Expression of intermediate-conductance, Ca\(^{2+}\)-activated K\(^+\) channel (KCNN4) in H441 human distal airway epithelial cells


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Wilson, S. M., S. G. Brown, N. McTavish, R. P. McNeill, E. M. Husband, S. K. Inglis, R. E. Olver, and M. T. Clunes. Expression of intermediate-conductance, Ca\(^{2+}\)-activated K\(^+\) channel (KCNN4) in H441 human distal airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 291: L957–L965, 2006.—Electrophysiological studies of H441 human distal airway epithelial cells showed that thapsigargin caused a Ca\(^{2+}\)-dependent increase in membrane conductance (G\(_{\text{Na}}\)) and hyperpolarization of membrane potential (V\(_{\text{m}}\)). These effects reflected a rapid rise in cellular K\(^+\) conductance (G\(_{\text{K}}\)) and a slow fall in amiloride-sensitive Na\(^+\) conductance (G\(_{\text{Na},\text{m}}\)). The increase in G\(_{\text{tot}}\) was antagonized by Ba\(^{2+}\), a nonselective K\(^+\) channel blocker, and abolished by clotromazine, a KCNN4 inhibitor, but unaffected by other selective K\(^+\) channel blockers. Moreover, 1-ethyl-2-benzimidazolone (1-EBIO), which is known to activate KCNN4, increased G\(_{\text{K}}\) with no effect on G\(_{\text{Na},\text{m}}\). RT-PCR-based analyses confirmed expression of mRNA encoding KCNN4 and suggested that two related K\(^+\) channels (KCNN1 and KCNNMA1) were absent. Subsequent studies showed that 1-EBIO stimulates Na\(^+\) transport in polarized monolayers without affecting intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), suggesting that the activity of KCNN4 might influence the rate of Na\(^+\) absorption by contributing to G\(_{\text{K}}\). Transient expression of KCNN4 cloned from H441 cells conferred a Ca\(^{2+}\)- and 1-EBIO-sensitive K\(^+\) conductance on Chinese hamster ovary cells, but this channel was inactive when [Ca\(^{2+}\)] was <0.2 μM. Subsequent studies of amiloride-treated H441 cells showed that clotromazine had no effect on V\(_{\text{m}}\) despite clear depolarizations in response to increased extracellular K\(^+\) concentration ([K\(^+\)]\(_{o}\)). These findings thus indicate that KCNN4 does not contribute to V\(_{\text{m}}\) in unstimulated cells. The present data thus establish that H441 cells express KCNN4 and highlight the importance of G\(_{\text{K}}\) to the control of Na\(^+\) absorption, but, because KCNN4 is quiescent in resting cells, this channel cannot contribute to resting G\(_{\text{K}}\) or influence basal Na\(^+\) absorption.

Airway Na\(^+\) transport; cloning; Chinese hamster ovary cell expression; 1-ethyl-2-benzimidazolone

The epithelia that line the airways spontaneously absorb Na\(^+\) from the overlying film of surface liquid, and this process, which is vital to the integrated function of the respiratory tract (see, for example, Refs. 3, 26), occurs via a “leak-pump” mechanism in which Na\(^+\) first crosses the apical membrane by diffusing down the inwardly directed electrochemical gradient and is then extruded from the cell by the basolateral Na\(^+\) pump (37). An important feature of this model is that the transepithelial Na\(^+\) transport rate is restricted by the rate of apical entry, implying that Na\(^+\) absorption can be controlled by agents that regulate apical Na\(^+\) conductance (G\(_{\text{Na}}\)). However, early studies of absorptive tissues also revealed a correlation between the Na\(^+\) transport rate and the magnitude of the basolateral K\(^+\) conductance (G\(_{\text{K}}\)), and it is now clear that changes in apical G\(_{\text{Na}}\) and basolateral G\(_{\text{K}}\) are coordinated to prevent large excursions in membrane potential (V\(_{\text{m}}\)). This interrelationship allows the driving force for Na\(^+\) entry (V\(_{\text{Na}}\)) to be maintained, thus permitting sustained Na\(^+\) absorption, and also implies that Na\(^+\) absorption can be controlled by agents that regulate basolateral G\(_{\text{K}}\) (6, 9, 10). However, despite this importance, the K\(^+\) channels underlying basolateral G\(_{\text{K}}\) in absorptive airway epithelia have not been identified, although 1-ethyl-2-benzimidazolone (1-EBIO), which activates intermediate-conductance Ca\(^{2+}\)-dependent K\(^+\) channels (KCNN4) (9), has been shown to stimulate Na\(^+\) absorption in such tissues. This suggests that these channels, which are widely expressed in epithelial tissues (see, for example, Refs. 5, 7, 14, 28, 34), might be involved in the maintenance of V\(_{\text{Na}}\), and so the aim of the present study was to establish the extent to which KCNN4 activity can influence the rate of Na\(^+\) absorption in a cell line derived from the distal airway epithelium (H441; see, e.g., Refs. 4, 24, 32, 33).

