Cloning and expression of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger from Amphiuma RBCs: resemblance to mammalian NHE1

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McLean, Lee Anne, Shaheen Zia, Fredric A. Gorin, and Peter M. Cala. Cloning and expression of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger from Amphiuma RBCs: resemblance to mammalian NHE1. Am. J. Physiol. 276 (Cell Physiol. 45): C1025–C1037, 1999.—The cDNA encoding the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) from Amphiuma erythrocytes was cloned, sequenced, and found to be highly homologous to the human NHE1 isoform (hNHE1), with 79% identity and 89% similarity at the amino acid level. Sequence comparisons with other NHEs indicate that the Amphiuma tridactylum NHE isoform 1 (atNHE1) is likely to be a phylogenetic progenitor of mammalian NHE1. The atNHE1 protein, when stably transfected into the NHE-deficient AP-1 cell line (37), demonstrates robust Na\textsuperscript{+}-dependent proton transport that is sensitive to amiloride but not to the potent NHE1 inhibitor HOE-694. Interestingly, chimeric NHE proteins constructed by exchanging the amino and carboxy termini between atNHE1 and hNHE1 exhibited drug sensitivities similar to atNHE1. Based on kinetic, sequence, and functional similarities between atNHE1 and mammalian NHE1, we propose that the Amphiuma exchanger should prove to be a valuable model for studying the control of pH and volume regulation of mammalian NHE1. However, low sensitivity of atNHE1 to the NHE inhibitor HOE-694 in both native Amphiuma red blood cells (RBCs) and in transfected mammalian cells distinguishes this transporter from its mammalian homologue.

The ubiquitously expressed Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) has been shown to participate in a variety of physiological processes, including sodium absorption, cell development, intracellular pH (pHi) regulation, and cell volume regulation. Abnormalities in NHE function have been implicated in such pathophysiological conditions as diabetic nephropathy, hypertension, ischemia-reperfusion injury, and tumor proliferation (24, 36, 41–43). The first demonstration in mammals of the obligate counterexchange of equimolar Na\textsuperscript{+} for H\textsuperscript{+} was performed in 1976 on isolated brush-border membranes from rat intestine and kidney (30) and suggested that NHE participates in epithelial Na\textsuperscript{+} absorption. During that same year, Johnson and Epel (26) demonstrated that postfertilization activation of sea urchin eggs was associated with intracellular alkalization mediated by Na\textsuperscript{+}/H\textsuperscript{+} exchange. Subsequent studies by Thomas and Aickin directly implicated NHE-dependent regulation of pHi in snail neurons (44) and mouse soleus muscle (1). The role of the NHE in the regulation of cell volume was first demonstrated by our laboratory, in 1980, in studies using red blood cells (RBCs) from the giant salamander, Amphiuma tridactylum (9).

These initial studies underscored the broad distribution and versatility of the NHE, yet they raised questions regarding the molecular equivalence of the proteins performing the various functions. Distinct differences in substrate affinity and inhibitor potency led many to suspect that the Na\textsuperscript{+}/H\textsuperscript{+} exchange functions may be carried out by related rather than identical proteins. This has since been proven to be the case, with five distinct mammalian plasma membrane isoforms, NHE1–NHE5 (27, 35, 40, 45, 46), identified thus far, in addition to the cAMP-activated βNHE isoform from trout RBCs (5). More detailed information concerning the structural and functional similarities and differences among the various vertebrate NHE isoforms can be obtained from several excellent, recently published reviews (31, 33, 50, 52).

The present study is focused on the cloning and functional expression of the NHE from Amphiuma RBCs. The Amphiuma exchanger performs the same housekeeping roles as the mammalian isoform NHE1, functioning in both volume and pH regulatory capacities (1, 3, 9, 10). Consistent with its role in mediating volume and pH regulation, the Amphiuma NHE, like other NHE1 isoforms, is a highly regulated, inducible transporter. Activation by hyperosmotic shrinkage or intracellular acidification can result in an increase of NHE1 activity by one to two orders of magnitude, with restoration to prestimulus levels as volume or pH is regulated. The NHE-mediated pH and volume regulatory functions in Amphiuma RBCs are remarkably robust and are strikingly similar to those of mammalian systems (8–12). These kinetic and functional similarities between the Amphiuma RBC NHE and mammalian NHE1 led us to postulate that the primary structures of these transport proteins would be similar and therefore that control mechanisms would also be comparable for the two transport proteins. On the basis of the striking conservation of primary structure between the Amphiuma and human NHE1 isoforms and the high levels of NHE expression in the Amphiuma RBCs, we conclude that the Amphiuma NHE is a useful model in which to study the biochemical and molecular details of mammalian NHE1 volume and pH-dependent regulation.

**MATERIALS AND METHODS**

RNA isolation. Total intracellular RNA was isolated from Amphiuma RBCs, heart, lung, liver, and kidney using a...
modification of the one-step procedure of Chomczynski and Sacchi (14). Very high RNase activities in Amphiuma RBCs necessitated use of a lysis buffer that contained 14 M guanidinium and urea (Ultraspec, Biotecx Laboratories). RBCs were separated from plasma using low-speed centrifugation (1,000 g) and washed twice in ice-cold 120 mM NaCl before addition of lysis buffer, while other Amphiuma tissues were homogenized with a polytron. Poly(A)$^+$ RNA was isolated from total cellular RNA following two successive enrichments using oligo(dT)-cellulose (39).

RNA blot analysis. Poly(A)$^+$ RNA (5 µg) from each Amphiuma tissue was size fractionated on a 1.2% agarose-2.2 M formaldehyde gel with RNA markers ranging from 0.24 to 9.5 kb (Bethesda Research Lab). Fractionated poly(A)$^+$ RNA was transferred to a nylon membrane by capillary action and cross-linked to the membrane by ultraviolet light (Stratalinker 1800, Stratagene). Membranes were prehybridized in 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaHPO$_4$, and 0.001 M EDTA, pH 7.4), 2× Denhardt’s solution, and 0.1% SDS for 6 h at 42 °C; hybridized for 16 h with radiolabeled cDNA probes (21), and washed in 0.1% SDS and 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at 60 °C. Radioactive bands on the RNA blots were digitized using a PhosphorImager with ImageQuant software (Molecular Dynamics). The scanned autoradiographs were imported directly from ImageQuant into Adobe Photoshop without altering the relative intensities of the radioactive bands (29).

