Acid and base secretion in the Calu-3 model of human serous cells

Mauri E. Krouse, Jason F. Talbott, Martin M. Lee, Nam Soo Joo, and Jeffrey J. Wine


SUBMUCOSAL GLANDS ARE THE PRIMARY SOURCE OF AIRWAY MUCUS, A RICH MIXTURE OF WATER, SALTS, MUCINS, AND THE “ANTI-” COMPOUNDS: ANTIMICROBIALS, ANTIENZYMES, AND ANTIANTIGENS. AIRWAY MUCUSHelps maintain sterility of the lungs, but in CF the loss of functional CFTR causes initial secretions to be underhydrated and lower in pH. We hypothesized that the resulting mucus is thicker and that there is reduced bioavailability of serous cell antimicrobials. Hence airway innate defenses, which depend on both mucus clearance and a chemical shield, are rendered inadequate (36). Tests of this hypothesis have been hampered by difficulties in studying the molecular mechanisms of gland secretion in situ.

The Calu-3 cell line was introduced as a serous cell model based on evidence that it expressed the same range of proteins as natural serous cells, including CFTR (11), and formed functional, polarized monolayers that secreted anions in response to elevations of intracellular cAMP concentration ([cAMP]). The Calu-3 cell line (hereafter termed model 1) leads to several predictions about secretions from Calu-3 cells and, by extension, from airway glands. Model 1 predicts that CF gland secretions should be more acidic than normal, and gland mucus produced by elevations of [Ca²⁺] should be much more acidic than mucus produced by elevation of [cAMP]. However, the pH of pig gland mucus is ~7.0 when stimulated via [Ca²⁺]-elevating (acetylcholine, ACh) or [cAMP]-elevating (VIP/forskolin) pathways (18), and pH is also ~7.0 in mucus from ACh-stimulated glands of either CF or normal human subjects (16). To explain these results, it has been hypothesized that secondary processes in the gland modify secretions homeostatically as they pass from the serous acini along the mucous tubules and ducts, so that the pH of the final secretion is adjusted to near pH 7 regardless of the starting pH (36).

What about the initial serous cell secretions? Here, our results agree with the model qualitatively, but not quantitatively. During preliminary tests of the pH of Calu-3 secretions produced by different stimuli (Krouse ME, unpublished observations), we did not see the extreme pH values expected by a switch between 150 and 0 mM HCO₃⁻ concentrations as predicted by model 1 (Fig. 1, Ref. 8). Also, Lee et al. (20) used isotopic flux measurements to determine that elevation of HCO₃⁻ raised the pH of pig gland secretions by 0.7 in response to 0.1 mM HCO₃⁻.

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inhibited by apical ouabain and by removal of apical K which we trace to the activity of an apical process that is essential to HCO₃ secretion, whereas Cl by NKP predicts extremes of pH and further suggest that secretions may be hypotonic.

METHODS

We studied Calu-3 monolayers in Ussing chambers using two paradigms. Short-circuit (Isc) experiments, where the transcellular voltage is clamped to zero, have the advantage of maximizing anion transport. To obtain a separate measure of base (HCO₃) secretion, we also used the pH stat method in conjunction with Isc measurements.

Solutions

The standard solution was Krebs-Henseleit (K-H) solution, which contains (in mM): NaCl, 128; KCl, 4.6; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; and glucose, 11.2. The K-H solution contains both Cl and HCO₃ anions. In Cl⁻–free solution, NaCl was replaced with Na-isethionate, all other Cl− salts were replaced with nitrate salts, and 3 M KCl bridges were replaced by 1 M Na-isethionate bridges. For HCO₃⁻–free solution, NaHCO₃ was replaced with Na-HEPES and bubbled with O₂. For standard pH stat experiments, the apical solution was as follows (in mM): NaCl, 150; KCl, 5; CaCl₂, 2.5; and MgSO₄, 1.2. For pH stat experiments with K⁺–free apical solutions, the K⁺ salts were replaced with Na⁺ salts, and 1 M NaCl was replaced with 3 M KCl in the agar bridges. All solutions were made up at 80% volume, and distilled water was added until the osmolality was 320 mosM, as measured on a vapor pressure osmometer (Wescor 5500; Wescor, Logan, UT). The pH of each solution was adjusted to 7.4 at 37°C when bubbled with the appropriate gas.

Cell Culture

Monolayers of Calu-3 cells were grown on human placental collagen (HPC; Sigma, St. Louis, MO)–coated Snapwell inserts (1.13-cm² area) for 4 wk (28 ± 12 days). The cells were fed from the basolateral side only and grown with an air interface.

Isc and pH Stat

The inserts were mounted in an Ussing chamber (12-mL volume apical and basolateral chamber), the voltage was short circuited with a Physiologic Instrument (San Diego, CA) VCC600, and the current was recorded on a Macintosh computer using MacLab. HCO₃⁻–containing solutions were bubbled with 95% O₂-5% CO₂, and no-HCO₃ and pH stat solutions were bubbled with 100% O₂. The Ussing chambers were kept at 37°C with a temperature-controlled circulator (VWR Scientific, West Chester, PA). The voltage offset/function potential and fluid resistance were measured and subtracted electronically before each experiment. Snapwells were mounted and allowed to reach a steady state (~30 min) before the experiment was begun. A voltage pulse was passed across the monolayer every 20 s to measure monolayer resistance.

The pH stat method estimates the net acid or base secretion across a monolayer by measuring the amount of acid or base that must be added to the unbuffered apical solution to clamp its pH to 7.4. In our experiments, the unbuffered apical solution always alkalinized to varying degrees, so we added appropriate volumes of 3 mM acid (HCl or HNO₃ in Cl⁻–free solutions) manually to keep the apical pH within the range 7.37–7.43. Net base secretion was then converted into an equivalent current (Ieq) on the assumption that one net unit of base carried one net negative charge. Figure 2 shows two raw pH traces from a Calu-3 monolayer that had been pretreated with bumetanide, an inhibitor of the Na⁺–K⁺–2Cl⁻ cotransporter (NKCC). Acid was added near the start of the trace, and the subsequent slow alkalinization of the apical solution (dark line) was taken to reflect a low net level of secretion of base, which was assumed to be HCO₃⁻. Stimulation with 1-EBIO caused the solution to alkalinize more quickly (dotted line), as seen by the increased slope of alkalinization and the increased rate of acid addition. This increase in acid addition rate is seen as an increase in Ieq.

