Bicarbonate and Chloride Secretion in Calu-3 Human Airway Epithelial Cells

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ABSTRACT Serous cells are the predominant site of cystic fibrosis transmembrane conductance regulator expression in the airways, and they make a significant contribution to the volume, composition, and consistency of the submucosal gland secretions. We have employed the human airway serous cell line Calu-3 as a model system to investigate the mechanisms of serous cell anion secretion. Forskolin-stimulated Calu-3 cells secrete HCO$_3^-$ by a Cl$^-$-independent, serosal Na$^+$-dependent, serosal bumetanide-insensitive, and serosal 4,4'-dinitrostilben-2,2'-disulfonic acid (DNDS)-sensitive, electrogenic mechanism as judged by transepithelial currents, isotopic fluxes, and the results of ion substitution, pharmacology, and pH studies. Similar studies revealed that stimulation of Calu-3 cells with 1-ethyl-2-benzimidazolinone (1-EBIO), an activator of basolateral membrane Ca$^{2+}$-activated K$^+$ channels, reduced HCO$_3^-$ secretion and caused the secretion of Cl$^-$ by a bumetanide-sensitive, electrogenic mechanism. Nystatin permeabilization of Calu-3 monolayers demonstrated 1-EBIO activated a charybdotoxin- and clotrimazole-inhibited basolateral membrane K$^+$ current. Patch-clamp studies confirmed the presence of an intermediate conductance inwardly rectified K$^+$ channel with this pharmacological profile. We propose that hyperpolarization of the basolateral membrane voltage elicits a switch from HCO$_3^-$ secretion to Cl$^-$ secretion because the uptake of HCO$_3^-$ across the basolateral membrane is mediated by a 4,4'-dinitrostilben-2,2'-disulfonic acid (DNDS)-sensitive Na$^+$.HCO$_3^-$ cotransporter. Since the stoichiometry reported for Na$^+$.HCO$_3^-$ cotransport is 1:2 or 1:3, hyperpolarization of the basolateral membrane potential by 1-EBIO would inhibit HCO$_3^-$ entry and favor the secretion of Cl$^-$ Therefore, differential regulation of the basolateral membrane K$^+$ conductance by secretory agonists could provide a means of stimulating HCO$_3^-$ and Cl$^-$ secretion. In this context, cystic fibrosis transmembrane conductance regulator could serve as both a HCO$_3^-$ and a Cl$^-$ channel, mediating the apical membrane exit of either anion depending on basolateral membrane anion entry mechanisms and the driving forces that prevail. If these results with Calu-3 cells accurately reflect the transport properties of native submucosal gland serous cells, then HCO$_3^-$ secretion in the human airways warrants greater attention.

KEY WORDS: submucosal glands • cystic fibrosis • cystic fibrosis transmembrane conductance regulator • sodium bicarbonate cotransporter • serous cells

INTRODUCTION

The inherited disease cystic fibrosis (CF) is characterized by secretion of a thick viscous mucus that plugs the submucosal glands and small airways. This leads to chronic airway infections and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) (Boat et al., 1989). The predominant site of CFTR expression in the human lung is the serous cells of the submucosal glands (Jacquot et al., 1993; Engelhardt et al., 1994). Serous cells account for 60% of the cellular volume of the submucosal gland in human airways (Basbaum et al., 1990). Stimulation of an isotonic fluid secretion from the serous cells contributes to the hydration of the secretions from the mucous cells, thereby forming the low viscosity mucus that lines the conducting airways. Serous cells are also a major source of antimicrobial enzymes and peptides that help maintain an aseptic environment in the lungs (Basbaum et al., 1990). Salt concentration can influence the activity of these antimicrobial agents and it was recently suggested that altered salt concentration in the airway surface fluid may contribute to chronic airway infection in CF (Smith et al., 1996). Thus, the serous cells make a significant contribution to the volume, composition, and consistency of the submucosal gland secretions and represent a potentially important target in CF therapy. These considerations indicate the importance in understanding the mechanisms of fluid and electrolyte transport by serous cells.

Shen et al. (1994) screened 12 cell lines derived from lung adenocarcinomas in an attempt to identify a cell
line that displayed electrophysiological properties consistent with human airway serous cells. They identified the Calu-3 cell line as being serous cell in nature, forming a monolayer with a transepithelial resistance of ~100 Ω cm², expressing high levels of CFTR and responding to both cAMP- and Ca²⁺-mediated agonists with changes in net transepithelial ion transport as measured by short circuit current (Isc) (Finkbeiner et al., 1993; Shen et al., 1994). Several studies have produced variable results in the basal and stimulated transport properties of the Calu-3 cells and the ionic basis of the responses to secretory agonists remains unsettled (Shen et al., 1994; Illek et al., 1997; Moon et al., 1997; Singh et al., 1997; Lee et al., 1998). In this report, we present studies with Calu-3 cells that displayed a low basal Isc (13 μA cm⁻²) and robust sustained responses to secretory agonists enabling the measurement of isotopic fluxes. The results demonstrate that Calu-3 cells, when stimulated by forskolin, secrete HCO₃⁻ by a Cl⁻-dependent, Na⁺-dependent, 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS)-sensitive, electrogenic mechanism. Secondly, when stimulated by 1-ethyl-2-benzimidazoline (1-EBIO), an activator of the basolateral membrane Ca²⁺-activated K⁺ channels (KCa) (Devor et al., 1996), HCO₃⁻ secretion is reduced and the Calu-3 cells secrete predominately Cl⁻ by a bumetanide-sensitive, electrogenic mechanism.

**Methods**

**Cell Culture**

Calu-3 cells were grown in Dulbecco’s modified Eagle’s medium and Ham’s F-12 (1:1) supplemented with 15% fetal bovine serum and 2 mM glutamine. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Measurements of short-circuit current (Isc), Calu-3 cells were seeded onto Costar Transwell cell culture inserts (0.33 cm²) or Snapwell inserts (1.1 cm²). Both the Transwell and Snapwell inserts were collagen-coated overnight with 0.01% human placenta collagen type VI (Sigma Chemical Co.). On day one, the medium bathing the apical surface was removed to establish an air interface. Apical medium was removed and the cells fed every 48 h. After ~7-14 d, the cells formed a confluent monolayer that held back fluid, thus forming an apical air interface. Short circuit current measurements were performed after an additional 14-28 d in culture. Patch-clamp experiments were performed on single cells plated onto glass cover slips 18-48 h before use.

**Solutions**

For measurements of Isc, the bath solution contained (mM): 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 10 glucose. Mannitol was substituted for glucose in the mucosal solution to eliminate the contribution of Na⁺ gluconate cotransport to Isc as previously reported by Singh et al. (1997). The pH of this solution was 7.4 when gassed with a mixture of 95% O₂-5% CO₂ at 37°C. For the Cl⁻-free solution, equimolar Na-gluconate replaced NaCl, 1 mM Mg-gluconate replaced MgCl₂, and 4 mM Ca-gluconate replaced CaCl₂. Calcium was increased to 4 mM to compensate for the Ca²⁺ buffering capacity of the gluconate. The HCO₃⁻-free buffer consisted of (mM): 145 NaCl, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, 10 HEPES, pH adjusted with NaOH, 10 glucose or mannitol and was gassed with air. For the Na⁺-free Cl⁻-free solution, equimolar N-methyl-d-glucamine-gluconate replaced NaCl, choline-HCO₃ replaced NaHCO₃, 1 mM Mg-gluconate replaced MgCl₂, and 4 mM Ca-gluconate replaced CaCl₂. This solution contained 10 μM atropine to block the cholinergic effect of choline (Muallem et al., 1988).

The effects of forskolin and 1-EBIO on apical membrane Cl⁻ currents (Isc) were assessed after permeabilization of the serosal membrane with nystatin (360 μg/ml), and the establishment of a mucosa-to-serosa Cl⁻ concentration gradient. Serosal NaCl was replaced by equimolar Na-gluconate and Ca²⁺ was increased to 4 mM with Ca-gluconate. Nystatin was added to the serosal membrane 15-30 min before the addition of drugs. Successful permeabilization of the basolateral membrane was based upon the recording of a current consistent with the mucosal-to-serosal flow of negative charge. The effect of 1-EBIO on basolateral membrane K⁺ currents (Isc) was assessed after permeabilization of the apical membrane with nystatin (180 μg/ml) for 15-30 min, and establishment of a mucosa-to-serosa K⁺ concentration gradient. For measurements of Isc, mucosal NaCl was replaced by equimolar K-gluconate, while serosal NaCl was substituted with equimolar Na-gluconate. Calcium and Mg²⁺ salts were replaced as above.

