Determinants of Cation Permeation and Drug Sensitivity in Predicted Transmembrane Helix 9 and Adjoining Exofacial Re-Entrant Loop 5 of Na+/H+ Exchanger NHE1

Tushare Jinadasa1‡, Colin B. Josephson1,2‡, Annie Boucher1 and John Orlowski1†

From the 1Department of Physiology, McGill University, Montréal, Québec, H3G 1Y6, Canada and 2Division of Clinical Neurosciences, University of Calgary Foothills Medical Centre, Calgary, Alberta, T2N 2T9

*Running Title: Determinants of cation and drug sensitivity of NHE1

‡T.J. and C.B.J made equal contributions

†To whom correspondence should be addressed: John Orlowski, Department of Physiology, McGill University, Bellini Life Sciences Building, Room 166, 3649 Promenade Sir-William-Osler, Montreal, Quebec, H3G 0B1, Canada; Email: john.orlowski@mcgill.ca.

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Background: NHE1 is a therapeutic drug target, yet knowledge of sites involved in cation translocation and drug binding remains incomplete.

Results: Mutagenesis analyses identified residues in transmembrane helix M9 and exofacial re-entrant loop EL5 that affect substrate affinities and/or drug sensitivity.

Conclusion: M9 and EL5 form part of the cation permeation pathway.

Significance: These findings provide new insight into the structure-function domains of NHE1.

ABSTRACT

Mammalian Na+/H+ exchangers (NHEs) regulate numerous physiological processes and are involved in the pathogenesis of several diseases, including tissue ischemia and reperfusion injuries, cardiac hypertrophy and failure, and cancer progression. Hence, NHEs are being targeted for pharmaceutical-based clinical therapies, but pertinent information regarding the structural elements involved in cation translocation and drug binding remains incomplete. Molecular manipulations of the prototypical NHE1 isoform have implicated several predicted membrane-spanning (M) helices, most notably M4, M9 and M11, as important determinants of cation permeation and drug sensitivity. Here, we have used substituted-cysteine accessibility mutagenesis and thiol-modifying methanethiosulphonate (MTS) reagents to further probe the involvement of evolutionarily-conserved sites within M9 (residues 342-363) and the adjacent exofacial re-entrant loop 5 between M9 and M10 (EL5; residues 364-415) of a cysteine-less variant of rat NHE1 on its kinetic and pharmacological properties. MTS treatment significantly reduced the activity of mutants containing substitutions within M9 (H353C, S355C, and G356C) and EL5 (G403C and S405C). In the absence of MTS, mutants S355C, G403C and S405C showed modest to significant decreases in their apparent affinities for Na+ and/or H+. In addition, mutations Y370C and E395C within EL5, while failing to confer sensitivity to MTS, nevertheless reduced the affinity for Na+, but not for H+. The Y370C mutant also exhibited higher affinity for ethylisopropylamiloride, a competitive antagonist of Na+ transport. Collectively, these results further implicate helix M9 and EL5 of NHE1 as important elements involved in cation transport and inhibitor sensitivity which may inform rational drug design.

Electroneutral countertransport of alkali cations such as Na+, K+ and Li+ for H+ across membranes of mammalian cells are catalyzed by a heterogeneous family of at least eleven secondary active solute carriers (SLC9 gene family) generically termed Na+/H+ exchangers or antiporters (NHE/NHA)† (1,2). The activities of...
these transporters are tightly controlled and important for efficient execution of numerous physiological processes, ranging from cellular and systemic pH and volume homeostasis (3-5) to the regulation of cell shape (6), migration (7-10) and mitosis (11), amongst others (12-17). On the other hand, aberrant over-activation of NHE activity - notably the ubiquitous NHE1 isoform - occurs in several pathophysiological conditions and contributes to disease progression, including tissue injuries following ischemic and hemorrhagic stroke (18-20), acute myocardial infarction (21-23), cardiac hypertrophy and failure (24-27), and cancer metastasis and invasion (28-31); damages that can be mitigated in animal models by pharmacological inhibition of NHE1 activity. However, in the case of heart disease, attempts to translate some of these promising experimental findings into clinical therapies have thus far proven inconclusive due to modest efficacy and adverse side effects of the tested compounds (32-35). Despite these setbacks, an improved understanding of the molecular determinants that underlie the catalytic and pharmacological properties of NHE1 could assist in the development of more efficacious drugs and treatment regimens.

Information regarding the structural and functional properties of the mammalian NHEs has been derived mainly from analyses of the ubiquitous NHE1 and epithelial NHE3 isoforms. Early computational modelling and substituted-cysteine accessibility mutagenesis studies of human NHE1 (hNHE1) by Wakabayashi et al. (36) predicted a configuration of twelve membrane-spanning (M) helices at its N-terminus (~400-500 amino acids) involved in cation translocation and drug binding, and a cytoplasmically-oriented segment at its C-terminus (~350 amino acids) that confers responsiveness to various regulatory stimuli (illustrated in Fig. 1A). Biochemical (37-44) analyses indicated that the exchanger most likely assembles as a homodimer, though higher-ordered structures have also been proposed (45).

To identify sites involved in Na⁺ binding and translocation, initial studies (46-48) took advantage of pyrazine- (e.g., amiloride and ethylisopropylamiloride (EIPA)) or benzoylguanidinium-based (e.g., HOE642 and HOE694) compounds that inhibit NHE activity by acting as competitive or mixed-type antagonists of Na⁺ influx, suggesting that they bind at or near sites involved in Na⁺ binding. Random or site-directed mutagenesis of NHE1 followed by functional selection for altered drug sensitivity identified several residues located in the predicted second exofacial loop (EL2) (49) and two membrane-spanning segments, M4 (50-54) and M9 (49,55,56), that are significant determinants of drug recognition and/or transport velocity. However, only mutations at Phe¹⁶² in M4 of hNHE1 were found to appreciably reduce Na⁺ affinity (52). Additional mutations of conserved residues within the fifth intracellular loop (IL5) (i.e., Arg⁴⁴⁶) and adjacent transmembrane helix M11 (Gly⁴⁴⁵, Gly⁴⁴⁶) of hNHE1 decreased and increased, respectively, its sensitivity to intracellular H⁺ (57,58). Other mutagenesis analyses have confirmed the importance of M4 (59), M9 (60) and M11 (61), and further implicated residues in M6 (62) and M7 (63) as pore-lining elements.