Methods

Cell culture. Standard techniques were used to maintain stocks of H441 cells in RPMI medium supplemented with 8.5% fetal bovine serum (FBS), 8.5% newborn calf serum (NCS), 2 mM glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and an antibiotic-antimycotic mixture (Sigma, Poole, UK; catalog no. A9595). For experiments, cells were removed from culture flasks with trypsin-EDTA, resuspended in standard medium, and plated onto glass coverslips or Costar Snapwell culture membranes (Corning, Schipol-Rijk, The Netherlands) (~100 cells/cm\(^2\)). For experiments in which short-circuit current (I\(_{\text{SC}}\)) and [Ca\(^{2+}\)]\(_{i}\) were measured simultaneously, the Snapwell membranes were cut into small pieces that were glued to Perspex disks with 1-mm holes drilled though them to form small wells into which the cells were seeded (21). Once cellular attachment occurred (2–3 h), the plating medium was replaced with medium identical to that described above except that FBS and NCS were replaced with 8.5% FBS that had been dialyzed to remove hormones and growth factors. Unless otherwise stated, this medium was supplemented with 0.2 μM dexamethasone, a synthetic glucocorticoid known to induce a Na\(^+\)-absorbing phenotype in these cells (4, 30, 32). Stocks of Chinese hamster ovary (CHO) cells were maintained in Ham’s F-12 Glutamax medium (GIBCO, Paisley, UK) containing antibiotics and 10% FBS.
Membrane currents in H441 cells. Membrane currents were recorded (–22°C) from single cells or small groups of H441 cells with the perforated-patch recording technique (Axopatch 200B amplifier, Digidata 1322A data acquisition board; Axon Instruments, Foster City, CA), in which electrical access to the cell interior is gained by including nystatin (0.5 mg/ml) in the pipette filling solution to render the patch of membrane spanning the pipette tip permeable to K⁺, Na⁺, and Cl⁻ (15). This allows experimental control over the internal concentrations of these ions but prevents the loss of higher-molecular-weight substances and allows [Ca²⁺], to be regulated by normal physiological mechanisms. The pipette filling solution contained (in mM) 10 NaCl, 18 KCl, 92 K gluconate, 0.5 MgCl₂, 1 EGTA, and 10 HEPES; pH was adjusted to 7.2 with KOH, which brought K⁺ concentration ([K⁺]) to 113.3 mM. The standard bath solution contained (in mM) 140 NaCl, 4.5 KCl, 1 MgCl₂, 2.5 CaCl₂, 10 HEPES, and 5 glucose; pH was adjusted to 7.4 with NaOH, which brought Na⁺ concentration ([Na⁺]) to 144.4 mM. These solutions were designed to maintain quasi-physiological ionic gradients, and the equilibrium potentials for Na⁺, K⁺, and Cl⁻ (Eₐ, Eₖ, and Eₐ, respectively) were normally +68, –82, and –42 mV, respectively. Modifications to the bath solution are detailed below. The recording pipettes had resistances of 2–5 MΩ, and once seals were obtained the nystatin-induced fall in access resistance (Rₛ) was monitored with the standard features of pCLAMP 9 and experiments were initiated once Rₛ had fallen to a stable value below 35 MΩ. In each experiment Rₛ and input capacitance (Cᵢₛ) were noted and necessary compensations were applied to the recording circuitry. These parameters were monitored carefully, and the development of a large change in Rₛ or Cᵢₛ led to the discontinuation of the experiment; therefore, all data are from preparations in which these parameters were stable. In studies of cells held under voltage clamp, Vₘ was inferred from the reversal potential [i.e., the value of holding potential (Vᵢₛ佬d) at which membrane current was zero], but in some instances Vₘ was measured directly by monitoring the zero-current potential in cells held under current clamp. Cited values of Vᵢₛ佬d and Vₘ have been corrected for liquid junction potentials (1), and values of membrane current and conductance have been normalized to the value of Cᵢₛ associated with a single cell (39 pF). These data are therefore presented as picoampere or nanoampere per cell and picosiemens or nanosiemens per cell, respectively.

Electrophysiological properties of cultured epithelia. Assays of transepithelial ion transport were undertaken (37°C), using confluent H441 cells grown to confluence on Millipore filters (Millipore, Bedford, MA). The epithelial monolayer was then lifted from the filters, transferred to a chamber placed on the stage of an inverted microscope, where the cells were superfused with standard bath solution containing (in mM): 117 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, and 11 n-glucose; pH 7.3–7.5 when bubbled with 5% CO₂ that was continually circulated with gas lifts. Transepithelial resistance (Vₛ) was initially monitored under open-circuit conditions, and once this stabilized (20–30 min), Vₛ was clamped at 0 mV (DVC 1000 Voltage/Current Clamp; World Precision Instruments, Stevenage, UK) and the current needed to hold this potential (Iₛₚ) was monitored and recorded (4 Hz) with a PowerLab interface (AD Instruments, Hastings, UK). Transepithelial resistance (Rₛ) was determined from the expression Rₛ = Vₛ/Iₛₚ. To measure Iₛₚ and [Ca²⁺], simultaneously, confluent cells were loaded with fura-2 by incubation (~40 min, 37°C) in medium containing the acetoxymethyl ester form (3 μM) of this Ca²⁺-sensitive fluorescent dye together with Pluronic F127 (1.8 μM), a nonionic detergent, and probenecid (2.5 mM), an inhibitor of organic cation extrusion systems. Confluent H441 cells take up little dye under standard conditions, and inclusion of these compounds was necessary for adequate dye loading, but we (3a) have shown that these substances have no effect on Rₛ or Vₛ. The fura-2-loaded epithelia were mounted in a miniature Ussing chamber (21, 23) in which the basolateral and apical sides of the cell layer were independently superfused (~3 ml/min) with physiological saline (see above). Solenoid-operated valves allowed these solutions to be independently switched, while thermostatically controlled in-line heaters (Warner Instrument Dual Automatic Temperature Control System) maintained the temperature at 37°C. The chamber was mounted on the stage of a Nikon inverted microscope equipped with extra long-working-distance fluorescence optics (Nikon CFI Plan Fluor ELWD, 0.6 numerical aperture) and a Cairn Research (Faversham, UK) Optoscan UV light source so that the cells could be alternatively illuminated at 340 and 380 nm. The intensity of fluorescence (510 nm) evoked at these voltages (F₃₄₀ and F₃₈₀, respectively) was often used as an indicator of [Ca²⁺]. Most such experiments are undertaken with cells plated onto glass coverslips while confluent cells take up less dye than single cells, and a substantial amount of light is scattered by the culture membrane. The fractions of F₃₄₀ and F₃₈₀ due to background can thus be substantial under these conditions, and, because fura-2 fluorescence declines as the dye is bleached or extruded from the cell (12), the relative contribution of this background will change throughout the experiments. To ensure that this did not confound analysis of the present data, cells were exposed to thapsigargin (1 μM, bilateral) at the end of each experiment. This evokes a substantial rise in [Ca²⁺] (see Ref. 36), and once this response was fully established the cells were exposed to 10 mM MnCl₂. Because the thapsigargin-activated Ca²⁺-influx pathway is Mn²⁺ permeable (see, e.g., Ref. 18), and because Mn²⁺ rapidly quenches fura-2 fluorescence, Mn²⁺ application caused a rapid fall in F₃₄₀ and F₃₈₀ and the Mn²⁺-resistant component of each signal was assumed to indicate the cation-insensitive background fluorescence present throughout the preceding experiment. This allowed background corrections to be made before F₃₄₀/F₃₈₀ was calculated.

