Electrogenic bicarbonate secretion by prairie dog gallbladder

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Although gallbladder disease is the most frequent indication for abdominal surgery in the United States (19), the factors that confer susceptibility to gallstones remain poorly understood. Prairie dogs have been widely studied as an experimental model of human cholelithiasis due to their unique propensity for developing gallstones on high-cholesterol chow (7). Prior studies in cholesterol-fed prairie dogs (4) have demonstrated that gallbladder salt and water transport are altered before gallstones form, a phenomenon that may cause cholesterol to precipitate in the gallbladder lumen. The potential for targeting gallbladder ion transport as a strategy for preventing cholelithiasis is underscored by data showing that amiloride inhibits the formation of gallstones in cholesterol-fed prairie dogs (56).

To use the prairie dog as a model to investigate this event, we performed a detailed characterization of gallbladder ion transport in animals fed control chow.

Classic studies in Necturus and rabbit gallbladders established the paradigm for electroneutral ion transport across an electrically leaky epithelium but made no attempt to identify the role of the gallbladder in gallstone formation (9, 11, 44). In addition to low transepithelial resistance (Rt), both Necturus and rabbit gallbladders generated negligible short-circuit current (ISc < 20 μA/cm2) as a result of minimal ionic conductance at the apical membrane (Ga) (40, 41). Nominal ISc and lumen-positive potential difference in these species was attributed to the back-diffusion of Na+ across a cation-selective paracellular junction (32). Significant electrogenic movement of Na+, Cl−, and HCO3− was excluded by means of pharmacological blockers, ion substitution, and microelectrode im- pallaments. In particular, transepithelial bicarbonate transport was mediated by electroneutral parallel Na+/H+ and Cl−/HCO3− exchangers, although electrogenic HCO3− secretion (<60 μA/cm2) has been observed following maximal PGE2 simulation in guinea pigs (55).

Recent observations in human and primate gallbladders challenge the prevailing concept that the gallbladder is an electrically silent absorptive organ that concentrates bile between meals and suggest the intriguing possibility that bicarbonate transport is an important cause of gallstone susceptibility. Using in vivo methods to analyze bile composition, Igimi et al. (14) and Svanvik et al. (58) found that human and monkey gallbladders absorb electrolytes at night but secrete bicarbonate-rich fluid after meals. The association of bicarbonate secretion with high values for transepithelial potential difference and ISc reported in these species suggests that bicarbonate secretion might be electrogenic and introduces the possibility that bicarbonate secretion may be an important etiologic factor in gallstone susceptibility (12, 45). Moreover, a link between aberrant luminal pH and gallstones has been observed in humans (50) as well as cholesterol-fed prairie dogs (25). Our own electrophysiological observations in normal human (A. J. Moser, unpublished data) and prairie dog gallbladders (33) demonstrate basal ISc exceeding 130 μA/cm2, a value that is inconsistent with the established paradigm for electroneutral gallbladder ion transport. The ionic basis for the significant ISc and the shared susceptibility of human and prairie dog gallbladders to develop gallstones in response to dietary cholesterol have not been investigated.

We hypothesize that the gallbladder is an integral component of the biliary tree that maintains a nonlithogenic environ-
ment for the transport of bile and cholesterol into the digestive tract. Alterations in gallbladder concentrating capacity and biliary pH as a result of bicarbonate secretion may be critical factors causing gallstone susceptibility during periods of excess dietary cholesterol intake. As a prelude to studies in cholesterol-fed animals, we performed a detailed investigation of electrogenic ion transport in normal prairie dogs by means of transepithelial currents, isotopic fluxes, ion substitution, pharmacology, impedance analysis, and molecular biology.

**MATERIALS AND METHODS**

Surgical procedures and animal care. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Adult male prairie dogs were caught in the wild under an FDA Special Permit and were fed a nutritionally complete diet (http://www.labdiet.com) containing trace cholesterol (0.02%). After a 16-h fast with water ad libitum, animals were anesthetized with intramuscular ketamine (100 mg/kg) and xylazine (10 mg/kg), and cholecystectomy was performed. Gallbladders were opened longitudinally and were rinsed with warm PBS at pH 7.4 to remove adherent bile. Tissues were mounted in 5.0-ml Ussing chambers (Navicyte, San Diego, CA) with 0.64-cm² apertures and were heated to 37°C. Chambers were sealed with silicone grease and were gassed continuously with 95% O₂-5% CO₂ or 100% O₂. Parafilm gaskets were used to minimize evaporative losses.

Electrophysiological measurements. Intact gallbladders were used because the mucosa is damaged by stripping the thin serosa. Tissues were short-circuited with fluid resistance (Rmf) compensation by using automatic voltage clamps (Department of Bioengineering, University of Iowa, Iowa City, IA) connected to a computer interface (National Instrument) running Labview data-acquisition software. Voltage and Iw measurements were made with glass electrodes (University of Pittsburgh Machine Shop) containing Ag wire electroplated with AgCl and filled with 1 M KCl (8). Rw was calculated every 20 s after a 2-mV bipolar pulse. Electrode drift was checked at the conclusion of experiments by adding 1 mM serosal ouabain. Data were excluded from statistical analysis when electrode drift exceeded 2 mV.

Unidirectional ion fluxes. After Iw stabilized, radioisotopes (22Na and 36Cl or 86Rb) were added to the appropriate bath. Fluxes were measured in only one direction during each experiment to minimize errors, i.e., mucosa to serosa (ms) or serosa to mucosa (sm). Samples (400 μl) were taken in duplicate every 15 min from the unlabeled side, and volume was replaced with fresh unlabeled solution. Samples were weighed in tared scintillation vials, and the calculated volumes were used to correct the chamber volume (8). For experiments using both 22Na and 36Cl, samples were analyzed in both β-(liquid scintillation analyzer; Packard Instrument) and γ-(Auto-Gamma counting system; Packard Instrument) counters. Activities of the individual isotopes were derived from the β- and γ-decay energies of 22Na and 36Cl and the efficiencies of the respective counters. Unidirectional fluxes were calculated by using standard equations (3), and mean unidirectional ion flux was calculated from pooled data. Net flux (Jnet) was calculated [Jnet = Jms - Jsm, where i represents the given isotope] as long as mean Rw differed by <20% between the unidirectional groups. Net residual ion flux (Jrk) was calculated: Jrk = Iwsc - (Jms + Jsc + Jrb).

Transepithelial impedance analysis. Impedance was measured by published methods using the same Ussing chambers and electrodes employed during flux studies (52). Total epithelial capacitance was measured at five selectable frequencies (2, 4.1, 8.2, 11.0, and 16.5 kHz) in response to 99 computerized sine waves, whereas the mean voltage across the tissue (Vt) was clamped to 0 V. The fundamental frequency was 1 Hz, and the frequency range was 1 Hz–22 kHz.

Curve-fitting routines derived from a one- or two-membrane equivalent circuit were used to estimate individual membrane parameters from the acquired data (52). We modeled the intact gallbladder as a series arrangement of two independent resistor-capacitor elements representing the apical (Rp and Cb) and basolateral (Rf and Cf) membranes in parallel with a shunt resistance (Rs), representing the paracellular resistance. The term Rseries represented the sum of the bathing solution (Rsol) and subepithelial connective tissue (Rsub) resistances. This two-membrane equivalent circuit is the simplest morphologically correct model of epithelial structure and has been used previously for studies of human bronchial epithelial cells (20), T84 cells (52), and Necturus gallbladder (16, 47). Conversely, the one-membrane equivalent circuit modeled a single cellular membrane capacitance in parallel with a low-resistance shunt.

