cell line. Am J Physiol Cell Physiol 283: C1705–C1714, 2002. First published August 14, 2002; 10.1152/ajpcell.00245.2002.—The cell regulatory volume decrease (RVD) response triggered by hypotonic solutions is mainly achieved by the coordinated activity of Cl$^-$ and K$^+$ channels. We now describe the molecular nature of the K$^+$ channels involved in the RVD response of the human bronchial epithelial (HBE) cell line 16HBE14o-. These cells, under isotonic conditions, present a K$^+$ current consistent with the activity of maxi K$^+$ channels, confirmed by RT-PCR and Western blot. Single-channel and whole cell maxi K$^+$ currents were readily and reversibly activated following the exposure of HBE cells to a 28% hypotonic solution. Both maxi K$^+$ current activation and RVD response showed calcium dependency, inhibition by TEA, Ba$^{2+}$, iberitoxin, and the cationic channel blocker Gd$^{3+}$ but were insensitive to clotilum, clotrimazole, and apamin. The presence of the recently cloned swelling-activated, Gd$^{3+}$-sensitive cation channels (TRPV4, also known as OTRPC4, TRP12, or VR-OAC) was detected by RT-PCR in HBE cells. This channel, TRPV4, which senses changes in volume, might provide the pathway for Ca$^{2+}$ influx under hypotonic solutions and, consequently, for the activation of maxi K$^+$ channels.

The maintenance of several epithelial functions (e.g., Cl$^-$ secretion, Na$^+$ absorption, and cell volume regulation) requires the coordinated regulation of apical membrane ion channels (e.g., cystic fibrosis transmembrane conductance regulator Cl$^-$ channel (CFTR), Ca$^{2+}$-dependent Cl$^-$ channel, and epithelial Na$^+$ channel (ENaC)) and basolateral membrane K$^+$ channels (37, 57, 61). A vast account of the activity and regulation of the epithelial Cl$^-$ and Na$^+$ channels required in such epithelial functions exists in the literature (4, 5), whereas little is known about the K$^+$ channels involved in these ion transport processes. Several types of K$^+$ channels have been identified in epithelial cells, and their functional roles are just starting to emerge (14). In the case of the airways, the molecular identities of the different K$^+$ channels associated with each type of cellular function remain largely unknown.

In many different types of cells, exposure to hypotonic solutions induces cell swelling, followed by the return of the cell volume to a set point close to the original value. This response, known as regulatory volume decrease (RVD), reflects the cell’s adjustments to the osmotic stress. The response involves the loss of osmolytes and osmotically obliged water (27, 35). The general mechanism that allows cells to lose osmolytes has been outlined in many different cell types, including epithelial cells, and typically employs the activation of K$^+$ and Cl$^-$ channels or transporters, as well as organic osmolyte pathways (27, 35). However, despite the major impetus for the study of epithelial volume-sensitive Cl$^-$ and K$^+$ channels, little advance has been made in the identification of their molecular nature. Moreover, we do not know whether epithelial cells use specific volume-sensitive ion channels for cell volume regulatory purposes or whether they recruit ion channels that are also responsible for other cellular functions. In the present study we have investigated the nature of the K$^+$ channels involved in the control of human airway epithelial volume with special emphasis on their regulation by increases in intracellular Ca$^{2+}$ and the molecular identity of the possible Ca$^{2+}$ entry pathway.

METHODS

Cells

Human bronchial epithelial cells 16HBE14o- (15) were grown in modified Eagle’s medium with Earle’s salts (GIBCO), 10% fetal bovine serum (GIBCO), and 1% gentamicin (Sigma). Cells were grown on flasks coated with a solution containing 1 mg of human fibronectin (Stratech), 0.33 mg of Vitrogen (Imperial Laboratories), and 10 mg of bovine serum albumin.
albumin (ICN Flow) in 100 ml of modified Eagle's medium with Earle's salts. The 25-cm² plastic flasks were coated overnight (37°C) with the fibronectin-based solution. Any excess fibronectin was aspirated before cell seeding. For the experiments, cells were seeded onto plastic dishes or glass coverslips and used within 2 days.

