Electrogenic bicarbonate secretion by prairie dog gallbladder

A. James Moser,1,2 A. Gangopadhyay,1 N. A. Bradbury,3 K. W. Peters,2 R. A. Frizzell,2 and R. J. Bridges3

Departments of 1Surgery and 2Cell Biology and Physiology, University of Pittsburgh
School of Medicine, Pittsburgh, Pennsylvania; and 3Department of Physiology and Biophysics, Rosalind Franklin School of Medicine, North Chicago, Illinois

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Moser AJ, Gangopadhyay A, Bradbury NA, Peters KW, Frizzell RA, Bridges RJ. Electrogenic bicarbonate secretion by prairie dog gallbladder. Am J Physiol Gastrointest Liver Physiol 292: G1683–G1694, 2007. First published March 15, 2007; doi:10.1152/ajpgi.00268.2006.—Pathological rates of gallbladder salt and water transport may promote the formation of cholesterol gallstones. Because prairie dogs are widely used as a model of this event, we characterized gallbladder ion transport in animals fed control chow by using electrophysiology, ion substitution, pharmacology, isotopic fluxes, impedance analysis, and molecular biology. In contrast to the electroneutral properties of rabbit and Necturus gallbladders, prairie dog gallbladders generated significant short-circuit current (Isc; 171 ± 21 μA/cm2) and lumen-negative potential difference (−10.1 ± 1.2 mV) under basal conditions. Unidirectional radioisotopic fluxes demonstrated electroneutral NaCl absorption, whereas the residual net ion flux corresponded to Isc. In response to 2 μM forskolin, Isc exceeded 270 μA/cm2, and impedance estimates of the apical membrane resistance decreased from 200 Ω·cm² to 13 Ω·cm². The forskolin-induced Isc was dependent on extracellular HCO₃⁻ and was blocked by serosal 4,4'-dinitrostilben-2,2'-disulfonic acid (DNDS) and acetazolamide, whereas serosal bumetanide and Cl⁻ ion substitution had little effect. Serosal trans-6-cyano-4-(N-ethylsulfonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-choran and Ba⁡⁺ reduced Isc, consistent with the inhibition of cAMP-dependent K⁺ channels. Immunoprecipitation and confocal microscopy localized cystic fibrosis transmembrane conductance regulator protein (CFTR) to the apical membrane and subapical vesicles. Consistent with serosal DNDS sensitivity, pancreatic sodium-bicarbonate cotransporter protein pNBC1 expression was localized to the basolateral membrane. We conclude that prairie dog gallbladders secrete bicarbonate through cAMP-dependent apical CFTR anion channels. Basolateral HCO₃⁻ entry is mediated by DNDS-sensitive pNBC1, and the driving force for apical anion secretion is provided by K⁺ channel activation.

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 (Rₑ), 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 (Gₑ) (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 impalements. In particular, transepithelial bicarbonate transport was mediated by electroneutral parallel Na⁺/H⁺, 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. Paraffin gaskets were used to minimize edge damage. Glass condensers reduced 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 (Rsf) compensation by using automatic voltage clamps (Department of Bioengineering, University of Iowa, Iowa City, IA) connected to a computer interface (National Instruments) running Labview data-acquisition software. Voltage and Isc measurements were made with glass electrodes (University of Pittsburgh Machine Shop) containing Ag wire electroplated with AgCl and filled with 1 M KCl (8). Ri 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 Isc stabilized, radioisotopes (²²Na and ¹⁸⁶Cl 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 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 (Jₚₛ) 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 Rsf. Because the passive flux of Na⁺ is equal to the partial ionic conductance of Na⁺ (49), the maximum estimate of Rsf was 169 ± 16 mS/cm (Rsf = 1.00/Rₚₛ). Given the relationship between Ri and the cellular (Rcell) and shunt resistances (1/Ri = 1/Rcell + 1/Rs; equation 1), the minimum value of Ri in each experiment was equal to Rsf. Although Isc and Ri changed dramatically in response to indomethacin and forskolin, mean Jₚₛ varied by <10%, suggesting that Ri remained constant.

