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Functional Characterization of Na^+/H^+ Exchangers in Primary Cultures of Prairie Dog Gallbladder

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Abstract. Gallbladder Na^+ absorption is linked to gallstone formation in prairie dogs. We previously reported Na^+/H^+ exchanger (NHE1-3) expression in native gallbladder tissues. Here we report the functional characterization of NHE1, NHE2 and NHE3 in primary cultures of prairie dog gallbladder epithelial cells (GBECs). Immunohistochemical studies showed that GBECs grown to confluency are homogeneous epithelial cells of gastrointestinal origin. Electron microscopic analysis of GBECs demonstrated that the cells form polarized monolayers characterized by tight junctions and apical microvilli. GBECs grown on Snapwells exhibited polarity and developed transepithelial short-circuit current, I_{sc}, (11.6 ± 0.5 $\mu A \cdot cm^{-2}$), potential differences, V_t (2.1 ± 0.2 mV), and resistance, R_t (169 ± 12 $\Omega \cdot cm^2$). NHE activity in GBECs assessed by measuring dimethylamiloride-inhibitable $^{22}Na^+$ uptake under a H^+ gradient was the same whether grown on permeable Snapwells or plastic wells. The basal rate of $^{22}Na^+$ uptake was 21.4 ± 1.3 nmol $\cdot$ mg prot^{-1} $\cdot$ min^{-1}, of which 9.5 ± 0.7 ($\sim$45%) was mediated through apically-restricted NHE. Selective inhibition with HOE-694 revealed that NHE1, NHE2 and NHE3 accounted for $\sim$6%, $\sim$66% and $\sim$28% of GBECs' total NHE activity, respectively. GBECs exhibited saturable NHE kinetics (V_{max} 9.2 ± 0.3 nmol $\cdot$ mg prot^{-1} $\cdot$ min^{-1}; K_m 11.4 ± 1.4 mM Na^+). Expression of NHE1, NHE2 and NHE3 mRNAs was confirmed by RT-PCR analysis. These results demonstrate that the primary cultures of GBECs exhibit Na^+ transport characteristics similar to native gallbladder tissues, suggesting that these cells can be used as a tool for studying the mechanisms of gallbladder ion transport both under physiologic conditions and during gallstone formation.

Key words: Sodium/hydrogen antiporter — Epithelial sodium transport — Electrophysiology — Ussing chambers — Primary cultures — Gallstones

Introduction

The prairie dog (*Cynomys ludovicianus*) is widely used as an animal model for the investigation of human cholesterol gallstone disease [19, 20, 25, 27]. The prairie dog is unique as a model of human cholesterol gallstone disease because its biliary lipid composition is similar to that of humans [4, 13]. Moreover, prairie dogs fed a cholesterol-enriched diet develop cholesterol gallstones in a manner that recapitulates events known to occur in humans with cholelithiasis [21]. Electrophysiologic studies suggest that the prairie dog gallbladder exhibits a prominent transepithelial short-circuit current (I_{sc}) and lumen-negative potential difference (V_t), and thus the ion transport across this tissue is said to be electrogenic [36] resembling human gallbladder [35]. This is in contrast to the gallbladders of most other species, such as *Necturus* [34], rabbit [12, 14], and guinea pig [41], which exhibit V_t less than 2 mV and demonstrate no significant I_{sc}, such that NaCl transport in these tissues is nearly electroneutral. Its electrically prominent V_t and I_{sc} closely approximate human gallbladder parameters and thus this model provides us with an opportunity to study the effects of luminal and pharmacological

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factors on gallbladder ion transport during gallstone formation.

Studies conducted in this animal model [10, 17] and in others [26] have shown that gallbladder Na^+ and water absorption is increased in the early stage of gallstone formation and may promote cholesterol nucleation. Administration of amiloride, an inhibitor of Na^+ transport, has resulted in a significant decrease in the incidence of diet-induced gallstones in prairie dogs, suggesting that alterations in Na^+ transport may have a pathogenic role in cholelithiasis [39]. Previous studies in a variety of species indicate that gallbladder Na^+ transport is predominantly mediated through apically restricted Na^+/H^+ exchange (NHE) [2, 12, 34]. Recently, we have reported the expression of NHE isoforms (NHE1, NHE2 and NHE3) in native prairie dog gallbladder epithelium and have shown that both NHE2 and NHE3 are involved in vectorial Na^+ transport in this tissue [1]. However, because native gallbladder tissues are an inherently heterogeneous population of cells, this is a limiting factor for further studies of NHE kinetics and the cellular elements that regulate NHE activity under physiological and pathological conditions. Thus, to get greater insight into the cellular and molecular mechanisms of apical Na^+ transport under defined conditions, we deemed it necessary to use a more homogeneous population of gallbladder epithelial cells.

Although several investigators have developed primary cultures of gallbladder epithelial cells from various species [18, 24, 31–33], including prairie dogs [7], only Gunter-Smith and coworkers have used primary cultures of gallbladder epithelial cells to study ion transport [18]. These authors demonstrated that primary cultures of guinea pig gallbladder epithelial cells, when grown on permeable supports, develop R_t and I_{sc} comparable to the native epithelium and are responsive to secretagogues, such as ionomycin and forskolin. However, to our knowledge, there are no reports in which gallbladder primary cell cultures have been used to study Na^+ transport and its regulation. Thus, the goal of our study was to characterize Na^+/H^+ exchange in primary cultures of prairie dog gallbladder epithelial cells and to establish that these cells exhibit Na^+ transport characteristics comparable to native gallbladder tissues. These cells will allow us to plan a systematic study of the factors that regulate Na^+ transport during gallstone formation.

Materials and Methods

Materials

$^{22}Na^+$ was purchased from New England Nuclear (Boston, MA). HOE-694 (3-methylsulfonyl-4-piperidinobenzoyl guanidine) was a gift of Dr. H. J. Lang (Hoechst, Frankfurt, Germany) and dimethylamiloride (DMA) was from Sigma Chemicals (St. Louis, MO). Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA), and all other reagents were selected from vendors as noted in the text. Oligonucleotide primers were obtained from Express Genetics (Princeton, NJ). Avian myelomablastosis virus reverse transcriptase (RT), *Taq* DNA polymerase, and polymerase chain reaction (PCR) reagents were purchased from Perkin-Elmer (Norwalk, CT). One hundred-base pair DNA size ladder was purchased from GIBCO (Grand Island, NY). The mouse anti-cytokeratin-7/17, goat anti-cytokeratin-20 and goat anti-vimentin antibodies were obtained from Santa Cruz Biotechnologies, (Santa Cruz, CA). The mouse anti-cytokeratin-22 (pan-cytokeratin) antibody was purchased from Biomeda (Foster City, CA). The donkey anti-goat secondary antibody conjugated to rhodamine (TRITC) and goat anti-mouse secondary antibody conjugated to TRITC were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Gallbladder Harvesting

Adult male black-tailed prairie dogs (*Cynomys ludovicianus*) trapped in the wild and obtained from Flyers Speciality Pets (Lubbock, TX), were caged individually in a 23°C thermoregulated room. The animals were fed normal laboratory chow (Purina Laboratory Chow, Ralston-Purina, St. Louis, MO), sufficient to maintain body weight. After a 16-h fast with water *ad libitum*, animals were anesthetized with ketamine (100 mg/kg body weight) and xylazine (1.5 mg/kg) intramuscularly, cholecystectomy was performed via a midline laparotomy, and gallbladders were harvested. Use of animals followed the prior approval by the Institutional Animal Care and Use Committee (IACUC) of Drexel University College of Medicine.

Preparation of Gallbladder Epithelial Cells (GBECs)

Harvest and isolation of GBECs were performed following a modification of the method described by Chapman et al. [7]. The changes made in the methodology were necessary to adapt to our system and the modifications improved the yield and homogeneity of isolated cells. Briefly, gallbladders were opened longitudinally in a small sterile petri dish (35 × 10 mm, Becton Dickinson Labware, Bedford, MA), bile was collected, and tissues were rinsed free of bile with cold phosphate buffered saline (PBS). Gallbladders were transferred to a new petri dish, with the mucosal side face down over 0.3 ml of 0.25% Ca^{2+}/Mg^{2+} free trypsin (GIBCO), and incubated at 37°C for 45 min. The trypsinization reaction was inactivated by adding 5 ml of Dulbecco's MEM-Ham's F-12 (1:1) medium (DMEM) supplemented with 10% FBS (Sigma), ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenium from GIBCO, and 2 mM glutamine (Fisher Scientific). Following digestion with trypsin, the mucosa was scraped gently with a sterile scraper under aseptic conditions to isolate epithelial cells. The isolated cells were washed three times in sterile Minimum Essential Medium (MEM). Cell viability following isolation, as assessed by Trypan Blue exclusion, was >95%. Cells isolated from one gallbladder yielded sufficient cells to seed 18 wells of a 24-well plate (1 cm^2, Costar, Cat. # 3526) or 18 Snapwells filters (1 cm^2, Costar, Cat. # 3407) at a density of ~0.25×10^6 cells/well in 500 μl of MEM. Cultures were maintained in a 5% CO_2 atmosphere at 37°C in DMEM, and exchanges of Medium were performed every 48 h until performance of selected experiments.

