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**The Role of Histidine Residues in the pH_i Sensitivity of the
 Na^+/H^+ Exchanger**

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ABSTRACT

Na^+/H^+ exchanger (NHE) is a major contributor in controlling intracellular pH. The activity of this protein is allosterically modified by intracellular H^+ . Histidine residues of the NHE that face the cytoplasm may be involved in determining the intracellular pH set point, with their state of protonation influencing the rate of Na^+/H^+ exchange. To test this hypothesis, histidine residues in the ubiquitously expressed NHE isoform (NHE1) that are relatively conserved amongst members of the NHE gene family were substituted by site-directed mutagenesis and the mutants were stably transfected into mammalian cells that are deficient in endogenous Na^+/H^+ exchange activity. The pH_i sensitivity of each mutant was evaluated by measuring the rate of $^{22}\text{Na}^+$ influx as a function of the intracellular H^+ concentration. Mutation of the histidines located at the putative cytoplasmic face of the N-terminal transmembraneous domain of NHE1 did not show any significant effect on the pH_i sensitivity of the protein. By contrast, substitution of histidines located in the C-terminal cytoplasmic tail activated the exchanger by increasing its sensitivity to H^+ . These mutants were no longer activated in response to protein kinase C, when compared to wild type. Taken together, these data support the hypothesis that some of the relatively conserved histidine residues in the C-terminal cytoplasmic tail of NHE1 may be involved in determining the pH_i "threshold" or "set point" of the transporter.

RÉSUMÉ

L'échangeur Na^+/H^+ (NHE) contribue de façon importante au contrôle du pH intracellulaire (pH_i). L'activité de cette protéine est modifiée de manière allostérique par les protons intracellulaires. Les résidus histidines du NHE orientés vers le cytoplasme pourraient être impliqués dans la détermination du réglage du pH_i , leur état de protonation influençant le taux d'échange Na^+/H^+ . Afin de tester cette hypothèse, j'ai travaillé avec l'isoforme NHE1 qui est exprimée de manière ubiquitaire. J'y ai substitué par mutagenèse dirigée les résidus histidines qui sont conservés parmi les membres de la famille des gènes NHE. Les mutants ont été transfectés stablement dans des cellules de mammifères dont l'activité endogène de l'échangeur Na^+/H^+ est déficiente. La sensibilité au pH_i de chaque mutant a été évaluée en mesurant le taux d'influx de $^{22}\text{Na}^+$ en fonction de la concentration des protons intracellulaires. Les mutations des histidines localisées dans la région supposée cytoplasmique du domaine transmembranaire amino-terminal de l'NHE1 n'ont pas eu d'effet significatif sur la sensibilité de la protéine au pH_i . Au contraire, la substitution des histidines de la queue C-terminale cytoplasmique a activé l'échangeur en augmentant sa sensibilité aux protons. Par comparaison avec le type sauvage, ces mutants n'étaient pas activés en réponse à la protéine kinase C. Dans l'ensemble, ces résultats soutiennent l'hypothèse que certains des résidus histidines conservés dans la queue C-terminale cytoplasmique sont impliqués dans la détermination du seuil du pH_i ou du réglage du transporteur.

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INTRODUCTION

General Description and Historical Background of the Na⁺/H⁺ Exchanger

Eukaryotic Na⁺/H⁺ exchangers are a family of integral membrane proteins that catalyze the electroneutral exchange of extracellular sodium for intracellular proton. To date, six different isoforms of the protein have been identified by molecular cloning and functional expression studies (Orlowski, 1993; Yu et al., 1993; Levine et al., 1993; Bookstein et al., 1994).

The NHE isoforms, depending on the cell type and their membrane localization, participate in several diverse cellular functions, including regulation of intracellular pH (pH_i), control of cell volume, and in some epithelia, transcellular transport of salt, water and acid-base equivalents. The exchanger also plays a role in facilitating mitogenesis in response to growth factors [reviewed by (Grinstein et al., 1989), (Sardet et al., 1990)]. In addition, the mitochondrion-localized NHEs are believed to influence Ca²⁺ and volume homeostasis of this organelle (Numata et al., 1998; Garlid and Beavis, 1985).

The history of identification of NHE activity dates back to 1961 when Mitchell proposed the necessity for cation/proton exchangers in cells (Mitchell, 1961) and, later on, experimentally demonstrated their presence in mitochondria (Mitchell and Moyle, 1969). In 1972, Na⁺/H⁺ exchange activity was first demonstrated in the bacterium *Streptococcus faecalis* (Harold and Papineau, 1972). Subsequently, in 1976, Mürer *et al.* (Murer et al., 1976) revealed the presence of a mammalian NHE in brush border

membrane vesicles of rabbit small intestine and kidney. It was not until 1989 that the cDNA of the first isoform of mammalian Na^+/H^+ exchangers, namely NHE1, was cloned by Sardet and colleagues (Sardet et al., 1989). Their strategy was based on first developing an exchanger-deficient mouse fibroblast cell line, then transfecting these cells with human genomic DNA, followed by selection of cells that overexpressed a functional human exchanger by their ability to survive repeated "acid-loads" (refer to the "Experimental Procedures").

The existence of additional isoforms of the protein was suggested by noted variations in the hormonal regulation and pharmacological features of Na^+/H^+ exchange. Using the NHE1 cDNA as a hybridization probe, the epithelial isoforms NHE2 to NHE4 (Tse et al., 1992; Tse et al., 1993a; Wang et al., 1993; Orłowski et al., 1992), and the nonepithelial NHE5 isoform (Klanke et al., 1995; Baird et al., 1999; Sun et al., 1998) were subsequently cloned. NHE6 was recently identified and is believed to be involved in cation translocation in mitochondria (Numata et al., 1998).

Functional Characteristics

Intracellular pH regulation

As mentioned before, the rate of Na^+ influx by NHE depends on the intracellular pH; however, above neutral pH_i , the Na^+/H^+ exchange process is almost inactive. Once the cytoplasm is acidified, the exchanger becomes active in a positive cooperative manner that is fine-tuned by a putative intrinsic H^+ -sensor site. Different factors can alter this tuning by shifting the pH_i set-point towards more alkaline (e.g., growth factors or

osmotic cell shrinkage) or acidic values (e.g., ATP depletion), which render the protein more or less sensitive to the $[H^+]_i$, respectively.

Cell volume regulation

Cell volume is controlled by ion transport, followed by displacement of water across the cell membrane. The major ions that contribute to the osmolar gradients in tissues are Na^+ , K^+ , and Cl^- . If these ions were allowed to equilibrate unchecked across the plasma membrane, cells would tend to swell due to the Donnan osmotic component of nondiffusible intracellular macromolecules. In steady state, most cells maintain their volume primarily by controlling electrolyte levels via the Na^+/K^+ -ATPase (Macknight and Leaf, 1977; Grinstein et al., 1984). In the event of acute alterations in cell volume, other ion transporters play larger roles. In this regard, osmotic swelling of cells is prevented by loss of K^+ and Cl^- through ion channels, and cell shrinkage is counteracted by parallel operation of the Na^+/H^+ and the Na^+ -independent Cl^-/HCO_3^- exchangers (Cala, 1983; Grinstein et al., 1984). Cl^- influx also appears to be a secondary consequence of pH_i change induced by NHE (Cala, 1983; Grinstein et al., 1984). However, it should be noted that these transport mechanisms are not the exclusive regulators of cell volume (Hoffmann and Simonsen, 1989).

It has been shown that cell shrinkage causes an alkaline shift in the pH_i set point of the Na^+/H^+ exchanger, thereby activating transport (Grinstein and Rothstein, 1986; Grinstein et al., 1985). Little is known about the mechanism of perception of volume change by cells and transmission of this information to the operator antiporters.

Transepithelial transport of water and electrolytes

The composition of fluids bathing the two sides of epithelial cells is different. Na^+/H^+ exchanger is present in the apical and/or basolateral membranes of epithelial cells, where it is involved in transepithelial transport of sodium, as well as translocation of acid-base equivalents (Aronson, 1983; Nakhoul and Boron, 1988). The apical NHE (NHE3) isoform has been found to play an indirect role in HCO_3^- reabsorption by the proximal tubules of the kidney. Secondary active excretion of H^+ by the luminal NHE leads to the accumulation of HCO_3^- inside the cell, a process that is a consequence of hydration of CO_2 and is catalyzed by carbonic anhydrase. This accumulation of HCO_3^- creates an outward gradient for the HCO_3^- across the basolateral membrane. Cotransport of Na^+ and HCO_3^- across this membrane completes the process of HCO_3^- reabsorption [reviewed by Grinstein *et al.* (Grinstein *et al.*, 1989)].

NHE plays additional indirect roles in the translocation of other ions across different epithelia. In the epithelial cells that possess both Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers in their apical membranes, H^+ excretion can cause Cl^- reabsorption. In this case, the H^+ extrusion by NHE and the consequent intracellular HCO_3^- accumulation cause reabsorption of Cl^- into the cell. An increase in the intracellular Cl^- concentration adds up to the conductive gradient towards extrusion of Cl^- ions across the basolateral membrane (Aronson, 1983; Nakhoul and Boron, 1988).

Na^+/H^+ exchanger can also play a role in the transepithelial transport of weak organic acids. These acidic compounds are protonated by the H^+ ions that are excreted by

NHE across the luminal membrane. The resultant compounds, which are lipid-soluble, readily enter the cells by non-ionic diffusion mechanism (Aronson, 1983; Nakhoul and Boron, 1988).

In cells that possess a $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism, the NHE can also indirectly affect the intracellular Ca^{2+} homeostasis. By changing the intracellular Na^+ concentration, the NHE is expected to change the rate and potentially even the direction of $\text{Na}^+/\text{Ca}^{2+}$ exchange, thereby influencing the intracellular Ca^{2+} concentration (Shigekawa et al., 1996).

As mentioned above, NHE also influences the movement of water, which passively follows solutes, across the epithelial barriers.

Facilitation of cell proliferation

The Na^+/H^+ exchanger is involved in stimulation, or at least facilitation, of cell proliferation. This hypothesis was proposed following identification of Na^+/H^+ exchange-mediated alkalinization as one of the early events in the process of cell growth and development. The lines of evidence supporting the role of Na^+/H^+ exchange in cell proliferation [reviewed extensively by Shrode *et al.* (Shrode et al., 1997a)] include the following: 1) Na^+/H^+ exchange is activated by growth factors and mitogens; 2) cell proliferation is dependent on extracellular sodium; 3) cell proliferation can be induced by cytoplasmic alkalinization in the absence of mitogens; 4) amiloride and its analogues

(inhibitors of NHE) can inhibit cell proliferation; and 5) proliferation is reduced in cells that are devoid of Na^+/H^+ exchange function.

Nevertheless, there are some arguments against the “causative” role of NHE in cell proliferation and mitogenesis. For example, in the early studies that demonstrated the requirement of NHE for cell growth and proliferation, the experiments were often performed under conditions precluding the operation of other pH regulatory mechanisms, e.g., in the absence of bicarbonate. It was therefore plausible that the inability of the cells to proliferate reflected simply a sub-optimal intracellular pH, which is essential for appropriate function of most enzymatic operations. In support of this conjecture, antiporter-deficient mutants were found to grow at normal rates, provided that bicarbonate is included to support normal pH maintenance by alternative pathways (Kapus et al., 1994).

The information available at present suggests that although cytosolic pH may play a permissive role in cellular growth and proliferation, but it is neither a trigger nor an essential step in the mitogenic signal transduction cascade (Shrode et al., 1997a).

Tissue Distribution

Na^+/H^+ exchange activity exists in the surface of virtually all cell types, including prokaryotes and eukaryotes. In the case of polarized epithelial cells, the exchange process has been found in both basolateral and apical membranes, although not necessarily in the same cells (Mahnensmith and Aronson, 1985; Seifter and Aronson,

1986). Despite this ubiquitous expression, the population of transporters, estimated indirectly from the rates of transport, varies greatly among different cell types. For example, the level of Na^+/H^+ exchange per unit surface area in human blood lymphocytes is several hundred times more than in red blood cells (Grinstein et al., 1984; Escobales and Canessa, 1985). In some polarized cell types, even the distribution of the transporter in different parts of the membrane of the same cell is unequal; e.g., in hepatocytes, Na^+/H^+ exchange is abundant in the basolateral, but barely detectable in the canalicular membrane (Moseley et al., 1986). The presence of NHE has also been demonstrated in intracellular organelles such as mitochondria (Numata et al., 1998), renal endocytic membranes (Gurich and Warnock, 1986) and the phagosomal membrane of intact neutrophils (Grinstein et al., 1988).

The NHE1 isoform is ubiquitously expressed and fulfills a housekeeping role in controlling pH_i and cell volume. In polarized cells, NHE1 resides along the basolateral membrane. An exception is human placenta, where NHE1 is localized at the apical membrane (Kulanthaivel et al., 1992).

NHE2 to NHE5 isoforms have more restricted patterns of distribution that vary somewhat between species. For instance, in rabbit, NHE2 is present predominantly in the epithelium of kidney, intestine and adrenal glands but has a low level of expression in skeletal muscle (Tse et al., 1993a). In rat, the NHE2 mRNA is expressed mainly in small intestine, colon, and stomach, but at a much lower level in skeletal muscle, kidney, brain,

testis, uterus, heart, and lung (Wang et al., 1993). In polarized cells, this isoform is predominantly expressed at the apical membrane.

NHE3 has the most defined pattern of expression. In human (Brant et al., 1995), rat (Orlowski et al., 1992), and rabbit (Tse et al., 1992), it has been shown to be localized mainly to the apical membrane of renal and intestinal epithelium.

NHE4 transcript, in rat, is mostly abundant in stomach and at minor levels in several other tissues (Orlowski et al., 1992), including the collecting ducts of renal medulla (Bookstein et al., 1994) where it has been shown to play a major role in cell volume homeostasis.

NHE5 is expressed mainly in the brain (Baird et al., 1999) and, to some extent, in spleen and testes and skeletal muscles. NHE6 has a wide tissue distribution; however, it is more expressed in mitochondrion-rich tissues such as brain, skeletal muscles and heart (Numata et al., 1998).

