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


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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 μg/ml insulin; 5 μg/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 (~10⁵ cells/cm²) 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 (~22°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 (Cᵢn) of each preparation, which provides an indicator of membrane area, was noted carefully, and all cited values of Iₘ were normalized to the average value of Cᵢn associated with a single cell (~35 pf). Such data are therefore presented as picocapillarities per average-sized cell (pA/cell). The pipette filling solution always contained in mM 10 NaCl, 18 KCl, 92 potassium gluconate, 0.5 MgCl₂, 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 [K⁺] to 113.3 mM. The standard bath solution contained in mM 140 NaCl, 4.5 KCl, 1 MgCl₂, 2.5 CaCl₂, 5 HEPES, and 5 glucose, and its pH was adjusted to 7.4 with NaOH, which brought [Na⁺] to 144.4 mM. Under these quasi-physiological ionic conditions, the equilibrium potentials for Na⁺, K⁺, and Cl⁻ (Eₑ₂, Eₓ₁, and Eₓ₂, 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/AgCl pellet connected to the recording chamber via a salt bridge filled with 3 M KCl/4% agar, the solution was bubbled with 5% CO₂ while transepithelial potential difference (Vₑ) from cells bathed with the standard physiological salt solution (containing in mM 112 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 11.6 Tris-glucose, pH 7.3-7.5 when bubbled with 5% CO₂) while transepithelial potential difference (Vₑ) was monitored (DVC-1000 voltage/current clamp; World Precision Instruments, Stevenage, Hertfordshire, United Kingdom). Once this parameter had stabilized, Vₑ was clamped to 0 mV (DVC-1000 voltage/current clamp), and the current needed to maintain this potential (short-circuit current, Iₛ𝑐) 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 potassium gluconate, 11 calcium gluconate, 1.2 MgSO₄, 1.2 KH₂PO₄). The concentration of Cl⁻ in the apical and basolateral baths was then 30 mM. The apical membrane was then permeabilized by adding 200 μM 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 Vₑ was held at 0 mV (basolateral membrane current, Iₘ). At intervals throughout all experiments, the cells were briefly returned to open circuit conditions so that Vₑ could be measured to allow transepithelial resistance (Rₑ) 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 ± SE, and values of n refer to the number of times a protocol was repeated using cells at different passage numbers.

**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 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 on agarose gels 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

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

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<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product size, bp</th>
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<td>TWIK-1</td>
<td>GTCCTGGAGGAGTACGCTGGA</td>
<td>GGCTGATTTCGCTCTGTC</td>
<td>346</td>
</tr>
<tr>
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<td>AGGTGGCCAGCTGAGCTGAGGAGG</td>
<td>TGATTCAGGCGGACGAATGA</td>
<td>447</td>
</tr>
<tr>
<td>TREK-1</td>
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<td>GCAATTCTCTACAGCAACACA</td>
<td>298</td>
</tr>
<tr>
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<td>GCAGTGCTGGAAAGGAGTGAGG</td>
<td>GCACATGGCAGAGGAGGAG</td>
<td>316</td>
</tr>
<tr>
<td>TASK-1</td>
<td>ACCAGAGACGCACAGACGCACAGAC</td>
<td>TCAAAACAT2TCTCCACACCTC</td>
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<tr>
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<td>TASK-3</td>
<td>GACAGCTGCTGATAAGCTGTT</td>
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<tr>
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<td>GGCTCCACCCACGAGTCTC</td>
<td>312</td>
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<tr>
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<tr>
<td>TALK-1</td>
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<tr>
<td>γENaC</td>
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<td>AGGTTAGCTGCTCTTGGA</td>
<td>461</td>
</tr>
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</table>

ENaC, epithelial Na⁺ channel.
solution showed that $V_m$ was normally $-27 \pm 1.7$ mV, and, as anticipated by earlier work (6), lowering extracellular Na concentration ([Na]$\textsubscript{i}) to 10 mM by iso-osmotically replacing this ion with N-methyl-D-glucammonium (NMDG$^+$) inhibited the inward currents flowing at negative values of $V_{\text{Hold}}$ and hyperpolarized $V_m$ to $-60 \pm 2.8$ mV ($P < 0.001$). This confirms that $G_{\text{Na}}$ 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 $V_{\text{Hold}}$.

