NHE1 Inhibition by Amiloride- and Benzoylguanidine-type Compounds

INHIBITOR BINDING LOCI DEDUCED FROM CHIMERAS OF NHE1 HOMOLOGUES WITH ENDOGENOUS DIFFERENCES IN INHIBITOR SENSITIVITY*

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Stine F. Pedersen§, Scott A. King†, Eva B. Nygaard§, Robert R. Rigor†, and Peter M. Cala§†

From the §Department of Physiology and Membrane Biology, University of California, Davis, California 95616 and the †Department of Molecular Biology, University of Copenhagen, DK-2100 Copenhagen, Denmark

The interaction of the ubiquitous Na\(^+\)/H\(^+\) exchanger, NHE1, with its commonly used inhibitors, amiloride- and benzozyguanidine (Hoechst type inhibitor (HOE))-type compounds, is incompletely understood. We previously cloned NHE1 from Amphiuma tridactylum (AtNHE1) and Pleuromonectes americanus (PaNHE1). Although highly homologous to the amiloride- and HOE-sensitive human NHE1 (hNHE1), AtNHE1 is insensitive to HOE-type and PaNHE1 to both amiloride- and HOE-type compounds. Here we generated chimeras to “knock in” amiloride and HOE sensitivity to PaNHE1, and we thereby identified several NHE1 regions involved in inhibitor interaction. The markedly different inhibitor sensitivities of hNHE1, AtNHE1, and PaNHE1 could not be accounted for by differences in transmembrane (TM) region 9. Replacing TM10 through the C-terminal tail of PaNHE1 with the corresponding region of AtNHE1 partially restored sensitivity to amiloride and the related compound 5‘-(N-ethyl-N-isopropyl)amiloride (EIPA) but not to HOE694. This effect was not due to the tail region, but it was dependent on TM10–11, because replacing only this region with that of AtNHE1 also partially restored amiloride and EIPA but not HOE sensitivity. The converse mutant (TM10–11 of AtNHE1 replaced with those of PaNHE1) exhibited even higher amiloride and EIPA sensitivity and was also HOE-sensitive. Replacing an LFFFY motif in TM region 4 of PaNHE1 with the corresponding residues of hNHE1 (VFFLF) or AtNHE1 (TFFLF) greatly increased sensitivity to both amiloride- and HOE-type compounds, despite the fact that AtNHE1 is HOE694-insensitive. Gain of amiloride sensitivity appeared to correlate with increased Na\(^+\)/H\(^+\) exchange rates. It is concluded that regions within TM4 and TM10–11 contribute to amiloride and HOE sensitivity, with both regions imparting partial inhibitor sensitivity to NHE1.

The ubiquitous plasma membrane Na\(^+\)/H\(^+\) exchanger, NHE1, plays a major role in the regulation of cellular pH, cell volume, and cytoskeletal organization in most cell types, and it has been implicated in the control of a wide variety of cellular functions, including migration, proliferation, and cell death (1–4). Accordingly, NHE1 is often referred to as the “housekeeping” isomorph. Consistent with its widespread roles, excessive NHE1 activity is a central factor in several clinically important pathophysiological states, including the growth advantage and invasive properties of many cancer cells (5–8) and the cell damage following cardiac ischemia/reperfusion (9–11).

The ability to specifically interfere with NHE1 function, and to develop high affinity and selective NHE1 inhibitors, is therefore of considerable clinical interest. All known mammalian NHE1s are sensitive to amiloride and to 5‘-N-substituted amiloride derivatives such as 5‘-(N-ethyl-N-isopropyl)amiloride (EIPA), as well as to the Hoechst-type inhibitors HOE642 (cariporide) and HOE694, which are benzoylguanidinium compounds with some structural resemblance to amiloride and its derivatives (12). In contrast, NHE3, which is the Na\(^+\) uptake pathway located in the apical membrane of many epithelial cells, is essentially insensitive to these compounds (13).

Hydropathy analyses suggest a membrane topology for NHE1 of 12 transmembrane (TM) domains and a large C-terminal cytoplasmic region. Additionally, cysteine accessibility studies have pointed to the presence of one large and two smaller re-entrant loops, partially embedded in the membrane (14). The two smaller loops are located intracellularly between TM4 and -5 (i.e. intracellular loop (IL) 2) and TM8 and -9 (i.e. IL4), respectively, whereas the large loop, the fifth extracellular loop (EL5), which resembles the pore-forming loops found in voltage-gated ion channels, has an extracellular origin and presumably inserts into the exofacial surface between TM9 and -10 (2, 14). These putative re-entrant regions are highly conserved among hNHE1, AtNHE1,

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†To whom correspondence should be addressed: Dept. of Physiology and Membrane Biology, School of Medicine, University of California Davis, One Shields Ave., Davis, CA 95616. Tel.: 530-752-1285; Fax: 530-752-5423; E-mail: pmcala@ucdavis.edu.

§From the Department of Physiology and Membrane Biology, University of California, Davis, California 95616.

§§The abbreviations used are: EIPA, 5‘-(N-ethyl-N-isopropyl)amiloride; AtNHE1, A. tridactylum NHE1; PaNHE1, P. americanus NHE1; hNHE1, human NHE1; HOE, Hoechst type inhibitor; TM, transmembrane; RBC, red blood cell; BCECF-AM, 2‘,7‘-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester; NMDG, N-methyl-D-glucamine or N-methyl-D-glucamine-Cl; NHE, Na\(^+\)/H\(^+\) exchanger; IL, intracellular loop; EL, extracellular loop.

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and PaNHE1, supporting the notion that they are critical for NHE1 function.

