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A Ba²⁺-resistant, acid-sensitive K⁺ conductance in Na⁺-absorbing H441 human airway epithelial cells

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Abstract

By analysis of whole cell membrane currents in Na⁺-absorbing H441 human airway epithelial cells, we have identified a K⁺ conductance (G_K) resistant to Ba²⁺ but sensitive to bupivacaine or extracellular acidification. In polarized H441 monolayers, we have demonstrated that bupivacaine, lidocaine, and quinidine inhibit basolateral membrane K⁺ current (I_{BL}) whereas Ba²⁺ has only a weak inhibitory effect. I_{BL} was also inhibited by basolateral acidification, and, although subsequent addition of bupivacaine caused a further fall in I_{BL} , acidification had no effect after bupivacaine, demonstrating that cells grown under these conditions express at least two different bupivacaine-sensitive K⁺ channels, only one of which is acid sensitive. Basolateral acidification also inhibited short-circuit current (I_{SC}), and basolateral bupivacaine, lidocaine, quinidine, and Ba²⁺ inhibited I_{SC} at concentrations similar to those needed to inhibit I_{BL} , suggesting that the K⁺ channels underlying I_{BL} are part of the absorptive mechanism. Analyses using RT-PCR showed that mRNA encoding several two-pore domain K⁺ (K2P) channels was detected in cells grown under standard conditions (TWIK-1, TREK-1, TASK-2, TWIK-2, KCNK-7, TASK-3, TREK-2, THIK-1, and TALK-2). We therefore suggest that K2P channels underlie G_K in unstimulated cells and so maintain the driving force for Na⁺ absorption. Since this ion transport process is vital to lung function, K2P channels thus play an important but previously undocumented role in pulmonary physiology.

Keywords

airway sodium channel transport; patch clamping; Ussing chamber

The integrated functioning of the respiratory tract is dependent on the controlled absorption of Na⁺ from the liquid film that covers the lung/airway epithelia (see, e.g., Refs. 3 and 25), and this absorptive process occurs by a “leak-pump” mechanism (38). An important feature of this model is that the overall rate of Na⁺ transport is restricted by the rate of apical entry, and since this step is passive, this influx rate is determined by the product of the apical Na⁺ conductance (G_{Na}) and the electrochemical driving force for Na⁺ entry (Φ_{Na} , i.e., the difference between membrane potential, V_m , and the Na⁺ equilibrium potential, E_{Na}). It is now clear that G_{Na} is normally restricted by the continual removal of epithelial Na⁺ channels (ENaC) from the apical membrane and that many of the hormones/neurotransmitters that control Na⁺ absorption do so by activating signaling pathways that regulate this ENaC internalization mechanism (see, e.g., Refs. 10 and 36). However, classical studies of absorptive tissues identified the basolateral K⁺ conductance (G_K) as another important control point. Early studies of absorptive epithelia,

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such as frog skin, turtle colon, and toad bladder (see, e.g., Refs. 9,13, and 15), thus revealed a correlation between the rate of Na^+ transport and the amplitude of the basolateral G_K , and it is now clear that this conductance normally stabilizes the membrane potential, and this allows Φ_{Na} , and hence the rate of Na^+ absorption, to be maintained. Moreover, agents that increase basolateral G_K can stimulate Na^+ transport by hyperpolarizing V_m and so increasing Φ_{Na} (9, 12,13).

The K^+ channels that underlie Na^+ absorption in lung/airway epithelia have yet to be identified (see Ref. 41), and the aim of the present study was therefore to characterize G_K in such cells. We have chosen as our experimental model the H441 cell line derived from the human distal airway, which, when treated with glucocorticoids, absorbs Na^+ from the apical solution by a process dependent on ENaC. Several groups have used these cells as an experimental system to explore the molecular basis of this physiologically important absorptive process (6,22, 31-33,35), but, so far, the K^+ channels that underlie this absorptive process have not been identified.

METHODS

Cell culture

Standard techniques were used to maintain stocks of H441 cells in serial culture; the medium used was as follows: RPMI supplemented with 8.5% fetal bovine serum (FBS); 8.5% newborn calf serum (NCS); 2 mM glutamine; 5 $\mu\text{g}/\text{ml}$ insulin; 5 $\mu\text{g}/\text{ml}$ transferrin; 5 ng/ml selenium; and an antibiotic-antimycotic mixture (Sigma Chemical, Poole, Dorset, United Kingdom). For experiments, cells removed from culture flasks using trypsin-EDTA were plated ($\sim 10^6$ cells/ cm^2) onto glass coverslips or Costar Snapwell membranes (Corning BV, Schiphol-Rijk, The Netherlands). These cells were maintained in medium identical to that described above except that FBS and NCS were replaced by FBS (8.5%) that had been dialyzed to remove hormones/growth factors. This medium was supplemented with 0.2 μM dexamethasone, a synthetic glucocorticoid known to induce a Na^+ -absorbing phenotype in these cells (6,31,33).