Amphiuma NHE cDNAs were amplified by PCR to generate a gene-specific cDNA library. Poly(A)$^+$ RNA (2 µg) from Amphiuma RBCs was annealed with an oligo(dT)18 primer, and first-strand cDNA was transcribed using Moloney murine leukemia viral RT (Superscript II, BRL). Resultant cDNA-RNA hybrids were incubated with RNaseH and diluted 10-fold for subsequent PCR. A partial NHE cDNA (clone I) was amplified by PCR using primers that contained sequences conserved in human, pig, and trout NHE1 cDNA (Fig. 1, Table 1). Two additional contiguous cDNA regions (clones II and III) were successively generated by PCR to provide the 3′ untranslated nucleotide sequence necessary for a primer-specific NHE cDNA library (clone IV).

Nested PCR conditions for clone I (1077 bp) and clone II (772 bp). The first-strand cDNA templates were amplified in the presence of primers (100 nM), 1 mM Tris·HCl, 0.01% Triton X-100, 5 mM KCl, 150 mM MgCl$_2$, 20 nM dNTP, and 7.5 units of Taq DNA polymerase (Promega). The reaction mixture was denatured at 95 °C for 3 min, followed by two cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 2 min. The double-stranded DNA was then amplified with the initial primer pair for 20 cycles at 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 2 min, followed by a final 10-min extension at 72 °C. This PCR reaction was diluted 1:25 (vol/vol) and amplified with a nested, forward primer and the same reverse primer (Fig. 1, Table 1).

Clone III was generated by 3′ cDNA extension of clone II. Rapid amplification of cDNA ends (3′ RACE) was accomplished by ligating adapters AP1 and AP2 (Marathon cDNA amplification kit, Clontech) to clone II cDNA (Fig. 1). Two gene-specific, nested forward primers, Fd5 and Fd6, were derived from the clone II cDNA sequence (Table 1). The RACE-PCR contained primers (0.2 µM each), 0.2 mM dNTP, 25 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; pH 9.3 at 25 °C), 50 mM KCl, 2 mM MgCl$_2$, 1 mM 2-mercaptoethanol, and 2.5 units of TakaRa-Ex-taq DNA polymerase (Oncor). The initial PCR amplification using Fd5 and AP2 was as follows: 1 cycle at 94 °C for 2 min; 5 cycles at 94 °C for 30 s and 72 °C for 4 min; 5 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 4 min; 15 cycles at 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 4 min; and a final cycle of 72 °C for 10 min. The PCR amplification using nested primers Fd6 and AP1 was as follows: 1 cycle at 94 °C for 2 min; 25 cycles at 94 °C for

![Fig. 1. Cloning strategy for isolation of Amphiuma tridactylum Na$^+$/H$^+$ exchanger isoform 1 (atNHE1) cDNA. Open reading frames (open bars) and untranslated regions (UTR; solid bars) are depicted based on transcript size. Predicted transmembrane domains (shaded blocks) are labeled and correspond with other NHEs. Clones I and II were obtained by RT-PCR of poly(A)$^+$ RNA, and clone III was obtained from 3′ extension of cDNA of clone II (3′ RACE). Nucleotide sequence of clone III was used for generation of a sequence-specific, primed cDNA library, from which clone IV was isolated. Primers used for RT-PCR are indicated next to arrows. Primer D5 was used to construct gene-specific cDNA library.](http://ajpcell.physiology.org/)
specific cDNA library was constructed with 5 µg of poly(A) RNA from Amphiuma for 10 min. 30 s, 65°C for 30 s, and 72°C for 4 min; and a final cycle of 72°C for 10 min.

Generation of an NHE-specific cDNA library. A primer-specific cDNA library was constructed with 5 µg of poly(A)+ RNA from Amphiuma RBCs by using the D5 oligomer (Table 1, Fig. 1). First-strand cDNA synthesis utilized RNaseH digestion, with the second strand synthesized by Escherichia coli T4 DNA polymerase (Superscript Choice System, GIBCO BRL). Double-stranded cDNA was ligated to precut script Choice System, GIBCO BRL. Double-stranded cDNA was synthesized by RT followed by RNaseH digestion, with the second strand being added to the 5′ end of the first strand. The second strand was then ligated to pre-cuts of the D5 oligomer (Table 1, Fig. 1). First-strand cDNA synthesis utilized RNaseH digestion, with the second strand synthesized by Escherichia coli T4 DNA polymerase (Superscript Choice System, GIBCO BRL). Double-stranded cDNA was ligated to precut script Choice System, GIBCO BRL. Double-stranded cDNA was synthesized by RT followed by RNaseH digestion, with the second strand being added to the 5′ end of the first strand. The second strand was then ligated to pre-cuts of the D5 oligomer (Table 1, Fig. 1).

Table 1. Primers used in generation of Amphiuma NHE cDNA

<table>
<thead>
<tr>
<th>5′ Primers</th>
<th>NHE cDNA</th>
<th>3′ Primers</th>
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<tr>
<td>Clone I (nested PCR)</td>
<td>Fd1 (5′-GGATTGTGAGTACGAGGAGAACCA-3′)</td>
<td>D1 (5′-ACCAGCTCGATGGCTGCTTCATGTCAT-3′)</td>
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<tr>
<td>Clone II (nested PCR)</td>
<td>Fd2 (5′-TTCCGAGCATGACTCTGGCCGGAGAC-3′)</td>
<td>D2 (5′-CTCAGTACCGGCGCCACacaACG-3′)</td>
</tr>
<tr>
<td>Clone III (3′ RACE)</td>
<td>Fd4 (5′-CATGATGCATCCAGGAGAC-3′)</td>
<td>AP1 (Marathon, Clontech)</td>
</tr>
<tr>
<td>Clone IV (primed cDNA library)</td>
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<td>AP2 (Marathon, Clontech)</td>
</tr>
<tr>
<td>Untagged</td>
<td>D6 (5′-GGATTGTGAGTACGAGGAGAACCA-3′)</td>
<td>D5 (5′-CTCAGTACCGGCGCCACacaACG-3′)</td>
</tr>
<tr>
<td>Amino-terminal cMyc</td>
<td>dMyc (5′-CGGATCCGCTGAGAGAAGAGCTTCATCCAGAAGAC-3′)</td>
<td>T3 primer of plasmid pBK-CMV (5′-ATTACCCCTCACATAAGGA-3′)</td>
</tr>
<tr>
<td>Carboxy-terminal FLAG</td>
<td>dMyc (5′-CGGATCCGCTGAGAGAAGAGCTTCATCCAGAAGAC-3′)</td>
<td>T3 primer of plasmid pBK-CMV (5′-ATTACCCCTCACATAAGGA-3′)</td>
</tr>
<tr>
<td>Epitopically labeled cDNAs for PCR modification of clone IV</td>
<td>FLAG (5′-GGCTGCAGAGAGTCCATCCTGCTGCTGCTCGTCTGTCG-3′)</td>
<td>FLAG (5′-GGCTGCAGAGAGTCCATCCTGCTGCTGCTCGTCTGTCG-3′)</td>
</tr>
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NHE, Na+/H+ exchanger.