During inside-out patch-clamp recordings, the bathing solution contained (mM): 145 K-gluconate, 5 KCl, 1 MgCl₂, 1 EGTA, 0.78 CaCl₂, (free Ca²⁺ = 400 nM), and 10 HEPES, pH adjusted to 7.2 with KOH. The pipette solution contained (mM): 140 K-gluconate, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES, pH adjusted to 7.2 with KOH. For outside-out recordings, the bathing solution contained 1 mM CaCl₂ in the absence of any added EGTA, while the pipette solution Ca²⁺ was buffered to 200 mM with EGTA (0.71 mM Ca²⁺, 1 mM EGTA).

**Short-Circuit Current (Isc) Measurements**

Transwell inserts were mounted in an Ussing chamber (Jim’s Instruments). Snapwell inserts were mounted in Ussing chambers (NavिCyte), and the monolayers were continuously short-circuited after fluid resistance compensation using automatic voltage clamps (558C-5; Iowa Bioengineering). Transepithelial resistance (Rt) was measured by open-circuiting the monolayer, or with a 2-mV bipolar pulse and the resistance calculated by Ohm’s law. Forskolin, 1-EBIO, clotrimazole, 293B, and acetazolamide were added to both sides of the monolayers at the indicated concentrations. Bumetanide and charybdotoxin (CTX) were added only to the serosal bathing solution.

**Unidirectional Ion Fluxes**

20 min after the Snapwell filters were mounted in Ussing chambers, isotopes (³⁶Cl, ²²Na, or ⁸⁶Rb) were added to the bath solution on one side of the monolayers. After an additional 20 min, by which time isotopic fluxes had reached a steady state, two 0.4 ml samples were taken from the unlabelled side and fresh unlabelled solution of equal volume was added. This time was considered time = 0 (T₀), and samples were taken thereafter at 15-min intervals for the next 75 min. When the effects of forskolin, 1-EBIO, or forskolin plus 1-EBIO were studied, the drugs were added to the serosal and mucosal sides at T₃₀ and fluxes before (T₃₀ - T₀) and 15 min after the drug additions (T₇₅ - T₃₀) were compared. Isotope activities were determined in a Packard liquid scintillation counter. All samples were weighed and these volumes were used to correct the chamber volume and to calculate the unidirectional ion fluxes using standard equations (Bridges et al., 1983). The net residual ion flux (Jₑₛₑ) was calculated from
the difference in $I_g$ and the net fluxes of $\text{Cl}^-$, $\text{HCO}_3^-$, $\text{Na}^+$, $\text{K}^+$, and $\text{Rb}^+$, $J_{\text{net}}$, where $J_{\text{net}} = I_g - (J_{\text{Rb}^+} + J_{\text{K}^+} - J_{\text{Cl}^-})$.

### Single Channel Recording

Single channel currents were recorded in the inside-out and outside-out patch-clamp recording configuration using a List EPC-7 amplifier (Medical Systems) and recorded on videotape for later analysis as described previously (Devor and Frizzell, 1993). Pipettes were fabricated from KG-12 glass (Willmard Glass Co.). All recordings were done at a holding voltage of $-100$ mV. The voltage is referenced to the extracellular compartment as the standard method for membrane potentials. Inward currents are defined as the movement of positive charge from the extracellular compartment to the intracellular compartment, and are presented as downward deflections from baseline in all recording configurations.

Single channel analysis was performed on records sampled after low-pass filtering at 400 Hz. Data records for all experimental conditions were at least 60 s long. The $n_P$, the product of the number of channels, $n$, and the channel open probability, $P_o$, of the channels was determined using Biopatch software (3.11; Molecular Kinetics). $n_P$ was calculated from the mean total current (i) divided by the single channel current amplitude (i), such that $n_P = I_i/i$. $i$ was determined from the amplitude histogram of the current record.

### Chemicals

Nystatin was a generous gift from Dr. S. Lucania (Bristol Meyers-Squibb). 293B (trans-6-cyano-4-(N-ethylsulfonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chroman) was a generous gift from Dr. Rainer Greger (Albert-Ludwigs-Universitat, Freiberg, Germany). 1-EBIO, 293B, and clotrimazole were made as a 10 mg/ml stock solution in DMSO and sonicated for $15$ min. 1-EBIO was obtained from Aldrich Chemical Co. Acetazolamide, charybdotoxin, and bumetanide were obtained from Sigma Chemical Co. Forskolin was obtained from Calbiochem. NDNS was from Pfaltz and Bauer. Charybdotoxin was obtained from Accurate Chemical and Scientific Corp. and made as a 10 μM stock solution in standard bath solution. 1-EBIO, 293B, and clotrimazole were made as >1,000-fold stock solutions in DMSO. Nystatin was made as a 180 mg/ml stock solution in DMSO and sonicated for 30 s just before use. Forskolin and bumetanide were made as 1,000-fold stock solutions in ethanol. Cell culture medium was obtained from GIBCO BRL.

### Data Analysis

All data are presented as means ± SEM, where $n$ indicates the number of experiments.

### Results

#### Effects of Forskolin on $I_g$

In total, we evaluated 216 filters with standard bath solutions on the mucosal and serosal membrane surfaces. The basal $I_g$ and $R_T$ under these conditions averaged $13 \pm 0.8$ μA · cm$^{-2}$ (range 2–21 μA · cm$^{-2}$) and $353 \pm 14$ Ωcm$^2$ (range 187–667 Ωcm$^2$), respectively. Forskolin (2–10 μM) induced, in all filters tested ($n = 109$), a damped oscillatory response that became stable and sustained after 5–10 min at a plateau value of $66 \pm 4$ μA · cm$^{-2}$ (range 50–103 μA · cm$^{-2}$). A representative current trace is shown in Fig. 1 A. The increase in $I_g$ caused by forskolin was accompanied by a decrease in $R_T$ to an average of $189 \pm 7$ Ωcm$^{-2}$ (range 111–333 Ωcm$^{-2}$). Bumetanide (20 μM), an inhibitor of the NaK2Cl cotransporter, caused a small inhibition of the forskolin-stimulated $I_g$ ($\Delta = -4.9 \pm 1.3$ μA · cm$^{-2}$, $n = 11$). The failure of bumetanide to inhibit the forskolin-stimulated increase in $I_g$ suggests that the NaK2Cl cotransporter does not contribute to the $I_g$, and this raised the question whether the $I_g$ was due to $\text{Cl}^-$ secretion. Additional experiments were performed to establish the ionic basis of the forskolin-stimulated $I_g$.

### Effects of Forskolin on Isotopic Fluxes

To help elucidate the ionic basis of the forskolin-induced increase in $I_g$, we performed unidirectional ion flux measurements with $^{36}\text{Cl}$, $^{22}\text{Na}$, or $^{86}\text{Rb}$; the latter was used as a measure of $K^+$ movements. The $\text{Cl}^-$ flux studies are shown in Fig. 1 B and are summarized together with $\text{Na}^+$ and $\text{Rb}^+$ fluxes in Table I. As in the previous experiments, there was a small basal $I_g$ under control conditions of $\sim$8 μA · cm$^{-2}$ (i.e., 0.3 μEq · cm$^{-2}$ · h$^{-1}$) that was stimulated 6–10-fold by forskolin in the subset of 36 filters used for the flux studies. Under control conditions, there was no net movement of $\text{Cl}^-$ or $\text{Rb}^+$ and a small net absorption of $\text{Na}^+$. Forskolin increased both unidirectional fluxes of $\text{Cl}^-$ four- to fivefold (Fig. 1 B). Both $\text{Rb}^+$ fluxes were increased 1.5-fold, but forskolin had no effect on the fluxes of $\text{Na}^+$ (Table I). Because both unidirectional fluxes of $\text{Cl}^-$ and $\text{Rb}^+$ were increased to a similar extent, there was no net flux of $\text{Cl}^-$ or $\text{Rb}^+$ caused by forskolin. The difference between $I_g$ and the net flux of each ion was calculated and is given in Table I as $J_{\text{net}}$. Because there was no net flux of $\text{Cl}^-$ or $\text{Rb}^+$ under control or forskolin conditions, neither of these ions account for the basal or forskolin-stimulated $I_g$. However, the net absorption of $\text{Na}^+$ fully accounts for the control, basal $I_g$, and a small portion (15%) of the $I_g$ in the forskolin-stimulated cells. When the flux studies for $\text{Cl}^-$, $\text{Na}^+$, and $\text{Rb}^+$ were combined to calculate the $J_{\text{net}}$ using the mean $I_g$ (control 0.31 ± 0.053 μEq · cm$^{-2}$ · h$^{-1}$; forskolin 2.60 ± 0.144 μEq · cm$^{-2}$ · h$^{-1}$, $n = 36$) for the studies in Table I, the control $J_{\text{net}}$ was $-0.12 \pm 0.11$ μEq · cm$^{-2}$ · h$^{-1}$, and the forskolin $J_{\text{net}}$ was $2.37 \pm 0.189$ μEq · cm$^{-2}$ · h$^{-1}$. These results demonstrate that the forskolin-induced increase in $I_g$ cannot be accounted for by the net transepithelial secretion of $\text{Cl}^-$ or the absorption of $\text{Na}^+$ or $K^+$. Rather, the increase in $I_g$ caused by forskolin must be attributed to the net movement of an unmeasured ion, often referred to as the net residual ion flux, $J_{\text{net}}^R$. Because $\text{HCO}_3^-$ is the only remaining ion of significant concentration, $J_{\text{net}}^R$ is likely to be due to the net secretion of $\text{HCO}_3^-$ and additional experiments were performed to test this hypothesis.
Ion Substitution Studies