Membrane currents in single cells/small groups of cells

Membrane currents (I_m) were recorded (Axopatch 200B amplifier and Digidata 1322A data acquisition board, Axon Instruments, Foster City, CA) from single cells or groups of 2–6 cells ($\sim 22^\circ\text{C}$) using the perforated patch recording technique 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^- (16). The input capacitance (C_m) of each preparation, which provides an indicator of membrane area, was noted carefully, and all cited values of I_m have been normalized to the average value of C_m associated with a single cell (~ 35 pF). Such data are therefore presented as picoamperes per average-sized cell (pA/cell). The pipette filling solution always contained in mM 10 NaCl, 18 KCl, 92 potassium gluconate, 0.5 MgCl_2 , 1 5,5 ethylene glycol-bis-(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), and 10 HEPES, and its pH was adjusted to 7.2 with KOH, which brought $[\text{K}^+]$ to 113.3 mM. The standard bath solution contained in mM 140 NaCl, 4.5 KCl, 1 MgCl_2 , 2.5 CaCl_2 , 10 HEPES, and 5 glucose, and its pH was adjusted to 7.4 with NaOH, which brought $[\text{Na}^+]$ to 144.4 mM. Under these quasi-physiological ionic conditions, the equilibrium potentials for Na^+ , K^+ , and Cl^- (E_{Na} , E_K , and E_{Cl} , respectively) were +68 mV, -82 mV, and -42 mV, respectively. Modifications to these standard recording conditions are detailed in the text. All cited voltages have been corrected for the liquid junction potential between the bath and pipette filling solution (see Ref. 1), and since the bath was always grounded using a Ag/AgCl₂ pellet connected to the recording chamber via a salt bridge filled with 3 M KCl/4% agar, the solution changes imposed in the present study had negligible (<1 mV) effects on this potential. Currents were evoked by imposing ramp changes in holding

potential (V_{Hold}) on cells held under voltage clamp (see Ref. 6), and, for analysis, plots showing the relationship between I_m and V_{Hold} were constructed using spreadsheet software (Microsoft Excel). V_m was inferred from the value of V_{Hold} at which I_m is zero, whereas values of membrane conductance (G_m) were derived by linear regression (i.e., $\Delta V_m/\Delta V_{\text{Hold}}$) of data collected at negative values of V_{Hold} . All data are shown as mean $\pm$ SE, and the cited values of n refer to the number of cells in each group. All reported phenomena were observed in cells at least three different passage numbers.

Electrometric studies of polarized cells

Snapwell membranes bearing confluent H441 cells were mounted in Ussing chambers and initially bathed (37°C) with bicarbonate-buffered physiological salt solution (containing in mM 112 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 11.6 D-glucose, pH 7.3–7.5 when bubbled with 5% CO₂) while transepithelial potential difference (V_t) was monitored (DVC-1000 voltage/current clamp; World Precision Instruments, Stevenage, Hertfordshire, United Kingdom). Once this parameter had stabilized, V_t was clamped to 0 mV (DVC-100 voltage/current clamp), and the current needed to maintain this potential (short-circuit current, I_{SC}) was recorded directly to computer disk (4 Hz, PowerLab interface; AD Instruments, Hastings, East Sussex, United Kingdom). In all such studies, positive current was defined as that current carried by cations moving from the apical to the basolateral bath.

To study the conductive properties of the basolateral membrane, the cultured epithelia were exposed to a basolaterally directed [K⁺] gradient that was imposed under open circuit conditions by replacing the basolateral solution with a Na⁺-rich solution containing gluconate as the principal anion (composition in mM: 82 sodium gluconate, 30 NaCl, 4.7 potassium gluconate, 25 NaHCO₃, 11 calcium gluconate, 1.2 MgSO₄, 1.2 KH₂PO₄) while the apical saline was replaced with a similar solution that contained K⁺ as the principal cation (composition in mM: 6.7 sodium gluconate, 80 potassium gluconate, 30 KCl, 25 KHCO₃, 11 calcium gluconate, 1.2 MgSO₄, 1.2 KH₂PO₄). The concentration of Cl[−] in the apical and basolateral baths was thus 30 mM. The apical membrane was then permeabilized by adding 200 μ M amphotericin B to the solution bathing this side of the cultured epithelium (4), and the conductive properties of the intact, basolateral membrane were then studied by recording the current flow across the permeabilized epithelial layer while V_t was held at 0 mV (basolateral membrane current, I_{BL}). At intervals throughout all experiments, the cells were briefly returned to open circuit conditions so that V_t could be measured to allow transepithelial resistance (R_t) to be measured. In some experiments, the pH of the basolateral saline was monitored using a pH electrode connected to the PowerLab interface via an AD Instruments pH Pod. All data are means $\pm$ SE, and values of n refer to the number of times a protocol was repeated using cells at different passage number.

Isolation/analysis of RNA

Total RNA was isolated (SV Total RNA Isolation Kit, Promega) from cells cultured in standard flasks or on Costar Transwell membranes. The extracted RNAs were subjected to RT-PCR analysis using gene-specific primers for most known tandem pore domain K⁺ (K2P) channels (Table 1) or a GAPDH control. PCR was carried out using aliquots of cDNA corresponding to 1 ng (GAPDH), 5 ng (TWIK-1, TASK-2, and TWIK-2), 10 ng (TREK-2, KCNK-7), or 25 ng of RNA (all others) using GoTaq Taq polymerase (Promega). All PCR reactions were allowed to proceed for 36 denaturing (95°C, 2 min)/annealing (60°C, 30 s)/extension (68°C, 60 s) cycles, and the resultant products were fractionated by agarose gel electrophoresis and visualized by staining with ethidium bromide. The identity of all products was confirmed by sequencing.

RESULTS

Membrane currents in single cells/small groups of cells

Experiments in which membrane currents were recorded ($n = 6$) from cells bathed with the standard physiological salt solution showed that V_m was normally -27 ± 1.7 mV, and, as anticipated by earlier work (6), lowering extracellular Na^+ concentration ($[\text{Na}^+]_o$) to 10 mM by iso-osmotically replacing this ion with *N*-methyl-D-glucammonium (NMDG^+) inhibited the inward currents flowing at negative values of V_{Hold} and hyperpolarized V_m to -60 ± 2.8 mV ($P < 0.001$). This confirms that G_{Na} is significant in H441 cells cultured under the present conditions (i.e., dexamethasone treated), and our (6) earlier studies revealed substantial cell-to-cell variations in the magnitude of this conductance, implying that an inward Na^+ current of unpredictable magnitude will flow at negative values of V_{Hold} . To ensure that this did not confound analysis of the present data, the Na^+ content of the bath solution used in all subsequent experiments was reduced to 10 mM Na^+ by iso-osmotically replacing this cation with NMDG^+ or K^+ . The use of such solutions ensured that the membrane Na^+ currents would be $< \sim 5$ pA/cell at physiologically relevant values of V_{Hold} (see Ref. 6) and allowed us to modify the cationic composition of the bath while E_{Na} remained at ~ 0 mV, which ensured that the magnitude and polarity of any Na^+ current will remain constant throughout each experiment.