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 $< -5$ pA/cell at physiologically relevant values of $V_{\text{Hold}}$ (see Ref. 6) and allowed us to modify the cationic composition of the bath while $E_{\text{Na}}$ remained at $\sim 0$ 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 $-60.4 \pm 2.8$ 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 ([K]$\textsubscript{i}) augmented the inward currents flowing at negative potentials and depolarized ($P < 0.001$) $V_m$ to $-33.5 \pm 7.6$ mV, confirming (41) that these cells express a conductance that allows a depolarizing K$^+$ current to flow as [K]$\textsubscript{i}$ is raised. However, although the K$^+$-rich saline used in these experiments was designed to shift $E_K$ to $\sim 0$ 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 $-25.8 \pm 2.2$ mV under these conditions. This potential is more negative than $E_K$ ($P < 0.001$) and, since $E_{\text{Na}}$ was always 0 mV (see METHODS), this discrepancy between $V_m$ and $E_K$ implies that the cellular Cl$^-$ conductance ($G_{\text{Cl}}$) 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, $-26.1 \pm 2.4$ mV; Ba$^{2+}$, $-30.0 \pm 2.7$ mV). In contrast, bupivacaine (3 mM) reduced the magnitude of the currents flowing at positive and negative values of $V_{\text{Hold}}$ (Fig. 2A) and hyperpolarized $V_m$ to a value close to $E_{\text{Cl}}$ (control, $-22.9 \pm 1.6$ mV; bupivacaine, $-50.8 \pm 3.9$ mV; $P < 0.001$). These effects were fully reversible (data not shown). Further analysis of these data showed that bupivacaine reduced $G_{\text{Na}}$ from 0.89 $\pm$ 0.12 to 0.38 $\pm$ 0.35 pS/cell ($P < 0.01$), demonstrating that $\sim$50% of $G_{\text{Na}}$ 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 ($I_{\text{Bupiv}}$) displayed slight inward rectification and reversed at a potential essentially identical to $E_K$ ($\sim 0$ mV; Fig. 2B). Interestingly, the value of reversal potential ($V_{\text{Rev}}$) associated with the bupivacaine-resistant component of $I_m$ lay close to $E_{\text{Cl}}$, suggesting that the bupivacaine-sensitive and bupivacaine-resistant currents are carried by K$^+$ and Cl$^-$, respectively.

To test the hypothesis that $I_{\text{Bupiv}}$ was a K$^+$ current, the ionic composition of the K$^+$-rich bath solution was further modified by lowering extracellular Cl$^-$ concentration ([Cl]$\textsubscript{i}) to 26.5 mM (gluconate substitution). Analysis of currents recorded under these conditions (Fig. 2C) showed that $V_m$ was less negative ($-7.1 \pm 3.8$ mV; $P < 0.001$) than at 151.5 mM Cl$^-$ ($-25$ mV, see above). Reducing [Cl]$\textsubscript{i}$ in this way thus depolarized the cells, which confirms (21, 41) that $G_{\text{Cl}}$ is significant, although $V_m$ did not fully depolarize to $E_{\text{Cl}}$, suggesting that the anion channels underlying this conductance have a significant permeability to gluconate. Application of 3 mM bupivacaine caused a fall in $G_{\text{Cl}}$ (control, $0.808 \pm 0.253$ nS/cell; bupivacaine, $0.529 \pm 0.189$ 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 [Cl]$\textsubscript{i}$ was low, and, although this response was smaller ($P < 0.05$) than that seen at 151.1 mM [Cl]$\textsubscript{i}$ (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 [Cl]$\textsubscript{i}$ had no effect on $I_{\text{Bupiv}}$ (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 [K]$\textsubscript{i}$ to 20 mM (NMDG$^+$ substitution) to selectively shift $E_K$ to $-43$ mV. Analysis of these data showed that this reduction in [K]$\textsubscript{i}$ shifted the value of $V_{\text{Rev}}$ associated with $I_{\text{Bupiv}}$ in a manner that almost perfectly matched the hyperpolarization of $E_K$. This finding thus demonstrates that $I_{\text{Bupiv}}$ 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 indi-
cated that this acidification caused a 47.4 ± 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 ($I_{\text{Acid}}$) reversed at a potential essentially 
identical to $E_K$ and was qualitatively and quantitatively similar 
to $I_{\text{bupiv}}$ (Fig. 2, B and D). Further experiments therefore 
explored the possibility that bupivacaine and bath acidification 
amay 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, $1.06 ± 0.16$ 
3 mM) at 