Electron Microscopy

GBECs were grown in T-75 flasks for 7 days until confluent and fixed in Trumps fixative (4% formaldehyde, 1% glutaraldehyde) for 1 h at room temperature, after which the fixative was decanted and the cells were washed 3 times with Millonigs phosphate buffer (pH 7.4). Cells were post-fixed in 1% OsO_4 in Syn-Collidine buffer for 1 h, washed in Millonigs Phosphate buffer and dehydrated in a graded alcohol series. Following a second 100% alcohol dehydration and removal of alcohol, Epon resin mixture was immediately added to the flask until a level of approximately 4–6 mm of material covered the cells. Covered GBECs were incubated at room temperature overnight and then oven-heated (60°C) for 24–48 h. Treated GBECs were examined with an inverted phase-contrast microscope, and confluent regions suitable for electron microscopic analysis were marked on the flask and then removed with a small coping saw. All flask material was separated from the embedded cells, and GBECs were thick- and thin-sectioned, with thin sections picked up on uncoated formvar-cover single-hole grids. All sections were stained with alcoholic uranyl acetate and Reynolds lead citrate, and all grid were examined in a Zeiss 10A electron microscope operated at 80 kV accelerating voltage.

Immunofluorescence

Individual chambers of glass 4-chamber slides were coated with 500 μl Cell-Tak adhesive following the manufacturer's protocol (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA), air-dried at RT, and briefly rinsed with serum-free MEM prior to plating of GBECs at a density of $\sim 0.25 \times 10^6$ cells/well. GBECs were grown for 7 days until confluent. Confluent GBECs were fixed in 100% methanol at −20°C for 5 min and then washed 3 times for 5 min with PBS (pH 7.4). Nonspecific binding was blocked by a single wash in either 1% donkey or goat serum in PBS for 30 min at room temperature, depending upon the secondary antibody to be used. GBECs were incubated for 1 h at room temperature with primary antibody. Primary antibodies were used at the following dilutions; Mouse anti-cytokeratin-7/17 (CK-7/17), 1:100; mouse anti-cytokeratin-22 (CK-22), pre-diluted; goat anti-cytokeratin-20 (CK-20), 1:100; and goat anti-vimentin, 1:100. GBECs were then washed with PBS three times over 15 min and blocked in either 1% donkey or goat serum for 30 min at room temperature. GBECs were then incubated for 1 h at 37°C in the dark with the appropriate TRITC-conjugated secondary antibody (1:800 dilution), washed with PBS three times for 15 min, counter-stained with bisbenzamide at 10 μg/ml in PBS for 3–5 min, and quickly rinsed twice with PBS. Following a quick rinse with distilled water, all slides were mounted with an aqueous mounting medium (DAKO). Slides were viewed on a Nikon E800 fluorescent microscope, and images captured with a Spot RT camera (40×) for analysis with Image Pro Plus 4.5 imaging software.

Measurement of $^{22}Na^+$ Uptake

Na^+/H^+ exchange activity was measured in GBECs grown on both, 24-well plastic plates and permeable Snapwells. Briefly, the MEM was aspirated and the cells were acidified by incubation for 1 h in NH_4Cl buffer containing (in mM) 50 NH_4Cl, 90 TMA chloride, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 15 HEPES, adjusted to pH 6.4. After 1 h, the NH_4Cl buffer was aspirated and replaced with Na^+-free buffer containing (in mM) 140 TMA chloride, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 15 HEPES, adjusted to pH 7.4, with 1 mM ouabain and 0.1 mM bumetanide, either alone or containing selected inhibitors of ion transport, including dimethylamiloride (DMA; 1–1000 μM), HOE-694 (HOE; 1–1000 μM), hydrochlorothiazide (100 μM), or dichlorobenzamil (100 μM), for 5 min at 37°C. The Na^+-free buffer was then replaced with transport buffer containing (in mM) 20 NaCl, 120 TMA chloride, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 15 HEPES, adjusted to pH 7.4, with 1 mM ouabain, 0.1 mM bumetanide, and ~1 μCi/ml $^{22}NaCl$, either alone or with the selected inhibitors at the concentrations utilized during the pre-incubation step. In all inhibitor studies, 100 μM bumetanide was included in both the Na^+-free and transport buffers. Assessment of the specific contribution of the Na^+-K^+-$2Cl^-$ co-transporter to $^{22}Na^+$ uptake was examined in parallel experiments that compared uptake in the presence of bumetanide with uptake in GBECs exposed to Na^+-free and transport buffers without bumetanide. Uptake of ^{22}Na from the transport buffer was conducted for exactly 3 min at 37°C, after which the buffer was aspirated and wells were quickly rinsed 3 times over 30 s with 1 ml ice-cold wash buffer, containing (in mM, pH 7.4) 140 NaCl, 5 KCl, 1 Na_2HPO_4, and 15 HEPES. The cells were then lysed in 1 ml 1N NaOH for 30 min at room temperature, and a 100 μl aliquot of the lysates was used to determine the protein concentration. The remaining lysates were neutralized with 1 ml of 1N HCl and assessed for radioisotope activity in a Beckman liquid scintillation counter (LS 600 TA, Beckman Instruments, Fullerton, CA). DMA-inhibitable NHE activity was determined as the difference in Na^+ uptake at baseline minus the Na^+ uptake in the presence of 100 μM DMA (IC_{50} of DMA for NHE1, NHE2 and NHE3 is 0.1 μM, 0.7 μM, and 11 μM, respectively). The relative contribution of NHE1, NHE2 and NHE3 to total NHE activity was determined by their differential sensitivity to HOE-694 (IC_{50} of HOE-694 for NHE1, NHE2 and NHE3 is 0.16 μM, 5 μM, and 650 μM, respectively) [11, 42]. The contributions of the coupled Na^+-Cl^- transporter, the Na^+-K^+-$2Cl^-$ co-transporter, and Na^+/Ca^{2+} exchangers to total Na^+ uptake was determined by differential sensitivity to hydrochlorothiazide, bumetanide and dichlorobenzamil, respectively. Additional experiments were performed to assess the functional polarization of GBECs, in which $^{22}Na^+$ uptake was measured by addition of tracer $^{22}Na^+$ to the basolateral surfaces of GBECs plated in Snapwells.

Transepithelial Electrical Parameters

GBECs grown on Snapwells for 7 days were mounted in a two-piece Lucite Ussing chamber (Diffusion chambers, Costar). Mucosal and serosal surfaces were perfused with equal volumes (5 ml) of modified Ringer's solution of the following composition (in mM): 140 Na^+, 124 Cl^-, 21 HCO_3^-, 5.4 K^+, 1.3 Ca^{2+}, 1.2 Mg^{2+}, 2.4 HPO_4^{2-}, 0.6 $H_2PO_4^-$, 5 HEPES, and 10 glucose, adjusted to pH 7.4. The chamber was maintained at 37°C and gassed with 95% O_2/5% CO_2. Open-circuit transepithelial voltage (V_t) and short-circuit current (I_{sc}) were measured following standard 4-electrode techniques using an automatic voltage current clamp (VCC 600, Physiologic Instruments, San Diego, CA), as previously described [36]. Transepithelial resistance (R_t) was determined by recording the potential deflections in response to a bipolar current of ±10 μA passed across the monolayers by a pulse generator via Ag-AgCl electrodes. Compensations for fluid resistance and voltage electrode asymmetry were made prior to mounting monolayers in the chamber, using a blank Snapwell. Direct current was continually passed through the monolayers by means of Ag-AgCl electrodes in order to nullify the spontaneous V_t. Measurements of R_t and I_{sc} were normalized to chamber surface area.

Protein Assay

Protein concentration in the cell lysates was measured by the Bradford method [3], using Bio-Rad dye reagent (Bio-Rad Labs, Hercules, CA).

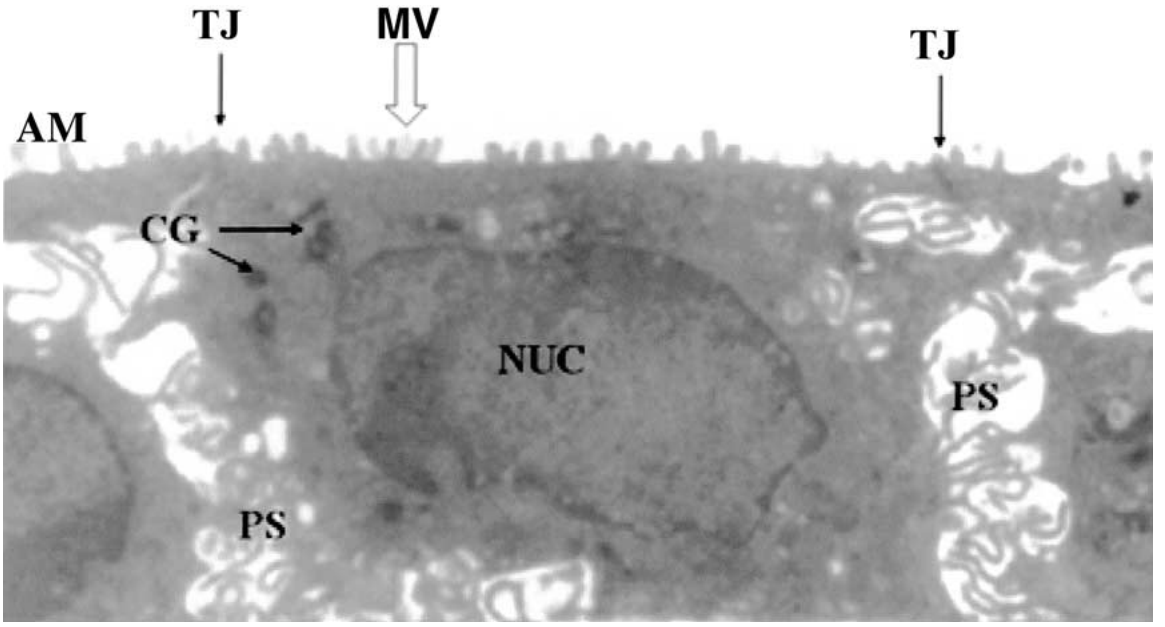

Fig. 1. Transmission electron microscopic features of primary cultures of prairie dog GBECs. GBECs demonstrate growth as confluent monolayer of polarized cuboidal cells with basally oriented nuclei (*NUC*) and numerous cytoplasmic granules (*CG*) clustered near the apical surfaces. The apical membrane (*AM*) surface is characterized by numerous microvilli (*MV*, *white arrow*) and by the presence of tight junctional complexes (*TJ*, *black arrows*). *PS*, peripheral space (×18,900).

RNA Isolation

Total RNA was extracted from GBECs grown to confluence on 24-well plates using a commercially available RNA isolation kit (Stratagene, San Diego, CA), according to the method of Chomczynski and Sacchi [9]. RNA concentration and purity were assessed by spectrophotometry at 260 nm/280 nm. Samples not used immediately were suspended in either 0.25 M sodium acetate/ethanol or DEPC-treated water prior to storage at −70°C.

RT-PCR Analysis of NHE Isoform Expression

Approximately 200 ng of total RNA isolated from GBECs was used for reverse transcriptase (RT)-PCR assay following the manufacturer's protocol (Perkin-Elmer). The RT reaction was performed in a 20 μl volume containing 1 mM deoxynucleotide triphosphate, 50 mM KCl, 50 μg/ml actinomycin D, 4 μM antisense primer (oligo dT), RT buffer (100 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$ and 10 mM dithiothreitol), and 10 U avian myelomablastosis virus RT. After 1 h of incubation at 42°C, samples were precipitated with 100% ethanol. Amplification of complementary DNA (cDNA) was performed with *Taq* polymerase and reagents from Perkin-Elmer. Isoform-specific primers of NHE1, NHE2 and NHE3 were designed for prairie dogs on the basis of maximum homology among published nucleotide sequence for rats, rabbits, and humans, as described previously [1]. Briefly, all of the cDNA products were used for PCR reaction with the protocol recommended by the manufacturer. All reagents were kept on ice, and the mixture was overlaid with mineral oil and then amplified with a PTC-100 Programmable Thermal Cycler (MJ Research, Watertown, MA). The 30-cycle amplification profile involved denaturation for 1 min at 94°C, primer annealing for 1 min at 53°C, and extension for 1 min at 72°C. The elongation phase was lengthened by 1 s per cycle. Aliquots of the PCR products were analyzed by electrophoresis on 1% agarose gels in 1 × Tris-borate/EDTA buffer with 0.05% ethidium bromide for visualization. PCR products corresponding to the predicted size for NHE1, NHE2 and NHE3 were ligated into the PCR 2.1 vector using the TA one-shot Cloning Kit from Invitrogen (San Diego, CA). The transformed *E. coli* cells were identified via blue/white plaque screening and grown in LB + ampicillin medium overnight, and the plasmids were isolated using the Qiagen miniprep kit (Chatsworth, CA). The NHE1, NHE2 and NHE3 plasmids were subsequently sequenced by the Sanger dideoxynucleotide method, and the amino-acid sequences of the amplified regions were determined.

Statistical Analysis

Data are presented as means ± SEM as needed. Statistical comparisons between groups were made by analysis of variance (ANOVA) followed by Fisher's exact test. Student's paired *t*-test was also used when appropriate.

Results

Electron Microscopy

Analysis by transmission electron microscopy of thin sections of primary cultures of GBECs demonstrated the presence of cytological features consistent with that of gallbladder epithelia. GBECs in culture were observed to be predominantly cuboidal, and a representative section through a confluent monolayer of GBECs cultured for 7 days is shown in Fig. 1. The figure further demonstrates the polarized nature of cultured GBECs, with basally oriented nuclei and numerous cytoplasmic granules that increase in

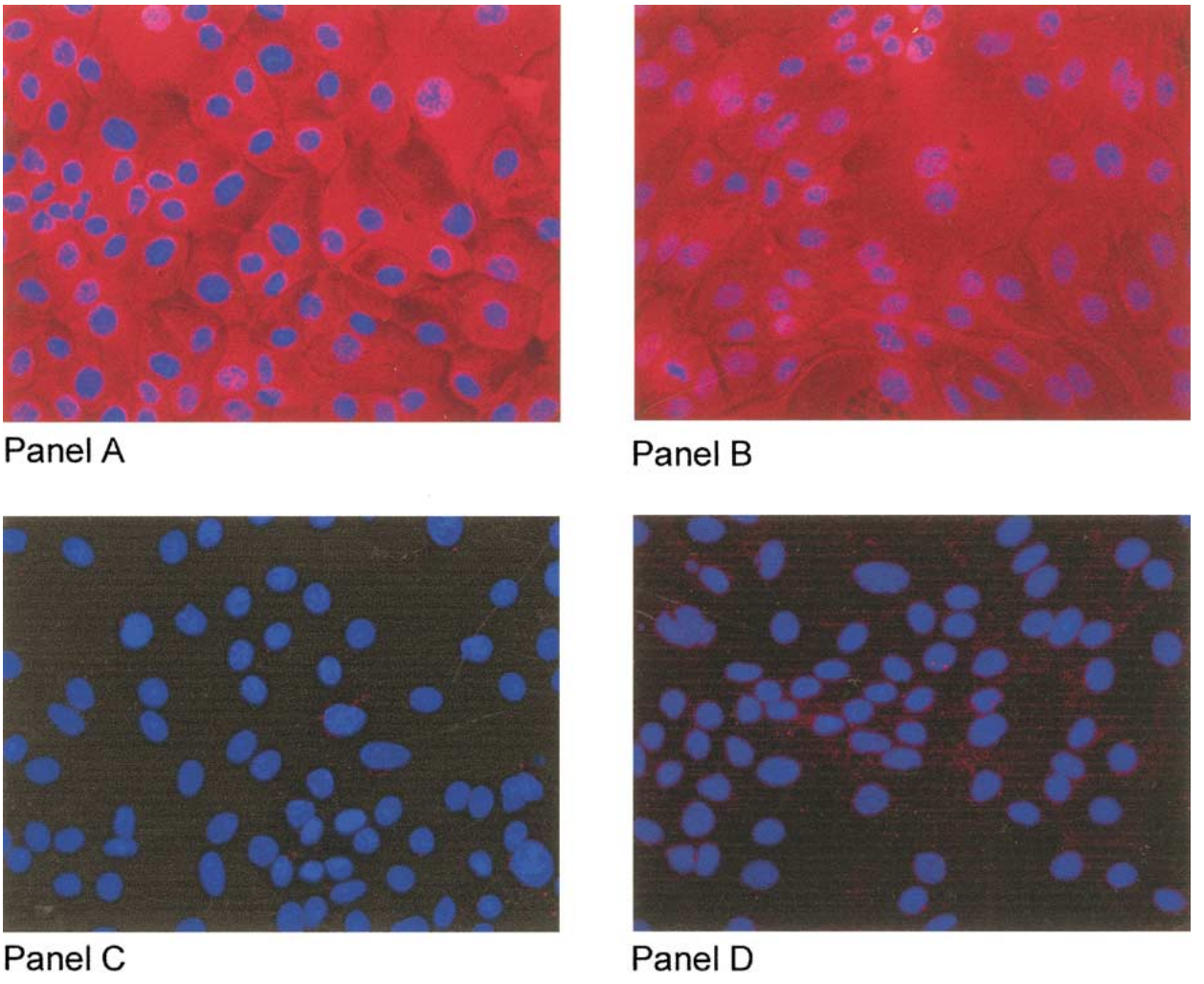

Fig. 2. Immunofluorescent labeling of primary culture of prairie dog GBECs. GBECs grown to confluence were immunolabeled with anti-cytokeratin and anti-vimentin antibodies. Positive staining for pan-specific (CK-22; Panel *A*) and gastrointestinal epithelium-specific (CK-20; Panel *B*) cytokeratins was observed. Staining was not observed for cytokeratins of extra-gastrointestinal origin (CK-7/17; Panel *C*) or vimentin (Panel *D*). Nuclei were counter-stained with bis-benzamide (×40).

number near the apical surfaces. Detailed examination of the apical surfaces of fixed GBECs demonstrates the presence of prominent microvilli and tight junctions (Fig. 1), suggesting that both functional and structural polarity of gallbladder epithelial cells is preserved in primary culture.

Immunofluorescence

Immunolabeling using antibodies for specific cytokeratin (CK) species of cytoskeletal proteins was performed to demonstrate that prairie dog GBECs in primary culture are epithelial in nature and of gastrointestinal origin. An antibody to vimentin, an intermediate filament specific to cells of mesenchymal lineage, such as fibroblasts, was used to determine the extent of fibroblast cell contamination in primary culture of prairie dog GBECs. As observed in Fig. 2, positive labeling with antibodies to panCK-specific (CK-22, Panel *A*) and gastrointestinal epithelium-specific (CK-20, Panel *B*) cytokeratins demonstrated that GBECs retained the characteristics of epithelial cells of gastrointestinal origin. In contrast, antibodies not specific for epithelial cells of the gastrointestinal tract (CK-7/17, Panel *C* and vimentin, Panel *D*; Fig. 2) were non-reactive with the cell cultures, demonstrating that contamination with other cell types, including fibroblasts, was not present in our primary cultures of prairie dog GBECs.

$^{22}Na^+$ Uptake in Cultured Monolayers of GBECs

To first determine if $^{22}Na^+$ uptake into the cells is dependent on a H^+ gradient and to establish the duration for which $^{22}Na^+$ uptake into the cells is

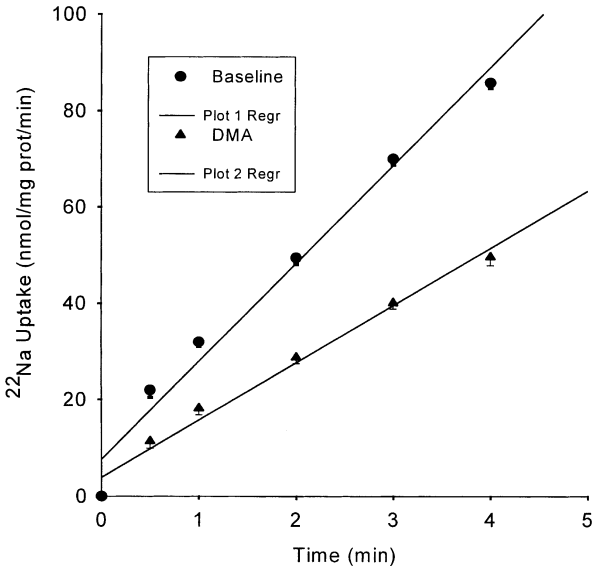

Fig. 3. Initial rates of $^{22}Na^+$ uptake by cultured GBECs. $^{22}Na^+$ uptake in the presence of an outwardly directed H^+ gradient was measured in the presence (▲) or absence (●) of 100 μM DMA at various time intervals starting from 0.5 to 4.0 min in cultured GBECs grown on 24-well plates. The uptake is linear over 4 min. Results are mean ± SEM of 3 identical experiments using 4–5 wells at each data point.

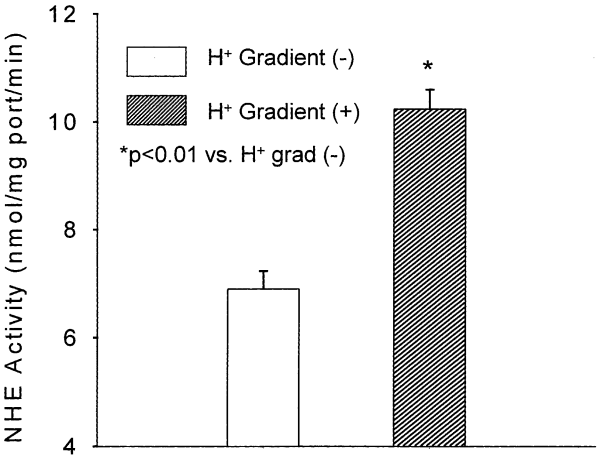

Fig. 4. Effect of H^+ gradient on $^{22}Na^+$ uptake in GBECs. ^{22}Na uptake was measured in cultured GBECs grown on 24-well plates for 3 min in the absence (*H^+ gradient* (−)) or presence of an outwardly directed H^+ gradient (*H^+ gradient* (+)). Na^+ uptake was stimulated by ~48% ($p < 0.01$) in the presence of the H^+ gradient. Results are mean ± SEM of 4 identical experiments using 4–5 wells in each experiment.

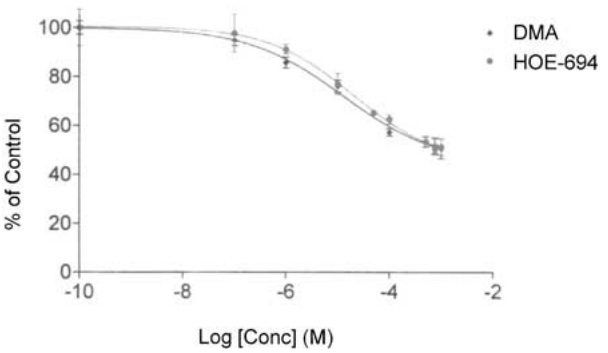

Fig. 5. DMA and HOE-694 dose-response curve in cultured GBECs. DMA and HOE-sensitive ^{22}Na uptake was measured in cultured GBECs grown on 24-well plates at varying concentrations of DMA (0.1–1000 μM) in the presence of an H^+ gradient. Results were normalized to basal $^{22}Na^+$ uptake in the absence of inhibitors (19.5 ± 1.6 nmol · mg prot^{-1} · min^{-1}). $^{22}Na^+$ uptake was inhibited by ~45% at 100 μM DMA and 500 μM HOE-694, respectively. Graph represents the results of triplicate experiments using 4–5 wells at each data point.

linear, $^{22}Na^+$ uptake into GBECs grown on 24-well plates was determined using an outwardly directed proton gradient. As shown in Fig. 3, $^{22}Na^+$ uptake in both the presence and absence of DMA was linear up to 4 min, and it was stimulated by ~48% ($p < 0.01$) by the pH gradient (Fig. 4). Stimulation of Na^+ uptake by an H^+ gradient suggests the presence of a carrier-mediated transport process that is dependent on the transmembrane H^+ gradient.

We have previously shown that apical $^{22}Na^+$ uptake by native prairie dog gallbladder is inhibited by DMA and HOE-694, inhibitors of Na^+/H^+ exchange. To determine if $^{22}Na^+$ uptake into primary cultures of gallbladder epithelial cells is sensitive to both DMA and HOE-694, $^{22}Na^+$ uptake was measured in cultured monolayers of GBECs grown on 24-well plates in the presence of varying concentrations of DMA and HOE-694. $^{22}Na^+$ uptake was reduced by ~6% by treatment of cultured GBECs with 1 μM HOE-694, at which concentration NHE1 activity is fully blocked. As the concentrations of the inhibitors increased, $^{22}Na^+$ uptake was progressively inhibited to a maximum of ~45% at ~100 μM DMA and ~500 μM HOE-694, respectively (Fig. 5). These results are consistent with our previous observations that both HOE-sensitive NHE2 and HOE-insensitive NHE3 contribute to total NHE activity in GBECs of prairie dogs. Hill-plot analysis of these data yielded IC_{50} and Hill coefficient of 13.0 ± 4.7 μM and 0.47 ± 0.09 for DMA and 20.2 ± 3.5 μM and −0.53 ± 0.04 for HOE-694, respectively.

Since only ~45% of H^+ gradient-driven Na^+ uptake was mediated through DMA-inhibitable Na^+/H^+ exchange, we investigated if other Na^+ transporters are involved and contribute significantly to the H^+ gradient-driven Na^+ uptake in the GBECs. As shown in Fig. 6, only DMA and HOE-694 (an amiloride analog that inhibits NHE2 at the present dose) significantly inhibited H^+-gradient-driven $^{22}Na^+$ uptake, whereas the effects of other inhibitors, bumetanide (Na^+-K^+-$2Cl^-$ cotransport inhibitor), hydrochlorothiazide (coupled Na^+-Cl^- transport inhibitor) and dichlorobenzamil (Na^+

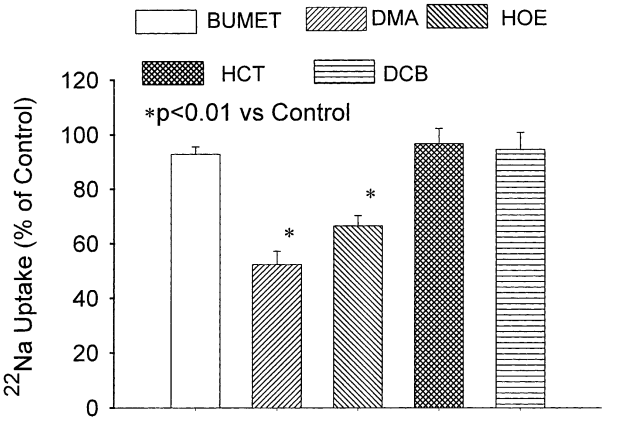

Fig. 6. Effects of Na^+ transport inhibitors on $^{22}Na^+$ uptake in cultured GBECs. ^{22}Na uptake was measured for 3 min in the presence of an outwardly directed H^+ gradient in GBECs exposed to specific inhibitors of Na^+ transporters. Basal Na^+ uptake was inhibited ~48% by 100 μM 5-(N, N-dimethyl) amiloride (*DMA*) and ~33% by 50 μM 3-methylsulfonyl-4-piperidinobenzoyl guanidine (*HOE*). Basal Na^+ uptake was not significantly inhibited by 100 μM bumetanide (*BUMET*), 100 μM hydrochlorothiazide (*HCT*) or 100 μM dichlorobenzamil (*DCB*). Results are mean ± SEM of 4 identical experiments with 4–5 wells for each inhibitor in each experiment.

channel and Na^+/Ca^{2+} exchange inhibitor), were minimal and not significant.

Kinetics of Na^+/H^+ Exchange in Cultured Monolayers of GBECs

$^{22}Na^+$ uptake into cultured monolayers of GBECs grown on 24-well plates was measured for 3 min at different external Na^+ concentrations in the absence or presence of 100 μM DMA. Na^+/H^+ exchange was defined as DMA-inhibitable $^{22}Na^+$ uptake. Maximal rate of enzyme reactions (V_{max}) and Michaelis-Menten constant ($K_m^{Na^+}$) were determined by Lineweaver-Burk transformations via computer-assisted nonlinear regression (ENZFITTER). The $^{22}Na^+$ uptake in the cultured cells conformed to simple Michaelis-Menten kinetics and Lineweaver-Burk analysis demonstrated a V_{max} of 9.2 ± 0.3 nmol · mg prot^{-1} · min^{-1} and an apparent $K_m^{Na^+}$ of 11.4 ± 1.4 mM (Fig. 7). The apparent K_m value observed in the GBECs is comparable with the values for NHE1 (10.2 mM), NHE2 (11.6 mM) and NHE3 (16.6 mM), but the V_{max} value is severalfold lower than the corresponding values for the NHEs in PS120 cells transfected with NHE isoform specific cDNAs [6].

NHE mRNA Expression in GBECs

Having established that the primary cultures demonstrated $^{22}Na^+$ uptake sensitive to both DMA and HOE-694, the expression of NHE messenger RNA was examined in cultured monolayers of GBECs grown on 24-well plates by RT-PCR analysis, using NHE isoform-specific primers described earlier [1]. Figure 8 shows an ethidium bromide-stained 1.5% agarose gel of amplified cDNA products of NHE1 through NHE-3 isoforms. The sizes of the RT-PCR products matched with the predicted sizes and were identical to the corresponding values of NHE1, NHE2 and NHE3 observed in native gallbladder epithelia. The NHE1, NHE2, and NHE3 RT-PCR products were sequenced to confirm their authenticity, and the corresponding deduced amino-acid sequences were identical to the NHE isoform values reported in native gallbladder epithelia [1].

Comparison of $^{22}Na^+$ Uptake by Plate- and Filter-grown GBECs

When the primary cultures were grown to confluency on Snapwell filters and mounted in diffusion chambers (bathed with symmetrical Ringer's solution), they developed a transepithelial short-circuit current (I_{sc}) of 11.6 ± 0.5 μA · cm^{-2}, an apical negative potential difference (V_t) of 2.1 ± 0.2 mV and a resistance (R_t) of 169 ± 12 Ω · cm^2 (Table 1). Although electrical polarity of the monolayers is comparable to the native epithelium, the numerical values of I_{sc} and V_t are lower and the R_t is higher in comparison to the values observed in native gallbladder tissues of prairie dogs [36]. The electrical parameters exhibited by the monolayers were sensitive to amiloride. Mucosal exposure to 100 μM dimethylamiloride (DMA) resulted in ~29% ($p < 0.001$) and 24% ($p < 0.001$) decrease in I_{sc} and V_t, respectively, and ~12% ($p < 0.003$) increase in R_t, as compared to baseline. These DMA effects were similar to those observed in native gallbladder tissues of prairie dogs. I_{sc} exhibited by native gallbladder epithelium originates through a combination of net mucosal Na^+ absorption (46% of measured I_{sc}) and net Cl^- secretion (42% of measured I_{sc}). The remaining 12% of measured I_{sc} is unaccounted for by Na^+ and Cl^- flux [36].

When $^{22}Na^+$ uptake by GBECs grown to confluency on Snapwell filters and 24-well plates was measured for 3 min (Fig. 3) using a proton gradient, the values obtained in these two preparations were almost identical (Fig. 9). The basal rate of apical $^{22}Na^+$ uptake in the cultures grown on Snapwell filters was 21.8 ± 1.7 nmol · mg prot^{-1} · min^{-1} and the apical uptake rate in the cultures on plastic wells was 21.4 ± 1.3 nmol · mg prot^{-1} · min^{-1}. Approximately 45% of Na^+ uptake was mediated through 100 μM DMA-inhibitable Na^+/H^+ exchange. These DMA-inhibitable Na^+ uptake values measured in GBECs are in good agreement with ~43% of apical Na^+ uptake mediated through Na^+/H^+ exchange in native gallbladder tissues of prairie dogs [1]. In parallel experiments, the basolateral uptake rate in the

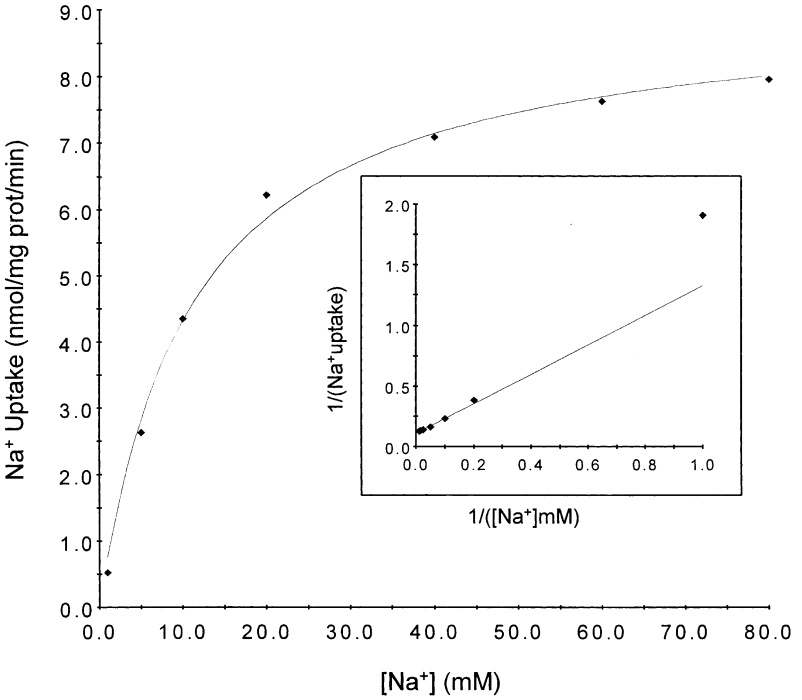

Fig. 7. Kinetics of Na^+/H^+ exchange in cultured GBECs. $^{22}Na^+$ uptake into cultured monolayers of GBECs grown on 24-well plates was measured for 3 min at different external Na^+ concentrations in the absence or presence of 100 μM DMA. Na^+/H^+ exchange was defined as DMA-sensitive $^{22}Na^+$ uptake. Maximal rate of enzyme reactions (V_{max}) and Michaelis-Menten constant ($K_m^{Na^+}$) were determined by Lineweaver-Burk transformations via computer-assisted nonlinear regression (ENZFITTER). Na^+ uptake via NHE in cultured GBECs conformed to simple Michaelis-Menten kinetics. *Inset*: Lineweaver-Burk transformation to predict the maximal rate of enzyme reaction (V_{max}) and apparent affinity constant for external Na^+ ($K_m^{Na^+}$) for NHE. Lineweaver-Burk analysis demonstrated a V_{max} of 9.2 ± 0.3 nmol · mg prot^{-1} · min^{-1} and an apparent $K_m^{Na^+}$ of 11.4 ± 1.4 mM. Results are mean ± SEM of 3 identical experiments with 4–5 wells per external Na^+ concentration in each experiment.

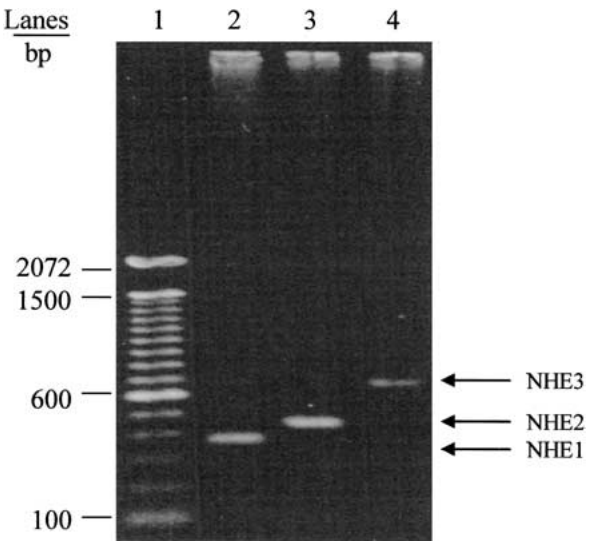

Fig. 8. Expression of NHE isoform mRNA in cultured GBECs. Ethidium bromide staining of 1.5% agarose gel of amplified cDNAs obtained by RT-PCR analysis of GBEC RNA using isoform-specific NHE primers designed for prairie dogs on the basis of high homology of nucleotide sequence amongst rats, rabbits and humans. Lane *1*: 100 bp DMA ladder; lane *2*: NHE 1 (383 bp) lane *3*: NHE2 (415 bp); lane *4*: NHE 3 (683 bp). These sizes are identical to the values observed in native gallbladder tissues.

cultures grown on Snapwell filters was observed to be 22.13 ± 1.89 nmol · mg prot^{-1} · min^{-1}. Approximately 6% of basolateral Na^+ uptake was mediated through 100 μM DMA-inhibitable Na^+/H^+ exchange, compared to 45% of apical uptake (Fig. 10). These DMA-inhibitable Na^+ uptake values suggest that the cultured GBECs maintain functionally separate apical and basolateral membrane domains, with NHE predominantly operational at the apical surfaces.

Contribution of NHE1, NHE2 and NHE3 to Total NHE Activity in GBECs

Total NHE activity was measured by the difference in the basal rate of Na^+ uptake minus the uptake in the presence of 100 μM DMA, and the contribution of NHE1, NHE2 and NHE3 to total NHE activity was determined by their differential sensitivity to HOE-694, as previously reported [1, 11]. Total NHE activity in GBECs grown on Snapwells was 9.5 ± 0.7 nmol · mg prot^{-1} · min^{-1} (mean ± SEM), which was identical to the values observed in GBECs grown on 24-well plates (9.5 ± 0.7 nmol · mg prot^{-1} · min^{-1}). The contribution of NHE1 to total NHE activity, defined as the difference in the basal rate of apical Na^+ uptake minus the uptake in the presence of 1 μM HOE-694, was examined and was found to be ~6% of total NHE activity. HOE-sensitive NHE2 activity was 6.3 ± 0.5 nmol · mg prot^{-1} · min^{-1} in GBECs on Snapwells and 6.4 ± 0.5 nmol · mg prot^{-1} · min^{-1} in GBECs on 24-well plates, respectively (Fig. 11). The contribution of NHE2 to total NHE activity was ~66% in the GBECs grown on either Snapwells or 24-well plates. The contribution of NHE3 to total NHE was ~28% (Fig. 11). These relative contributions of NHE2 and NHE3 to the total NHE activity are similar to the values previously reported in native gallbladder tissues of prairie dogs [1]. These results

Table 1. Electrical properties of primary cultures of prairie dog GBECS

	Baseline	DMA (100 μM)	% change
I_{sc} ($\mu A \cdot cm^{-2}$)	11.6 ± 0.5	8.2 ± 0.4**	−29.3
V_t (mV)	2.1 ± 0.2	1.6 ± 0.2**	−24.3
R_t ($\Omega \cdot cm^2$)	169 ± 12	189 ± 14*	+11.8

Results represent means ± SEM of electrical parameters measured on 3 separate days using 4–5 Snapwells mounted in diffusion chambers.
*$p < 0.003$, **$p < 0.001$ *vs.* corresponding baseline (Student's paired *t*-test).

show that the primary cultures of gallbladder epithelial cells grown to confluency exhibit Na^+ transport characteristics similar to native gallbladder epithelia.

Discussion

The prairie dog is widely used as an animal model for the study of human cholesterol gallstone disease [19, 20, 25, 27]. Previous studies have linked alterations in gallbladder Na^+ absorption to the pathogenesis of cholesterol gallstones [10, 17, 26]. We have recently identified the NHE isoforms involved in Na^+ transport in the native gallbladder tissues of prairie dogs [1]. However, native gallbladder tissues, because of their cellular heterogeneity, have become a limiting factor for further studies of Na^+/H^+ exchange kinetics and the factors that regulate its activity under both physiological and pathological conditions. Although previous investigators have established primary cultures of gallbladder epithelia from several species [18, 24, 31–33], including prairie dogs [7], no systematic studies to characterize Na^+ transport have been undertaken using GBECs. In the present study, we have shown that primary cultures of prairie dog GBECs are an appropriate model to characterize Na^+ transport. We have demonstrated that these cells exhibit ultrastructural features consistent with gastrointestinal epithelia and become polarized when grown to confluency on permeable supports, as well as exhibit mucosal negative potential difference, short-circuit current and transepithelial resistance responsive to amiloride, similar to intact gallbladder tissues. Functional assessment of Na^+/H^+ exchange in GBECs has demonstrated that Na^+/H^+ exchange accounts for ~45% of apical Na^+ uptake, similar to native gallbladder tissues. Further studies with HOE-694 have revealed that the relative contributions of NHE2 and NHE3 to total NHE activity in cultured GBECs are identical to the values observed in native gallbladder tissues. In addition, we have shown that these GBECs exhibit saturable NHE kinetics in apical Na^+ uptake with a K_m for Na^+ of 11.4 ± 1.4

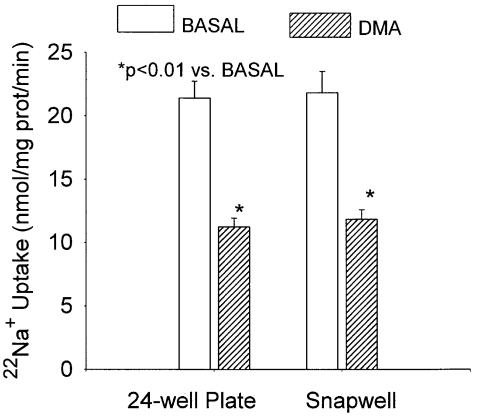

Fig. 9. Comparison of $^{22}Na^+$ uptake in GBECs cultured on 24-well plates and permeable supports. Basal and DMA-inhibitable ^{22}Na uptake was measured for 3 min in the presence of an outwardly directed H^+ gradient in GBECs cultured on 24-well plates and permeable Snapwells. There was no significant difference in basal or DMA-inhibitable $^{22}Na^+$ uptake rates in GBECs grown on either culture support. Approximately 45% of observed $^{22}Na^+$ uptake was mediated through 100 μM DMA-inhibitable Na^+/H^+ exchange. Results are mean ± SEM of 4–5 identical experiments with 4–5 wells at each data point.

mM and a V_{max} of 9.2 ± 0.3 nmol · mg $prot^{-1}$ · min^{-1}. Furthermore, our RT-PCR analysis has confirmed that the cultured cells express Na^+/H^+ exchanger isoforms NHE1, NHE2 and NHE3 similar to native gallbladder tissues. Taken together, these findings demonstrate that the GBECs from prairie dogs are an important tool to study gallbladder ion transport under physiological conditions and to examine the mechanisms of alterations in ion transport that occur during gallstone formation.

The observation that the GBECs grown to confluency on permeable supports develop mucosal-negative potential difference, short-circuit current and transepithelial resistance suggests that an electrogenic transport system exists in the cultured cells similar to that present in the native gallbladder tissues of prairie dogs [36]. The component of I_{sc} in GEBECs has not been determined but is expected to be similar to that of native gallbladders. Most of the I_{sc} exhibited by native gallbladder epithelium originates through a combination of net mucosal Na^+ absorption and net Cl^- secretion. The effects of the amiloride analog DMA on the electrical parameters of GBECs are similar to its observed effects on these parameters in native gallbladder tissues. This indicates that primary cultures of GBECs can be used as a model system to examine the regulation of transepithelial Na^+ transport across gallbladder epithelial cells. This is in good agreement with the findings of Gunter-Smith and coworkers, who have shown that the range of responses of primary cultures of guinea pig gallbladder epithelial cells to various secretagogues are

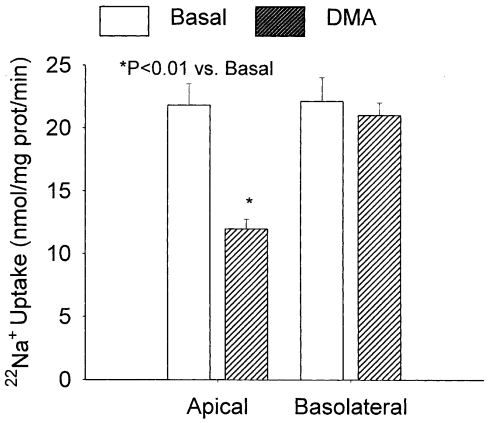

Fig. 10. Comparison of apical and basolateral $^{22}Na^+$ uptake in GBECs cultured on permeable supports. Basal and DMA-inhibitable ^{22}Na uptake was measured for 3 min in the presence of an outwardly directed H^+ gradient by application of tracer to the apical or basolateral surfaces of GBECs cultured on permeable Snapwells. There was no significant difference in basal apical or basolateral $^{22}Na^+$ uptake rates in GBECs grown on permeable supports. Approximately 45% of observed apical $^{22}Na^+$ uptake was mediated through 100 μM DMA-inhibitable Na^+/H^+ exchange. In contrast, only 6% of observed basolateral $^{22}Na^+$ uptake was mediated through 100 μM DMA-inhibitable Na^+/H^+ exchange. Results are mean ± SEM of 4–5 identical experiments with 4–5 wells at each data point.

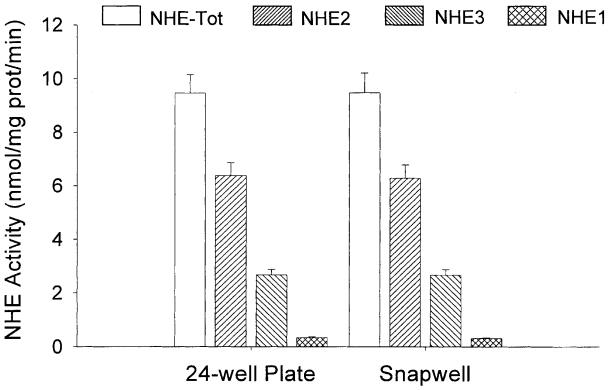

Fig. 11. Contributions of NHE isoforms to total NHE activity in cultured GBECs. Total NHE activity in cultured GBECs was determined as the difference in the basal rate of apical Na^+ uptake minus the uptake in the presence of 100 μM DMA. The contributions of NHE2 and NHE3 to total NHE activity were determined by their differential sensitivity to 50 μM HOE-694. Total NHE activity in GBECs was identical whether grown on 24-well plates (9.5 ± 0.7 nmol · mg prot^{-1} · min^{-1}) or permeable Snapwells (9.5 ± 0.7 nmol · mg prot^{-1} · min^{-1}). The relative contributions of NHE1 (~6%), NHE2 (~66%) and NHE3 (~28%) to total NHE activity were also not significantly different between GBECs grown either on 24-wells or permeable Snapwells. Results are mean ± SEM of 4–5 identical experiments with 4–5 wells of cells at each data point.

similar to the values observed in the native gallbladder tissues [18].

The proposal that Na^+/H^+ exchange is involved in Na^+ transport in GBECs is based upon the findings that: i) the cultured cells exhibit DMA-inhibitable $^{22}Na^+$ uptake; ii) $^{22}Na^+$ uptake is stimulated by an outwardly directed H^+ gradient; iii) apical $^{22}Na^+$ uptake demonstrates Michaelis-Menten saturable kinetics for external Na^+; and iv) the cultured cells express the mRNAs coding for NHE isoforms NHE1-3.

The functional role of Na^+/H^+ exchange was assessed by measuring DMA-inhibitable $^{22}Na^+$ uptake in GBECs grown on both permeable supports (Snapwells, Costar) and 24-well plastic plates. The NHE activity was identical for both culture conditions. The finding that Na^+/H^+ exchange mediates ~45% of basal Na^+ uptake in GBECs is strikingly similar to the ~43% of apical Na^+ uptake mediated through Na^+/H^+ exchange observed in native gallbladder tissues [1]. These values are also in good quantitative agreement with the 40% of apical Na^+ entry through Na^+/H^+ exchange observed in *Necturus* gallbladders [40]. To investigate if other epithelial Na^+ transporters are involved and contribute significantly to the observed GBEC Na^+ uptake, we examined the Na^+ uptake effects of other inhibitors, bumetanide (Na^+-K^+-$2Cl^-$ cotransport inhibitor), hydrochlorothiazide (coupled Na^+-Cl^- transport inhibitor) and dichlorobenzamil (Na^+ channel and Na^+/Ca^{2+} exchange inhibitor), as well as HOE-694 (an amiloride analog that selectively inhibits NHE2). The data indicate that only DMA and HOE-694 significantly inhibit H^+-gradient-driven Na^+ uptake, whereas the other inhibitors do not significantly affect Na^+ uptake, suggesting that Na^+/H^+ exchange is the major pathway for apical Na^+ uptake in GBECs similar to native gallbladder tissues. Analysis of apical and basolateral Na^+ uptake further suggests that the cultured GBECs maintain functionally separate apical and basolateral membrane domains with NHE predominantly functional at the apical surfaces.

Data from the HOE-694 experiments demonstrate that both NHE2 and NHE3 are major contributors to Na^+/H^+ exchange activity in GBECs, similar to native gallbladder tissues. The relative contributions of NHE2 (~66%) and NHE3 (~28%) to total NHE activity in GBECs are identical to the relative contributions of these isoforms to NHE activity in native gallbladder tissues. This further suggests that the cultured cells retain the Na^+ transport characteristics of native gallbladder tissues. It should be stressed that the prairie dog appears to be unique in the expression and involvement in Na^+ transport of both NHE2 and NHE3 in the gallbladder epithelium. Previous studies by Silviani and coworkers using rabbit and human gallbladders have suggested that only NHE1 and NHE3 are expressed by gallbladder epithelium, as NHE2 expression could not be

detected by RT-PCR [37, 38]. However, NHE2 expression has been detected by RT-PCR in rat bile duct epithelial cells [28]. The presence of the NHE2 and NHE3 in prairie dog GBECs raises the possibility of cooperation between these exchangers in apical Na^+ transport. It is possible that one isoform may be involved in basal Na^+ transport, while the other isoform may become differentially functional in response to external signals under physiological or pathophysiological states. Such a potential cooperation between NHE2 and NHE3 and their differential regulation have been described in other Na^+-absorptive epithelia where NHE2 is coexpressed with NHE3 and is involved in Na^+ transport [8, 15, 16, 23, 29, 42]. It also has relevance to our previous finding that Ca^{2+}-calmodulin and protein kinase C are involved in regulating gallbladder Na^+ transport in prairie dogs [5, 30].

The ability to study Na^+/H^+ exchange kinetics in GBECs offers an additional and important advantage over the native gallbladder tissues to gain insight into the possible mechanisms for alterations in Na^+ transport observed during gallstone formation. We have shown that Na^+ uptake in the cultured cells conforms to simple Michaelis-Menten kinetics with a V_{max} of 9.2 ± 0.3 nmol · mg prot^{-1} · min^{-1} and an apparent $K_m^{Na^+}$ of 11.4 ± 1.4 mM. The apparent K_m for Na^+ in the GBECs is comparable to the values for NHE1 (10.2 mM), NHE2 (11.6 mM) and NHE3 (16.6 mM), but the V_{max} value is several-fold lower than the corresponding values for the NHEs in PS120 cells transfected with NHE isoform-specific cDNAs [6]. Studies with ileal and colonic brush border membrane vesicles in chicken [15] and colonic apical membrane vesicles in rats [22] have shown that V_{max} and K_m are differentially affected by dietary NaCl. Sodium depletion results in a several-fold increase in V_{max} without affecting K_m, resulting in a significant increase in NHE activity in these species. Cholesterol diet may cause similar changes in the kinetic parameters of gallbladder Na^+/H^+ exchange, which may partly explain the mechanisms for increased Na^+ absorption observed during gallstone formation in prairie dogs.

Finally, the results of RT-PCR analysis confirmed the mRNA expression coding for NHE2 and NHE3, in addition to NHE1, in the cultured cells. The sizes of the RT-PCR products matched the predicted sizes and were identical to the corresponding sizes of NHE1, NHE2 and NHE3 observed in native gallbladder epithelia. The NHE2 and NHE3 RT-PCR products were sequenced to confirm their authenticity, and the corresponding deduced amino-acid sequences were also identical to the NHE isoform values reported in native gallbladder epithelia [1].

In conclusion, we have shown that primary cultures of prairie dog gallbladder epithelium exhibit Na^+ transport characteristics similar to native gallbladder tissues. We have also shown that Na^+/H^+ exchange is the major pathway for apical Na^+ uptake in the cultured cells, and the relative contributions of NHE2 and NHE3 to total NHE activity are identical to the values observed in native gallbladder tissues. Furthermore, we have demonstrated that the Na^+ uptake by the cultured cells conforms to simple Michaelis-Menten kinetics. These primary cultures of gallbladder epithelial cells can be used as a tool for studying the mechanisms of gallbladder ion transport under normal physiologic conditions and during gallstone formation.