Structural Features

General characteristics

The mammalian Na^+/H^+ exchanger isoforms are polypeptides of ~70-110 kDa (depending on the isoform and its state of glycosylation) that consist of two distinct structural and functional domains: an amino-terminal hydrophobic transmembraneous domain and a carboxy-terminal hydrophilic cytosolic tail. The specific roles of these two

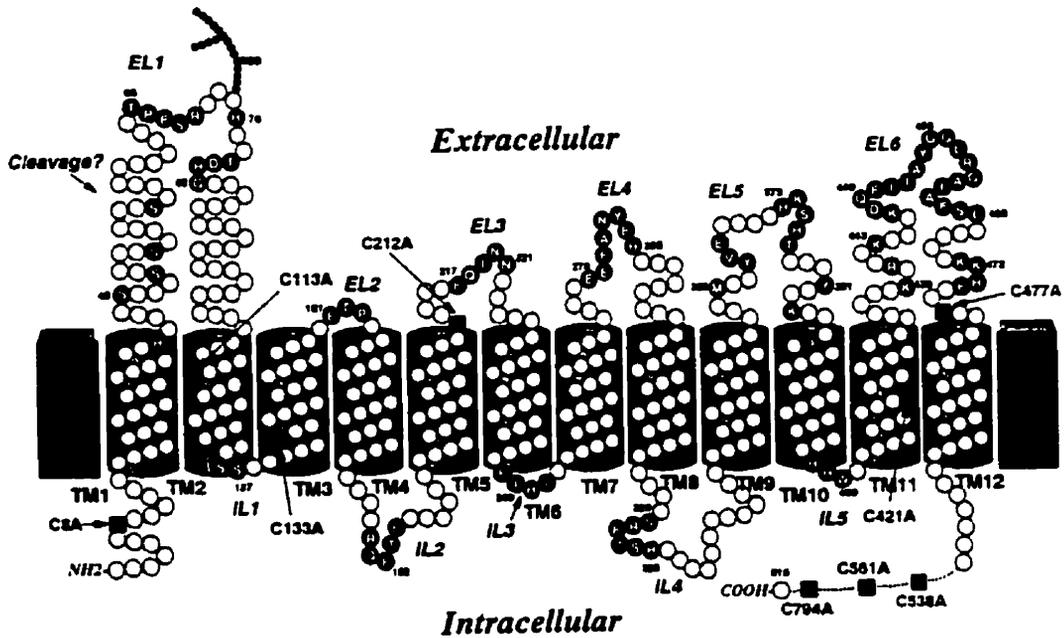
domains have been studied extensively by creating a series of deletion and point mutations. The N-terminal transmembraneous domain is considered to contain the cation-binding site, since it seems to have all the features required for catalyzing the exchange of cations across the cell membrane. By contrast, the cytosolic tail appears to function as the regulatory domain of the exchanger that is involved in determining the pH_i set point of the protein. Wakabayashi *et al.* demonstrated (Wakabayashi *et al.*, 1992a) that complete removal of the cytosolic domain preserved cation exchange activity and that the N-terminal domain is sufficient for both insertion of the antiporter into the plasma membrane and exchange of cations. However, this deletion markedly reduced the pH_i sensitivity of the protein and abolished the growth factor-induced cytoplasmic alkalinization. Furthermore, Winkel *et al.* (Winkel *et al.*, 1993) showed that microinjection of an antibody that was raised against the 157 amino acid stretch of the C-terminal end of NHE1 blocked activation of the protein by certain hormones. Studies of chimeric molecules between human NHE1 and the orthologous gene in trout, β -NHE (Boyarsky *et al.*, 1990), as well as chimeric molecules created by swapping the N-terminal and C-terminal domains of mammalian NHE1 and NHE3 (Wakabayashi *et al.*, 1995; Cabado *et al.*, 1996; Kandasamy *et al.*, 1995; Yun *et al.*, 1995) have also increased our understanding of the involvement of the C-terminal domain in regulating exchanger activity. For example, Wakabayashi *et al.* demonstrated that swapping the cytoplasmic tail of NHE1 with that of NHE3 can confer Ca^{2+} responsiveness to the latter isoform (Wakabayashi *et al.*, 1995). Similar results have been obtained by constructing chimeras of other NHE isoforms (Donowitz and Welsh, 1987). Comparable studies have also been performed with unrelated exchangers. For example, deletion of the N-terminal

cytoplasmic domain of the erythrocyte anion ($\text{Cl}^-/\text{HCO}_3^-$) exchanger, which is analogous to the C-terminal cytoplasmic tail of NHE, can retain the anion exchange activity (Kopito et al., 1989).

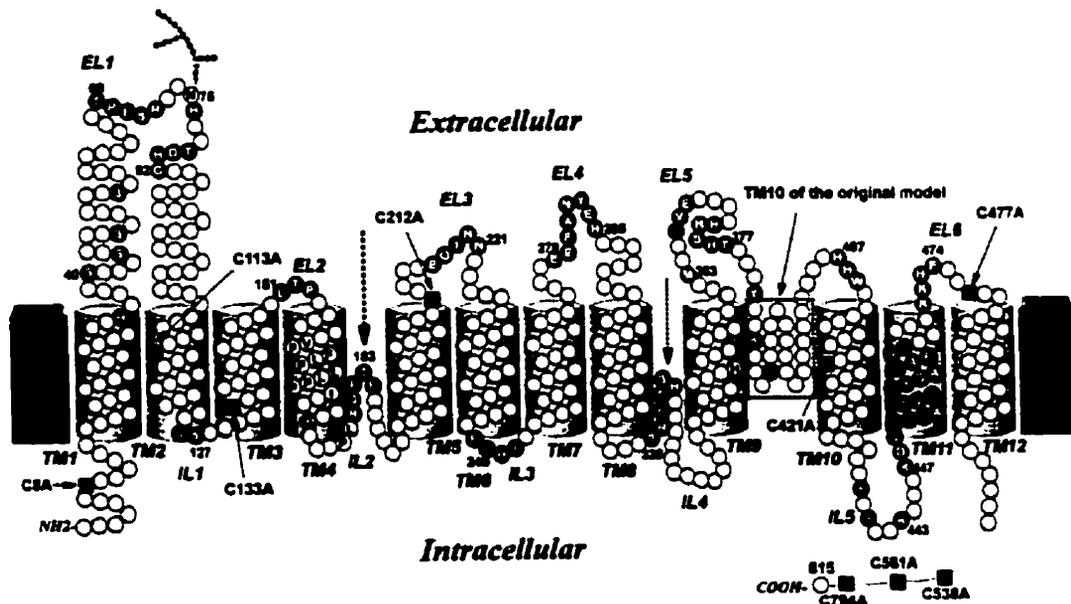
The size of NHE1 ranges from 815 to 820 amino acids amongst the species. The overall degree of amino acid identity between the isoforms is about 20-60%. The N-terminal domain has the highest percentage of identity (~60%) amongst the isoforms, consistent with this region playing a role in cation selectivity. In contrast, the C-terminal cytoplasmic tail exhibits the greatest divergence in amino acid sequence (~20% identity), which likely accounts for their variable responsiveness following activation of different signaling pathways. Sequence similarity suggests that NHE2 and NHE4 are phylogenetically more closely related to each other than to NHE1, and that NHE3 is more closely related to NHE5. NHE6, the mitochondrially-targeted isoform, is least homologous to other isoforms.

Membrane topology

The topology of the transmembrane domain of NHE is poorly understood but, based on hydropathy plot analysis and depending on which method is used, the polypeptide is predicted to span the membrane 12 times (Counillon et al., 1994; Dibrov and Fliegel, 1998; Orłowski et al., 1992) (Sardet et al., 1989). Wakabayashi *et al.* recently proposed a new model for the membrane topology of NHE1 that is based on the position of individual cysteine residues introduced into a functional cysteineless NHE1 mutant (Wakabayashi et al., 2000).



A topological model of NHE1 predicted from hydropathy analysis. This model is based on the hydropathy analysis of the primary amino acid sequence of NHE1. *EL1-EL6*, putative extracellular loops connecting Transmembrane (TM) loops 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12, respectively; *IL1-IL5*, putative intracellular loops connecting TMs 2 and 3, 4 and 5, 6 and 7, 8 and 9, and 10 and 11, respectively. [Obtained from Wakabayashi *et al.*, J Biol Chem, 275(11), 7942-7949, 2000].



The new topology model of NHE1, proposed by Wakabayashi *et al.* This model is based on the position of the position of individual cysteine residues introduced into a functional cysteineless NHE1 mutant. [Obtained from Wakabayashi *et al.*, J Biol Chem, 275(11), 7942-7949, 2000].

This model is different from the model derived from hydropathy analysis in several aspects. In this model, NHE1 is composed of 12 transmembrane domains with the N- and C- termini located in the cytosol. The large, last extracellular loop in the membrane domain of the original model is suggested to comprise an intracellular loop, a new transmembrane segment (transmembrane segment 11), and an extracellular loop, in the new model. They also demonstrated that cysteines at 183 and 184 and at 324 and 325 mapped to intracellular loops connecting transmembrane segments 4 and 5, and 8 and 9, respectively, were accessible to reagents from the outside. These results suggest that part of intracellular loops connecting transmembrane segments 4 and 5, and 8 and 9 may be located in a pore-lining region that is accessible from either side of the membrane and, therefore, involved in ion transport.

The most N-terminal sequence of NHE contains a putative signaling peptide that is presumed to be cleaved during protein maturation (Wakabayashi et al., 1992a). The central part of the N-terminal domain (the putative transmembranous domains 6 and 7 in NHE1) is highly conserved in all the NHE isoforms (95% similarity) and contains negatively-charged amino acids, suggesting that this region may constitute sites involved in cation translocation [for review see (Orlowski and Grinstein, 1997)]. In fact, it has been demonstrated in our laboratory that replacement of these amino acids with neutral or positively-charged residues renders NHE1 nonfunctional (unpublished data). Similar residues have been proven to play the same role in various ion transporters, including *E. coli* H⁺/lactose cotransporter (Kaback, 1988), the sarcoplasmic reticulum Ca²⁺ pump (Clarke et al., 1989), and the anion (Cl⁻/HCO₃⁻) exchanger (Jennings, 1989).

The C-terminal tail of NHE is hydrophilic and displays a low degree of similarity amongst isoforms. This region is inaccessible to extracellular antibodies (Sardet et al., 1990) and proteases (Shrode et al., 1998b) and is believed to be located in the cytosol. However, Biemesderfer *et al.* (Biemesderfer et al., 1998) demonstrated, by using an extracellular antibody, that a small segment of the cytosolic tail of NHE3 is in fact located outside the cell. More recently, Wakabayashi *et al.* (Wakabayashi et al., 2000) examined this possibility extensively and proposed a new model of NHE1 with 12 transmembrane segments with both the N- and C-termini oriented intracellularly.

Tertiary and quaternary structures

Not much is known about tertiary and quaternary structures of NHEs. There is some evidence suggesting that the NHE exists as a homodimer in the plasma membrane (Fliegel et al., 1993; Counillon et al., 1994); however, the exact contact sites remain to be determined. It has been proposed that the contact sites may reside in the N-terminal transmembranous domain and may be linked by disulfide bonding (Fafournoux et al., 1994).

Williams and co-workers (Williams et al., 1999) recently demonstrated, by electron crystallography, the two-dimensional structure of the bacterial Na^+/H^+ antiporter isoform A (NhaA) located in the inner membrane of *E.coli*. This study, which provided the first direct glimpse into the structure of a secondary transporter, indicated that the protein also spans the membrane 12 times. They also showed that NhaA exists in a tightly packed dimer form. Thus, while the bacterial transporter shares minimal sequence

identity to its mammalian homologues, they appear to share similar higher-ordered structural characteristics.

Glycosylation

NHE molecules are also subject to post-translational modification in an isoform-specific manner. There are several potential glycosylation sites in the Na^+/H^+ exchanger molecule. NHE1 appears to contain both *N*- and *O*-linked glycosylation restricted to its first N-terminal extracellular domain (Counillon et al., 1994). Rabbit NHE2 bears only *O*-linked glycosylation (Tse et al., 1994) and NHE3 does not seem to be glycosylated at all (Counillon et al., 1994; Biemesderfer et al., 1993). In NHE1 and NHE2, the first extracellular loop is glycosylated, which suggests an extracellular orientation. The state of glycosylation of NHE4, NHE5 and NHE6 remains to be revealed.

Drug Inhibition

The drug amiloride (a potassium-sparing diuretic) and its analogues are effective inhibitors of Na^+/H^+ exchangers, although their potency varies depending on the isoform. The mechanism of action of these drugs is not completely understood. Amiloride is a weak base with $\text{p}K_a$ of 8.7 and exists essentially as a monovalent cation within the physiological pH range. Kinetic analyses indicate that the drugs compete with Na^+ for binding at the extracellular side of the protein (Grinstein et al., 1989). This led to the suggestion that they may act at or near the Na^+ -binding sites. While mutagenesis studies have identified certain residues that reduce sensitivity to amiloride, they do not affect Na^+

affinity (Pouysségur et al., 1987). This suggests that the inhibitors likely bind to additional sites that are involved in transport.

Counillon *et al.* demonstrated that Phe165 and Phe167, which are located in the middle of the fourth putative transmembrane segment of NHE1, are critically involved in inhibition of NHE1 by MPA, an amiloride derivative (Counillon et al., 1993). They showed that mutation of these residues to leucine and tyrosine, respectively, greatly reduces the sensitivity of NHE1 to MPA.

The IC₅₀ value of NHE1 for MPA is 10- and 200-fold lower than those of NHE2 and NHE3, respectively (Counillon et al., 1993), and the IC₅₀ value of NHE1 for amiloride is 50-fold lower than that of NHE3. Interestingly, Counillon *et al.* demonstrated that replacing the two amino acids in NHE1 (Leu167-Phe168) with Leu-Tyr (NHE2 type) or Phe-Tyr (NHE3 type) conferred NHE1 resistance to inhibition by MPA and amiloride (Counillon et al., 1993).

Accordingly, Yun and coworkers (Yun et al., 1993) found that Leu143 in the 4th transmembrane segment of NHE2 is also critical for amiloride sensitivity. Putting these data together, it may be suggested that the middle region of the fourth transmembrane segment forms at least part of the amiloride binding site. More recently, Wang *et al.* (Wang et al., 1995) produced point mutations of His residues within the transmembrane helices of NHE1 and found that substitution of His349 with Gly or Leu causes a modest reduction (2-fold) in sensitivity to amiloride, whereas replacement with Tyr or Phe

increases drug sensitivity by a similar amount. Thus, this His residue within the transmembrane segment 9 may also be involved in the interaction of amiloride with NHE1. Orłowski and Kandasamy, in an attempt to define structural domains that confer differential sensitivity of the isoforms to antagonists, exploited the striking pharmacological differences and extensive sequence homology in the N-terminal transmembranous regions between NHE1 and NHE3 to create chimeric exchangers (Orłowski and Kandasamy, 1996). They demonstrated the transmembranous domain 9 may be a major site of interaction with the antagonists. More recently, random mutagenesis studies carried out in our laboratory, also demonstrated important residues in the putative transmembrane segments 4 and 9 that are involved in amiloride sensitivity of NHE1 (unpublished data).

Kinetic Properties

The Na^+/H^+ exchange process is driven by the relative concentration gradients of Na^+ and H^+ , without the direct requirement for ATP as an energy source. Modeling studies suggest that sodium/proton exchange occurs by a ping-pong mechanism; i.e., translocation of ions is performed individually and consecutively at separate steps. Therefore, existence of only one binding site for both ions would be sufficient for the exchange [reviewed by (Wakabayashi et al., 1997) and (Wakabayashi et al., 1992b)]. This putative single cation-selective binding site is suggested to perform the exchange function by making alternative changes in the conformation of the protein. As indicated earlier (the reader is referred to "Structural Features"), a good candidate for this single binding site is a highly conserved segment within the putative transmembrane segments 6

and 7 of NHE1 that contains several negatively-charged amino acids (refer to “Membrane Topology”).

Kinetic and thermodynamic studies indicate that the stoichiometry of Na^+/H^+ exchange is one-to-one, and therefore electroneutral (Aronson, 1985; Grinstein et al., 1984; Paris and Pouysségur, 1983). As such, the activity of the protein is not associated with any change in the membrane potential. In native systems, dependence of the rate of NHE1, NHE2 and NHE3 on the extracellular Na^+ concentration follows simple Michaelis-Menten hyperbolic kinetics, suggesting the involvement of a single Na^+ -binding site. The kinetics of the inhibitory effect of extracellular H^+ and Li^+ on Na^+ uptake also suggests interaction at a single site (Aronson, 1985). The affinity of the NHE isoforms for extracellular Na^+ ranges between 5 and 50 mM (Orlowski, 1993; Levine et al., 1993; Bookstein et al., 1996).