While there is general support for this model, the precise arrangement of transmembrane helices remains controversial (64-66). High-resolution crystal structures of the bacterial Escherichia coli Na⁺/H⁺ antiporter NhaA (67,68) that is structurally (~10% amino acid identity) and kinetically (i.e., electrogenic) distinct from NHE1 also indicated a twelve-transmembrane structure that assembles as a dimer. Such parallels prompted Landau et al. (65) and Nygaard et al. (69) to use the atomic coordinates of NhaA as a template to generate a three dimensional model of hNHE1. However, while the model proposed by Nygaard et al. (69) closely mirrors that of Wakabayashi et al. (36), Landau and colleagues derived a novel membrane topology that challenges this view (Fig. 1B). Notably, in their new model, the original transmembrane helices M1 and M2 were repositioned intracellularly and the catalytically important M9 helix was reconfigured to form two short intramembrane helices relabelled as M7 and M8, while the adjoining exofacial loop 5 (EL5, amino acids 364-415) between M9 and M10 formed a new intracellular loop and a new transmembrane segment M9 that is largely inaccessible to external reagents. While the computationally-derived reorientation of M1 and M2 seems unlikely given that the intervening EL1 segment was shown previously to undergo N- and
O-linked glycosylation and therefore places this segment extracellularly (70), the topology of the M9-EL5 region remains uncertain. This region is of particular interest because the putative EL5 loop was originally postulated to invaginate into the membrane (36) in a manner analogous to the pore-lining loops present in ion channels (71,72), and therefore may contribute to cation permeation and drug sensitivity.

Based on discordances between these models, we have further probed the potential contributions of evolutionarily-conserved as well as certain known drug-sensitive sites located in the originally designated M9 and adjacent re-entrant loop EL5 segments (as depicted in Fig. 1A) to cation translocation using the substituted-cysteine accessibility method (73). The data revealed additional amino acids in both M9 and EL5 that contribute to the affinities of the exchanger for substrates and pharmacological antagonists and hence are likely to line the cation permeation pathway. The relevance of these findings to the two different membrane topologies proposed for NHE1 is discussed.

EXPERIMENTAL PROCEDURES

Materials – Carrier free ²²NaCl (radioactivity, 5 mCi/ml) was obtained from PerkinElmer Life Sciences (Woodbridge, ON). Amiloride, ouabain, and nigericin were purchased from Sigma-Aldrich (Oakville, ON, USA). α-Minimal Essential Medium, fetal bovine serum, G418®, and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Murine monoclonal HA antibody was purchased from BabCo (Richmond, CA) while horseradish peroxidase-conjugated goat α-mouse IgG was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). [2-sulfonatoethyl]methylenebisulfonate (MTSES) and [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) were purchased from Toronto Research Chemicals (North York, ON). All other chemicals and reagents used in these experiments were purchased from Fisher Scientific and were of the highest grade available.

Construction of Na⁺/H⁺ Exchanger Mutants – The rat cDNA, engineered to contain a series of unique restriction endonuclease sites that provide convenient DNA cassettes for mutagenesis, was subcloned into a mammalian expression vector under the control of the enhancer/promoter region from the immediate early gene of human cytomegalovirus (pCMV), as previously described (56). A single copy of an influenza virus hemagglutinin (HA) peptide (YPYDVPDYA) was inserted at the C-terminus of NHE1 using polymerase chain reaction (PCR) mutagenesis (the construct is referred to herein as NHE1HA). NHE1HA∆C was constructed by substituting the eight endogenous cysteine residues (amino acids 117, 137, 216, 425, 481, 542, 565, 799) with the structurally conservative residue serine. The substitution of serine did not generate any obvious consensus motifs for phosphorylation by known serine/threonine kinases. Single cysteine residues were introduced into this cysteine-less background using the QuikChange™ Site-Directed Mutagenesis system purchased from Stratagene (La Jolla, CA, USA). All mutant constructs were sequenced to verify their fidelity.

Stable Transfection and Expression of the Na⁺/H⁺ Exchanger cDNAs– Chinese hamster ovary cells (AP-1 cells), a chemically mutagenized cell-line devoid of endogenous plasmalemmal NHE activity (74), were transfected with mammalian expression plasmids containing either wild type NHE1HA or NHE1HA∆C-based constructs using Lipofectamine™. Cells were maintained in complete α-minimal essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 25 mM NaHCO₃, pH 7.4, and incubated in an humidified atmosphere of 95% air, 5% CO₂ at 37°C. Starting 48 h after transfection, the AP-1 cells were selected for survival in response to repeated (~ 6 times over a two week period) acute NH₄Cl-induced acid loads (i.e., H⁺-killing technique) (75,76) in order to discriminate between NHE1 positive and negative transfectants. Six clonal isolates per mutant were routinely selected and the one exhibiting the highest amiloride-sensitive H⁺-activated ²²Na⁺ uptake was subjected to further analyses.

Measurement of Na⁺/H⁺ Exchanger Activity and Covalent Modification with Sulfhydryl-Reactive Reagents– Clonal cells were grown to confluence in 24-well plates and then acidified (to ~pH 6.0) using the NH₄Cl pre-pulse technique (75,76). Briefly, the cell culture medium was aspirated and replaced by isotonic NH₄Cl medium (50 mM NH₄Cl, 70 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose,
20 mM HEPES-Tris, pH 7.4). Cells were incubated in this media for 30 min at 37 °C in a nominally CO₂-free atmosphere. After acid loading, the monolayers were rapidly washed twice with isotonic choline chloride solution (125 mM choline chloride, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). ²²Na⁺ influx assays were initiated by incubating the cells in isotonic choline chloride solution containing 1 μCi ²²NaCl/ml (~120 nM ²²NaCl, carrier-free). The assay medium was K⁺-free to prevent ²²Na⁺ influx and efflux by the Na⁺/K⁺-Cl⁻ cotransporter and the Na⁺/K⁺-ATPase, respectively. Influx of ²²Na⁺ was terminated by rapidly washing the cell monolayers three times with four volumes of ice-cold isotonic saline solution (130 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM HEPES-Tris, pH 7.4). The washed cell monolayers were solubilized in 0.25 ml of 0.5 N NaOH and the wells were washed with 0.25 ml of 0.5 N HCl. Both the solubilized cell extract and wash solutions were added to vials and radioactivity was assayed using a liquid scintillation counter.

Under the conditions of H⁺-activated NHE activity as assessed using a liquid scintillation counter, the activity of the Na⁺/H⁺ exchanger were determined as the difference between the initial rates of H⁺-activated ²²Na⁺ influx and the Na⁺/K⁺-ATPase, respectively. Influx of ²²Na⁺ was terminated by rapidly washing the cell monolayers three times with four volumes of ice-cold isotonic saline solution (130 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM HEPES-Tris, pH 7.4). The washed cell monolayers were solubilized in 0.25 ml of 0.5 N NaOH and the wells were washed with 0.25 ml of 0.5 N HCl. Both the solubilized cell extract and wash solutions were added to vials and radioactivity was assayed using a liquid scintillation counter. Under the conditions of H⁺-loading used in this study, uptake of ²²Na⁺ was linear with time for 8 to 10 min (at low Na⁺ concentrations, 22 °C). Therefore, ²²Na⁺ uptakes were measured after 5 min except when examining the kinetics of NHE activity as a function of the extracellular Na⁺ (Na⁺ₐ) concentration.