Ion substitution experiments were performed to help further establish the ionic basis of the forskolin-stimulated $I_{sc}$. Consistent with the failure of bumetanide to inhibit the forskolin-stimulated $I_{sc}$ and the $J_{cl}$ of 0.09 ± 0.09 mEq/cm² h⁻¹, substitution of Cl⁻ with gluconate caused only a partial reduction of the response to forskolin (Fig. 2 A). Similar to the control response, the $I_{sc}$ response to forskolin in Cl⁻-free solution was rapid in onset with a transient peak and a sustained plateau of 46 ± 1.6 μA cm⁻² ($n$ = 24) (Fig. 2 A). The subsequent addition of Cl⁻ (30-60 mM) to the mucosal or serosal solution did not cause a further increase in $I_{sc}$ (data not shown). As in the Cl⁻ containing solution, bumetanide (20 μM serosal) had no effect on the forskolin-stimulated $I_{sc}$ ($Δ$ 0.15 ± 0.76 μA cm⁻², $n$ = 6) (Fig. 3). In contrast, removal of HCO₃⁻ from the mucosal and serosal bathing solutions resulted in a greatly diminished response to forskolin (Fig. 2 B). After a transient response, $I_{sc}$ was increased by only 4 ± 1 μA cm⁻² ($n$ = 10) in HCO₃⁻-free solutions. Substitution of Na⁺ with N-methyl-D-glucamine, Cl⁻ with gluconate, and NaHCO₃ with choline HCO₃ also resulted in a greatly reduced response to forskolin.

### Table I

| Flux Type      | Jₘₘ | Jₘₙ | Jₙₘ | Iₘ | Rₜ | Jₙₑ |
|----------------|-----|-----|-----|----|----|-----|
| Chloride Fluxes|     |     |     |    |    |     |
| Control        | 0.53 ± 0.059 | 0.51 ± 0.091 | +0.02 ± 0.108 | 0.27 ± 0.045 | 339 ± 24 | 0.29 ± 0.117 |
| Forskolin      | 2.53 ± 0.151 | 2.44 ± 0.208 | +0.09 ± 0.257 | 2.55 ± 0.109 | 202 ± 14 | 2.64 ± 0.279 |
| Sodium Fluxes  |     |     |     |    |    |     |
| Control        | 2.27 ± 0.154 | 1.81 ± 0.184 | +0.46 ± 0.239 | 0.37 ± 0.074 | 294 ± 21 | 0.09 ± 0.250 |
| Forskolin      | 2.22 ± 0.205 | 1.89 ± 0.211 | +0.33 ± 0.294 | 2.36 ± 0.186 | 154 ± 17 | 2.03 ± 0.347 |
| Rubidium Fluxes|     |     |     |    |    |     |
| Control        | 0.03 ± 0.005 | 0.04 ± 0.003 | +0.01 ± 0.020 | 0.29 ± 0.03 | 454 ± 27 | 0.30 ± 0.036 |
| Forskolin      | 0.05 ± 0.008 | 0.06 ± 0.005 | +0.01 ± 0.006 | 2.9 ± 0.128 | 190 ± 10 | 2.91 ± 0.129 |

Flux values and $I_{sc}$ are in μEq cm⁻² h⁻¹, and resistance ($Rₜ$) in Ω cm². Measurements were made before and after the addition of forskolin (2 μM) to the mucosal (m) and serosal (s) solutions. Values are the mean ± SEM, $n$ = 6 for each unidirectional flux and 12 for $J_{net}$, $I_{sc}$, $Rₜ$, and $J_{net}$. See text for explanation of $J_{net}$.
caused a transient increase in $I_{sc}$ without a sustained plateau in the Na$^+$-, Cl$^-$-, HCO$_3^-$-containing solution (Fig. 2 C), which resembles the response in Na$^+$-free media. However, the subsequent addition of Na$^+$ (30 mM) to the serosal but not the mucosal solution caused a sustained increase in $I_{sc}$ of $24 \pm 1.0 \mu A \cdot cm^{-2} (n = 12)$ in forskolin-stimulated cells (Fig. 4). Addition of Na$^+$ (30 mM) to the serosal solution before forskolin caused a small decrease in $I_{sc}$ $\Delta -7.6 \pm 0.2 \mu A \cdot cm^{-2} (n = 12)$ as expected for the serosal-to-mucosal diffusion of a cation. This decrease in $I_{sc}$ was reversed and $I_{sc}$ rose to a sustained level of $23 \pm 0.8 \mu A \cdot cm^{-2} (n = 12)$ with the subsequent addition of forskolin. Thus, the forskolin-stimulated increase in the $I_{sc}$ was Cl$^-$ independent but Na$^+$ and HCO$_3^-$ dependent.

Pharmacology Studies

The above results are consistent with forskolin-stimulated net secretion of HCO$_3^-$. To further test this hy-
Hypothesis, the pharmacological sensitivity to various inhibitors of \( \text{HCO}_3^- \) transport were evaluated. The carbonic anhydrase inhibitor, acetazolamide (1 mM mucosal and serosal), caused a 27% decrease (a reduction of 13 ± 1 \( \mu \text{A cm}^{-2} \), \( n = 6 \)) in the forskolin-stimulated \( I_{sc} \) in \( \text{Cl}^- \)-free solutions (Fig. 3). DNDS (3 mM), an inhibitor of \( \text{Cl}^-/\text{HCO}_3^- \) exchangers and \( \text{Na}^+/\text{K}^+ \) cotransporters, was without effect when added to the mucosal solution (\( \Delta = 0.2 \mu \text{A cm}^{-2}, n = 6 \)), but caused an inhibition of 56% (\( \Delta = 26 \pm 1 \mu \text{A cm}^{-2}, n = 6 \)) when added to the serosal side in \( \text{Cl}^- \)-free solutions. Similar results were obtained in \( \text{Cl}^- \)-containing solutions (\( \Delta = 2.5 \pm 1.3 \mu \text{A cm}^{-2}, n = 6 \) mucosal; \( \Delta = 27 \pm 2 \mu \text{A cm}^{-2}, n = 6 \) serosal). The half maximal inhibitory concentration (\( K_i \)) for serosal DNDS was 300 \( \mu \text{M} \).

The inhibitory effects of serosal DNDS and acetazolamide were additive, together causing a 75% decrease in \( I_{sc} \). The \( \text{Na}^+/\text{K}^- \)-ATPase inhibitor, ouabain (100 \( \mu \text{M} \)), caused an immediate and complete inhibition of the forskolin-stimulated \( I_{sc} \). Neither CTX (50 nM), a blocker of \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channels (Garcia et al., 1995), nor 293B (100 \( \mu \text{M} \)), a blocker of the cAMP/PKA activated \( \text{K}^+ \) channel (KvLQT1; Lohrmann et al., 1995; Loussouarn et al., 1997) inhibited the forskolin-stimulated \( I_{sc} \). The nonselective \( \text{K}^+ \) channel blocker, \( \text{Ba}^{2+} \) (5 mM serosal side), inhibited the forskolin-stimulated \( I_{sc} \) by only 10 ± 2 \( \mu \text{A cm}^{-2} \) (\( n = 6 \)).