Figure 1 shows data recorded under these conditions. Initially, the cells were bathed with the NMDG^+ -rich salt solution containing 5 mM K^+ ; V_m was -60.4 ± 2.8 mV under these conditions, essentially identical to the value reported above. Figure 1 also shows currents subsequently recorded from the same cells after the external solution had been replaced with the K^+ -rich (134.5 mM) bath solution. This increase in extracellular K concentration ($[\text{K}^+]_o$) augmented the inward currents flowing at negative potentials and depolarized ($P < 0.001$) V_m to -33.5 ± 7.6 mV, confirming (41) that these cells express a conductance that allows a depolarizing K^+ current to flow as $[\text{K}^+]_o$ is raised. However, although the K^+ -rich saline used in these experiments was designed to shift E_K to ~ 0 mV, analysis of the pooled data from all experiments in which membrane currents were recorded from cells bathed with this solution ($n = 28$) showed that V_m was -25.8 ± 2.2 mV under these conditions. This potential is more negative than E_K ($P < 0.001$) and, since E_{Na} was always 0 mV (see METHODS), this discrepancy between V_m and E_K implies that the cellular Cl^- conductance (G_{Cl}) must also be large enough to influence V_m (see also Ref. 41).

Subsequent experiments ($n = 4$) showed that Ba^{2+} (5 mM), a divalent cation that blocks many different types of K^+ channel, had no discernible effect on the membrane currents recorded from cells bathed with the K^+ -rich saline, and analysis of these data confirmed that Ba^{2+} also caused no significant shift in membrane potential (control, -26.1 ± 2.4 mV; Ba^{2+} , -30.0 ± 2.7 mV). In contrast, bupivacaine (3 mM) reduced the magnitude of the currents flowing at positive and negative values of V_{Hold} (Fig. 2A) and hyperpolarized V_m to a value close to E_{Cl} (control, -22.9 ± 1.6 mV; bupivacaine, -50.8 ± 3.9 mV; $P < 0.001$). These effects were fully reversible (data not shown). Further analysis of these data showed that bupivacaine reduced G_m from 0.89 ± 0.12 to 0.38 ± 0.35 pS/cell ($P < 0.01$), demonstrating that $\sim 50\%$ of G_m can be attributed to the expression of ion channels that are sensitive to bupivacaine but not Ba^{2+} . Moreover, further analysis of these data showed that the bupivacaine-sensitive component of the membrane current (I_{Bupiv}) displayed slight inward rectification and reversed at a potential essentially identical to E_K (~ 0 mV; Fig. 2B). Interestingly, the value of reversal potential (V_{Rev}) associated with the bupivacaine-resistant component of I_m lay close to E_{Cl} , suggesting that the bupivacaine-sensitive and bupivacaine-resistant currents are carried by K^+ and Cl^- , respectively.

To test the hypothesis that I_{Bupiv} was a K^+ current, the ionic composition of the K^+ -rich bath solution was further modified by lowering extracellular Cl^- concentration ($[\text{Cl}^-]_o$) to 26.5 mM

(gluconate substitution). Analysis of currents recorded under these conditions (Fig. 2C) showed that V_m was less negative (-7.1 ± 3.8 mV; $P < 0.001$) than at 151.5 mM Cl^- (~ 25 mV, see above). Reducing $[\text{Cl}^-]_o$ in this way thus depolarized the cells, which confirms (21,41) that G_{Cl} is significant, although V_m did not fully depolarize to E_{Cl} , suggesting that the anion channels underlying this conductance have a significant permeability to gluconate. Application of 3 mM bupivacaine caused a fall in G_m (control, 0.808 ± 0.253 nS/cell; bupivacaine, 0.529 ± 0.189 nS/cell; $P < 0.05$) essentially identical to that seen in Cl^- -rich saline. Interestingly, bupivacaine still hyperpolarized V_m ($\Delta V_m = 13.3 \pm 4.3$ mV; $P < 0.002$) when $[\text{Cl}^-]_o$ was low, and, although this response was smaller ($P < 0.05$) than that seen at 151.1 mM $[\text{Cl}^-]_o$ (Fig. 2A; $\Delta V_m = 22.9 \pm 1.6$ mV), this hyperpolarization provides further evidence that gluconate can carry inward current under these conditions. However, the most important result to emerge from these experiments was that this reduction in $[\text{Cl}^-]_o$ had no effect on I_{Bupiv} (Fig. 2D), establishing that this current is independent of Cl^- . Subsequent experiments explored the effects of further modifying the ionic composition of the low Cl^- saline by reducing $[\text{K}^+]_o$ to 20 mM (NMDG⁺ substitution) to selectively shift E_K to -43 mV. Analysis of these data showed that this reduction in $[\text{K}^+]_o$ shifted the value of V_{Rev} associated with I_{Bupiv} in a manner that almost perfectly matched the hyperpolarization of E_K . This finding thus demonstrates that I_{Bupiv} is a K^+ -selective current.