**Estimation of Rp.** The use of curve-fitting routines to analyze impedance data requires an independent estimate of one circuit element. Because mammalian gallbladder is notoriously difficult to impale with microelectrodes, we estimated Rp by linear regression analysis of the transepithelial conductance (Gt) and Iw data in each experiment (61). To confirm constant Rp, we first measured mucosa-to-serosa fluxes of [³H]mannitol under basal conditions and found <20% variability (n = 3; data not shown). We subsequently measured the passive unidirectional flux of 22Na (J22Na) during the basal period (5.9 ± 0.6 μeq·cm⁻²·h⁻¹; n = 8) and in response to both 10 μM indomethacin (6.2 ± 0.7 μeq·cm⁻²·h⁻¹; n = 8) and 2 μM forskolin (6.4 ± 0.6 μeq·cm⁻²·h⁻¹; n = 8) to obtain limiting estimates of Rp. Because the passive flux of Na⁺ is equal to the partial ion conductance of Na⁺ (49), the maximum estimate of Rp was 169 ± 16 mS/cm (Rpmax = 1,000/Rsol). Given the relationship between Rp and the cellular (Rcell) and shunt resistances (1/Rp = 1/Rcell + 1/Rs; equation 1), the minimum value of Rp in each experiment was equal to Rs. Although Iw and Rs changed dramatically in response to indomethacin and forskolin, mean J22Na varied by <10%, suggesting that Rp remained constant.

Having defined the boundaries of Rp (169 ± 16 mS/cm > Rp > Rs), we plotted unique estimates of Rp during each experiment by linear regression analysis of the Gt and Iw data according to Gt = Iw/Vt + Gp (equation 2), where Gp is paracellular conductance, and used this value in curve-fitting analysis. Despite having low Rp, prairie dog gallbladder satisfied the major methodological requirements for estimating the individual membrane parameters by impedance techniques: 1) the plot of Gt vs. Iw was linear in each experiment, and r = 0.84 ± 0.05 (P < 0.0001) in 10 experiments; 2) Rp remained constant at different values of Iw as demonstrated by stable passive fluxes of [³H]mannitol and 22Na; and 3) the best-fit impedance function correlated well with measured impedance under all experimental conditions, as reflected by the low normalized error (0.047 ± 0.008) of the curve-fitting routine. The suitability of intact gallbladder to impedance measurements may be attributed to its homogeneous cell population, limited epithelial folding, and the minimal constant Rs measured during instrument setup and Rs measured during impedance measurements at different values of Iw.

Power law dependence and curve-fitting parameters. Deviations of actual impedance from the best-fit ideal were assessed by using the power law factor (γ) and the curve-fitting parameters normalized error (norm) and r. The γ measured the Cole-Cole power law dependence of the membrane dielectric and described the divergence of the impedance locus from a circular arc (15, 52). The norm was a measure of the percentage difference between the observed and fitted impedance at each frequency. Although we assumed that intact gallbladder would exhibit complex dielectric properties manifested by center suppression of the impedance locus as well as frequency-dependent dispersions of the complex capacitance from the best-fit ideal, actual impedance deviated minimally from the best-fit curves below 7 Hz and above 6 kHz (data not shown). This observation suggested minimal dispersion of the membrane dielectric throughout the frequency range, consistent with calculated values of γ close to unity (see Table 4).
Because we did not observe center suppression of the impedence locus or deviations of the high- and low-frequency data from the best-fit ideal indicating a capacitative property of the junctional complex, we concluded that the junctional pathway could be represented as a single resistive element in the equivalent circuit without oversimplifying the electrical behavior of the tight junction and lateral intercellular space (16, 36, 60). This observation permitted the classic two-membrane equivalent circuit to be used as a model of epithelial impedance. The low resistance of the paracellular shunt ($R_p < 10 \Omega \cdot cm^2$) reduced the shape of the Nyquist plots toward a single semicircle. Because the leaky nature of the gallbladder required a wide range of frequencies to calculate impedance, we focused this report on estimates of the $R_a$ and $R_b$, given the potential for capacitance to be frequency-dependent when $R_a \approx R_p, R_b$ (36, 37). Unlike capacitance, individual membrane resistance is calculated from the real term of impedance ($Z_R$) and is frequency-independent (1, 23, 29) given the relationship $Z (\omega) = V / I$, which simplifies to $Z_R = R = (Z_a R_b)/(Z_a + R_b)$. 

**Bathing solutions.** The composition of the bicarbonate Ringer solution was (in mM): 114 NaCl, 25 NaHCO₃, 4.8 KCl, 2.4 Na₂HPO₄, 0.6 KH₂PO₄, 1.2 MgCl₂, 1.2 CaCl₂, 10 mucosal manniol, and 10 serosal glucose, pH 7.4. For bicarbonate-free experiments, 25 mM Na-gluconate was substituted for NaHCO₃, 10 mM HEPES was added, and the solution was gassed with 100% O₂. For Cl⁻-free experiments, equimolar gluconate salts of Na⁺, K⁺, and Mg²⁺ replaced NaCl, KCl, and MgCl₂. Ca-gluconate (4 mM) replaced 1.2 mM CaCl₂ to compensate for the Ca²⁺-buffering capacity of gluconate.

**Chemicals.** 4,4’-Dinitrostilbene-2,2’-disulfonic acid (DNDS) was synthesized by Tokyo Kasei Kogyo (Tokyo, Japan). Trans-6-cyano-4-(N-ethylsulfonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chroman (293B) was a gift from Dr. Rainer Greger (Albert-Ludwigs-Universität, Freiberg, Germany). Forskolin and apamin were purchased from Calbiochem. Azetazolamide, amiloride, BaCl₂, bumetanide, DIDS, indomethacin, ouabain, phosphoridzin, sodium taurocholate, and tetrodotoxin were purchased from Sigma. Amiloride, tetrodotoxin, phosphoridzin, sodium taurocholate, BaCl₂, and ouabain were made as stock solutions in H₂O. DNDS and DIDS were dissolved in buffer immediately prior to addition. Forskolin, azetazolamide, and 293B were dissolved in DMSO, whereas bumetanide was dissolved in ethanol. Charybdotoxin was obtained from Accurate Chemical and Scientific and was made as a 10 μM stock solution in buffer. All compounds prepared in ethanol or DMSO were made as 1,000-fold stock solutions to keep solvent concentration in the bath at <0.1%. 22Na, 36Cl, and 86Rb were purchased from NEN Life Science Products (Boston, MA).

**RT-PCR.** Total RNA was extracted from intact gallbladders by using Tri-Reagent (Sigma) and was treated with DNase I (Ambion). First-strand cDNA synthesis and PCR were performed by published methods (21) using a Robo ThermoCycler (Stratagene). Cystic fibrosis transmembrane conductance regulator (CFTR)-specific primers (forward: 5’-TTGGAATGCAGATGAGAATACC-3’; reverse: 5’-CCCTGAGAAGAAGAAGGCTGA-3’) amplified a 499-bp region of the published prairie dog sequence (GenBank accession no. AF012893). Pancreatic sodium-bicarbonate cotransporter (pNBC1) primers (forward: 5’-GGATGAAGCTGTCCTGGACAG-3’; reverse: 5’-CCAAGAGCTGGCATCAGTGCC-3’) amplified a 1.7-kb region of the published prairie dog sequence (GenBank accession no. DQ431115). Annealing temperatures for the CFTR and pNBC1 reactions were 59°C and 63°C, respectively. Controls were performed by omitting reverse transcriptase or by adding only water to the reaction.