**Solutions**

The isotonic bathing solution contained (mM) 140 NaCl, 2.5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES, pH 7.25 adjusted with Tris (osmolality: 303 ± 4 mosmol/kgH₂O; n = 10 observations). The hypotonic bathing solution (osmolality: 215 ± 8 mosmol/kgH₂O; n = 12) was prepared by omitting 50 mM NaCl from the isotonic Hank's solution and adjusting the osmolality with D-mannitol when necessary. All chemicals were purchased from the Sigma-Aldrich except ibetorix (Alomone Laboratories) and clofibrate (RBI).

**Morphometric Analysis**

Cell volume experiments were performed at room temperature. Cells were grown on 35-mm cell culture dishes, bathed in isotonic solution, and observed under phase-contrast optics with an inverted microscope (Leica DMIL). The individual cell volume was calculated as described previously (8, 39, 67) and normalized to that measured at time 0 (t = 0).

**Electrophysiology**

Ionic currents were measured by using the whole cell, cell-attached, or excised inside-out recording mode of the patch-clamp technique (24). Strathclyde Electrophysiological Software (written by J. Dempster, University of Strathclyde, Glasgow, Scotland) or pCLAMP8 (Axon Instruments, Foster City, CA) was used for pulse generation, data acquisition through an Axon Digitida analog-to-digital interface, and subsequent analysis. Cells were plated in 35-mm plastic dishes and mounted on the stage of inverted Olympus IX70 or Leica DMIL microscopes.

Whole cell ionic currents were measured by using borosilicate glass electrodes (2–4 MΩ) filled with a solution containing (mM) 140 KCl, 1.2 MgCl₂, and 10 HEPES, pH 7.3. The intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ), calculated using EqCal (Biosoft, Cambridge, UK), was adjusted to the desired values by adding different combinations of CaCl₂ and EGTA to the intracellular solution: 66 mM Ca²⁺ solution, 0.15 mM CaCl₂ and 0.5 mM EGTA; or 200 nM Ca²⁺ solution, 0.7 mM CaCl₂ and 1 mM EGTA. The osmolality of the intracellular solutions was adjusted to 290 ± 8 mosmol/kgH₂O (n = 23). ATP and GTP were not added to the pipette solution to delay and reduce the activation of swelling-activated Cl⁻ channels (7, 19, 22). Isotonic and hypertonic extracellular solutions were as described above. Ca²⁺-free extracellular hypotonic solution (0 [Ca²⁺]), containing 0 Ca²⁺, 1.7 mM MgCl₂, and 1 mM EGTA, was also used in several experiments. Occasionally, the isotonic solution contained 100 mM NaCl plus 80 mM n-mannitol instead of 140 mM NaCl, and the hypotonic solution was obtained by omitting n-mannitol. Whole cell currents were recorded with an Axon 200A amplifier. Cells were clamped at −80 mV and pulsed for 400 ms from −100 to +100 mV in 20-mV steps. Alternatively, whole cell current-voltage curves were obtained by applying a ramp of voltage from −120 to +100 mV over a 4000 ms period.

Cell-attached single-channel recordings were carried out by using borosilicate glass electrodes (4–6 MΩ) filled with a solution containing (mM) 100 NaCl, 5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES, pH 7.25 (osmolality adjusted with D-mannitol: 300 ± 5 mosmol/kgH₂O; n = 15), and the isotonic bathing solution contained (mM) 105 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose, 10 HEPES, and 80 n-mannitol, pH 7.25 (osmolality: 305 ± 4 mosmol/kgH₂O; n = 10). The hypotonic bathing solution (osmolality: 215 ± 7 mosmol/kgH₂O; n = 10) was prepared by omitting 80 mM n-mannitol from the isotonic solution. Single-channel currents were obtained by clamping the cells at different potentials (−20, −40, −80, and −100 mV) for 30 s. Excised inside-out single-channel currents were recorded by using extracellular (pipette) solutions containing (mM) 140 KCl, 0.7 MgCl₂, 10 HEPES, and 4.25 CaCl₂ (1.6 µM free Ca²⁺), pH 7.25 (osmolality: 300 mosmol/kgH₂O). Currents were low-pass filtered at 1 kHz and sampled at 10 kHz. Single-channel current levels were measured via all-points histograms, and open probability, Pₒ or Npₒ, was calculated from 30-s recordings at the indicated potential in patches containing only one channel or more than one channel, respectively.