For pH stat experiments, there can be no buffer in the apical solution, especially HCO₃⁻. Therefore, we used K-H solution where all salts were Cl⁻ salts and no buffers were present (NaHCO₃ replaced by NaCl). The nominally unbuffered apical solution was further conditioned by bubbling with pure O₂ for ~1 h before the experiment.
RESULTS

Ussing Chamber Experiments in Normal K-H (Cl⁻ + HCO₃⁻) Solutions

Figure 3A illustrates the main features of Calu-3 Iₛᵣ that we are attempting to understand. In the absence of any apparent stimulation, Calu-3 monolayers develop a basal Iₛᵣ that varies widely in different laboratories and sometimes within laboratories at different times, with mean values in the literature from 13 to 35 μA/cm² (6, 8, 23). Mean basal Iₛᵣ values in our experiments clustered in two groups of 56.3 ± 7.4 μA/cm² (n = 11, high basal current) and 21.2 ± 2 μA/cm² (n = 28, low basal current) when tested with phlorizin or zero apical glucose to eliminate the Na⁺-glucose cotransporter (34). In cells with high basal Iₛᵣ, forskolin had a minimal effect (for example, Fig. 3A), showing on average a 6% increase to 58.2 ± 10.1 μA/cm² (n = 23), whereas in cells with low basal Iₛᵣ, forskolin caused a 100% increase in Iₛᵣ to 43.6 ± 3.8 μA/cm² (n = 17). Thus forskolin stimulation eliminated the significant difference between the groups for Iₛᵣ (P > 0.05), resistance (250 ± 40 % for high basal vs. 350 ± 50 Ω·cm² for low basal, P > 0.05), and the proportion of Iₛᵣ eliminated by bumetanide (20 ± 4% for high basal, n = 14, vs. 12 ± 3% for low basal, n = 10). We

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did not determine the source(s) of variation in basal $I_{sc}$, but these results suggest that Calu-3 cell sheets with high basal $I_{sc}$ resemble those that have been forskolin stimulated, both having an $I_{sc}$ of ~50 $\mu$A/cm$^2$.

Stimulation with either thapsigargin or 1-EBIO, each of which results in open basolateral K$^+$ channels (2, 7), should increase the driving force for anion secretion and produce a proportional increase in $I_{sc}$. That was observed, with the responses peaking and then falling to a plateau (Fig. 4). Plateau was $+177 \pm 72\%$ ($n = 4$) for high basal and $+156 \pm 61\%$ ($n = 7$) for low basal (Table 1), with corresponding decreases in transepithelial resistance (~190 $\Omega \cdot \text{cm}^2$ for both high and low basal). Addition of bumetanide just after the peak $I_{sc}$ caused an immediate inhibition of most of the stimulated $I_{sc}$ (Fig. 3A; Table 1). However, note in Fig. 3A that the $I_{sc}$ after thapsigargin stimulation and bumetanide inhibition was larger than before stimulation. That was observed consistently; the $\Delta I_{sc}$ to K$^+$ channel openers in the presence of bumetanide was $11 \pm 3 \mu$A/cm$^2$ for high basal ($n = 12$) and $12 \pm 4 \mu$A/cm$^2$ for low basal ($n = 17$). It will be shown below that this $\Delta I_{sc}$ represents increased HCO$_3^-$ secretion.

To determine the basis of the bumetanide-insensitive $I_{sc}$, acetalazolamide was added to inhibit carbonic anhydrase, and DNDS was added to inhibit the basolateral NBC. Before 1-EBIO stimulation (but after bumetanide inhibition), DNDS (3 mM) inhibited $12.2 \pm 2.5 \mu$A/cm$^2$ of the $I_{sc}$ (high basal, $n = 10$, Table 1), and after 1-EBIO stimulation, DNDS inhibited less of the $I_{sc}$ (7.8 $\pm 2.2 \mu$A/cm$^2$, high basal, $n = 10$). Under the same conditions (high basal cells), acetalazolamide inhibited significantly more $I_{sc}$ before than after stimulation ($16.2 \pm 3.5 \mu$A/cm$^2$, $n = 8$, Table 1, vs. $30.5 \pm 2.7 \mu$A/cm$^2$, $P < 0.05$, $n = 11$).

The contribution of DNDS- and acetalazolamide-sensitive transport to the bumetanide-insensitive $I_{sc}$ varied with stimulation (Fig. 3B). After forskolin, similar proportions of the $I_{sc}$ were inhibited by DNDS (20%) or acetalazolamide (27%), but after 1-EBIO, there was a large disparity in the proportion of the $I_{sc}$ inhibited by DNDS (10%) vs. acetalazolamide (37%). The DNDS-sensitive component of the $I_{sc}$ decreased after 1-EBIO stimulation ($P = 0.03$), and the acetalazolamide-sensitive component of the $I_{sc}$ increased after 1-EBIO stimulation.

These results suggest four provisional conclusions. 1) Elevation of [Ca$^{2+}$], or stimulation with 1-EBIO stimulates a large increase in Cl$^-$ secretion and a small increase in HCO$_3^-$ secretion. 2) Most of the HCO$_3^-$-dependent secretion after stimulation is mediated by carbonic anhydrase-generated HCO$_3^-$ and CO$_2$. 3) The contribution of NBC is minor. 4) After 1-EBIO stimulation, NBC is not working in reverse, because if it were DNDS should have increased the $I_{sc}$.