Having defined the boundaries of Ri (169 ± 16 mS/cm > Rsf > Rs), we plotted unique estimates of Ri during each experiment by linear regression analysis of the Gi and Isc data according to Gi = Isc/Vsc + Gp (equation 2), where Gp is paracellular conductance, and used this value in curve-fitting analysis. Despite having low Rs, prairie dog gallbladder satisfied the major methodological requirements for estimating the individual membrane parameters by impedance techniques: 1) the plot of Gi vs. Isc was linear in each experiment, and r = 0.84 ± 0.05 (P < 0.0001) in 10 experiments; 2) Ri remained constant at different values of Isc 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 Rsub (6.2 ± 1.9 Ω·cm²). Rsub was calculated (Rsub = Rseries - Rcell) by measuring Rcell during instrument setup and Rseries during impedance measurements at different values of Isc.

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 percent 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 impedance 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 ($R_s < 100 \Omega$·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 $R_s$ and $R_b$, given the potential for capacitance to be frequency-dependent when $R_s \equiv R_b$ ($R_s$, 36, 37). Unlike capacitance, individual membrane resistance is calculated from the real term of impedance ($Z_b$) and is frequency-independent (1, 23, 29) given the relationship $Z' (\omega) = V / I$, which simplifies to $Z_b = R (C)$. 

**Bathing solutions.** The composition of the bicarbonate Ringer solution was (in mM): 114 NaCl, 25 NaHCO$_3$, 4.8 KCl, 2.4 Na$_2$HPO$_4$, 0.6 KH$_2$PO$_4$, 1.2 MgCl$_2$, 1.2 CaCl$_2$, 10 mucosal mannitol, and 10 serosal glucose, pH 7.4. For bicarbonate-free experiments, 25 mM Na-glucuronate was substituted for NaHCO$_3$, 10 mM HEPES was added, and the solution was gassed with 100% O$_2$. For Cl$^-$-free experiments, equimolar glucuronate salts of Na$^+$, K$^+$, and Mg$^{2+}$ replaced NaCl, KCl, and MgCl$_2$. Ca-glucuronate (4 mM) replaced 1.2 mM CaCl$_2$ to compensate for the Ca$^{2+}$-buffering capacity of glucuronate.

**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, BaCl$_2$, bumetanide, DIDS, indomethacin, ouabain, sodium taurocholate, and tetrodotoxin were purchased from Sigma. Charybdotoxin was obtained from Accurate Chemical and Scientific (293B) was a gift from Dr. Rainer Greger (Albert-Ludwigs-Universität, Freiberg, Germany). Forskolin and apamin were purchased from Calbiochem. Azetazolamide, amiloride, BaCl$_2$, bumetanide, DIDS, indomethacin, ouabain, sodium taurocholate, and tetrodotoxin were purchased from Sigma. Charybdotoxin was obtained from Accurate Chemical and Scientific (293B) was a gift from Dr. Rainer Greger (Albert-Ludwigs-Universität, Freiberg, Germany). Forskolin and apamin were purchased from Calbiochem.

**RT-PCR.** Total RNA was extracted from intact gallbladders by using Tri-Reagent (Sigma) and was treated with DNase 1 (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'-TTGAATGCAGATGAGAATACC-3'; reverse: 5'-CCCTGAAAGAAGAGGCTGA-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'-CCAAGAAGCTGCGATCAGGTC-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 $^{32}$P 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 overdose. 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 NH$_2$ 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 I$_{sc}$.** We studied 72 gallbladders from prairie dogs fed nonlithogenic chow. Gallbladders were mounted in bicarbonate Ringer solution. $I_{sc}$ ($79 \pm 22 \mu A/cm^2$) and $G_S$ ($13.0 \pm 0.8 mS/cm^2$) remained stable for an average of 7 min with a calculated lumen-negative $V_{L}$ of $-6.2 \pm 1.8 mV$ (Table 1). Afterward, both $I_{sc}$ and $G_S$ increased spontaneously for ~30 min (range, 4–46 min) before stabilizing at $171 \pm 21 \mu A/cm^2$ ($P < 0.01$, ANOVA) and $16.3 \pm 1.5 mS/cm^2$. Regression analysis of $G_S$ 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/cm$^2$, equal to 84 ± 3% of total $G_S$. Because stimulatory VIPergic neurons have been reported in guinea pig gallbladder submucosa (39), we added 3 mM tetrodotoxin to the serosal bathing solution.
cin and forskolin replicated the transport phenotypes observed compared with the indomethacin period. Because indomethacin reduced 
Isc (118 ± 11 μA/cm²) persisted in forskolin-treated tissues. This finding suggested residual