**Expression of Maxi K⁺ and TRPV4 Channels in 16HBE140− Cells**

**RT-PCR.** Total RNA was extracted from 16HBE140− and HEKhSlo cells [human embryonic kidney cells permanently transfected with hSlo (1)] when they were 80% confluent by using the Nucleospin RNA II kit (Macherey-Nagel), which also degrades the genomic DNA. Total RNA (1–2 µg) was reverse transcribed to obtain cDNA. After 2 min of denaturation at 90°C, the RNA was incubated for 1 h at 35°C in 20 µl of reverse transcriptase reaction mix containing 1× RT buffer (Promega), 2 µM oligo(dT₁₂) primer, 125 µM pooled dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). One microliter of cDNA product was sufficient to amplify by PCR a fragment of 479 bp that corresponds to nucleotides 2675 to 3154 of the hSlo complete coding sequence (U11717), within a conserved non-alternative spliced region encompassing the S10 region of the gene, which is not shared with other known genes (confirmed by a BLASTN search), by using the following primers: forward 5'-ACCAAGAGGATGATGATGACC-3' and reverse 5'-AGCAGAAAGATCAGTGTCGTC-3'. The profile was as follows: 95°C for 45 s, 60°C for 1 min, and 72°C for 60 s (30 cycles).

The detection of transient receptor potential cation channel (TRPV4) transcripts in 16HBE140− cells was carried out by PCR on two different regions of the cDNA, one of 323 bp from nucleotides 362 to 685 and another one of 500 bp that corresponds to nucleotides 1064 to 1564 of the TRPV complete coding sequence (AF258465). Both are gene-specific sequences containing several introns to rule out the possibility of amplifying genomic DNA. The following primers were used: forward 5'-AGGAGTTGGAGAAAGATC-3' and reverse 5'-GTTAATGACCCCGCATG-3' for the first segment (PCR1); and forward 5'-CTCTTCCCAGACGACAC-3' and reverse 5'-CCCCAGTGAAGGCGTAAATG-3' for the second segment (PCR2). The PCR profile is the same as that given above but with an annealing temperature of 58°C.

**Membrane preparations of 16HBE140− and HEK hSlo cells.** A detailed description of the membrane preparation protocol has been previously published (23), and only minor modifications have been made to the basic protocol. Briefly, after the cells were washed with Ca²⁺- and Mg²⁺-free PBS, they were incubated in the same medium with 0.1 mM EGTA at 4°C for 10 min, scraped, and centrifuged at 800 g for 5 min.
The pellet was then resuspended in (mM) 137 NaCl, 5.6 dextrose, 1 EGTA, and 5 HEPES at pH 7.4 and centrifuged as above. The pellet was then homogenized in (mM) 5 Tris·HCl, 5 MgCl$_2$, and 1 EGTA at pH 7.5. The homogenate was centrifuged at 100,000 g for 1 h to obtain crude membranes. Membranes were resuspended in 50 mM Tris·HCl, 5 mM MgCl$_2$, and 1 mM EGTA at pH 7.6 supplemented with 10 μg/ml leupeptin, 10 μg/ml aprotonin, and 10 μg/ml phenylmethylsulfonyl fluoride and stored at −70°C. Protein content was determined using the Lowry method (Bio-Rad).