Effects of bath pH on membrane currents in single cells/small groups of cells

Figure 3A shows data from cells bathed with the K^+ - and Cl^- -rich saline, which establish that reducing bath pH from 7.4 to 6.4 also inhibits the membrane currents recorded under these conditions. Analysis of these data indicated that this acidification caused a $47.4 \pm 10.5\%$ reduction ($P < 0.05$) in G_m that was associated with a hyperpolarization of V_m (control, -23 ± 3.1 mV; pH 6.4, -51 ± 7.7 mV; $P < 0.05$). These effects were fully reversible (data not shown). Figure 3B shows that the acid-sensitive component of the total membrane current (I_{Acid}) reversed at a potential essentially identical to E_K and was qualitatively and quantitatively similar to I_{Bupiv} (Fig. 2, B and D). Further experiments therefore explored the possibility that bupivacaine and bath acidification may act on the same population of ion channels. The first such studies ($n = 4$) confirmed that bath acidification caused a fall in G_m (control, 1.53 ± 0.14 nS/cell; pH 6.4, 1.06 ± 0.16 nS/cell; $P < 0.02$) and a hyperpolarization of V_m (control, -27.0 ± 1.6 mV; pH 6.4, -54.0 ± 6.7 mV; $P < 0.05$) but established that subsequent addition of bupivacaine (3 mM) at pH 6.4 had no further effect on these parameters (G_m , 0.843 ± 0.137 nS/cell; V_m , -50.3 ± 4.2). The effects of bupivacaine were similarly confirmed in separate experiments ($n = 3$), which established that reducing bath pH to 6.4 in the continued presence of this drug had no further effect on the either G_m or V_m . Further analysis of these data showed that reducing bath pH to 6.4 essentially abolished ($\sim 90\%$ inhibition) I_{Bupiv} (Fig. 3C) while the application of 3 mM bupivacaine essentially abolished I_{Acid} (Fig. 3D). Bupivacaine and extracellular acidification thus appear to reduce G_m by acting on the same populations of K^+ -selective ion channels.

Studies of polarized cells

Cells grown to confluence ($R_t = 281 \pm 11 \Omega\text{cm}^2$, $n = 94$) on permeable culture membranes generated a V_t of -12 ± 0.6 mV when bathed symmetrically with standard physiological saline. Exposing these cultured epithelia to a basolaterally directed $[\text{K}^+]$ gradient (see METHODS) increased R_t to $576 \pm 22 \Omega\text{cm}^2$ ($P < 0.05$) and depolarized V_t to -8.4 ± 0.2 mV ($P < 0.05$) while subsequently permeabilizing the apical membrane so that the conductive properties of the intact, basolateral membrane could be studied (4), hyperpolarized V_t to -12 ± 0.3 mV ($P < 0.05$), and reduced R_t to $493 \pm 20 \Omega\text{cm}^2$ ($P < 0.05$). Measurements made while V_t was held at 0 mV showed that the mean current flowing under these conditions (I_{BI}) was $26 \pm 1.0 \mu\text{A}/\text{cm}^2$, and since there is no chemical or electrical driving force for anionic movement, the fact

that this current is outwardly (i.e., basolaterally) directed shows that it must reflect a net efflux of K^+ across the intact basolateral membrane (4).

Table 2 shows the result of experiments that explored the effects of several putative K^+ channel blockers on this current. Although the tested compounds were all used at concentrations thought likely to be maximally effective, clotrimazole, apamin, iberiotoxin, and chromanol 293B all had no effect, while Ba^{2+} , quinidine, lidocaine, and clofilium caused only 10–20% inhibition. Bupivacaine was the only tested compound that caused a substantial (~50%) fall in I_{BI} (Table 2), and this effect was confirmed by a subsequent series of experiments in which the basolateral concentration of bupivacaine was increased progressively so that the kinetics of inhibition could be studied (Fig. 4B). This analysis indicated that a maximally effective concentration of bupivacaine would inhibit I_{BI} by $57 \pm 3.4\%$ whereas the concentration needed for a half maximal effect (EC_{50}) was $113 \pm 2.5 \mu M$. Lidocaine and quinidine also caused concentration-dependent inhibition of I_{BI} , and the EC_{50} values for these compounds were 5.5 ± 0.2 and 2.5 ± 0.2 mM, respectively (Fig. 4C). However, whereas the slope factors for bupivacaine and quinidine were close to unity (0.9 ± 0.02 and $0.8 \pm 0.02 \text{ mol}^{-1}$, respectively), the value for lidocaine was only $0.4 \pm 0.001 \text{ mol}^{-1}$, suggesting that the inhibitory action of this drug may not involve equilibrium binding to a single site. The pharmacological basis of this effect was not investigated further. These experiments also confirmed (see Table 2) that high concentrations of Ba^{2+} were needed to inhibit I_{BI} (Fig. 4A). Indeed, even at 30 mM, Ba^{2+} caused less inhibition than 3 mM bupivacaine. The EC_{50} for Ba^{2+} was thus >10 mM.

Figure 4D shows the effects of increasing concentrations of basolateral bupivacaine, quinidine, lidocaine, and Ba^{2+} on the spontaneous I_{SC} recorded from intact cells bathed symmetrically with physiological saline. These K^+ channel blockers clearly inhibited this current, and analysis of these data indicated that a maximally effective concentration of bupivacaine would cause $51.2 \pm 5.1\%$ inhibition, and the EC_{50} value for this substance was calculated to be $163 \pm 11.4 \mu M$. Quinidine and lidocaine also caused ~50% inhibition but were 10-to-15-fold less potent than bupivacaine (EC_{50} values were 1.6 ± 0.1 and 1.9 ± 0.05 mM, respectively), whereas Ba^{2+} had relatively little effect, and the EC_{50} value for this cation was thus >10 mM.