A number of studies have implicated sites in TM4 and TM9 in amiloride-type inhibitor binding to NHE1. Early mutagenesis studies of hamster NHE1 established important roles of Phe165 and Leu167 of TM4 (15), and the apparent role of TM4 was substantiated by later studies of both NHE1 (16) and NHE2, which also exhibits substantial sensitivity to these compounds (17). Similar investigations of human NHE1 pointed to the involvement of Gly349 in TM9 (18) in amiloride sensitivity. Roles for Glu391 of hNHE1, which resides in the presumed re-entrant loop between TM9 and TM10 (19), and for residues in the extracellular loop between TM3 and TM4 (20), have also been proposed, indicating that association with inhibitors involves multiple NHE1 regions. The apparent major role of TM9 was supported by the findings that replacement of 66 amino acids in and around TM9 of NHE1 with the corresponding residues of NHE3 decreased inhibitor sensitivity (21), and that targeted replacement of Glu350 (rat)/Glu346 (human) or Gly356 (rat) in TM9 reduced sensitivity to both amiloride- and HOE-type compounds (20, 22).

A limitation shared by all of the above studies is that when studying loss-of-function mutants, specific effects of the mutated residue generally can not be separated from broader conformational changes indirectly affecting the property of interest. A more ideal system would allow gain-of-function mutants to be created from homologues of high sequence similarity. Although NHE3 is amiloride-insensitive, the low sequence homology of this isoform to NHE1 (39% for the human homologues) severely compromises its use for gain-of-function studies of inhibitor sensitivity. To our knowledge, the only previous such studies involved replacement of all or part of the NHE3 TM9 with the corresponding residues of NHE1, manipulations which only partially conferred HOE694 sensitivity to NHE3 (20, 21). Although these findings might indicate that the role of TM9 in inhibitor binding is only partial, this conclusion cannot be separated from effects simply reflecting the low sequence similarity between NHE1 and NHE3. The two NHE1 homologues cloned from the giant salamander Amphiuma tridactylum (AtNHE1, see Ref. 23) and the winter flounder, Pleuronectes americanus (PaNHE1, see Ref. 24), respectively, exhibit very high sequence homology to hNHE1 (79 and 65% overall amino acid identity to hNHE1, respectively). It was therefore exciting to find that despite this high homology, AtNHE1 and PaNHE1 differ dramatically from hNHE1 with respect to inhibitor sensitivity. The AtNHE1 is amiloride-sensitive yet HOE-insensitive (23), and, unique among the NHE1s, PaNHE1 is completely insensitive to amiloride, EIPA, and HOE (24, 25). Consequently, PaNHE1 is an ideal background for identification of regions necessary for inhibitor binding by “knock-in” of inhibitor sensitivity. Another interesting aspect of this comparative model is that TM9 is identical between hNHE1 and AtNHE1 and differs by only two similar residues in PaNHE1, indicating that other regions in addition to TM9 must influence inhibitor binding.

Therefore, the aim of this study was to further define regions of NHE1 responsible for inhibitor binding, using PaNHE1 as a background for the construction of gain-of-function NHE1 chimeras exhibiting inhibitor sensitivity. In this manner, we confirmed the important role of TM4 in determining the inhibitor sensitivity of NHE1, and we demonstrated for the first time that regions in TM10–11 and/or the associated intracellular loops (IL5 and EL6) play a major role in NHE1 inhibitor sensitivity. Parts of these findings have been published previously in abstract form (25).

**EXPERIMENTAL PROCEDURES**

**Reagents and Solutions**

Unless otherwise stated, chemicals were of analytical or molecular biology grade and were obtained from Sigma or Fisher. Amiloride and EIPA were obtained from Sigma and were dissolved at 0.5 mM in Me2SO. The 4-isopropyl-3-methylsulfonylbenzoylguanidine, methane sulfonate (HOE642, cariporide) and 3-methylsulfonyl-4-piperidinobenzoylguanidine, methane sulfonate (HOE694) were kind gifts from Aventis Pharma, Frankfurt am Main, Germany, and both these compounds were dissolved at 0.1 mM in Me2SO. HOE642 and HOE694 are structurally closely related and exhibit comparable IC50 values for NHE1 (reported at 26 nM for HOE642 and 130 nM for HOE694, respectively, for inhibition of EIPA-sensitive 22Na+ uptake into rabbit RBCs) (26). Because in this study much higher concentrations of these compounds (>20 μM) were employed to answer the yes/no question of whether a given construct was refractory to inhibition by these compounds, their effects on NHE1 are assumed to be similar, and consequently we did not test both compounds in all experiments. 2’,7’-bis(2-carboxyethyl)-5(6)-Carboxyfluorescein, acetoxymethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR) and was dissolved at 1 mM in Me2SO. Ouabain was from Sigma and, where used, was dissolved directly into the Ringer solutions at 1 mM. Restriction enzymes were from New England Biolabs (Ipswich, MA), and T4 ligase was from Invitrogen.

**Preparation of Red Blood Cells**

*Amphiuma* tridactylum (giant salamanders) were obtained from Atchafalaya Bio (Raceland, LA) and maintained in fresh water tanks at room temperature. *Amphiuma* RBCs were harvested by cardiac puncture with a heparinized syringe. The cells were separated from the plasma by gentle centrifugation (1000 × g, 45 s) and washed three times in 10–15 volumes of isotonic Ringer (in mM: NaCl 90–110, KCl 3; MgCl2 1; CaCl2 0.5, HEPES 18; glucose 5, pH 7.65), matched to the plasma osmolality of the animal from which blood was obtained (220–250 mosm).

*Pleuronectes americanus* (winter flounder) were caught off the coast of Maine and kept in sea water at 10°C. *Pleuronectes* RBCs were harvested by caudal vein puncture with a heparinized syringe. The cells were separated from the plasma by gentle centrifugation (1000 × g, 60 s), and the cells were washed three times in 8–10 volumes of isotonic Ringer (in mM: NaCl 148, KCl 3; MgCl2 1; CaCl2 0.5, HEPES 30, pH 7.65, 360 mosm).