Western blots. 16HBE14o—membrane proteins (20 μg) were separated on 8% SDS-polyacrylamide gels under reducing conditions and electrotransferred to nitrocellulose paper. Blots were blocked with TBST (100 mM Tris·HCl, 150 mM NaCl, and 0.1% Tween 20, at pH 7.5) containing 5% nonfat dry milk overnight at 4°C. Blots were then incubated with 1:400 affinity-purified anti-maxi K channel α-subunit polyclonal antibody (62) in TBST-5% nonfat dry milk for 2 h at room temperature, washed with TBST-5% nonfat dry milk five times for 10 min each time, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000) for 1 h. After being washed, blots were treated at room temperature, washed with TBST-5% nonfat dry milk five times for 10 min each time, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The currents recorded resembled the activity of maxi K$^+$ channels; therefore, we explored the presence of maxi K$^+$ channels in HBE cells by RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B). Figure 2A shows that a single amplicon of about 500 bp was obtained from HBE cells. Subsequent sequencing of the band confirmed that it indeed corresponded to the maxi K$^+$ sequence. Western blot analysis revealed a band with a molecular mass of ~120 kDa, consistent with the presence of maxi K$^+$ α-subunit (42, 62). HEK-293-hSlo cells expressing human maxi K$^+$ [hSlo, KCNMA1 (1)] were included as a positive control for both the RT-PCR and Western blot.

The electrophysiological (Fig. 1) and molecular data (Fig. 2) support the presence of hSlo in HBE cells. To further characterize the nature of the whole cell currents recorded in HBE cells, we used different inhibitors of K$^+$ channels (Fig. 3), some of which were wide spectrum [tetraethylammonium (TEA) and Ba$^{2+}$ (36, 69)] whereas others were more specific for a particular type of K$^+$ channel: iberiotoxin and apamin are inhibitory toxins specific for the large (maxi K$^+$, or BK) and small-conductance Ca$^{2+}$-dependent K$^+$ channels (SK)

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**RESULTS**

Identification of K$^+$ Channels in Human Bronchial Epithelial Cells

Human bronchial epithelial (HBE) cells were routinely clamped at −80 mV, and whole cell currents were elicited by pulsing from −100 to +100 mV. The currents recorded showed a strong outward rectification (Fig. 1), and the magnitude of the currents was dependent on [Ca$^{2+}$]. The currents recorded resembled the activity of maxi K$^+$ channels; therefore, we explored the presence of maxi K$^+$ channels in HBE cells by RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B). Figure 2A shows that a single amplicon of about 500 bp was obtained from HBE cells. Subsequent sequencing of the band confirmed that it indeed corresponded to the maxi K$^+$ sequence. Western blot analysis revealed a band with a molecular mass of ~120 kDa, consistent with the presence of maxi K$^+$ α-subunit (42, 62). HEK-293-hSlo cells expressing human maxi K$^+$ [hSlo, KCNMA1 (1)] were included as a positive control for both the RT-PCR and Western blot.

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to a 28% hypotonic bathing solution in 19 of 28 cells. The current increase was reversed upon removal of the hypotonic condition (washout). The increase in K⁺ channel activity following hypotonic shock was inhibited by 100 nM iberiotoxin (Fig. 4B), confirming that the K⁺ channel activated by hypotonic solutions in HBE cells is a maxi K⁺ channel. In the presence of iberiotoxin, a small volume-sensitive Cl⁻ current, with the characteristic inactivation at positive potentials (64), was visible. The effect of iberiotoxin on hypotonically activated maxi K⁺ currents is summarized in Fig. 4C. Figure 5A shows cell-attached single-channel recordings obtained from HBE cells exposed to either isotonic or hypotonic conditions. Maxi K⁺ channels increased their activity in response to hypotonic conditions (∆Pₒ = 0.23 ± 0.10, n = 7) without changing the unitary conductance (~150 pS; Fig. 5B).