The requirement for serosal \( \text{Na}^+ \), the inhibition by ouabain, and the partial inhibition by serosal DNDS suggests some of the secreted \( \text{HCO}_3^- \) is mediated by the uptake of \( \text{HCO}_3^- \) across the basolateral membrane on a \( \text{Na}^+:\text{HCO}_3^- \) cotransporter.\(^2\) The partial inhibition of \( I_{sc} \) by acetazolamide suggests some of the secreted \( \text{HCO}_3^- \) originates from a metabolic source. The \( \text{Cl}^- \) independence and the failure of mucosal DNDS to inhibit \( I_{sc} \) suggests the exit of \( \text{HCO}_3^- \) across the apical membrane is not mediated by a \( \text{Cl}^-/\text{HCO}_3^- \) exchanger.

**pH Studies**

The above results are consistent with the conclusion that forskolin stimulation causes the electrogenic secretion of \( \text{HCO}_3^- \). To further test this hypothesis, we performed experiments to determine whether forskolin caused an alkalinization of the apical solution. Calu-3 cells were studied under open circuit conditions with a small volume of fluid (100 \( \mu \text{l} \)) on the apical surface (1.1 \( \text{cm}^2 \)) and 5 ml of continuously gassed (95% O\(_2\)/5% CO\(_2\)) NaCl, NaHCO\(_3\) buffer, pH 7.4, on the serosal side. Cells were incubated without or with forskolin (2 \( \mu \text{M} \)) and the apical solution collected after 90 min. The apical sample was thoroughly gassed before mea-

\(^2\)White (1989) has reported a complete inhibition by 1 mM DNDS of a \( \text{Na}^+:\text{HCO}_3^- \) cotransporter in the basolateral membrane of salamander intestine. Newman (1991) has reported a 73% inhibition by 2 mM DNDS of a \( \text{Na}^+:\text{HCO}_3^- \) cotransporter in retinal glial cells of the salamander. Although perhaps not directly comparable, Boron and Knakal (1989) reported a DNDS \( K_i \) of 300 \( \mu \text{M} \) of a \( \text{Na}^+/-\text{Cl}^- \)-dependent \( \text{HCO}_3^- \) cotransporter in the squid axon.
suring its pH with a miniature pH electrode. Studied in this manner, we found forskolin caused an alkalinization of the apical solution to a pH of 7.8 ± 0.06 (n = 6), whereas control untreated filters showed a small acidification of the apical solution, pH 7.3 ± 0.05 (n = 6). The forskolin-stimulated alkalinization of Δ0.5 pH over a 90-min period corresponds to the net movement of $\text{HCO}_3^-$ of 1.7 μeq·cm$^{-2}·h^{-1}$ or 46 μA·cm$^{-2}$, a value in good agreement with the forskolin-stimulated increase in $I_{c}$ of 53 μA·cm$^{-2}$ under short circuit conditions. Based on these pH measurements, the ion flux measurements, the ion substitution studies, and the pharmacology studies, we conclude that the forskolin-induced $I_{c}$ response in Calu-3 cells is due to the net secretion of $\text{HCO}_3^-$ by a $\text{Cl}^-$-independent $\text{Na}^+$-dependent, and DNDS-sensitive electrogenic mechanism.

Effects of 1-EBIO on Calu-3 Cells

We previously demonstrated that the novel benzimidazolone, 1-EBIO, induced a sustained transepithelial $\text{Cl}^-$ secretory response in rat colonic mucosa, human colonic T84 cells, and murine airway epithelia (Devor et al., 1996). CTX and clotrimazole inhibited the 1-EBIO-stimulated $\text{Cl}^-$ secretion consistent with the activation of basolateral membrane $\text{K}^+$ channels that was confirmed in permeabilized monolayers (Devor et al., 1996, 1997). Moreover, patch clamp studies demonstrated 1-EBIO activates an inwardly rectifying, calcium activated, CTX, and clotrimazole-sensitive $\text{K}^+$ channel (Devor et al., 1996, 1997). Permeabilized monolayers revealed 1-EBIO also activates an apical membrane $\text{Cl}^-$ conductance (Devor et al., 1996). The studies reported here were performed to determine if 1-EBIO would have similar effects on Calu-3 cells.

In 46 experiments, 1-EBIO (1 mM) increased $I_{c}$ from a basal value of 8 ± 0.8 to 62 ± 4 μA·cm$^{-2}$ with only a modest decrease in $R_T$ (control 397 ± 21 Ω·cm$^2$ vs. 1-EBIO 336 ± 20 Ω·cm$^2$). A current trace of a typical $I_{c}$ response to 1-EBIO is shown in Fig. 5 A. The response was rapid in onset and sustained over a long period. Dose-response studies revealed the half maximal effective concentration of 1-EBIO was ~500 μM. Consistent with the activation of the $K_{Ca}$ channels, CTX (50 nM) inhibited 47% of the 1-EBIO-stimulated $I_{c}$. The half maximal effective concentration of CTX was 3.2 nM (n = 4). Clotrimazole (10 μM), a nonpeptide inhibitor of $K_{Ca}$, also inhibited 87.6 ± 1.9% (n = 5) of the response to 1-EBIO with a $K_i$ of 1.2 μM (n = 5). Bumetanide (20 μM) inhibited ~50% of the 1-EBIO-stimulated $I_{c}$ (Table II). DNDS and acetazolamide caused only small (<10%) decreases in the 1-EBIO-stimulated $I_{c}$.

Unidirectional fluxes of $\text{HCO}_3^-$ revealed that 1-EBIO caused the net secretion of $\text{Cl}^-$ (Fig. 5 B and Table II). As in previous experiments (Fig. 1 B), there was no net

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**Figure 5.** Effects of 1-EBIO and bumetanide on Calu-3 cells $I_{c}$ and $\text{Cl}^-$ fluxes (A) short-circuit current trace demonstrating the increase in $I_{c}$ in response to 1-EBIO (1 mM) and the inhibition by bumetanide (20 μM). (B) Unidirectional and net ion fluxes of $\text{Cl}^-$ are shown for time periods before and after the addition of 1-EBIO. Note that 1-EBIO caused a sixfold increase in $J_{ms}$, no change in $J_{sm}$, and a net secretion of $\text{Cl}^-$ that, as summarized in Table II, was nearly equal to the increase in $I_{c}$. The 1-EBIO-stimulated secretion of $\text{Cl}^-$ was inhibited by bumetanide (Table II), as anticipated from the inhibition in $I_{c}$. Fluxes are plotted as the absolute values.
secretion of Cl\(^{-}\) in control monolayers. 1-EBIO caused a sixfold increase in the serosal-to-mucosal flux of Cl\(^{-}\) without altering the mucosal-to-serosal flux leading to net Cl\(^{-}\) secretion. Moreover, the net secretion of Cl\(^{-}\) fully accounted for the increase in \(I_{sc}\) caused by 1-EBIO, leaving a \(J_{R_{net}}\) of only 0.25 ± 0.055 μEq · cm\(^{-2}\) · h\(^{-1}\). Bumetanide inhibited the serosal-to-mucosal flux of Cl\(^{-}\) and thereby caused a 70% inhibition in \(I_{sc}\) in 1-EBIO-stimulated monolayers.

**Effects of Forskolin and 1-EBIO on \(I_{sc}\)**

The above results demonstrate Calu-3 cells secrete HCO\(_3\) when stimulated by forskolin and Cl\(^{-}\) when stimulated by 1-EBIO. In the next series of experiments, we evaluated the effects of 1-EBIO on forskolin stimulated monolayers. As in the previous experiments, forskolin increased \(I_{sc}\) from a control value of 6.8 ± 0.7 to 67 ± 4.3 μA · cm\(^{-2}\) (\(n = 12\)) without causing the net secretion of Cl\(^{-}\) and leaving a \(J_{R_{net}}\) nearly equal to the change in \(I_{sc}\) (Fig. 6 and Table III). 1-EBIO further increased \(I_{sc}\) to 114 ± 5 μA · cm\(^{-2}\) (Fig. 6 and Table III). Similar results were obtained if the order of the addition of forskolin and 1-EBIO were reversed. CTX inhibited 79 ± 2% (\(n = 8\)) and bumetanide inhibited 80 ± 1% (\(n = 5\)) of the forskolin plus 1-EBIO-stimulated \(I_{sc}\). When added to the forskolin-stimulated cells, 1-EBIO caused a twofold increase in the serosal-to-mucosal flux of Cl\(^{-}\) and a \(J_{R_{net}}\) that was nearly equal to the \(I_{sc}\) (Fig. 6 and Table III). Thus, 1-EBIO caused a 70% decrease in the forskolin-stimulated \(J_{R_{net}}\). These results suggest 1-EBIO can switch the forskolin-stimulated Calu-3 cells from HCO\(_3\)- to Cl\(^{-}\)-secreting cells.