When assessing the $K_m$ for [Na⁺ₐ], a modified version of the aforementioned protocol was applied. Previous measurements indicated that when [Na⁺ₐ] is increased to 100-125 mM, ²²Na⁺ uptake is linear for several min. Therefore, when conducting this kinetic analysis in which [Na⁺ₐ] concentration ranges from 1.25 mM to 120 mM, ²²Na⁺ uptake was terminated after 1 min. Measurements of ²²Na⁺ influx specific to the Na⁺/H⁺ exchanger were determined as the difference between the initial rates of H⁺-activated ²²Na⁺ influx in the absence and presence of either 2 mM amiloride or 100 μM EIPA (concentrations sufficient to inhibit NHE1 under these experimental conditions) and expressed as amiloride- or EIPA-inhibitable ²²Na⁺ influx. To make quantitative comparisons of the intrinsic rates of transport of the various NHE constructs in stably transfected cells, the cellular rates of drug-sensitive H⁺-activated ²²Na⁺ influx were measured under near maximal acid-load conditions and normalized per mg of total cellular protein per abundance of the fully-glycosylated NHE1 protein present at the plasma membrane (assessed by Western blotting and densitometry), as described previously (49).

To examine NHE activity as a function of intracellular H⁺ concentration, pHᵢ was set over the range of 5.4-7.4 using the K⁺-nigericin method as previously described (77,78). Briefly, the confluent monolayers were washed with isotonic N-methyl-D-glucamine (NMG)-chloride solution (140 mM NMG-Cl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 10 mM HEPES-Tris, pH 7.4), and then incubated for 4 min at room temperature in NMG-balanced salt solutions specific for each pHᵢ (pHᵢ-clamp solutions). All solutions contained 2 mM NaCl, 1 mM MgCl₂, and 10 mM HEPES-Tris, pH 7.4, varying concentrations of K⁺ (1.4 to 140 mM KCl/K⁺-glutamate, adjusted as needed with NMG-glutamate to bring the final concentration to 140 mM), and the K⁺/H⁺ exchange ionophore, nigericin (10 μM). These pHᵢ-clamp solutions are designed to fix the pHᵢ at a desired level by adjusting the extracellular K⁺ concentration as described (78). In essence, the desired pHᵢ can be established according to the following equation: $[K^+]/[K^-] = [H^+]/[H^+]_0$, assuming the intracellular K⁺ concentration is 140 mM and the extracellular H⁺ concentration is set at 7.4. ²²Na⁺ uptake was initiated in the same pHᵢ-clamp solutions supplemented with 1 μCi/mL of ²²Na⁺ and 1 mM ouabain in the absence or presence of 2 mM amiloride. Uptake occurred for a period of 10 minutes and was terminated in the same fashion as described above. Protein content was assessed using the Bio-Rad DC protein assay kit as per the manufacturer’s protocol. Rates were expressed as nmol Na⁺/min/mg protein.

To evaluate the effect of the methanethiosulfonate derivatives (73) on NHE1 activity, cell monolayers were incubated in phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂, pH 7.2, with or without 1 mM MTSET or 10 mM MTSES for 30 min at 37 °C. Cells were washed twice with isotonic choline chloride solution and then intracellular pH was clamped at pH 5.6 using the K⁺-nigericin method. ²²Na⁺ uptake was initiated using the same clamp solution supplemented with 1 μCi of ²²Na⁺ and 1 mM ouabain in the absence or presence of 2 mM amiloride or 100 μM EIPA. ²²Na⁺ uptake was
terminated after 10 min using ice-cold isotonic saline solution in the same fashion as mentioned above. Residual NHE1 activity was determined as the ratio of amiloride or EIPA-inhibitable $^{22}\text{Na}^+$ uptake in the presence and absence of MTSET or MTSES.

**Immunoblotting**– Stably transfected cells were grown to confluence in 10 cm plates and were lysed using 1% Triton X-100. Total cellular protein extracts (30 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF Hybond-P™ membranes (Amersham Pharmacia Biotech). The blots were rinsed briefly with PBST (1 x PBS containing 0.1% Tween 20), blocked with 5% non-fat skim milk in PBST, and then incubated with a murine monoclonal anti-HA antibody (dilution 1:5,000). Following extensive washing with PBST, blots were incubated with goat anti-murine IgG secondary antibody conjugated with horseradish peroxidase (dilution 1:5,000). Immunoreactive bands were visualized using enhanced chemiluminescence (Perkin Elmer) and recorded on X-ray film.

**Statistics**– The data was analyzed using Origin and Microsoft Excel. The data from the sodium affinity, drug resistance and pH profile experiments was fitted with a Hill function, while the EIPA and pH profile data was fitted with a dose-response function. Unless otherwise stated, error bars represent the standard error of the mean and statistical analysis was performed by using the one-way ANOVA Tukey post hoc test, with a significance level of 0.05.

**RESULTS**

**Characterization of NHE1$_{\Delta C}$ and Single-Substituted Cysteine Mutants**: To further explore the structure-function domains of NHE1 in the M9-EL5 region (the nomenclature used will follow the Wakabayashi model illustrated in Fig. 1A), we used the substituted-cysteine accessibility method (73). This method relies on covalent interaction between the ionized sulfhydryl group of cysteines located in a water-accessible environment, such as the pore, with membrane-impermeant sulfhydryl-reactive reagents that have the potential to irreversibly impair transmembrane ion fluxes by either steric blockage or charge attraction/repulsion. Such covalent modifications might also hinder critical conformation changes that occur during cation translocation either at the cation pore itself or at a more distant site that nevertheless impacts cation translocation.

To this end, a cysteine-less hemagglutinin (HA) epitope-tagged version of rat NHE1 (NHE1$_{\Delta C_{HA}}$) was constructed by replacing the eight native cysteines in NHE1 (indicated in Fig. 2A) with serine, a conservative substitution that maintains amino acid polarity and side-chain length and hence minimizes potential structural perturbations of the transporter. The NHE1$_{\Delta C_{HA}}$ was stably expressed in mutagenized Chinese hamster ovary AP-1 cells devoid of endogenous NHE1 as previously described (75). Levels of protein expression for NHE1$_{HA}$ wild-type (WT) and $\Delta C$ are displayed in the inset of Fig. 2B. Previous studies demonstrated that the slower migrating band at $\sim$100 kDa represents the fully-glycosylated form of the protein present at the cell surface, whereas the faster migrating band at $\sim$75 kDa represents the partially processed or core-glycosylated form of the protein that is largely retained in the endoplasmic reticulum (79). Importantly, substitution of the cysteine residues with serine did not impair the biosynthesis and maturation of the transporter.