Cloning and characterization of KCNN4. To obtain cDNA encoding the human intermediate-conductance Ca²⁺-activated K⁺ channel (KCNN4), RNA was extracted from H441 cells with an SV total RNA isolation Kit (Promega) and reverse transcribed with oligo(dT) primers (Sigma) and avian myeloblastosis virus reverse transcriptase (Sigma). First-strand cDNA was subjected to PCR amplification using primers (5′-GTGCTCAGACGAAATTGTC-3′ and 5′-CTACTTG-GACTGCTGCTGG-3′) designed with the Primer 3 PCR primer design program (Whitehead Institute for Biomedical Research). The 1,416-bp product was cloned into the pCR2.1 plasmid (Invitrogen) and clones were sequenced at the DNA Analysis Facility, Ninewells Hospital and Medical School, University of Dundee. This plasmid was then digested with EcoRI, and a 1,432-bp fragment containing the KCNN4 sequence was subcloned into the same site in pCDNA3 (Invitrogen). To characterize this cloned channel, CHO cells were plated on glass coverslips on six-well plates and, after 24–48 h, Lipofectamine transfection reagent (Invitrogen) was used to cotransfect the cells with pcDNA3-KCNN4 (1–1.5 μg) in conjunction with a second plasmid (pEGFP, 0.1 μg) encoding green fluorescent protein (GFP). After a further 20–48 h, coverslips bearing transfected cells were mounted into a small chamber attached to the stage of an inverted microscope, where the cells were superfused with standard bath solution (composition in mM: 140 NaCl, 1 CaCl₂, 5 KCl, 5 n-glucose, 1 MgCl₂, and 10 HEPES; pH adjusted to 7.4 with NaOH) and viewed with fluorescence optics. Transfected cells were identified by GFP fluorescence, and the properties of the expressed channel were characterized by comparing the electrophysiological properties of KCNN4-transfected cells with those of cells transfected with GFP alone. Such experiments were undertaken with the standard whole cell recording configuration in which the patch of membrane spanning the pipette tip is physically ruptured by gentle suction. This allows the diffusible components of the cytoplasm and pipette filling solution to equilibrate, and [Ca²⁺] was therefore under experimental control in these studies (13). The pipette filling solution used in these experiments contained (in mM) 10 NaCl, 20 KCl, 110 K gluconate, 5 EGTA, and 2 Mg-ATP; pH was adjusted to 7.2 with KOH. Sufficient CaCl₂ (determined with REACT software; see Ref. 8) was added to this solution to raise [Ca²⁺] to 0.05, 0.2, or 0.5 μM. Recorded currents
were normalized to the mean value of $C_m$ associated with single cells (19 pF).

**RESULTS**

Thapsigargin-evoked membrane currents in H441 cells. Initial experiments explored the effects of thapsigargin on the conductive properties of H441 cells by recording membrane currents from groups of two to four voltage-clamped cells (see METHODS). In these experiments $V_{\text{Hold}}$ was normally $-40$ mV, but every 15 s this potential was driven through a “staircase” containing eight distinct voltage steps ($-92, -82, -62, -42, -13, 17, 47, \text{and } 68 \text{ mV})$ of 0.5-s duration. The mean membrane current flowing at each such value of $V_{\text{Hold}}$ was subsequently determined and plotted against time. These data show that acute application of thapsigargin (1 $\mu$M) increased the membrane current flowing at values of $V_{\text{Hold}}$ more positive than approximately $-80$ mV (Fig. 1A), and analysis of data collected once this response was fully developed (2–10 min) showed that $G_{\text{Tot}}$ had increased approximately sixfold (Fig. 1B) while $V_m$ had shifted to a value close to $E_K$ (Fig. 1C). These effects could be sustained for at least 15 min (not shown), but withdrawal of extracellular Ca$^{2+}$ caused $G_{\text{Tot}}$ and $V_m$ to fall to their respective control levels (Fig. 1, A–C) and readmitting extracellular Ca$^{2+}$ restored the stimulated values within 1–2 min (not shown). In subsequent studies of thapsigargin-stimulated cells membrane currents were successively recorded under standard conditions and after [K$^+$]$_o$ had been raised to 31, 57, 84, and 110 mM by isosmotically replacing Na$^+$. Such increases in [K$^+$]$_o$ had no effect on $G_{\text{Tot}}$ but depolarized $V_m$ by 52.5 ± 1.2 mV per tenfold rise in [K$^+$]$_o$, which is essentially identical to the shift predicted by the Nernst equation for a selective K$^+$ conductance (Fig. 1D). Ba$^{2+}$ (5 mM) caused ~75% inhibition of the thapsigargin-evoked increase in $G_{\text{Tot}}$, whereas clotrimazole (1 $\mu$M) essentially abolished the response (Fig. 1E). Chromanol 293B, iberiotoxin, and apamin, which block cAMP-activated K$^+$ channels, large-conductance Ca$^{2+}$-activated K$^+$ channels (KCNM1), and small-conductance Ca$^{2+}$-activated K$^+$ chan-

![Fig. 1. Thapsigargin-evoked membrane currents in H441 cells.](image)