A rise in [Ca²⁺]ᵢ has been implicated in the cell swelling-mediated modulation of different ion channels (44, 48). To evaluate whether similar mechanisms are operative in the activation of maxi K⁺ channels in HBE cells, we recorded whole cell currents under conditions in which the extracellular Ca²⁺ concentration had been regulated.

**Modulation of Maxi K⁺ Currents by Hypotonic Cell Swelling**

K⁺ currents were recorded under isotonic conditions in HBE cells dialyzed with solutions containing 66 nM free Ca²⁺ (Fig. 4A). A substantial increase in K⁺ currents was achieved within 3 min of exposure of the cell to a 28% hypotonic bathing solution in 19 of 28 cells. The current increase was reversed upon removal of the hypotonic condition (washout). The increase in K⁺ channel activity following hypotonic shock was inhibited by 100 nM iberiotoxin (Fig. 4B), confirming that the K⁺ channel activated by hypotonic solutions in HBE cells is a maxi K⁺ channel. In the presence of iberiotoxin, a small volume-sensitive Cl⁻ current, with the characteristic inactivation at positive potentials (64), was visible. The effect of iberiotoxin on hypotonically activated maxi K⁺ currents is summarized in Fig. 4C. Figure 5A shows cell-attached single-channel recordings obtained from HBE cells exposed to either isotonic or hypotonic conditions. Maxi K⁺ channels increased their activity in response to hypotonic conditions (∆Pₒ = 0.23 ± 0.10, n = 7) without changing the unitary conductance (~150 pS; Fig. 5B).

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modified (Fig. 6). HBE cells dialyzed with a pipette solution containing ≤100 nM free Ca\(^{2+}\) and bathed in isotonic and hypotonic solutions containing 1.2 mM Ca\(^{2+}\) showed an increase in maxi K\(^+\) current (Fig. 6A). Removal of extracellular Ca\(^{2+}\) (0 [Ca\(^{2+}\)]\(_o\)) prevented the increase in maxi K\(^+\) current under hypotonic conditions (Fig. 6B). Figure 6C shows the time course of [Ca\(^{2+}\)]\(_i\) in response to hypotonic solutions measured as the 340/380 fluorescence ratio. The mean increase in the fluorescence ratio in response to hypotonic solutions was 0.33 ± 0.09 \((n = 17)\). The increase in [Ca\(^{2+}\)]\(_i\), was mainly due to entry of extracellular Ca\(^{2+}\), because removal of extracellular Ca\(^{2+}\) (Fig. 6D) greatly reduced [Ca\(^{2+}\)]\(_i\), increase (0.07 ± 0.02; \(n = 7\); \(P < 0.01\)). Figure 6E shows superimposed time courses for the increase in [Ca\(^{2+}\)]\(_i\), and the activation of maxi K\(^+\) current in response to hypotonic swelling.

The fact that exposure to hypotonic solutions triggered an increase in [Ca\(^{2+}\)]\(_i\) (Fig. 6C) and that removal of extracellular Ca\(^{2+}\) impairs the activation of the maxi K\(^+\) channel (Fig. 6B) suggests the participation of a Ca\(^{2+}\) entry pathway, activated in response to hypotonic shocks. This Ca\(^{2+}\) pathway has typically been associated with stretch-activated ion channels (52, 54). Indeed, the recent cloning of new members of the family of transient receptor potential cation channels has provided the molecular basis for at least one of the pathways for Ca\(^{2+}\) entry following hypotonic shock (38, 60, 71, 75). To test the involvement of these cation channels in the activation of RVD mechanisms, we carried out experiments in the presence of Gd\(^{3+}\), a well-known blocker of stretch- and swelling-activated cation channels (77). Figure 7A shows the time course of a typical experiment in which the holding current and the current measured at +100 mV were continuously recorded under control conditions as well as in the presence of Gd\(^{3+}\) and/or a hypotonic solution. The presence of 100 μM Gd\(^{3+}\) did not block maxi K\(^+\) channels under isotonic conditions (see also Fig. 7B), but it did prevent the further increase in maxi K\(^+\) channel activity in response to a hypotonic solution. Upon removal of Gd\(^{3+}\), the maxi K\(^+\) current was readily increased in the presence of a hypotonic solution. Figure 7B shows the mean current-voltage curves obtained from HBE cells in response to hypotonic shocks in the presence or absence of 100 μM Gd\(^{3+}\). To discard the possibility that the absence of maxi K\(^+\) channel activation could be due to a direct inhibition of maxi K\(^+\) by