To determine whether prior treatment with bumetanide influenced the DNDS results, we compared the inhibitory effect of DNDS on the 1-EBIO-stimulated $I_{sc}$ in paired experiments with and without bumetanide (low basal cells). DNDS alone inhibited 14.6 $\pm$ 0.1% of 1-EBIO-stimulated $I_{sc}$ and this was reduced to 9.1 $\pm$ 0.5% after bumetanide ($n = 10$, $P < 0.001$, Fig. 3B). The significant reduction in DNDS inhibition after bumetanide inhibition of NKCC might arise if the diminished intracellular $\Delta I_{sc}$ results in hyperpolarization of the cells, which would then reduce the transport of HCO$_3^-$ by the NBC (see DISCUSSION).

A substantial $I_{sc}$ (32% of forskolin-stimulated $I_{sc}$, Table 1) remained after combined treatment with bumetanide, DNDS, and acetalazolamide. Evidence to be presented below suggests that this $I_{sc}$ includes a component of HCO$_3^-$ secretion derived from the uncatalyzed conversion of CO$_2$ + H$_2$O to HCO$_3^-$, which still proceeds relatively rapidly in the absence of carbonic anhydrase, with a half-time of ~5 s (12).

**Analysis of $I_{sc}$ with pH Stat Experiments**

Measurement of $I_{sc}$ under pH stat conditions is designed to maximize ionic currents while providing an additional measure of acid/base movement, and no other method can provide the same kind of information. However, pH stat results have been questioned because the technique creates an artificially large HCO$_3^-$ gradient across the cells and across the paracellular space. If any of the drugs caused a change in the paracellular pathway’s HCO$_3^-$ conductance, this might lead to a noncellular response. To estimate the magnitude of any artifact that might have arisen from the HCO$_3^-$ gradient, we measured the $I_{sc}$ in paired Ussing (apical and basolateral solutions clamped to 25 mM HCO$_3^-$) and pH stat experiments (zero apical HCO$_3^-$). Any change of HCO$_3^-$ movement across the paracellular pathway will pro-

![Fig. 4. Results from a typical pH stat experiment. Top trace (○) is $I_{sc}$, and bottom trace (●) is $I_{sc}$ calculated as explained in Fig. 2. Voltage deflections that measure the transepithelial conductance were eliminated for clarity. This monolayer was prestimulated with forskolin, and the forskolin-stimulated $I_{sc}$ was ~35 $\mu$A/cm$^2$ vs. $I_{sc}$ of ~20 $\mu$A/cm$^2$. 1-EBIO (600 $\mu$M) stimulated a large increase in $I_{sc}$ and a small decrease in $I_{sc}$. Bumetanide inhibited $I_{sc}$ and increased $I_{sc}$, bringing them into closer correspondence, but with a gap still present (see text). Response delays in the pH stat measurements are primarily an artifact of the time it takes to eliminate CO$_2$ from the apical solution. After bumetanide, most of the $I_{sc}$ was accounted for by HCO$_3^-$ secretion; notice that $I_{sc}$ and $I_{sc}$ are nevertheless increased over initial values. DNDS (3 mM) inhibited only slightly, but acetalazolamide (100 $\mu$M) inhibited most of the $I_{sc}$ and $I_{sc}$.

### Table 1. $I_{sc}$ with both Cl$^-$ and HCO$_3^-$: K-H solution

<table>
<thead>
<tr>
<th>Stimulus Condition</th>
<th>$I_{sc}^*$, $\mu$A/cm$^2$</th>
<th>$\Delta I_{sc}^*$, $\mu$A/cm$^2$</th>
<th>$\Delta I_{sc}$, $%$</th>
<th>$n$</th>
<th>$\Delta\text{TER}, \Omega \cdot \text{cm}^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>65.7 ± 4.6</td>
<td>45</td>
<td>289.6 ± 38</td>
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<td></td>
</tr>
<tr>
<td>Basal + phlorizin</td>
<td>56.2 ± 7.4</td>
<td>+0.43 ± 0.9</td>
<td>+0.6 ± 11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Forskolin</td>
<td>+3.4 ± 2.8</td>
<td>+9</td>
<td>+23 ± 39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>+106.8 ± 23.9</td>
<td>+205</td>
<td>+8 ± 137</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-EBIO</td>
<td>+109.7 ± 44.8</td>
<td>177</td>
<td>4 ± 189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetalazolamide</td>
<td>-16.2 ± 3.0</td>
<td>-27</td>
<td>8 ± 43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNDS</td>
<td>-12.2 ± 2.5</td>
<td>-21</td>
<td>10 ± 1</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>Bumetanide</td>
<td>-16.2 ± 3.5</td>
<td>-20</td>
<td>14 ± 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gilbenclamide</td>
<td>-62.0 ± 2.0</td>
<td>-100</td>
<td>5 ± 156</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPAC</td>
<td>-62.0 ± 2.5</td>
<td>-100</td>
<td>5 ± 336</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SE. Responses to first agonist or antagonist addition to unstimulated (basal) Calu-3 cells (high basal). $I_{sc}$, short-circuit current; TER, transepithelial resistance; 1-EBIO, 1-ethyl-2-benzimidazolincarbonic anhydrase, with a half-time of ~5 s (12).

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duce an increase or decrease in $I_{sc}$. For each condition tested (7 different conditions), there was no significant difference between the $I_{sc}$ measured with the Ussing technique and that with the pH stat technique (data not shown). For example, the maximum $HCO_3^-$ current after 1-EBIO and bumetanide was 41 ± 3 $\mu$A/cm² in the pH stat experiments and 52 ± 6 $\mu$A/cm² in conventional Ussing experiments ($n = 11, P > 0.08$). As a second example, we can compare the magnitude of $HCO_3^-$ current in each condition that was blocked by acetazolamide + DNDS. These agents blocked 26 ± 2 $\mu$A/cm² of the $HCO_3^-$ $I_{sc}$ in pH stat experiments and 32 ± 3 $\mu$A/cm² in conventional Ussing chamber experiments ($n = 6, P > 0.1$).