Following harvest and wash, the RBCs were suspended at a hematocrit of 10% and incubated for 1.5 (*Amphiuma*) or 2 h (*Pleuronectes*) in the dark prior to experiments. Hypertonic Ringer solutions were obtained by increasing the concentration of NaCl.
Regions of NHE1 Conferring Inhibitor Sensitivity

Net Flux Measurements of Na⁺, K⁺, Cl⁻, and H₂O in RBCs

All net flux experiments were conducted at room temperature (20–25 °C), and all flux media contained ouabain (1 mM) to prevent contributions from the Na⁺/K⁺-ATPase. The RBC content of Na⁺, K⁺, Cl⁻, and H₂O was determined from 400-μl aliquots of cell suspensions prepared as above, as described previously for *Amphiuma* (27, 28) and *Pleuronectes* (24, 29). To initiate an experiment, cells were separated from suspension media by gentle centrifugation, and experimental medium was added to the cell pellet to yield a hematocrit of 10%. 400-μl samples were removed at the indicated time points, transferred to preweighed polyethylene tubes (Stockwell Scientific, Monterey Park, CA), and centrifuged for 4 min at 12,000 × g (*Amphiuma*) or 16,750 × g (*Pleuronectes*). The wet cell pellet was weighed and lysed by mechanical disruption in ~250 μl of lysis buffer (4.6 mM MgSO₄ and 40 mM ZnSO₄). The lysate was centrifuged at 4 min at 12,000 × g (*Amphiuma*) or two times for 15 min at 16,750 × g (*Pleuronectes*), and the pellet was separated from the supernatant. Cellular Na⁺ and K⁺ contents were determined by flame photometry (model 443, Instrumentation Laboratories, Boston) and Cl⁻ content by coulometric titration using a Buchler chloridometer (Searle Diagnostics Inc., Fort Lee, NJ). The pellets were dried to constant weight (for a minimum of 18 h at 60–70 °C) and weighed, and cellular H₂O content was calculated as the difference between wet and dry weight of the cell pellets. All measurements were corrected for trapped extracellular medium as described previously for *Amphiuma* (28) and *Pleuronectes* (24).

Cell Culture

AP1 cells (a Chinese hamster ovary-derived cell line with no endogenous NHE activity (30)) were the kind gift from Dr. S. Grinstein, Hospital for Sick Children, Ontario, Canada. NHE1-transfected and -untransfected AP1 cells were grown at 37 °C, 5% CO₂, 95% humidity in α-modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin and streptomycin (all from Invitrogen). Media for transfected cells additionally contained 600 μg/ml G418 sulfate (Invitrogen). Every 3–4 days, the cells were passaged by gentle trypsinization. Only passages 5–30 were used for experiments.

Constructs and Stable Transfection of Wild Type and Mutant NHE1

Wild type *A. tridactylum* and *P. americanus* NHE1 were cloned and inserted into the mammalian expression vector pcDNA3.1 as described previously (23, 31). The At/PaNHE1 chimeras were constructed using common restriction enzyme sites as follows. The PaTM7CAt (replacement of residues 240 through the end of the C terminus with the corresponding residues of AtNHE1) was made using the two BsgI sites, and the PaTM10CAt chimera (replacement of residues C-terminal to 489 with the corresponding residues of AtNHE1) was made using the PshAl site. The PaTM10–12At chimera (residues 403–488 replaced by the corresponding residues of AtNHE1) was made by digesting PaTM10CAt and PaNHE1 with BsgI and ligating the appropriate fragments. The

AtTM10–12Pa chimera (residues 420–503 of AtNHE1 replaced by the corresponding residues of PaNHE1) was constructed using the same sites. The PaTM4h (LFFFY of TM4 replaced by the corresponding residues of hNHE1, VFFLF) and PaTM4At constructs (LFFFY in TM4 replaced by the corresponding residues of AtNHE1, TFLLF) were made by point mutagenesis using the QuikChange Multi (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing prior to transfection (DBS Sequencing Facility, University of California Davis). Transfectants were selected for resistance to 600 μg/ml genetecin (G418), and NHE1 expression was verified by Western blotting using the 4e9 mouse monoclonal NHE1 antibody. Clonal selection was carried out by limiting dilution, and stably transfected clones were used in all experiments. All the chimeras employed in the study were functional. For most constructs,
FIGURE 2. Inhibitor sensitivity of wild type hNHE1, PaNHE1, and AtNHE1 in AP1 cells. AP1 cells, either untransfected (A) or stably transfected with wild type hNHE1 (B), PaNHE1 (C), or AtNHE1 (D), were loaded with BCECF and mounted either on a Zeiss Axiovert S100 microscope (A–D) or in a PTI fluorescence spectrophotometer (E and F) as described under "Experimental Procedures." All experiments were carried out in nominally HCO3⁻/H⁺-free HEPES-buffered Ringer, and the cells were continuously perfused at 1.6 ml/min with the indicated Ringer, in the absence or presence of inhibitors. The cells were initially perfused with IR, which, at the times indicated, was replaced first with a Ringer of similar composition but containing 10 mM NH4Cl, then an NH4Cl-containing Ringer, in the absence or presence of inhibitors as indicated. Inhibitor concentrations were as follows: 500 μM amiloride and 100 μM HOE694. Data shown are mean ± S.E. of 18 cells in an experiment representative of six experiments (A), 12 cells in an experiment representative of three experiments (B), 16 cells in an experiment representative of four experiments (C), 22 cells in an experiment representative of four experiments (D), and 3–4 independent population experiments per cell line and inhibitor concentration (E and F). To obtain the data in E and F, rates of pH recovery (in pH units/min⁻¹) were calculated from the initial linear part of the pH recovery curves. IC₅₀ values were calculated in GraphPad Prism 4 by nonlinear regression, using the following equation: $y = 1/(1 + 10^{(x-x_{50})})$ x HillSlope), where $x$ is the logarithm of the inhibitor concentration, and $y$ is the normalized pH recovery rate; see also Table 1). The line shows the fit of this equation to the mean data.

recovery rates are given in Table 1, whereas for PaTM7CaT, PaCaT, and PaTM4At, these data are not shown.