30 s, 65°C for 30 s, and 72°C for 4 min; and a final cycle of 72°C for 10 min.

Generation of an NHE-specific cDNA library. A primer-specific cDNA library was constructed with 5 µg of poly(A)+ RNA from Amphiuma RBCs by using the D5 oligomer (Table 1, Fig. 1). First-strand cDNA synthesis utilized RNaseH RT followed by RNaseH digestion, with the second strand synthesized by Escherichia coli T4 DNA polymerase (Superscript Choice System, GIBCO BRL). Double-stranded cDNA was ligated to precut EcoRI adapters, purified by column fractionation, and cloned into an EcoRI-digested ZAP Express vector (Stratagene). Approximately 8 × 10⁶ plaques were identified and cloned into the plasmid pBK-CMV phagemid vector (Stratagene). These clones were verified to be overlapping by bidirectional dideoxynucleotide sequencing using an automated, fluorescent dideoxynucleotide sequencer (Applied Biosystem, Gene Sequencing Lab, University of California, Davis, CA).

Clone IV consisted of a 3160-bp insert that contained the entire Amphiuma triactylum NHE isoform 1 (atNHE1) open reading frame flanked by 5′ and 3′ untranslated regions (UTR).

Construction of NHE expression clones. Expression constructs from clone IV used PCR to introduce nucleotide sequences that encoded cMyc and FLAG epitopes at the amino and carboxy termini, respectively. The cMyc-atNHE1 construct replaced the first three codons of atNHE1 with nucleotides encoding the cMyc epitope (Met-Glu-Glu-Lys-Leu). The cMyc-atNHE1-FLAG construct inserted the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Lys) followed by a termination codon at the end of the cMyc epitope coding region (bp 1501–2415 of atNHE1) with a BsgI/XhoI fragment of atNHE1. The hNHE1 cDNA was cloned from a human astrocytoma cell line, and its identity was verified by double-stranded, automated nucleotide sequencing as was done for the atNHE1 and chimeric NHE constructs.

Fig. 2. Construction of chimeric NHEs. Two Amphiuma-human chimeras were constructed by interchanging amino-terminal (including all putative transmembrane domains) and carboxy-terminal domains of atNHE1 and human NHE1 (hNHE1). A native BsgI restriction endonuclease site is located immediately following 12th transmembrane segment in both atNHE1 (bp 1525) and hNHE1 (bp 1501) sequences. atNhC (Amphiuma amino terminus, human carboxy terminus) chimera was constructed by replacement of nucleotide coding region 1525–2469 of atNHE1 with a BsgI/XhoI fragment of hNHE1 that was generated by partial digestion. Similarly, the hNatC (human amino terminus, Amphiuma carboxy terminus) chimera was similarly constructed by replacement of nucleotide coding region 1501–2415 of hNHE1 with corresponding BsgI/XhoI fragment from atNHE1. AA, amino acids.
Nucleotide sequence analysis. Analyses and comparisons of nucleic acid sequences were carried out using the Wisconsin package Genetic Computer Group (GCG) software. Alignments of multiple protein sequences were performed using CLUSTAL W (version 3.0, NCSA, University of Illinois). Phylogenetic trees were derived from the aligned sequences using the protein sequence parsimony method (PROTPARS, PHYLIP, version 3.5c, NCSA, University of Illinois) (22) and by the neighbor-joining method of Saitou and Nei (38). Distance matrices were bootstrapped 1,000 times by sampling sites at random with replacement. The resultant distance matrices were used to revise the phylogenetic relationships and obtain confidence values (PAUP program).

Cell culture. The mutant Chinese hamster ovary (CHO) cell line AP-1, which lacks endogenous Na+/H+ exchange activity (37), was used for stable transfection of plasmids containing Amphiuma, human, and chimeric NHE cDNA. Cells were maintained in complete MEM (Cellgro) supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Cellgro) at 37°C, 95% humidity, and 10% fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Cellgro). All solutions were nominally HCO3- free to exclude the effects of HCO3- dependent mechanisms on pH recovery.

Preparation of cell extracts and immunoblot analysis. Membrane-enriched fractions utilized for epitope determination were prepared by washing confluent cells twice with a solution containing PBS (145 mM NaCl, 20 mM KH2PO4, adjusted to pH 7.45 with KOH), 2 mM EDTA, and 4% (vol/vol) Triton X-100. The membrane-enriched pellet was solubilized in 200 µl of sample buffer (1% NP-40, 1 mM Na3PO4, 100 mM phenylmethylsulfonyl fluoride, aprotinin, 4-amidino-7-chloromethyl ketone, 100 µM Nα-tosyl-L-lysine chloromethyl ketone, 100 µM N-tosyl-L-phenylalanine chloromethyl ketone, and 0.1–1 µg/ml leupeptin, antipain, bestatin, chymostatin, pepstatin A, phosphoramidon, 4-(aminophenyl)fluoride, 4-amidino-phenylmethanesulfonyl fluoride, and benzamidine; Sigma). The cells were gently scraped in 1–2 ml of rinse solution, pelleted at 6,000 g for 10 min at 4°C, resuspended in 1 ml of rinse solution, and then lysed by sonication. Large cytosolic debris was pelleted by centrifugation at 12,000 g for 10 min, and the supernatant was subjected to 150,000 g for 45 min at 4°C. The membrane-enriched pellet was solubilized in 200 µl of 50 mM NaCl, 20 mM Tris, and 1% SDS, plus the aforementioned protease inhibitors. Whole cell lysates were prepared from confluent cells harvested by gentle pipetting, pelleted by centrifugation at 4°C, and lysed directly in sample buffer (1% SDS, 6 M urea, 72 mM Na2HPO4, 25 mM NaH2PO4, 0.015% wt/vol bromphenol blue, and 2% vol/vol 2-mercaptoethanol). Total protein concentrations were determined using a bicinchoninic acid assay system with BSA as a standard (Pierce).

Protein homogenates were size fractionated through 7.5% SDS-PAGE, and electroblotted to polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore). Immunoblots were incubated in blocking buffer (3% dry milk, PBS, and 0.5% Tween-20) for 1 h before incubation with the primary monoclonal antibodies anti-NHE1 (MAb 4E9), anti-cMyc, or anti-FLAG (Eastman-Kodak) for 1 h at 22°C. After several washes with PBS plus 0.5% Tween-20, immunoblots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Zymed) for 1 h at 22°C. Blots were developed by enhanced chemiluminescence detection (SuperSignal, Pierce). Before blotting with subsequent primary antibodies, blots were incubated in stripping buffer (62.5 mM Tris at pH 6.8, 2% SDS, and 0.7% 2-mercaptoethanol) at 65°C for 30 min.