Fig. 1. Thapsigargin-evoked membrane currents in H441 cells. A: cells were continuously superfused with physiological saline and, every 15 s, holding potential ($V_{\text{Hold}}$) was driven through a staircase containing 8 distinct voltage steps ($-92, -82, -62, -42, -13, 17, 47, \text{and } 68 \text{ mV})$. The mean current flowing at each such value of $V_{\text{Hold}}$ was then determined and plotted against time; cells were exposed to thapsigargin (1 $\mu$M) and nominally Ca$^{2+}$-free saline as indicated. $I$: current; $B$: pooled data (n = 9) showing the relationship between $I$ (means ± SE) and $V_{\text{Hold}}$ during superfusion with standard saline and at the peak of the response to thapsigargin; data averaged from 4–6 consecutive pulse protocols. C: means ± SE values of membrane conductance ($G_{\text{Tot}}$) and membrane potential ($V_m$) determined from regression analysis of data collected under control conditions, at the peak of the response to thapsigargin, and after 10- to 15-min exposure to Ca$^{2+}$-free solution. Statistically significant deviations from control (Student’s paired t-test): ***$P < 0.001$, **$P < 0.01$. D: relationship between extracellular K$^+$ concentration ([K$^+$]$_o$) and $V_m$ (inferred from $V_{\text{Na}}$) in thapsigargin-stimulated cells (n = 5, means ± SE); the solid line was fitted to the data by least-squares regression ($R^2 = 0.997$, $P < 0.001$), whereas the dashed line shows the solution to the Nernst equation for a selective K$^+$ conductance. E: cells were stimulated with thapsigargin as shown in A and, once the characteristic rise in $G_{\text{Tot}}$ and hyperpolarization of $V_m$ were fully developed, exposed to putative K$^+$ channel antagonists (Ba$^{2+}$, clotrimazole, cromakalim 293B, iberiotoxin, or apamin). The effects of these compounds of were quantified (% inhibition) by determining the extent to which they blocked the current flowing at 68 mV ($I_{68\text{mV}}$), a potential chosen because it equates to the equilibrium potential for Na$^+$ ($E_{N\text{a}}$), implying that $I_{68\text{mV}}$ will not contain a component carried by Na$^+$. The results of this analysis are shown as means ± SE for values of $n$ indicated beside each column. Statistically significant inhibitory effects (Student’s paired t-test): **$P < 0.005$. 

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nals (KCNN1–KCNN3), respectively, were without effect (Fig. 1E).

Lowering [Na+]o to 10 mM by isosmotically substituting NMDG+ hyperpolarized unstimulated cell Vm by ~35 mV (control ~33 ± 8 mV, NMDG+ ~66 ± 7 mV; n = 4, P < 0.05) but had no effect once the thapsigargin-evoked (1 μM, ~4 min) hyperpolarization (Vm = ~79 ± 2 mV; P < 0.02) was fully developed, suggesting that increased [Ca2+]i might also inhibit GNa. We therefore explored the effects of thapsigargin (1 μM) on the membrane currents that persisted in the presence of sufficient clotrimazole (3 μM) to block the rise in GTot described above. Vm was approximately ~25 mV under these conditions, and, as anticipated (4, 33), amiloride (10 μM) reduced inward current and hyperpolarized Vm (Fig. 2A); analysis of these data showed that GTot and GNa were ~850 and ~300 pS/cell, respectively. Once these measurements were completed, amiloride was washed from the bath and the cells were exposed to thapsigargin (1 μM, 4 min) before the measurements were repeated. Analysis of these data revealed a rise in GTot (unstimulated 853 ± 169, thapsigargin stimulated 1,207 ± 174 pS/cell; P < 0.001), but this was <5% of control (see Fig. 1) and occurred with no change in Vm (Fig. 2B), confirming that the thapsigargin-evoked increase in GTot is essentially abolished by clotrimazole. The physiological basis of this small, clotrimazole-resistant rise in GTot was not investigated further. The effects of amiloride (10 μM) on the thapsigargin-stimulated cells were essentially identical to control, indicating that thapsigargin has no effect on GNa (control 323 ± 173, thapsigargin 410 ± 184 pS/cell) over the timescale of this experiment. Subsequent experiments therefore studied the effects of thapsigargin (1 μM) over a longer time period by measuring GNa at 2- to 4-min intervals over 16 min. These experiments revealed a progressive fall in GTot (control 1,031 ± 335, 16-min thapsigargin 785 ± 137 pS/cell; P < 0.01) that occurred with no change in the amiloride-resistant component of GTot (control 596 ± 289, 16-min thapsigargin 578 ± 44 pS/cell) but could be attributed to a decline in GNa (Fig. 2, C and D).

1-EBIO-evoked membrane currents in H441 cells. 1-EBIO (1 mM) increased GTot and hyperpolarized Vm, although these effects were not as well sustained as the response to thapsigargin because the increase in GTot decayed back toward the starting value in the continued presence of 1-EBIO (Fig. 3). Analysis of data collected at the peak of this response (15–30 s) showed that the current recorded from 1-EBIO-stimulated cells reversed at a potential close to EK (Fig. 3). Parallel studies of clotrimazole-treated (3 μM) cells showed that 1-EBIO had no effect on GNa (control 495 ± 145, 1-EBIO 339 ± 104 pS/cell; n = 7).

Effects of 1-EBIO on polarized H441 cells. Experiments in which ISC and [Ca2+]i were recorded simultaneously from confluent cells showed that 1-EBIO (1 mM, bilateral) evoked a reversible rise in ISC (∆ISC = 4.2 ± 0.8 μA/cm²; n = 6, P < 0.01) but had no effect on [Ca2+]i (Fig. 4). This effect was confirmed in further studies of cells mounted in standard Ussing chambers in which 1-EBIO (1 mM) increased ISC from 36.4 ± 3.6 to 40.9 ± 3.6 μA/cm² (∆ISC = 4.5 ± 0.5 μA/cm²; n = 10, P < 0.001). Parallel studies of age-matched cells at identical passage confirmed that 10 μM amiloride (n = 10), an epithelial Na+ channel antagonist, caused ~95% inhibition of this spontaneous ISC (see also, e.g., Refs. 30, 32) and showed that 1 mM 1-EBIO had no effect on the small ISC that persisted under these conditions (control 2.7 ± 2.2, 1-EBIO 3.5 ± 2.5 μA/cm²; n = 10).