Because the stoichiometry is one-to-one, a single proton must therefore be translocated out of the cell in each cycle. However, the effect of intracellular H^+ on the rate of Na^+/H^+ exchange is sigmoidal, suggesting the existence of additional cytoplasmic H^+ -binding site(s) which sense the intracellular pH and allosterically modify the activity of the exchanger (Aronson et al., 1982). The concept of such a regulatory mechanism is consistent with inactivation of the protein above neutral pH_i , despite a large inward Na^+ gradient. This mechanism suggests the involvement of a “threshold” or “set point” that is a subject to change by the intracellular proton concentration. Below this pH_i threshold, protonation of the sensor/modifier site may alter the conformation of the protein and

stimulate the activity of the exchanger (Grinstein et al., 1989). Although the concept of this proton sensor site has been evoked as a major regulatory mechanism to explain the proton sensitivity of the Na^+/H^+ exchanger, other hypotheses for characterization of this cooperative pattern of regulation cannot be ruled out. For example, based on biochemical evidence that NHE exists as a homodimer, the increased affinity of the protein for intracellular proton can be attributed to the cooperative behavior of its monomer units (Fafournoux et al., 1994).

Regulation of Na^+/H^+ Exchanger

Na^+/H^+ exchange function is regulated by a wide variety of molecular signals. It is generally believed that most of the regulatory factors exert their effects through the cytoplasmic tail of the NHE (Ikeda et al., 1997; Wakabayashi et al., 1992a). A list of the main regulators of NHE and their postulated mechanism of action follows.

Calcium

Villereal and co-workers demonstrated that the calcium ionophore A23187 stimulates sodium influx in human foreskin fibroblasts (Owen and Villereal, 1982; Vicentini and Villereal, 1985; Villereal, 1981). These studies provided the first evidence that intracellular Ca^{2+} can stimulate the Na^+/H^+ exchanger. This stimulation can be either direct or indirect as a consequence of cell acidification. Calcium induces cell acidification by two mechanisms: 1) competition with protons for binding sites within the cell interior (Meech and Thomas, 1977), and 2) activation of the $\text{Ca}^{2+}/\text{H}^+$ exchange catalyzed by the plasma membrane Ca^{2+} pump (Niggli et al., 1982). The effect of

calcium in activating the exchanger can also be attributed to its direct interactions with calmodulin (Wakabayashi et al., 1994b).

Calmodulin

The cytoplasmic tail of NHE1 contains two putative calmodulin-binding domains: a high affinity calmodulin-binding site (CaM-A) with a K_d of ~20 nM and a low affinity calmodulin-binding site (CaM-B) with a K_d of ~350 nM (Bertrand et al., 1994; Wakabayashi et al., 1994b). These are amphiphilic regions rich in amino acids with basic side chains that are likely to assume an α -helical structure. The high affinity CaM-A domain (residues 636-656) is thought to have a role in regulating the NHE1 activity. Deletion of this segment renders the protein constitutively active, as if the intracellular Ca^{2+} concentration were constantly elevated. Therefore, it can be concluded that, at basal $[\text{Ca}^{2+}]$ levels, the unoccupied CaM-A binding domain exerts an autoinhibitory effect on NHE1 that is relieved upon ligand binding (Bertrand et al., 1994). Only NHE1 has been convincingly shown to be regulated by calmodulin. As Wakabayashi *et al.* (Wakabayashi et al., 1995) demonstrated, insertion of the CaM-binding domain of NHE1 into NHE3 conferred calcium sensitivity to the latter isoform.

Calcineurin B homology protein (CHP)

Another Ca^{2+} -binding protein that has been found to interact with and regulate NHE1 is calcineurin B homology protein (CHP) (Lin and Barber, 1996). The site of interaction of CHP with NHE1 is near the site of emergence of the cytoplasmic tail of the exchanger from the plasma membrane (residues 567-635) (Lin and Barber, 1996). It has

been shown that CHP can bind and inhibit NHE1 in its phosphorylated state, although the role of calcium in this process is not clear. CHP appears to be constitutively phosphorylated and stimulation of the exchange is accompanied by its dephosphorylation. This suggests that the phosphorylated form of CHP is normally associated with the exchanger and exerts an inhibitory effect, and that dissociation upon dephosphorylation may lead to activation of the exchanger.

Phosphorylation mechanisms

There is considerable evidence supporting the involvement of protein kinases in regulating the NHE isoforms. These include kinases that are linked to the G protein-coupled and tyrosine kinase receptor signaling pathways.

Sardet *et al.* were the first to demonstrate that NHE1 is a phosphorylated glycoprotein (Sardet *et al.*, 1990). They showed that two distinct mitogens, epidermal growth factor and thrombin, are able to increase the phosphorylated state of NHE1 in association with increased transport activity. Biochemical analysis revealed that this phosphorylation occurs exclusively on serine residues in NHE1, and that the phosphopeptide maps are the same with both epidermal growth factor and thrombin, suggesting that they both induce phosphorylation at the same sites.

A) protein kinase C (PKC) – Peptide hormone activation of G protein-coupled receptors leads to stimulation of different protein kinases, including protein kinase C. A large body of evidence suggests a role for PKC in activation of NHE1. Diacylglycerol (DAG) and phorbol esters, compounds that cause cell growth through PKC activation, are

able to stimulate NHE1 (Kandasamy et al., 1995; Moolenaar, 1986; Moolenaar et al., 1983), an effect that is abolished in PKC-depleted cells (Huang et al., 1987). Also, inhibitors of protein kinase C have been shown to prevent the activation of the exchanger (Lowe et al., 1990).

It has been proposed that protein kinase C directly phosphorylates and activates NHE1. The fact that phorbol esters increase the phosphorylation state of NHE1 in some cell, such as fibroblasts (Sardet et al., 1990) supports this assumption. Also consistent with this result, Wakabayashi *et al.* demonstrated that NHE1 has consensus protein kinase C phosphorylation sites that lie in the region of amino acids 636-815 (Wakabayashi et al., 1994a). In fact, Ser648, which is located in a putative PKC motif, had been suggested as the main candidate for direct phosphorylation of the protein by PKC. However, replacement of this residue with alanine caused no significant reduction in cytoplasmic alkalinization in response to thrombin and phorbol esters (Wakabayashi et al., 1994a). Recent studies have also provided evidence that the activity of NHE1 may not be regulated exclusively by direct phosphorylation: 1) part of the regulation by growth factors persists in truncated mutants lacking the major phosphorylation sites (Wakabayashi et al., 1992a; Wakabayashi et al., 1994a); 2) some stimuli activate the exchanger without detectable changes in the phosphorylation (Grinstein et al., 1992; Goss et al., 1994). In fact, in some cell lines, such as granulocyte HL-60 cells, phorbol esters cause a decrease, rather than increase, in the phosphorylated state of NHE1 (Rao et al., 1992). Therefore, growth factor-induced activation of NHE1 occurs in part by a mechanism that does not involve direct phosphorylation of the exchanger. It is

hypothesized that the phosphorylation-independent activation of NHE1 is mediated through an interaction of the region of amino acids 567-635 (known as the regulatory domain) with an “accessory or regulatory” protein, which then interacts with a H⁺ sensor located within the N-terminal membraneous domain (Wakabayashi et al., 1994a).

The mechanism by which protein kinases regulate the exchange activity is not completely understood. In most studies, NHE1 has been shown to be activated by an increase in its affinity for cytosolic H⁺ (Paris and Pouysségur, 1984; Moolenaar, 1986; Moolenaar et al., 1984; Moolenaar et al., 1983); however, Vigne *et al.* demonstrated a change in the maximal activity (V_{max}) in skeletal cells that expressed mainly NHE1 (Vigne et al., 1985). V_{max} alteration can be explained by either a change in the density of functional exchangers localized to the plasma membrane or an alteration in the turnover rate of the rate-limiting step of the exchange.

In contrast to NHE1, NHE3 is inhibited by PKC (Tse et al., 1993b). In fact, the PKC regulation of NHE3 is attributable to changes in the V_{max} of the exchange, rather than changes in the affinity for intracellular H⁺ (Tse et al., 1993b; Levine et al., 1993).

B) cAMP-dependent protein kinase A (PKA) – Unlike PKC, adenosine 3', 5'-cyclic monophosphate (cAMP) has been found to have either no effect (Grinstein et al., 1992; Wakabayashi et al., 1994a) or to stimulate (Azarani et al., 1995b) NHE1 activity depending on the cell type. For example, a rise of cAMP has been reported to activate NHE1 in Chinese hamster ovary cells (Kandasamy et al., 1995) and rat osteoblastic cells

UMR-106 (Gupta et al., 1994). A regulatory role for cAMP has also been demonstrated in murine macrophages (Kong et al., 1989), parotid rat acinar cells (Manganel and Turner, 1989), primary rat hepatocytes (Moule and McGivan, 1990), and rat brown adipose tissue (Giovannini et al., 1988). The mechanism of NHE1 activation by cAMP is unknown. Interestingly, mammalian NHE1 lacks obvious potential cAMP phosphorylation sites, suggesting that the effects may be indirect.

On the other hand, inhibition of Na^+/H^+ exchange by cAMP has been reported in specific cell types such as pig kidney LLC-PK₁ cells (Casavola et al., 1992) and opossum kidney (OK) cells (Cano et al., 1993; Casavola et al., 1992), with the latter known to endogenously express NHE3 (Azarani et al., 1995a). Cabado *et al.* demonstrated that agents known to increase cAMP levels inhibit NHE3 function (Cabado et al., 1996) in transfected Chinese hamster ovary cells. Subsequent studies showed that cAMP-dependent protein kinase down-regulates NHE3 in part by direct phosphorylation (Kurashima et al., 1997). In addition, ancillary factors such as NHE Regulatory Factors 1 and 2 (NHERF1 and 2) have also been implicated in cAMP-mediated inhibition of NHE3 (Weinman et al., 2000).

There are also some data supporting the existence of species differences in cAMP sensitivity of the Na^+/H^+ exchange. For instance, trout erythrocyte exchanger (β -NHE), which displays the strongest homology to NHE1, is known to be upregulated by cAMP and other activators of PKA in PS120 cells (Busch et al., 1995; Grinstein et al., 1985). In fact, the amino acid sequence of the cloned β -NHE contains two potential protein kinase

A phosphorylation sites within its cytoplasmic domain (Borgese et al., 1992). Removal of these phosphorylation sites by deletion or single or double mutation almost completely abolishes cAMP-mediated activation of the protein (Borgese et al., 1994) (Borgese et al., 1992). Furthermore, a substitution of human NHE1 C-terminal cytoplasmic region with that of β -NHE creates a chimeric exchanger that is sensitive to increased cAMP levels, indicating that the cytoplasmic region contains elements that confer hormonal regulation on the exchanger (Borgese et al., 1994).

C) ATP depletion – Na^+/H^+ exchange activity does not seem to directly require energy in the form of ATP; nevertheless, several studies have shown that cellular ATP depletion caused by metabolic inhibitors reduces the activity of the transporter (Cassel et al., 1986; Weissberg et al., 1989; Tse et al., 1991; Little et al., 1988; Brown et al., 1991; Burns et al., 1991; Goss et al., 1994; Wakabayashi et al., 1992a). In addition, Na^+/H^+ exchange in OK cells has been reported to be inhibited by acute phosphate depletion, which may lead to a reduction in cellular ATP pool (Green et al., 1993). Inhibition of the exchanger by ATP depletion may be the result of a reduction in the basal phosphorylation of the protein, considering that NHE1 is constitutively phosphorylated in resting cells. However, experiments have shown that acute ATP depletion does not decrease the level of phosphorylation of NHE1. The inhibition of NHE by ATP depletion is associated with either a reduced sensitivity of the exchanger for internal H^+ (Grinstein et al., 1989; Goss et al., 1994; Cabado et al., 1996; Cassel et al., 1986; Wakabayashi et al., 1992a) or a decrease in the V_{max} (Goss et al., 1994; Weissberg et al., 1989) or both (Kapus et al., 1994; Levine et al., 1993). In stably transfected CHO cells that are devoid of endogenous

Na^+/H^+ exchange activity (AP-1 cells) (Kapus et al., 1994) and PS-120 cells (Levine et al., 1993) expressing NHE1, NHE2, or NHE3 ectopically, an acidic shift in pH_i dependence and/or a decreased V_{max} have been reported to contribute to the inhibition of the exchanger activity following cellular depletion of ATP.

RATIONALE AND OBJECTIVES

The presence of Na⁺/H⁺ exchangers in all eukaryotic and prokaryotic cells studied thus far highlights the importance of this protein for proper cell function.

The Na⁺/H⁺ exchangers serve a diverse variety of functions, one of the most important of which is intracellular pH regulation. At physiologic intracellular pH, the antiport is largely quiescent, but becomes activated when the cytosol is acidified. This behavior, consistent with NHE's role in pH_i homeostasis, is primarily dictated by the pH_i set point (or threshold) of the protein. Since 1982 when Aronson and colleagues (Aronson et al., 1982) demonstrated the cooperative pattern of activation of NHE by intracellular protons, researchers have been trying to understand more about the processes that underlie this regulatory mechanism and locate the amino acids involved in this task. However, the advancements have not been overwhelming, in part because of the absence of a detailed three dimensional structure of the protein. Nevertheless, the development of heterologous cell expression systems lacking endogenous NHEs has facilitated investigations of the structure-function relationships of the NHEs without the complicating presence of other endogenous isoforms. Although the regulatory mechanisms expressed in this type of study may not be quite similar to what is seen in native systems, findings can still be incorporated in more advanced model systems.

As indicated, regulation of NHE often involves changes in its proton sensitivity and we were interested in defining residues that are involved in this process. Amino acid

histidine has been shown to play role in this task in different unrelated proteins that are sensitive to proton concentration (Gerchman et al., 1993; Echtay et al., 1998; Coulter et al., 1995; Morley et al., 1996; Ek et al., 1994).

The general objective of this thesis is to investigate the involvement of relatively conserved histidines of the Na^+/H^+ exchanger isoform 1 in determining the pH sensitivity of the protein. To accomplish this, we mutated those histidines predicted to reside on the cytoplasmic side of the plasma membrane by site-directed mutagenesis and tested the activity of the mutants at different intracellular proton concentrations. Then we examined the response of our mutants to protein kinase C, and compared it to that of the wild type NHE1.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free $^{22}\text{NaCl}$ (range of specific activity, 900-950 mCi/mg; concentration, ~10 mCi/ml) was obtained from NEN Life Science Products (Mandel Scientific, Guelph, Ontario). Amiloride, ouabain, and PMA (Phorbol 12-myristate 13-acetate) were purchased from Sigma Chemical Co. (St. Louis, MO). Nigericin was purchased from Molecular Probes Inc. (Eugene, OR). α -Minimal essential medium, fetal bovine serum and trypsin-EDTA were from Life Technologies, Inc (Burlington, ON). Polyclonal rabbit α -NHE1 antibody was a generous gift from Dr. S Grinstein (Hospital for the sick children, Toronto, Canada) and the horseradish conjugated goat α -rabbit antibody was purchased from New England BioLabs (Beverly, MA). Cell culture dishes and flasks were purchased from BectonDickinson and Corning (Montreal, Québec). All other chemicals and reagents used in these experiments were from British Drug House (BDH) Inc. (St. Laurent, Québec) or Fisher Scientific, and were of the highest grade available.

Stock Solutions

Stock solutions of PMA (1 mM) and amiloride (0.5 M) were prepared in dimethyl sulfoxide (DMSO) and stored in -20°C . Nigericin (10 mM) was dissolved in ethanol and stored at -20°C . Ouabain was dissolved in deionized distilled H_2O in a concentration of 10 mM and stored at 4°C .

Construction and Stable Expression of the Mutants

The mutations were made by the site-directed mutagenesis technique of Deng and Nickoloff (Deng and Nickoloff, 1992). The oligomers were synthesized by Life Technologies, Inc. Initially, the mutations were made on a full-length rat NHE1, while later mutations were performed using a full-length rat NHE1 containing an influenza virus hemagglutinin (HA) epitope tag (Wilson et al., 1984) inserted at the extreme C-terminus. This epitope was introduced to facilitate detection of the protein in cellular extracts. The function of the wild type NHE1 was indistinguishable from the HA-tagged NHE1 (data not shown). The mutant NHE1s were sequenced to ensure that no additional mutations were introduced into the cDNA in the process of site-directed mutagenesis. Plasmid DNA used for cell transfection was isolated from bacteria (DH5 α strain) using the "midiprep" kit (Promega).