We next compared the kinetic properties of the WT and $\Delta C$ transporters. The initial rates of NHE1 activity as a function of the Na$_o$ concentration were examined using solutions containing tracer amounts of the radioisotope $^{22}\text{Na}^+$ added to Na$_o$ concentrations ranging from 2.5 to 120 mM following an imposed NH$_4$Cl-induced intracellular acidification. The data for both WT and $\Delta C$ were best fit to a Hill equation and revealed an apparent sigmoidal dependence on the Na$_o$ concentration for both constructs, indicative of positive cooperative binding with comparable Hill coefficients of $\sim$1.5 and 1.7, respectively (Table 1). Replacement of the cysteine residues also did not alter its maximal velocity ($V_{\text{max}}$, nmol/min/mg protein: WT, 105.5 ± 1.0; $\Delta C$, 105.0 ± 0.7), but did cause a reduction ($\sim$36%) in its affinity for Na$_o$ ($K_{\text{Na}}$: WT, 29.5 ± 0.1; $\Delta C$, 46.1 ± 0.9; $p < 0.05$) (Fig. 2B, Table 1). To determine their affinities for intracellular H$, the H$_i$ concentration was adjusted by clamping pH$_i$ at specific levels within the range of 5.4-7.4 using the K'/H$ ionophore nigericin, and measuring the rate of $^{22}\text{Na}^+$ influx. As shown in
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Fig. 2C, the WT and ΔC transporters exhibited similar affinities for $H^+$ ($K_{\text{i}, pH}$: WT, 6.14 ± 0.05; ΔC, 6.26 ± 0.03; $p > 0.05$). On the other hand, ΔC showed an approximate 8-fold decrease in sensitivity to the pharmacological antagonist EIPA ($IC_{50}$, nM: WT, 5.4 ± 1.2; ΔC, 40.7 ± 10.4; $p < 0.05$) (Fig. 2D). Based on these data, the endogenous cysteines moderately influenced, either directly or indirectly, the $Na^+$ and drug affinities of the transporter, but they were not structurally essential. Hence, NHE1 ΔC could serve as a suitable template for further cysteine-substitution mutagenesis and functional analyses.

To rationalize potential targets for cysteine substitution, certain sites in M9 (Glu350, His353 and Gly356) (49,55,56) were selected based on their known involvement in conferring sensitivity to amiloride- or benzoylguanidinium-based compounds that act as either simple or mixed competitors of $Na^+$ binding and therefore are likely in close proximity to the permeation pathway. Additional amino acids in M9 and EL5 were chosen based on their conservation amongst the plasmalemmal NHE isoforms (NHE1-NHE5) (Figs. 3A and 4A), their relative predicted position in a transmembrane α-helix (Fig. 3B), and their hydrophilic nature which likely orients them in an aqueous environment and potentially accessible to the thiol-modifying reagents. In total, 20 single-cysteine substitutions were generated in M9 (S342C, Y343C, Y346C, S348C, E350C, H353C, S355C, G356C, A359C) and EL5 (Y370C, N374C, S376C, T381C, Y385C, S392C, E395C, L397C, G403C, S405C, W415C). The resulting constructs were then stably expressed in AP-1 cells based on their ability to confer cell survival followed repeated intracellular acid loads that are lethal to AP-1 cells lacking a functional transporter.

To make initial estimates of the relative activities of parental and single-cysteine substituted mutants of NHE1 ΔC in stably transfected cells, the cellular rates of amiloride-sensitive $H^+$-activated $22Na^+$ influx were measured in the presence of nominal non-radioactive $Na^+$ (~120 nM $22NaCl$, carrier- free; 5 min uptake) and then normalized for the level of fully glycosylated NHE1 protein at the cell surface as determined by immunoblotting and densitometry. Levels of protein expression for NHE1 ΔC and the single-cysteine substituted mutants are displayed in Fig. 3C and Fig. 4B. Within M9, the relative activity levels of the single cysteine-substituted mutants were generally higher (i.e., ~2 fold) than the parental NHE1 ΔC (Fig. 3C), with the notable exception of S348C which, while not highly expressed, exhibited an apparent 24-fold increase in its rate of transport. However, because of its low level of expression, the calculated rate of activity per unit NHE1 protein is more subject to error and hence the determined value may overestimate its actual rate of transport. By comparison, the majority of the mutants in EL5 exhibited lower rates of transport with exception of L397C which displayed a 2.5-fold increase, though its expression level was also quite low which made an accurate assessment of its relative rate of transport more difficult (Fig. 4B). These initial findings implicate both regions as important elements in cation translocation.

MTS Inhibition of the M9 and EL5 Single-Cysteine Mutants- To further probe the involvement of these regions in cation permeation, the cysteine-substituted transporters were subjected to chemical modification using the membrane-impermeant sulfhydryl-active reagents MTSET and MTSES that are positively- and negatively-charged, respectively, and assessed for their effects on NHE1 activity. The activities of the majority of the M9 mutants were unaffected by the MTS-derivatives, suggesting that the sites are either inaccessible to these reagents or alternatively that they did react with the compounds but that this did not lead to inhibition (Fig. 3D). However, three mutants containing neighbouring substitutions, H353C, S355C and G356C showed approximately 40%, 30% and 98% inhibition of activity, respectively, following treatment with MTSET (Fig. 3D). The negatively-charged MTSES caused a similar reduction (~30%) in the activity of the S355C mutant, but had a considerably lesser effect on H353C and G356C. Since both reagents are similar in size, the differing degrees of inhibition caused by the respective MTS reagents are likely due to charge effects rather than steric hindrance. Collectively, these data suggest that amino acids His353, Ser355, and Gly356 (equivalent to hNHE1 His349, Ser351, and Gly352) comprise an accessible segment that
faces an aqueous environment and are potentially involved in cation translocation.

Likewise, the majority of the EL5 mutants were unaffected by exposure to the MTS-derivatives, with the exceptions of G403C and S405C which showed significant reductions (~30% and 70%, respectively) in 22Na+ influx in the presence of MTSET, and to a lesser extent in the presence of MTSES (Fig. 4C).

Kinetic Properties of Mutants Sensitive to Thiol-Modification- Having identified sites where cysteine substitutions confer sensitivity to thiol-reactive compounds, as revealed by reductions in transport activity, we next investigated whether the mutations alone (i.e. without MTS treatment) influenced the kinetic properties of the exchanger.

For sites in M9 (i.e., H353C, S355C, and G356C), calculation of their maximal velocities as a function of the external Na+ concentration (Fig. 5A-C) revealed a significant reduction in transport for H353C (~42%) (V_max, nmol/min/mg protein: 60.9 ± 3.1; p < 0.05) and to a lesser extent for G356C (~20%) (84.3 ± 3.1; p < 0.05), whereas the apparent V_max for S355C increased by ~36% (S355C, 143.1 ± 3.0; p < 0.05) compared to control NHE1 ΔC (V_max: WT, 105.0 ± 0.7). The curve obtained for H353C also displayed a reduction in cooperative Na+ binding (Hill coefficient (n): ΔC, 1.73 ± 0.02; H353C, 1.13 ± 0.02; p < 0.05), whereas those for S355C (n = 1.72 ± 0.01) and G356C (n = 1.62 ± 0.04) were unchanged (Table 1). However, calculation of the apparent Na+ affinity constants (K_Na) for H353C and G356C yielded values similar to that determined for the parental ΔC construct (K_Na, mM: ΔC, 46.1 ± 0.9; H353C, 43.6 ± 2.6; G356C, 40.1 ± 1.6) (Table 1), suggesting that these amino acids, while important for maximal transport velocity, are not critical determinants of Na+ binding. By contrast, the transport velocity of the S355C mutant did not approach saturation within the Na+ concentration range tested and exhibited a significant reduction (~53%) in Na+ affinity (K_Na, 70.7 ± 0.6 mM, p < 0.05) (Table 1). This finding is particularly striking given the conservative nature of the S355C substitution, and therefore implicates Ser355 as an important determinant of Na+ transport. On the other hand, measurement of the intracellular H+ affinities of H353C, S355C and G356C were equivalent to parental ΔC (K_H, pH: ΔC, 6.26 ± 0.03; H353C, 6.20 ± 0.07; S355C, 6.37 ± 0.11; G356C, 6.39 ± 0.13; p > 0.05) (Figs. 5D-F; Table 1). Collectively, these findings suggest that cysteine substitutions at these sites have a preferential impact on V_max, with certain sites also affecting the cooperativity (H353C) or affinity (S355C) for Na+ binding.