Neither of these measures showed a significant increase in $HCO_3^-$ secretion during the pH stat method; in fact, the trend was toward a smaller $I_{sc}$ when the apical solution lacked $HCO_3^-$. This independence from the $HCO_3^-$ gradient can be explained if there is minimal movement of $HCO_3^-$ across the tight junctions, and if the limiting factor for $HCO_3^-$ $I_{sc}$ is not dependent on either the apical $HCO_3^-$ conductance or on gradient-sensitive mechanisms for $HCO_3^-$ accumulation (e.g., NBC). Instead, the rate-limiting step in maximally stimulated cells appears to be the gradient-insensitive and voltage-insensitive production of $HCO_3^-$ from $CO_2$. The $CO_2$ inside the Calu-3 cells in pH stat experiments is likely to be <5%, because the basolateral $CO_2$ is 5% and the apical $CO_2$ is 0%. Because $CO_2$ and $HCO_3^-$ are in equilibrium inside the cell (assuming constant intracellular pH), the lower $CO_2$ level leads to a lower $HCO_3^-$ level and less $HCO_3^-$ current.

The power of the pH stat technique is that it provides an estimate of the net base ($HCO_3^-)$ movement across the monolayer. The assumption underlying these experiments was that Calu-3 cell $I_{sc}$ is the sum of NKCC-mediated $Cl^-$ secretion plus $HCO_3^-$ secretion, but it quickly became apparent that the assumption was not valid. Instead, an unidentified process was producing a discrepancy between $I_{sc}$ and $I_{eq}$ measurements.

Forskolin-stimulated monolayers in the pH stat experiments had an $I_{sc}$ of 65 ± 5 $\mu$A/cm² ($n = 15$, high basal) or 44 ± 2 $\mu$A/cm² ($n = 15$, low basal), and the $I_{eq}$ was approximately two-thirds of the $I_{sc}$ in both high and low basal conditions (Figs. 4 and 5). Bumetanide blocked 18 ± 3% ($n = 11$) of the $I_{sc}$ and none of the $I_{eq}$ leaving 13% of the forskolin-stimulated $I_{sc}$ unexplained (Fig. 5). Stimulation with 1-EBIO after forskolin produced a further large increase in $I_{sc}$ (110 ± 21%, $n = 6$), but, surprisingly, the $I_{eq}$ remained stable (increase of 2.5 ± 5%, $n = 6$, Fig. 4). We had expected an increase in $I_{eq}$ on the basis of the evidence just presented that 1-EBIO increased the bumetanide-insensitive and acetazolamide-sensitive $I_{sc}$, consistent with increased driving force on carbonic anhydrase-produced $HCO_3^-$. On the other hand, if $HCO_3^-$ secretion depended entirely on an NBC with 2:1 stoichiometry, it should have been eliminated under these conditions (8). Neither expectation was met.

Furthermore, in the presence of bumetanide, 1-EBIO produced a significant increase in $I_{sc}$ but not in $I_{eq}$. This is shown most strikingly in Fig. 5, where, in the presence of bumetanide, 1-EBIO stimulates a 20 $\mu$A/cm² increase in $I_{sc}$ but a slight decrease in $I_{eq}$. On average, in the presence of bumetanide 1-EBIO increased the $I_{sc}$ by 27 ± 9% ($n = 13$) and the $I_{eq}$ by 11 ± 7% ($n = 9$, not significantly different from 0), further increasing the discrepancy between $I_{sc}$ and $I_{eq}$ so that $I_{eq}$ was on average only 70 ± 4% of $I_{sc}$ ($n = 9$, Figs. 4 and 5).

Bovine tracheal epithelial cells (28), Calu-3 cells (14), and cultured human bronchial epithelium (4) each express $H^+\cdotK^+\cdotATPase$, and, at least in the human cells, expression in the apical membrane is only of the nongastric form. These results suggest the possibility that some of the $HCO_3^-$ based $I_{eq}$ was being neutralized by $K^+$-dependent $H^+$ secretion. To test this idea, ouabain (10–20 $\mu$M), a nonspecific $H^+\cdotK^+\cdotATPase$ inhibitor, was added apically after 1-EBIO stimulation and bumetanide inhibition (Fig. 5). Apical ouabain had no effect on the $I_{sc}$ ($n = 9$, showing that it did not have access to the basolateral bath but instead caused a dramatic increase in $I_{eq}$ from 70 ± 4 to 95 ± 1% of the $I_{sc}$ ($n = 9, P < 0.0001$, paired) (see Figs. 5 and 6B).

As an additional test of a role for a putative $H^+\cdotK^+\cdotATPase$, we omitted $K^+$ from the apical solution. With a nominally $K^+$-free apical solution, the $I_{sc}$ and the $I_{sc}$ after 1-EBIO in the presence of bumetanide were equivalent (94 ± 4%, $n = 6, P < 0.0001$ vs. control), and apical ouabain had no further effect on $I_{eq}$ ($n = 6$, Fig. 6A). Similarly, when cells were stimulated with 1-EBIO in the presence of apical ouabain and bumetanide, the $I_{sc}$ and $I_{eq}$ rose together, and $I_{eq}$ was 100 ± 4% of $I_{sc}$ ($n = 9, P < 0.001$ vs. control, Fig. 6B). These results indicate that a ouabain-sensitive, $K^+$-dependent nonelectrogenic process of acid secretion is stimulated in Calu-3 cells by 1-EBIO in parallel with increased $Cl^-$ and $HCO_3^-$ secretion. One possible mechanism for such secretion is the nongastric form of $H^+\cdotK^+\cdotATPase$, which others have shown to be expressed in Calu-3 cells (14, 28).

To determine whether ouabain-sensitive $H^+$ secretion was also stimulated by forskolin, ouabain was added apically during forskolin-stimulated $I_{sc}$ but before 1-EBIO addition. Ouabain did not alter either $I_{sc}$ or $I_{eq}$ in forskolin-stimulated cells ($n = 9$, Fig. 6B), suggesting that $H^+\cdotK^+\cdotATPase$ is not activated by forskolin stimulation.