The wild type and mutant constructs were transfected into chemically mutagenized Chinese hamster ovary cells (AP-1 cells) devoid of endogenous Na⁺/H⁺ exchange activity (Rotin and Grinstein, 1989) using the calcium phosphate-DNA precipitation method (Chen and Okayama, 1987). Starting 48 hours after transfection, the cells were selected for survival in response to repeated (5-6 times over a 2-week period) acute NH₄Cl-induced acid loads (Orlowski, 1993; Franchi et al., 1986) in order to select for NHE-positive stable transfectants. Several clonal cell lines were screened for their level of NHE1 expression by measuring H⁺-activated ²²Na⁺ influx. The cell clones that had the highest level of NHE1 activity were then used between passages 2 and 8. They were maintained in complete α -minimal essential medium supplemented with 10%

fetal bovine serum and 25 mM NaHCO₃, pH 7.4, and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Immunoblotting

Stably transfected cells were grown to confluence in 10 cm dishes and lysed with 1% Triton X-100. Protein extracts of 30 µg were subjected to SDS-PAGE in 6% polyacrylamide gels and transferred onto PVDF membranes. Blots were blocked with 5% fat-free milk in PBST (phosphate-buffered saline with 0.1% Tween 20) and exposed to the primary antibody (monoclonal mouse anti-HA, dilution 1: 5000). Horseradish peroxidase conjugated goat anti-mouse IgG (dilution 1: 2,0000) was applied as the secondary antibody. The immunoreactive bands were visualized using chemiluminescence (Amersham Corp.).

Measurement of ²²Na⁺ Influx

For experimentation, the cells were subcultured at 5×10^4 cells/well in 24-well plates and grown to confluence. Na⁺/H⁺ exchange activity was assayed by measuring amiloride-inhibitable H⁺-dependent ²²Na⁺ influx.

In experiments where the pH_i profile of the wild type and mutant exchangers was determined, pH_i was clamped at different levels by incubating the cells in solutions of various K⁺ concentrations, containing 10 µM of the K⁺/H⁺ exchange ionophore nigericin, as well as 2 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM Ouabain, 10 mM HEPES, and

various concentrations of KCl, K-glutamate, and N-Methyl D-Glucamine, pH 7.4 (Table 1). Because at equilibrium, $[K^+]_i / [K^+]_o = [H^+]_i / [H^+]_o$, the desired pH_i can be calculated from the imposed $[K^+]$ gradient and the extracellular pH ($pH_o = 7.4$), assuming an intracellular $[K^+]$ of 140 mM. Briefly, confluent cell monolayers in 24-well plates were washed twice with a Na^+ -free "choline chloride" solution, containing 125 mM CholineCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris (pH 7.4). Then the cells were preincubated in the so-called " pH_i -clamp" solutions for 4 min. This solution was then replaced with fresh " pH_i -clamp" solution supplemented with 1 $\mu Ci/ml$ $^{22}NaCl$, and incubated for 10 minutes in the presence or absence of 2 mM amiloride. $^{22}Na^+$ uptake was then stopped by washing the cells three times with an ice-cold $NaCl$ "stop" solution, containing 130 mM $NaCl$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Glucose, and 20 mM Hepes, pH 7.4.

To extract the radiolabel, 0.25 ml of 0.5 N $NaOH$ was added to each well and the cells were lysed and collected. Then the wells are washed with 0.25 ml of 0.5 N HCl and the solution is collected again. Both the solubilized cell extract and wash solution were suspended in 5 ml of scintillation fluid, and the radioactivity was assayed by liquid scintillation spectroscopy. Protein content was determined using the Bio-Rad DC Protein Assay procedure.

Measurements of $^{22}Na^+$ influx specific to the Na^+/H^+ exchanger were determined as the difference between the initial rates of H^+ -activated $^{22}Na^+$ influx in the absence or

presence of 2 mM amiloride, a concentration sufficient to inhibit NHE1 under these experimental conditions) and expressed as amiloride-inhibitable $^{22}\text{Na}^+$ influx.

To determine the maximum rate of transport of the mutants, the intracellular pH was clamped arbitrarily at 5.4 (as maximum) and the $^{22}\text{Na}^+$ influx was measured over 10 min, as indicated above. The maximum specific rate of transport of $^{22}\text{Na}^+$ (the amount of $^{22}\text{Na}^+$ uptake per milligram of protein per minute) was measured based on the amount of protein in corresponding wells.

In experiments where the response of the mutants to PMA was evaluated, the cells were serum-deprived overnight. Then they were washed twice with "Na⁺-saline" solution, containing 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM Glucose, and 20 mM HEPES, pH 7.4, and preincubated in the same solution supplemented with 1 μM PMA for 15 min at 37°C. Then the cells were washed twice with the "choline chloride" solution, and incubated in the same solution supplemented with 1 μCi/ml $^{22}\text{NaCl}$ and 1 mM ouabain, in the presence or absence of 2 mM amiloride for 5 min, and the $^{22}\text{Na}^+$ uptake was stopped as indicated above.

Data Analysis

The data represent the average of two to four experiments, each performed in quadruplicate. The significance of the data was analyzed by measuring the "*p* value", using student *t*-test. The mean value of each data point in the mutant curves was

calculated and compared with the corresponding point in the wild type curve. The significance of difference of the two means was then calculated at the $V_{\max}/2$ level.

RESULTS

Mutational Analysis of the Conserved Histidine Residues of NHE1 - The C-terminal cytoplasmic tail of the Na⁺/H⁺ exchanger is known to play a crucial role in regulating transporter activity (Ikeda et al., 1997; Wakabayashi et al., 1992a). Recent deletion mutation analyses have suggested that the pH_i sensor may reside in a small region immediately adjacent to the transmembranous domain (Ikeda et al., 1997).

The amino acid histidine, because of its chemical properties, is a likely candidate residue that could serve as a H⁺ sensor within the physiological pH range. This amino acid has a side chain that contains an imidazole ring with a pK of approximately 6.5 that titrates in the physiological pH range.

In order to define potential histidines involved in pH_i sensitivity of the NHE, different NHE isoforms within and between species were aligned to highlight conserved sites. Fig. 1 illustrates the alignment of rat NHE1 to NHE5, as well as human and xenopus NHE1 sequences. This analysis revealed several histidines in intracellular loops between the transmembranous helices (H254, H329, and H411) and in the cytoplasmic tail (H527, H533, H544, and the triple His at positions 547 to 549) that are relatively conserved.

To assess the significance of these residues to pH_i sensitivity, we initially substituted them with Ala, a neutral amino acid that is nonpolar and incapable of

hydrogen-binding. The pH_i sensitivity of the mutants and the wild type NHE1 were compared by plotting the transport activity of the proteins as a function of intracellular pH over the range of pH_i 5.4-7.4. Nigericin, an ionophore that couples the K^+ and H^+ gradients across the plasma membrane, was used to clamp the intracellular pH at different values in the stably transfected AP-1 cells. The Na^+/H^+ exchange activity was then assessed by measuring amiloride-inhibitable $^{22}\text{Na}^+$ influx.

Fig. 2A demonstrates the schematic location of H254, H329, and H411 in the intracellular loops between the transmembranous domains 6 and 7, 7 and 8, and 9 and 10, respectively.

The effect on pH_i -dependence of His to Ala substitution at positions 254, 329 and 411 are represented in Figures 2B, 2C and 2D, respectively. These mutants displayed a pH_i -dependence that resembled that of the wild type, with minimal activity at pH 7.4 and a marked increase in activity thereafter as the pH_i was further decreased. These specific mutations did not significantly alter the H^+ -dependence of the exchanger, as shown by the lack of a significant shift in the pH_i profile of mutants, compared to wild type. These data indicate that the proton sensor is unlikely to involve histidines in the transmembranous region.

Mutation of the triple His at positions 547 to 549 of the cytoplasmic tail also had no significant effect on the pH_i sensitivity of NHE1 (Fig. 3B). By contrast, replacing the H544 with Ala caused an alkaline shift in the pH_i sensitivity of the transporter (Fig. 3C).

In addition, replacement of the more N-terminal H533 with Ala caused an even greater alkaline shift of the curve, compared to H544A (Fig. 3D).

Interestingly, replacement of the most N-terminal conserved His residue (H527) with Ala rendered the protein nonfunctional, inasmuch as it did not survive the NH_4Cl -induced H^+ -suicide (described in "Experimental Procedures"), suggesting that the His at this position might be critical for proton sensitivity.

Based on the above data, H527, H533, and H544 were substituted with other amino acids in order to evaluate the importance of side-chain charge, size, and polarity. In comparison to H544A (Fig. 4B), substitution of H544 with Asp (Fig. 4C), Arg (Fig. 4D), or Tyr (Fig. 4E) did not significantly affect pH_i sensitivity of NHE1. Similarly, replacing H533 with Tyr (Fig. 5E) had no effect, while Asp (Fig. 5C) and Arg (Fig. 5D) substitutions caused minor shifts in the pH_i profile of the protein, but these were not statistically significant.

Unlike the H527A mutation, replacement of H527 with polar residues Asp, Arg, and Tyr, resulted in a functional transporter. While the pH_i profile of the H527D (Fig. 6B) was similar to wild type, both Arg (Fig. 6C) and Tyr (Fig. 6D) replacement of this amino acid shifted the set point of the protein to the alkaline side by 0.25 and 0.3 pH units, respectively. These results suggest that size and/or polarity, but not charge, at positions 527, 533 and 544 are important structural determinants of the pH_i sensor.

The effect of mutations at positions H544, H533 and H527, respectively, suggests that this region may play a critical role in determining the pH_i set point of NHE1. Further examination of this region revealed a cluster of negatively charged amino acids (D532, E539, and D540) that were highly conserved amongst the isoforms. To assess whether these residues also contributed to the pH_i sensitivity of NHE1, the amino acids at these positions were substituted with Ala. As shown in Fig. 7, while D532A (Fig. 7B) mutation had almost no effect on the pH_i sensitivity of NHE1, the ED539-40AA double mutation (Fig. 7C) caused a major alkaline shift in the pH_i profile. Hence, specific charged residues in this region appear to contribute to the pH_i sensitivity of NHE1.

Maximum Rate of Transport Activity of the Wild Type and Mutant NHE1 - The above data for all mutations was presented as a percentage of the maximal activity of the transporter at pH_i 5.4. While this type of analysis is required to compare the effect of the mutations with that of the wild type with regard to pH_i -dependence, it does not allow one to evaluate the effect on other kinetic properties such as V_{max} . In order to determine the maximum activity (V_{max}) of those mutants, their level of activity was assessed at an intracellular pH of 5.4 (arbitrarily taken as the pH_i corresponding to the V_{max}). This V_{max} was then normalized with respect to protein expression levels.

Western blot analysis was employed to determine the level of expression of NHE1 by each stable cell line. In this experiment, 30 μg protein extract of each transfectant was loaded on a 6% polyacrylamide gel and the proteins were probed by anti-HA tag antibody (Fig. 8). NHE1 appears as two distinct bands by SDS-PAGE, an

~80 kDa core glycosylated band that is believed to be the partially processed form of the protein (possibly located in the endoplasmic reticulum) and a ~110 kDa fully glycosylated band that is located in the plasma membrane. A third band with a size of ~200 kDa is also detectable in some lanes that is modestly stable in SDS detergent and is believed to represent the homodimer form of fully-processed NHE1. Such a band has been reported in other studies (Fliegel and Fröhlich, 1993; Fafournoux et al., 1994). An additional band of unknown nature is detected in the lane corresponding to the mutant H533D. The two bands in the lane that corresponds to the mutant H527D show an anomalous migration that can not be explained at this point; however, the transport function of the protein was not noticeably affected.

Densitometry analysis of the fully glycosylated band was conducted in order to compare the relative activity of those mutants that caused the greatest shift in the pH_i profile with that of the wild type NHE1 (Fig. 9). Normalization of the radioisotope flux rates of the mutants to their respective plasma membrane protein levels showed that these mutants had relatively higher specific activities when compared to wild type.

The Activation Response of the wild type and Mutant NHE1 to Phorbol Ester - As mentioned in the introduction, NHE1 is activated by various extracellular stimuli, including phorbol esters (which activate PKC) and growth factors, which cause an increase in the affinity of the transporter for intracellular protons (in other words, a shift in the pH_i set point towards more alkaline values) (Paris and Pouysségur, 1984; Grinstein et al., 1985). Therefore, assuming that the mutants that shifted the pH_i set point of NHE1

are part of the pH_i sensor unit, it was of interest to determine whether these mutants could be further activated by these stimuli.

To this end, we tested the response of H527Y and ED539-40AA to PMA in comparison with that of wild type NHE1. As demonstrated in Fig. 10, the response of the wild type NHE1 to a 15-minute stimulation with 1 μM PMA was a ~50% increase in basal activity, as previously reported (Kandasamy et al., 1995; Moolenaar, 1986; Moolenaar et al., 1983), whereas the mutants showed no stimulation of activity in response to PMA. These results suggest that these residues may contribute to the changes in pH_i sensitivity induced by phorbol esters and possibly other stimulatory agents.

DISCUSSION

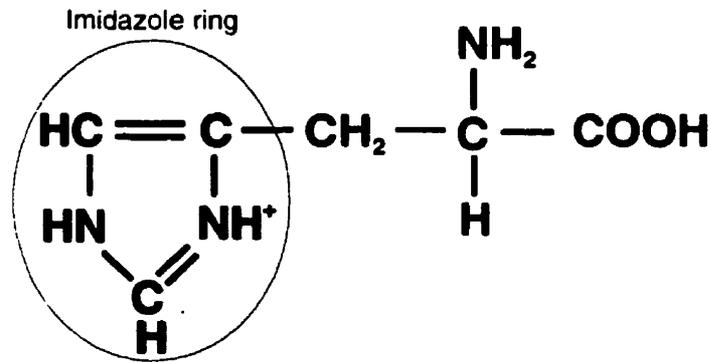
The data in this study indicate that conserved residues, both positively and negatively charged, in the juxtamembranous region of the cytoplasmic tail of NHE1, play an important role in the pH_i sensitivity of the transporter.

One of the proposed mechanisms for the regulation of NHE by intracellular H^+ is based on the existence of a putative H^+ sensor, possibly a His residue, located in the cytoplasmic tail region of the transporter. Changes in the state of protonation of this sensor presumably cause a conformational change in the molecule that stimulates ion translocation. Phosphorylation of the very C-terminal end of NHE1 by growth factors and other mitogens such as PMA are also believed to alter the pH sensitivity of the H^+ sensor.

Another model proposes that the H^+ sensor is located in the N-terminal transmembrane domain of the protein. According to this model, the cytoplasmic tail of NHE plays the role of an integrator that detects and processes the stimulatory (or inhibitory) signal and transmits it to the pH_i sensor in the transmembrane domain (Wakabayashi et al., 1992a).

Since the imidazole ring of His (pK_a in solution $\sim 6.0 - 6.5$) (Gerchman et al., 1993) is the principle ionizable group amongst the side chains of amino acids that is

titratable at near-neutral pH (Grillo and Aronson, 1986), His residues predicted to reside on the cytoplasmic side of the membrane were examined in search for the H⁺ sensor.