**Measurements of Intracellular pH in AP1 Cells**

**Single Cell Measurements**—AP1 cells stably transfected with the NHE1 constructs were grown on 25-mm round collagen-coated glass coverslips to a confluency of ~50%. Cells were rinsed in isotonic Ringer (IR, 130 mM NaCl, 3 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM NaOH, 10 mM glucose, pH 7.4, 300 mosM) then incubated in IR with 1 μM BCECF-AM for 30 min at 37 °C in a CO₂-free incubator. The cells loaded with BCECF were then mounted in a Warner RC-21B perfusion chamber of a Zeiss Axiovert S100 microscope equipped with a 63× Plan Apochromat objective. BCECF was excited at 490 and 440 nm wavelengths, and emission was measured at 530 nm. Data were acquired using Openlab software (Improvision, Lexington, MA). Intracellular pH was determined by radiometric analysis of emissions from 490 nm excitation (the pH-sensitive) to the 440 nm excitation (the pH-insensitive) wavelength, emission was measured at 530 nm. Two means of activating NHE1 were applied as follows: intracellular acidification and hypertonic challenge. To acidify the cells and measure pH recovery, we initially perfused the cells with IR to determine base-line pH and then alkalinized the cells by perfusing with an NH₄⁺/NH₃ solution (125 mM NaCl, 3 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM NaOH, 10 mM glucose, 10 mM NH₄Cl, pH 7.4, 310 mosM) followed by perfusion with an ammonium- and Na⁺⁻free solution (127.5 mM NMDG-Cl, 3 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 15 mM NMDG-OH, 10 mM glucose, pH 7.4, 300 mosM) to cause rapid acidification as NH₃ diffused out of the cells, leaving H⁺ behind. The acidified cells were then perfused with IR plus inhibitors (unless otherwise indicated concentrations were 500 μM amiloride and 100 μM HOE694 or HOE642) and finally with IR alone. It may be noted that there was zero recovery after acid loading in untransfected AP1 cells (see Fig. 2A); hence, the initial rate of pH recovery in the NHE1-transfected cells can be taken to reflect NHE1 activity.

To test response to hypertonic challenge, we initially perfused with IR to measure base-line pH and then perfused the cells with a Na⁺⁻free hypertonic solution (210 mM NMDG-Cl, 3 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 15 mM NMDG-OH, 10 mM glucose, pH 7.4, 490 mosM), followed by Na⁺⁻containing hypertonic solution (210 mM NaCl, 3 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM NaOH, 10
mM glucose, pH 7.4, 490 mosM) containing inhibitors. Finally, the cells were perfused with hypertonics solution in the absence of inhibitors. Positive controls for inhibitor efficacy were always run as parallel experiments. Each experiment was concluded by perfusing the cells with a high K⁺/H11001 solution (95 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM KOH, 35 mM NMDG-Cl, 10 mM glucose; pH 7.0, 300 mosM) containing 10 μM nigericin. This final treatment was used to calibrate the data to pH values, the 490/440 nm ratio (R) was calculated for each experiment after subtraction of background fluorescence (coverslip with unloaded cells in the appropriate Ringer). Finally, the pHi values were calculated from normalized R values (Rₙ) using the equation pHᵢ = 1.1 × Rₙ³ − 5.2 × Rₙ² + 9.1 × Rₙ + 1.6. The R values were normalized by adding the difference between the calculated value at pH 7.0 and the value of the curve at pH 7.0 (1.32 ± 0.0055, n = 63). This procedure allowed for greater range of ratio data and was shown to correspond well to the Boyarsky method of 7-point high K⁺/nigericin calibration (32).

Cell Population Measurements and Estimation of IC₅₀ Values—Measurements of pHᵢ in cell populations were carried out essentially as described previously (23, 31). Briefly, cells were seeded on collagen-coated coverslips and were ~90% confluent at the day of the experiment. Cells were loaded with 2 μM BCECF-AM in IR for 30 min at 37°C, washed twice, and incubated another 15 min in IR prior to use. The cells were mounted in the temperature-controlled cuvette house of a PTI Ratiomaster spectrofluorometer (PTI, Lawrenceville, NJ) and perfused with 37°C IR at a flow rate of 1.6 ml/min. During Ringer replacement, the flow rate was increased to 3.5 ml/min. Where indicated, the perfusate contained amiloride, EIPA, HOE642, or HOE694 at the relevant concentrations. BCECF fluorescence was measured at 525 nm after excitation at 445 and 495 nm. For acid loading, the NH₄Cl prepulse technique was applied as described above. Cells were exposed to the ammonium prepulse for exactly 5 min, and there were no significant differences in the pHᵢ values at which the results were obtained for the individual chimeras. Background fluorescence for each solution was measured on each experimental day on unloaded cells and subtracted before calculation of the BCECF ratio (F₁/F₀). 7-Point calibration using the high K⁺/nigericin technique was performed, essentially as in Ref. 32. At the end of each experiment, a single point calibration was made using 10 μM nigericin in a high K⁺ solution of pH 7.0. This calibration point was used to normalize the excitation ratio (Rₙ) and to convert the measurements into pHᵢ values using the following equation:

\[
\text{pH}_i = 1.1 \times R_n^3 - 5.2 \times R_n^2 + 9.1 \times R_n + 1.6
\]

Regional of NHE1 Confering Inhibitor Sensitivity

**FIGURE 3.** Amino acid sequences of the amphipathic region of hNHE1, PaNHE1, and AtNHE1. The alignment was made using the published sequences for hNHE1, PaNHE1, and AtNHE1, and Genetics Computer Group (GCG) software, employing a BLOSUM62 amino acid substitution matrix. Also shown is the 100% consensus sequence. Gray shading indicates the probable location of the TM domains, based on the topology model proposed by Ref. 14 and on hydropathy analyses of AtNHE1 and PaNHE1. The TM4 LFFFY and corresponding motifs and the two residues in TM9 discussed in the text are boxed in black. See text for details.
equation: $pH = pK_a + \log \left(\frac{(R_N + (-1 + a \times b))/((1 + b - b \times a) - R_N)}{}\right)$, where $a = (10^{-(pH)}/(1 + 10^{-pH})$. Rates of pH recovery (in pH units/min$^{-1}$) were calculated from the initial linear part of the pH recovery curves.