**Immunoprecipitation and phosphorylation of CFTR.** CFTR immunoprecipitation was performed on gallbladder tissue homogenates by using monoclonal anti-CFTR antibody, N-Terminus (L12B4; Upstate Biotechnology, Lake Placid, NY) as described (2, 13). Wild-type HEK-293 cells transfected with CFTR served as a positive control. Negative controls included untransfected HEK-293 cells in addition to prairie dog skeletal muscle and liver as a test of nonspecific binding.

Immunoprecipitates were labeled by 32P phosphorylation, and the reaction products were resolved on 6% SDS-PAGE.

**Immunofluorescence of CFTR and pNBC1.** Freshly collected tissues were washed with ice-cold PBS and were fixed with 2% paraformaldehyde for 2 h at 4°C, followed by treatment with 30% sucrose overnight. Cryostat sections were blocked with 2% BSA for 40 min, followed by incubation with primary antibody in 0.5% BSA for 1 h. Sections were labeled with the following antibodies: 1) 1:100 dilution of rabbit polyclonal anti-pNBC1 antibody (21); and 2) 1:100 dilution of mouse monoclonal antibody against the NH₂-terminus of CFTR (L12B4). After three washes with 0.5% BSA, the sections were further incubated with secondary antibody, goat anti-rabbit Alexa 488 (1:500; Molecular Probes) for pNBC1 or goat anti-mouse Alexa 488 (1:500; Molecular Probes) for CFTR. Samples were counterstained with rhodamine phalloidin (red) for 40 min at room temperature to label the apical membrane. Nonimmune isotype IgG followed by exposure to secondary antibody served as a control. Prairie dog heart was used as a second negative control for CFTR expression. Slides were visualized by using an Olympus Fluoview 500 confocal microscope.

**RESULTS**

**Basal Isc.** We studied 72 gallbladders from prairie dogs fed nonlithogenic chow. Gallbladders were mounted in bicarbonate Ringer solution. $I_{sc}$ (79 ± 22 μA/cm²) and $G_i$ (13.0 ± 0.8 mS/cm²) remained stable for an average of 7 min with a calculated lumen-negative $V_{l}$ of −6.2 ± 1.8 mV (Table 1). Afterward, both $I_{sc}$ and $G_i$ increased spontaneously for ~30 min (range, 4–46 min) before stabilizing at 171 ± 21 μA/cm² ($P < 0.01$, ANOVA) and 16.3 ± 1.5 mS/cm². Regression analysis of $G_i$ vs. $I_{sc}$ during the spontaneous rise in $I_{sc}$ was a linear function with a slope $G_p$ of 12.3 ± 1.0 mS/cm², equal to 84 ± 3% of total $G_i$. Because stimulatory VIPergic neurons have been reported in guinea pig gallbladder submucosal (39), we added 3 μM tetrodotoxin to the serosal bathing solution.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{sc}$, μA/cm²</th>
<th>$G_i$, mS/cm²</th>
</tr>
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<tbody>
<tr>
<td>Basal Period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>79 ± 22</td>
<td>13.0 ± 0.8</td>
</tr>
<tr>
<td>Peak</td>
<td>171 ± 21</td>
<td>16.3 ± 1.5</td>
</tr>
<tr>
<td>Indomethacin, 10 μM</td>
<td>77 ± 18</td>
<td>11.6 ± 1.0</td>
</tr>
<tr>
<td>Forskolin, 200 nM</td>
<td>125 ± 28</td>
<td>15.7 ± 2.3</td>
</tr>
<tr>
<td>Forskolin, 2 μM</td>
<td>241 ± 21</td>
<td>23.1 ± 2.0</td>
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</tbody>
</table>

Data are means ± SE; $n$ indicates the number of experiments. Comparisons between groups were made by ANOVA followed by Fisher’s exact test or by Student’s paired $t$-test as appropriate by using StatView software. Significance was assumed when $P < 0.05$. For net flux data, experimental error was calculated by the method of error propagation. Curve-fitting of the impedance data was performed by using BLIMP (52) or a curve-fitting routine written for Micromath Scientist.

**Table 1. Electrical parameters during the basal period and in response to 10 μM indomethacin and increasing doses of forskolin**
(n = 3; data not shown) to block potential neuronal stimulation of $I_{sc}$ but observed no effect. Similarly, adding 10 mM glucose (n = 5), 100 µM phlorizin (n = 3), or 30 mM taurine acid (n = 4) to the mucosal solutions to probe for electrogenic Na$^+$-coupled glucose or bile salt cotransport had no effect on $I_{sc}$.

We therefore hypothesized that cholecystectomy triggered endogenous prostaglandin synthesis and added 10 µM indomethacin to the bathing solutions once $I_{sc}$ stabilized (33, 35). As expected, indomethacin reduced $I_{sc}$ and $G_{t}$ to 77 ± 18 µA/cm$^2$ (n = 10) and 11.6 ± 1.0 mS/cm$^2$ (n = 10) over 26 min (range, 13–41 min), in keeping with the inhibition of a prostaglandin-dependent ionic conductance. To reproduce the stimulatory effects of prostaglandins on $I_{sc}$, we used increasing doses of forskolin to raise cAMP production. $I_{sc}$ increased almost immediately, achieved a transient peak within 3 min, and then settled back to a sustained plateau. Forskolin (2 µM) was a maximal stimulus, raising $I_{sc}$ to 241 ± 21 µA/cm$^2$ and $G_{t}$ to 23.1 ± 2.0 mS/cm$^2$ in 10 experiments ($P < 0.01$ vs. indomethacin, ANOVA). Forskolin (200 nM) had intermediate effects on $I_{sc}$ (124.6 ± 27.9 µA/cm$^2$) and $G_{t}$ (15.7 ± 2.3 mS/cm$^2$) that were statistically significant ($P = 0.05$, ANOVA) compared with the indomethacin period. Because indomethacin and forskolin replicated the transport phenotypes observed before and after the spontaneous rise in $I_{sc}$, we used 10 µM indomethacin and 2 µM forskolin in subsequent experiments to create stable conditions for mechanistic studies.

Ionic basis of the forskolin-induced $I_{sc}$. To determine the ionic basis of the forskolin-induced current, we first tested a series of apical cation-channel blockers. Gallbladders were exposed to both low (10 µM) and intermediate doses (50 µM) of the Na$^+$-channel blocker amiloride under four conditions: during the spontaneous rise in $I_{sc}$, after 10 µM indomethacin, and after both 200 nM and 2 µM forskolin. Neither 10 µM nor 50 µM amiloride had any effect on $I_{sc}$ or $G_{t}$ under any of the four experimental conditions (n = 9; data not shown). Mucosal application of Ba$^{2+}$ (2 mM BaCl$_2$), a nonselective K$^+$-channel blocker, also had no effect on $I_{sc}$ (n = 4).