Measurement of pHi. Cells on coverslips were loaded for 30 min with 1 µM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM (Molecular Probes) in HEPES-buffered Ringer (HR) at 37°C, in the absence of HCO3- and CO2. HR was composed of (in mM) 130 NaCl, 3 KCl, 20 HEPES, 1 MgCl2, 0.5 CaCl2, 10 glucose, and 10 NaOH, adjusted to pH 7.4 at 37°C with NaOH or HCl. Coverslips were washed in HR three times, incubated in HR for an additional 30 min at 37°C and 0% CO2, and then transferred to polystyrene cuvettes that permitted continuous perfusion of solution. Cells were maintained at 37°C in the spectrophotometer (F-2000,Hitachi Instruments), and BCECF fluorescence was measured at an emission wavelength of 535 nm, using optimized excitation wavelengths of 507 and 440 nm. Cells were acidified by perfusion with 10 mM NH4Cl-HR (in mM: 125 NaCl, 3 KCl, 20 HEPES, 1 MgCl2, 0.5 CaCl2, 10 glucose, 10 NaOH, and 10 mM NH4Cl) for 5 min, followed by washout in NH4Cl-free and Na+-free media for 5 min. The impermeant cation N-methyl-2-glucamine (NMDG) was used to substitute for Na+ in the Na+-free solution (NMDG-HR), which was prepared by using NMDG-Cl and NMDG-OH in place of NaCl and NaOH, respectively. Cells were then perfused with HR or HR+amiloride (Sigma) or HOE-694 (Hoechst), and the recovery rate (ΔpH/Δt) was determined during the initial linear portion of recovery from the acid load. Amiloride was dissolved in DMSO and HOE-694 was dissolved in distilled H2O to stock concentrations of 500 mM and 20 µM, respectively, and both were diluted to final concentrations in HR as indicated.

Intrinsic buffering capacity (mM H+/ΔpH) was determined over the range of pH studied by perfusing cells with progressively decreasing concentrations of NH4+-HR (10, 4, 2, 1, 0.5, and 0 mM NH4Cl) (6). Calibration of the fluorescence ratios was performed using high-K+ solutions of known extracellular pH (in mM: 30 KCl, 110 potassium gluconate, 10 HEPES, 1 MgCl2, 0.5 CaCl2, and 10 glucose, adjusted to pH values of 6.2, 6.6, 7.0, 7.4, and 7.8 at 37°C using NMDG-OH or HCl) in conjunction with 5 µM nigericin (Sigma), as described by Boyarsky et al. (6). All solutions were nominally HCO3-free to exclude the effects of HCO3-dependent mechanisms on pH recovery.

RESULTS

Cloning Amphiuma NHE cDNA. An initial NHE cDNA probe was amplified from Amphiuma RBC poly(A)+ RNA using oligonucleotide primers that correspond to highly conserved nucleotide sequences in the human and pig NHE1 and trout βNHE isoforms (clone I; Fig. 1, Table 1). Nucleotide sequencing of clone I demonstrated 74 and 60% nucleotide identity with the nNHE1 (40) and trout βNHE (5) isoforms, respectively. Radiolabeled clone I was hybridized with an RNA blot containing poly(A)+ RNA from Amphiuma tissues, and a transcript of ~7 kb was detected (Fig. 3). The open reading frames of other cloned NHE cDNAs are encompassed by ~2.5 kb, so that the putative high-molecular-mass transcript of Amphiuma NHE indicated the likelihood of large flanking 5′ and 3′ UTR. This large transcript size suggested that an Amphiuma cDNA...
library constructed from the polyadenylated 3' terminus might not extend sufficiently upstream to contain the nucleotide sequence complementary to clone I (Fig. 1). Accordingly, nested primers and polymerase chain amplification were used to extend 3' from the nucleotide sequence of clone I to encompass the entire coding region of the NHE carboxy terminus (clones II and III; Fig. 1).

A gene-specific, unamplified cDNA library was then synthesized using a primer (D5) corresponding to the proximal 3' UTR of clone III. The longest clone (clone IV) isolated from the cDNA library was 3160 bp and was demonstrated by nucleotide sequencing to contain the entire open reading frame. The nucleotide and predicted amino acid sequences of clone IV are depicted in Fig. 4. Successive rehybridization of the RNA blot with radiolabeled cDNA probes corresponding to the 3' UTR, the carboxy-terminal region, and a transmembrane region confirmed the presence of 6.8- to 7.1-kb NHE transcripts in Amphiuma RBCs, heart, lung, liver, and kidney (Fig. 3).

Comparisons of atNHE1 with other NHE sequences. The Amphiuma exchanger exhibits 79% identity and 89% similarity with the hNHE1 isoform at the amino acid level (Table 2, see also Fig. 6). The atNHE1 protein is 80% identical to the other cloned amphibian NHE from Xenopus laevis oocyte (XI-NHE) (7) and 63% identical to the βNHE (5) isoform isolated from trout RBCs (Table 2). The Amphiuma NHE is more similar to the NHE1 isoforms than to the other mammalian isoforms (NHE2–NHE5), as shown by the phylogenetic tree of aligned, full-length sequences (Fig. 5). The atNHE1 and hNHE1 amino acid sequences (Fig. 6) retain a high degree of homology throughout the putative membrane-spanning domains M2–M12 (87% amino acid identity), as well as the proximal 200 amino acids of the carboxy terminus (90% identity). The largest divergence in sequence homology exists at the initial, amino-terminal 90 amino acids that include the putative M1 transmembrane domain (17% amino acid identity), which may be cleaved as a signal peptide, and at the last carboxy-terminal 100 amino acids (52% identity).