Histidine Structure

Similar pH regulatory mechanisms have been reported in other ion transport systems. Bacterial Na⁺/H⁺ antiporter isoform A (NhaA) is a pH-sensitive protein that is critically involved in adaptation of *E.coli* to higher extracellular pH's. It has been suggested that when the extracellular pH increases, the antiporter is activated so that it can acidify the cytoplasm back to the "resting pH". Interestingly, H226 has been suggested to be part of the pH sensor of NhaA (Gerchman et al., 1993). It was also demonstrated that replacing this His with Cys (H226C) or Ser (H226S) retained both normal activity and pH sensitivity of the protein, Asp (H226D) shifted the pH profile towards basic pH, and Ala (H226A) inactivated the carrier at all pH values (Rimon et al., 1995). Based on this mutational analysis, this residue may be postulated to play the same role as the H527 in the mammalian NHE1; however, more detailed analysis is required to reach a solid conclusion.

The nucleotide binding to uncoupling protein (UCP-1) (from brown adipose tissue) is another example of regulation that is highly sensitive to pH. Echtay and co-workers showed that H214 in this protein functions as a pH sensor (Echtay et al., 1998). Also, it has been demonstrated that the inward rectifier potassium channel (HIR), present in heart and central nervous system, is modulated by extracellular pH in the physiologic range. Coulter *et al.*, showed that H117 confers pH sensitivity to this molecule (Coulter et al., 1995).

Cardiac gap junction protein connexin43 (Cx43), a gap junction channel that allows for the passage of ions and small molecules between adjacent cells, is also regulated by pH. Acidification-induced closure of cardiac gap junction channels has been extensively documented (Spray and Bennett, 1985; Bennett and Verselis, 1992). Although the molecular mechanisms mediating this process are not fully understood, Morley *et al.* demonstrated that a region including H95 plays a major role in pH gating of Cx43 (Morley et al., 1996; Ek et al., 1994).

Based on the above examples, we postulated that the mammalian NHE has a built-in sensor which contains His as its main operator. In search for such an element, we looked at the His residues that presumably face the intracellular side of the membrane, starting by those residing in the N-terminal transmembranous domain, i.e., H254, H329, and H411. Our results indicate that these residues are not significantly involved in determining the pH_i set point of the protein, since substitution of these residues with a neutral amino acid (Ala) did not influence the pH_i profile of the protein. This suggested

that the sensor is likely to be residing in the cytoplasmic tail of NHE1. In fact, the regulatory factors of the NHE are known to exert their function through the C-terminal domain (Ikeda et al., 1997; Wakabayashi et al., 1997; Wakabayashi et al., 1994a). In agreement with this hypothesis, the cytoplasmic domain of NHE shows the greatest structural divergence among NHE isoforms (Sardet et al., 1989; Tse et al., 1992; Orłowski et al., 1992; Tse et al., 1993a; Wang et al., 1993) and complete deletion of this domain in NHE1 isoform causes a constitutive acidic shift of pH_i -dependence of Na^+/H^+ exchange (Wakabayashi et al., 1992a; Wakabayashi et al., 1994a).

Analysis of His residues residing in the C-terminal cytoplasmic tail of NHE1 showed different results. While mutation of the triple His cluster at positions 547 to 549 did not have any effect on the pH_i sensitivity of the protein, substitution of H544 to Ala activated NHE1 by shifting the pH_i sensitivity of the protein towards more alkaline values, suggesting that this residue might be part of the proton sensor. However, the involvement is probably minor, judging from the minor extent of the shift. Interestingly, neighboring N-terminal histidines showed gradually increasing involvement in the pH_i sensitivity of NHE1. Replacement of H533 with Ala not only shifted the pH_i profile of NHE1 towards more alkaline values, but also the extent of the shift was greater than that of H544A. In the case of H527, which is the most proximal His residue in that specific stretch of amino acids, its replacement with Ala resulted in a nonfunctional protein. This may suggest that this mutant is no longer sensitive to intracellular acidification inasmuch as it does not survive the "acid-selection" procedure. This suggests that it may play a critical role in determining the pH_i sensitivity of the protein. Alternatively, mutation at

this site may have caused improper processing and degradation of the protein. However, this is less likely since other mutations at this site result in a functional protein and complete removal of the tail region also preserves the transport capabilities of NHE1 (Ikeda et al., 1997; Wakabayashi et al., 1994a).

To test whether the charge, polarity, or hydrogen-binding capability of residues at positions 527, 533 and 544 of NHE1 are involved in its role in pH_i determination, the histidines at these positions were substituted with other amino acids. Substitution of H544 and H533 with Asp (negatively-charged), Arg (positively-charged) and Tyr (capable of hydrogen-binding) did not have much of an effect on the pH_i profile of the protein. However, in case of H527, which seems to be the most important position in determining the pH_i set point of NHE1, size and perhaps polarity and/or hydrogen-binding capability, but not the charge, of the amino acid is critical.

As mentioned before, Ala replacement of the H226 in bacterial NhaA also completely inactivates the carrier (Rimon et al., 1995). This suggests that the H527 in mammalian NHE1 and the H226 in bacterial NhaA may fulfill similar structural roles in determining the pH sensitivity of their corresponding proteins. Replacing the H527 in NHE1 with Arg (H527R) shifted the pH profile towards the *alkaline* side by more than half a pH unit, rendering the protein more active. In contrast, NhaA bearing the same mutation at position 226 (H226R) had a pH profile shifted to the *acidic* pH range by half a pH unit. This difference may be explained by the fact that the bacterial antiporter is

more active in *higher extracellular* pH values, while its mammalian counterpart is activated in *lower intracellular* pH range.

Taken together, the above data suggest that the model proposing the involvement of protonation of histidines as a means of determining the pH_i set point may be simplistic and there are probably a number of amino acids that constitute the H^+ -sensor of NHE. However, the underlying H^+ -relay mechanism remains obscure. These amino acids are most likely located in the juxtamembrane segment of the C-terminal tail. Other relatively conserved amino acids that are capable of hydrogen-binding or are ionizable within the physiological range as a consequence of their close interaction with other residues, might be part of this built-in H^+ -sensor. The aspartic acid at position 532 and a pair of negatively-charged residues, a glutamic acid and an asparic acid, residing at positions 539 and 540 are significantly conserved amongst species and have the above-mentioned characteristics.

One of the prominent features of NHE1 is that it is rapidly activated in response to various extracellular stimuli, including growth factors, Ca^{2+} -releasing agonists, and hyperosmotic stress (Paris and Pouyssegur, 1984; Grinstein et al., 1985). This activation is believed to occur through alterations in the pH_i -sensitivity of NHE1. By contrast, growth factor activation of the epithelial isoforms NHE2 and NHE3 was reported to be due to an increase in the maximal activity (V_{max}) (Levine et al., 1993; Tse et al., 1993b). Our results suggest that while the mutants are more sensitive to the intracellular pH and

therefore more active (compared to the wild type) at the same pH values, their maximal activity or turnover rate is also increased (Fig. 9).

Wakabayashi *et al.* demonstrated that complete removal of the cytoplasmic tail greatly reduces the H^+ sensitivity of NHE1 and abolishes growth factor-induced cytoplasmic alkalinization (Wakabayashi *et al.*, 1992a; Wakabayashi *et al.*, 1994a). They proposed that mitogen activation of NHE1 occurs through the reversible phosphorylation-dependent coupling of the C-terminal cytoplasmic domain with the H^+ sensor site. This proposal is mainly based on the fact that growth factor activation of NHE1 coincides with increased phosphorylation of the antiporter at a common critical site (Sardet *et al.*, 1990; Sardet *et al.*, 1991) and that deletion of the cytoplasmic domain reduces the affinity of the H^+ sensor site. Their interpretation was that the deletion has uncoupled the cytoplasmic effector domain from the H^+ sensor site. Alternatively, one might argue that complete removal of the cytoplasmic domain has resulted in a large structural alteration of the N-terminus of the protein, which irreversibly altered H^+ sensitivity. However, this is unlikely, because the affinity of the antiporter for the extracellular Na^+ , amiloride, and MPA were minimally affected by the deletion of the C-terminal domain (Wakabayashi *et al.*, 1992a).

Phorbol ester was used to test the activation response of those mutants that caused significant shifts on the pH_i dependence. Interestingly, the mutants were unresponsive to phorbol ester, perhaps because they are already activated. This result further supports the contribution of these residues in proton sensitivity of NHE1. Alternatively,

unresponsiveness of the mutants to PKC may have resulted from altered phosphorylation. To test this possibility, *in vivo* phosphorylation was performed by incubating the stably transfected cells expressing the wild type and mutant exchanger in a solution containing [³²P] orthophosphate with or without PMA. The cells were then lysed and the protein extract was subjected to SDS-PAGE and transferred onto a PVDF membrane. The quantity of phosphorylated protein was assessed using a phosphorimager (data not shown). Interestingly, neither the wild type nor the mutant Na⁺/H⁺ exchangers showed an appreciable difference in the phosphorylation level of the PMA treated and untreated samples. It is not clear whether the effect of phorbol ester on NHE1 is through phosphorylation or other mechanisms; however, the present literature suggests the process to be dependent on the cell expression system. For example, it has been shown that phorbol ester increases phosphorylation of NHE1 in fibroblastic cells (Sardet et al., 1990) but in granulocytes it actually causes a decrease, rather than increase, in phosphorylation (Rao et al., 1992). Wakabayashi *et al.* (Wakabayashi et al., 1994a) demonstrated that deletion of all major phosphorylation sites preserved 50% of the exchanger activation in response to several growth promoting agents, including phorbol ester, and thereby concluded that growth factor activation of NHE1 occurs at least partly by a mechanism not involving direct phosphorylation of NHE1 molecule.

CONCLUDING REMARKS

Regulation of intracellular pH is absolutely necessary for proper functioning of cells. The Na^+/H^+ exchanger plays an important role in this process. The results of this study provide new insights into the location of this sensor and cast doubt on the earlier hypothesis that simple ionization of histidine residues is sufficient to explain the pH-sensitivity of the exchanger. Rather, the data suggest that while histidines in the C-terminal tail are important, additional residues or regions of the protein are likely to be interacting with these histidines to form the pH-sensor.

	pH _i												
	7.4	7.2	7.0	6.8	6.6	6.4	6.2	6.1	6.0	5.9	5.8	5.6	5.4
KCl	14	14	14	14	14	14	8.83	7.02	5.57	4.43	3.52	2.22	1.4
K-glutamate	126	75	42	21	8	0	0	0	0	0	0	0	0
NMG	24	75	108	129	142	150	155	157	158	159	160	162	163
NaCl	2	2	2	2	2	2	2	2	2	2	2	2	2
MgCl₂	1	1	1	1	1	1	1	1	1	1	1	1	1
Hepes	10	10	10	10	10	10	10	10	10	10	10	10	10
CaCl₂	1	1	1	1	1	1	1	1	1	1	1	1	1
Orobain	1	1	1	1	1	1	1	1	1	1	1	1	1
Nigericin	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Table 1: The composition of pH_i-clamp solutions. Depending on the desired intracellular pH, the cells were incubated in solutions of various K⁺ concentrations. Because at equilibrium, $[K^+]_i / [K^+]_o = [H^+]_i / [H^+]_o$, the desired pH_i can be calculated from the imposed [K⁺] gradient and the extracellular pH (pH_o = 7.4), assuming an intracellular [K⁺] of 140 mM. The numbers in the boxes represent mM concentrations. After mixing the above components, the pH of solutions was adjusted on 7.4, using methane sulfonic acid.

A

	254	329	411
1 RATNHE1	EEI H INE	FTS H IRV	AGS H QWN
2 RATNHE2	ENI H VNE	FTH N IRV	GKN H EWN
3 RATNHE3	EEV H VNE	FTK H VRI	D-P V IWT
4 RATNHE4	EEA R VNE	FTQ N ISA	GKN H EWN
5 RATNHE5	EEV H VNE	FTK R VRI	D-S S KWA
6 HUMNHE1	EEI H INE	FTS H IRV	AGS H HWN
7 XENNHE1	EEI H INE	FTS H IRV	AGP H KWN
8 Consensus	*EI H V**	** H IRV	SHK*N

B

	527	533	539	544	548
1 RATNHE1	EEI H TQFL D HLLTG I E D ICG H Y G HH H W K D				
2 RATNHE2	EEI H CRFF D HVKTG I E D VCG H WG H N F WR D				
3 RATNHE3	EKL H GRA F D HILSA I E D ISG Q IG H NY L R D				
4 RATNHE4	EEL H IRL M D H L KAG I E D VCG Q WS H Y Q VR D				
5 RATNHE5	QEL H EHT F D H I LA A V E D V V G HH G Y H Y W R D				
6 HUMNHE1	EEI H TQFL D HLLTG I E D ICG H Y G HH H W K D				
7 XENNHE1	EEI H SEFL D HLLTG I E D ICG H Y G HH H W K D				
8 Consensus	EEI* RFF**LLTGI**IC* H G HH WR*				

Fig. 1: The alignment of amino acid sequences of domains that contain the relatively conserved histidines in rat NHE1 to NHE5, as well as human and xenopus NHE1. **A.** Residues located in the N-terminal transmembranous domain. **B.** Residues located in the C-terminal cytoplasmic tail.

Fig. 2: Transport activity of the wild type and mutant rat NHE1 as a function of the intracellular H⁺ concentration. AP-1 cells expressing wild type and mutant rat NHE1 were grown to confluence in 24-well plates. Initial rates of H⁺-activated ²²Na⁺ influx were measured at various intracellular H⁺ concentrations over the range of pH_i 5.4 to 7.4. The intracellular H⁺ concentrations were adjusted by K⁺-nigericin method as described in "Experimental Procedures". The schematic location of histidines 254, 329 and 411 is presented in (A). pH_i profiles for H254A, H329A, and H411A mutants are presented in (B), (C), and (D) respectively. Data were normalized as a percentage of the maximal rate of H⁺-activated ²²Na⁺ influx at pH_i 5.4. Values represent the average of three experiments each performed in quadruplicates. (Solid line: wild type; dashed line: mutant). In B, C and D, *P*>0.05.