One hypothesis to explain the effects of 1-EBIO on Calu-3 cells is the activation of basolateral membrane K\(^{+}\) channels that would tend to hyperpolarize the membrane potential. The inhibition of the 1-EBIO response by CTX and clotrimazole support this hypothesis.

**Table II**

| Experiment  | \(J_{ms}\) (μA · cm\(^{-2}\)) | \(J_{sm}\) (μA · cm\(^{-2}\)) | \(J_{net}\) (μEq · cm\(^{-2}\) · h\(^{-1}\)) | \(I_{sc}\) (μA · cm\(^{-2}\)) | \(R_{T}\) (Ω · cm\(^{-2}\)) | \(J_{R_{net}}\) (μEq · cm\(^{-2}\) · h\(^{-1}\)) |
|-------------|-------------------------------|-------------------------------|---------------------------------|-----------------------------|--------------------------|-------------------------------|
| Control     | 0.64 ± 0.07                   | 0.67 ± 0.085                 | -0.03 ± 0.081                   | 0.40 ± 0.061                 | 388 ± 21                  | 0.37 ± 0.072                  |
| 1-EBIO      | 0.72 ± 0.068                  | 3.18 ± 0.411                 | -2.46 ± 0.305                   | 2.71 ± 0.213                 | 273 ± 14                  | 0.25 ± 0.263                  |
| Bumetanide  | 0.59 ± 0.150                  | 1.33 ± 0.054                 | -0.74 ± 0.109                   | 1.25 ± 0.058                 | 280 ± 17                  | 0.51 ± 0.987                  |

Units are as given in Table I. Measurements were made before and after the addition of 1-EBIO (1 mM) to the mucosal (m) and serosal (s) solutions and bumetanide (20 μM) to the serosal solution. Values are the mean ± SEM, \(n = 6\) for each unidirectional flux and 12 for \(J_{R_{net}}\), \(I_{sc}\), \(R_{T}\), and \(J_{R_{net}}\).

**Figure 6.** Effects of forskolin and 1-EBIO on Calu-3 cell \(I_{sc}\) and Cl\(^{-}\) fluxes. (A) Short-circuit current trace demonstrating the increase in \(I_{sc}\) in response to forskolin (2 μM) and 1-EBIO (1 mM). (B) Unidirectional and net Cl\(^{-}\) fluxes are shown for the forskolin and forskolin plus 1-EBIO time periods. As in the previous experiments, forskolin increased both unidirectional fluxes without causing a net Cl\(^{-}\) secretion. 1-EBIO further increased \(J_{ms}\) and caused a net secretion that accounted for 83% of the \(I_{sc}\). Fluxes are plotted as the absolute values and are also summarized in Table III.
sis. Hyperpolarization of the membrane potential would increase the driving force for anion exit of both HCO$_3^-$ and Cl$^-$ across the apical membrane. However, hyperpolarization of the basolateral membrane potential would also tend to decrease the driving force for basolateral membrane HCO$_3^-$ entry on the Na$^+$:HCO$_3^-$ cotransporter, whose Na$^+$ to HCO$_3^-$ stoichiometry is reported to be 1:2 or 1:3 in various cell types (Boron and Boulpaep, 1989). A second hypothesis, and one that is not mutually exclusive with the former hypothesis, is that 1-EBIO activates apical membrane anion channels that were not activated by forskolin and that the 1-EBIO-activated channels allow for the preferential exit of Cl$^-$ over HCO$_3^-$. To test these hypotheses, we performed studies on permeabilized monolayers.

The pore forming antibiotic nystatin was used to permeabilize the apical membrane and a transepithelial mucosal-to-serosal K$^+$ gradient was established. After permeabilization, 1-EBIO increased $I_{K}$, and this was inhibited by both CTX (Fig. 7 A) and clotrimazole (B).

In 17 experiments, 1-EBIO (1 mM) increased $I_{K}$ an average of 91 ± 9 μA · cm$^{-2}$ and this was inhibited 66 ± 2% by CTX (50 nM, $n = 10$) and 95 ± 2% by clotrimazole (10 μM, $n = 7$). Thus, 1-EBIO does activate basolateral membrane K$^+$ channels. In contrast, forskolin (2 μM) failed to cause an increase in $I_{K}$. After the establishment of a mucosal-to-serosal Cl$^-$ gradient, the addition of nystatin to the serosal membrane elicited an absorptive $I_{Cl}$ of 58 ± 9 μA · cm$^{-2}$ ($n = 24$, Fig. 8). Thus, in contrast to the measurements of $I_{K}$, treatment of the monolayers with nystatin appears to uncover or activate a substantial basal $I_{Cl}$. Similar results were observed in T84 cells studied under the same experimental conditions (Devor et al., 1996). Therefore, this effect of nystatin is not unique to Calu-3 cells. The mechanisms involved in this nystatin induced increase in $I_{Cl}$ are unknown. The subsequent addition of forskolin (10 μM) to the nystatin-treated monolayers increased $I_{Cl}$ by an additional 186 ± 15 μA · cm$^{-2}$ ($n = 7$) (Fig. 8 A). 1-EBIO failed to cause any further increase in $I_{Cl}$ in the

| Forskolin | 1-EBIO |
|-----------|--------|
| $I_{Cl}$  | $I_{Cl}$ |
| 2.30 ± 0.106 | 2.60 ± 0.190 |
| $J_{Cl}$ | $J_{Cl}$ |
| 2.57 ± 0.096 | 6.14 ± 0.582 |
| $J_{Cl}$ | $J_{Cl}$ |
| -0.27 ± 0.105 | -3.54 ± 0.452 |
| $I_{K}$ | $I_{K}$ |
| 2.49 ± 0.158 | 4.24 ± 0.192 |
| $R_T$ | $R_T$ |
| 180 ± 7 | 173 ± 9 |
| $J_{net}$ | $J_{net}$ |
| 2.22 ± 0.134 | 0.72 ± 0.347 |

Units are as given in Table I. Measurements were made before and after the addition of 1-EBIO (1 mM) to the mucosal and serosal solutions of forskolin (2 μM)-treated monolayers. Values are the mean ± SEM, $n = 6$ for each unidirectional flux and 12 for $J_{net}$, $I_{K}$, $R_T$, and $J_{net}$.

Table III

Effects of Forskolin and 1-EBIO on Unidirectional and Net Cl$^-$ Fluxes Across Calu-3 Cell Monolayers

Figure 7. Effect of 1-EBIO, CTX, and clotrimazole on basolateral membrane K$^+$ currents ($I_{K}$) after establishment of a mucosal-to-serosa K$^+$ gradient and permeabilization of the mucosal membrane with nystatin. (A) Effects of 1-EBIO (600 μM) and CTX (50 nM), and (B) effects of 1-EBIO and clotrimazole (10 μM) on $I_{K}$. Monolayer illustration indicates the direction of the ion gradient and the dashed line in the monolayer the permeabilization of the apical membrane with nystatin. Current traces are representative of six experiments with similar results.

Figure 8. Effect of 1-EBIO, CTX, and clotrimazole on basolateral membrane K$^+$ currents ($I_{K}$) after establishment of a mucosal-to-serosa K$^+$ gradient and permeabilization of the mucosal membrane with nystatin. (A) Effects of 1-EBIO (600 μM) and CTX (50 nM), and (B) effects of 1-EBIO and clotrimazole (10 μM) on $I_{K}$. Monolayer illustration indicates the direction of the ion gradient and the dashed line in the monolayer the permeabilization of the apical membrane with nystatin. Current traces are representative of six experiments with similar results.
forskolin treated monolayers. However, 1-EBIO alone when added to the nystatin-treated monolayers increased $I_{Cl}$ by an additional $74 \pm 11 \mu A \cdot cm^{-2}$ ($n = 6$) and forskolin further increased $I_{Cl}$ by an additional $110 \pm 12 \mu A \cdot cm^{-2}$ ($n = 6$; Fig. 8 B).