Expression of Amphiuma NHE in AP-1 cells. The epitope tags cMyc and FLAG were respectively ligated to the amino and carboxy termini of the atNHE1 cDNA to investigate the potential posttranslational processing of the Amphiuma NHE protein. AP-1 cells were stably transfected to express the following atNHE1 cDNA constructs: atNHE1, cMyc-atNHE1, and cMyc-atNHE1-FLAG. Whole cell lysates of the transfected cells were screened for expression with an anti-NHE1 antibody (MAb 4E9) on immunoblots. Multiple clones, with varying levels of expression, were found to express atNHE1 for each of the three cDNA constructs. The retention of the cMyc and FLAG epitope tags was evaluated using a membrane-enriched protein fraction that was isolated from nontransfected cells and each of the transfected AP-1 cell lines. The proteins were size-fractionated by SDS-PAGE, electrotransferred to PVDF membrane, and successively probed with monoclonal antibodies to NHE1, cMyc, and FLAG (Fig. 7). Immunoblots probed with the anti-NHE1 antibody (Fig. 7A) show that atNHE1 is expressed in each of the transfected AP-1 cell lines, with bands of comparable size shown for each of the three constructs (atNHE1, cMyc-atNHE1, and cMyc-atNHE1-FLAG). The anti-NHE1 MAb detects two bands that correspond with the unmodified protein with a predicted molecular mass of 90.6 kDa and a posttranslationally modified protein at ~110–115 kDa (Fig. 7A). There are two potential N-glycosylation sites for atNHE1 in the first amino-terminal extracellular loop (Asn-80 and Asn-84), compared with a single site that is glycosylated at Asn-75 in hNHE1 (17) (Fig. 6), and preliminary studies from our laboratory indicate that atNHE1 is also N-glycosylated (unpublished observations). Both the cMyc-atNHE1 and cMyc-atNHE1-FLAG transfectedants expressed high levels of NHE protein, although expression was still less than that seen in native Amphiuma RBCs (Fig. 7A, lane 7). The high abundance of NHE protein in the Amphiuma RBCs is also demonstrated by comparison with a nontransfected human astrocytoma cell line (Fig. 7A, lane 8) that expresses endogenous NHE1 protein.

The immunoblot in Fig. 7A was stripped and reprobed with an anti-cMyc antibody (Fig. 7B), using a membrane-enriched fraction from the KCC2-1CT cell line (lane 6) that expressed a cMyc-tagged K⁺-Cl⁻ cotransporter for a positive control. Neither of the epitope-tagged AP-1 transfectedants expressed a detectable cMyc epitope (Fig. 7B), but a strong signal was detected from the expressed cMyc-tagged K⁺-Cl⁻ cotransporter protein. Prolonged exposure of this immunoblot beyond the linear range of enhanced chemiluminescence detection did not reveal any additional signal from the AP-1 transfectedants (data not shown). These data suggest that a region of the amino terminus containing the cMyc epitope tag is cleaved from the mature protein, consistent with the hypothesis that part of the amino terminus functions as a signal peptide sequence (17). Reprobing of the same blot with the anti-FLAG antibody demonstrated retention of the carboxy-terminal epitope in the AP-1 transfectedants (Fig. 7C). There was some nonspecific binding of the anti-FLAG antibody to other AP-1 cell proteins, but the
CLONING AND EXPRESSION OF AMPHIUMA NHE
Table 2. Sequence comparisons between Amphiuma NHE and other NHE proteins

<table>
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<th>NHE Isoform</th>
<th>% Sequence Identity</th>
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atNHE1, Amphiuma tridactylum NHE1; hNHE1, human NHE1; XI-NHE, Xenopus laevis NHE1; nNHE1–NHE4, rat NHE1–NHE4.

unique NHE proteins detected by the anti-NHE1 antibody were also clearly observed using the anti-FLAG antibody (lane 3, Fig. 7C).

Expression of hNHE1 and chimeric NHEs in AP-1 cells. cDNA encoding the full-length sequence of hNHE1 and two chimeric Amphiuma-human exchangers (atNhc and hNatc) were stably transfected into AP-1 cells. The atNhC chimera consists of the amino-terminal domain of the Amphiuma exchanger, including the entire transmembrane-spanning region M1–M12, and the carboxy-terminal domain of the hNHE1 protein, whereas the hNatC contains the amino-terminal and transmembrane domains from human and the carboxy-terminal region of Amphiuma (see Fig. 2). As was observed with the atNHE1 constructs, multiple clones for each cDNA construct were found to express an NHE1 protein when probed by immunoblot analysis. Several clones from each cDNA construct were tested for function by monitoring recovery of pH, following acidification using the fluorescent ratio dye BCECF, and all these clones exhibited significant NHE activity. A single clone from each of the NHE-transfected AP-1 cells was selected for more detailed analysis.

Functional characterization of Amphiuma, human, and chimeric NHE transfectants. Na+/H+ exchange activity was evaluated by measuring the maximal ΔpH/Δt following an acid load, as described in Measurement of pH,. Cells were acidified to a mean pH, of 6.95 ± 0.06, with no significant difference between the various cell lines. The rate of H+ efflux (mM H+/min) was calculated by multiplying the rate of recovery by the intrinsic buffering capacity (mM H+/ΔpH), which was empirically derived from separate experiments. The buffer capacity was linearly dependent on pH, with a similar trend for both the transfected and nontransfected AP-1 cells (data not shown).

The typical recovery from intracellular acidification, under nominally HCO3-free conditions for transfected and control AP-1 cells is shown in Fig. 8. Nontransfected AP-1 cells (Fig. 8A) exhibited modest Na+-

Table 2.

Fig. 4. Nucleotide sequence of atNHE1 cDNA and deduced amino acid sequence of protein. Nucleotides are numbered at right of sequence relative to putative translation initiation site. Amino acids are numbered at left of sequence and are shown by their single-letter abbreviations. Presumptive TATA boxes are underlined. *In-frame stop codon.
cells was 24 µM, nearly an order of magnitude higher than that reported elsewhere. This disparity, however, primarily reflects the different experimental conditions between our studies (external Na\(^+\) concentration 140 mM) and those of others (external Na\(^+\) concentration ~0 mM). Using the \(^{22}\)Na uptake protocol of Orlowski and Kandasamy (34), in nominally Na\(^+\)-free medium, we measured amiloride IC\(_{50}\) values of 4.7 ± 0.5 µM for hNHE1-transfected AP-1 cells and 1.3 ± 0.2 µM for atNHE1-transfected AP-1 cells (data not shown).

Recent studies have shown that HOE-694 is more selective and more potent than amiloride for inhibiting the NHE1 isoform (18, 34). Counillon and co-workers (18) reported an IC\(_{50}\) of 0.16 µM when using HOE-694 to inhibit PS120 cells that were stably transfected with hNHE1, compared with an IC\(_{50}\) of 3 µM for amiloride. In the present study, HOE-694 inhibited activity of the hNHE1 transfectant to a greater degree than amiloride, with a calculated IC\(_{50}\) of 3.200 µM for atNHE1-transfected AP-1 cells. Due to precipitation of HOE-694 from the HR solution at concentrations greater than 200 µM, we were not able to obtain a full dose response curve for this compound. As with the atNHE1-transfected AP-1 cells, HOE-694 sensitivity of native atNHE1 in intact Amphiuma RBCs was also minimal (data not shown).