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**Fig. 5. Activation of maxi K\(^+\) channels by hypotonic shock in nondialyzed HBE cells. Top: representative single-channel current recordings from a cell-attached patch under isotonic and hypotonic conditions at a voltage command (V\(_{cmd}\)) of ~80 mV. Bottom: unitary current-voltage relationship under isotonic (○) and hypotonic (●) conditions. Values are means ± SE; \(n = 7–9\) observations for each data point.**

**Fig. 6. Effect of extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) on swelling-activated maxi K\(^+\) currents in HBE cells. A: current-voltage relationship of maxi K\(^+\) currents recorded in the presence of extracellular Ca\(^{2+}\) under isotonic (○) or hypotonic (●) conditions (\(n = 7\) observations for each condition). B: maxi K\(^+\) currents recorded under isotonic (○) or hypotonic (●) conditions in the absence of extracellular Ca\(^{2+}\) (\(n = 7\) observations for each condition). Time courses of the changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in HBE cells were measured as the ratio of emitted fluorescence (340/380) in response to normal Ca\(^{2+}\)-containing (C) and Ca\(^{2+}\)-free (D) hypotonic solutions. E: superimposition of the time courses for the fluorescence signal ([Ca\(^{2+}\)]\(_i\), measured as 340/380 ratio) and the activation of maxi K\(^+\) currents (measured at +100 mV every 10 s; see METHODS) in response to hypotonic solutions.**

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Gd$^{3+}$, we measured single-channel maxi K$^+$ currents using the inside-out configuration of the patch-clamp technique in the presence or absence of 100 μM Gd$^{3+}$ in the pipette and 1.6 μM Ca$^{2+}$ in the bathing solution (Fig. 7, C and D). Neither the channel conductance nor the open probability was altered in the presence of Gd$^{3+}$. These results are consistent with the hypothesis that points to a Gd$^{3+}$-sensitive, swelling-activated Ca$^{2+}$ influx as the trigger of maxi K$^+$ activation. The recent molecular identification of a cation channel, TRPV4, which is rapidly activated under hypotonic conditions when expressed heterologously (38, 60, 71, 75), allowed us to design primers to check its presence in HBE cells. RT-PCR produced two amplicons of the expected size (see METHODS), which, after sequencing of the bands, were confirmed to indeed correspond to the TRPV4 coding sequence.

**RVD in HBE Cells**

Relative cell volume changes of HBE cells in response to hypotonic solutions were measured as described in METHODS. Superfusion of HBE cells with a 28% hypotonic solution resulted in a clear increase in cell size, followed by the return to near-original size in isotonic solutions (Fig. 8A). The RVD response observed under control conditions was prevented when the experiments were carried out in the absence of extracellular Ca$^{2+}$ (Fig. 8B) or in the presence of extracellular Ca$^{2+}$ and 10 μM Gd$^{3+}$ (Fig. 8C). To determine whether the activation of maxi K$^+$ channels plays a role in the RVD response of HBE cells, we monitored cell volume changes in response to hypotonic solutions and the effect on RVD of various potassium channel blockers previously tested on the K$^+$ channels of epithelia identified K$^+$ (see Fig. 3). Of all the compounds tested, only those shown to block maxi K$^+$ channel activity [5 mM Ba$^{2+}$ (Fig. 9A), 5 mM TEA (Fig. 9B), and 100 nM iberiotoxin (Fig. 9C)] were effective as inhibitors of the RVD response. On the other hand, 100 nM clotrimazole (n = 3), 1 μM apamin (n = 7), and 100 μM apamin (n = 4) did not block the RVD response (results not shown).