anion secretion insensitive to the pharmacological inhibitors. To test the hypothesis that residual Isc 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 Isc significantly (38.8 ± 16.5 μA/cm²; n = 4; P = 0.05, paired t-test), whereas 20 mM serosal charybdoxotoxin (Ca²⁺-sensitive K⁺ channel blocker) and 100 mM serosal ampin (SK channel blocker) had no effect (data not shown). Serosal BaCl₂ (2 mM) inhibited Isc transiently (24.4 ± 3.7 μA/cm²; n = 7; P = 0.05, ANOVA), a finding that we attributed to the precipitation of insoluble Ba²⁺ salts in phosphate- and HCO₃⁻-buffered Ringer. All of the residual Isc was eliminated by 1 mM serosal ouabain (-13 ± 15 μA/cm²; P < 0.0001, ANOVA), confirming the dependence of Isc on serosal Na⁺-K⁺-ATPase activity (Fig. 1).

Effects of ion substitution on forskolin-induced Isc. To identify the transporters mediating basolateral anion entry, we repeated these pharmacological studies in HCO₃⁻-free buffer. As shown in Table 2, the forskolin response in HCO₃⁻-free buffer was reduced by >80% compared with standard conditions (ΔIsc = 26 ± 3.5 μA/cm² vs. 170 ± 35 μA/cm²; n = 5; P < 0.001, ANOVA), and 20 μM serosal bumetanide blocked Isc completely. Neither 3 mM serosal DNDS nor 100 μM acetazolamide had any effect on Isc in the absence of HCO₃⁻. 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 DNDS required extracellular HCO₃⁻, 3) metabolic HCO₃⁻ production did not contribute to Isc in the absence of exogenous CO₂, and 4) removing HCO₃⁻ from the buffer solution inhibited the forskolin-induced Isc significantly more than the sequential additions of serosal DNDS and acetazolamide in standard buffer (P < 0.008, ANOVA). We inferred from this observa-

<table>
<thead>
<tr>
<th>n</th>
<th>Isc (μA/cm²)</th>
<th>10 μM forskolin</th>
<th>2 μM forskolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>241 ± 24</td>
<td>170 ± 17</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3</td>
<td>241 ± 24</td>
<td>170 ± 17</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3</td>
<td>241 ± 24</td>
<td>170 ± 17</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3</td>
<td>241 ± 24</td>
<td>170 ± 17</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Fig. 1. Current trace from a typical experiment using pharmacological blockers to inhibit the forskolin-stimulated short-circuit current (Isc). Gallbladders were pretreated with 10 μM indomethacin and 2 μM forskolin. Pharmacological inhibitors were added to the indicated solutions while Isc and transepithelial resistance (Rt) were measured. Ouabain was used to measure electrode drift. DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; M, mucosal; S, serosal.