To further examine the importance of Ser355 within the context of WT NHE1 (as opposed to the ΔC variant), Ser was replaced with Ala (S355A), a non-polar residue whose side-chain length is comparable in size. This mutant was properly synthesized and processed at levels comparable to WT, ΔC and S355C (Fig. 6A). However, unlike S355C, its maximal velocity was decreased slightly, albeit significantly, compared to WT (V_max: WT, 105.5 ± 1.1; S355A, 89.9 ± 1.0; p < 0.05). Nevertheless, S355A, like S355C, showed a significant reduction (~73%) in Na+ affinity (K_Na, mM: WT, 29.5 ± 0.1; S355A, 50.8 ± 1.0, p < 0.05) as well as a decrease in cooperative Na+ binding (Hill coefficient (n): WT, 1.53 ± 0.01; S355A, 1.13 ± 0.02; p < 0.05) (Fig. 6B and Table 1). S355A was also more active at more alkaline pH values compared to WT, resulting in an apparent 58% increase in H+ affinity (K_H, pH: WT, 6.14 ± 0.05; S355A, 6.34 ± 0.03; p < 0.05) (Fig. 6C and Table 1). Thus, these findings corroborate a critical role for Ser355 in cation translocation.

Kinetic analyses of the two MTS-sensitive mutants in EL5, G403C and S405C, revealed decreases in their maximal velocities, especially for S403C (V_max: ΔC, 105.0 ± 0.7; S403C, 25.6 ± 0.7; S405C, 89.0 ± 2.3, p < 0.05) (Fig. 7A, B; Table 1). In the case of G403C, this was accompanied by a marked reduction in its affinity for H+ (i.e., acidic shift) (K_H, pH: ΔC, 6.26 ± 0.03; G403C, 5.82 ± 0.04, p < 0.05) (Fig. 7E and Table 1) while its affinity for Na+ was apparently unaffected (K_Na, mM: ΔC = 46.1 ± 0.9; S403C = 41.9 ± 1.2, p > 0.05) (Fig. 7A; Table 1). By contrast, S405C exhibited reductions in affinities for both Na+ (K_Na, mM: ΔC = 46.1 ± 0.9; S405C, 68.0 ± 3.4, p < 0.05) and H+ (K_H, pH: ΔC, 6.26 ± 0.03; S405C, 6.00 ± 0.04, p < 0.05 (Fig. 7B, F; Table 1). Thus, Gly403 and Ser405 are also important for cation binding and permeation.

The majority of the other Cys substitutions in the predicted re-entrant loop EL5 did not confer
sensitivity to the MTS compounds. Nevertheless, we explored whether some of these sites (i.e., Y370C, N374C, S376C, T381C, Y385C, S392C, E395C and L397C) could contribute to substrate affinities in some cases to their sensitivity to inhibition by EIPA. The majority of the mutations caused marked reductions in $V_{\text{max}}$ and cooperativity of Na$^+$ binding (Table 1). We were unable to determine the kinetic properties of the N374C and L397C mutants because while their velocities increased as a function of the Na$^+$ concentration, the values were erratic and did not display saturable binding kinetics (data not shown). Amongst these mutations, only Y370C and E395C exhibited marked reductions in Na$^+_o$ affinity ($K_{Na}$ mM: ΔC, 46.1 ± 0.9; Y370C, 92.8 ± 2.0, $p < 0.05$; E395C, 65.7 ± 3.6, $p < 0.05$) (Fig. 7C, D; Table 1), but no significant shift in H$^+$ affinity (Fig. 7G, H; Table 1). When analyzed for sensitivity to inhibition by EIPA, Y370C exhibited a significant 4-fold increase ($K_{0.5}$ nM: ΔC, 40.7 ± 9.1, $n = 11$; Y370C, 9.6 ± 3.2, $n = 8$; $p < 0.05$) (Fig. 8) whereas E395C was not significantly altered ($K_{0.5}$ nM: 67.6 ± 17.3, $n = 8$; $p > 0.05$). This suggests that while the Y370C and E395C mutants were unaffected by the MTS derivatives, the sites nevertheless contributed to cation permeation and partly to drug sensitivity.

**DISCUSSION**

In this study, the substituted-cysteine accessibility method (73) was applied to rNHE1 to identify candidate amino acids in two segments of the transporter - the predicted transmembrane helix M9 (rNHE1 342-363; equivalent to hNHE1 338-359) and the adjoining exofacial re-entrant loop between M9 and M10 (EL5; rNHE1 364-415 or hNHE1 360-411) - that had previously been implicated in conferring sensitivity to competitive antagonists of Na$^+_o$ binding and therefore may potentially line the cation permeation pathway of the transporter (49,56,60). The data show that of the twenty cysteine substitutions made at a select number of known drug-sensitive or evolutionarily-conserved sites, only a limited number of these in M9 (i.e., rat H353C, S355C, G356C; human H349C, S351C, G352C) and EL5 (i.e., rat G403C and S405C; human G399C and S401C) caused marked reductions in NHE1 activity upon modification with MTS sulfhydryl reagents. Significantly, kinetic measurements of unmodified rNHE1 S355C, G403C and S405C as well as two other substitutions in EL5 (rat Y370C and E395C; human Y366C and E391C) showed reduced affinities for Na$^+_o$ and/or H$^+$. Interestingly, the Y370C mutant, while possessing a 2-fold decrease in Na$^+$ affinity, exhibited a 4-fold increase in affinity for the competitive antagonist EIPA. This Tyr residue is highly conserved in the mammalian NHEs and therefore it is tempting to speculate that it may serve as a common binding site for Na$^+_o$ and sidechain substituents of amiloride derivatives. Other cysteine substitutions within EL5 also significantly impaired the maximal activity of the transporter, though they did not alter the apparent affinities for Na$^+_o$ or H$^+$, suggesting that they may fulfill other structural roles in ion translocation. Collectively, these data implicate these amino acids as important elements involved in cation permeation and drug sensitivity.