**DISCUSSION**

Basolateral K$^+$ channels are critical for epithelial function (14, 16). Recent work carried out in airway epithelia identified K$^+$ channels that are involved in the maintenance of the resting cell membrane poten-
tial as well as ion transport processes: intermediate-conductance Ca$^{2+}$-dependent K$^+$ channels (hIK, KCNN4) (11, 18, 43, 67) and small-conductance K$^+$ channel, known as K$_v$LQT1 (KCNQ1) (6, 70). The latter can form a channel complex with a regulatory subunit, KCNE1 (also named MinK or IsK) or KCNE3 (MiRP2) (6). However, little is known about the regulation and molecular identity of volume-sensitive K$^+$ channels despite their key role in epithelial physiology.

Our results indicate that the 16HBE14o− cells possess a K$^+$ current that, on the basis of electrophysiological and pharmacological profiles, is consistent with the activity of Ca$^{2+}$-dependent maxi K$^+$ channels (Figs. 1 and 3). No indication of the presence of functional Ca$^{2+}$-dependent K$^+$ channels of intermediate conductance [sensitive to clotrimazole (30)], small conductance [sensitive to apamin (33)], or KvLQT1 channels [sensitive to clofilium (9)] was obtained in the 16HBE14o− cells. The presence of maxi K$^+$ channels in 16HBE14o− cells was further confirmed by RT-PCR and Western blot (Fig. 2).

The present and previous (39, 67) studies have established that airway epithelial cells undergo an RVD response following a hypotonic challenge. The RVD response is typically mediated by the loss of cytosolic K$^+$ and Cl$^-$, via the coordinated activation of K$^+$ and Cl$^-$ channels (27, 35). Swelling-activated Cl$^-$ channels are present in most cell types. Their regulation and biophysical properties are well known, but their molecular identity has been particularly difficult to resolve, and no clear candidates exist to date (46, 59, 63, 74).

Different swelling-sensitive K$^+$ channels have been described in several cell preparations (48, 58, 72), although according to Hoffmann and Dunham (27), “there are no K$^+$ channels which are directly activated by swelling, meaning that activation of K$^+$ channels following cell swelling is secondary to the membrane depolarization or the production of intracellular signals.” Exceptions to that statement are the K$^+$ channels directly activated by membrane stretch (2, 53).

Among the numerous K$^+$ channels associated with the mechanisms of cell volume control, only a handful have been identified at the molecular level: 1) voltage-gated K$^+$ channels, including Kv1.3 (17), Kv1.5 (21), and KCNQ/KCNE complex (10, 39); 2) background TREK-1 (49), TRAAK (41), and TASK-2 (45) channels, with TREK-1 and TRAAK being mechanosensitive; and 3) Ca$^{2+}$-dependent K$^+$ channels of large (2) and intermediate conductance (67).

Cell volume regulation following hypotonic or isotonic swelling in epithelial cells is normally associated with changes in intracellular Ca$^{2+}$ concentrations (44, 48). However, the source of Ca$^{2+}$ appears to be different, depending on the original stimulus, i.e., hypotonic cell swelling typically involves extracellular Ca$^{2+}$, whereas isotonic swelling following nutrient absorption involves mobilization of intracellular Ca$^{2+}$ (40), although descriptions of Ca$^{2+}$ release from intracellular stores under hypotonic conditions also exist (29, 76). Our results with HBE cells showing no increase of intracellular Ca$^{2+}$ in Ca$^{2+}$-free hypotonic conditions resembled those reported by Ishii et al. (29) on Intestine 407 epithelial cells. On the contrary, RVD in nonepithelial cells normally shows no Ca$^{2+}$ dependence (3).