- VOL 292 • JUNE 2007 • www.ajpgi.org

AJP-Gastrointest Liver Physiol • VOL 292 • JUNE 2007 • www.ajpgi.org
Table 2. Effects of pharmacological blockers and ion substitution on forskolin-induced $I_{sc}$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HCO$_3^-$</th>
<th>HCO$_3^-$ free</th>
<th>Cl$^-$ free</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 $\mu$m Indomethacin</td>
<td>100±32</td>
<td>55±8</td>
<td>28±9*</td>
</tr>
<tr>
<td>2 $\mu$m Forskolin</td>
<td>269±25</td>
<td>81±8*</td>
<td>194±22*</td>
</tr>
<tr>
<td>20 $\mu$m 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>9±5*</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 $I_{sc}$ ($\mu$A/cm$^2$) ± SE; $n$, number of experiments. *$P < 0.05$ (ANOVA) vs. 10 $\mu$m indomethacin in standard buffer; $P = 0.001$ (ANOVA) vs. 2 $\mu$m forskolin in both standard and Cl$^-$free buffer; $P < 0.02$ (ANOVA) vs. 2 $\mu$m forskolin in standard buffer; $P < 0.001$ (ANOVA) vs. 20 $\mu$m bumetanide in both standard and Cl$^-$free buffer; $P < 0.05$ vs. 20 $\mu$m bumetanide in standard buffer, $P = 0.001$ (ANOVA) vs. 20 $\mu$m bumetanide in Cl$^-$free buffer; $P = 0.0005$ (ANOVA) vs. 20 $\mu$m bumetanide in standard buffer; $P < 0.001$ (paired t-test) vs. 2 $\mu$m forskolin standard buffer.

Table 3. Unidirectional fluxes of Na$^+$ and Cl$^-$ during the basal period

<table>
<thead>
<tr>
<th>$J_{in}$</th>
<th>$J_{cm}$</th>
<th>$J_{out}$</th>
<th>$I_{sc}$</th>
<th>$G_{i}$</th>
<th>$P_{R}$</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>
</tr>
<tr>
<td>Chloride</td>
<td>7.0±0.8</td>
<td>2.7±0.7</td>
<td>4.3±1.0</td>
<td>5.2±1.2</td>
<td>8.4±1.0</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; $n = 8$. Units are $\mu$eq·cm$^{-2}$·h$^{-1}$ (flux and $I_{sc}$) and nEq/cm$^2$(Gs). $^{22}$Na and $^{86}$Rb fluxes were measured simultaneously in each experiment. $P_{R}$ was calculated from the unidirectional mucosa-serosa (ms) and serosa-mucosa (sm) fluxes during 30-min periods of stable $I_{sc}$. $P_{R} = P_{ms} - P_{sm}$, where positive $P_{R}$ denotes absorption. Mean $G_{i}$, different 20% between the unidirectional flux groups.
After indomethacin, we concluded that indomethacin raised and increasing concentrations of forskolin (Forsk). After gallbladder was mounted at experiments, we inferred that the mean values for subsequent additions of indomethacin and forskolin.

Because 2 μM forskolin reduced $R_t$ by 40%, we next performed a range analysis to test the hypothesis that $R_t$ 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 μM forskolin rested on the absence of a statistically significant change in the unidirectional fluxes of $[^3H]$mannitol and $J_{\text{eq}}^\text{sm}$ and the constant slope of the $G_t$-vs-$I_{sc}$ 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 $J_{\text{eq}}^\text{sm}$. Assuming a constant standard deviation ($S = 1.7 \mu\text{eq cm}^{-2}\text{h}^{-1}$) of mean $J_{\text{eq}}^\text{sm}$, a 40% drop in $R_p$ (corresponding to the 40% drop in $R_t$ after forskolin) would increase predicted $J_{\text{eq}}^\text{sm}$ to 9.0 ± 1.7 μeq cm$^{-2}$h$^{-1}$ according to equation 2, a value that is significantly greater ($P = 0.004$) than observed $J_{\text{eq}}^\text{sm}$ (6.4 ± 1.7 μeq cm$^{-2}$h$^{-1}$) after forskolin. Given this potentially significant change in $J_{\text{eq}}^\text{sm}$, 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><strong>Basal period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>203 ± 81</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$^b$</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>Indomethacin, 10 μM</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 μM</td>
<td>12.6 ± 2.0$^a$</td>
<td>83 ± 15$^b$</td>
<td>43 ± 4$^b$</td>
<td>0.96 ± 0.00</td>
</tr>
<tr>
<td>Plotted $R_p$</td>
<td>13.3 ± 2.2$^c$</td>
<td>367 ± 85$^c$</td>
<td></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 Ω•cm$^2$; $\gamma$ quantitates center suppression of the impedance locus caused by dispersions of the membrane dielectric (see METHODS). The basal period corresponds to the time between tissue mounting (Initial) and the peak of the spontaneous $I_{sc}$ (Peak). After exposure to 10 μM 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. $^aP < 0.01$ (ANOVA), $^bP < 0.005$ (paired t-test) vs. corresponding Initial; $^cP < 0.005$ (ANOVA), $^dP = 0.05$ (paired t-test) vs. corresponding Peak; $^eP < 0.005$, $^fP < 0.05$ (ANOVA) vs. corresponding 10 μM indomethacin; $^gP = 0.0001$ (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 $R_b$ included all of the values: 85 Ω•cm$^2$ > $R_b$ > 51 Ω•cm$^2$, where the standard deviation of mean $R_p$ was 21 Ω•cm$^2$.