Effects of basolateral acidification on ion transport in polarized cells

Subsequent experiments explored the extent to which I_{BI} was sensitive to basolateral pH. Since this solution was HCO_3^-/CO_2 -buffered, its pH was reduced by increasing the CO_2 content of the gas mixture used to bubble the bath to ~40%. In all experiments, the apical bath was continually bubbled with 5% CO_2 . This increase in CO_2 consistently reduced the pH of the basolateral bath by ~1 pH unit, and Fig. 5A shows that this acidification was associated with a fall in I_{BI} ($\Delta I_{BI} = 2.7 \pm 0.5 \mu A/cm^2$; $P < 0.01$). However, subsequent addition of basolateral bupivacaine (3 mM) caused further inhibition ($\Delta I_{BI} = 5.5 \pm 1.3 \mu A/cm^2$; $P < 0.05$), indicating that the acidification had not completely blocked the K^+ channels underlying this current. Figure 5B confirms that bupivacaine (3 mM) normally inhibits I_{BI} ($\Delta I_{BI} = 3.4 \pm 0.4 \mu A/cm^2$; $P < 0.005$) but shows that basolateral acidification has no further effect on the current that persisted in the presence of this K^+ channel blocker.

Directly analogous experiments explored the effects of basolateral acidification on the spontaneous I_{SC} generated by intact epithelia. This spontaneous current was normally ~30 $\mu A/cm^2$, which is in accord with our previous studies (see, e.g., Ref. 31), and Fig. 5C shows that basolateral acidification caused a clear inhibition of this current ($\Delta I_{SC} = 2.6 \pm 0.7 \mu A/cm^2$; $P < 0.05$) but that subsequent addition of basolateral bupivacaine (3 mM) consistently caused further inhibition ($\Delta I_{SC} = 11 \pm 2.9 \mu A/cm^2$; $P < 0.05$). Figure 5D confirms (Fig. 3D) that basolateral bupivacaine (3 mM) normally inhibits I_{SC} ($\Delta I_{SC} = 12 \pm 3.7 \mu A/cm^2$; $P < 0.05$) and shows that basolateral acidification had no further effect on the residual current.

Analysis of extracted RNA

RT-PCR-based analysis of extracted RNA using primers designed to amplify sequences specific for K2P (Table 1) showed that mRNA transcripts encoding TWIK-1, TREK-1, TASK-2, TWIK-2, TASK-3, THIK-1, TALK-2/TASK-4, TREK-2, and KCNK-7 were all present in cells grown in culture flasks or on Transwell membranes (Fig. 6). Parallel analyses using appropriate primers (Table 1) confirmed that PCR products encoding sequences specific to α -, β -, and γ -ENaC were also produced under these assay conditions (data not shown).

DISCUSSION

K^+ channels can be grouped into three structurally distinct families that are respectively defined by the presence of 2, 4, or 6 transmembrane domains (2, 4, or 6TM; Ref. 18), and studies of airway epithelia have shown that G_K can be controlled by hormones/neurotransmitters that signal via $[Ca^{2+}]_i$ and by those which activate cAMP/PKA. These cellular signals appear to activate physiologically distinct K^+ conductances that correspond to different 6TM K^+ channel subtypes (7,11,14,23,26,27,42). Ca^{2+} -coupled agents thus activate KCNN4 K^+ channels that are characteristically sensitive to clotrimazole and Ba^{2+} , whereas the cAMP/PKA-regulated conductance seems to depend on KCNQ1/KCNE3, which form a PKA/cAMP-regulated K^+ channel that is blocked by Ba^{2+} , chromanol 293B, and clofilium (7,14,23,24,34). Our (41) earlier work showed that H441 cells express KCNN4 and demonstrated that these channels allow thapsigargin, which causes a large and sustained rise in $[Ca^{2+}]_i$ (37), to evoke large increases in G_K . However, these experiments also showed that KCNN4 is inactive at the levels of $[Ca^{2+}]_i$ found in resting cells (see also Ref. 2), indicating that this channel does not contribute to resting G_K and thus cannot be part of the mechanism underlying spontaneous Na^+ transport (41). Having excluded the possibility that KCNN4 channels determine G_K in unstimulated cells, we undertook the present study to identify alternative candidates.

Whole cell membrane currents

Experiments in which cells were initially bathed with a bath solution containing NMDG⁺ as the principal cation showed that increasing $[K^+]_o$ caused a clear and consistent depolarization demonstrating that G_K is significant under these conditions. However, irrespective of whether the cells were bathed with NMDG⁺- or K^+ -rich solutions, there was always a discrepancy between V_m and E_K , which implies that G_{Cl} must also be large enough to influence V_m (see also Ref. 41). Indeed, V_m in cells bathed with the K^+ -rich salt solution lay about halfway between E_{Cl} and E_K , indicating that G_K and G_{Cl} must be of approximately equal magnitude. At least one earlier study (21) has indicated that H441 cells express the cAMP-regulated Cl^- channels encoded by the gene that is mutated in cystic fibrosis (CFTR). However, studies undertaken in this laboratory indicate that the channels that underlie G_{Cl} in H441 cells are more permeable to I^- than to Cl^- , whereas the Cl^- channels associated with CFTR expression characteristically display a low permeability to this anion (see, e.g., Ref. 19). While it is clear that H441 cells do express a significant Cl^- conductance, the underlying channels have yet to be identified. Interestingly, recent studies (39,41) of lens fiber epithelial cells have identified an anion conductance with properties very similar to those of the conductance described here.