$P < 0.02$) 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 (~90% inhibition) $I_{\text{bupiv}}$ 
(Fig. 3C) while the application of 3 mM bupivacaine essen-
tially abolished $I_{\text{Acid}}$ (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 ($R_i = 
281 ± 11 \Omega \text{cm}^2$, $n = 94$) on permeable culture membranes 
generated a $V_i$ of $-12 ± 0.6$ mV when bathed symmetrically 
with standard physiological saline. Exposing these cultured epithelia to 
a basolaterally directed [K+] gradient (see METHODS) increased $R_i$ to $576 ± 22 \Omega \text{cm}^2 (P < 0.05)$ and depolarized $V_i$ 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 $V_i$ 
to $-12 ± 0.3$ mV ($P < 0.05$), and reduced $R_i$ to 493 ± 20 
$\Omega \text{cm}^2 (P < 0.05$). Measurements made while $V_i$ was held at 0 
$mV$ showed that the mean current flowing under these condi-
tions ($I_{bupiv}$) was $26 ± 1.0 \mu\text{A/cm}^2$, 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 Ba2+, 
quinidine, lidocaine, and clofilium caused only 10–20% inhibi-
tion. Bupivacaine was the only tested compound that caused a 
substantial (~50%) fall in $I_{bupiv}$ (Table 2), and this effect was 
confirmed by a subsequent series of experiments in which the 
basolateral concentration of bupivacaine was increased pro-
gressively so that the kinetics of inhibition could be studied 
(Fig. 4B). This analysis indicated that a maximally effective 
concentration of bupivacaine would inhibit $I_{bupiv}$ by 57 ± 3.4% 
whereas the concentration needed for a half maximal effect 
(EC$_{50}$) was 113 ± 2.5 $\mu\text{M}$. Lidocaine and quinidine also 
caused concentration-dependent inhibition of $I_{bupiv}$, 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 ± 0.02 $\text{mol}^{-1}$, respectively), the value for lidocaine was 
only 0.4 ± 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 investi-
gated further. These experiments also confirmed (see Table 2) that high concentrations of Ba$^{2+}$ were needed to inhibit $I_{B1}$ (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 standard physiological saline.

**Table 2. Effects of putative K$^+$ channel blockers on $I_{B1}$**

<table>
<thead>
<tr>
<th>Blocker</th>
<th>$n$</th>
<th>Control</th>
<th>Experimental</th>
<th>Inhibition, %</th>
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<tbody>
<tr>
<td>Ba$^{2+}$, 5 mM</td>
<td>6</td>
<td>23.2 ± 3.1</td>
<td>19.3 ± 3.2</td>
<td>17.8 ± 3.4</td>
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<td>Clotrimazole, 100 µM</td>
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<tr>
<td>Apamin, 1 µM</td>
<td>7</td>
<td>36.0 ± 6.0</td>
<td>35.3 ± 6.0</td>
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<tr>
<td>Iberiotoxin, 0.1 µM</td>
<td>8</td>
<td>31.7 ± 5.8</td>
<td>30.7 ± 5.2</td>
<td>—</td>
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<tr>
<td>Clofibrate, 100 µM</td>
<td>7</td>
<td>20.9 ± 1.1</td>
<td>15.4 ± 1.8</td>
<td>26.7 ± 5.6</td>
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<tr>
<td>Chromanol 293B, 10 µM</td>
<td>3</td>
<td>34.3 ± 5.4</td>
<td>33.2 ± 5.0</td>
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<tr>
<td>Quinidine, 3 mM</td>
<td>6</td>
<td>18.9 ± 1.6</td>
<td>12.8 ± 0.7 *</td>
<td>30.9 ± 3.4</td>
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<tr>
<td>Bupivacaine, 3mM</td>
<td>4</td>
<td>26.2 ± 0.6</td>
<td>12.5 ± 2.0</td>
<td>52.7 ± 14.3</td>
</tr>
<tr>
<td>Lidocaine, 3 mM</td>
<td>3</td>
<td>21.0 ± 1.0</td>
<td>15.9 ± 0.7 ‡</td>
<td>24.2 ± 1.3</td>
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Values are means ± SE. Basolateral membrane K$^+$ current ($I_{B1}$) was monitored in apically permeabilized cells exposed to outwardsly 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). Ba$^{2+}$ was added as the acetate salt.