Amongst the three MTS-sensitive residues within M9, the G356C mutation was the most reactive. The activity of G356C was almost completely inhibited (~98%) in the presence of positively-charged MTSET. Likewise, transport activity was also significantly reduced by negatively-charged MTSES, although to a lesser extent (~40%). Mechanistically, thiol-modification of G356C may block cation transport by sterically hindering critical conformation changes that occur during cation transport, either at the cation pore itself or at a more remote site that nevertheless influences cation translocation. However, the observed sizeable differences in the degree of inhibition elicited by the two oppositely-charged, but similarly-sized, MTS reagents also implicates the involvement of electrostatic forces on the flow of cations through the protein, suggesting that Gly$^{356}$ is more likely in close proximity to the cation translocation pathway. Thus, aside from possible steric effects, the positive charge of MTSET could further impede the flow of Na$^+$ by electrostatic repulsion, whereas the negatively-charged MTSES reagent could act as an attractant and retard passage of Na$^+$ ions through the pore, but with less efficiency than the repelling effects of MTSET. We have also reported (49) that this residue is an important determinant of the high sensitivity of NHE1 to inhibition by both amiloride- and benzylguanidinium-based compounds, further
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Aside from G356C, cysteine-substitution of the two adjacent N-terminal residues, H353C and S355C, also rendered the transporter moderately sensitive to inhibition by the MTS reagents. The reactivity of S355C agrees with an earlier study showing that the equivalent mutation in a cysteine-less variant of hNHE1, S351C, is also sensitive to MTS (60). The lesser reactivity of these two residues to MTS reagents might be expected if they lie deeper within the membrane, as predicted by the Wakabayashi model (see Fig. 9A) and their thiol side-chains would be directed more towards the lipid bilayer, assuming 3.6 amino acids per turn of an α-helix relative to position Gly356 and thereby making them less available for modification by the MTS reagents. This might also explain why MTS reagents did not have a detectable effect on rNHE1 mutants containing cysteine-substitutions N-terminal to His353, though one previous study (60) reported that E350C (equivalent to hE346C) was sensitive to MTSET. The basis for the variance is unclear.

While His353 and Ser355 are hydrophilic residues, it is perhaps initially unexpected to find that a hydrophobic residue such as Gly356 would also directly face the aqueous pore. However, since Gly contains a hydrogen atom as its side-chain group, it is only moderately nonpolar and confers flexibility to the peptide backbone structure that may be important for conformational changes that ensue during cation translocation. Moreover, previous studies have demonstrated that hydrophobic residues often found to line the translocation pores of transporters and channels where they are postulated to provide an inert surface that facilitates ion diffusion (80-82). Kinetic analyses, however, did not reveal significant changes in the cation affinities for H353C and or G356C, but did show a marked reduction in Na+ affinity for S355C which agrees with a previous observation (60). This finding was further corroborated in native NHE1, where substitution of Ser355 with Ala also reduced Na+ affinity by approximately 2-fold. Taken together, these data are consistent with the notion that M9, and these three residues in particular, constitute an integral part of the cation translocation pore.

The adjacent putative EL5 R-loop between M9 and M10 was also found to contribute significantly to transport activity and drug sensitivity. Indeed, manipulation of sites in EL5 often had more profound effects on transporter function than sites in M9. The R-loop shares structural similarities to P-loops that are known to invaginate into the membrane bilayer and facilitate ion flow through channels and pumps (83-85). These structural elements are responsible for mediating antagonist binding, ion selectivity, and conductance. Regarding NHE1, the vast majority of single-cysteine substitutions in EL5 significantly decreased maximal transport velocity. In most cases (i.e., Y370C, S376C, Y385C, E395C, G403C and S405C), these changes were associated with a reduction in positive cooperativity of Na+ binding, as defined by the Hill coefficient. Previous kinetic analyses of the Na+ dependence of the NHEs have provided mixed results with some studies reporting simple Michaelis-Menten kinetics (86,87) while others describing cooperative activating effects of Na+ (37,39,88,89). While the basis for these differences is not fully understood, part of these differences may relate to assay conditions and/or cell type. In a detailed study by Fuster et al. (89), they observed that the Hill coefficient for extracellular Na+ dependence was dependent on cytoplasmic pH. Specifically, the extracellular Na+ concentration dependence was sigmoidal at a cytoplasmic pH of 7.2 with a Hill coefficient of ~1.8, whereas this cooperativity was diminished at more acidic values. The extracellular Na+ dependencies of NHE1 were explained equally by either a parallel or serial model of dimer coupling with a 2Na+/2H+ stoichiometry of the monomer, with cooperative Na+_o binding dependent on the H+_i concentration. Our mutagenesis data suggest that EL5 may play an important role in the cooperative activating effects of Na+_o.

In the case of four mutants in EL5, Y370C, E395C, G403C and S405C, the changes in activity also correlated with marked reductions in Na+ affinity (Y370C, E395C, and S405C) and/or H+ affinity (G403C and S405C). Both the G403C and S405C mutants were also sensitive to modification by MTS reagents, suggesting that they were accessible from the extracellular medium. Furthermore, the Y370C mutation exhibited increased sensitivity to inhibition by EIPA. While other mutations in this region did not appear to affect ion or drug binding, the impairment in V_max...
suggests that these sites may nevertheless be important for the ensuing conformational changes required for optimal transport activity.

Presently, two different transmembrane topologies have been postulated for NHE1. The first model developed by Wakabayashi et al. (36) using the substituted-cysteine accessibility mutagenesis approach predicted twelve membrane-spanning helices with M9 encompassing amino acids 342-363 of rat NHE1 (human 338-359) followed by a re-entrant loop structure extending from amino acids 364 to 415 (human 360-411), as illustrated in Figs. 1A and 9A. Subsequently, Landau et al. (65) proposed a different helical arrangement (Fig. 1B and 9B) based on comparisons of evolutionary conserved sequences and secondary structure predictions, and then mapped the optimized alignment to the crystal structure of the E. coli NhaA antiporter. In this alternate model, the N-terminal segment encompassing the original helices M1 and M2 (residues ~1-150) was located instead in the cytoplasm and postulated to serve as part of a truncated signal sequence, with the first transmembrane segment commencing at the former M3 helix. However, this seems unlikely given that the predicted EL1 segment between M1 and M2 is known to undergo N- and O-linked glycosylation (70) as it transits through the endoplasmic reticulum and Golgi compartments and therefore should be oriented extracellularly when the transporter is inserted into the plasma membrane. The M9 helix was also rearranged to form two short helices renamed M7 (amino acids 335-348) and M8 (amino acids 353-366), while the neighboring extracellular re-entrant loop 5 (EL5) was predicted to form a new intracellular loop (amino acids 367-377) and new M9 helix (amino acids 378-402) that was predicted to be largely inaccessible to external reagents, followed by residues that again were accessible to external reagents (amino acids 403-418).