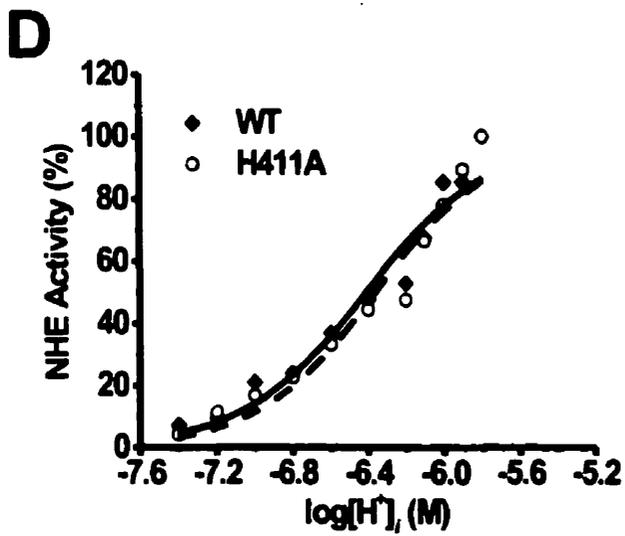
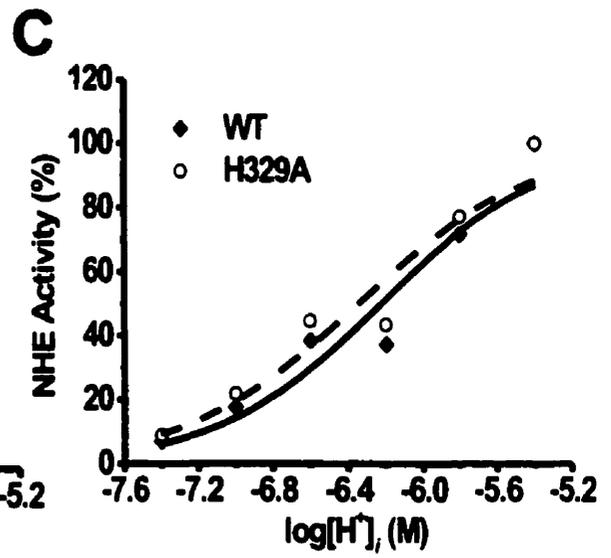
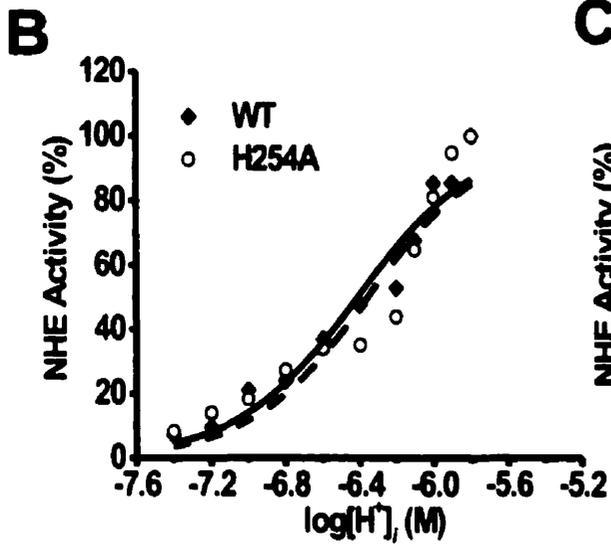
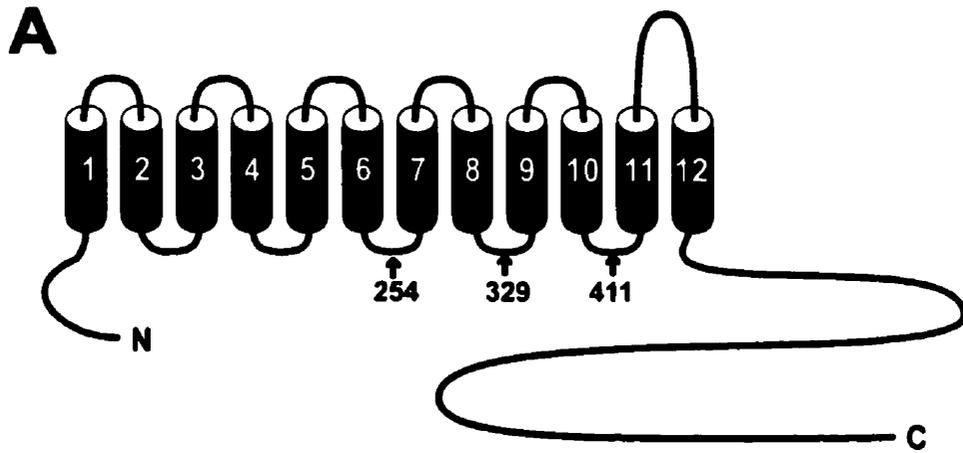


Fig. 3: Transport activity of the wild type and mutant rat NHE1 as a function of the intracellular H⁺ concentration. AP-1 cells expressing wild type and mutant rat NHE1 were grown to confluence in 24-well plates. Initial rates of H⁺-activated ²²Na⁺ influx were measured at various intracellular H⁺ concentrations over the range of pH_i 5.4 to 7.4. The intracellular H⁺ concentrations were adjusted by K⁺-nigericin method as described in "Experimental Procedures". The schematic location of His547-9, His544 and His533 is presented in (A). pH_i profiles for HHH547-9AAA, H544A, and H533A mutants are presented in (B), (C), and (D) respectively. Data were normalized as a percentage of the maximal rate of H⁺-activated ²²Na⁺ influx at pH_i 5.8. Values represent the average of three experiments each performed in quadruplicates. (Solid line: wild type; dashed line: mutant). In B and C, *P* > 0.05; in D, *P* < 0.05.

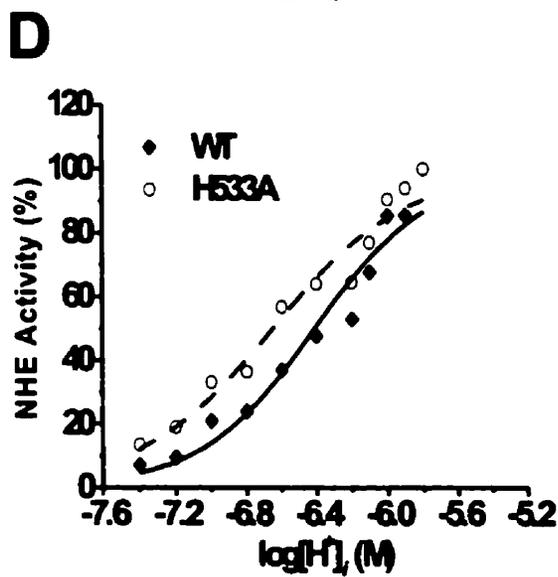
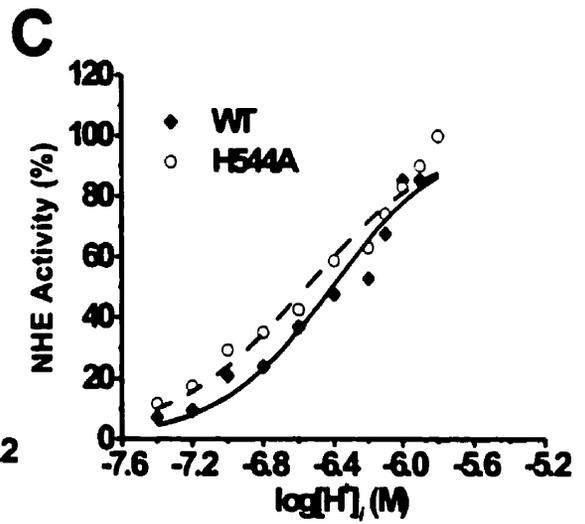
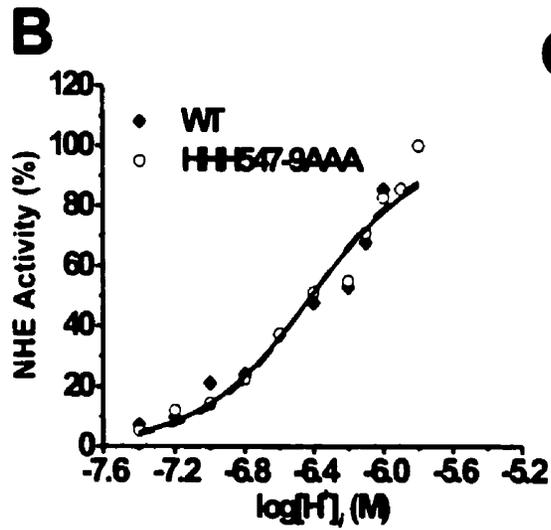
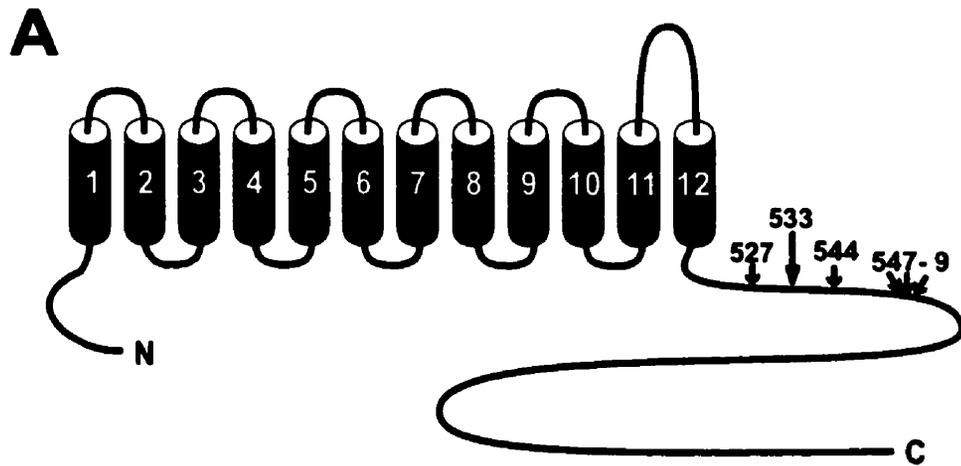


Fig. 4: Transport activity of the wild type and mutant rat NHE1 as a function of the intracellular H⁺ concentration. AP-1 cells expressing wild type and mutant rat NHE1 were grown to confluence in 24-well plates. Initial rates of H⁺-activated ²²Na⁺ influx were measured at various intracellular H⁺ concentrations over the range of pH_i 5.4 to 7.4. The intracellular H⁺ concentrations were adjusted by K⁺-nigericin method as described in "Experimental Procedures". The schematic location of histidine 544 is presented in (A). pH_i profiles for H544A, H544D, H544R and H544Y mutants are presented in (B), (C), (D) and (E) respectively. Data were normalized as a percentage of the maximal rate of H⁺-activated ²²Na⁺ influx at pH_i 5.4. Values represent the average of three experiments each performed in quadruplicates. (Solid line: wild type; dashed line: mutant). In B, C, D and E, *P* > 0.05.

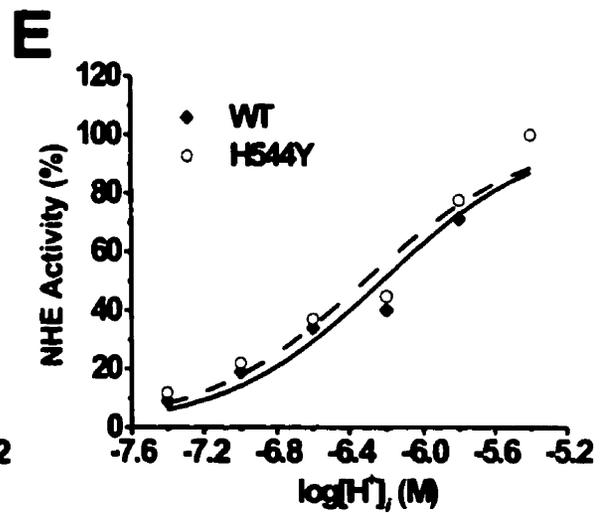
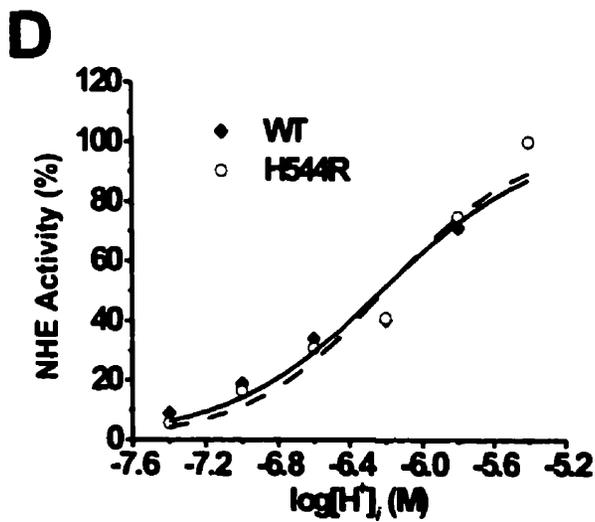
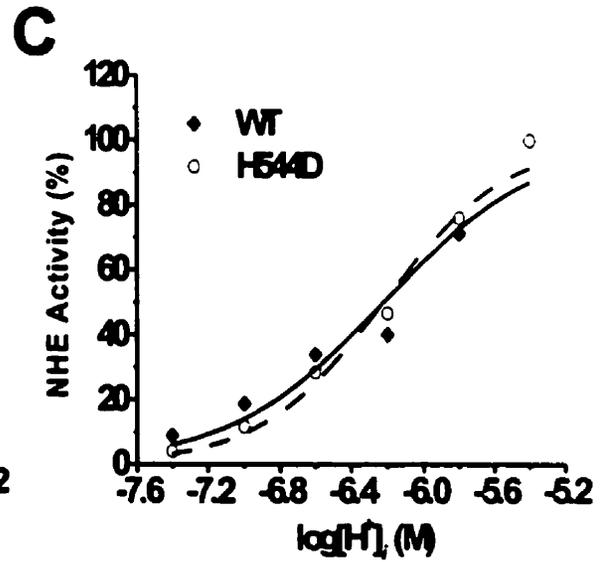
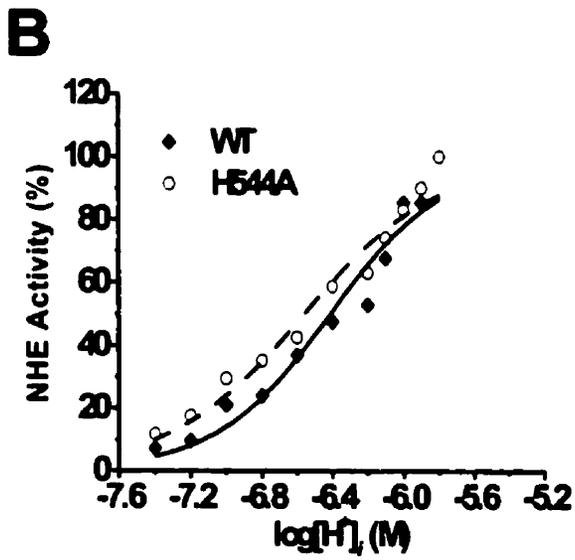
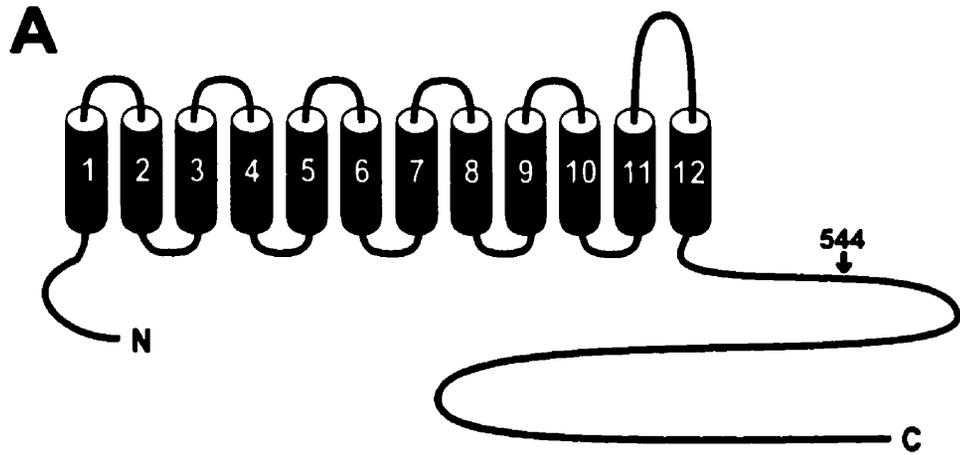


Fig. 5: Transport activity of the wild type and mutant rat NHE1 as a function of the intracellular H⁺ concentration. AP-1 cells expressing wild type and mutant rat NHE1 were grown to confluence in 24-well plates. Initial rates of H⁺-activated ²²Na⁺ influx were measured at various intracellular H⁺ concentrations over the range of pH_i 5.4 to 7.4. The intracellular H⁺ concentrations were adjusted by K⁺-nigericin method as described in "Experimental Procedures". The schematic location of histidine 533 is presented in (A). pH_i profiles for H533A, H533D, H533R and H533Y mutants are presented in (B), (C), (D) and (E) respectively. Data were normalized as a percentage of the maximal rate of H⁺-activated ²²Na⁺ influx at pH_i 5.4. Values represent the average of three experiments each performed in quadruplicates. (Solid line: wild type; dashed line: mutant). In B, *P* < 0.05; in C, D and E, *P* > 0.05.