Fig. 5. pH stat evidence for 1-EBIO stimulation of $HCO_3^-$ secretion and its masking by $H^+$ secretion via $H^+\cdotK^+\cdotATPase$. As in Fig. 4, the top trace is $I_{eq}$ and the bottom trace is computed $I_{sc}$. The monolayer was prestimulated with 10 $\mu$M forskolin. The $Cl^-$ component of the $I_{sc}$ was inhibited with bumetanide but remained larger than the $I_{eq}$. Stimulation with 1-EBIO in the presence of bumetanide increased the $I_{sc}$ by ∼50%, but there was little change in $I_{eq}$. The large gap between $I_{sc}$ and $I_{eq}$ induced by 1-EBIO is caused by concomitant stimulation of $H^+$ secretion, as shown by the effect of apical ouabain (10–20 $\mu$M), which inhibits $H^+\cdotK^+\cdotATPase$ and caused the $I_{eq}$ to increase until $I_{eq} = I_{sc}$. Thus all $I_{eq}$ after bumetanide could be accounted for by $HCO_3^-$ secretion, which was increased by 1-EBIO and unmasked (in terms of $I_{eq}$) by apical ouabain. Subsequent addition of acetazolamide caused a large and equivalent inhibition of both $I_{sc}$ and $I_{eq}$. The remaining secretion of $HCO_3^-$ is proposed to represent the uncatalyzed formation of $HCO_3^-$ from $CO_2$ (see text).
Table 2. $I_{sc}$ with Cl$^-$ as the only permeant anion (zero HCO$_3^-$): NaCl solution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{sc}^*$, $\mu$A/cm$^2$</th>
<th>$\Delta I_{sc}^*$, $\mu$A/cm$^2$</th>
<th>$\Delta I_{sc}$, %</th>
<th>$\Delta$TER, $\Omega$cm$^2$</th>
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<tbody>
<tr>
<td>Basal (+HEPES)</td>
<td>0</td>
<td>7</td>
<td>2,000</td>
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</tr>
<tr>
<td>Initial (+HEPES)</td>
<td>45.1 ± 4.4</td>
<td>30</td>
<td>198.9 ± 25</td>
<td></td>
</tr>
<tr>
<td>Initial + phlorizine</td>
<td>35.1 ± 4.7</td>
<td>-0.2 ± 1.3</td>
<td>-1.7 14</td>
<td>+35</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>35.0 ± 4.2</td>
<td>+102</td>
<td>-110</td>
<td></td>
</tr>
<tr>
<td>1-EBIO</td>
<td>64.2 ± 4</td>
<td>+136</td>
<td>-81</td>
<td></td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>-2.2 ± 0.6</td>
<td>-6</td>
<td>13 +6</td>
<td></td>
</tr>
<tr>
<td>DNDS</td>
<td>+3.2 ± 2.8</td>
<td>+7</td>
<td>10 -2</td>
<td></td>
</tr>
<tr>
<td>Bumetanide</td>
<td>-47.6 ± 8.1</td>
<td>-92</td>
<td>12 -8</td>
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</tr>
<tr>
<td>Glibenclamide</td>
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<td>-98</td>
<td>4 +112</td>
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<tr>
<td>DPAC</td>
<td>-24.6 ± 6.8</td>
<td>-94</td>
<td>5 +180</td>
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*Values are means ± SE. Responses to first agonist or antagonist addition to unstimulated (basal) Calu-3 cells (high basal). Except for the first condition, all other conditions included HEPES buffer. Vehicle (ethanol) decreased $I_{sc}$ by 1.5 ± 0.5 $\mu$A/cm$^2$, -3%.

(n = 12, Table 2) and 100% of the $I_{sc}$ stimulated by either 1-EBIO or thapsigargin (n = 7). Thus NKCC is the major pathway for transporting Cl$^-$ into the cell under all the conditions we tested.

Acetazolamide and DNDS had negligible effects on $I_{sc}$ (Fig. 7 and Table 2). The lack of inhibition by these agents is an important result because it indicates that their ability to inhibit $I_{sc}$ when HCO$_3^-$ is present (34) (Fig. 2 and Table 1) is not a nonspecific effect. This further supports a role for CO$_2$ conversion as well as basolateral HCO$_3^-$ uptake in HCO$_3^-$ secretion (20).

The $I_{sc}$ properties of Calu-3 cells when Cl$^-$ is the only CFTR-permeant anion resemble the properties of T84 cells in normal media, where inhibition of NKCC with bumetanide eliminates essentially all $I_{sc}$, as reported in a long series of experiments by Dharmasathaphorn et al. (9) and others (3).

$I_{sc}$ in Cl$^-$-Free Solutions (HCO$_3^-$ is the Only Permeant Anion)

In these experiments, we attempted to isolate the pathways responsible for HCO$_3^-$ secretion and further assess the relative contributions of CO$_2$ conversion and Na$^+$-HCO$_3^-$ cotransport.

When all Cl$^-$ in the bathing solutions was replaced with isethionate, leaving 25 mM HCO$_3^-$ as the only CFTR-permeant