Recalculating the impedance data in each experiment with this unique range of $R_b$ values (Table 4) demonstrated that a 40% drop in $R_p$ had no significant effect on the impedance estimates of $R_a$ and produced 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 $R_t$ in magnitude. In prairie dog gallbladder, forskolin’s effect predominated at the apical membrane, where $R_t$ fell into a range of values between 12.6 ± 2.0 and 13.3 ± 2.2 Ω•cm$^2$ as $I_{sc}$ exceeded 250 μA/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 μM indomethacin, indicating that cAMP activated highly significant ionic conductances in the apical and basolateral membranes. Furthermore, the estimated values of $R_b$ at peak forskolin effect (13 ± 2 Ω•cm$^2$) were commensurate with the remarkable rate of HCO$_3^-$ secretion demonstrated by $I_{sc}$ (267 ± 36 μA/cm$^2$; $n = 10$). Although estimates of $R_b$ resulting from the $R_p$ range analysis were less robust than $R_p$, 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 serum Ba$^{2+}$.

Membrane localization of CFTR and pNBC1. Activation of cAMP-dependent G$\alpha$ 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, DNDsensitive 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.
**DISCUSSION**

Ion transport across prairie dog gallbladders differed significantly from the electro-neutral model established by previous studies in rabbits, *Necturus*, and guinea pigs. Despite sharing high $G_a$ $(12.3 \pm 1.0 \, \text{mS/cm}^2)$ with the gallbladders of these other species, prairie dog gallbladders generated remarkable basal $I_{sc}$ $(79 \pm 22 \, \mu \text{A/cm}^2)$, which rose spontaneously to $171 \pm 21 \, \mu \text{A/cm}^2$ 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 $\sim 200 \, \Omega \cdot \text{cm}^2$ to $\sim 50 \, \Omega \cdot \text{cm}^2$ and $R_b$ decreased from $800 \, \Omega \cdot \text{cm}^2$ to $200 \, \Omega \cdot \text{cm}^2$. 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 $\text{HCO}_3^-$ secretion.** The combination of low apical membrane resistance and high resting $I_{sc}$ in prairie dog gallbladder is a unique finding within 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 $\text{HCO}_3^-$ secretion $(89\% ; 249 \pm 27 \, \mu \text{A/cm}^2)$ with a smaller rate of $\text{Cl}^-$ secretion $(11\% ; 24 \pm 4.8 \, \mu \text{A/cm}^2)$. 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 $\mu$eq·cm$^{-2}$·h$^{-1}$ in *Necturus*, resting $\text{HCO}_3^-$ current in prairie dog gallbladder was $4.0 \pm 1.4 \, \mu$eq·cm$^{-2}$·h$^{-1}$ and increased to $9.3 \pm 1.0 \, \mu$eq·cm$^{-2}$·h$^{-1}$ after 2 $\mu$M forskolin (16–18). The cAMP response of the $G_a$ pathway coupled with its apparent permeability to both $\text{HCO}_3^-$ and $\text{Cl}^-$ 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 $R_a$ $(13 \pm 2 \, \Omega \cdot \text{cm}^2)$ 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 $\text{HCO}_3^-$ secretion gives physiological relevance to our identification of three different mechanisms for $\text{HCO}_3^-$ uptake, including basolateral $\text{Na}^+\text{-HCO}_3^-$ cotransport, $\text{Cl}^-/\text{HCO}_3^-$ exchange, and metabolic production by carbonic anhydrase. Given the mag-
magnitude 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 electroneutral HCO$_3^-$ secretion via apical Cl$^-$/HCO$_3^-$ exchange at 1.0 $\mu$eq·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 cells in our experiments as well as the reduction of HCO$_3^-$ secretion by acetazolamide in both murine (31) and mouse (34).