Expression of mRNA encoding Ca2+-activated K+ channels in H441 cells. RT-PCR based analysis of RNA extracted from polarized H441 cells showed that these cells expressed mRNA transcripts encoding KCNN4 but provided no evidence for the expression of mRNA encoding large (KCNNMA1)- or small (KCNN1)-conductance Ca2+-activated K+ channels (Fig. 5).
Functional characteristics of KCNN4. To establish the extent to which the expression of KCNN4 (Fig. 5) could account for the Ca$^{2+}$ (Fig. 2)- and 1-EBIO (Fig. 3)-evoked currents described above, this channel was cloned from H441 cells and its properties were studied by recording membrane currents from CHO cells transfected with this cDNA sequence (see METHODS). In these experiments the background currents attributable to endogenous CHO cell conductances were first characterized in cells expressing GFP because this fluorescent protein served as a marker of transfection in all such experiments. Data recorded with pipette filling solutions designed to hold [Ca$^{2+}$]$_i$ at 0.5 μM (Fig. 6A) or 0.05 μM (not shown) showed that conductance of control cells was so low that only negligible currents were recorded at all test potentials. Moreover, irrespective of [Ca$^{2+}$]$_i$, 1-EBIO (1 mM, 1–2 min) had no effect on the currents recorded from such cells, indicating that this substance does not activate an endogenous conductance. Subsequent studies of KCNN4-expressing cells using a pipette solution designed to maintain [Ca$^{2+}$]$_i$ at 0.5 μM showed that channel expression was associated with large membrane currents that reversed at a potential close to $E_K$ (Fig. 6A). Clotrimazole (10 μM) caused substantial inhibition of this current (Fig. 6B), and analysis of these data showed that KCNN4-transfection conferred ~40 nS/cell of clotrimazole-sensitive conductance on the cells (Fig. 6B). Subsequent experiments using a wider range of putative K$^+$ channel blockers confirmed the inhibitory effect of clotrimazole and showed that Ba$^{2+}$ also caused substantial inhibition of the KCNN4-associated K$^+$ conductance; iberiotoxin and apamin were both ineffective (Fig. 6C). Experiments in which Na$^+$ was isosmotically replaced by K$^+$ showed that increasing [K$^+$]$_o$ depolarized $V_m$ in KCNN4-expressing CHO cells in the manner predicted (Nernst equation) for a selective K$^+$ conductance (Fig. 6D), demonstrating that the current associated with KCNN4 expression is K$^+$ selective.

Only small currents could be recorded from such KCNN4-expressing cells when pipette solutions designed to hold [Ca$^{2+}$]$_i$ at 0.05 or 0.2 μM were used, although 1-EBIO consistently caused clear increases in membrane conductance in these cells (Fig. 7). The currents recorded from 1-EBIO-

Figure 3. 1-ethyl-2-benzimidazolinone (1-EBIO)-evoked membrane currents. A: $I-V$ relationships (means ± SE; n = 6) determined by using the staircase protocol (see Fig. 2) to record membrane currents under control conditions, after 15- to 30-min exposure to 1-EBIO (1 mM), and 2–3 min after this substance had been washed from the bath. B: time course showing the 1-EBIO-evoked change in $V_m$. C: time course showing the 1-EBIO-induced change in $G_{ton}$. 

Figure 4. Effects of 1-EBIO on short-circuit current ($I_{sc}$) and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in polarized H441 cells. Top: $I_{sc}$ recorded from confluent H441 cells under control conditions and during exposure to a pulse of 1-EBIO (1 mM). Bottom: simultaneously measured records of ratio of fura-2 fluorescence at 340 and 380 nm (F$_{340}$/F$_{380}$), which provides an indicator of [Ca$^{2+}$]$_i$. Both records are means ± SE (n = 6).

Figure 5. Expression of mRNA encoding different K$^+$ channel subunits. RT-PCR-based analyses (n = 4) of RNA isolated from confluent, dexamethasone-treated cells were undertaken with primers designed to amplify sequences specific to KCNN1 (small-conductance Ca$^{2+}$-activated K$^+$ channel), KCNMA1 (large-conductance Ca$^{2+}$-activated K$^+$ channel), and KCNN4 (intermediate-conductance Ca$^{2+}$-activated K$^+$ channel). In each such experiment the amplification procedure was verified with primers specific for actin, while omission of the reverse transcriptase step (No RT) and inclusion of a water control ensured that positive results could not be attributed to contamination from genomic DNA or contamination of the reagents, respectively. All products were isolated from the gels and sequenced to verify their origin.
KCNN4-expressing cells showing the relationship between caused a rapid increase in However, in both groups of cells acute application of 1-EBIO increase in outward K current was maintained throughout a 45 s. This rapidly developing response when \([\text{Ca}^{2+}]_o\) was 0.2 \(\mu\text{M}\) (Fig. 7D), these effects were not statistically significant.