Thus, both forskolin and 1-EBIO when added alone can activate an apical membrane $Cl^-$ conductance in nystatin-treated Calu-3 monolayers. Forskolin caused a 2.5-fold greater increase in $I_{Cl}$ compared with the 1-EBIO response. The lack of specific $Cl^-$ channel blockers (Schultz et al., 1999) prevents us from determining whether the same channel or different $Cl^-$ channels are activated by forskolin and 1-EBIO. However, when forskolin and then 1-EBIO was added, the effects on $I_{Cl}$ were not additive, suggesting that forskolin alone can maximally activate the apical $Cl^-$ conductance. Therefore, the effect of 1-EBIO in causing the switch from $secretion to $Cl^-$ secretion appears to result from the activation of basolateral membrane $K^+$ channels and decreased driving force for $HCO_3^-$ entry across the basolateral membrane. This hypothesis will be considered further in the discussion.

Excised Patch Single Channel Records

The above results indicate that Calu-3 cells express $K^+$ channels with similar pharmacological characteristics to the $K^+$ channels we described previously in T84 cells (Devor and Frizzell, 1993; Devor et al., 1996, 1997) and that this conductance may be important in altering the driving force for $HCO_3^-$ entry across the basolateral membrane that elicits $Cl^-$ secretion in Calu-3 cells.

Thus, we wished to characterize this $K^+$ channel at the single channel level. Inward and outward single-channel currents observed on excision of membrane patches into a symmetric $K^+$ bath containing 400 nM free $Ca^{2+}$ are shown in Fig. 9 A. Channel activity showed no obvious voltage dependence and required $Ca^{2+}$ in the bath (data not shown). The average current-voltage for four such patches is shown in Fig. 9 B.

A

B

Figure 8. Effects of forskolin and 1-EBIO on apical membrane $Cl^-$ currents ($I_{Cl}$) after establishment of a mucosa-to-serosa $Cl^-$ gradient and permeabilization of the basolateral membrane with nystatin. (A) Effects of forskolin (2 $\mu M$) and 1-EBIO (1 mM), and (B) effects of 1-EBIO and forskolin on $I_{Cl}$. The monolayer illustration indicates the direction of the ion gradient and the dashed line in the monolayer indicates the permeabilization of the basolateral membrane with nystatin. Current traces are representative of 24 experiments with similar results.
Calu-3 cells. The effect of 1-EBIO (200 μM) on one patch is shown in Fig. 10. Under control conditions (400 nM free Ca\textsuperscript{2+} in the bath), minimal K\textsubscript{Ca} channel activity was observed. 1-EBIO produced a large increase in channel activity that was readily reversible after washout of the 1-EBIO. In 14 inside-out recordings, 1-EBIO increased nP\textsubscript{0} from 0.08 ± 0.02 to 1.68 ± 0.39. These results indicate that this channel, as in T84 cells, is responsible for the increase in the basolateral membrane K\textsuperscript{+} conductance and I\textsubscript{sc} during an 1-EBIO–mediated secretory response.

Effect of K\textsuperscript{+} Channel Blockers

We demonstrate above that the 1-EBIO–induced basolateral membrane K\textsuperscript{+} conductance is sensitive to block by CTX and clotrimazole (Fig. 7). We therefore determined whether these inhibitors would block the channel in excised outside-out and inside-out patches. The effect of CTX (50 nM) on K\textsubscript{Ca} in an outside-out patch is shown in Fig. 11A. When holding the patch at −100 mV, addition of CTX to the outside of the channel resulted in a complete inhibition of channel activity. This block was voltage dependent and was partially relieved by voltage clamping the patch to +100 mV. The inhibition by CTX was completely reversible. Similar results were obtained in three additional outside-out patches. Clotrimazole (10 μM) also completely inhibited K\textsubscript{Ca} activity, reducing nP\textsubscript{0} from 1.59 ± 0.24 to 0.05 ± 0.02 (n = 6; Fig. 11B). Thus, results from these K\textsuperscript{+} channel blocker experiments further indicate that 1-EBIO is activating this inwardly rectifying Ca\textsuperscript{2+}-activated K\textsuperscript{+} conductance in Calu-3 monolayers resulting in the stimulation of Cl\textsuperscript{−} secretion and the inhibition of HCO\textsubscript{3}\textsuperscript{−} secretion.

discussion

The results of our studies with Calu-3 cells demonstrate that forskolin stimulates the net secretion of HCO\textsubscript{3}\textsuperscript{−}.
Forskolin consistently caused an increase in $I_{sc}$ to a new sustained plateau. Ion flux studies revealed that this increase in $I_{sc}$ could not be explained by the net transport of Na$^{+}$, Rb$^{+}$, or Cl$^{-}$, leaving HCO$_3^-$ secretion as the likely basis for the increase in $I_{sc}$. Ion substitution experiments demonstrated HCO$_3^-$ secretion, but not Cl$^{-}$, was required to elicit a sustained increase in $I_{sc}$ with forskolin. In addition, Na$^{+}$ was required in the serosal bath to elicit a forskolin response. Inhibitor studies revealed that the forskolin response was sensitive to ouabain, indicating a role for the Na$^{+}$/K$^{+}$-ATPase. The forskolin response was also sensitive to DNDS on the serosal side but not the mucosal side, indicating a role for a basolateral membrane Na$^{+}$:HCO$_3^-$ cotransporter or Cl$^{-}$:HCO$_3^-$ exchanger. However, because Cl$^{-}$ was not required and serosal Na$^{+}$ was, the effects of DNDS are likely to result from the inhibition of a basolateral membrane Na$^{+}$:HCO$_3^-$ cotransporter. Acetazolamide caused a partial inhibition of the forskolin response, consistent with some of the secreted HCO$_3^-$ arising from metabolic sources. The ion flux studies failed to show evidence of net secretion of Cl$^{-}$ in response to forskolin, and bumetanide did not inhibit the $I_{sc}$ response. Thus, forskolin did not cause the net secretion of Cl$^{-}$ across Calu-3 cells under short circuit conditions. Rather, we conclude forskolin causes the net secretion of HCO$_3^-$ by a Cl$^{-}$-independent, Na$^{+}$-dependent, and DNDS-sensitive electrogenic mechanism in Calu-3 cells. The forskolin-stimulated alkalization of the mucosal bathing solution of Calu-3 cells, studied under open circuit conditions, lends further support to this conclusion.

Although forskolin did not stimulate the net secretion of Cl$^{-}$, it did cause a fivefold increase in both unidirectional fluxes of Cl$^{-}$ (Fig. 1B and Table I) and it is of interest to understand the mechanisms that underlie these changes. Our first interpretation was that forskolin increased the transcellular passage of Cl$^{-}$ in both directions. Thus, the opening of CFTR would allow for both the exit and entry of Cl$^{-}$ across the apical membrane. The NaK2Cl cotransporter in the basolateral membrane would allow the entry of Cl$^{-}$ leaving one to explain how Cl$^{-}$ exits the cell in the serosal-to-mucosal direction. However, bumetanide did not alter the unidirectional fluxes, consistent with the lack of change in the forskolin-stimulated $I_{sc}$. Thus, the NaK2Cl cotransporter does not appear to mediate the entry of Cl$^{-}$ across the basolateral membrane in the forskolin-stimulated monolayers. We next entertained the possibility that Cl$^{-}$ may move across the basolateral membrane on a Cl$^{-}$:HCO$_3^-$ exchanger. However, the increases in both unidirectional fluxes in response to forskolin were still observed in HCO$_3^-$-free buffer. Thus, the increased fluxes do not depend on extracellular HCO$_3^-$, Because this experiment does not exclude the possibility that a basolateral membrane anion exchanger is operating in a Cl$^{-}$:Cl$^{-}$ exchange mode, we examined the effects of serosal DNDS (1 mM) on the Cl$^{-}$ fluxes. DNDS cause a 70% decrease in both unidirectional fluxes in the forskolin-stimulated monolayers. Therefore, the increase
in Cl⁻ fluxes caused by forskolin can largely be accounted for by a Cl⁻:Cl⁻ exchange across the basolateral membrane and the exit and entry of Cl⁻ via CFTR across the apical membrane.