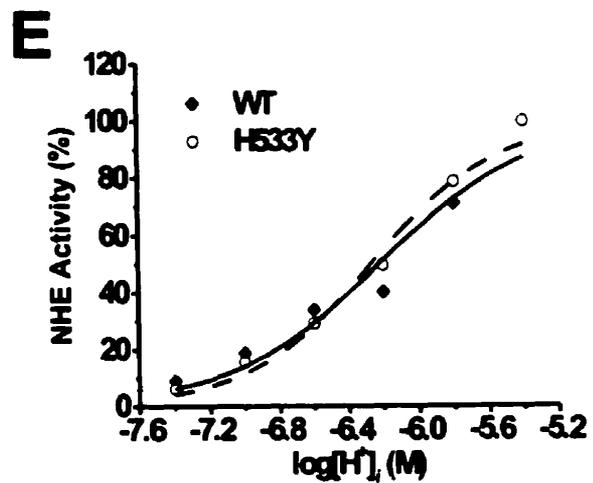
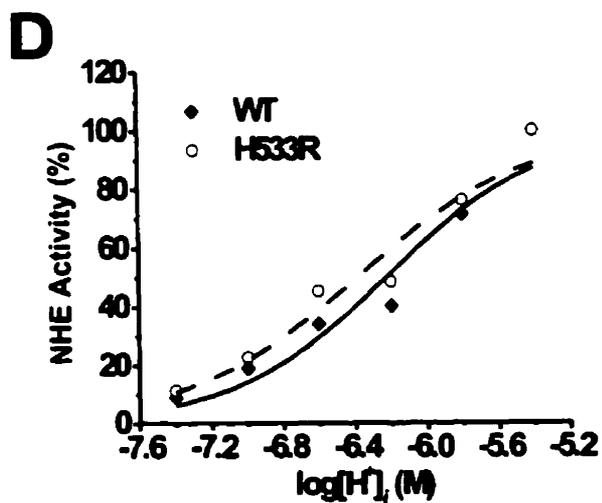
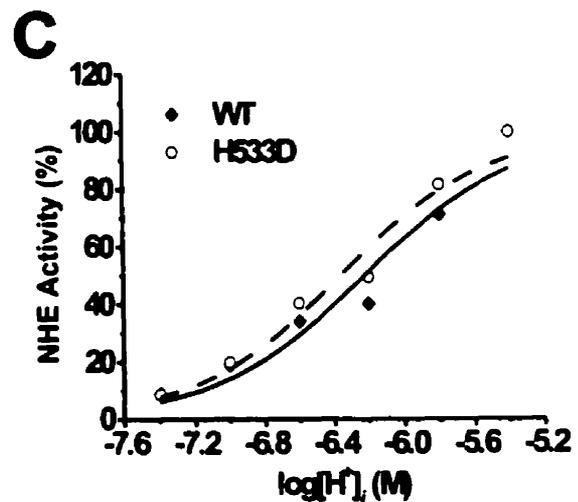
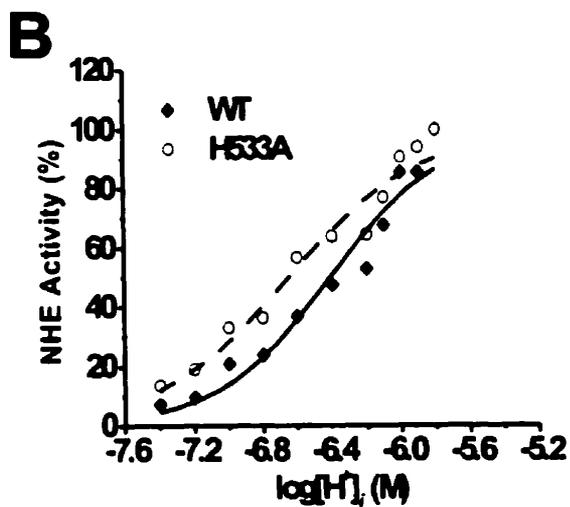
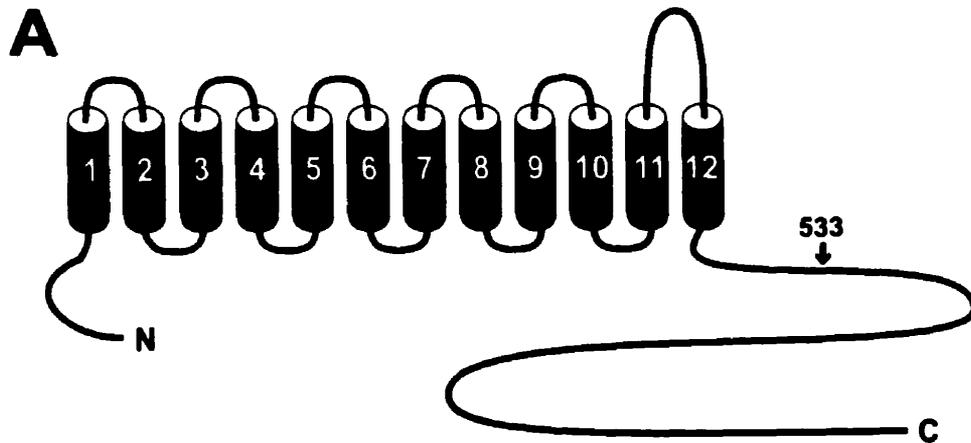
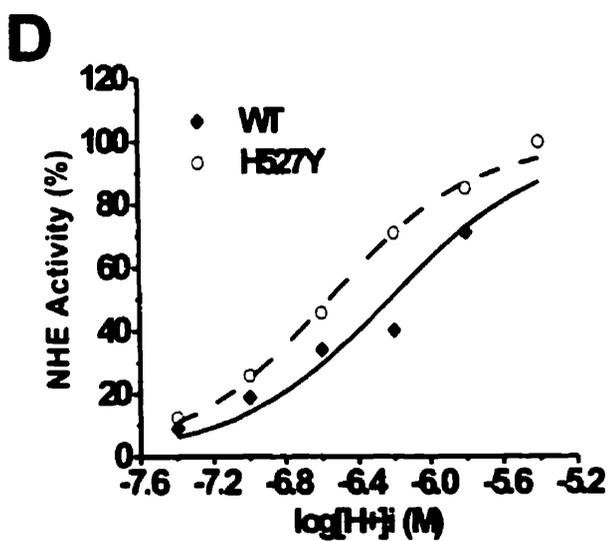
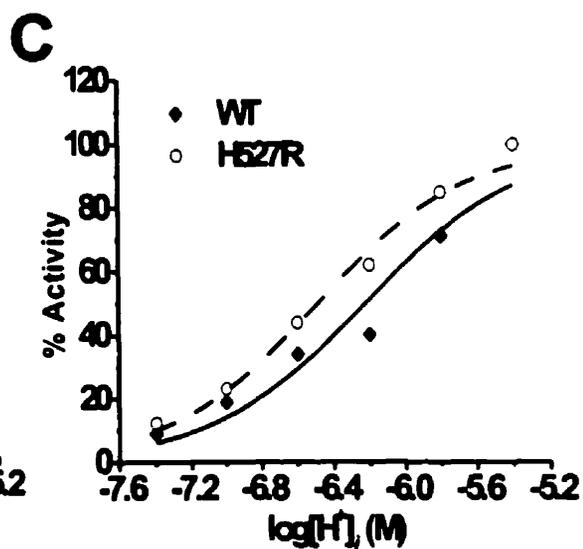
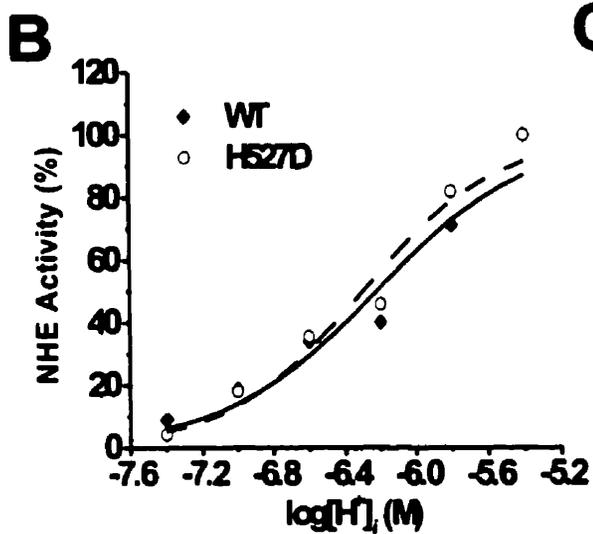
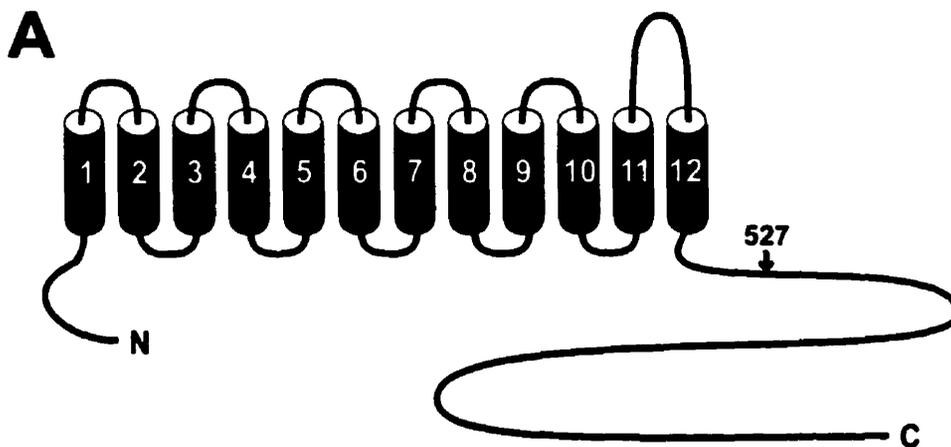


Fig. 6: Transport activity of the wild type and mutant rat NHE1 as a function of the intracellular H⁺ concentration. AP-1 cells expressing wild type and mutant rat NHE1 were grown to confluence in 24-well plates. Initial rates of H⁺-activated ²²Na⁺ influx were measured at various intracellular H⁺ concentrations over the range of pH_i 5.4 to 7.4. The intracellular H⁺ concentrations were adjusted by K⁺-nigericin method as described in "Experimental Procedures". The schematic location of histidine 527 is presented in (A). pH_i profiles for H527D, H527R and H527Y mutants are presented in (B), (C) and (D) respectively. Data were normalized as a percentage of the maximal rate of H⁺-activated ²²Na⁺ influx at pH_i 5.4. Values represent the average of three experiments each performed in quadruplicates. (Solid line: wild type; dashed line: mutant). In B, *P* < 0.05; in C and D, *P* > 0.05.



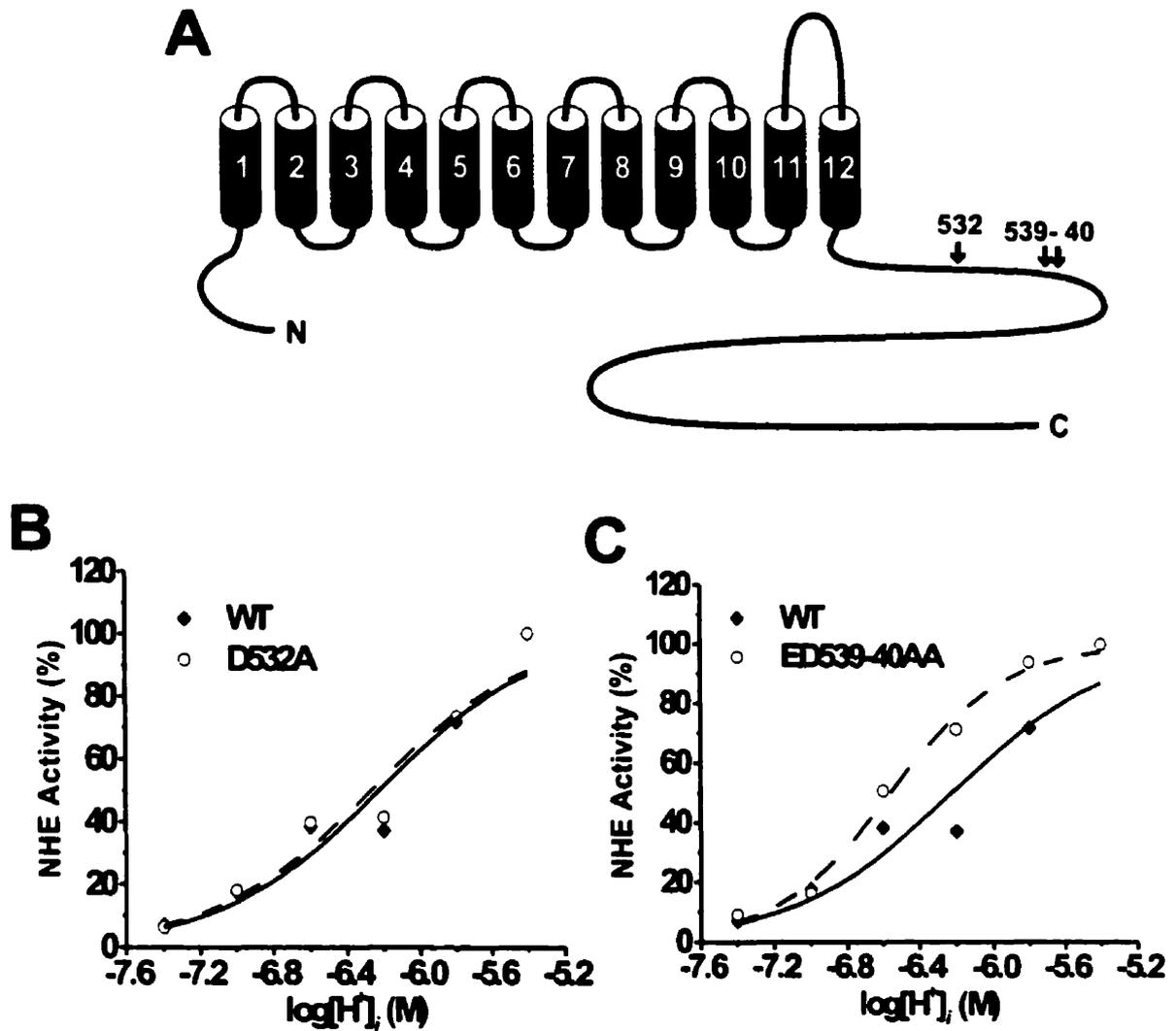


Fig. 7: Transport activity of the wild type and mutant rat NHE1 as a function of the intracellular H^+ concentration. AP-1 cells expressing wild type and mutant rat NHE1 were grown to confluence in 24-well plates. Initial rates of H^+ -activated $^{22}Na^+$ influx were measured at various intracellular H^+ concentrations over the range of pH, 5.4 to 7.4. The intracellular H^+ concentrations were adjusted by K^+ -nigericin method as described in "Experimental Procedures". The schematic location of Asp532 and Glu539Asp540 is presented in (A). pH_i profiles for D532A and ED539-40AA mutants are presented in (B) and (C), respectively. Data were normalized as a percentage of the maximal rate of H^+ -activated $^{22}Na^+$ influx at pH_i 5.4. Values represent the average of three experiments each performed in quadruplicates. (Solid line: wild type; dashed line: mutant). In B, $p > 0.05$; In C: $P < 0.05$.