Data were normalized to the value in the control experiment (i.e. in the absence of inhibitor) on that day, and IC$_{50}$ values were calculated in GraphPad Prism 4 by nonlinear regression, using the equation: $y = 1/(1 + 10^{(\log IC_{50} - x) \times \text{HillSlope}})$, where $x$ is the logarithm of the inhibitor concentration, and $y$ is the normalized pH recovery rate.

**Statistical Analysis**

Data are shown either as individual experiments representative of at least three independent experiments, or as means $\pm$ S.D. or S.E. as indicated. Student’s $t$ test was used to test for statistically significant differences, with $p < 0.05$ as the significance level.

**RESULTS**

**Inhibitor Affinity in Wild Type NHE1 Homologues from Different Species**—We first characterized the inhibitor sensitivity of the *Amphiuma* and *Pleuronectes* NHE1 homologues (PaNHE1 and AtNHE1, respectively) in the native RBCs. The NH$_4$Cl prepulse protocol is the most widely used method for (PaNHE1 and AtNHE1, respectively) in the native RBCs. The NH$_4$Cl prepulse protocol is the most widely used method for stimulation, the PaNHE1 in native RBCs was stimulated by

**TABLE 1**

| Construct     | PaNHE1 regions replaced | Recovery rate$^b$ | Inhibitor profile/IC$_{50}$ values |
|---------------|-------------------------|-------------------|-----------------------------------|
|               |                         | pH units min$^{-1}$ | Amiloride | EIPA | HOE642/HOE694 |
| WT$^a$ PaNHE1 |                         | 0.14 ± 0.018       | 98 ± 8.1 | 8.7 ± 0.91 | Insensitive |
| WT AtNHE1     |                         | 0.60 ± 0.017       | 60 ± 7.8 | 0.8 ± 0.04 | Insensitive |
| WT hNHE1      |                         | 0.40 ± 0.058       | 491 ± 112 | 37 ± 10.2 | Insensitive |
| PaTM7CAt      | TM7 (position 240) through end of C-terminal tail replaced with AtNHE1 | ND$^c$ | Sensitive | ND | ND |
| PaTM10CAt     | TM10 (position 403) through end of C-terminal tail replaced with AtNHE1 | 0.37 ± 0.023 | 137 ± 26 | 11 ± 3.5 | Insensitive |
| PaCat         | C-terminal tail (from position 489) replaced with AtNHE1 | ND | Insensitive | ND | ND |
| PaTM10–12At   | TM10–12 (position 403–488) replaced with AtNHE1 | 0.45 ± 0.037 | 38 ± 4.5 | 5.8 ± 0.84 | Sensitive |
| AtTM10–12Pa   | TM10–12 (position 420–503) of AtNHE1 replaced with PaNHE1 | 0.46 ± 0.037 | 118 ± 17 | 0.9 ± 0.14 | Sensitive |
| PaTM4h        | LFFFY in TM4 replaced with VFFLF | 0.46 ± 0.052 | 118 ± 17 | 0.9 ± 0.14 | Sensitive |
| PaTM4At       | LFFFY in TM4 replaced with TFFLF | ND | Sensitive | ND | ND |

$^a$ WT indicates wild type.
$^b$ Data are mean ± S.E. of 3–4 independent fluorescence spectroscopy experiments for each condition for recovery rates; IC$_{50}$ values were also obtained from these 3–4 experiments per condition, and the remaining data are based on 3–5 independent fluorescence microscopy experiments per condition. Rates of pH recovery (in pH units/min$^{-1}$) were calculated from the initial linear part of the pH recovery curves. Data were normalized to the value in the control experiment (i.e. in the absence of inhibitor) on that day, and IC$_{50}$ values were calculated in GraphPad Prism 4 by nonlinear regression, using the equation: $y = 1/(1 + 10^{(\log IC_{50} - x) \times \text{HillSlope}})$, where $x$ is the logarithm of the inhibitor concentration, and $y$ is the normalized pH recovery rate.

$^c$ ND indicates not determined.

has been demonstrated previously to reflect Na$^+$/H$^+$ exchange (24, 27). As seen in Fig. 1A, the shrinkage-induced AtNHE1 activity in native *Amphiuma* RBCs was inhibited by amiloride (1 mM) in agreement with previous findings (23), yet was insensitive to HOE649 in concentrations up to 100 $\mu$M. When similarly stimulated, the PaNHE1 in native Pleuronectes RBCs was insensitive to amiloride (1 mM), as well as to HOE649 (20 $\mu$M) (Fig. 1B). The AtNHE1 in native RBCs was also inhibited by the 5'-N-substituted amiloride derivative EIPA (22Na$^+$ influx measurements), whereas EIPA was without effect on PaNHE1 in RBCs in concentrations of up to 100 $\mu$M ($n = 3$ at 20 $\mu$M, and $n = 3$ at 100 $\mu$M; data not shown). It is noteworthy that the magnitude of the NHE1-mediated net Na$^+$ flux is substantially greater in *Amphiuma* than in *Pleuronectes* RBCs.