**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 $I_{B1}$ 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 $I_{B1}$ 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 ± 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 asymmetrically with standard physiological saline.
cally 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 \(\sim\)50\% inhibition but were 10-to-15-fold less potent that bupivacaine (EC\(_{50}\) values were 1.6 \(\pm\) 0.1 and 1.9 \(\pm\) 0.05 mM, respectively), whereas Ba\(^{2+}\) had relatively little effect, and the EC\(_{50}\) value for this cation was thus \(\sim\)10 mM.

Effects of basolateral acidification on ion transport in polarized cells. Subsequent experiments explored the extent to which pH was sensitive to basolateral pH. Since this solution was HCO\(_3\)--buffered, its pH was reduced by increasing the CO\(_2\) content of the gas mixture used to bubble the bath to \(\sim\)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 \(\sim\)1 pH unit, and Fig. 5A shows that this acidification was associated with a fall in \(I_B\) (\(\Delta I_B\) = 2.7 \(\pm\) 0.5 \(\mu\)A/cm\(^2\); \(P < 0.01\)). However, subsequent addition of basolateral bupivacaine (3 mM) caused further inhibition (\(\Delta I_B\) = 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 \(I_B\) (\(\Delta I_B\) = 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 \(\sim\)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-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 \(\alpha\)-, \(\beta\)-, and \(\gamma\)-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/KCN3E, which form a PKA/cAMP-regulated K\(^+\) channel that is blocked by Ba\(^{2+}\), chro- manol 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 \(G_{Cl}\) and \(G_{K}\) 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 \(E_K\). Further analysis of these data showed that \(I_{\text{Bupiv}}\) 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 \(I_{\text{Bupiv}}\), 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 \(V_{Rc}\), associated with \(I_{\text{Bupiv}}\) that was in good accord with the accompanying hyperpolarization of \(E_K\). This result therefore shows that \(I_{\text{Bupiv}}\) 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 (KCNO1/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 KCNO1/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 \(I_{\text{Bll}}\) 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 ∼120 μM. Such experiments also showed that lidocaine and quinidine also cause ∼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 \(I_{\text{SC}}\), and, for each inhibitor, the concentrations needed to inhibit this transepithelial current were very similar to the concentrations needed to inhibit \(I_{\text{Bll}}\). This excellent correlation thus suggests that the K\(^{+}\) channels that determine \(I_{\text{Bll}}\) are part of the mechanism underlying basolateral Na\(^{+}\) absorption.

Lowering basolateral pH also inhibited \(I_{\text{Bll}}\), 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

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distinct K⁺ channel populations determine basolateral Gk, 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_H, suggesting that these two effects may have a similar underlying mechanism.

Possible molecular basis of the observed K⁺ conductance. Ba²⁺ had no effect on the K⁺ currents recorded from single cells even at very high concentrations and caused only very weak inhibition of I_H 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 Ba²⁺ (see, e.g., Ref. 18). By inference, our data thus imply that resting Gk 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 Ba²⁺ 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 O₂-sensitive K⁺ conductance identified in neuronal epithelial 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 PO₂ (29, 30).

The present data thus show that Gk in resting H441 cells is determined by K⁺ channels that are blocked by bupivacaine or extracellular acidification but only very weakly inhibited by Ba²⁺. 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.

GRANTS

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REFERENCES