Having excluded amiloride and Ba$^{2+}$-sensitive apical cation channels, we hypothesized that forskolin stimulated cAMP-dependent anion secretion ineptitive to the pharmacological inhibitors. To test the hypothesis that residual $I_{sc}$ depended on basolateral membrane hyperpolarization caused by K$^+$ efflux, we added 100 µM serosal 293B to block the cAMP/PKA-dependent K$^+$ channel KvLQT1 (27). 293B reduced $I_{sc}$ significantly (38.8 ± 16.5 µA/cm$^2$; n = 4; P = 0.05, paired t-test), whereas 20 nM serosal charybdotoxin (Ca$^{2+}$-sensitive K$^+$ channel blocker) and 100 nM serosal ampin (SK channel blocker) had no effect (data not shown). Serosal BaCl$_2$ (2 mM) inhibited $I_{sc}$ transiently (24.4 ± 3.7 µA/cm$^2$; n = 7; P = 0.05, ANOVA), a finding that we attributed to the precipitation of insoluble Ba$^{2+}$ salts in phosphate- and HCO$_3^-$-buffered Ringer. All of the residual $I_{sc}$ was eliminated by 1 mM serosal ouabain (−13 ± 15 µA/cm$^2$; P < 0.0001, ANOVA), confirming the dependence of $I_{sc}$ on serosal Na$^+$-K$^+$-ATPase activity (Fig. 1).

Effects of ion substitution on forskolin-induced $I_{sc}$. To identify the transporters mediating basolateral anion entry, we repeated these pharmacological studies in HCO$_3^-$-free buffer. As shown in Table 2, the forskolin response in HCO$_3^-$-free buffer was reduced by $>$80% compared with standard conditions ($\Delta I_{sc} = 26 ± 3.5$ µA/cm$^2$ vs. $170 ± 35$ µA/cm$^2$; n = 5; $P < 0.001$, ANOVA), and 20 µM serosal bumetanide blocked $I_{sc}$ completely. Neither 3 mM serosal DNS nor 100 µM acetazolamide had any effect on $I_{sc}$ in the absence of HCO$_3^-$. We concluded that 1) forskolin activated a cAMP-dependent Cl$^-$ current dependent on basolateral Na-K-2Cl cotransport, 2) the inhibitory effect of 3 mM serosal DNS required extracellular HCO$_3^-$, and 3) metabolic HCO$_3^-$ production did not contribute to $I_{sc}$ in the absence of exogenous CO$_2$, and 4) removing HCO$_3^-$ from the buffer solution inhibited the forskolin-induced $I_{sc}$ significantly more than the sequential additions of serosal DNS and acetazolamide in standard buffer ($P < 0.008$, ANOVA). We inferred from this observa-
Table 2. Effects of pharmacological blockers and ion substitution on forskolin-induced Isc

<table>
<thead>
<tr>
<th>Treatment</th>
<th>25 mM HCO₃⁻</th>
<th>HCO₃⁻ free</th>
<th>Cl⁻ Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM Indomethacin</td>
<td>100±32</td>
<td>55±8</td>
<td>28±9*</td>
</tr>
<tr>
<td>2 μM Forskolin</td>
<td>269±25</td>
<td>81±8*</td>
<td>194±22</td>
</tr>
<tr>
<td>20 μM Bumetanide</td>
<td>249±27⁺⁺</td>
<td>17±6*</td>
<td>185±25</td>
</tr>
<tr>
<td>DNDS + Acetazolamide</td>
<td>118±11'</td>
<td>5±9</td>
<td>81±10⁺⁺</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are mean Isc (μA/cm²) ± SE; n, number of experiments. *P < 0.05 (ANOVA) vs. 10 μM indomethacin in standard buffer; †P < 0.01 (ANOVA) vs. 2 μM forskolin in both standard and Cl⁻/free buffer; ‡P < 0.02 (ANOVA) vs. 20 μM bumetanide in standard buffer; †P > 0.001 (ANOVA) vs. 20 μM bumetanide in both standard and Cl⁻/free buffer; *P < 0.05 vs. 20 μM bumetanide in standard buffer, †P ≤ 0.001 (ANOVA) vs. 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) + acetazolamide in standard buffer and Cl⁻/free solution; ‡P < 0.01 (ANOVA) vs. DNDS + Acetazolamide in standard buffer; †P ≤ 0.01 (ANOVA) vs. 20 μM bumetanide in Cl⁻/free buffer; ‡P ≤ 0.005 (paired t-test) vs. 2 μM forskolin standard buffer.

...ion that DNDS and acetazolamide did not completely block HCO₃⁻ secretion under standard conditions, a finding that accounted for residual Isc at the conclusion of the blocker studies.

Because the magnitude of the forskolin-induced Isc decreased significantly in the absence of HCO₃⁻, we hypothesized that cAMP stimulated HCO₃⁻ secretion in preference to Cl⁻ under standard conditions. To determine the basolateral entry mechanism for HCO₃⁻, we removed Cl⁻ from the bath to block Cl⁻/HCO₃⁻ exchange as well as Na-K-2Cl cotransport. As shown in Table 2, removing Cl⁻ from the bathing solution inhibited the forskolin-induced Isc (P < 0.02) significantly more than 20 μM serosal bumetanide did in standard buffer, suggesting that DNDS-sensitive Cl⁻/HCO₃⁻ exchange contributed to the forskolin-induced Isc in standard buffer. In Cl⁻/free solution, serosal bumetanide had no effect, whereas 3 mM serosal DNDS and 100 μM acetazolamide inhibited >50% of forskolin-induced Isc (P < 0.01, ANOVA). This inhibitor profile in HCO₃⁻/free solution was consistent with transepithelial HCO₃⁻ secretion driven by carbonic anhydrase and Cl⁻/HCO₃⁻ secretory systems and DNDS-sensitive HCO₃⁻ entry characteristic of basolateral Na⁺-HCO₃⁻ cotransport.

Unidirectional ion fluxes. To determine the ionic basis of Isc in standard buffer, we measured unidirectional ⁴¹Na, ⁸⁶Cl, and ⁸⁶Rb fluxes in 16 normal gallbladders (Table 3). After the spontaneous run-up in Isc, galbladders generated stable current (166 ± 18 μA/cm² or 6.2 ± 0.7 μeq·cm⁻²·h⁻¹) with a calculated lumen-negative potential difference of −10.5 ± 0.9 mV. J⁺,net was twice the rate of passive J⁺,net under these conditions, generating net Na⁺ absorption at 6.5 ± 1.0 μeq·cm⁻²·h⁻¹. Similarly, J⁺,net significantly exceeded J⁺,min, resulting in net Cl⁻ absorption (4.3 ± 1.0 μeq·cm⁻²·h⁻¹) nearly equal to the rate of net Na⁺ absorption. Subsequent measurements of unidirectional ⁸⁶Rb fluxes excluded net ⁸⁶Rb movement as a component of Isc (data not shown). Given minor differences between the rates of net Na⁺ and Cl⁻ absorption, we attributed Isc primarily (70%) to the net flux of unmeasured ions across the epithelium (J⁺,net). Because HCO₃⁻ was the only unmeasured ion in abundance and J⁻,net was not significant, we concluded that transepithelial HCO₃⁻ secretion accounted for J⁺,net. Moreover, the drop in Isc caused by adding DNDS and acetazolamide (115 ± 15 μA/cm²) was equal to the measured value of J⁺,net (4.0 ± 1.4 μeq·cm⁻²·h⁻¹ or 107 ± 37 μA/cm²), further evidence that Isc was attributable to HCO₃⁻ secretion.