The studies with 1-EBIO demonstrated the Calu-3 cells are not limited to the secretion of Cl⁻, but rather they can also be stimulated to secrete Cl⁻. 1-EBIO, like forskolin, consistently caused a sustained increase in $I_{sc}$. $^{36}$Cl flux studies showed the 1-EBIO-stimulated increase in $I_{sc}$ could be fully accounted for by the net secretion of Cl⁻. In addition, both the increase in $I_{sc}$ and the net secretion of Cl⁻ were inhibited by bumetanide. Studies on permeabilized Calu-3 monolayers revealed 1-EBIO activates both a basolateral membrane K⁺ conductance and an apical membrane Cl⁻ conductance as previously shown in studies on T84 cells (Devor et al., 1996). CTX and clotrimazole both inhibited the 1-EBIO $I_{sc}$ response as well as the 1-EBIO-activated K⁺ current in permeabilized monolayers. Patch-clamp studies demonstrated the presence of an intermediate conductance, inwardly rectified, Ca⁺-activated K⁺ channel in Calu-3 cells that was activated by 1-EBIO and blocked by CTX and clotrimazole. We and others have also identified a Ca⁺-activated K⁺ channel with identical biophysical properties and pharmacological profile in T84 cells (Devor and Frizzell, 1993; Tabcharani et al., 1994; Roch et al., 1995; Devor et al., 1996). Moreover, Welsh and McCann (1985) and McCann et al. (1990) have already shown that this channel is expressed in native airway epithelial cells and is therefore not just in

![Figure 11. Inhibition of $K_{Ca}$ by CTX and clotrimazole. (A) Addition of CTX (50 nM) to the extracellular side of $K_{Ca}$ in an excised, outside-out patch resulted in a complete inhibition of channel activity when the patch was voltage clamped to −100 mV (inside negative). Patch pipette contained 400 nM free Ca²⁺. (B) Addition of clotrimazole (3 μM) to the cytoplasmic side of $K_{Ca}$ in an excised inside out patch resulted in a complete inhibition of channel activity at a holding potential of −100 mV. Other conditions as indicated in Fig. 10, the arrows indicate the closed state of the channel.](image-url)
epithelial cell lines. Recently, three different groups have cloned the same \( K^+ \) channel, variously referred to as hIK-1, hSK4, and hIK (Ishii et al., 1997; Joiner et al., 1997; Jensen et al., 1998). These channels have identical biophysical properties and pharmacological profile to the channel observed in canine tracheocytes, T84 cells, and Calu-3 cells. Northern blot analysis has confirmed the presence of the mRNA for hIK-1 in T84 and Calu-3 cells (Devor, D.C., unpublished results). Thus, we conclude that one site of action of 1-EBIO is the activation of hIK-1 in the basolateral membrane of Calu-3 cells. Permeabilization of monolayers demonstrated 1-EBIO also activates an apical membrane \( Cl^- \) channel; however, the identity of the apical membrane \( Cl^- \) channel that is activated by 1-EBIO is less certain. Haws et al. (1994) have reported the predominant \( Cl^- \) channel observed in Calu-3 cells is a low conductance channel with properties consistent with those of CFTR. 1-EBIO is a benzimidazolinone and other benzimidazolinones (e.g., NS004 and NS1619) have been reported to activate CFTR (Gribkoff et al., 1994; Champigny et al., 1995). Thus, it is possible that the \( Cl^- \) channel activated by 1-EBIO in Calu-3 cells is CFTR. However, further studies will be necessary to confirm this hypothesis.

Calu-3 cells secrete \( HCO_3^- \) in response to forskolin and \( Cl^- \) in response to 1-EBIO. However, when the two agonists are added together, anion secretion is dominated by \( Cl^- \) secretion and there is a decrease in the net secretion of \( HCO_3^- \). Studies with primary cultures of human bronchial epithelial cells lead Smith and Welsh (1992) to suggest that airway epithelia may also switch between \( HCO_3^- \) and \( Cl^- \) secretion. Ashton et al. (1991) have also suggested that pancreatic ductal epithelial cells can be differentially stimulated to secrete \( HCO_3^- \) or \( Cl^- \). The mechanisms that underlie the switch between \( HCO_3^- \) and \( Cl^- \) secretion are largely unknown. Our results with Calu-3 cells offer some insight and suggest a model (Fig. 12) to explain how the same cell can secrete \( HCO_3^- \) when stimulated by forskolin and \( Cl^- \) when stimulated by 1-EBIO or 1-EBIO plus forskolin.

The first tenet of the model is the presence of an anion channel in the apical membrane that can conduct both \( HCO_3^- \) and \( Cl^- \). Whether there are two separate channel types, one favoring \( HCO_3^- \) and activated by forskolin and one favoring \( Cl^- \) and activated by 1-EBIO, or a single channel type that conducts both \( HCO_3^- \) and \( Cl^- \) is not clear at this time. Nonselective anion channels have been reported but to our knowledge an epithelial anion channel that favors \( HCO_3^- \) over \( Cl^- \) has

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**Figure 12.** Model for Calu-3 anion secretion. Stimulation by cAMP causes the activation of an apical membrane anion channel that conducts both \( HCO_3^- \) and \( Cl^- \). We propose this anion channel is CFTR. Activation of CFTR will tend to bring the apical membrane potential to \( E_{Cl^-} \) (about \(-35 \text{ mV}\)), a value greater than the equilibrium potential for \( HCO_3^- \) \( (E_{HCO_3^-} = -13 \text{ mV}) \) and thereby provides the driving force for \( HCO_3^- \) exit across the apical membrane. Stimulation by cAMP (forskolin) alone leaves the basolateral membrane potential \( (\psi_{bl}) \) less hyperpolarized than the reversal potential of the DNDS-sensitive \( Na^+:HCO_3^- \) cotransporter \( (E_{revNaHCO_3}) \) and \( HCO_3^- \) is secreted. Activation of \( K_{Ca} \) by 1EBIO hyperpolarizes \( \psi_{bl} \) so that \( \psi_{bl} > E_{revNaHCO_3} \) and this inhibits \( HCO_3^- \) uptake by the \( Na^+:HCO_3^- \) cotransporter but provides the driving force for \( Cl^- \) secretion. Whether cAMP activates the \( Na^+:HCO_3^- \) cotransporter is unknown. The stoichiometry, 1:2 or 1:3, of the Calu-3 cell \( Na^+:HCO_3^- \) cotransporter is also unknown.
not yet been described in the literature. Because HCO$_3^-$ secretion is stimulated by forskolin, the anion channel mediating the secretion of HCO$_3^-$ is likely to be activated by cAMP and PKA, as is CFTR. CFTR is highly expressed in Calu-3 cells (Finkbeiner et al., 1993; Shen et al., 1994) and activated by forskolin when measured by anion efflux methods and patch clamp analysis (Haws et al., 1994). Preliminary studies using impedance analysis have shown forskolin does activate an apical membrane anion conductance in Calu-3 cells (Bridges, R.J., unpublished observations). Patch-clamp anion selectivity studies have shown CFTR can conduct HCO$_3^-$, although at a fraction (0.15–0.25) of the Cl$^-$ conductance (Gray et al., 1990; Poulsen et al., 1994; Linsdell et al., 1997). Heterologous expression of wt-CFTR but not ΔF508-CFTR in NIH3T3 fibroblasts and C127 mammary cells was shown to confer the cells with a Na$^+$-independent, HCO$_3^-$-dependent, forskolin-regulated intracellular pH recovery mechanism (Poulsen et al., 1994). Illek et al. (1997) have shown, in α-toxin-permeabilized monolayers of Calu-3 cells, the activation of a HCO$_3^-$ current by cAMP with a similar HCO$_3^-$ to Cl$^-$ selectivity as observed in the patch-clamp studies. In addition, Smith and Welsh (1992) demonstrated cAMP-stimulated HCO$_3^-$ secretion across normal but not CF airway epithelia and they suggested HCO$_3^-$ exit across the apical membrane is through the Cl$^-$ channel that is defectively regulated in CF. Thus, we propose that CFTR mediates the exit of HCO$_3^-$ across the apical membrane of Calu-3 cells.

The involvement of an anion channel in HCO$_3^-$ secretion is not a new concept. However, previous models have proposed the anion channel acts as a shunt pathway mediating the exit of Cl$^-$ from the cell (Stetson et al., 1985). Luminal Cl$^-$ is then thought to be used by an apical membrane Cl$^-$:HCO$_3^-$ exchanger that mediates the exit of HCO$_3^-$ from the cell. Thus, this model for HCO$_3^-$ secretion necessitates the presence of luminal Cl$^-$ for the apical membrane exit of HCO$_3^-$.