Despite the clear depolarization evoked by increasing $[K^+]_o$, Ba^{2+} had no effect on the membrane currents recorded from cells bathed with the K^+ -rich salt solution, indicating that the K^+ channels underlying this depolarization are insensitive to this cation. This was surprising since Ba^{2+} is widely used as a nonspecific inhibitor of many different epithelial K^+ channels; indeed, the concentration of Ba^{2+} used here (5 mM) was sufficient to cause essentially complete blockade of all 2TM or 6TM K^+ channels. However, the present data also show that bupivacaine, a local anesthetic known to block certain K^+ channel types, consistently caused a substantial (~50%) fall in G_m that was associated with a hyperpolarization to E_{Cl} . Further

analysis of these data showed that I_{Bupiv} displayed slight inward rectification and, most importantly, reversed at a potential identical to E_K , suggesting that this current is carried by K^+ . Further evidence of this came from ionic substitution experiments, which showed that lowering $[Cl^-]_o$ to 21.5 mM, while $[K^+]_o$ remained at 134.5 mM, depolarized the cells. Whereas this provides further evidence that G_{Cl} is significant, this reduction in $[Cl^-]_o$ had no discernible effect on I_{Bupiv} , which establishes that this current is independent of external anions. Further modifying the bath solution by reducing $[K^+]_o$ to 20 mM caused a shift in the value of V_{Rev} associated with I_{Bupiv} that was in good accord with the accompanying hyperpolarization of E_K . This result therefore shows that I_{Bupiv} is K^+ selective.

Further experiments showed that reducing bath pH to 6.4 fully mimicked these effects of bupivacaine and experiments in which cells were sequentially exposed to bupivacaine/acid-external pH indicated that these maneuvers inhibited G_m by blocking the same population of ion channels. These data thus show that G_K in dexamethasone-treated H441 cells is determined by a population of K^+ channels that are insensitive to Ba^{2+} but which can be blocked by bupivacaine or by extracellular acidification.

Currents across polarized monolayers

Subsequent studies characterized the K^+ channels underlying basolateral G_K by monitoring K^+ currents in apically polarized cells exposed to basolaterally directed $[K^+]_o$ gradients (see Ref. 4). The first such experiments explored the effects of several putative K^+ channel blockers at concentrations thought likely to induce maximal effects and showed that inhibitors of Ca^{2+} -activated K^+ channels (clotrimazole: KCNN4; apamin: KCNN1, 2, and 3; and iberiotoxin: KCNMA1) had no effect, indicating that these channels do not contribute to basolateral G_K . Chromanol 293B was also without effect, indicating that PKA/cAMP-regulated K^+ channels (KCNQ1/KCNE3) also do not contribute to the basal K^+ conductance of this membrane. This contrasts with the situation in the mouse trachea where chromanol 293B reduces G_K and inhibits amiloride-sensitive Na^+ absorption, suggesting that the K^+ conductance associated with KCNQ1/KCNE3 may be part of the absorptive mechanism (14). Interestingly, we (17) have recently shown that activation of PKA/cAMP does increase basolateral G_K in H441 cells and so, although PKA/cAMP-regulated K^+ channels do not contribute to basal G_K , increased activity of these channels may well contribute to the PKA/cAMP-mediated stimulation of Na^+ transport that has been documented in these cells (5,31).

The present study also showed that Ba^{2+} caused only modest inhibition of I_{B1} despite being used at a very high concentration (5 mM), while lidocaine, quinidine, and clofilium, which also block several types of K^+ channels, also caused only 10–20% inhibition. Indeed, the only tested compound that caused substantial inhibition was bupivacaine, and this effect was confirmed by more detailed studies that demonstrated an EC_{50} of $\sim 120 \mu\text{M}$. Such experiments also showed that lidocaine and quinidine also cause $\sim 50\%$ inhibition if used at a high enough concentration, but these inhibitors were 20-to-50-fold less potent than bupivacaine. However, even when used at very high concentrations, Ba^{2+} caused only modest inhibition and was at least 100-fold less potent than bupivacaine.

Basolateral G_K in polarized monolayers is thus determined by a population of K^+ channels sensitive to bupivacaine and certain other compounds, but only weakly inhibited by Ba^{2+} , a situation similar, although not identical, to that documented in single cells (see above). Further studies in intact monolayers showed that basolateral bupivacaine, lidocaine, quinidine, and Ba^{2+} also inhibited the spontaneous I_{SC} , and, for each inhibitor, the concentrations needed to inhibit this transepithelial current were very similar to the concentrations needed to inhibit I_{B1} . This excellent correlation thus suggests that the K^+ channels that determine I_{B1} are part of the mechanism underlying basal Na^+ absorption.

Lowering basolateral pH also inhibited I_{BI} , which accords well with our data from single cells (see above), but, in polarized cells, subsequent addition of bupivacaine caused a further fall in current not seen in single cells. Thus, whereas our studies of single cells suggest that G_{K} is dependent on a single population of acid- and bupivacaine-sensitive K^+ channels, our studies of polarized cells suggest that two functionally distinct K^+ channel populations determine basolateral G_{K} , one that can be inhibited by basolateral acidification and is sensitive to bupivacaine, and a second that is bupivacaine-sensitive and remains active after acidification. This expression of additional channels in polarized cells is not surprising since previous studies of many different epithelial cell types have shown that the formation of a functionally polarized epithelial layer is associated with increased expression of ion channels (e.g., ENaC; see Ref. 20) and cell surface receptors (e.g., P2Y receptors; see Ref. 40).

A similar picture emerged from experiments that explored the effects of basolateral acidification and/or bupivacaine on the I_{SC} generated by intact epithelia. Although we cannot specifically exclude the possibility that basolateral acidification may inhibit I_{SC} by changing intracellular pH or by some other undefined mechanism, this seems unlikely since this maneuver had essentially no effect on bupivacaine-treated cells. Indeed, acidification and/or bupivacaine had very similar effects on I_{SC} and I_{BI} , suggesting that these two effects may have a similar underlying mechanism.