Transepithelial impedance analysis. To estimate the resistances of the individual membranes at varying levels of Isc, we performed impedance analysis during the basal period and in response to indomethacin and forskolin. Fourteen normal tissues were evaluated in standard buffer. After tissues were mounted, Isc rose spontaneously and stabilized within 40 min (Fig. 2A). The corresponding Nyquist plots (Fig. 2B) were shifted to the right of the origin of the real axis of impedance by a R series equal to the sum of Rtot and Rsub. The statistical correlation between measured impedance and the fitted Nyquist plots was significant at all time points (norm < 0.05), indicating that the classic two-membrane equivalent electrical circuit was a valid model of the intact gallbladder. The impedance locus at time 0 described a semicircle with slight flattening of the low-frequency arc and minimal center suppression (γ = 0.976) compared with the ideal (γ = 1.0, dashed line). The high-conductance shunt Rs magnified the impedance locus of the apical membrane relative to that of the higher-resistance basolateral membrane (36).

The spontaneous rise of Isc after mounting was accompanied by progressive flattening of the low-frequency impedance arc and falling Rs, where Rs was represented by the intersection of the low-frequency impedance data with the real axis. These changes in impedance were observed in all experiments and indicated that the Gs and Gb increased as Isc rose (Table 4). The change in conductance was most pronounced at the apical membrane, where Rs decreased from 203 ± 81 Ω·cm² to 47 ± 9 Ω·cm² (Table 4). Rising Gs (falling Rs) was consistent with basolateral K⁺ channel activation demonstrated by the inhibitory effects of serosal 293B and Ba2⁺ on Isc. Neither 10 nor 50 μM amiloride had any affect on Rs to indicate amiloride-sensitive Na⁺ channel expression (data not shown). Despite falling Rs, Rsub (6.2 ± 1.9 Ω·cm²) remained constant across the range of Isc, indicating that the subepithelial connective tissue was not affected by the prostaglandin stimulation associated with mounting tissues in the chamber. These changes in transepithelial impedance were reversed by adding 10 μM indomethacin (Fig. 2C) to the bathing solutions. Indomethacin inhibited Isc and shifted the low-frequency data to higher impedance, consistent with rising Rs.

The corresponding Nyquist plots were consistent with the two-membrane electrical circuit model for an average of 11 min in all experiments. In five tissues, the Nyquist plots adopted a single impedance locus as Isc approached its minimum value, meaning that the two-membrane equivalent electrical circuit was a valid model of the intact gallbladder. The impedance locus at time 0 described a semicircle with slight flattening of the low-frequency arc and minimal center suppression (γ = 0.976) compared with the ideal (γ = 1.0, dashed line). The high-conductance shunt Rs magnified the impedance locus of the apical membrane relative to that of the higher-resistance basolateral membrane (36).

Table 3. Unidirectional fluxes of Na⁺ and Cl⁻ during the basal period

<table>
<thead>
<tr>
<th></th>
<th>J⁺</th>
<th>J⁻</th>
<th>J⁺,net</th>
<th>J⁻,net</th>
<th>Isc</th>
<th>Gs</th>
<th>J⁺,net</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>12.5±0.9</td>
<td>5.9±0.6</td>
<td>6.5±1.0</td>
<td>6.2±0.7</td>
<td>17.2±1.1</td>
<td>4.0±1.4</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>7.0±0.8</td>
<td>2.7±0.7</td>
<td>4.3±1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 8. Units are μeq·cm⁻²·h⁻¹ (flux and Isc) and mS/cm² (Gs). ⁴¹Na and ⁸⁶Cl fluxes were measured simultaneously in each experiment. J⁺,net was calculated from the unidirectional mucosal serosa (ms) and serosa-to-mucosa (sm) fluxes during 30-min periods of stable Isc. [J⁺,net = J⁺,ms - J⁺,sm] where positive J⁺,ms denotes absorption]. Mean Gs, different 20% between the unidirectional flux groups.

...
After gallbladder was mounted at time 0, $I_{mc}$ rose spontaneously for 35 min and stabilized prior to the subsequent additions of indomethacin and forskolin. 

Because curve-fitting analysis could not be performed at nadir $I_{mc}$ in all experiments, we inferred that the mean values for $R_a$ and $R_b$ in Table 4 probably underestimated indomethacin's maximum effect. Nonetheless, $R_a$ increased from $47 \pm 9 \, \Omega \cdot \text{cm}^2$ to at least $157 \pm 61 \, \Omega \cdot \text{cm}^2$, whereas $R_b$ increased from $229 \pm 73 \, \Omega \cdot \text{cm}^2$ to $1,102 \pm 223 \, \Omega \cdot \text{cm}^2$. Because stable values of $R_{series}$ and $J_{sm}^{\text{Na}}$ demonstrated that the $R_p$ and $R_{sub}$ remained constant after indomethacin, we concluded that indomethacin raised $R_t$ and reduced $I_{mc}$ by inhibiting the prostaglandin-dependent $G_a$ and $G_b$ that carried the transepithelial anion current.

Conversely, forskolin stimulated $I_{mc}$ and restored the Nyquist plots to the shape observed during the spontaneous rise in $I_{mc}$. These data demonstrate stimulation of the $G_a$ and $G_b$ by cAMP. Forskolin (200 nM) reduced total impedance, flattened the low-frequency arc, and decreased $R_t$. Forskolin (2 $\mu M$) was a maximum stimulus to $I_{mc}$ and generated two distinct impedance semicircles with visually identifiable characteristic frequencies (Fig. 2D) for the apical and basolateral membranes.