To verify that these unusual inhibitor sensitivity patterns were intrinsic to the NHE1 proteins, AtNHE1, PaNHE1, and hNHE1 were expressed in AP1 cells, which lack endogenous NHE activity (30). The cells were suspended in HEPES-buffered, nominally HCO$_3^-$-free Ringer solutions, and NHE1 activity was estimated using the pH$_i$-sensitive fluorescent dye, BCECF, and the NH$_4$Cl prepulse protocol, which is readily applicable in these cells. Following the initial acidification, all three transfected cell lines exhibited pH$_i$ recovery in the absence of inhibitors, whereas no recovery was detectable in the untransfected cells (Fig. 2). Thus, under these conditions, the pH$_i$ recovery rate is a measure of NHE1 activity. Similar to the pattern seen in the native RBCs, hNHE1-mediated recovery was sensitive to amiloride, EIPA, and HOE642; AtNHE1-mediated recovery was amiloride- and EIPA-sensitive but HOE642-insensitive; and PanNHE1-mediated recovery was insensitive to either of these compounds (Fig. 2 and Table 1). IC$_{50}$ values for inhibition by amiloride were about 60 $\mu$M for hNHE1 and significantly higher ($n = 4$, $p < 0.05$), about 100 $\mu$M, for AtNHE1. The corresponding values for EIPA were about 0.8 and 9 $\mu$M, in hNHE1 and AtNHE1, respectively (significantly different, $p < 0.0005$, $n = 4$ for each condition). In contrast, PanNHE1 was fully insensitive to both compounds (Table 1).

$^3$ R. Rigor and P. M. Cala, unpublished data.
To further substantiate these findings, we also assessed inhibitor sensitivity after osmotic shrinkage in AP1 cells. Exposure of AP1 cells to hypertonic Ringer (1.6 times the isotonic osmolarity by NaCl addition) elicited a Na\(^+\)/H\(^+\)-dependent intracellular alkalinization in all three NHE1-transfected cell lines, which was absent in untransfected cells and hence reflected shrinkage-induced activation of NHE1. Confirming the above findings, the pattern of NHE1 inhibition by amiloride, EIPA, and HOE694 was the same after osmotic shrinkage as after intracellular alkalinization (5–8 independent experiments for each cell line and inhibitor; data not shown).

Regions of NHE1 Confering Inhibitor Sensitivity

Inhibitor Affinity in NHE1 Chimeras; Regions Confering Inhibitor Sensitivity to PaNHE1—Having established that the unique inhibitor sensitivity profiles of AtNHE1 and PaNHE1 seen in native RBCs are preserved in AP1 cells and hence are intrinsic to these proteins, we proceeded to address the question of which regions of NHE1 are important for inhibitor binding. To this end, we constructed a series of chimeric proteins, using the inhibitor-insensitive PaNHE1 as a background for knock-in of sensitivity to amiloride- and Hoechst-type inhibitors. An amino acid sequence alignment of the relevant part of the amphipathic region of PaNHE1, AtNHE1, and hNHE1 is shown in Fig. 3, and the chimeras constructed are shown in Fig. 4.

We first replaced all six C-terminal TM segments (TM7–TM12) as well as the tail region of PaNHE1 with the corresponding segments of AtNHE1. In contrast to native PaNHE1, the resulting chimera, PaTM7Cat, was inhibited by 500 \(\mu\)M amiloride (Fig. 5A), demonstrating that some part of this region is important for inhibitor action. Of the NHE1 sequence, the C-terminal cytosolic tail region exhibits by far the lowest homology between the three homologues, and we therefore addressed the possible impact of this region on inhibitor sensitivity. However, when only the tail of PaNHE1 was replaced with that of AtNHE1 (PaCAt), the resulting NHE1 chimera remained amiloride-insensitive (Fig. 5B); hence the tail region...
is not responsible for the gain of inhibitor sensitivity in PaTM7CAt.

Because the TM7–TM12 region contains TM9, which has previously been implicated in inhibitor binding (18, 20), we next addressed the effect of regions distal to TM9 in isolation. When the region from the start of TM10 through the C-terminal cytoplasmic region of PaNHE1 was replaced with the corresponding region of AtNHE1, the chimera, PaTM10CAt, was inhibited by amiloride and EIPA albeit with much lower sensitivity than that of wild type AtNHE1 and hNHE1 (IC50 ~ 500 and ~40 μM, respectively; significantly different from the values in AtNHE1 and hNHE1, p < 0.05 and n = 4 for each condition; Fig. 6, A–D, and Table 1). The PaTM10CAt chimera remained insensitive to HOE694 (Fig. 6E).

Thus, these data in conjunction with the lack of ability of the AtNHE1 tail region to impart inhibitor sensitivity to PaNHE1 strongly indicate that the gain of amiloride and EIPA sensitivity in PaTM10CAt is caused by the TM10–12 region (or rather, it is caused by TM10 and/or TM11, because TM12 is identical among the NHE1 homologues studied; see “Discussion”). To further address the role of this region, we next replaced only TM10–12 of PaNHE1 with the corresponding region in AtNHE1, resulting in the chimeric protein PaTM10–12At (the identical TM12 was only swapped for practical reasons, because of the presence of a convenient restriction site). Interestingly, for this chimera, IC50 values were about 140 μM for amiloride and about 11 μM for EIPA (Fig. 7, A and B, and Table 1). In other words, amiloride sensitivity was further increased when compared with the effect of replacing the entire TM10 onward (PaTM10CAt; p < 0.05, n = 4), and the same trend was seen for EIPA, albeit not quite statistically significant. This implies that the tail of the AtNHE1 not only does not impart inhibitor sensitivity but may in fact interfere with inhibitor binding (see “Discussion”). To verify the importance of TM10–12, we also constructed the reverse chimera, AtTM10–12Pa, replacing TM10–12 of AtNHE1 with the corresponding region of PaNHE1. For this chimera, IC50 values were about 40 μM for amiloride and 6 μM for EIPA, respectively (Fig. 7, A and B), in fact a modest increase in sensitivity compared with the wild type AtNHE1 protein (n = 4, p < 0.05 for amiloride, not significantly different for EIPA). Notably, although the PaTM10–12At chimera remained HOE642-insensitive, AtTM10–12Pa was HOE642-sensitive (Fig. 7, C and D, and Table 1).
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The recent three-dimensional structure of the bacterial Na⁺/H⁺ exchanger NhaA points to a close proximity of regions within TM11 and TM4 (34), and as noted above, TM4 has previously been assigned important roles in NHE1 inhibitor sensitivity (15, 16). Upon cloning of PaNHE1 (24), it was therefore exciting to find a 2-amino acid motif (LFFFYL) in TM4 of this homologue, which is identical to the corresponding region in the amiloride- and HOE694-insensitive NHE3. The sequence in hNHE3 is VFFFYLL, whereas the corresponding sequence in hNHE1 is VFFLELL. To address the role of this motif, we constructed the chimera PaTM4h, by replacing the LFFFY motif with the corresponding residues in hNHE1 (VFFLF), i.e. the total change encompassed three amino acids. The PaTM4h protein exhibited partial amiloride and full EIPA sensitivity compared with hNHE1, with IC₅₀ values of about 120 and 0.9 μM, respectively, and was also HOE642-sensitive. When the PaNHE1 LFFFY motif was instead replaced with the corresponding residues in AtNHE1 (TFFFL), which differs by only one amino acid from that of hNHE1 (VFFLF), the resulting chimera (PaTM4a) was also inhibited by both amiloride and HOE642 (Table 1). Taken together, these findings demonstrate the importance of the VFFLF motif in TM4 for NHE1 inhibitor sensitivity.