**Fig. 6.** pH stat with zero K$^+$ in the apical solution to inactivate H$^+$-K$^+$-ATPase. A: in this experiment, basal $I_{sc}$ was low and computed $I_{inj}$ was near zero. Bumetanide had little effect, and forskolin subsequently stimulated a large increase in both $I_{sc}$ and $I_{inj}$ when K$^+$ was absent from the apical solution, as expected if all of the $I_{sc}$ was accounted for by HCO$_3^-$ secretion now unmasked by inactivation of H$^+$-K$^+$-ATPase. Experiments like those provide additional evidence that H$^+$-K$^+$-ATPase is responsible for the $I_{inj}$-to-$I_{sc}$ gap seen in normal K-H solution and further show that H$^+$-K$^+$-ATPase is stimulated by 1-EBIO. DNDS (4 mM basolateral) had only a small inhibitory effect after 1-EBIO stimulation (see text). In this experiment, a large gap separated $I_{inj}$ and $I_{sc}$ until after 1-EBIO stimulation. The nature of this gap is not yet established, but notice that it cannot represent H$^+$ secretion via H$^+$-K$^+$-ATPase because no K$^+$ was present in the apical solution. B: summary of evidence for H$^+$ secretion via H$^+$-K$^+$-ATPase. The bar graph shows $I_{inj}$ as a percentage of $I_{sc}$ in monolayers pretreated with bumetanide and then stimulated with 1-EBIO or thapsigargin, either in control conditions (normal pH stat apical fluid, n = 9) or in conditions in which the putative H$^+$-K$^+$-ATPase was disabled either with apical ouabain (10–20 $\mu$M) added simultaneously (prestim; n = 9) or after 1-EBIO (poststim; n = 9) or K$^+$-free apical fluid (n = 6). Each condition is significantly different from control (P < 0.001).

**Ussing Chamber Experiments in HCO$_3^-$-Free Media (Cl$^-$ as the Only Permeant Anion)**

The results so far indicate that HCO$_3^-$ is secreted by Calu-3 cells under all of the conditions we tested and is derived to a large extent from conversion of CO$_2$ to HCO$_3^-$, because $I_{sc}$ is inhibited by acetazolamide. However, acetazolamide might have other effects including inhibition of NKCC. To study the NKCC transporter in relative isolation, we performed Ussing chamber experiments on Calu-3 cells in HEPES-buffered HCO$_3^-$-free media, where Cl$^-$ is the only CFTR-permeant anion (Table 2).

Figure 7 shows a typical experiment under these conditions. In unstimulated (high basal) or forskolin-stimulated cells, the initial $I_{sc}$ was 45 ± 4 $\mu$A/cm$^2$ if glucose was present in the apical solution and was decreased to 35 ± 5 $\mu$A/cm$^2$ by addition of phlorizin (Table 2). 1-EBIO caused a large sustained increase in $I_{sc}$ of 64 ± 4 $\mu$A/cm$^2$ (n = 4) (Fig. 7 and Table 2). Thapsigargin increased the $I_{sc}$ by 35 ± 4 $\mu$A/cm$^2$ (n = 5). Bumetanide inhibited ~90% of the unstimulated $I_{sc}$ by 10.2 ± 0.3 $\mu$A/cm$^2$ on March 30, 2017.
acinetazolamide inhibited 26 ± 3% of the basal $I_{sc}$ ($n=9$), and DNDS inhibited 25 ± 4% ($n=9$, Table 3). After stimulation with 1-EBIO, the acetazolamide inhibition increased to 45 ± 6% (significant increase, $P=0.005$), and the DNDS inhibition decreased to 11 ± 8% (decrease not significant). The substantial $I_{sc}$ that remained after inhibition with bumetanide, acetazolamide, and DNDS (30 ± 6%, $n=6$) of stimulated $I_{sc}$ could represent $Na^+$ absorption via unknown pathways or continued secretion of $HCO_3^-$ from unknown sources. To decide among these possibilities, glibenclamide and N-phenyl-lanthranilic acid (DPAC), which are frequently used to inhibit CFTR (but have other actions), were tested for their ability to inhibit $I_{sc}$ in $Cl^-$-free media. Each produced nearly complete inhibition of $I_{sc}$ and increased the monolayer resistance ($n=8$), implying that all the $I_{sc}$ was current through CFTR.

The results in zero $Cl^-$ make three points. 1) Because DNDS inhibited the $I_{sc}$, the NBC was still causing an influx of $HCO_3^-$ under these conditions. This could be because $[Na^+]_i$ was reduced secondary to NKCC inactivity, but the magnitude of DNDS inhibition did not differ from that observed in K-H solution. 2) The large effect of acetazolamide emphasizes the importance of $CO_2$ conversion in $HCO_3^-$ secretion. 3) The substantial $I_{sc}$ that remains after both pathways for generating intracellular $HCO_3^-$ concentration are inhibited probably represents continued secretion of $HCO_3^-$ produced by the uncatalyzed conversion of $CO_2$ to $HCO_3^-$, rather than $Na^+$ absorption, because the residual $I_{sc}$ was blocked by glibenclamide and DPAC.

Evidence for a Single Exit Pathway for $HCO_3^-$ and $Cl^-$

CFTR conducts both $Cl^-$ and $HCO_3^-$ (15, 21), and glibenclamide blocks $I_{sc}$ carried by either ion (30, 33). Although glibenclamide is not specific for CFTR (e.g., see Refs. 1, 22), its effect on CFTR might be sufficient to test the hypothesis that both $Cl^-$ and $HCO_3^-$ exit Calu-3 cells via CFTR. Glibenclamide blocked the $I_{sc}$ with identical dose-inhibition curves ($IC_{50}$ of 120 μM) in K-H solution or when $Cl^-$ or $HCO_3^-$ was the only permeant anion (Fig. 9). The conductance dose-

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Table 3. $I_{sc}$ with $HCO_3^-$ as the only permeant anion (zero $Cl^-$, replaced with isethionate): Na$HCO_3^-$ solution (+isethionate)