Because 2 $\mu M$ forskolin reduced $R_t$ by 40%, we next performed a range analysis to test the hypothesis that $R_p$ was altered by forskolin as well. In proportion to the gallbladder's low baseline $R_p$, small changes in absolute $R_p$ have a large relative effect on the individual membrane resistances estimated by curve-fitting algorithms. The assumption that $R_p$ remained constant after 2 $\mu M$ forskolin rested on the absence of a statistically significant change in the unidirectional fluxes of $[{}^3\text{H}]$mannitol and $J_{sm}^{\text{Na}}$ and the constant slope of the $G_t$-vs.-$I_{mc}$ plots. We therefore picked a range of potential $R_p$ values that were statistically equivalent due to experimental errors in the unidirectional mannitol flux and $J_{sm}^{\text{Na}}$. Assuming a constant standard deviation ($S = 1.7 \, \mu \text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) of mean $J_{sm}^{\text{Na}}$, a 40% drop in $R_p$ (corresponding to the 40% drop in $R_t$ after forskolin) would increase predicted $J_{sm}^{\text{Na}}$ to $9.0 \pm 1.7 \, \mu \text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ according to equation 2, a value that is significantly greater ($P = 0.004$) than observed $J_{sm}^{\text{Na}} (6.4 \pm 1.7 \, \mu \text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1})$ after forskolin. Given this potentially significant change in $J_{sm}^{\text{Na}}$, we concluded that 40% was the maximum possible effect of forskolin on $R_p$. Our subsequent range
Table 4. Impedance estimates of individual membrane resistance during the basal period and in response to both indomethacin and forskolin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( R_a )</th>
<th>( R_b )</th>
<th>( R_t )</th>
<th>( \gamma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>203 ± 61</td>
<td>811 ± 191</td>
<td>77 ± 5</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>Peak</td>
<td>47 ± 9(^a)</td>
<td>229 ± 73(^b)</td>
<td>62 ± 6(^a)</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>Indomethacin, 10 ( \mu M )</td>
<td>157 ± 61(^b)</td>
<td>1,102 ± 223(^b)</td>
<td>86 ± 8(^b)</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>Forskolin, 200 ( nM )</td>
<td>74 ± 21(^d)</td>
<td>250 ± 87(^c)</td>
<td>64 ± 9(^d)</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>Forskolin, 2 ( \mu M )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plotted ( R_p )</td>
<td>12.6 ± 2.0(^e)</td>
<td>83 ± 15(^c)</td>
<td>43 ± 4(^b)</td>
<td>0.96 ± 0.00</td>
</tr>
<tr>
<td>( R_p = R_t )</td>
<td>13.3 ± 2.2(^c)</td>
<td>367 ± 85(^e)</td>
<td>71 ± 0.1(^e)</td>
<td>0.97 ± 0.00</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE at the indicated time points; 14 tissues were studied, except for 200 nM forskolin (\( n = 5 \)). The units of resistance (\( R \)) were \( \Omega \cdot \text{cm}^2 \); \( \gamma \) quantitates center suppression of the impedance locus caused by dispersions of the membrane dielectric (see METHODS). The basal period corresponded to the time between tissue mounting (Initial) and the peak of the spontaneous \( \text{I}_{\text{sc}} \) (Peak). After exposure to 10 \( \mu M \) indomethacin, only 5 tissues maintained impedance characteristics compatible with the 2-membrane model. As a result, reported parameters probably underestimate the maximum effect of indomethacin. \(^a\) \( P < 0.01 \) (ANOVA), \(^b\) \( P < 0.0005 \) (paired \( t \)-test) vs. corresponding Initial; \(^c\) \( P < 0.005 \) (ANOVA), \(^d\) \( P = 0.05 \) (paired \( t \)-test) vs. corresponding Peak; \(^e\) \( P < 0.05 \), \(^f\) \( P < 0.005 \) (ANOVA) vs. corresponding 10 \( \mu M \) indomethacin; \(^g\) \( P = 0.0001 \) (paired \( t \)-test) vs. corresponding 200 nM forskolin.

Analysis accounted for a potential 40% decrease in \( R_p \) in each experiment subject to the constraints of equation 1, which requires that measured \( R_t \) always be the minimum value of \( R_p \). The analyzed range of \( R_p \) included all of the values: 85 \( \Omega \cdot \text{cm}^2 \) > \( R_p \) > 51 \( \Omega \cdot \text{cm}^2 \), where the standard deviation of mean \( R_p \) was 21 \( \Omega \cdot \text{cm}^2 \).

Recalculating the impedance data in each experiment with this unique range of \( R_p \) values (Table 4) demonstrated that a 40% drop in \( R_p \) had no significant effect on the impedance estimates of \( R_a \) and \( R_b \) at a relatively narrow range of \( R_b \) estimates. These results are similar to the analysis of rabbit colon by Wills and Clausen (60), in which impedance estimates of \( R_a \) and \( R_b \) were reasonably robust against errors in \( R_p \) up to 2SD of mean \( R_p \) in magnitude. In prairie dog gallbladder, forskolin’s effect predominated at the apical membrane, where \( R_p \) fell into a range of values between 12.6 ± 2.0 and 13.3 ± 2.2 \( \Omega \cdot \text{cm}^2 \) as \( \text{I}_{\text{sc}} \) exceeded 250 \( \mu A/cm^2 \) (Table 4). The ranges of \( R_a \) and \( R_b \) values resulting from this analysis were significantly lower than estimates obtained in 10 \( \mu M \) indomethacin, indicating that cAMP activated highly significant ionic conductances in the apical and basolateral membranes. Furthermore, the estimated values of \( R_p \) at peak forskolin effect (13 ± 2 \( \Omega \cdot \text{cm}^2 \)) were commensurate with the remarkable rate of \( \text{HCO}_3^- \) secretion demonstrated by \( \text{I}_{\text{sc}} \) (267 ± 36 \( \mu A/cm^2 \); \( n = 10 \)). Although estimates of \( R_b \) resulting from the \( R_p \) range analysis were less robust than \( R_a \), changes in \( R_b \) were still statistically significant compared with the indomethacin values and were consistent with the activation of cAMP-dependent basolateral \( K^+ \) channels sensitive to both 293B and serosal \( Ba^{2+} \).

Membrane localization of CFTR and pNBC1. Activation of cAMP-dependent \( G_a \) is consistent with the function of CFTR protein. RT-PCR analysis of total gallbladder RNA with CFTR-specific primers demonstrated a 499-bp cDNA with 90% sequence homology to human CFTR (Fig. 3A). Subsequent immunoprecipitation with monoclonal anti-CFTR antibody (L12B4) confirmed the synthesis of CFTR protein. As shown in Fig. 3B, both prairie dog gallbladder (lane 3) and CFTR-transfected HEK-293 cells (lane 1) expressed a 190-kDa protein corresponding to fully-glycosylated band C CFTR (13). The 190-kDa band was not observed in untransfected HEK-293 cells (lane 2) or the negative prairie dog controls. Given the specificity of the L12B4 monoclonal antibody for prairie dog CFTR, we performed confocal microscopy (Fig. 4) on intact tissues and observed CFTR immunoreactivity primarily at the apical membrane, with diffuse staining of the apical cytoplasm corresponding to the intracellular distribution of CFTR in subapical vesicles (59). The absence of apical signal with idiotypic antibody (Fig. 4C) on prairie dog skeletal muscle (Fig. 4D) excluded nonspecific antibody binding.

To ascertain the mechanism for \( Cl^- \)-independent, DNDS-sensitive \( \text{HCO}_3^- \) movement across the basolateral membrane, we next used RT-PCR to probe for pNBC1 expression. pNBC1-specific primers amplified a 1.7-kb cDNA with 94% homology to human pNBC1 but not the renal splice variant kNBC1 (Fig. 5A). As shown by immunofluorescence microscopy (Fig. 5, B–D), prairie dog gallbladder localized pNBC1 protein to the basolateral membrane in keeping with the site of DNDS sensitivity. The negative isotype IgG control confirmed that antibody binding was specific. Furthermore, the deduced amino acid sequence of the prairie dog pNBC1 cDNA was 100% identical to the 22-amino acid human pNBC1 polypeptide used to generate the rabbit polyclonal antibody.