Recovery after Intracellular Acidification; Relation to Inhibitor Affinity?—The question of the relationship between ion binding and inhibitor binding to NHE1 was not addressed in this study. However, the pHᵢ recovery data obtained in the absence of inhibitors are interesting in this regard. Thus, as seen in Table 1, the rate of NHE1-mediated recovery after an acid load was substantially lower in the inhibitor-insensitive wild type PaNHE1 compared with that of wild type hNHE1 and AtNHE1, in congruence with the net Na⁺ flux data in native RBCs (Fig. 1). Although we have not quantified expression of NHE1 in the various cell lines, and hence cannot directly compare absolute recovery rates, it is notable that the gain of inhibitor sensitivity in a given chimera that was consistently associated with increased pHᵢ recovery rates. This points to a correlation between these parameters, indicating that both the LF motif in TM4 and residues in TM10 and/or TM11 are likely to be important for ion translocation via NHE1.

DISCUSSION

NHE1 plays important roles in the pathophysiology of cancer and ischemia (8, 11); hence, further understanding of the interaction of NHE1 with efficient inhibitors of its activity is of substantial clinical interest. The aim of this study was to increase the understanding of the structural basis for the inhibitor sensitivity of NHE1 by the use of a comparative gain-of-function approach. Previous studies have largely addressed this question based on the identification of point mutations associated with reduced inhibitor sensitivity in the mammalian NHE1. These studies primarily point to regions in TM4 and TM9 as important for inhibitor sensitivity (15, 17, 18, 20–22, 33). In loss-of-function studies, however, it is difficult to distinguish between a direct role of the amino acids in question and global changes resulting in deterioration of protein integrity. We therefore decided to take another approach, using the uniquely amiloride-, EIPA-, and HOE-insensitive PaNHE1 (24) and the HOE-insensitive AtNHE1 (23) to identify NHE1 regions necessary for inhibitor binding by knock-in. In this manner, we confirmed the important role of TM4 and demonstrated for the first time that regions in TM10–11 and/or the associated intra- and extracellular loops (IL5 and EL6) play a major role in determining NHE1 inhibitor sensitivity.

To narrow in on regions involved in inhibitor sensitivity, we first replaced the entire region distal to TM6 of PaNHE1 with
The corresponding region of AtNHE1, resulting in an amiloride- and EIPA-sensitive chimera (PaTM7CAT). Notably, TM9 was not responsible for this gain of inhibitor sensitivity, because replacing only the region from TM10 onward (PaTM10CAT) also leads to amiloride and EIPA sensitivity. In congruence with this, TM9, as defined in the topological model proposed by Wakabayashi et al. (14), is identical between hNHE1 and the HOE694-insensitive AtNHE1, and deviates from this by only two residues in the amiloride-, EIPA-, and HOE-insensitive PaNHE1 (a Leu-Val and an Ala-Ser change compared with hNHE1; see Fig. 4). Specifically, the Glu and Gly residues of TM9, which were assigned important roles in determining inhibitor sensitivity in the rat NHE1 (20, 22), are identical among PaNHE1, AtNHE1, and hNHE1. Clearly then, TM9 is unlikely to be responsible for the differences in inhibitor sensitivity between these three NHE1 homologues, and NHE1 sensitivity to amiloride- and HOE-type inhibitors can be modulated independently of TM9.

Replacing only the C-terminal cytosolic region of PaNHE1 with that of AtNHE1 (PaCAT) did not confer amiloride sensitivity to PaNHE1. As TM12 is identical among all three NHE1 homologues tested, this implicated TM10–11 and/or the associated intra- and extracellular loops (IL5 and EL6). To our knowledge, this is the first demonstration that regions distal to the extracellular loop between TM9 and TM10 play a role in NHE1 inhibitor sensitivity. To confirm this further, we replaced only TM10–12 (as noted above, the identical TM12 was replaced for practical reasons only) of PaNHE1 with the corresponding region of AtNHE1 (PaTM10–12At) and, conversely, TM10–12 of AtNHE1 with the corresponding region of PaNHE1 (AtTM10–12Pa). Both constructs exhibited amiloride and EIPA sensitivity. However, PaTM10–12At was less sensitive than AtTM10–12Pa and, at least with respect to EIPA, also substantially less sensitive than hNHE1. Thus, from these data it is concluded that TM10–11 and/or IL5 and EL6 play a major role in determining NHE1 inhibitor sensitivity.