Ionic basis of \(V_m\) in H441 cells. Previous studies (4) showed that \(V_m\) in unstimulated H441 cells is influenced by \(G_{\text{Na}}\) and established that cell-to-cell variability in the magnitude of this conductance leads to considerable variability in \(V_m\). The present experiments sought to identify the other membrane conductances that contribute to \(V_m\), and, to ensure that this variability in \(G_{\text{Na}}\) did not confound analysis of these data, the cells used in these experiments were treated with 10 \(\mu\text{M}\) amiloride to block this conductance (4, 33). Analysis of zero-current potentials recorded from cells held under current clamp showed that clotrimazole had no effect on \(V_m\) under these conditions (control 47.5 \(\pm\) 6.7, clotrimazole 48.0 \(\pm\) 6.8 \(\mu\text{M}\)), although subsequently raising \([\text{K}^+]_o\) from 4.7 to 113 \(\mu\text{M}\), the concentration in the pipette filling solution, consistently caused depolarization (control 52.8 \(\pm\) 6.8, high \(K^+\) 25.7 \(\pm\) 4.0 \(\mu\text{M}\); \(P < 0.001\)). Anion substitution experiments showed that lowering \([\text{Cl}^-]_o\) from 152.5 to 29 \(\mu\text{M}\) depolarized \(V_m\) if nominally impermeant \(\text{Cl}^-\) substitutes were used but caused hyperpolarization when \(\text{Cl}^-\) was replaced with \(I^-\) (Table 1). The anionic selectivity of the channels underlying the anion conductance in H441 cells is thus \(I^- < \text{Cl}^- < \text{gluconate} < \text{methanesulfonate}\). Diphenylamine carboxylate (DPC; 1 \(\mu\text{M}\)), a relatively nonselective anion channel blocker, depolarized amiloride-treated cells by \(-6 \mu\text{V}\) (control \(-42 \pm 12.6\), DPC \(-36.2 \pm 12.2\) \(\mu\text{V}\); \(n = 6\), \(P < 0.05\)), whereas glibenclamide stimulated cells were qualitatively similar to those described above and reversed at potentials close to \(E_K\) (Fig. 7, A and B), although 1-EBIO had no statistically significant effect on the spontaneous membrane current seen when \([\text{Ca}^{2+}]_o\) was 0.5 \(\mu\text{M}\) (Fig. 7C). Further experiments explored the effects of 1-EBIO on the membrane current needed to clamp \(V_m\) at \(-40\) \(\mu\text{V}\) (I–40mV): this potential equates to \(E_K\), implying that \(K^+\) is the only ion able to carry outward current. Only very small currents were recorded during the initial period of superfusion with control saline when \([\text{Ca}^{2+}]_o\) was 0.05 or 0.2 \(\mu\text{M}\) (Fig. 7D), confirming that KCNN4 expression has no overt effect on the conductive properties of CHO cells when \([\text{Ca}^{2+}]_o\) is low. However, in both groups of cells acute application of 1-EBIO caused a rapid increase in \(I_{-40mV}\) that became apparent with no discernible latency and reached a peak after \(-45\) s. This increase in outward \(K^+\) current was maintained throughout a 2-min exposure to 1-EBIO, but the response decayed back toward its control value once this drug was withdrawn. Although this drug seemed to evoke a slightly larger and more

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**Fig. 6.** Membrane currents associated with heterologous expression of KCNN4. A: whole cell current records from control [i.e., green fluorescent protein (GFP) transfected] and KCNN4-transfected Chinese hamster ovary (CHO) cells under quasi-physiological ionic conditions. Currents were evoked by stepping (200 ms) \(V_{\text{hold}}\) from 0 mV to a series of values between \(-110\) and 30 mV; pipette \([\text{Ca}^{2+}]_o\) was 0.5 \(\mu\text{M}\), and arrows indicate the zero-current level. B: relationship between \(I\) (means \(\pm\) SE) and \(V_{\text{hold}}\) for KCNN4-expressing cells with the pipette filling solution in which \([\text{Ca}^{2+}]_o\) was buffered to 0.5 \(\mu\text{M}\). Data were first recorded under control conditions and then after the application of 10 \(\mu\text{M}\) clotrimazole (\(n = 6\)). C: extent to which Ba\(^{2+}\), clotrimazole, amin, and iberiotoxin blocked the currents recorded from KCNN4-associated conductance CHO was determined by quantifying the inhibition of the membrane current required to hold \(V_m\) at 30 mV (\(I_{30mV}\)). ***\(P < 0.005\). D: data (\(n = 3\)); mean values, errors lie within symbols) from KCNN4-expressing cells showing the relationship between \(V_m\) and \([\text{K}^+]_o\): the solid line was fitted to the experimental data by linear regression, whereas the dashed line shows the relationship predicted (Nernst equation) for a selective \(K^+\) conductance.

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**Fig. 7.** 1-EBIO-evoked membrane currents in KCNN4-expressing CHO cells. A: relationships between \(V_{\text{hold}}\) and \(I\) derived from studies of KCNN4-transfected cells undertaken with a pipette filling solution designed to hold \([\text{Ca}^{2+}]_o\) at 0.05 \(\mu\text{M}\). Data (means \(\pm\) SE; \(n = 5\)) were obtained during superfusion with control saline and after 1- to 2-min exposure to 1 mM 1-EBIO. B: data from directly analogous experiments (\(n = 6\)) undertaken with a pipette filling solution designed to hold \([\text{Ca}^{2+}]_o\) at 0.2 \(\mu\text{M}\). C: pooled data showing the effects of \([\text{Ca}^{2+}]_o\) and 1-EBIO on \(I_{30mV}\). Statistically significant effects of 1-EBIO (Student’s paired \(t\)-test): ***\(P < 0.005\). D: continuous records (means \(\pm\) SE) showing the effects of 1-EBIO on the current required to hold \(V_m\) at \(-40 \mu\text{V}\) (\(I_{-40mV}\)) with pipette filling solutions designed to hold \([\text{Ca}^{2+}]_o\) at 0.2 (\(n = 4\)) or 0.05 (\(n = 5\)) \(\mu\text{M}\). This potential was chosen as it equates to the equilibrium potential for \(\text{Cl}^-\) (\(E_{\text{Cl}}\)), implying that \(K^+\) is the only ion able to carry outward current under the present conditions.
Table 1. Effects of lowering \([\text{Cl}^-]_{o}\) on \(V_m\)

<table>
<thead>
<tr>
<th>Replacement Anion</th>
<th>(n)</th>
<th>Control</th>
<th>Low (\text{Cl}^-)</th>
<th>(\Delta V_m), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconate</td>
<td>17</td>
<td>40.6±3.4</td>
<td>30.9±4.8‡</td>
<td>9.7±2.5</td>
</tr>
<tr>
<td>Methanesulfonate</td>
<td>7</td>
<td>53.3±5.5</td>
<td>37.5±4.5*</td>
<td>15.8±5.2</td>
</tr>
<tr>
<td>I</td>
<td>7</td>
<td>33.5±6.9</td>
<td>44.1±9.1†</td>
<td>−10.6±2.8</td>
</tr>
</tbody>
</table>

Data are means ± SE for \(n\) cells in each group. Values of membrane potential \((V_m)\) measured under zero current clamp from amiloride-treated (10 \(\mu\)M) cells superfused successively with control saline \([\text{Cl}^-]_{o}\) concentration \((\langle [\text{Cl}^-]_{o}\rangle) = 151.5\,\text{nM}\) and with saline in which extracellular \([\text{Cl}^-]_{o}\) had been lowered to 29 nM by replacing this anion with gluconate, methanesulfonate, or I. Shifts in \(V_m\) \((\Delta V_m)\) were quantified by subtracting the value measured at low \([\text{Cl}^-]_{o}\) from that measured in the same cells under control conditions. Significant effects of low \([\text{Cl}^-]_{o}\) (Student’s paired \(t\)-test): *\(P < 0.05\), ‡\(P < 0.005\), †\(P < 0.002\).