<table>
<thead>
<tr>
<th>Stimulus Condition</th>
<th>$I_{sc}$, μA/cm²</th>
<th>$\Delta I_{sc}$, μA/cm²</th>
<th>$\Delta I_{sc}$, %</th>
<th>n</th>
<th>δTER, Ω·cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>-42.8 ± 2.8</td>
<td></td>
<td></td>
<td>44</td>
<td>350 ± 49</td>
</tr>
<tr>
<td>Initial + thapsigargin</td>
<td>30.2 ± 4.2</td>
<td>-5.3 ± 1.6</td>
<td>-28</td>
<td>15</td>
<td>+28</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>+52.6 ± 5.1</td>
<td>+107</td>
<td>4</td>
<td>-245</td>
<td></td>
</tr>
<tr>
<td>1-EBIO</td>
<td>+64.2 ± 19.1</td>
<td></td>
<td>163</td>
<td>5</td>
<td>-216</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>-12.9 ± 1.4</td>
<td>-26</td>
<td>9</td>
<td>-51</td>
<td></td>
</tr>
<tr>
<td>DNDS</td>
<td>-12.3 ± 2.1</td>
<td>-25</td>
<td>9</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>Bumetanide</td>
<td>-4.5 ± 2.6</td>
<td>-8</td>
<td>21</td>
<td>-20</td>
<td></td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>-28.9 ± 9.4</td>
<td>-95</td>
<td>6</td>
<td>+67</td>
<td></td>
</tr>
<tr>
<td>DPAC</td>
<td>40.3 ± 2.4</td>
<td>-101</td>
<td>6</td>
<td>+158</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SE. Responses to first agonist or antagonist addition to unstimulated (basal) Calu-3 cells (high basal).
inhibition curves mirrored the \( I_{sc} \) dose-inhibition curves in all three solutions. The Hill slope of these curves was 1, suggesting that one molecule of glibenclamide blocks the relevant channel. (Because the effect of glibenclamide on intracellular ATP concentration occurs at concentrations > 1 mM, this action should not affect a process with an IC\(_{50}\) of 120 \( \mu \)M.)

We conclude that the most likely effect of glibenclamide in these experiments is blockade of a single type of channel that conducts both Cl\(^-\) and HCO\(_3^-\). Presumably, that conductance is CFTR. Because glibenclamide blocks >90% of the \( I_{sc} \), there is no need to propose additional apical Cl\(^-\) or HCO\(_3^-\) conductances or electrogenic transporters. However, these results cannot eliminate electrically silent transport processes, such as an apical anion exchanger, because an electroneutral HCO\(_3^-\) exchanger produces no \( I_{sc} \).

**Estimation of HCO\(_3^-\)/Cl\(^-\) Conductance**

Direct comparisons of the \( I_{sc} \) in Cl\(^-\)-only and HCO\(_3^-\) solutions cannot be made because the driving forces in each condition are not known. However, a comparison is possible if we make the simplifying assumption that, at the peak of the 1-EBIO response, the cell voltage goes to \( E_K \). In HCO\(_3^-\)-only solutions, the initial cell voltage is determined by the HCO\(_3^-\) reversal potential (\( E_{HCO3} \)), the HCO\(_3^-\) conductance (\( G_{HCO3} \)), the \( E_K \), and the initial K\(^+\) conductance (\( G_K \)). The fold increase in the driving force after 1-EBIO stimulation is proportional to (\( G_{HCO3} \)/\( G_K \)). Thus the ratio of the peak fold increase stimulated by 1-EBIO in HCO\(_3^-\)-only solutions and Cl\(^-\)-only solutions should reflect the HCO\(_3^-\)-to-Cl\(^-\) permeability ratio (Fig. 10, A and B).

The peak fold increase in the HCO\(_3^-\)-only solution was 2 (Fig. 10C, open bar) vs. a peak fold increase of 7 in the Cl\(^-\)-only solution (Fig. 10C, solid bar). [The peak fold increase in K-H solution that contains both HCO\(_3^-\) and Cl\(^-\) current was intermediate (Fig. 10C, gray bar), consistent with 1-EBIO stimulation causing both Cl\(^-\) and HCO\(_3^-\) efflux through CFTR.] The ratio of the fold increases was 2/7, giving a HCO\(_3^-\) conductance that is 29% of the Cl\(^-\) conductance, a value similar to estimates for CFTR arrived at by other methods (15, 21, 27). It is possible that the cell voltage does not actually reach \( E_K \) because of the apical anion conductance through CFTR, and this effect will be greatest in Cl\(^-\)-only solutions. Thus 29% of Cl\(^-\) conductance is an upper limit for HCO\(_3^-\) conductance through CFTR.

**DISCUSSION**

Calu-3 cells secrete a HCO\(_3^-\)-rich fluid when stimulated with agents that elevate [cAMP], and a Cl\(^-\)-rich fluid when stimulated with agents that open basolateral K\(^+\) channels (8). The main new result from this paper is that K\(^+\) channel openers also stimulate HCO\(_3^-\) secretion, but the effect is masked by concomitant stimulation of H\(^+\) secretion via an apical mechanism that has the properties of a nongastric H\(^+\)-K\(^+\)-ATPase. A similar mechanism has been proposed by Furukawa et al. (13), where H\(^+\) secretion via Na\(^+\)/H\(^+\) exchanger-3 masks some of the HCO\(_3^-\) secretion by CFTR in rat intestine. It is not yet known whether serous cells in submucosal glands, for which Calu-3 cells are models, possess a similar apical mechanism.

**Evaluation of Model 1**

We began these studies to examine perceived inconsistencies between model 1 (Fig. 1) and results from experiments in our laboratory. We considered three features of the model. 1) Is most HCO\(_3^-\) derived from the activity of an NBC? 2) Does the NBC have a stoichiometry that causes it to be reversed by cellular hyperpolarization, i.e., does it export HCO\(_3^-\) basolaterally after stimulation? 3) As predicted by these features of the model, does activation of basolateral K\(^+\) channels with 1-EBIO cause Calu-3 cells to secrete a highly acidic fluid, essentially devoid of HCO\(_3^-\), with Cl\(^-\) as the only anion (8)? Our results lead us to answer “no” to all three questions.