It is not straightforward to explain why the replacement of TM10–11 of AtNHE1 with those of the less amiloride-sensitive PaNHE1 yielded a chimera that was more sensitive to amiloride than wild type AtNHE1. TM10 is almost identical in AtNHE1 and hNHE1, yet differs substantially in PaNHE1. It is tempting to speculate that although not the site of competition between Na+ and inhibitor, such residues in TM10 of PaNHE1 may facilitate or stabilize the association of amiloride-type inhibitors with the exchanger, such that the interaction of these compounds with the inhibitory site(s) elsewhere on the protein is facilitated. In other words, compared with the corresponding residues in PaNHE1, residues in AtNHE1 different from the
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inhibitory site(s) per se may interfere with inhibitor binding (perhaps by spatially restricting access to the inhibitor binding pocket). Upon replacement of TM10–11 with those of PaNHE1, this interference may be lifted, resulting in increased inhibitor sensitivity. However, this remains to be experimentally addressed, and full understanding of the phenomenon will require more information about the three-dimensional structure of NHE1 than is currently available, as well as a comprehensive knowledge of all of the sites important for inhibitor binding.

The role of TM4 was addressed next. Replacing the LFFFY motif in TM4 of PaNHE1 with the corresponding motif (VFLLF) from hNHE1 (PaTM4h) also rendered PaNHE1 amiloride- and EIPA-sensitive. Amiloride sensitivity in this chimera was approximately similar to that induced in PaNHE1 by the AtTM10–11 substitution, whereas the EIPA sensitivity approached that of wild type hNHE1. These data are in good agreement with previous observations pointing to a major role for the Leu in this motif (Leu^{163} in hNHE/Leu^{167} in hamster NHE1/Leu^{443} in NHE2) in conferring amiloride sensitivity to both NHE1 and NHE2 (15, 17). However, the fact that we were able to knock-in inhibitor sensitivity in PaNHE1 by replacing the LFFFY motif with the corresponding hNHE1 motif provides much stronger evidence for the necessary involvement of this region in inhibitor binding.

Thus, we have shown that both the TM10–11 region and TM4 are involved in determining inhibitor sensitivity in NHE1. Interestingly, our findings indicate that the regions modulating sensitivity to HOE compounds are partially different from those controlling amiloride and EIPA sensitivity. Thus, wild type AtNHE1 has the same LF motif in TM4 as does hNHE1 yet is insensitive to HOE694. Moreover, whereas PaNHE1 and AtNHE1 lack HOE sensitivity, both AtTM10–12Pa (TM10–11 of AtNHE1 replaced with those of PaNHE1) and PaTM4at (the TM4 motif of PaNHE1 replaced with that of AtNHE1) are HOE-sensitive. Accordingly, it would seem that there are yet undefined regions in these NHE1 homologues that interfere with HOE compound binding, an effect that was lifted in the HOE-sensitive chimeras. The fact that amiloride and EIPA sensitivity was increased in the absence of the AtNHE1 tail (compare PaTM10CaAt to PaTM10–12At), in conjunction with our previous observation that HOE sensitivity of hNHE1 is lost when the tail of this homologue is replaced with that of AtNHE1 (23), may point to an interaction with the tail region in this phenomenon; however, this remains to be determined.

Notably, the conclusion that both TM4 and TM10–11 are major determinants of inhibitor sensitivity is strongly supported by the structural studies of the bacterial NHE1, NhaA, in which TM4 and TM11 are proposed to directly interact in the central funnel structure (34). However, it is also possible that the nonpolar Leu (PaNHE1) for the polar Ser (AtNHE1 and hNHE1) substitution in TM10 may play a role in rendering PaNHE1 amiloride- and EIPA-insensitive. In congruence with this, this Ser residue (Ser^{416} of hNHE1) is close to the extracellular face of TM10 and in a position where it is likely to interact with the re-entrant loop between TM9 and TM10, for which a role in ion translocation has been suggested (see Ref. 2). Moreover, not only the amino acid side chains need to be taken into account. As suggested previously by Counillon et al. (16), it is also possible that the free backbone carbonyls can provide hydrogen bonding for interactions with inhibitors as well as with other NHE1 regions. Clearly, multiple noncontiguous regions in NHE1 are of importance for inhibitor binding, and substantial further advances in the understanding of how these compounds spatially interact with NHE1 will likely require information about the three-dimensional structure of the vertebrate NHE1 and the dynamic changes that the protein undergoes upon inhibitor binding.

Finally, although it was not a specific aim of the study to address this issue, it is noteworthy that our findings point to a strong correlation between inhibitor sensitivity and ion translocation by NHE1. Thus, all mutations that conferred inhibitor sensitivity to PaNHE1 also increased the rate of pH recovery after an acid load. This is again in excellent agreement with the NhaA model pointing to the localization of TM4 and TM11 in conjunction with TM9 in the ion translocation pathway, as well as with previous findings indicating at least partial competition between Na\(^+\), and both amiloride- and HOE-type inhibitors for binding to NHE1 (35, 36). Nonetheless, mutations in TM4 and TM9 associated with reduced inhibitor sensitivity have, in many cases, been reported to be without apparent effect on Na\(^+\) binding (15, 18, 20; see Ref. 22 for a contrasting view), although some were found to affect catalytic turnover (20). Hence, the relationship between Na\(^+\) binding, ion translocation, and inhibitor sensitivity in NHE1 is far from understood, and future studies should specifically address this question taking into account the emerging three-dimensional information on Na\(^+\)/H\(^+\) exchanger proteins.

In conclusion, by constructing gain-of-function chimeras conferring inhibitor sensitivity to an endogenously inhibitor-insensitive NHE1, we have confirmed the important role of TM4 in determining the inhibitor sensitivity of NHE1. Moreover, we demonstrate here for the first time that regions in TM10–11 and/or the associated intra- and extracellular loops (IL5 and EL6) play a major role in NHE1 inhibitor sensitivity.

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