DISCUSSION

Our laboratory has previously shown that the NHE expressed in the RBCs of the giant salamander, A. tridactylum, is especially active in both pH and volume regulation (9–11). We have measured amiloride-sensitive Na\(^+\) uptake rates of >30 mM·min\(^{-1}\)·kg dry cell solid\(^{-1}\) in Amphiuma RBCs maximally stimulated by the addition of the phosphatase inhibitor calyculin A (1 µM; unpublished observations). Consistent with the high level of functional NHE activity, these RBCs express exceptionally large quantities of the NHE protein (Fig. 7A). In this study, the Amphiuma NHE (atNHE1) was cloned and sequenced to ascertain its similarity to the mammalian NHE1 isoforms and to provide the foundation for developing sequence-specific antibodies for use in studies of atNHE1 control. When the kinetic, functional, and structural similarities between atNHE1 and mammalian NHE1 are considered, together with the low tonic activity of the Amphiuma NHE in Amphiuma RBCs, the Amphiuma NHE is a valuable model for studying the control of pH and volume regulation by mammalian NHE1.
KCC2-1CT cell line overexpresses a cMyc epitope-tagged K
immunoblot was stripped and reprobed with an anti-cMyc antibody.
to somewhat lower levels of expression relative to other constructs.
transfected AP-1 cells) is shown with an increased exposure time due
expressing endogenous NHE1 (U251 astrocytoma; Amphiuma
comparison, 50 µg of membrane-enriched protein from wild-type
lanes 5 and 6), cMyc-atNHE1 transfected AP-1 clone A4 (lane 3), and untagged
untransfected AP-1 clone A4 (lane 4). Positive controls for cMyc
antibodies were run in lanes 5 and 6, respectively. For
untagged atNHE1 and from nontransfected mammalian cells
(NHE1 monoclonal antibody (MAb 4E9). A
on a separate immunoblot.
KCC2-1CT cell line expresses endogenous NHE1 (U251 astrocytoma; lane 8) were run
on a separate immunoblot. A: Immunoblotts were probed with anti-
NHE1 monoclonal antibody (MAb 4E9). Lane 4 (untagged atNHE1-
transfected AP-1 cells) is shown with an increased exposure time due to
somewhat lower levels of expression relative to other constructs. B:
immunoblot was stripped and reprobed with an anti-cMyc antibody.
KCC2-1CT cell line overexpresses a cMyc epitope-tagged K+−Cl−
cotransporter, and a membrane-enriched protein fraction (1 µg) was
included as a positive control (lane 6). C: same blot was restripped
and reprobed with an anti-FLAG antibody, with a bacterial alkaline
phosphatase-FLAG fusion protein (0.5 µg) serving as a positive
control (lane 5). Marker sizes are in kDa.

Patterns of transcript and protein expression. In
Amphiuma, the atNHE1 isoform isolated from the
RBCs was detected in all other tissues examined,
including heart, lung, kidney, and liver (Fig. 3). The
transcript size for atNHE1 (~7 kb) is larger than those
reported for the human and rat NHE1 isoforms, 5.1 kb
(23) and 4.8 kb (35), respectively. The transcript size of
the recently cloned Xl-NHE isoform from
X. laevis oocytes (7) has not been described, but a recent report
describes a 6-kb mRNA for a reptilian NHE homologue
(23). Our cloning strategy was directed toward obtaining
and expressing the coding region of the cDNA, and
therefore we did not sequence the entire 7-kb transcript
once the complete open reading frame was identified.
However, 3′ RACE did indicate that the 3′ UTR exceeded 1.1 kb. The significance of these large UTRs for
some NHE transcripts is unknown, but regions within the 3′ UTR have been shown to regulate transcript
stability and intracellular localization in some genes.

Fig. 7. Immunoblot analysis of atNHE1 expressed in AP-1 cells.
Crude membrane extracts (~100 µg) from each of following cell
extracts was resolved by 7.5% SDS-PAGE: nontransfected AP-1 cells
(lane 1), cMyc-atNHE1 transfected AP-1 clone G6 (lane 2), cMyc-
atNHE1-FLAG transfected AP-1 clone C5 (lane 3), and untagged
atNHE1 transfected AP-1 clone A4 (lane 4). Positive controls for cMyc
and FLAG antibodies were run in lanes 5 and 6, respectively. For
comparison, 50 µg of membrane-enriched protein from wild-type
Amphiuma RBCs (lane 7) and from nontransfected mammalian cells
expressing endogenous NHE1 (U251 astrocytoma; lane 8) were run
on a separate immunoblot. A: Immunoblotts were probed with anti-
NHE1 monoclonal antibody (MAb 4E9). Lane 4 (untagged atNHE1-
transfected AP-1 cells) is shown with an increased exposure time due to
somewhat lower levels of expression relative to other constructs. B:
immunoblot was stripped and reprobed with an anti-cMyc antibody.
KCC2-1CT cell line overexpresses a cMyc epitope-tagged K+−Cl−
cotransporter, and a membrane-enriched protein fraction (1 µg) was
included as a positive control (lane 6). C: same blot was restripped
and reprobed with an anti-FLAG antibody, with a bacterial alkaline
phosphatase-FLAG fusion protein (0.5 µg) serving as a positive
control (lane 5). Marker sizes are in kDa.

Table 3. Comparison of maximum $J_{\text{H}+}$ and inhibition
constants for transfected and nontransfected AP-1 cells
following acidification