The results from our analyses are partially consistent with both models, but overall are better accounted for by the Wakabayashi model. The susceptibility of both G403C and S405C to modification by MTS reagents places these residues in an environment that is accessible to the extracellular milieu, in agreement with both models. The MTS-sensitivity of H353C, S355C and G356C, with the latter residue being the most reactive, also positions these amino acids extracellularly and possibly facing the external funnel of the ion permeation pathway. Based on the hierarchy of their MTS-reactivity, these amino acids could be positioned according to the Wakabayashi model, but seem at odds with the Landau model which places Gly356 deep within the membrane. We further found that mutations to Ser355 affected substrate affinities, especially for Na+, and maximal transport velocity, consistent with an earlier finding (60). This suggests that this residue faces the ion pore and is catalytically important. In the Landau et al. model (65), the side-chain of Ser355 was oriented into the lipid bilayer which, in principle, would make it less accessible. However, Landau et al. (65) postulated that this helical segment could potentially rotate 180° around its axis to allow this residue to participate in cation permeation. Notwithstanding, the new proposed orientation of this segment also placed Tyr370 within an intracellular loop. Such a position would make it more difficult to account for our observation that Tyr370 is involved in external Na+ binding and drug sensitivity. These data are explained more simply by positioning this residue in the extracellular milieu, but which is unaffected by MTS reagents for reasons that remain obscure. Since Gly356 is also known to confer sensitivity to NHE antagonists (49), these residues may be in close proximity to each other. More recently, Reddy et al. (60) determined the structure of a peptide representing amino acids 342-369 (human 338-365) by high resolution nuclear magnetic resonance (NMR) in detergent micelles. The structure contained two helical regions (amino acids Met344-Ser348 and Ile357-Ser363) separated by a sharp, potentially flexible, segment (amino acids Ala349-Gly356) that bends immediately N-terminal to Ser355, resulting in a kinked “L”-shaped structure. If such an arrangement exists in the native transporter, then it has the potential to position Tyr370 as well as neighboring amino acids in an intramembranous, albeit externally-facing, environment that constitutes part of the pore funnel involved in ion coordination.

In summary, residues in M9 and EL5 comprise important structural elements for cation translocation in NHE1. As a crystal structure has yet to be achieved, additional biochemical, biophysical and structural analyses using
approaches that combine substituted cysteine accessibility mutagenesis with electron paramagnetic resonance spectroscopy and NMR may help to shed further structural and mechanistic insight into this catalytically important region of the transporter. Such information should prove valuable in the design and development of more efficacious NHE1-specific drugs to prevent or lessen tissue damage arising from certain cardio- and cerebrovascular diseases and cancer progression.

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Author Contributions: JO conceived and coordinated the study and JO and TJ wrote the paper. TJ designed, performed and analyzed the experiments shown in Figures 2D, 4B, 5A-C, 6-8. CBJ designed, performed and analyzed the experiments shown in Figures 2A-C, 3, 5D-F. AB provided technical assistance. JO, TJ and CBJ contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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**FOOTNOTES**

1The abbreviations used are: NHE/NHA, Na\(^+\)/H\(^+\) exchanger or antiporter; EIPA, ethylisopropylamiloride; AP-1, a chemically mutagenized Chinese hamster ovary cell line that is devoid of plasma membrane Na\(^+\)/H\(^+\) exchange activity; MTSES, [2-sulfonatoethyl] methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl] methane-thiosulfonate)
FIGURE LEGENDS

Fig. 1. Schematic illustration of the predicted membrane topology of mammalian NHE1. Transmembrane organization of NHE1 according to the models proposed by (A) Wakabayashi et al. (36) and (B) Landau et al. (65). The black shading highlights regions that differ between the two models.

Fig 2. Functional characterization of a cysteine-less variant of NHE1. A. Schematic representation of the membrane topology of rat NHE1 according to Wakabayashi et al. (36) and sites of endogenous cysteines replaced with serine to form the cysteine-less (\(\Delta C\)) NHE1. B-D, NHE1 wild type (WT) and \(\Delta C\) were stably expressed in AP-1 cells and assayed for their affinities for \(Na^+\) (B) and \(H^+\) (C) and their sensitivity to inhibition by ethylisopropylamiloride (EIPA) (D) according to protocols described in ‘Experimental Procedures’. Values represent the mean ± S.E. of 3-5 experiments, each performed in quadruplicate. Error bars smaller than the symbol are absent.

Fig. 3. Functional analysis of single cysteine substitutions in membrane-spanning segment M9. A, alignment of M9 sequences from rat (r) NHE1 to NHE5. Amino acids that are highly conserved are shaded in black while moderately conserved are shaded in gray. Asterisks indicate residues that were mutated to cysteine. B, helical wheel representation of M9 with hydrophobic and hydrophilic amino acids depicted by blue and red shading, respectively. C, western blot analysis displaying the protein expression levels of the cysteine-less NHE1HA (\(\Delta C\)) and the single-cysteine substituted mutations in M9. The slower migrating band represents the fully-glycosylated (fg) form of the protein, while the faster migrating band represents the core glycosylated (cg) form of the protein. D, NHE1 activity, defined as rates of amiloride-inhibitable \(H^+\)-activated \(^{22}\text{Na}^+\) influx, for WT, \(\Delta C\) and the single-cysteine substituted mutants in the presence of 10 mM MTSES or 1 mM MTSET. Results are expressed as a percentage of the uptake catalyzed by each mutant in the absence of the MTS compounds. Values represent the mean ± S.E. of at least 3 experiments, each performed in quadruplicate. Asterisks indicate statistical significance at the 0.05 level by Student’s t-test (\(p < 0.05\)).

Fig. 4. Functional analysis of single cysteine substitutions in the EL5 R-loop. A, alignment of the EL5 R-loop from rat (r) NHE1 to NHE5. Amino acids that are highly conserved are shaded in black while moderately conserved are shaded in gray. Asterisks indicate residues that were mutated to cysteine. B, western blot analysis displaying the protein expression levels of NHE1HA\(\Delta C\) (\(\Delta C\)) and the single-cysteine substituted mutations in EL5. C, NHE1 activity, defined as rates of amiloride-inhibitable \(H^+\)-activated \(^{22}\text{Na}^+\) influx, for WT, \(\Delta C\) and the single-cysteine substituted mutants in the presence of 10 mM MTSES or 1 mM MTSET. Results are expressed as a percentage of the uptake catalyzed by each mutant in the absence of the MTS compounds. Values represent the mean ± S.E. of at least 3 experiments, each performed in quadruplicate. Asterisks indicate statistical significance at the 0.05 level by Student’s t-test (\(p < 0.05\)).

Fig. 5. Kinetic analyses of NHE1 MTS-responsive mutants in helix M9. NHE1 \(\Delta C\) and mutants H353C, S355C and G356C were stably expressed in AP-1 cells and assayed for their affinities for \(Na^+\) (A-C) and \(H^+\) (D-F) according to protocols described in ‘Experimental Procedures’. Values represent the mean ± S.E. of 3-9 experiments, each performed in quadruplicate. Error bars smaller than the symbol are absent.