**Impedance measurements of $G_{m}$.** 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 $\mu$eq·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 ($R_d/R_b = 1.9$) in rabbit gallbladder that did not respond to 8-bromoadenosine-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 $\approx 0.5$ $\mu$eq·cm$^{-2}$·h$^{-1}$ after 2 $\mu$M forskolin. Unlike in rabbits, the rate-limiting step for HCO$_3^-$ secretion across *Necturus* gallbladder was not $G_{m}$. Using impedance techniques, Kottra et al. (17) demonstrated that $R_d$ fell from its basal value of 2,850 $\Omega$·cm$^2$ in *Necturus* gallbladder to 370 $\Omega$·cm$^2$ within 3 min of exposure to 2 $\mu$M forskolin and to 20 ± 5 $\Omega$·cm$^2$ within 30 min. Likewise, $R_b$ fell from 1,185 to 512 $\Omega$·cm$^2$, reducing $R_d/R_b$ 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 $R_d$ ($\approx 200$ $\Omega$·cm$^2$) under resting conditions but similar $R_b$ ($\approx 800$ $\Omega$·cm$^2$). In response to cAMP, $R_d$ fell precipitously to the remarkable value of 13 ± 2 $\Omega$·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 \( \text{HCO}_3^- \) uptake set the stage for \( \text{HCO}_3^- \) 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/cm², suggesting that a driving force of \( \approx 3.6 \) mV could generate the observed anion current of 269 \( \mu \)A/cm². 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 \( \text{Ba}^{2+} \) 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 \( \text{Ca}^{2+} \)-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 \( \text{Na}^+\text{-K}^+\text{-ATPase} \) 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 \( G_t \) 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 \( R_p \) 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 \textit{Necturus}. Our assumption of constant \( R_p \) is strengthened by data obtained in \textit{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 \textit{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 \( R_p \) 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 \( R/R_p \gg R_p \) 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 \( R_p \), confirming prior reports in \textit{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 \( \text{HCO}_3^- \) uptake constitutes a coordinated mechanism for electrogenic \( \text{HCO}_3^- \) secretion into the lumen of the prairie dog gallbladder under resting conditions that is directed opposite to the absorptive flux of \text{NaCl}. The bumetanide sensitivity of \( I_{sc} \) suggested that \( J^*_{sc} \) was the sum of oppositely directed \( \text{Cl}^- \) movements involving \( \text{Cl}^- \) secretion mediated by basolateral \text{Na}-K-2Cl cotransport at 1.0 \( \pm 0.2 \) \( \mu \)eq/cm²·h⁻¹ due to electroneutral \( \text{Cl}^- \) absorption at 3.3 \( \mu \)eq/cm²·h⁻¹. In the absence of amiloride-sensitive current or apical resistance, equal rates of net \text{Na}^+ \text{-Cl}^- exchange suggested that electroneutral parallel ion exchange or \text{Na}^+\text{-Cl}^- cotransport mediated \text{NaCl} absorption as described by Reuss (43) and Frizzell et al. (11) in \textit{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 \( \text{Cl}^- \) 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 \( \text{Cl}^- \), thereby limiting the driving force for \( \text{Cl}^- \) move-
ment. The majority of $I_{\text{sc}}$ 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 uptake: • VOL 292 • JUNE 2007 • www.ajpgi.org

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