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Fig. 8. Regulatory volume decrease (RVD) in HBE cells. A: time course of volume changes after exposure of HBE cells to a 28% hypotonic bath solution (n = 13). Removal of extracellular Ca$^{2+}$ (B; n = 10) or addition of 10 μM Gd$^{3+}$ to the hypotonic solution (C; n = 5) prevented RVD response.

Fig. 9. Effect of K$^+$ channel inhibitors on RVD response in HBE cells. Relative change in HBE cells’ volume before and after replacement of the isotonic solution containing the indicated inhibitor: 5 mM TEA, n = 6 (A); 5 mM Ba$^{2+}$, n = 5 (B); or 100 nM iberiotoxin, n = 4 (C). The blockers were added 3 min before the addition (at t = 0) of the hypotonic solution, which also contained the appropriate inhibitor.
As we discussed earlier, the RVD mechanisms in most cells involve the coordinated activation of Cl− and K+ channels. The swelling-activated Cl− channels are characteristically Ca2+- independent. Exceptions include swelling-sensitive Cl−/H11002 and the subsequent activation of maxi K+ channels, which has only started to emerge (see below), the Ca2+ (26, 50, 55, 67). Among the Ca2+-dependent K+ channels have been characterized in epithelia that also show Ca2+-dependent RVD (26, 50, 55, 67). Among the Ca2+-dependent K+ channels responding to cell swelling, maxi K+ channels have been identified in several epithelia, including proximal tubule of the kidney (20, 32), lacrimal gland (47), and airways (present study) as well as nonepithelial cells (73).

The activation of maxi K+ channels following hypotonic swelling of the 16HBE14o− cells is accompanied by increases in [Ca2+]i, requires extracellular Ca2+(Fig. 6), and is blocked by Gd3+, a blocker of stretch- and/or swelling-activated cation channels (Fig. 7). Similarly, RVD in 16HBE14o− cells is inhibited in the absence of extracellular Ca2+ or in the presence of Gd3+ (Fig. 8). These observations suggest that the mechanisms activated to achieve a complete RVD consist of an increase in Ca2+ entry, most likely via stretch- or swelling-activated cation channels, the nature of which has only started to emerge (see below), and the subsequent activation of maxi K+ channels, a mechanism previously characterized for other cell preparations (13, 28).

Several laboratories have identified a new TRP channel (TRPV4) that is responsive to changes in extracellular osmolarity (38, 60, 71, 75). RT-PCR experiments confirmed the presence of TRPV4 amplicons in HBE cells (Fig. 7E). Therefore, this TRPV4 channel could provide the Ca2+ influx pathway associated with maxi K+ channel activation and RVD response in HBE cells.

A similar series of events appears to underlie the activation of the RVD response in the human tracheal CFT1-LCFSN cells (67), although in this case the Ca2+-dependent K+ channel activated is of intermediate conductance (hIK, also known as KCNN4). The fact that 16HBE14o− cells (present study) and CFT1-LCFSN cells use different Ca2+-dependent K+ channels for the RVD response might be related to their different origin, with the former being bronchial and the later tracheal cells. It would be interesting to show whether native human airway tissue shows similar regional differences. The only study addressing the RVD capability of native airway epithelial cells was carried out on murine tracheal cells (39). That study demonstrated that in murine tracheal cells, the pathway for K+ efflux was via a cloflium-sensitive KCNQ K+ channel.

In summary, the human bronchial epithelial cell line 16HBE14o− shows Ca2+-dependent RVD. The swelling of 16HBE14o− triggers extracellular Ca2+ entry, most likely through stretch- or swelling-activated cation channels, with TRPV4 being a strong candidate for the Ca2+ pathway, and the activation of Ca2+-dependent maxi K+ channels. The identification of the mechanisms involved in cell volume regulation in epithelial cells is relevant to the pathophysiology of cystic fibrosis because it has been shown that RVD is impaired in both cystic fibrosis murine intestine (65, 66) and human airways (67).

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REFERENCES