<table>
<thead>
<tr>
<th></th>
<th>Maximum $J_{\text{H}+}$, mmol/min</th>
<th>IC50, µM</th>
<th>Amiloride</th>
<th>HOE-694</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>0.12 ± 0.01</td>
<td>7</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>atNHE1</td>
<td>6.58 ± 0.60</td>
<td>21</td>
<td>5.6</td>
<td>20.4</td>
</tr>
<tr>
<td>atNhC</td>
<td>3.25 ± 0.33</td>
<td>15</td>
<td>26.2</td>
<td>298</td>
</tr>
<tr>
<td>hNatC</td>
<td>5.09 ± 0.76</td>
<td>7</td>
<td>6.1</td>
<td>242</td>
</tr>
<tr>
<td>hNHE1</td>
<td>3.95 ± 0.35</td>
<td>24</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Values for $H^+$ flux rate ($J_{\text{H}+}$) are means ± SE; n = no. of
experiments. Values for IC50 were determined by fitting sigmoidal
inhibition data presented in Fig. 9 by equation
$y = J_{\text{max}} - (J_{\text{max}} - J_{\min})/\left(1 + IC50/x\right)$,
where $y$ is %inhibition, maximal flux ($J_{\text{max}}$) = 100%, minimal flux ($J_{\min}$) = 0%, and $x$ is concentration of
inhibitor. Cells were loaded with BCECF and acidified by 10 mM
$NH_4^+$ prepulse as described in MATERIALS AND METHODS.
Cells were acidified to an average intracellular pH (pHi) of 6.95, with no
significant difference in pHi between the various cell lines. Maximum
rate of $H^+$ efflux during recovery from acid load was determined by
multiplying $J_{\text{H}+}$ at by the intrinsic buffering capacity (mM/ΔpHi),
which was determined separately. Average flux rate for nontransfected
AP-1 cells was subtracted from that for each of transfected cells
correct for basal alkalinization in response to acid load. atNhC,
chimeric transporter with atNHE1 amino terminus and hNHE1
carboxy terminus; hNatC, chimeric transporter with hNHE1 amino
terminus and atNHE1 carboxy terminus.
The open reading frame of the atNHE1 transcript translates to a protein consisting of 813 amino acids with a predicted core molecular mass of 90.6 kDa (Fig. 4). Kyte and Doolittle hydropathy plots predict an approximately 500-amino acid amino-terminal region that contains 12 putative transmembrane domains followed by a large carboxy-terminal, cytoplasmic tail of ~300 amino acids (Fig. 6) (28). This secondary structure prediction is consistent with that predicted for the other members of the NHE supergene family (50, 52). The homologies between the Amphiuma, mammalian, and Xenopus NHE1 proteins exceed those observed between atNHE1 and the other mammalian isoforms NHE2–NHE5 (Table 2). Figure 5 depicts the phylogenetic relationship between atNHE1 and 12 other NHE proteins. Multiple protein alignments using CLUSTAL W were separately determined for the full-length NHE sequences, the conserved M2–M12 transmembrane regions, and the cytoplasmic carboxy-terminal regions. The phylogenetic relationships among the NHE proteins were the same for these comparisons and reflect the sequence conservation throughout the entire NHE sequence. These analyses also indicate that the amphibian NHE proteins, notably atNHE1, are likely to be progenitors of the mammalian NHE1.

Structure and function comparisons. The putative transmembrane domains M2–M12 are highly conserved among all of the various NHE isoforms, with the greatest divergence seen in membrane-spanning region M1 (50). Although this M1 domain is conserved among the different mammalian NHE1 isoforms, comparison of atNHE1 with hNHE1 reveals only 19% identity in this region. It has been proposed that this first putative membrane-spanning domain may function as a signal peptide that is cleaved from the membrane-associated NHE protein (17). As predicted for other NHE isoforms, the atNHE1 contains a potential peptide cleavage sequence immediately following the M1 transmembrane domain (47). In this study, AP-1 cells were transfected with atNHE1 cDNA constructs that contained an amino-terminal cMyc epitope tag (cMyc-atNHE1 and cMyc-atNHE1-FLAG). These transfected cells expressed functional atNHE1 protein, with an approximate molecular mass of 110–115 kDa, that was recognized by a monoclonal anti-NHE1 antibody (MAb 4E9; Fig. 7A) but not recognized by a monoclonal anti-cMyc antibody (Fig. 7B). Conceivably, amino-terminal glycosylation could interfere with recognition of the cMyc epitope, thus resulting in lack of signal at 110–115 kDa. However, in addition to the 110- to 115-kDa glycosylated protein, the anti-NHE1 antibody also recognized an 85- to 90-kDa unglycosylated core protein (Fig. 7A). The failure of the cMyc antibody to detect this core atNHE1 protein supports the signal peptide cleavage theory and indicates that the cMyc epitope is cleaved before glycosylation. The fact that the mature atNHE1 protein is glycosylated implies that the proposed signal peptide would have to be cleaved proximal to amino acid 80 or 84, the two potential N-glycosylation sites present in atNHE1 (Fig. 6).

The membrane-spanning regions M2–M12 share 95–100% homology between atNHE1 and hNHE1, with four of the transmembrane domains being identical (Fig. 6). The proposed ion transport region of the exchanger, spanning transmembrane domains M6 and M7 (also referred to as M5a and M5b), is highly conserved among all NHE isoforms (20, 31, 50) and is identical for the atNHE1 and hNHE1 isoforms with the exception of a single amino acid (Ser-232 of hNHE1 replaced by Ala in atNHE1). The putative amiloride binding site in transmembrane domain M4 for atNHE1 and hNHE1 also differs by only one amino acid (Val-160 of hNHE1 replaced by Thr in atNHE1) (15, 50, 51).

The carboxy-terminal domain of atNHE1, like the amino terminus (from M2 through M12), maintains a high degree of homology with hNHE1 and is 90% identical over the proximal 200 amino acids (Fig. 6), with the greatest similarity in regions of the carboxy terminus believed to regulate NHE activity. For example, the high-affinity Ca2+/calmodulin binding region [amino acids 636–656, hNHE1 (2)] is identical in atNHE1 and hNHE1, with the exception of Thr-645 (atNHE1) being replaced by an asparagine in the hNHE1 protein (Asn-637). The region between amino acids 567–635 (hNHE1) is believed to interact with the H+–sensing region of the transmembrane domain and to be crucial for stimulation by phorbol esters, thrombin, platelet-derived growth factor, and okadaic acid.
A comparison between hNHE1 and atNHE1 reveals only two dissimilar amino acids over this region (Leu-614 and Asp-626 of hNHE1 replaced by Lys and Val, respectively, in atNHE1).

The hNHE1 and atNHE1 proteins differ most notably over the terminal 100 amino acids (52% amino acid identity). Both hNHE1 and atNHE1 contain putative phosphorylation consensus sites in this region; however, no common sites are shared between the two transporters. Wakabayashi and co-workers (49) report that deletion of the last 117 carboxy-terminal amino acids does not interfere with NHE activity by growth factors, phorbol esters, or phosphatase inhibitors, nor does such modification result in an apparent alteration in pH set point. In fact, deletion of these amino acids resulted in a nearly threefold increase in exchanger activity. In the present study, we found that the Amphiuma NHE-transfected AP-1 cells exhibited activity nearly twice as high as that of the hNHE1 transfectants (Table 3). Interestingly, in response to intracellular acidification, the atNHc chimeric NHE (Amphiuma amino terminus, human carboxy terminus) displayed exchange activity similar to the hNHE1, whereas the hNatC chimera (human amino terminus, Amphiuma carboxy terminus) was more similar to the Amphiuma exchanger. This implies that the carboxy terminus, notably the terminal ~100 amino acids, plays some role in regulating exchanger activity in response to acidification. More specifically, the deletion data of Wakabayashi et al. (49) and our chimera data are consistent with the notion that the 117 terminal amino acids in the hNHE1 might serve to suppress exchanger activity in response to changes in pH. The fact that the phosphorylation sites in this carboxy-terminal region differ between the Amphiuma and human transporters suggests that a phosphorylation-dependent process could be involved in suppressing H⁺-induced exchanger activity. In addition to pH regulation, preliminary studies indicate that atNHE1 expressed in AP-1 cells also functions in a volume regulatory capacity, displaying amiloride-sensitive Na⁺ uptake in response to cell shrinkage (unpublished observations).