Possible molecular basis of the observed K^+ conductance

Ba^{2+} had no effect on the K^+ currents recorded from single cells even at very high concentrations and caused only very weak inhibition of I_{BI} in polarized cells (see above). This effectively excludes a role for all members of 6TM and 2TM K^+ channel families since, as far as we are aware, such channels are all potently blocked by Ba^{2+} (see, e.g., Ref. 18). By inference, our data thus imply that resting G_{K} is determined by 4TM K^+ channels. This structurally distinct group of K^+ channels includes over 20 members that are defined by the presence of two copies of the “ K^+ channel signature sequence,” a highly conserved amino acid motif that seems to underlie K^+ selectivity. For this reason, such channels are usually referred to as K2P channels, and a growing body of evidence indicates that such channels determine the “background” or resting K^+ conductance found in essentially all animal cells (18,28). It is well-documented that several such channels (TASK-1, TASK-2, and TASK-3) are inhibited by a fall in external pH, and although their pharmacology is not yet fully understood, many such channels are also inhibited by local anesthetics such as bupivacaine (e.g., TASK-2, TASK-3), and some are either only weakly inhibited by Ba^{2+} or completely resistant to this cation (e.g., TREK-2, TALK-2; Refs. 18 and 28). We therefore undertook RT-PCR-based analysis of mRNA extracted from H441 cells using primers designed to amplify sequences specific to virtually all known K2P channels. These studies showed clearly that H441 cells express mRNA encoding several such channels. As far as we are aware, the present study is therefore the first to report K2P channel expression in absorptive airway epithelial cells, although Davis and Cowley (8) showed that mRNA encoding TREK-1, TASK-2, TWIK-1, and TWIK-2 was present in a secretory cell line (Calu-3) thought to retain the physiological features of the submucosal gland. These authors suggest that tonic activity of such channels may provide the driving force for anion secretion in unstimulated cells (8). It has also been suggested that K2P channels can account for the O_2 -sensitive K^+ conductance identified in neuroepithelial bodies, discrete clusters of neurally derived cells found throughout the airways that are thought to play an important role in pulmonary physiology by sensing changes in airway Po_2 (29,30).

The present data thus show that G_{K} in resting H441 cells is determined by K^+ channels that are blocked by bupivacaine or extracellular acidification but only very weakly inhibited by Ba^{2+} . These data therefore raise the possibility that this resting K^+ conductance may be

determined by the activity of one or more K2P channels (see, e.g., Refs. 18 and 28). Such channels could thus play a previously undocumented role in pulmonary physiology by contributing to the driving forces that maintain spontaneous Na⁺ absorption.

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Fig. 1. Whole cell membrane currents (I_m) in single cells/small groups of cells. Shown here is the relationship between I_m and holding potential (V_{Hold}) in cells ($n = 6$) initially exposed to NMDG⁺-rich bath solution (5 mM $[K^+]_o$), which was subsequently exchanged for a K^+ -rich saline ($[K^+]_o = 130$ mM) prepared by iso-osmotically replacing NMDG⁺ with K^+ . $[Na^+]_o$ thus remained at 10 mM throughout the experiment.

Fig. 2.

Bupivacaine-sensitive membrane currents. *A*: current voltage relationships ($n = 6$) for cells bathed with the standard K^+ rich saline ($[Cl^-] = 151.5$ mM) both under control conditions and in the presence of bupivacaine (3 mM, 1–2 min). *B*: the membrane current that persisted in the presence of bupivacaine was digitally subtracted from the total current to isolate the bupivacaine-sensitive current (I_{Bupiv}), which is plotted against V_{Hold} . *C*: current voltage relationships ($n = 10$) for cells bathed with K^+ -rich saline that had been modified by lowering $[Cl^-]$ to 21 mM (gluconate substitution). Data were recorded under control conditions and in the presence of 3 mM bupivacaine. *D*: these data were further analyzed to isolate I_{Bupiv} , which has been plotted against V_{Hold} . This figure includes data from cells bathed with the standard K^+ -rich low Cl^- solution (134.5 mM K^+) and results obtained in a separate series of directly analogous experiments ($n = 5$) in which the composition of this low Cl^- saline was further modified by lowering $[K^+]$ to 20 mM (NMDG $^+$ substitution). All data are means $\pm$ SE.

Fig. 3.

Acid-sensitive membrane currents. *A*: current voltage relationships ($n = 4$) recorded under control conditions (pH 7.4) and after the external pH had been lowered to 6.4. *B*: the membrane current that persisted at pH 6.4 was subtracted from the corresponding control current to isolate the acid-sensitive current (I_{Acid}), which is plotted against V_{Hold} . *C*: data from a separate series of experiments in which membrane currents were again recorded from cells bathed with K⁺-rich saline ($[Cl^-]_o = 151.5$ mM) under standard conditions ($n = 3$) and during exposure to the acidified external solution; I_{Acid} was isolated by digital subtraction and quantified as the mean current flowing at values of V_{Hold} between -80 mV and -90 mV. The figure shows data from control and bupivacaine-treated (3 mM, 1–2 min) cells. *D*: membrane currents were recorded

from cells bathed with K⁺-rich bath solution ($[Cl^-]_o = 151.5$ mM) both under control conditions and in the presence of 3 mM bupivacaine so that the bupivacaine-sensitive component of the total membrane current (I_{Bupiv}) could be isolated by digital subtraction and quantified as the mean current flowing at values of V_{Hold} between -80 mV and -90 mV. The figure shows data from experiments undertaken at pH 7.4 (control) and pH 6.4. All data are means $\pm$ SE; asterisks denote statistically significant differences (Student's t -test) between the control and experimental values (* $P < 0.05$; ** $P < 0.02$).

Fig. 4.