(100 \(\mu\)M), another anion channel blocker, had no effect (control −48.7 ± 4.0, glibenclamide −48.4 ± 4.0 mV; \(n = 6\)).

DISCUSSION

**Thapsigargin-evoked rise in \(G_{\text{Tot}}\).** Because KCNN4 can be regulated via \([\text{Ca}^{2+}]_{i}\) (see, e.g., Refs. 2, 28) we first explored the possibility that this \(K^+\) channel might be present in H441 cells by studying the effects of thapsigargin, which characteristically causes a large and sustained rise in \([\text{Ca}^{2+}]_{i}\) (see, e.g., Ref. 36) on the membrane currents recorded from single cells or small groups of cells. These experiments revealed a \([\text{Ca}^{2+}]_{i}\)-dependent increase in \(G_{\text{Tot}}\) accompanied by a hyperpolarization of \(V_m\), and once these effects were established increasing \([\text{Ca}^{2+}]_{i}\) was inhibited by including nucleotides in the pipette filling solution (5, 28). Moreover, our studies of polarized cells also show that the \(1\text{-EBIO}\)-induced increase in \(G_{\text{Tot}}\) reflects KCNN4 activation.

**Effects of \(1\text{-EBIO}\) on \(G_{\text{Na}}\).** Whereas replacing \([\text{Na}^+]_{o}\) with a nominally impermeant cation \((\text{NMDG}^-)\) hyperpolarized unstimulated cells (see also Refs. 4, 33), this maneuver had no such effect after −4-min exposure to thapsigargin, indicating that the conductive properties of thapsigargin-stimulated cells are dominated by a \(K^+\) conductance. This contrasts with the situation in unstimulated cells, where \(V_m\) is clearly influenced by \(G_{\text{Na}}\) (4, 33), and subsequent experiments therefore explored the effects of thapsigargin on \(G_{\text{Na}}\) by characterizing the membrane current that persisted when KCNN4 was blocked with clotrimazole. These studies revealed a substantial (~50%) but slowly developing fall in \(G_{\text{Na}}\). Spontaneous rundown of ENaC has been documented in experiments in which \(G_{\text{Na}}\) was monitored by the standard whole cell recording technique, but this was inhibited by including nucleotides in the pipette filling solution (16) and our previously published data (4) show that, when the perforated-patch recording technique is used, \(G_{\text{Na}}\) is stable over the time course of this experiment. The present data thus indicate that, as well as increasing \(G_{\text{K}}\), thapsigargin causes a slowly developing fall in \(G_{\text{Na}}\), and this is consistent with data from several different systems, including lung and airway epithelia, which show that increases in \([\text{Ca}^{2+}]_{i}\) inhibit epithelial \(\text{Na}^+\) channels (ENaC) (17) and reduce \(\text{Na}^+\) absorption (11, 25, 27, 29, 38). The slow onset of this response was surprising because the hyperpolarizing response to \(\text{NMDG}^-\)-containing saline was lost after only ~4 min. However, the present data show that \(G_{\text{Tot}}\) rises rapidly in thapsigargin-stimulated cells, and once this response is established, \(G_{\text{K}}\) would be 20- to 25-fold greater than \(G_{\text{Na}}\). Examination of this problem with the Goldman-Hodgkin-Katz Equation predicts that, even before a fall in \(G_{\text{Na}}\) had occurred, exposure to the \(\text{NMDG}^-\)-containing solution would hyperpolarize \(V_m\) by only ~4 mV. Such a small response may not have been detected under the present conditions.

**Effects of \(1\text{-EBIO}\).** \(1\text{-EBIO}\) mimicked the effects of thapsigargin on \(G_{\text{Tot}}\) and \(V_m\), and analysis of data collected at the peak of this response showed that the \(1\text{-EBIO}\)- and thapsigargin-evoked membrane currents were qualitatively similar. Because \(1\text{-EBIO}\) is known to activate KCNN4 (28), this result provides further evidence for the expression of these \(K^+\) channels in H441 cells. The response to \(1\text{-EBIO}\) was less well sustained than the response to thapsigargin, and, although a clear fall in \(G_{\text{Tot}}\) occurred in the presence of this drug, \(V_m\) was still hyperpolarized by 10–15 mV after 3- to 4-min exposure to \(1\text{-EBIO}\). \(1\text{-EBIO}\) had no effect on \(G_{\text{Na}}\), because this parameter remained at ~300 pS/cell throughout the exposure to this drug, and these findings therefore predict that the \(1\text{-EBIO}\)-induced hyperpolarization would potentiate cellular \(\text{Na}^+\) current by −4 to −6 pA/cell [because \(I_{\text{Na}} + \Delta I_{\text{Na}} = (G_{\text{Na}} + \Delta G_{\text{Na}})(V_m + \Delta V_{\text{Na}})\). The confluent cultures used in the present study contained ~10⁶ cells/cm², and so this implies a 4 to 6 μA/cm² stimulation of \(I_{\text{SC}}\), which almost exactly matches the response observed in our studies of polarized cells. The effects of \(1\text{-EBIO}\) on the conductive properties of single cells can thus explain the stimulation of \(\text{Na}^+\) transport seen in cultured epithelia, and these data highlight the central importance of \(G_{\text{K}}\) to the control of epithelial \(\text{Na}^+\) absorption (6, 9, 10). Moreover, our studies of polarized cells also show that the \(1\text{-EBIO}\)-induced increase in \(I_{\text{SC}}\) occurs with no change in \([\text{Ca}^{2+}]_{i}\), and this is consistent with the view that \(1\text{-EBIO}\) activates KCNN4 by sensitizing these channels to \(\text{Ca}^{2+}\) (28).

**Role of KCNN4 in unstimulated cells.** Analysis of RNA extracted from H441 cells revealed mRNA transcripts encoding KCNN4 but indicated that two related \(K^+\) channels, one of which (KCNN1) is also activated by \(1\text{-EBIO}\), were not present. In subsequent experiments KCNN4 was therefore cloned from H441 cells and the corresponding cDNA sequence was expressed in CHO cells so that we could directly explore the physiological features of this gene product. Heterologous expression of KCNN4 was associated with a large and highly selective \(K^+\) conductance when \([\text{Ca}^{2+}]_{i}\) was 0.5 μM but had no overt effect on the conductive properties of CHO cells when \([\text{Ca}^{2+}]_{i}\) was 0.05 or 0.2 μM. However, \(1\text{-EBIO}\) consistently caused a rapid increase in \(G_{\text{K}}\) under these conditions, although this substance had little further effect on the large currents recorded when \([\text{Ca}^{2+}]_{i}\) was 0.5 μM. These findings are therefore consistent with the observation that \(1\text{-EBIO}\) sensitizes KCNN4 to \([\text{Ca}^{2+}]_{i}\) (28) and thus confirm that KCNN4 expression confers a \(\text{Ca}^{2+}\)- and \(1\text{-EBIO}\)-sensitive \(K^+\) current on the plasma membrane.
The responses to thapsigargin and 1-EBIO seen in H441 cells can thus be attributed to activation of KCNN4, but the fact that expression of this K\(^+\) channel gene had no effect on the conductive properties of the membrane unless [Ca\(^{2+}\)] was >0.2 \(\mu\)M suggests that KCNN4 is inactive at the values of [Ca\(^{2+}\)], typically found in resting cells. This accords with the data from experiments in which KCNN4 was expressed in oocytes (35), although studies using an alternative mammalian expression system (HEK 293 cells) indicate that KCNN4 displays significant activity when [Ca\(^{2+}\)] is \(\sim 0.1 \mu\)M (28). The discrepancy between these studies may reflect the fact that Ca\(^{2+}\) controls KCNN4 by activating calmodulin rather than by directly regulating channel gating, and the activity of this signaling pathway can be modulated via other signaling pathways such as calmodulin kinase (20). It is therefore possible that there may be differences between the concentrations of [Ca\(^{2+}\)] needed to activate KCNN4 in different cell types. We therefore undertook further studies of H441 cells that showed that increasing [K\(^+\)], consistently depolarized cells that had been treated with amiloride, establishing that G\(K\) is significant under these conditions. However, these experiments also showed that a concentration of clotrimazole sufficient to block KCNN4 had no effect on V\(_m\), and this finding, in common with our data from the cloned channel, suggests that KCNN4 does not contribute to G\(K\) in unstimulated cells.

The K\(^+\)-rich bath solution used in the present study was designed to shift \(E_K\) to 0 mV, but the experiments that explored the effects of this solution on amiloride-treated cells showed that V\(_m\) never reached this potential, and so at least one other ionic conductance must contribute to V\(_m\) under these conditions. Subsequent experiments showed that lowering [Cl\(^-\)], and thus shifting \(E_{Cl}\) to 0 mV, caused depolarization when nominally impermeant Cl\(^-\) substituents were used, and this shows that V\(_m\) is also influenced by the cellular Cl\(^-\) conductance (G\(Cl\)). Although the channels underlying G\(Cl\) are still to be identified, the fact that the \(\Gamma\)-rich solution caused hyperpolarization establishes that these channels are more permeable to \(\Gamma\) than to Cl\(^-\) [i.e., permeability (P\(_\Gamma\)) > P\(_{Cl}\)], and channels with this property have been described in rat cortical lens fiber cells (40). At least one earlier study has suggested that H441 cells express the Ca\(^{2+}\)-regulated anion channels (CFTR) encoded by the gene that is mutated in cystic fibrosis (22), but these channels characteristically display a lower permeability to \(\Gamma\) than to Cl\(^-\) (i.e., P\(_\Gamma\) > P\(_{Cl}\)) (see, e.g., Ref. 19), which does not accord with the present data. It thus appears that H441 cells do not express functional CFTR under the present experimental conditions, and further evidence of this came from the fact that DPC, a nonselective Cl\(^-\) channel blocker, caused depolarization whereas glibenclamide, which displays some selectivity for CFTR, did not (see, e.g., Ref. 19).

H441 cells have now been used in several recent studies of human airway epithelial Na\(^+\) transport (4, 24, 30–33), and the present data show clearly that these cells express KCNN4 and establish that the presence of this K\(^+\) channel can account for the thapsigargin- and 1-EBIO-evoked increases in G\(Tot\) seen in single cells and the 1-EBIO-evoked stimulation Na\(^+\) transport seen in cultured epithelia (see also Ref. 9). These findings highlight the importance of G\(_K\) to the control of Na\(^+\) absorption (6, 9, 10) and show that physiological activators of KCNN4 might contribute to the neurohormonal modulation of this physiologically important ion transport process (see also Ref. 9). However, our data also show that KCNN4 is inactive at resting [Ca\(^{2+}\)], and this is reminiscent of the situation described in the erythrocyte and colon, where KCNN4 expression confers a “latent” conductance on the membrane that can be activated by increases in [Ca\(^{2+}\)], or perturbations in cell volume (2, 39). In H441 cells, KCNN4 could well play an important role in mediating absorptive responses to hormones/neurotransmitters or in the control of cell volume during active ion transport. However, because this channel does not influence V\(_m\) in resting cells, KCNN4 cannot be part of the mechanism underlying spontaneous Na\(^+\) absorption. Indeed, our data show that V\(_Na\) is dependent on unidentified K\(^+\) and Cl\(^-\) channels and, given the importance of airway Na\(^+\) transport to lung function (see, for example, Refs. 3, 26), it is now important to identify the channels underlying these physiologically relevant conductances.

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