Fig. 6. Kinetic analysis of NHE1 mutant S355A. (A) NHE1 WT and S355A were stably expressed in AP-1 cells and their relative expression levels were compared to \(\Delta C\) and S355C by Western blot analysis. NHE1 WT and S355A were assayed for their affinities for \(Na^+\) (B) and \(H^+\) (C) according to protocols described in ‘Experimental Procedures’. Values represent the mean ± S.E. of 6-9 experiments, each performed in quadruplicate. Error bars smaller than the symbol are absent.
Fig. 7. Kinetic analyses of NHE1 mutants in re-entrant loop EL5. NHE1 ΔC and mutants Y370C, E395C, G403C and S405C were stably expressed in AP-1 cells and assayed for their affinities for Na⁺ (A-D) and H⁺ (E-H) according to protocols described in ‘Experimental Procedures’. Values represent the mean ± S.E. of 3-9 experiments, each performed in quadruplicate. Error bars smaller than the symbol are absent.

Fig. 8. Effect of Y370C on the drug sensitivity of NHE1. AP-1 cells stably expressing NHE1 ΔC or Y370C were grown to confluence in 24-well plates and their activities were measured in the presence of increasing concentrations of the NHE antagonist EIPA. Transport activity was measured as described in ‘Experimental Procedures’. Values represent the mean ± S.E. of 8 experiments, each performed in quadruplicate.

Fig. 9. Comparison of different models of the predicted transmembrane arrangement of the helix M9 and EL5 region of NHE1. Schematic representation of the transmembrane organization of NHE1 in the M9-EL5 region according to the models proposed by (A) Wakabayashi et al. (36) and (B) Landau et al. (65) that highlight functionally relevant amino acids identified in the present study. The red or green shading identifies sites that conferred sensitivity to MTS reagents or affected substrate affinities (Na⁺ and/or H⁺), respectively, when replaced with cysteine. Mutations of some sites affected both parameters and are dual labelled.
Table 1. Kinetic constants of wild-type and mutant NHE1.

| NHE1   | \( K_{\text{Na}} \) (nM) | \( \text{Na}^+ \) Hill coefficient | \( K_{\text{H}} \) (nM) | \( \text{Vmax} \) nmol/min/mg protein |
|--------|---------------------------|----------------------------------|-------------------------|------------------------------------|
| WT     | 29.5 ± 0.1 (9)            | 1.53 ± 0.01                       | 6.14 ± 0.05 (6)         | 105.5 ± 1.1                        |
| ΔC     | 46.1 ± 0.9*† (9)          | 1.73 ± 0.02                       | 6.26 ± 0.03 (6)         | 105.0 ± 0.7                        |
| H353C  | 43.6 ± 2.6 (9)            | 1.13 ± 0.02*                      | 6.20 ± 0.07 (3)         | 60.9 ± 3.1*                        |
| S355A  | 50.8 ± 1.0† (9)           | 1.13 ± 0.02†                      | 6.34 ± 0.03† (6)        | 89.9 ± 1.0†                        |
| S355C  | 70.7 ± 0.6* (9)           | 1.72 ± 0.01                       | 6.37 ± 0.11 (3)         | 143.1 ± 3.0*                       |
| G356C  | 40.1 ± 1.6 (9)            | 1.62 ± 0.04                       | 6.39 ± 0.13 (3)         | 84.3 ± 3.1*                        |
| Y370C  | 92.8 ± 2.0* (9)           | 1.21 ± 0.01*                      | 6.19 ± 0.03(5)          | 19.6 ± 0.2*                        |
| N374C  | n.d.                      | n.d.                             | n.d.                    | 31.6 ± 1.3*                        |
| S376C  | 58.2 ± 4.9 (9)            | 1.12 ± 0.03*                      | n.d.                    | 25.4 ± 3.9*                        |
| T381C  | 44.1 ± 3.8 (3)            | 2.01 ± 0.20*                      | n.d.                    | 30.5 ± 1.1*                        |
| Y385C  | 50.7 ± 1.9 (9)            | 1.42 ± 0.03*                      | 6.13 ± 0.05 (6)         | 53.2 ± 0.9*                        |
| S392C  | 41.5 ± 0.5 (9)            | 1.61 ± 0.02                       | 6.30 ± 0.02 (3)         | 19.8 ± 1.3*                        |
| E395C  | 65.7 ± 3.6* (10)          | 1.12 ± 0.02*                      | 6.19 ± 0.02 (4)         | 25.6 ± 0.7*                        |
| L397C  | n.d.                      | n.d.                             | n.d.                    | 89.0 ± 2.3*                        |
| G403C  | 41.9 ± 1.2 (9)            | 1.28 ± 0.02*                      | 5.82 ± 0.04* (12)       | 25.6 ± 0.7*                        |
| S405C  | 68.0 ± 3.4* (9)           | 1.25 ± 0.03*                      | 6.00 ± 0.04* (8)        | 89.0 ± 2.3*                        |

* indicates a significant difference of the mean from NHE1ΔC, where \( p < 0.05 \).
† indicates a significant difference of the mean from NHE1wt, where \( p < 0.05 \).
n.d., not determined
Values represent the mean ± S.E.M.
Figure 1

A Wakabayashi model

B Landau model
Figure 2

A

B

C

D

Determinants of cation and drug sensitivity of NHE1
Figure 3

A

B

C

D

Determinants of cation and drug sensitivity of NHE1
Figure 4

A

| M9   | R-Loop | M10 |
|------|--------|-----|
| 360  | 365    | 395 |
| 370  | 375    | 380 |
| 385  | 390    | 395 |
| 395  | 400    | 405 |
| 410  | 415    | 420 |

B

C

MTSES

MTSET
Determinants of cation and drug sensitivity of NHE1

Figure 5
Determinants of cation and drug sensitivity of NHE1

Figure 6

A

kDa

WT

ΔC

S355A

S355C

fg

cg

B

NHE1 Activity

(nmol/min/mg)

[Na⁺] mM

0

20

40

60

80

100

120

WT

S355A

C

NHE1 Activity (%)

5.2

5.6

6.0

6.4

6.8

7.2

7.6

pH_i

WT

S355A
Determined of cation and drug sensitivity of NHE1

Figure 7

A

B

C

D

E

F

G

H

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

NHE1 Activity (mmol/min/mg)

[Na+] mM

[Na+] mM

[Na+] mM

[Na+] mM

[Na+] mM

[Na+] mM

[Na+] mM

[Na+] mM

pH

pH

pH

pH

pH

pH

pH

pH

NHE1 (% Activity)

NHE1 (% Activity)

NHE1 (% Activity)

NHE1 (% Activity)

NHE1 (% Activity)

NHE1 (% Activity)

NHE1 (% Activity)

NHE1 (% Activity)

0 20 40 60 80 100 120

0 20 40 60 80 100 120

0 20 40 60 80 100 120

0 20 40 60 80 100 120

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6

5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6
Figure 8
Figure 9
Determinants of Cation Permeation and Drug Sensitivity in Predicted Transmembrane Helix 9 and Adjoining Exofacial Re-Entrant Loop 5 of Na+/H+ Exchanger NHE1

Tushare Jinadasa, Colin B. Josephson, Annie Boucher and John Orlowski

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