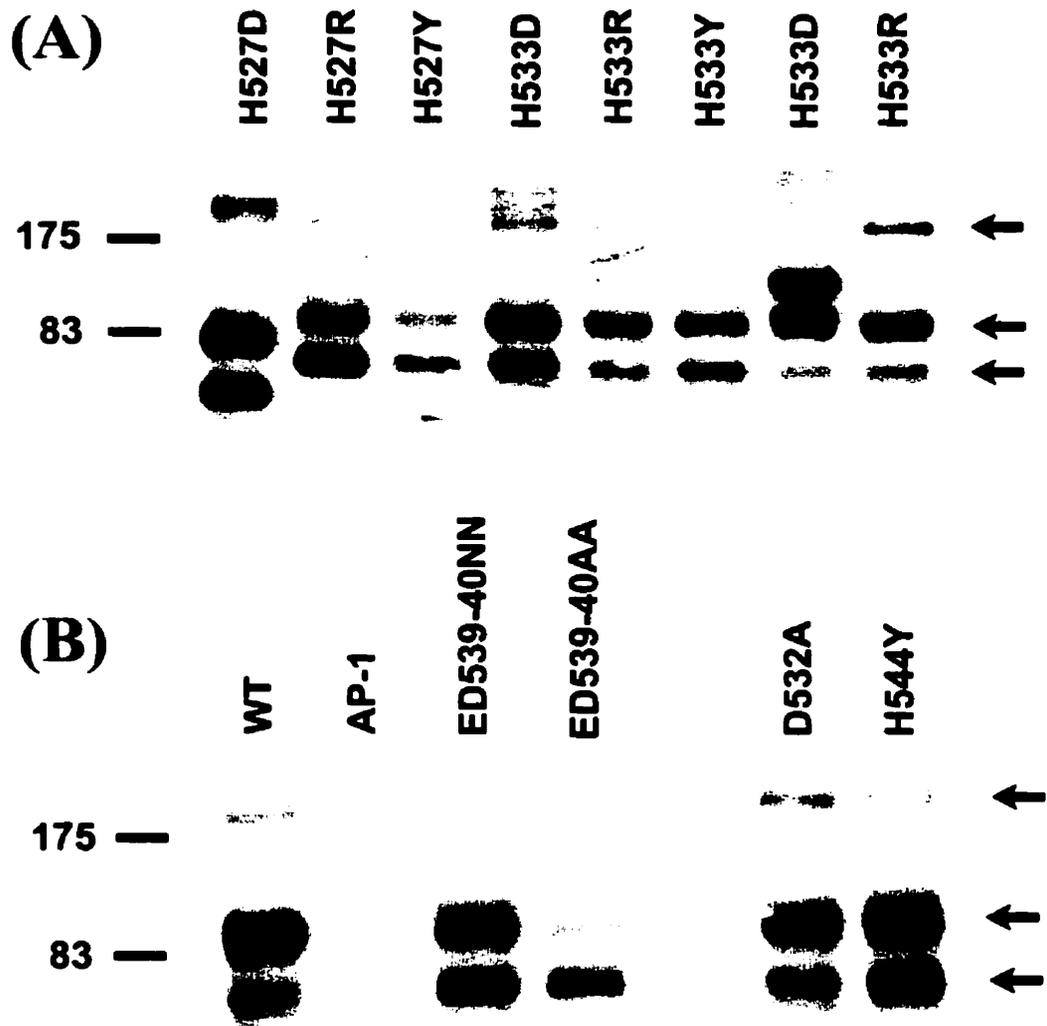


Fig. 8: Protein expression of the wild type and mutant rat NHE1. Stably transfected, fully confluent AP-1 cells were lysed and the protein extracts were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were then transferred onto PVDF membranes and probed with the primary and secondary antibodies and detected using chemiluminescence, as explained in the "Experimental Procedures". The monoclonal mouse anti-HA and the horseradish peroxidase conjugated goat anti-mouse IgG were used as the primary and the secondary antibodies, respectively.

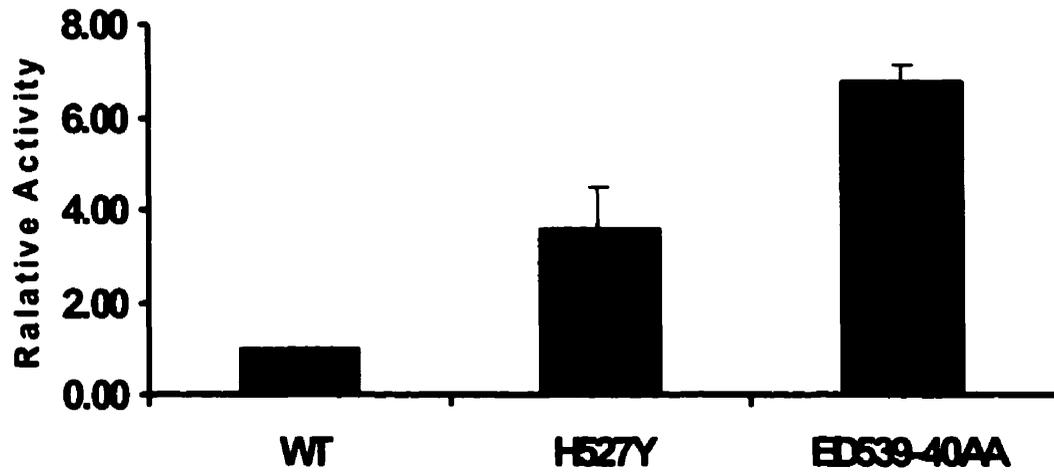


Fig. 9: Relative activity of the wild type and mutant rat NHE1 as a factor of protein level. The transport activity of the NHE1 wild type and two mutants that caused the greatest shift in the pH_i profile of the exchanger was measured as the rate of amiloride-inhibitable H⁺-activated ²²Na⁺ influx at the intracellular pH 5.4 and normalized to their respective plasma membrane protein levels. The data was further normalized to that of the wild type NHE1.

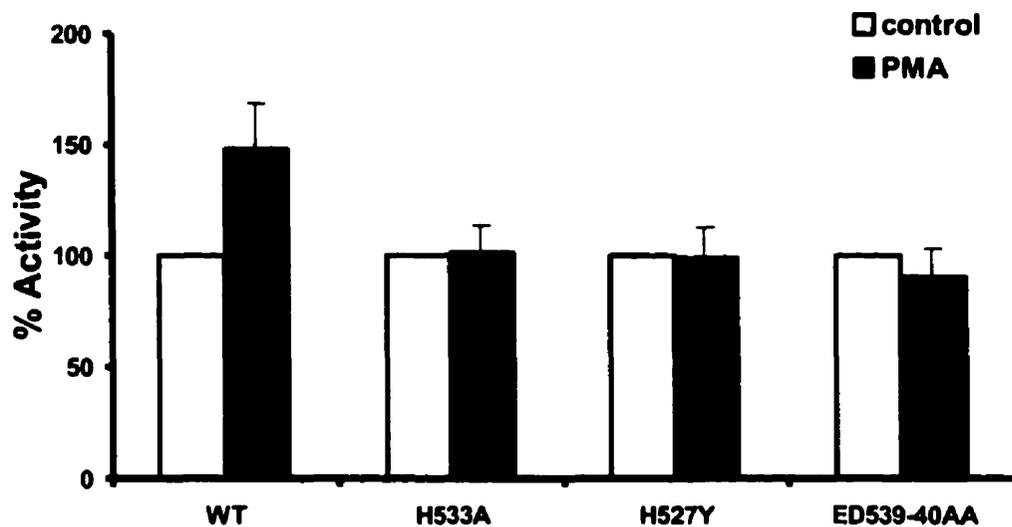


Fig. 10: The activation response of the wild type and mutant rat NHE1 to phorbol ester. AP-1 cells expressing the wild type and mutant NHE1 proteins were serum-deprived overnight. The transport activity of the exchanger was then measured as the rate of amiloride-inhibitable $^{22}\text{Na}^+$ influx in the presence or absence of PMA.

BIBLIOGRAPHY

- Aronson, P.S. (1983). Mechanisms of active H⁺ secretion in the proximal tubule. *Am J Physiol* 245, F647-F659
- Aronson, P.S. (1985). Kinetic properties of the plasma membrane Na-H exchanger. *Annu.Rev.Physiol.* 47, 545-560.
- Aronson, P.S., Nee, J., and Suhm, M.A. (1982). Modifier role of internal H⁺ in activating the Na⁺-H⁺ exchanger in renal microvillus membrane vesicles. *Nature* 299, 161-163.
- Azarani, A., Goltzman, D., and Orlowski, J. (1995a). Parathyroid hormone and parathyroid hormone-related peptide inhibit the apical Na⁺/H⁺ exchanger NHE-3 isoform in renal cells (OK) via a dual signalling cascade involving protein kinase A and C. *J.Biol.Chem.* 270, 20004-20010.
- Azarani, A., Orlowski, J., and Goltzman, D. (1995b). Parathyroid hormone and parathyroid hormone-related peptide activate the Na⁺/H⁺ exchanger NHE-1 isoform in osteoblastic cells (UMR-106) via a cAMP-dependent pathway. *J.Biol.Chem.* 270, 23166-23172.
- Baird, N.R., Orlowski, J., Szabó, E.Z., Zaun, H.C., Schultheis, P.J., Menon, A.G., and Shull, G.E. (1999). Molecular cloning, genomic organization, and functional expression of Na⁺/H⁺ exchanger isoform 5 (NHE5) from human brain. *J.Biol.Chem.* 274, 4377-4382.
- Bennett, M.V. and Verselis, V.K. (1992). Biophysics of gap junctions. *Semin Cell Biol* 3, 29-47.
- Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994). The Na⁺/H⁺ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites. *J.Biol.Chem.* 269, 13703-13709.
- Biemesderfer, D., DeGray, B., and Aronson, P.S. (1998). Membrane topology of NHE3. Epitopes within the carboxyl-terminal hydrophilic domain are exoplasmic. *J Biol Chem* 273, 12391-12396.
- Biemesderfer, D., Pizzonia, J., Abu-Alfa, A., Exner, M., Reilly, R., Igarashi, P., and Aronson, P.S. (1993). NHE3: A Na⁺ /H⁺ exchanger isoform of renal brush border. *Am.J.Physiol.* 265, F736-F742
- Bookstein, C., Musch, M.W., DePaoli, A., Xie, Y., Rabenau, K., Villereal, M., Rao, M.C., and Chang, E.B. (1996). Characterization of the rat Na⁺/H⁺ exchanger isoform NHE4 and localization in rat hippocampus. *Am.J.Physiol.Cell Physiol.* 271, C1629-C1638

- Bookstein, C., Musch, M.W., DePaoli, A., Xie, Y., Villereal, M., Rao, M.C., and Chang, E.B. (1994). A unique sodium-hydrogen exchange isoform (NHE-4) of the inner medulla of the rat kidney is induced by hyperosmolarity. *J.Biol.Chem.* 269, 29704-29709.
- Borgese, F., Malapert, M., Fievet, B., Pouysségur, J., and Motais, R. (1994). The cytoplasmic domain of the Na⁺/H⁺ exchangers (NHEs) dictates the nature of the hormonal response: Behavior of a chimeric human NHE1/trout βNHE antiporter. *Proc.Natl.Acad.Sci.USA* 91, 5431-5435.
- Borgese, F., Sardet, C., Cappadoro, M., Pouysségur, J., and Motais, R. (1992). Cloning and expression of a cAMP-activated Na⁺/H⁺ exchanger: Evidence that the cytoplasmic domain mediates hormonal regulation. *Proc.Natl.Acad.Sci.USA* 89, 6765-6769.
- Boyarsky, G., Ganz, M.B., Cragoe, E.J.J., and Boron, W.F. (1990). Intracellular-pH dependence of Na-H exchange and acid loading in quiescent and arginine vasopressin-activated mesangial cells. *Proc Natl Acad Sci U S A* 87, 5921-5924.
- Brant, S.R., Yun, C.H.C., Donowitz, M., and Tse, C.-M. (1995). Cloning, tissue distribution, and functional analysis of the human Na⁺/H⁺ exchanger isoform, NHE3. *Am.J.Physiol.* 269, C198-C206
- Brown, S.E.S., Heming, T.A., Benedict, C.R., and Bidani, A. (1991). ATP-sensitive Na⁺-H⁺ antiport in type II alveolar epithelial cells. *Am.J.Physiol.* 261, C954-C963
- Burns, K.D., Homma, T., and Harris, R.C. (1991). Regulation of Na⁺-H⁺ exchange by ATP depletion and calmodulin antagonism in renal epithelial cells. *Am.J.Physiol.* 261, F607-F616
- Busch, S., Wieland, T., Esche, H., Jakobs, K.H., and Siffert, W. (1995). G protein regulation of the Na⁺/H⁺ antiporter in *Xenopus laevis* oocytes. Involvement of protein kinases A and C. *J.Biol.Chem.* 270, 17898-17901.
- Cabado, A.G., Yu, F.H., Kapus, A., Gergely, L., Grinstein, S., and Orlowski, J. (1996). Distinct structural domains confer cAMP sensitivity and ATP dependence to the Na⁺/H⁺ exchanger NHE3 isoform. *J.Biol.Chem.* 271, 3590-3599.
- Cala, P.M. (1983). Cell volume regulation by *Amphiuma* red blood cells. The role of Ca²⁺ as a modulator of alkali metal/H⁺ exchange. *J Gen Physiol* 82, 761-784.
- Cano, A., Preisig, P., and Alpern, R.J. (1993). Cyclic adenosine monophosphate acutely inhibits and chronically stimulates Na/H antiporter in OKP cells. *J.Clin.Invest.* 92, 1632-1638.
- Casavola, V., Reshkin, S.J., Murer, H., and Helmle-Kolb, C. (1992). Polarized expression of Na⁺/H⁺ exchange activity in LLC-PK₁/PKE₂₀ cells: II. Hormonal regulation. *Pflügers Arch.* 420, 282-289.

- Cassel, D., Katz, M., and Rotman, M. (1986). Depletion of cellular ATP inhibits the Na^+/H^+ antiport in cultured human cells. Modification of the regulatory effect of intracellular protons on antiporter activity. *J.Biol.Chem.* 261, 5460-5466.
- Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol.Cell.Biol.* 7, 2745-2752.
- Clarke, D.M., Loo, T.W., Inesi, G., and MacLennan, D.H. (1989). Location of high affinity Ca^{2+} -binding sites within the predicted transmembrane domain of the sarcoplasmic reticulum Ca^{2+} -ATPase. *Nature* 339, 476-478.
- Coulter, K.L., Périer, F., Radeke, C.M., and Vandenberg, C.A. (1995). Identification and molecular localization of a pH-sensing domain for the inward rectifier potassium channel HIR. *Neuron* 15, 1157-1168.
- Counillon, L., Franchi, A., and Pouysségur, J. (1993). A point mutation of the Na^+/H^+ exchanger gene (*NHE1*) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis. *Proc.Natl.Acad.Sci.USA* 90, 4508-4512.
- Counillon, L., Pouysségur, J., and Reithmeier, R.A.F. (1994). The Na^+/H^+ exchanger NHE-1 possesses N- and O-linked glycosylation restricted to the first N-terminal extracellular domain. *Biochemistry* 33, 10463-10469.
- Deng, W.P. and Nickoloff, J.A. (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal.Biochem.* 200, 81-88.
- Dibrov, P. and Fliegel, L. (1998). Comparative molecular analysis of Na^+/H^+ exchangers: a unified model for Na^+/H^+ antiport? *FEBS Lett.* 424, 1-5.
- Donowitz, M. and Welsh, M.J. (1987). Regulation of mammalian small intestinal electrolyte transport. In *Physiology of the Gastrointestinal Tract*. L.R. Johnson, ed. (New York: Raven), pp. 1351-1388.
- Echtay, K.S., Bienengraeber, M., Winkler, E., and Klingenberg, M. (1998). In the uncoupling protein (UCP-1) His-214 is involved in the regulation of purine nucleoside triphosphate but not diphosphate binding. *J Biol Chem* 273, 24368-24374.
- Ek, J.F., Delmar, M., Perzova, R., and Taffet, S.M. (1994). