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Characterization of Na^+/H^+ Exchanger Isoform (NHE1, NHE2 and NHE3) Expression in Prairie Dog Gallbladder

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Abstract. Gallbladder Na^+ absorption is linked to gallstone formation in prairie dogs. Na^+/H^+ exchange (NHE) is one of the major Na^+ absorptive pathways in gallbladder. In this study, we measured gallbladder Na^+/H^+ exchange and characterized the NHE isoforms expressed in prairie dogs. Na^+/H^+ exchange activity was assessed by measuring amiloride-inhibitable transepithelial Na^+ flux and apical $^{22}Na^+$ uptake using dimethylamiloride (DMA). HOE-694 was used to determine NHE2 and NHE3 contributions. Basal J^{Na}_{ms} was higher than J^{Na}_{sm} with J^{Na}_{net} absorption. Mucosal DMA inhibited transepithelial Na^+ flux in a dose-dependent fashion, causing J^{Na}_{ms} equal to J^{Na}_{sm} and blocking J^{Na}_{net} absorption at 100 μM. Basal $^{22}Na^+$ uptake rate was 10.9 ± 1.0 $\mu mol \cdot cm^{-2} \cdot hr^{-1}$ which was inhibited by ~43% by mucosal DMA and ~30% by mucosal HOE-694 at 100 μM. RT-PCR and Northern blot analysis demonstrated expression of mRNAs encoding NHE1, NHE2 and NHE3 in the gallbladder. Expression of NHE1, NHE2 and NHE3 polypeptides was confirmed using isoform-specific anti-NHE antibodies. These data suggest that Na^+/H^+ exchange accounts for a substantial fraction of gallbladder apical Na^+ entry and most of net Na^+ absorption in prairie dogs. The NHE2 and NHE3 isoforms, but not NHE1, are involved in gallbladder apical Na^+ uptake and transepithelial Na^+ absorption.

Key words: Sodium proton exchanger — Epithelial sodium transport — Prairie dogs — Gallstones.

Introduction

The prairie dog has emerged as an important animal model for the study of human cholesterol gallstone disease. Gallbladder and hepatic bile compositions of prairie dogs are similar to those of humans [8]. Moreover, prairie dogs fed cholesterol-enriched diet develop cholesterol gallstones in a manner that recapitulates events known to occur in humans with cholelithiasis [23, 25]. Our Ussing-chamber studies suggest that 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 [44], resembling porcine [40] and human [43]. This is in contrast to the gallbladders of most other species, such as *Necturus* [42], rabbit [15, 16], and guinea pig [62], which exhibit V_t less than 2 mV and no significant I_{sc}, and thus NaCl transport in these tissues is nearly electroneutral. The short-circuit current observed in prairie dog gallbladder originates from a combination of net Na^+ absorption and net Cl^- secretion and not from amiloride sensitive apical Na^+ channels as reported in other electrogenic epithelia [2]. Nonetheless, the prairie dog is unique as a model of human cholesterol gallstone disease in having an electrically prominent gallbladder that closely resembles human, and thus provides an advantage to study the effects of luminal and pharmacological factors on gallbladder ion transport during gallstone formation.

Alterations in gallbladder ion (and H_2O) transport may have a pathogenic role in cholesterol gallstone formation [55]. In fact, gallbladder Na^+ and water absorption are increased prior to gallstone formation in prairie dogs and may promote cholesterol nucleation [13, 20]. The mechanisms by which gallbladder Na^+ and water transport are altered during gallstone formation are not clearly understood. It is believed that the Na^+ transport processes present in gallbladder epithelium must play a key role in regulating gallbladder absorption. Nonetheless, our ability to understand the observed alterations in gallbladder Na^+ and water transport during gallstone formation has been hampered by the paucity of data on the

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molecular mechanisms of ion transport in this animal model under physiological conditions. Although several studies from various animal species suggest that gallbladder Na^+ transport is mostly mediated through apically restricted Na^+/H^+ exchange [1, 15, 42], the isoforms of the Na^+/H^+ exchanger involved in Na^+ transport in gallbladder epithelium have not been fully characterized.

Recent molecular cloning experiments have identified several distinct isoforms of NHE, designated as NHE1–NHE5, in various animal species and in humans [7, 30, 41, 56, 57, 60]. These isoforms are tissue-specific and have multiple physiological functions and different regulatory mechanisms. NHE1 is a housekeeper isoform, expressed ubiquitously and localized at the basolateral membrane in epithelial cells, which helps maintain intracellular pH and cell volume [4, 47, 58]. Unlike NHE1, expression of other NHE isoforms exhibits tissue specificity. Current data show that NHE2 is expressed predominantly in kidney, intestine, and adrenal glands [57]; NHE3 is expressed in the kidney, gastrointestinal tract [3, 5, 56], and gallbladder [52, 53]; NHE4 is expressed in stomach, kidney medulla, and brain [6, 41]; and NHE5 is expressed in brain, testis, spleen and skeletal muscle [30]. Immunocytochemical studies indicate that NHE2 and NHE3 reside at the apical membrane of the intestine and renal proximal tubule, where they are involved in vectorial Na^+ transport [3, 5, 51, 57]. Moreover, apical NHEs are more resistant to amiloride and ethylisopropylamiloride (EIPA) inhibition than basolateral isoforms [24, 32, 57].

The objective of the present study was to investigate NHE-mediated Na^+ absorption and to characterize NHE1, NHE2, and NHE3 isoform expression in prairie dog gallbladders using combination of electrophysiologic, molecular biological, and immunochemical techniques. Relative distribution of these NHE isoforms may play an important role in regulating gallbladder basal Na^+ absorption, and alterations in their expression and/or regulation may alter gallbladder absorption inducing gallstone formation.

Materials and Methods

Materials

$^{22}Na^+$ and ^3H-mannitol were 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 (St Louis, MO). Electrophoretic 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). Rabbit polyclonal anti-NHE3 antibody [5, 38] was a gift from Dr. Eugene B. Chang (University of Chicago, IL). Rabbit polyclonal anti-NHE2 antibody and mouse monoclonal anti-NHE1 antibody were obtained from Chemicon (Temecula, CA).

Gallbladder Tissue

Adult male prairie dogs (*Cynomys ludovicianus*), trapped in the wild and obtained from Otto Marten Locke (New Braunfels, 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-hour fast with water *ad libitum*, animals were anesthetized with ketamine (100 mg/kg body weight) and xylazine (1.5 mg/kg intramuscularly), and cholecystectomy was performed via a midline laparotomy. Gallbladders were incised longitudinally and rinsed free of bile with standard Ringer's solution.

Transepithelial Na^+ Flux Measurement

Unidirectional Na^+ fluxes were measured under control conditions and after the mucosal addition of dimethylamiloride according to the methods previously described [44]. To assess the paracellular permeability of prairie dog gallbladder, we simultaneously measured unidirectional fluxes of Na^+ and mannitol under short-circuit conditions. Mannitol, a paracellular marker previously used by other investigators [18, 29], was chosen as a representative small, uncharged, hydrophilic solute that would be expected to diffuse only through the extracellular pathways of the prairie dog gallbladder, as was observed in other epithelia [18]. Briefly, gallbladders were mounted in a two-piece Lucite Ussing chamber with a 0.67 cm^2 circular aperture similar to that described by Schultz and Zalusky [50]. In order to minimize edge damage, chamber contact and seal were achieved with high vacuum silicone grease (Dow Corning, Midland, MI) [21]. Mucosal and serosal surfaces were perfused with equal volumes (10 ml) of modified Ringers' 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 (N-2-Hydroxyethyl-piperazine-N′-2-ethanesulfonic acid), and 10 glucose, adjusted to pH 7.4. The chamber was maintained at 37°C and gassed with 95% O_2-5% CO_2. After stabilization for 30 min, 1.5 μCi each of ^{22}Na and ^3H-mannitol was added to the mucosal side, and unidirectional mucosa to serosa fluxes of Na^+ (J^{Na}_{ms}) and mannitol (J^{Man}_{ms}) were measured for three 10-min flux periods by withdrawing 1 ml sample from the serosal side under basal conditions and following the mucosal addition of various doses of dimethylamiloride. Both surfaces of the tissue were then simultaneously washed with 500 ml warm Ringer's solution to remove traces of isotope from the chambers, and unidirectional serosa to mucosa of fluxes of Na^+ (J^{Na}_{sm}) and mannitol (J^{Man}_{sm}) were measured in an identical fashion. Background counts were determined in 1 ml aliquots obtained from the serosal or mucosal chambers before the addition of radioisotopes. The directionality of the flux was randomized (in some cases mucosa to serosa first and in others serosa to mucosa first) to determine if the tissues were becoming leaky over time, or not responding well. The directionality had no significant effect on unidirectional flux. All flux measurements were performed under short-circuit conditions. Radioisotopes were counted in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, CA) using dual isotope technique for ^{22}Na and ^3H. ^{22}Na counts were corrected for the fraction of the ^3H spectrum that was counted in the ^{22}Na window (3.9% of the total ^3H cpm). The ^{22}Na-counting efficiency in

Table 1. Isoform-specific NHE primers for RT-PCR analysis

NHE isoform	Primers	Product size	Region	Accession	Species
NHE-1	Sense: 5′-TGG CCT GCC TCA TGA AGA TAG G-3′	383	909–930	gb/S686616	Human
	Antisense: 5′-GTC GTA GTA GAG CCG GCA CCT G-3′		1270–1291	gb/S686618	Human
NHE-2	Sense: 5′-GAT CAC GTG AAG ACT GGG ATT GA(A/G) GA(T/C) GT-3′	415	1636–1664	gb/L13733	Rabbit
	Antisense: 5′-CT GCG CTC TCG AGG CAA GCT GTT (A/G)TC (T/C)TT-3′		2062–2090	gb/L13733	Rabbit
NHE-3	Sense: 5′-ATC TTC ATG TTC CTG GGC ATC TCG GC-3′	683	1127–1152	gb/M85300	Rat
	Antisense: 5′-GTG CTG AAG TCC ACA TTG ACC AT-3′		1778–1800	gb/M85300	Rat
NHE-4	Sense: 5′-CTT CTC CAT CAA GGA CCA GC-3′	759	1711–1730	gb/M85301	Rat
	Antisense: 5′-GGC TGA GGA TTG CTG TAA GG-3′		2450–2469	gb/M85301	Rat

Isoform-specific NHE primers for prairie dogs were designed based on the high homology of nucleotide sequence for rats, rabbits and humans.

the ^3H window was 1.1%. Unidirectional Na^+ and mannitol fluxes were calculated by a previously reported standard formula [50].

Tissue Viability

Tissue viability in the present experiment was assessed according to the following criteria established earlier for this model [48]: (1) the ability of the tissue to maintain active ion transport as indicated by stable electrophysiologic parameters such as short circuit current (I_{sc}) and transepithelial potential difference (V_t); (2) recovery of tissue I_{sc} and V_t after chamber wash following exposures to transport inhibitors; (3) stimulation of I_{sc} and V_t after bilateral exposure to 10 mM/l theophyline at the conclusion of individual experiments. Previous studies indicate that 10 mM/l theophylline elicits a maximal stimulus to ion transport in this tissue [48].

Apical $^{22}Na^+$ Uptake

Apical $^{22}Na^+$ uptake from the mucosal solution across the mucosal membrane into the epithelium (J_{me}) was measured using a method similar to that described by others [19, 49, 61]. Classical Ussing chambers were modified in such a way that the bathing solution could circulate through the mucosal chamber only, while the serosal chamber served as a base without any bathing solution. Gallbladder tissues were mounted in the modified Ussing chambers, which permitted exposure of the mucosal surface alone to the solutions of desired compositions. The serosal surface of the tissues rested on filter paper wetted with Ringer's, and was supported by the base of the Ussing chamber. The volume used for bathing mucosal surface of the tissues was 5 ml and could be easily removed by suction through the drain at the bottom of the chamber. After mounting, tissues were preincubated at 37°C with Ringer's solution of the same composition used in transepithelial flux measurements, and gassed with humidified 95% O_2 5% CO_2. After preincubation for 30 min, the mucosal solution was replaced with the Ringer's solution containing ~1μCi/ml of $^{22}NaCl$ and ^3H-mannitol. After a timed interval (1–2 min), the bathing solution was removed by suction, and the tissue was rinsed by washing 3 times with 5 ml ice-cold isotonic sucrose solution (0.3M). The gallbladder was then removed from the chamber, blotted gently and transferred to a scintillation vial. The tissue was digested by incubation at 65°C for 1 hr with 200 μl $HClO_4$ (70%), and 20 μl H_2O_2 (30%) [35]. After digestion and cooling, 8 ml of Bio-Safe II (Research Products International, Mount Prospect, IL) was added, and the sample was counted in a Beckman liquid scintillation counter (Beckman Instruments) using dual isotope technique for ^{22}Na and ^3H, as described above in unidirectional Na^+ flux measurement. ^{22}Na influx was corrected for the ^{22}Na present in the extracellular fluid contamination as estimated by the distribution of [^3H] mannitol, which was ~7% of the total ^{22}Na counts. Uptake rates were determined from 1-min exposures. When the effects of amiloride analogs, DMA and HOE-694, on ^{22}Na uptake were tested, the tissue was preincubated as described and exposed mucosally to drug-containing solution for 5 min prior to exposure to the drug-containing radioactive tracer solution.

RNA Isolation

RNA was extracted from prairie dog tissues using a commercially available RNA isolation kit (Stratagene), according to the method of Chomczynski and Sacchi [11] (for RT-PCR), or by using Trizol reagent (Gibco-BRL) (for Northern blot analysis). RNA concentration and purity were assessed by spectrophotometry at 260nm/280nm. Samples not used immediately were suspended in either 0.25 M sodium acetate/ethanol (for RT-PCR) or DEPC treated water (for Northern blot analysis) prior to storage at −70°C.

RT-PCR Analysis of NHE Isoform Expression

Approximately 200 ng of total RNA isolated from gallbladder tissue 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 hr of incubation at 42°C, samples were precipitated with ethanol. Amplification of complementary DNA (cDNA) was performed with *Taq* polymerase and reagents from Perkin-Elmer. Isoform-specific primers of NHE1, NHE2, NHE3 and NHE4 were designed for prairie dogs on the basis of maximum homology among published nucleotide sequences for rats, rabbits, and humans, described in Table 1. Briefly, all of the cDNA products were used for PCR reaction with the protocol recommended by the manufacturer. All of the 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). 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 sec per cycle. Aliquots of the PCR products were analyzed by electrophoresis on 1.5% 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.

Northern Blot Analysis

Twenty μg of total RNA from each tissue was denatured at 65°C for 15 min in 50% formamide and 6% formaldehyde [in 22.5 mM 3-(N-Morpholine) propanesulfonic acid (MOPS) with 1.2 mM EDTA], electrophoresed through 1.2% agarose-8% formaldehyde gels (in 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH. 7.0) and blotted to Nytran membranes (0.45 μm, Schleicher and Schuel, Keene, NH) with 20 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH. 7.0). RNA was crosslinked to the membranes by ultraviolet radiation in a Hoefer UVC 500 UV Crosslinker (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were prehybridized in Ultrahyb buffer (Ambion, Austin, TX) at 42°C for 30 min and then hybridized overnight in the same buffer at 42°C containing ^{32}P-labeled NHE2, NHE3 or 18S cDNA probes (10^6 cpm/ml). The blots were washed twice in 2× SSC, 0.1% SDS at 42°C (5 min/wash) and twice in 0.1× SSC, 0.1% SDS at 60°C (15 min/wash). Blots were exposed to x-ray film (Bio-Max MR film, Eastman Kodak, Rochester, NY) at −70°C, and films were developed at varying times. Individual blots were stripped and sequentially hybridized with NHE2- and NHE3-specific cDNA probes.

The 18S cDNA probe (18S DECA template) was obtained from Ambion. The NHE2 and NHE3 isoform specific cDNA probes (NHE2: *Pvu* II fragment encompassing nt 849–1590; NHE3: *Pst* I fragment encompassing nt 1241–2522) [27] were digested from their respective full-length rat NHE2 and NHE3 cDNAs, generously provided by Dr. Crescence Bookstein, University of Chicago. The digested products were separated by agarose gel electrophoresis, and fragments corresponding to the expected sizes were recovered from the gel using a gel extraction kit (Qiagen). The recovered NHE fragments and the 18S probe were subsequently labeled with ^{32}P-dCTP by random hexamer priming (Promega, Madison, WI) to a specific activity of >1 × 10^9 dpm/μg DNA.

Immunoblotting

Gallbladders were homogenized in an ice cold buffer that contained 300 mM mannitol, 175 μg/ml phenylmethylsulfonylfluoride (PMSF), complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), (5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N'-tetra-acetic acid, and 12 mM HEPES/Tris, pH 7.4, with a High Speed Homogenizer (Fisher Scientific, Pittsburgh, PA), followed by centrifugation in a refrigerated centrifuge at 2500 × g for 15 min. The pellet was discarded. The supernatant was collected and centrifuged at 40,000 × g for 1 hr. The resulting pellet was resuspended in ice cold buffer containing 150 mM NaH_2PO_4, 10 mM 3-[(-3-cholamidopropyl)dimethylammino]-1-propanesulfonate, 175 μg/ml PMSF and complete EDTA free protease inhibitor cocktail for 1 hr at 4°C. The protein concentration of this crude microsomal fraction was determined with the Bio-Rad detergent compatible protein assay kit following the reactions of Lowry et al. [34]. Proteins (~30 μg/lane) were separated by 7.5% SDS-PAGE, transferred to polyvinylidine fluoride membrane (PVDF) (Immobilon, Millipore, Bedford, MA) and probed with anti-NHE1, anti-NHE2, or anti-NHE3 antibodies followed by the appropriate secondary antibody coupled to alkaline phosphatase. The mouse monoclonal anti-NHE1 antibody (clone 4E9; Chemicon, Catalog number MAB3140; 1:1000 dilution) was generated against a maltose binding protein (MBP) fusion protein containing amino acids 514-818 of the C-terminal domain of porcine NHE1 [46]. The affinity-purified rabbit anti-NHE2 antibody (Chemicon, Catalog number 3083;1:1000 dilution) was generated against a 20-amino-acid synthetic peptide corresponding to the C-terminus of rat NHE2. The rabbit anti-NHE3 antibody (gift of Dr. E.B. Chang; 1:200 dilution) was generated against a GST fusion protein containing amino acids 528–648 in the C-terminus of rat NHE3 (4, 34). Bound antibodies were detected using a Western-Lite chemiluminescent detection system (Tropix, Bedford, MA). Controls consisted of substitution of an appropriate concentration of nonimmune IgG for the immune IgG.

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

Transepithelial Na^+ Flux Demonstrating DMA-Inhibitable Apical Na^+/H^+ Exchange

Measurement of unidirectional Na^+ flux across gallbladder epithelium mounted in Ussing chambers showed similar mucosa to serosa flux and a net Na^+ absorption under basal conditions as previously reported [44]. Mucosal exposure to a lower concentration (1 μM) of dimethylamiloride (DMA), a potent analog of amiloride and inhibitor of Na^+/H^+ exchanger, had no significant effect on transepithelial Na^+ flux measured under steady-state conditions in the presence of 140 mM Na^+ in the transport buffer. One μM DMA inhibited J^{Na}_{ms} only by ~4% (NS) and J^{Na}_{net} by ~9% (NS). However, at higher concentrations, mucosal DMA caused dose-dependent inhibition of J^{Na}_{ms} without a significant change in J^{Na}_{sm} (Table 2). J^{Na}_{ms} was inhibited by 18% at 10 μM, 27% at 100 μM, and 41% at 250 μM of mucosal DMA, respectively. Net Na^+ absorption was inhibited by 50% at 10 μM, 85% at 100 μM, and was completely blocked at 250 μM mucosal DMA. Serosal addition of 1–100 μM DMA had no effect on unidirectional Na^+ flux consistent with our previous studies using 1 mM amiloride [44].

To assess the paracellular permeability of prairie dog gallbladder, we simultaneously measured unidirectional fluxes of Na^+ and mannitol under short-circuit conditions. Under basal conditions, J^{Man}_{sm} was higher than the J^{Man}_{ms} resulting in J^{Man}_{net} secretion (Table 2). In contrast, J^{Na}_{ms} was higher than the J^{Na}_{sm} resulting in J^{Na}_{net} absorption of 5.4 ± 1.2 $\mu Eq \cdot cm^{-2} \cdot hr^{-1}$. The mannitol flux in mucosa to serosa direction was ~200

Table 2. Effects of mucosal exposure to dimethylamiloride (DMA) on Na^+ and mannitol fluxes in gallbladder epithelium

DMA (μM)	*n*	Flux					
		Na^+ (μEq · cm^{-2} · hr^{-1})			Mannitol (nmol · cm^{-2} · hr^{-1})		
		M→S	S→M	Net	M→S	S→M	Net
0	9	21.8 ± 2.5	16.4 ± 4.1	+5.4 ± 1.2	116 ± 15	170 ± 29	−53 ± 11
1	4	20.9 ± 2.0	16.0 ± 3.9	+4.9 ± 1.6	115 ± 11	161 ± 11	−46 ± 10
10	5	17.9 ± 1.8[b]	15.2 ± 3.8	+2.7 ± 1.5	103 ± 8	152 ± 28	−49 ± 11[a]
100	5	15.9 ± 2.2[b]	15.1 ± 3.7	+0.8 ± 1.4[a]	117 ± 17	131 ± 32[b]	−14 ± 11[a]
250	5	12.9 ± 2.1[b]	13.3 ± 3.1	−0.4 ± 2.7[a]	115 ± 14	121 ± 31[b]	−7 ± 14[b]

Results are means ± SEM; *n* = number of experiments.
[a]$P < 0.05$, [b]$P < 0.01$ *vs.* corresponding baseline (paired *t*-Test)

times less in magnitude compared to Na^+ flux and DMA had no effect on the mucosa to serosa mannitol flux. Mannitol secretions under short-circuit conditions have been reported in other leaky epithelia, such as rat proximal intestine [29]. The net mannitol secretion in prairie dog gallbladder (−53 ± 11 nmol · cm^{-2} · hr^{-1}) is comparable with the values reported in rat duodenum (−26.3 ± 8.3 nmol · cm^{-2} · hr^{-1}) and jejunum (−76.0 ± 29.6 nmol · cm^{-2} · hr^{-1}). Although exact mechanisms for mannitol secretions in prairie dog gallbladder under short-circuit conditions are not forthcoming from the present study, "anomalous solvent drag effect" [59] may be a possible explanation for favoring mannitol flux in the S→M direction resulting in mannitol secretion.

Apical $^{22}Na^+$ Uptake Showing DMA-Inhibitable and HOE-Sensitive Na^+/H^+ Exchange

The transepithelial fluxes were measured under steady-state conditions and thus DMA-inhibitable mucosa-to-serosa Na^+ flux might not give a true estimate of the magnitude of Na^+/H^+ exchange involved in gallbladder apical Na^+ entry. Therefore, we measured DMA-inhibitable apical Na^+ uptake to further demonstrate that Na^+/H^+ exchange plays a major role in gallbladder apical Na^+ entry. DMA-inhibitable Na^+/H^+ exchange activity was determined by the difference in basal rate of apical Na^+ uptake minus the uptake in the presence of mucosal 100 μM DMA (DMA K_i for NHE2, 0.7 μM; for NHE3, 11 μM, as determined in NHE-deficient PS 120 fibroblasts stably transfected with isoform-specific NHE cDNAs) [14]. The basal rate of apical Na^+ uptake measured at 37°C in a bathing solution containing 140 mM Na^+ was 10.9 ± 1.0 μmol · cm^{-2} · hr^{-1} (Fig. 1, Panel *A*). In the presence of 100 μM mucosal DMA, Na^+ uptake was reduced by ~57% (6.2 ± 0.7 μmol · cm^{-2} · hr^{-1}) representing ~43% inhibition, which is comparable with ~27% and ~41% inhibition of unidirectional J^{Na}_{ms} at 100 and 250 μM DMA, respectively. These values are in good quantitative agreement with the 40% inhibition by 1 mM amiloride of gallbladder apical Na^+ entry in *Necturus* [61]. These results suggest that Na^+/H^+ exchange mediates a sizable fraction of gallbladder apical Na^+ uptake in prairie dogs.

The contribution of NHE2 and NHE3 to gallbladder apical Na^+/H^+ exchange was determined by use of differential sensitivity to mucosal exposure to HOE-694 (HOE-694 K_i for NHE2, 5 μM; for NHE3, 650 μM) [14,63]. HOE-sensitive NHE activity was defined as the difference in basal Na^+ uptake minus the uptake in the presence of 100 μM mucosal HOE-694. Under basal conditions, ~70% of gallbladder total DMA-inhibitable Na^+/H^+ exchange (4.7 ± 0.4 μmol · cm^{-2} · hr^{-1}) was contributed by HOE-sensitive NHE2 (3.3 ± 0.3 μmol · cm^{-2} · hr^{-1}) and ~30% by HOE-insensitive NHE3 (1.4 ± 0.1 μmol · cm^{-2} · hr^{-1}) (Fig.1, Panel *B*).

NHE Messenger RNA Expression in Gallbladder Epithelium

The expression of NHE messenger RNA was examined in gallbladder epithelium by RT-PCR analysis using NHE isoform-specific primers described in Table 1. Figure 2 shows an ethidium bromide-stained 1.5% agarose gel of amplified cDNA products of NHE1 through NHE-3 isoforms (Panels *A* & *B*). The sizes of the RT-PCR products observed matched with those predicted and listed in Table 1. No expression of NHE4 mRNA was observed in gallbladder epithelium (*data not shown*). To eliminate the possibility of amplification of contaminating genomic DNA in gallbladder samples, PCR reactions were run with RNA that had not been reverse-transcribed. No PCR product was observed (Fig. 2, Panel *A*, lane *2*). The NHE1, NHE2, and NHE3 RT-PCR products were sequenced to confirm their authenticity, and the corresponding amino acid sequences were de-

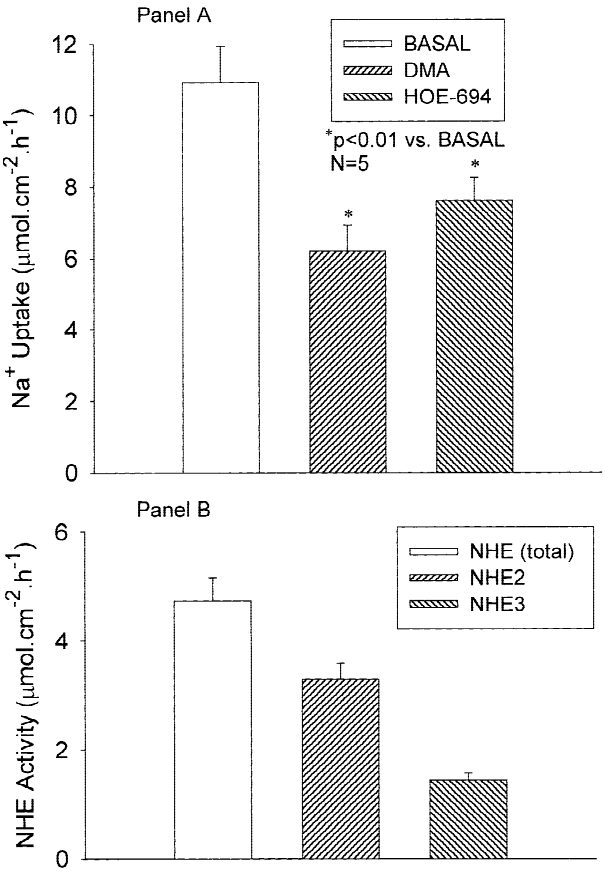

Fig. 1. $^{22}Na^+$ uptake demonstrating DMA-inhibitable and HOE-694-sensitive gallbladder apical Na^+/H^+ exchange. The influx of $^{22}Na^+$ from the mucosal solution into the epithelium was measured for 1 min in the presence or absence of either 100 μM mucosal DMA or HOE-694 using modified Ussing chambers with only mucosal side exposed to the Ringer's containing 140 mM Na^+. The tissue was pre-incubated with Ringer's for 30 min before addition of radioisotope-containing solution. To measure the effect of the drug on apical uptake, the tissue was exposed mucosally to drug-containing solution for 5 min prior to the addition of drug-containing radioisotope solution. (Panel *A*) Basal rate of apical $^{22}Na^+$ uptake was 10.9 ± 1.0 μmol · cm^{-2} · hr^{-2} (white bar), which was reduced by ~57% in the presence of 100 μM DMA (forward hatched bar), and ~70% in 100 μM HOE-694 (backward hatched bar). (Panel *B*). DMA-inhibitable Na^+/H^+ exchange activity (NHE-total) was determined by the difference in basal rate of apical Na^+ uptake minus the uptake in the presence of mucosal 100 μM DMA and represents a sizeable component (~43%) of gallbladder apical Na^+ entry (white bar). HOE-sensitive NHE activity was defined as the difference in basal Na^+ uptake minus the uptake in the presence of 100 μM mucosal HOE-694 and represents ~70% of gallbladder total DMA-inhibitable Na^+/H^+ exchange and may be contributed by HOE-sensitive NHE2 (forward hatched bar). Contribution by NHE3 is determined by the difference between the DMA-inhibitable NHE and the HOE-sensitive NHE and represents ~30% of total NHE (backward hatched bar).

duced. The results are summarized in Table 3. The partial nucleotide- and deduced amino-acid sequences of the prairie dog NHE2 and NHE3 have been deposited to the GenBank and their accession numbers are AF012894 and U75940, respectively.

Northern Blot Analysis

Northern blot analysis was used to corroborate the RT-PCR data demonstrating expression of NHE2 and NHE3 mRNA in the prairie dog gallbladder (Fig. 3). To confirm that the rat NHE2 and NHE3 cDNA probes specifically hybridized to NHE2 and NHE 3 mRNAs in prairie dog, a multiple tissue blot was probed. The NHE2 cDNA probe was observed to hybridize to an ~4.4 kb transcript in urinary bladder, distal colon, large intestine, duodenum, ileum, jejunum, stomach, and skeletal muscle (Fig. 3*A*). A faint ~6.5 kb transcript was also observed in some tissues. This larger-size NHE2 mRNA has been reported to be expressed in human tissue [36]. The 4.4 kb transcript was detected in gallbladder and kidney following long autoradiographic exposures (Fig. 3*B*). NHE2 abundance in the gallbladder, however, appears to be low when compared to other regions of the gastrointestinal tract. The NHE3 cDNA probe was observed to hybridize to an ~5.4 kb transcript in distal colon, large intestine, kidney and gallbladder (Fig. 3*C*). A weak signal was also present in skin and stomach. The sizes of the NHE2 and NHE3 transcripts and their tissue distributions are in agreement with those previously reported for NHE2 and NHE3 isoform mRNAs [5, 12, 27].

NHE Protein Expression in Gallbladder Epithelium

The expression of NHE protein in prairie dog gallbladder was examined by immunoblot analysis with anti-NHE1, anti-NHE2 or anti-NHE3 isoform-specific antibodies. The anti-NHE1 monoclonal antibody recognized an ~100 kDa polypeptide, whereas the anti-NHE2 and anti-NHE3 affinity-purified polyclonal antibodies both recognized polypeptides of ~85 kDa (Fig. 4, Panels *A–C*). The M_rs of these polypeptides are in good agreement with the reported M_r for NHE1 [5], NHE2 [26] and NHE3 [3]. When nonimmune serum was substituted for the NHE isoform-specific antibodies, none of these polypeptides were recognized (Fig. 4, Panels *A–C*). We have not been able to immunolocalize NHE1, NHE2, or NHE3 in the prairie dog gallbladder using these antibodies.

Discussion

The prairie dog is widely used as a model for human cholesterol gallstone research [23, 25]. Recent observations have linked alterations in gallbladder Na^+ and water absorption to the pathogenesis of cholesterol gallstones [13, 20]. Although several studies from various animal species suggest that gallbladder Na^+ transport is predominantly mediated through apically restricted Na^+/H^+ exchange [1, 15, 42], the molecular isoforms of the Na^+/

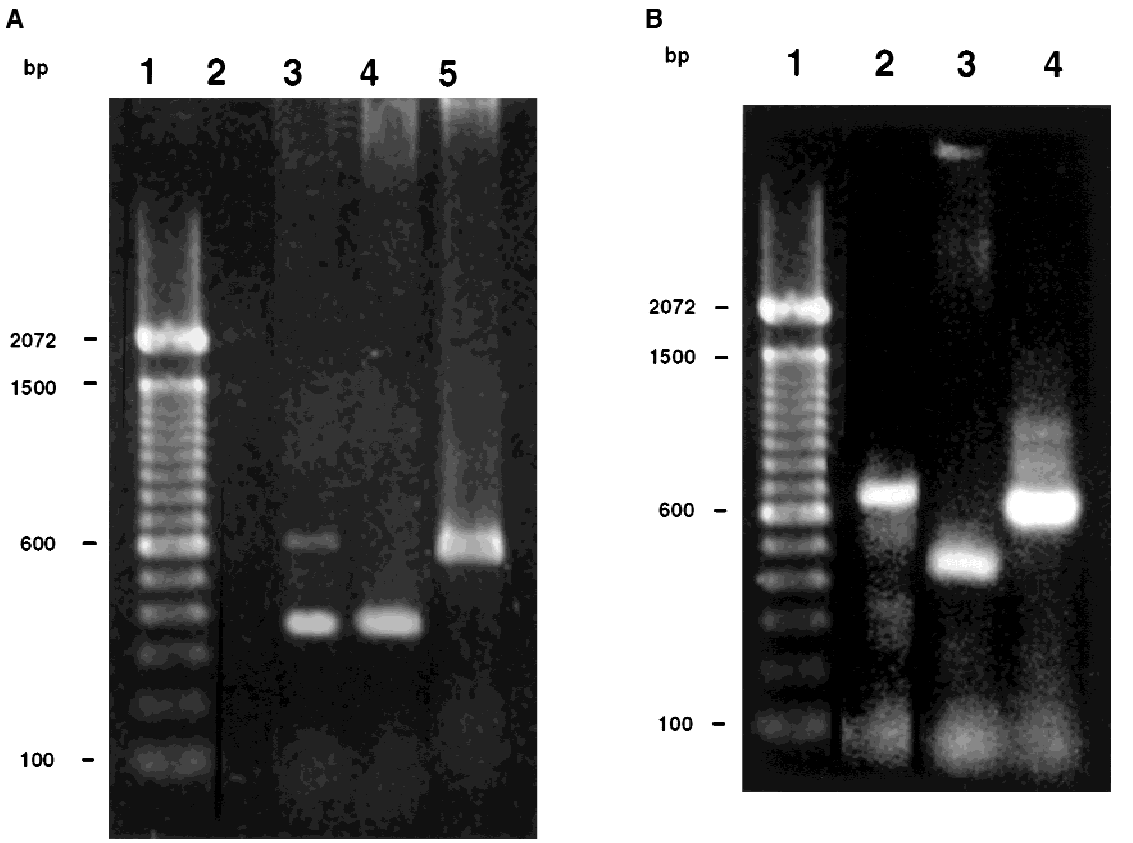

Fig. 2. RT-PCR analysis of prairie dog gallbladder epithelium. RNA isolated from gallbladder was reverse-transcribed with isoform-specific NHE primers designed for prairie dogs on the basis of high homology of nucleotide sequence between rats, rabbits, and humans (*see* Table 1). Ethidium bromide-stained 1.5% agarose gels of amplified cDNAs are shown. (Panel *A*) Lane 1: 100-bp DNA ladder; lane 2: PCR product without RT using β-actin primer; lane 3: NHE1 (383 bp, prairie dog cDNA); lane 4: NHE1 (383 bp, rabbit gallbladder cDNA); lane 5: β-actin (600 bp). (Panel *B*) Lane 1: 100-bp DNA ladder; lane 2: NHE3 (683 bp); lane 3: NHE2 (415 bp); and lane 4: β-actin (600 bp).

Table 3. Comparison of nucleotide sequence and deduced amino acid residues of cDNA fragments of NHE isoforms among prairie dog and other species

	NHE-1	NHE-2	NHE-3
Nucleotide base pairs	383	415	683
% homology to rats	91	87	88
rabbits	98	90	88
humans	94	84	87
Amino acid residues	127	138	227
% homology to rats	95	97	96
rabbits	98	98	91
humans	98	92	91

H^+ exchanger involved in Na^+ transport in gallbladder epithelium have not been fully characterized. In the present study, we have combined physiologic, molecular biological, and immunochemical techniques to identify and characterize the Na^+/H^+ exchanger isoforms involved in Na^+ transport in the prairie dog gallbladder. With classic Ussing chambers studies, we have shown that Na^+/H^+ exchange accounts for a substantial fraction of gallbladder apical Na^+ entry, and most of the transepithelial Na^+ absorption in prairie dogs. Reverse transcriptase-polymerase chain reaction and Northern blot analysis demonstrate that prairie dog gallbladder epithelium expresses mRNAs encoding for the NHE1, NHE2, and NHE3 Na^+/H^+ exchanger isoforms. In addition, we have used isoform-specific anti-NHE antibodies to demonstrate expression of the NHE1, NHE2, and NHE3 isoforms at the protein level.

The proposal that Na^+/H^+ exchange is involved in Na^+ transport in prairie dog gallbladder epithelium is based upon the demonstration of amiloride-inhibitable transepithelial Na^+ flux and apical $^{22}Na^+$ uptake in the presence of 140 mM NaCl in Ringer's bathing the tissues. Under basal conditions, prairie dog gallbladder exhibits a higher rate of J^{Na}_{ms} (21.8 ± 2.5 $\mu Eq \cdot cm^{-2} \cdot hr^{-1}$) than J^{Na}_{sm} (16.4 ± 4.1 $\mu Eq \cdot cm^{-2} \cdot hr^{-1}$) with a net Na^+ absorption ($+5.4 \pm 1.2$ $\mu Eq \cdot cm^{-2} \cdot hr^{-1}$) consistent with our previous results [20, 44]. Mucosal exposure to a

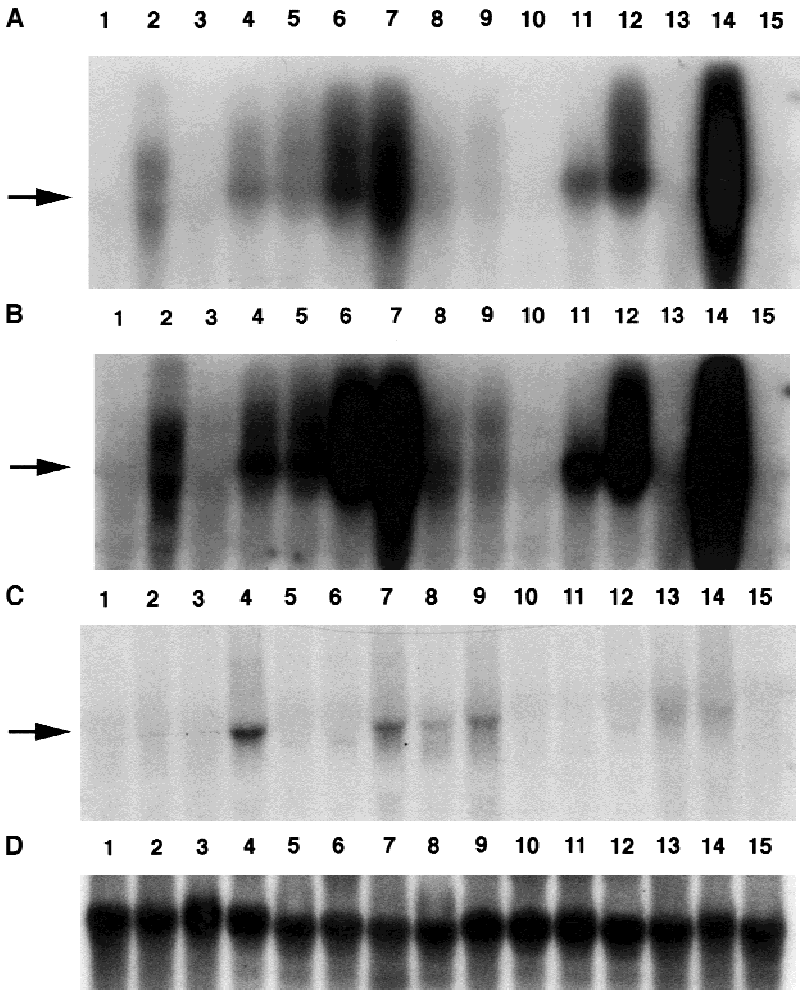

Fig. 3. Expression of NHE2 and NHE3 isoform mRNAs in prairie dog tissues. 20 μg of total RNA isolated from prairie dog tissues were analyzed by Northern blot analysis using NHE2 and NHE3 specific cDNA probes. (*A* and *B*). Blot was hybridized with NHE 2 isoform-specific cDNA probe followed by autoradiographic exposure for 24 (*A*) and 72 (*B*) hours. (*C*) Blot shown in *A* and *B* was stripped and hybridized with NHE3-specific cDNA probe. (*D*) Blot shown in *A*, *B*, and *C*, was stripped and hybridized with 18S probe. Arrows indicate predominant NHE2 and NHE3 transcripts. Lanes: 1, atria; 2, urinary bladder; 3, brain; 4, distal colon; 5, duodenum; 6, ileum; 7, intestine; 8, gallbladder; 9, kidney; 10, liver; 11, skeletal muscle; 12, jejunum; 13, skin; 14, stomach; 15, ventricle.

lower concentration (1 μM) of dimethylamiloride (DMA), a potent analog of amiloride and inhibitor of Na^+/H^+ exchange, had no effect on transepithelial Na^+ flux. However, at higher concentrations DMA caused dose-dependent inhibition of J^{Na}_{ms} without a significant change in J^{Na}_{sm} (Table 2). J^{Na}_{net} absorption was inhibited by 50% at 10 μM, 85% at 100 μM, and was completely prevented at 250 μM mucosal DMA. Serosal addition of 1–100 μM DMA had no effect on unidirectional Na^+ flux, consistent with our previous results using 1 mM amiloride [44]. The rate of Na^+ entry from the mucosal solution across the apical membrane into the epithelium was inhibited by 43% with 100 μM DMA compared to the basal rate (6.2 ± 0.7 *vs.* 10.9 ± 1.0 $\mu mol \cdot cm^{-2} \cdot hr^{-1}$, $P < 0.005$). This magnitude of inhibition of $^{22}Na^+$ uptake by 100 μM mucosal DMA is comparable to the 40% inhibition of apical Na^+ entry by 1 mM mucosal amiloride in *Necturus* gallbladder using similar techniques [61]. These data demonstrate that Na^+/H^+ exchange accounts for a significant fraction of apical Na^+ entry and is involved in transepithelial Na^+ transport in prairie dog gallbladder. Furthermore, they are consistent with data from studies examining Na^+ transport across *Necturus* and rabbit gallbladder epithelia [1, 15, 42].

Recent 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 [52, 53]. However, NHE2 expression has been detected by RT-PCR in rat bile duct epithelial cells [37]. In this study we have documented by RT-PCR and Northern blot analysis the expression of mRNAs encoding for NHE2 and NHE3, as well as for NHE1, in the prairie dog gallbladder epithelium. Sequence analysis of the RT-PCR products indicate that the nucleotide and deduced amino acid sequences of the amplified regions of prairie dog NHE1, NHE2, and NHE3 isoforms are highly homologous to the comparable regions of rat, rabbit, and human NHE1, NHE2 and NHE3. We have also demonstrated, using anti-NHE isoform-specific antibodies, the expression of NHE1, NHE2, and NHE3 polypeptides in prairie dog gallbladder epithelium. At present, the prairie dog appears to be unique in its expression

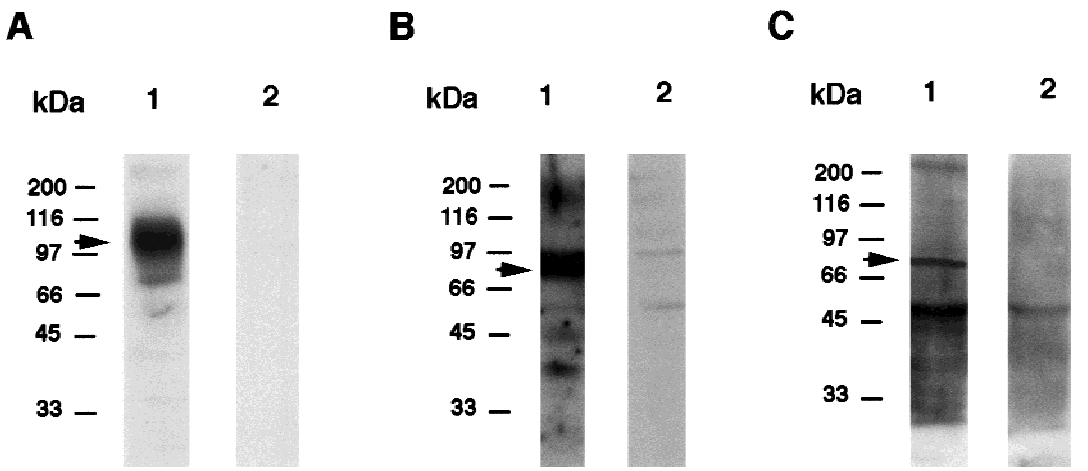

Fig. 4. Immunoblot analysis of NHE-isoform expression in prairie dog gallbladder epithelium. Approximately 30 μg of crude microsomal proteins isolated from prairie dog gallbladder prepared as described in Materials and Methods were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane. Membranes were probed with mouse monoclonal anti-NHE1 antibody (*A*), rabbit polyclonal anti-NHE2 antibody (*B*), or rabbit polyclonal anti-NHE3 antibody (*C*), and bound antibody was detected by chemiluminescence. (*A*) As shown in lane *1*, the anti-NHE1 monoclonal antibody recognized a polypeptide of ~100–110 kDa (*arrow*) corresponding in M_r to NHE1. This polypeptide was not recognized when nonimmune mouse IgG was substituted for the anti-NHE1 antibody (lane *2*). An ~75–80 kDa polypeptide, which may represent immature NHE1, was also recognized by the anti-NHE1 antibody. (*B*) The anti-NHE2 antibody specifically recognized a polypeptide of ~85 kDa (*arrow*) which corresponds to NHE2 (lane *1*). This polypeptide was not detected when nonimmune rabbit IgG was substituted for the anti-NHE2 antibody (lane 2). (*C*). The anti-NHE3 antibody recognized an ~85 kDa polypeptide (arrow), corresponding to the reported M_r of NHE3 (lane *1*). This polypeptide was not observed when nonimmune rabbit IgG was substituted for the anti-NHE 3 antibody (lane *2*).

of both NHE2 and NHE3 in the gallbladder epithelium, and it may underscore the relative contributions of these isoforms in gallbladder Na^+ absorption under physiological conditions, and specifically during gallstone formation in this organism.

We have assessed the functional roles of Na^+/H^+ exchanges in the prairie dog gallbladder by measuring amiloride-inhibitable transepithelial Na^+ flux and apical $^{22}Na^+$ uptake using DMA. DMA is a potent amiloride analog that displays 12-fold greater activity against Na^+/H^+ exchange than amiloride [31]. In addition, pharmacological studies in the PS120 fibroblast cell line stably expressing NHE1, NHE2, or NHE3 have demonstrated that DMA has more affinity for NHE1 (K_i of 0.1 μM) compared to NHE2 (K_i of 0.7 μM) and NHE3 (K_i of 11 μM) [14]. In prairie dog gallbladder, mucosal exposure to DMA had no effect on transepithelial Na^+ flux at lower concentrations, but it had significant inhibitory effects on transepithelial Na^+ flux and apical $^{22}Na^+$ uptake at higher concentrations. The finding that low concentration of DMA (1 μM) had no effect on transepithelial Na^+ flux suggests that the DMA-sensitive NHE1 isoform is not involved in gallbladder vectorial Na^+ absorption. This observation is consistent with basolateral distribution of NHE1 in epithelia and its role in cell "housekeeping" functions, activated only under conditions such as increased acid loads, cell volume changes and growth factor activation [22, 45].

The observations that higher concentrations of mucosal DMA inhibited transepithelial Na^+ flux in a dose-dependent fashion, causing J^{Na}_{ms} to almost equal J^{Na}_{sm} with 85% reduction in net Na^+ absorption at 100 μM, suggest that the apically restricted Na^+/H^+ exchanger isoforms NHE2 and NHE3, which are relatively resistant to amiloride, may be involved in vectorial Na^+ transport in gallbladder epithelia. The magnitude of Na^+/H^+ exchange involved in gallbladder apical Na^+ entry and the contribution of NHE2 and NHE3 were further demonstrated by $^{22}Na^+$ uptake studies using mucosal exposure to DMA and HOE-694 (HOE-694 K_i for NHE2, 5 μM; for NHE3, 650 μM) [14, 63]. The concentration of Na^+ in the transport buffer used to determine $^{22}Na^+$ uptake was 140 mM, identical to the concentrations used in our transepithelial Na^+ flux measurements. 100 μM DMA completely inhibited transepithelial net Na^+ absorption, and was used to inhibit both NHE2 and NHE3 in $^{22}Na^+$ uptake studies. We used 100 μM HOE-694 in $^{22}Na^+$ uptake studies to determine the contribution of NHE2. This dose is higher than the published values for HOE-694 used with transport buffers containing lower concentrations of Na^+. Indeed, there is a wide variation in the Na^+ concentration of the transport buffer that has been used to determine the contribution of HOE-sensitive component of NHE (NHE2) in various epithelial preparations. In general, Na^+ concentrations in the transport buffer for $^{22}Na^+$ uptake varied from 0.1–20 mM, and the dose of HOE-694 used to define the contribution of NHE2 lies between 25 and 30 μM [10, 27, 63]. In our present study,

the basal rate of gallbladder apical Na^+ uptake measured at 37°C was 10.9 ± 1.0 μmol · cm^{-2} · hr^{-1} (Fig. 1, Panel *A*). In the presence of 100 μM mucosal DMA, the uptake was reduced to 43%, which is in good quantitative agreement with the reduction to 40% by 1 mM amiloride of gallbladder apical Na^+ entry in *Necturus* [61]. These results suggest that Na^+/H^+ exchange mediates a sizable component of gallbladder apical Na^+ uptake in prairie dogs. Data from our HOE-694 experiments demonstrate that both NHE2 and NHE3 contribute to gallbladder apical Na^+/H^+ exchange under basal conditions (Fig. 1, Panel *B*). The presence of the NHE2 and NHE3 isoforms in prairie dog gallbladder raises the possibility of cooperation between these exchangers in transepithelial Na^+ absorption. This potential cooperation between NHE2 and NHE3 has been demonstrated in other absorptive epithelia where NHE2 is coexpressed with NHE3 and is involved in Na^+ absorption [17, 38]. The hypothesis of exchanger cooperation underscores the potential importance of differential regulation of NHE2 and NHE3 by external signals and second messengers [28, 33, 54], which have been shown to be involved in regulating gallbladder ion transport in prairie dogs [9, 39].

In conclusion, we have demonstrated that Na^+/H^+ exchange mediates a substantial fraction of apical Na^+ entry and accounts for most of transepithelial Na^+ absorption in prairie dog gallbladder. Furthermore, we have demonstrated that in addition to NHE1, the gallbladder epithelium expresses the mRNA transcripts and polypeptides for the apically restricted NHE isoforms NHE2 and NHE3. Both NHE2 and NHE3 contribute to gallbladder apical Na^+ uptake under basal conditions. The presence of NHE2 and NHE3 is consistent with the hypothesis that apically restricted NHE isoforms play an important role in vectorial Na^+ transport by absorptive epithelia. The presence of the two apical isoforms in prairie dog gallbladder epithelia raises the potential for cooperation between the exchangers in regulating Na^+ absorption under physiologic conditions, and especially during gallstone formation. Further studies are warranted to determine the relative expression and differential regulation of NHE2 and NHE3 in prairie dog gallbladder epithelium and whether their expression is altered causing changes in Na^+ absorption associated with cholesterol gallstone formation.

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This work is dedicated to the memory of Joel J. Roslyn, M.D., who devoted a good part of his life to understanding the mechanisms of cholelithiasis by linking gallbladder Na^+ absorption to gallstone formation in the prairie dog model.

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