Effects of K^+ channel blockers in polarized cells. *A*: experimental record showing the effects of progressively increasing the basolateral concentration of Ba^{2+} on I_{BI} measured in an apically polarized epithelial sheet exposed to a basolaterally directed $[K^+]$ gradient. *B*: data from an analogous experiment that explored the effect of bupivacaine. *C*: the values of I_{BI} that persisted in the presence of each tested concentration of bupivacaine ($n = 4$), quinidine ($n = 6$), lidocaine ($n = 4$), and Ba^{2+} ($n = 3$) were normalized to the initial control current and plotted (means $\pm$ SE) against the concentration of blocker used; sigmoid curves were fitted to the experimental data by least squares regression (GraFit 5; Erithacus Software, Staines, United Kingdom). *D*: data from studies of age-matched cells at identical passage that explored the effects of the K^+ channel blockers on the spontaneous I_{SC} generated by intact cells bathed symmetrically with standard physiological saline.

Fig. 5.

Effects of basolateral acidification on I_{BI} and I_{SC} . In all figures, the *top* shows a continuous record of basolateral pH, whereas the *bottom* shows simultaneous recordings of I_{BI} measured in apically permeabilized cells exposed to a basolaterally directed $[K^+]$ gradient (A and B) or I_{SC} , which was measured in intact cells bathed symmetrically with standard physiological saline (C and D). Cells were either exposed to basolateral acidification (40% CO_2) followed by 3 mM basolateral bupivacaine (A and C) or to 3 mM basolateral bupivacaine followed by basolateral acidification (B and D). All data are means $\pm$ SE, $n = 4$.

Fig. 6.

Expression of mRNA encoding tandem pore domain K^+ (K2P) channels. RNA extracted from H441 cells was analyzed by RT-PCR using gene-specific primers designed to amplify sequences specific to most known K2P channels (see Table 1) or GAPDH, which served as an internal control. All PCR products were sequenced to verify their origin, and, for all targets, similar results were obtained in 3 independent assays.

Table 1
Details of PCR primer sequences used in analyses of extracted RNA

Target	Forward Primer	Reverse Primer	Product size, bp
TWIK-1	GTCCTGGAGGATGACTGGAA	GGCTCATTTTGCTTCTGGTC	346
TWIK-2	AGCTTGCGAGTCCAGTGAGGT	TGATTCCAGGGCACAAACATA	447
TREK-1	AGGTGGGAGAGTTCAGAGCA	GCAATCTCTTCACCAGCACA	298
TREK-2	GCTCTGCCAAAAGGAATCAG	GCACATGCCAAAATGTCAAC	316
TASK-1	ACCAGAGAGCAAAAGGCAAAA	TCAAACAATCCTCCCACCTC	211
TASK-2	GGCCTTCCTAACCTTCCATC	GTAGTAGGTGCCCCGACCTGA	415
TASK-3	CAGCATGGTCATTACATCC	GGTGGTCGGTAAAGCTGTGT	287
TASK-5	GGCAAGGTCTTCTGCATGTT	GGTGAGGGTGATGAAGCAGT	278
THIK-1	GGGAACAAAAATTGCGAAGA	CCCTACACCACCATCTTGCT	312
THIK-2	AGAGCGAGTCCACGTAGTCC	GGGACCATCCTGTCTTCAA	276
TALK-1	CAAGTCACACCCAGGACTT	TCCCACACCTCTGTCCTTTC	242
TALK-2	TAGAGGCTGGGTGCAGCTAT	CAAACATTGCCGCTACTTCA	204
TRAAK	ATCTGGGGGAGAAGGACACT	CTGCCGATCCCTAACTGGTA	207
KCNK7	CAGCCTCCTTAGCTCTCGTG	GAGCGAGCTGAAGCAGAAAGT	238
α ENaC	TTCTGCAACACCACCAT	TTCCAGTCCTTCCAGTCCAC	484
β ENaC	CTGGACGTCATCGAGTCTGA	CCAGGAAGGAGAAAACCACA	404
γ ENaC	TGGACAGCTACTGCCAGATG	AGGGTCAGCTCTGTCTTGGA	461

ENaC, epithelial Na⁺ channel.

Table 2Effects of putative K⁺ channel blockers on I_{B1}

Blocker	n	I _{B1} , μA/cm ²		
		Control	Experimental	Inhibition, %
Ba ²⁺ , 5 mM	6	23.2±3.1	19.3±3.2‡	17.8±3.4
Clotrimazole, 100 μM	4	25.2±3.2	25.7±3.3	—
Apamin, 1 μM	7	36.0±6.0	35.3±6.0	—
Iberiotoxin, 0.1 μM	8	31.7±5.8	30.7±5.2	—
Clofilium, 100 μM	7	20.9±1.1	15.4±1.8*	26.7±5.6
Chromanol 293B, 10 μM	3	34.3±5.4	33.2±5.0	—
Quinidine, 3 mM	6	18.9±1.6	12.8±0.7*	30.9±3.4
Bupivacaine, 3mM	4	26.2±0.6	12.5±2.0†	52.7±14.3
Lidocaine, 3 mM	3	21.0±1.0	15.9±0.7‡	24.2±1.3

Values are means ± SE. Basolateral membrane K⁺ current (I_{B1}) was monitored in apically permeabilized cells exposed to outwardly directed [K⁺] gradients (see METHODS), and the tabulated data show I_{B1} recorded immediately before the basolateral addition of putative K⁺ channel blockers, and the corresponding values of I_{B1} were recorded 2–5 min later. Where there was a statistically significant difference between these values (**P* < 0.05, †*P* < 0.02, ‡*P* < 0.001, Student's paired *t*-test), the inhibitory effect was quantified and tabulated (% inhibition). Ba²⁺ was added as the acetate salt.