Fig. 3. Molecular detection of cystic fibrosis transmembrane conductance regulator (CFTR) in prairie dog gallbladder. A: amplified CFTR PCR product on 1.5% agarose gel stained with ethidium bromide. Lane 1, 100-bp DNA ladder; lane 2, water control; lane 3, no reverse transcriptase control; lane 4, CFTR PCR product at 499 bp. B: autoradiograph of 13\(^{2}P\)-labeled gallbladder immunoprecipitates using anti-CFTR L12B4 monoclonal antibody. Identical concentrations of extracted protein were loaded in each lane. Lane 1, wild-type HEK-293 cells transfected with CFTR showing fully glycosylated band C CFTR at 170–200 kDa (positive control); lane 2, untransfected HEK-293 cells (negative control); lane 3, intact prairie dog gallbladder showing the expected band at 170–200 kDa; lane 4, prairie dog skeletal muscle (negative control); lane 5, prairie dog liver (negative control).
DISCUSSION

Ion transport across prairie dog gallbladders differed significantly from the electroneutral model established by previous studies in rabbits, Necturus, and guinea pigs. Despite sharing high $G_a$ (12.3 ± 1.0 mS/cm²) with the gallbladders of these other species, prairie dog gallbladders generated remarkable basal $I_{sc}$ (79 ± 22 μA/cm²), which rose spontaneously to 171 ± 21 μA/cm² as $R_a$ fell. Impedance analysis correlated rising $I_{sc}$ with progressive activation of ion channels in the apical and basolateral membranes, during which $R_a$ decreased from ~200 Ω·cm² to ~50 Ω·cm² and $R_b$ decreased from 800 Ω·cm² to 200 Ω·cm². The changes in $I_{sc}$ and membrane conductance were reversed by indomethacin and were replicated by forskolin, consistent with cAMP-dependent activation of the relevant ionic conductances. Tetrodotoxin had no effect on $I_{sc}$ to suggest VIPergic neuronal stimulation within the intact submucosa. Prostaglandin synthesis was linked to cholecystectomy and probably represented an inflammatory response to tissue manipulation.

**Transepithelial HCO₃⁻ secretion.** The combination of low apical membrane resistance and high resting $I_{sc}$ in prairie dog gallbladder is a unique finding with the epithelium formerly regarded as electrically silent (5, 6). On the basis of results obtained with pharmacological blockers and ion substitution, we attributed the forskolin-induced $I_{sc}$ primarily to HCO₃⁻ secretion (89%; 249 ± 27 μA/cm²) with a smaller rate of Cl⁻ secretion (11%; 24 ± 4.8 μA/cm²). By itself, forskolin-sensitive $G_a$ is not a novel finding in the gallbladder and has been reported in both Necturus (5) and guinea pigs (62). By comparison with the modest forskolin-induced current of 0.5 μeq·cm⁻²·h⁻¹ in Necturus, resting HCO₃⁻ current in prairie dog gallbladder was 4.0 ± 1.4 μeq·cm⁻²·h⁻¹ and increased to 9.3 ± 1.0 μeq·cm⁻²·h⁻¹ after 2 μM forskolin (16–18). The cAMP response of the $G_a$ pathway coupled with its apparent permeability to both HCO₃⁻ and Cl⁻ in ion-substituted buffer was consistent with our molecular demonstration of CFTR protein and its localization to the apical membrane. Our findings corroborate previous reports of CFTR expression in human (10), mouse (38), and canine gallbladders (22). Although CFTR appears to be uniformly expressed among gallbladder epithelia, prairie dog gallbladders generated much larger forskolin-induced anion currents with lower $R_a$ (13 ± 2 Ω·cm²) than other species. In the absence of patch-clamp data, our identification of the $G_a$ as CFTR must be tempered by the observation that other channels, notably pCLCA1, can mediate cAMP-dependent anion secretion (26).

The observation of cAMP-dependent bicarbonate secretion across prairie dog gallbladder is more analogous to the function of pancreatic duct epithelium than the electrically silent gallbladders of Necturus and rabbits. The high rate of HCO₃⁻ secretion gives physiological relevance to our identification of three different mechanisms for HCO₃⁻ uptake, including basolateral Na⁺-HCO₃⁻ cotransport, Cl⁻/HCO₃⁻ exchange, and metabolic production by carbonic anhydrase. Given the mag-
nitude of the DNDS-sensitive $I_{sc}$ in Cl$^-$-free buffer, the majority of transepithelial HCO$_3^-$ secretion was mediated by basolateral sodium-bicarbonate cotransporter activity, consistent with our demonstration of basolateral pNBC1 expression. pNBC1 is the pancreatic isoform of the Na$^+$/HCO$_3^-$ cotransporter that mediates electrogenic HCO$_3^-$ uptake in pancreatic duct and Calu-3 cells, consistent with its role in transepithelial HCO$_3^-$ secretion. Basolateral Na$^+$/HCO$_3^-$ cotransport has been reported in guinea pig gallbladder, where it generates electrogenic HCO$_3^-$ secretion via apical Cl$^-$/HCO$_3^-$ exchange at 1.0 mEq·cm$^{-2}$·h$^{-1}$ (62). In addition to cotransporter activity, we also observed inhibition of the forskolin-induced $I_{sc}$ by Cl$^-$-free buffer, suggesting Cl$^-$-dependent HCO$_3^-$ exchange across the basolateral membrane. This functional evidence for a second HCO$_3^-$ uptake mechanism confirms our prior report that prairie dog gallbladder expresses anion exchanger 2 (33a), a basolateral isoform of the Cl$^-$/HCO$_3^-$ exchanger that forms membrane-bound complexes with carbonic anhydrase (57). Data showing that carbonic anhydrase is required for maximal Cl$^-$/HCO$_3^-$ exchanger activity in transfected HEK-293 cells (54) may explain the effects of acetazolamide on HEK-293 in our experiments as well as the reduction of HCO$_3^-$ secretion by acetazolamide in both murine (31) and mouse (34).