Sensitivity to amiloride and HOE-694. The highly similar sequences of atNHE1 and hNHE1 lead us to anticipate similar responses to known NHE1 antagonists. Specifically, amino acids proposed to be essential for amiloride sensitivity in hNHE1 [Leu-163 and Gly-174 in the M4 transmembrane domain (16) and His-349 in the M9 domain (51)] are retained in the atNHE1 sequence. Although amiloride sensitivities for atNHE1 and the two chimeric proteins were similar to what has been reported for mammalian NHE1 (Fig. 9A, Table 3), the measured IC₅₀ for our hNHE1 transfectant was nearly an order of magnitude higher. This discrepancy between our amiloride IC₅₀ values and those reported by others is essentially due to differences in experimental conditions. All previous reports of antagonist sensitivities have been performed under nominally Na⁺-free conditions (16, 18, 34), whereas the experiments in this study were performed under physiological conditions of 140 mM external Na⁺. Because amiloride displays competitive behavior with Na⁺, potency of amiloride inhibition should be an inverse function of external Na⁺ concentration. Indeed, ²²Na⁺ uptake studies performed in our laboratory under nominally Na⁺-free conditions resulted in amiloride IC₅₀ values of 4.7 µM for the hNHE1 transfectants and 1.3 µM for the atNHE1-transfected AP-1 cells. Thus it appears that there is an approximately fivefold increase in the amiloride IC₅₀ under conditions of physiological external Na⁺ compared with that in nominally Na⁺-free medium.

Although results with amiloride are readily explained on the basis of experimental conditions, the striking difference in response of the human and Amphiuma NHE isoforms to HOE-694 was unanticipated. We confirmed earlier studies demonstrating that HOE-694 was found to be a more potent inhibitor of the hNHE1 isoform than was amiloride (18), with an IC₅₀ of 1.2 µM (Fig. 9, Table 3). In contrast, the apparent IC₅₀ for HOE-694 inhibition of Amphiuma NHE1 in AP-1 cells exceeded 200 µM (Fig. 9B, Table 3), more closely resembling the IC₅₀ of 650 µM determined for inhibition of the mammalian NHE3 isoform (18). In addition, both of the Amphiuma-human chimeras exhibited an insensitivity to HOE-694 that was similar to the native Amphiuma exchanger.

A recent study by Orlowski and Kandasamy (34) utilized AP-1 cells transfected with cDNAs encoding chimeric NHE proteins that contained different transmembrane domains from mammalian NHE1 and NHE3. These investigators exploited the differential sensitivities displayed by the NHE1 (high-affinity) and NHE3 (low-affinity) isoforms to various NHE antagonists, including amiloride and HOE-694. They demonstrated that replacement of a 66-amino acid segment, spanning transmembrane domain M9, from NHE1 with the homologous segment of NHE3 resulted in a chimeric protein with drug sensitivities that approached that of NHE3. The converse was also true; replacement of this region of NHE3 with M9 from NHE1 resulted in drug sensitivities that were more similar to NHE1. These investigators concluded that the region between transmembrane domains M8 and M10 appears to be an important site of interaction with the HOE-694 compound. However, this region between M8 and M10 is identical in the atNHE1 and mammalian NHE1 isoforms (Fig. 6). Because both of our chimeric proteins were insensitive to HOE-694, this suggests that additional interactions between the amino terminus (including all transmembrane segments) and the carboxy terminus are contributing to specificity of hNHE1 for HOE-694. The variance in HOE-694 sensitivities between the native RBC atNHE1 and mammalian NHE1 proteins could be attributed to species-specific differences in membrane-associated accessory proteins or signal transduction pathways expressed in the two different cell types. However, when expressed in the mammalian AP-1 cell line, the Amphiuma transporter retains the same response to both HOE-694 and amiloride as in the native red blood cell, suggesting that species-specific differences are unlikely to be involved.
Furthermore, the fact that both the atNHE1 protein and the NHE proteins in the study of Orlowski and Kandasamy (34) were expressed in the same AP-1 cell line provides additional evidence that the differences in HOE-694 sensitivity are not due to interactions with accessory proteins.

A final interesting observation from our current study is the fact that the resting pH\textsubscript{i} of the atNHE1-transfected AP-1 cells (pH\textsubscript{i} 7.52 ± 0.01, 37°C) was considerably higher than that measured in intact Amphiuma RBCs (pH\textsubscript{i} 7.0 ± 0.1, 22°C) (11). Additional experiments were conducted on atNHE1-transfected AP-1 cells at room temperature to determine whether this was the cause of the discrepancy in resting pH\textsubscript{i}. Although decreasing the temperature did result in lower values of resting pH\textsubscript{i} (pH\textsubscript{i} 7.38 ± 0.09, 22°C), along with decreased activity in response to intracellular acidification, it could not account for the entire difference in steady-state pH\textsubscript{i} (data not shown). This suggests that the steady-state pH\textsubscript{i} set point of atNHE1 in these two systems is regulated by additional factors besides the tertiary structure of the core protein. Such factors could include interaction with ancillary regulatory proteins or intracellular differences in tonic kinase or phosphatase activity.

In conclusion, the high degree of similarity between hNHE1 and atNHE1 is reassuring, given the close correspondence in kinetics and regulation of the human and Amphiuma antiporters. The fact that the putative regulatory regions are nearly identical for atNHE1 and hNHE1 suggests that the Amphiuma RBC transport protein will continue to be a useful model for the study of mammalian NHE1 function and regulation. These structure-function observations are significant, since the Amphiuma RBC appears to be one of the most abundant sources of NHE1 protein yet described (Fig. 7A). Furthermore, due to its abundance and low tonic activity, it will be possible to utilize this system to address questions that are difficult to address in mammalian cells that have a relative paucity of antiporter protein and high levels of tonic NHE activity.

L. A. McLean and S. Zia contributed equally to this paper.

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