**Feature 1.** We found that most HCO\(_3^-\) was not derived from NBC-mediated transport but is instead generated by a carbonic anhydrase-dependent mechanism. In our experiments, the DNDs-sensitive pathway contributed ~20% of forskolin-stimulated HCO\(_3^-\) secretion and ~10% of 1-EBIO-stimulated HCO\(_3^-\) secretion (Fig 3B). Most of the HCO\(_3^-\) current was acetazolamide sensitive, suggesting that conversion of CO\(_2\) to HCO\(_3^-\) is the predominant mechanism for generating intracellular HCO\(_3^-\) concentration (Fig. 3B). An acetazolamide-insensitive, DNDs-insensitive source for HCO\(_3^-\) was also found, which could represent uncatalyzed conversion of CO\(_2\) to HCO\(_3^-\), a reaction that proceeds rapidly with a half-time of only ~5 s (12). The unidentified source for HCO\(_3^-\) may also include a contribution from anion exchanger-2, which exists in the basolateral membrane of Calu-3 cells (23). Because none of these pathways are voltage sensitive, they are well suited to mediating HCO\(_3^-\) transport in hyperpolarized cells.

**Feature 2.** We found that the NBC was not reversed by cellular hyperpolarization, because DNDs continued to inhibit \( I_{sc} \) after 1-EBIO stimulation. DNDs inhibition was significantly less after 1-EBIO. These results are consistent with recent experiments on two different NBC [rNKBC (31) and NBC4 (35)] that found that both transport HCO\(_3^-\) 2:1 over
Na+. In both cases, the predicted reversal potential for the NBC was more negative than −100 mV in normal Krebs ringer. If every cation in the cytosol were K+ (~150 mM [K+]i), the potential with a 5 mM extracellular K+ concentration can only reach a maximum negativity (Vm = EK) of −90 mV [log (5/150) × 61 mV], which is not enough to reverse an NBC with 2:1 stoichiometry but will reverse an NBC with a 3:1 stoichiometry. Because reversal was not seen, we propose that the NBC in Calu-3 cells has a stoichiometry not greater than 2:1.

Feature 3. Opening of K+ channels with 1-EBIO did not produce HCO3− -free secretion; instead, it increased HCO3− secretion. The result is a primary secretion consisting of Cl−, HCO3−, Na+, and K+, with the K+ then exchanged for H+ and the H+ then capable of combining with HCO3− to form CO2 and water. We did not attempt to measure K+ secretion, but evidence favors an apical K+ channel in Calu-3 cells (6), and theoretical considerations suggest that optimal secretion requires 10–20% of the K+ conductance to be apical (5). We have shown that when K+ is present in the apical solution, it is exchanged for H+, and, under the conditions of our pH stat experiments, it neutralizes part of the HCO3−.

In summary, our present results for hyperpolarized Calu-3 cells supply a mechanism to reconcile the increased HCO3− secretion measured by Lee et al. (20) with the lack of HCO3− secretion proposed by Devor et al. (8). As shown in Figs. 5–6, stimulation with 1-EBIO or thapsigargin in the presence of bumetanide increases Iw by increasing both HCO3− secretion and H+ secretion. H+ secretion was blocked either with apical ouabain or elimination of apical K+, suggesting that H+−K+−ATPase is responsible. An apical H+−K+−ATPase has been reported in Calu-3 cells (14) and in human surface airway epithelium (4). In the human airway, the activity of H+−K+−ATPase does not appear to be regulated (4). Furthermore, its sensitivity to ouabain (500 [μM] was used) may be less than we observed; the inhibitory curve for the nongastric H+−K+−ATPase protein expressed from ATP1AL1/βΗ,K (25) can accommodate both our observations and those in surface airway epithelium. Thus the ouabain-sensitive mechanism described here could also be a variant nongastric H+−K+−ATPase or yet another K+−dependent mechanism.

Role of CFTR

Model 1 proposes that both Cl− and HCO3− are conducted through CFTR, with no provision made for apical anion exchangers. Our results strongly support that aspect of model 1: either both anions are conducted through CFTR, or their secretion is mediated by a transporter that is completely dependent on CFTR. We show two CFTR channels in Fig. 1 for clarity and also because of evidence that Cl− and HCO3− conductances mediated by CFTR can be independently controlled (29).

Implications for Airway Submucosal Gland Mucus Secretion

The gland serous cells that are modeled by Calu-3 cells contribute ~50% of the secretory apparatus of glands, with the other 50% consisting of mucous cells that do not express CFTR. Secretions from both types of cells pass through a ducal system comprising at least two cell types, which presumably modifies the secretions in as-yet-unknown ways. We noted in the introduction that predictions from model 1 do not fit with observations of secretions from actual glands, which invariably have less than one-half as much HCO3− as the bath (mucus pH ~7.0 vs. bath pH of 7.4) under all stimulation conditions, across species and in both CP and control subjects (16, 18). As a further paradox, secretions from human airway glands are hypotonic (16, 19). To explain these results, we originally hypothesized that cells in the gland (mucous cells?) secrete HCO3− via a non-CFTR-dependent pathway and that secondary processes in the gland modified the secretions homeostatically so that the pH of the final secretion is adjusted to near pH 7, regardless of the starting secretion. We had no explanation for how gland secretions might become hypotonic.

A New Role for HCO3−: the Ion Subtraction Hypothesis

The discovery of a regulated apical pathway for H+ secretion (H+−K+−ATPase) provides one possible mechanism for adjusting the pH of the submucosal gland secretions. Furthermore, concordant H+ and HCO3− secretion provides a potential explanation for 1) pH regulation, 2) hypotonicity of gland secretions, and 3) the discordance between expected high levels of HCO3− secretion from gland serous cells and the observation that the final secretions from glands are essentially neutral. Unlike in the large volumes of an Ussing chamber, the secretions of gland cells completely determine the composition of their luminal fluid. The importance of controlling pH and osmolarity is likely to be crucial given the abundant quantities of diverse macromolecules that are also secreted by the gland cells. If the two-step conversion of K+−HCO3− to H+−HCO3− and then to CO2 + H2O that has been demonstrated here can be shown to apply to intact glands, it will provide a new control mechanism for both the tonicity and pH of airway surface liquid. It is apparent that this mechanism also alters the expected consequences of lost CFTR function.

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We thank Kim Winges and Dennis Lee for technical assistance and William Reenstra for discussions on uncatalyzed interconversion of CO2 and H2O to HCO3−.

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