Impedance measurements of $G_{\infty}$. Previous studies correlating membrane resistance with electrogenic HCO$_3^-$ secretion in rabbit and Necturus gallbladders reveal different limitations to conductive HCO$_3^-$ exit across the apical membrane. For example, rabbit gallbladder demonstrated a considerable rate (2.8 μEq·cm$^{-2}$·h$^{-1}$) of stilbene-sensitive HCO$_3^-$ uptake across the basolateral membrane but no electrogenic HCO$_3^-$ secretion. Corresponding microelectrode measurements showed a high resting membrane resistance ratio ($R_a/R_b = 1.9$) in rabbit gallbladder that did not respond to 8-bromo adenosine-cAMP as required for conductive HCO$_3^-$ exit (41). Similar studies in Necturus gallbladder showed negligible electrogenic HCO$_3^-$ secretion under basal conditions, with a slight increase to ≈0.5 μEq·cm$^{-2}$·h$^{-1}$ after 2 μM forskolin. Unlike in rabbits, the rate-limiting step for HCO$_3^-$ secretion across Necturus gallbladder was not $G_a$. Using impedance techniques, Kottra et al. (17) demonstrated that $R_a$ fell from its basal value of 2,850 Ω·cm$^2$ in Necturus gallbladder to 370 Ω·cm$^2$ within 3 min of exposure to 2 μM forskolin and to 20 ± 5 Ω·cm$^2$ within 30 min. Likewise, $R_b$ fell from 1,185 to 512 Ω·cm$^2$, reducing $R_a/R_b$ from 2.5 ± 0.3 to 0.1 ± 0.1. Considering the magnitude of the forskolin-induced $I_{sc}$ across Necturus gallbladder, the large change in the membrane resistance ratio indicated that driving forces limited the rate of transepithelial HCO$_3^-$ secretion. In contrast to both rabbits and Necturus, gallbladders demonstrated significantly lower $R_a$ (≈200 Ω·cm$^2$) under resting conditions but similar $R_b$ (≈800 Ω·cm$^2$). In response to cAMP, $R_a$ fell precipitously to the remarkable value of 13 ± 2 Ω·cm$^2$ within 4 ± 1 min of forskolin addition, whereas the membrane resistance ratio fell from 0.19 ± 0.04 to 0.13 ± 0.03 ($P < 0.04$) despite a simultaneous fall in $R_b$. These remarkable conductance estimates are commensurate with the degree of apical CFTR expression observed by immunoprecipitation and immunofluorescence microscopy.
Although high $G_a$ and redundant mechanisms for basolateral HCO$_3^-$ uptake set the stage for HCO$_3^-$ movement, the eventual rate of transepithelial secretion is determined by driving forces across the individual membranes. In response to forskolin, $G_a$ exceeded 75 mS/cm$^2$, suggesting that a driving force of $\approx$3.6 mV could generate the observed anion current of 269 $\mu$A/cm$^2$. Large changes in $R_b$ after exposure to indomethacin and forskolin indicated that a cAMP-dependent ionic conductance regulated the potential of the basolateral membrane and the resulting driving force for anion secretion across the apical membrane. Inhibitory effects of serosal Ba$^{2+}$ and 293B as well as the insensitivity to charybdotoxin and apamin demonstrated a basolateral K$^+$ conductance consistent with a cAMP-dependent K$^+$ channel like KvLQT1 rather than a Ca$^{2+}$-sensitive K$^+$ channel or SK type channel (28). Elimination of the residual $I_{sc}$ by serosal ouabain excluded systematic errors in the measurement of $I_{sc}$ in this leaky preparation and confirmed the dependence of anion transport on basolateral Na$^{+}$-K$^+$-ATPase activity.

We based our conclusions about the individual membrane resistances on the assumption that forskolin and indomethacin did not appreciably alter the $R_p$ during impedance experiments. Neither forskolin nor indomethacin altered the passive fluxes of nontransported substances, and the plots of $G_i$ vs. $I_{sc}$ remained linear across the range of $I_{sc}$ with a high correlation coefficient. These results supported the assumption that $R_p$ remained constant over the range of observed $I_{sc}$. Furthermore, recalculating impedance estimates of $R_a$ and $R_b$ over a range of potential $R_p$ values did not alter our conclusion that the individual membrane conductances in prairie dog gallbladder were significantly lower than in rabbits and Necturus. Our assumption of constant $R_p$ is strengthened by data obtained in Necturus gallbladder showing that cAMP had no effect on junctional resistance but gradually increased the resistance of the lateral intercellular spaces (18). The impact of including this resistance as a circuit element in a more complex, distributed equivalent electrical circuit of the prairie dog gallbladder has already been evaluated in Necturus. Although the distributed model reduced the magnitude of the deviation between the observed and fitted impedance functions compared with the classic equivalent electrical circuit used in our experiments, prior authors (16) observed no significant effect of the more complex circuit model on the magnitude of the calculated parameters like $R_a$ and $R_b$ derived from curve fitting.

A further test of the assumptions underlying our impedance analysis in this leaky preparation was the use of indomethacin to check for dielectric dispersions over the wide range of frequencies required during these experiments. Similar to the use of amiloride in frog skin, indomethacin blocked $G_a$ and $G_b$ in prairie dog gallbladder, raising the values of $R_a$ and $R_b$ relative to $R_p$ (1, 36). Under conditions of $R_a/R_b \gg R_p$ caused by indomethacin, we observed no significant change in the Cole-Cole power law factor $\gamma$ to indicate that dielectric dispersions affected impedance measurements, lending further credibility to our fitted estimates of $R_a$ and $R_b$. These data confirmed that impedance analysis could be successfully applied to prairie dog gallbladders despite their low $R_b$, confirming prior reports in Necturus gallbladder (15, 47) and human colon (48).

**Significance of apical CFTR and basolateral pNBC1 expression.** The combination of significant $G_a$ and redundant mechanisms for basolateral HCO$_3^-$ uptake constitute a coordinated mechanism for electrogenic HCO$_3^-$ secretion into the lumen of the prairie dog gallbladder under resting conditions that is directed opposite to the absorptive flux of NaCl. The bumetanide sensitivity of $I_{sc}$ suggested that $J_{sc}$ was the sum of oppositely directed Cl$^-$ movements involving Cl$^-$ secretion mediated by basolateral Na-K-2Cl cotransport at 1.0 $\pm$ 0.2 $\mu$eq-cm$^{-2}$-h$^{-1}$ offset by electroneutral Cl$^-$ absorption at 5.3 $\mu$eq-cm$^{-2}$-h$^{-1}$. In the absence of amiloride-sensitive current or apical resistance, equal rates of net Na$^+$ and Cl$^-$ absorption suggested that electroneutral parallel ion exchange or Na$^+$-Cl$^-$ cotransport mediated NaCl absorption as described by Reuss (43) and Frizzell et al. (11) in Necturus and rabbit gallbladders.

On the basis of the accumulated evidence, we propose the following model (Fig. 6) of the prairie dog gallbladder: CFTR mediates $G_a$ under resting conditions and is stimulated by cAMP and endogenous prostaglandins. The negligible value of bumetanide-sensitive Cl$^-$ secretion suggests that high resting $G_a$ depolarizes the apical membrane potential and reduces it to a value nearly equal to the electrochemical equilibrium potential for Cl$^-$, thereby limiting the driving force for Cl$^-$ move-
ment. The majority of $I_{nc}$ therefore represents electrogenic HCO$_3^-$ current maintained by three sources of HCO$_3^-$ uptake: basolateral pNBC1, basolateral Cl$^-$/HCO$_3^-$ exchange, and metabolic production from CO$_2$ via carbonic anhydrase. The driving force for HCO$_3^-$ secretion is provided by the activation of cAMP-dependent basolateral K$^+$ channels like KvLQT1 and is maintained by the ouabain-sensitive basolateral Na$^+$/K$^+$-$ATPase$. In prairie dog gallbladder, electrogenic HCO$_3^-$ secretion thereby exists in parallel with electroneutral NaCl absorption and is regulated by the cAMP-dependent conductances of the apical and basolateral membranes and the uptake of HCO$_3^-$ by multiple basolateral mechanisms.

The physiological relevance of electrogenic bicarbonate secretion in the prairie dog model has not been addressed in this study but may flush cholesterol and mucus out of the gallbladder lumen between meals as observed in humans and primates. We further speculate that HCO$_3^-$ secretion counteracts the fall in luminal pH resulting from the absorption of salt and water by the gallbladder epithelium and the resulting acidification of the lumen caused by rising bile acid concentration (42, 50). Defective HCO$_3^-$ secretion may permit the juxtaepithelial mucus layer to undergo a pH-dependent phase shift to the gel state that has been associated with the nucleation of cholesterol crystals from saturated bile (53). Indirect evidence for this hypothesis is the observation that gallstone risk is increased 10-fold among patients with cystic fibrosis (24) and reported links between CFTR and mucus hypersecretion during gallstone formation (51). Future studies in cholesterol-fed prairie dogs will determine the role of altered gallbladder HCO$_3^-$ secretion during the formation of gallstones.

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