The second tenet of the model (Fig. 12) is the presence of an electrogenic Na$^+:$HCO$_3^-$ cotransporter (NBC) in the basolateral membrane that mediates the entry of HCO$_3^-$ into the cell. Boron and Boulpaep (1983) were the first to describe an electrogenic NBC with Na$^+:$HCO$_3^-$ stoichiometry of 1:2 that mediates the exit of HCO$_3^-$ across the basolateral membrane in the proximal tubule of the tiger salamander Ambystoma tigrinum. Romero et al. (1997) using mRNA from the tiger salamander kidney have recently expressed this NBC. The cloning of a human homologue of the renal NBC has also recently been reported (Burnham et al., 1997), as has a unique human pancreatic isoform (Abuladze et al., 1996). The stoichiometries of the cloned NBCs have not yet been established but Xenopus oocyte expression studies have shown the renal NBC is electrogenic, Na$^+$- and HCO$_3^-$-dependent, Cl$^-$-independent, and disulfonic stilbene-sensitive (Romero et al., 1997). These characteristics are shared by NBCs studied in kidney, glial, liver, pancreas, and colon (Boron and Boulpaep, 1989). Our studies with Calu-3 cells demonstrate that forskolin-stimulated HCO$_3^-$ secretion also shares these characteristics, consistent with the presence of a NBC in the basolateral membrane. Preliminary reverse transcription-PCR and sequencing studies have shown Calu-3 cells express a NBC (Gangopadhyay and Bridges, unpublished observations) lending further support to this notion. Studies in progress are focused on ascertaining which of the NBC isoforms is expressed in Calu-3 cells as well as the membrane localization, apical versus basolateral, of the cotransporter. According to Fig. 12, we predict a basolateral membrane NBC with a Na$^+:$HCO$_3^-$ stoichiometry that favors the entry of HCO$_3^-$ when Calu-3 cells are stimulated by forskolin. Both the pancreatic and renal isoforms of the NBCs have consensus phosphorylation sites for protein kinase A and therefore may be regulated by cAMP-mediated agonists (Romero et al., 1997; Abuladze et al., 1998). Thus, in addition to the activation of an apical membrane anion channel (CFTR?), forskolin may also activate HCO$_3^-$ entry on the NBC.

Whether a NBC mediates entry or exit of HCO$_3^-$ depends on the stoichiometry of the transporter, the membrane potential, and the concentrations of Na$^+$ and HCO$_3^-$ inside and outside the cell. Sodium:HCO$_3^-$ stoichiometries of 1:2 and 1:3 have been reported (Boron and Boulpaep, 1989), indicating that turnover of the NBC may result in the transfer of one or two negative charges across the membrane at usual membrane voltages. The 1:2 stoichiometry is associated with NBC-mediated HCO$_3^-$ entry, whereas a 1:3 stoichiometry is consistent with HCO$_3^-$ exit. If one assumes typical ion concentrations of 145 mM Na$^+$, 25 mM HCO$_3^-$ outside, and 15 mM Na$^+$ and 15 mM HCO$_3^-$ inside, then HCO$_3^-$ will enter a cell on the NBC at membrane potentials...
less hyperpolarized than \(-85\) mV when the \(\text{Na}^+:\text{HCO}_3^-\) stoichiometry is 1:2 and \(-49\) mV when it is 1:3. Membrane potentials more hyperpolarized than these values will lead to \(\text{HCO}_3^-\) exit from the cells. Thus, the activation of basolateral membrane \(K^+\) channels by \(1\)-EBIO is expected to hyperpolarize the membrane potential, and this will inhibit the entry of \(\text{HCO}_3^-\) on the NBC. If the hyperpolarization is of sufficient magnitude, this change in driving force may drive \(\text{HCO}_3^-\) out of the cell across the basolateral membrane. Hyperpolarization will also tend to drive anions (\(\text{HCO}_3^-\) and \(\text{Cl}^-\)) out of the cell across the apical membrane. However, because basolateral membrane entry of \(\text{HCO}_3^-\) becomes inhibited, this apical membrane hyperpolarization will favor \(\text{Cl}^-\) secretion. Therefore, we propose that the switch between \(\text{HCO}_3^-\) secretion and \(\text{Cl}^-\) secretion is determined by the basolateral membrane potential. Differential regulation of the basolateral membrane potential by secretory agonists would provide a means of stimulating \(\text{HCO}_3^-\) or \(\text{Cl}^-\) secretion. As shown in Fig. 12, CFTR could serve as both a \(\text{HCO}_3^-\) and a \(\text{Cl}^-\) channel mediating the apical membrane exit of either anion depending on the nature of the anion provided by the basolateral membrane cotransporter mechanisms.

Why does forskolin fail to stimulate \(\text{Cl}^-\) secretion in Calu-3 monolayers? Cyclic AMP-stimulated \(\text{Cl}^-\) secretion is known to require the activation of both an apical membrane \(\text{Cl}^-\) conductance and a basolateral membrane \(K^+\) conductance; the former depolarizes and the latter repolarizes the membrane voltage to maintain a driving force for \(\text{Cl}^-\) exit (Halm and Frizzell, 1990). Permeabilization studies demonstrated forskolin does activate an apical membrane \(\text{Cl}^-\) conductance (Fig. 8), but that it fails to activate a basolateral membrane \(K^+\) conductance (Fig. 7). Thus, unless the basal \(K^+\) conductance can maintain the apical voltage above the \(\text{Cl}^-\) equilibrium potential (\(E_{\text{Cl}} < -35\) mV, assuming intracellular \(\text{Cl}^- = 30\) mM), \(\text{Cl}^-\) cannot be secreted. Indeed, the expected high \(\text{Cl}^-\) conductance of the apical membrane of forskolin-stimulated Calu-3 cells would set the apical membrane voltage at \(E_{\text{Cl}}\) and this would provide the driving force for \(\text{HCO}_3^-\) exit since \(E_{\text{HCO}_3^-} = -13\) mV (assuming intracellular \(\text{HCO}_3^- = 15\) mM and extracellular \(\text{HCO}_3^- = 25\) mM). This electrical coupling may explain the apparent \(\text{Cl}^-\) dependence of \(\text{HCO}_3^-\) secretion in some epithelia and further emphasizes the importance of CFTR in \(\text{Cl}^-\) and \(\text{HCO}_3^-\) secretion.

\(^4\)Together with the measured net secretion of \(\text{HCO}_3^-\) of \(\sim 60\) \(\mu\text{A} \cdot \text{cm}^{-2}\), one can use the values for \(E_{\text{HCO}_3^-} = -13\) mV and \(E_{\text{Cl}} = -35\) mV to obtain an estimate of the apical membrane \(\text{HCO}_3^-\) conductance (\(g_{\text{HCO}_3^-}\), where \(g_{\text{HCO}_3^-} = \left(E_{\text{Cl}} - E_{\text{HCO}_3^-}\right) / \text{HCO}_3^- = 2.7\) mS \(\cdot \text{cm}^{-2}\)). This estimation assumes the apical membrane is at \(E_{\text{Cl}}\). Results from impedance analysis on Calu-3 cells indicate forskolin increases the apical membrane conductance (\(g_{\text{apical}}\)) to \(\sim 20\) mS \(\cdot \text{cm}^{-2}\) (Bridges, R.J., unpublished observations). This remarkably high conductance would ensure the apical membrane potential is at or near \(E_{\text{Cl}}\), but also yields a \(\text{HCO}_3^-\) to \(\text{Cl}^-\) conductance ratio of \(\approx 0.15\) (where \(g_{\text{Cl}} = g_{\text{HCO}_3^-} / g_{\text{HCO}_3^-} = 20 - 27 = 17.3\) mS \(\cdot \text{cm}^{-2}\) so that \(g_{\text{HCO}_3^-} / g_{\text{Cl}} = 2.7/17.3 = 0.15\), a value in good agreement with the patch clamp estimates of 0.15–0.25 for CFTR. Moreover, an apical membrane \(g_{\text{Cl}}\) of 17.3 mS \(\cdot \text{cm}^{-2}\) means a driving force of only 3.5 mV is required to achieve a net \(\text{Cl}^-\) secretion of \(60\) \(\mu\text{A} \cdot \text{cm}^{-2}\).
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