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References

1. Abedin, M.Z., Giurgiu, D.I.N., Abedin, Z.R., Peck, E.A., Su, X., Smith, P.R. 2001. Characterization of Na^+/H^+ exchanger isoform (NHE1, NHE2, and NHE3) expression in prairie dog gallbladder. *J. Membrane Biol.* **182:**123–134
2. Baerentsen, H., Giraldez, F., Zeuthen, T. 1983. Influx mechanism for Na^+ and Cl^- across the brush border membrane of leaky epithelia: a model and microelectrode study. *J. Membrane Biol.* **75:**205–218
3. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254
4. Brenneman, D.E., Connor, W.E., Forker, E.L., DenBesten, L. 1972. The formation of abnormal bile and cholesterol gallstones from dietary cholesterol in the prairie dog. *J. Clin. Invest.* **51:**1495–1503
5. Cates, J.A., Abedin, M.Z., Saunders-Kirkwood, K.D., Moser, A.J., Giurgiu, D.I., Roslyn, J.J. 1995. Protein kinase C regulates prairie dog gallbladder ion transport. *Surgery* **117:**206–212
6. Cavet, M.E., Akhter, S., De Medina, F.S., Donowitz, M., Tse, C.M. 1999. Na^+/H^+ exchangers (NHE1-3) have similar turnover numbers but different percentages on the cell surface. *Am. J. Physiol.* **277:**C1111–C1121
7. Chapman, W.C., Fisk, J., Schot, D., Debelak, J.P., Washington, M.K., Bluth, R.F., Pierce, D., Williams, L.F. 1998. Establishment and characterization of primary gallbladder epithelial cell cultures in the prairie dog. *J. Surg. Res.* 80:35–435
8. Cho, J.H., Musch, M.W., Bookstein, C.M., McSwine, R.L, Rabenau, R., Chang, E.B. 1998. Aldosterone stimulates intestinal Na^+ absorption in rats by increasing NHE3 expression of the proximal colon. *Am. J. Physiol.* **274:**C586–C594
9. Chomczynski, P., Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanante-phenol-chloroform extraction. *Anal. Biochem.* **162:**156–159
10. Conter, R.L., Roslyn, J.J., Porter-Fink, V., DenBesten, L. 1986. Gallbladder absorption increases during early cholesterol gallstone formation. *Am. J. Surg.* **151:**184–191
11. Counillon, L., Scholz, W., Lang, H.J., Pouyssegur, J. 1993. Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Mol. Pharmacol.* **44:**1041–1045

12. Cremaschi, D., Meyer, G., Rosetti, C., Botta, G., Palestini, P. 1987. The nature of the neutral Na^+-Cl^- -coupled entry at the apical membrane of rabbit gallbladder epithelium I. Na^+/H^+, Cl^-/HCO_3^- double exchange and Na^+-Cl^- symport. *J. Membrane Biol.* **95:**209–218
13. DenBesten, L., Safaie-Shirazi, S., Connor, W.E., Bell, S. 1974. Early changes in bile composition and gallstone formation induced by a high cholesterol diet in prairie dogs. *Gastroenterology* **66:**1036–1045
14. Diamond, J.M. 1964. Transport of salt and water in rabbit and guinea pig gallbladder. *J. Gen. Physiol.* **48:**1–14
15. Donowitz, M., De La Horra, C.D., Calonge, M.L., Wood, I.S., Dyer, J., Gribble, S.M., Sanchez De Medina, F., Tse, C.M., Shirazi-Beechey, S.P., llundain, A.A. 1998. In birds, NHE2 is the major brush-border Na^+/H^+ exchanger in colon and is increased by a low-NaCl diet. *Am. J. Physiol.* **274:**R1659–R1669
16. Dudeja, P.K., Rao, D.D., Syed, I., Joshi, V., Dahdal, R.Y., Gardner, C., Risk, M.C., Schmidt, L., Bavishi, D., Kim, K.E., Harig, J.M., Goldstein, J.L., Layden, T., Ramaswamy, K. 1996. Intestinal distribution of human N^+/H^+ exchanger isoforms NHE-1, NHE-2, and NHE-3 mRNA. *Am. J. Physiol.* **271:**G483–G493
17. Giurgiu, D.I., Saunders-Kirkwood, K.D., Roslyn, J.J., Abedin, M.Z. 1997. Sequential changes in biliary lipids and gallbladder ion transport during gallstone formation. *Ann. Surg.* **25:**457–465
18. Gunter-Smith, P.J., Abdulkader, O., Hammonds-Odi, L., Scanlon, M., Terrel, R. 2000. A primary culture of guinea pig gallladder epithelial cells that is responsive to secretagogues. *Am. J. Physiol.* **279:**G866–G874
19. Gurll, N., DenBesten, L. 1978. Animal models of human cholesterol gallstone disease: a review. *Lab. Animal Sci.* **28:**428–432
20. Holzbach, R.T. 1984. Animal models of cholesterol gallstone disease. *Hepatology* **4:**191S–198S
21. Holzbach, R.T., Corbusier, C., Marsh, M., Naito, H.K. 1976. The process of cholesterol cholelithiasis induced by diet in the prairie dog: a physicochemical characterization. *J. Lab. Clin. Med.* **87:**987–998
22. Ikuma, M., Kashgarian, M., Binder, H.J., Rajendran, V.M. 1999. Differential regulation of NHE isoforms by sodium depletion in proximal and distal segments of rat colon. *Am. J. Physiol.* **39:**G539–G549
23. Kandasamy, R.A., Yu, F.H., Harris, R., Boucher, A., Hanrahan, J.W., Orlowski, J. 1995. Plasma membrane Na^+-H^+ exchanger isoforms (NHE-1, -2, -3) are differentially responsive to second messenger agonists of the protein kinase A and C pathways. *J. Biol. Chem.* **270:**29209–29216
24. Kuver, R., Savard, C., Nguyen, T.D., Osborne, W.R., Lee, S.P. 1997. Isolation and long-term culture of gallbladder cells from wild-type and mice. *In Vitro Cell Dev. Biol. Anim.* **33:**104–109
25. LaMorte, W., O'Leary, D., Booker, M., Scott, T. 1993. Increased dietary fat content accelerates cholesterol gallstone formation in the cholesterol-fed prairie dog. *Hepatology* **18:** 1498–1503
26. Lee, S.P. 1978. Enhanced fluid transport across gallbladder mucosa in experimental cholelithiasis. *Am. J. Physiol.* **234:** E575–E578
27. Lee, S.P., LaMont, J.T., Carey, M.C. 1981. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones:studies in the prairie dog. *J. Clin. Invest.* **67:**1712–1723
28. Marti, U., Elsing, C., Renner, E.L., Liechti-Gallati, S., Reichen, J. 1996. Differential expression of Na^+/H^+ antiporter mRNA in biliary epithelial cells and hepatocytes. *J. Hepatol.* **24:**489–502
29. McSwine, R.L, Musch, M.W., Bookstein, C., Xie, Y., Rao, M., Chang, E.B. 1998. Regulation of apical membrane Na^+/H^+ exchangers NHE2 and NHE3 in intestinal epithelial cell line C2/bbe. *Am. J. Physiol.* **275:**C693–C701
30. Moser, A.J., Abedin, M.Z., Abedin, Z.R., Roslyn, J.J. 1993. Ca^{2+} calmodulin regulates basal gallbladder absorption. *Surgery* **114:**300–307
31. Oda, D., Lee, S.P., Hayshi, A. 1991. Long-term culture and partial characterization of dog gallbladder epithelial cells. *Lab. Invest.* **682:**682–692
32. Plevris, J.N., Walker, S.W., Harrison, D.J., Dhariwal, A., Hayes, P.C., Bouchier, I.A. 1993. Primary culture of bovine gall bladder epithelial cells. *Gut* **34:**1612–1615
33. Purdum, P.P.O., Ulissi, A., Hylemon, P.B., Shiftman, M.L., Moore, E.W. 1993. Cultured human gallbladder epithelia. Methods and partial characterization of a carcinoma-derived model. *Lab. Invest.* **68:**345–353
34. Reuss, L. 1984. Independence of apical membrane Na^+ and Cl^- entry in *Necturus* gallbladder epithelium. *J. Gen. Physiol.* **84:**423–445
35. Rose, R.C., Gelarden, R.T., Narhwold, D.L. 1973. Electrical properties of isolated human gallbladder. *Am. J. Physiol.* **224:**1320–1326
36. Roslyn, J.J., Abedin, M.Z., Saunders, K.D., Cates, J.A., Strichartz, S.D., Alperin, M., Fromm, M., Palant, C.E. 1991. Uncoupled basal sodium absorption and chloride secretion in prairie dog (*Cynomys ludovicianus*) gallbladder. *Comp. Biochem. Physiol.* **100A:**335–341
37. Silviani, V., Colombani, V., Heyries, L., Gerolami, A., Cartouzou, G., Marteau, C. 1996. Role of the NHE3 isoform of the Na^+/H^+ by the rabbit gallbladder. *Pfluegers Arch. Eur. J. Physiol.* **432:**791–796
38. Silviani, V., Gastaldi, M., Planells, R., Marteau, C., Massacrier, A., Cohen, P., Cau, P., Gerolami, A. 1997. NHE-3 isoform of the Na^+/H^+ exchanger in human gallbladder: Localization of specific mRNA by *in situ* hybridization. *J. Hepatol.* **26:**1281–1286
39. Strichartz, S.D., Abedin, M.Z., Abdou, M.S., Roslyn, J.J. 1989. The effects of amiloride on biliary calcium and cholesterol gallstone formation. *Ann. Surg.* **209:**152–156
40. Weinman, S.A., Reuss, L. 1984. Na^+/H^+ exchange and Na^+ entry across the apical membrane of *Necturus* gallbladder. *J. Gen. Physiol.* **83:**57–74
41. Winterhager, J.M., Stewart, C.P., Heintze, K., Petersen, K.U. 1986. Electroneutral secretion of bicarbonate by guinea pig gallbladder epithelium. *Am. J. Physiol.* **250:**C617–C628
42. Wormmeester, L., De Medina, F.S., Kokke, F., Tse, C.M., Khurana, S., Bowser, J., Cohen, M.E., Donowitz, M. 1998. Quantitative contribution of NHE2 and NHE3 to rabbit ileal brush-border Na^+/H^+ exchange. *Am. J. Physiol.* **274:**C1261–C1272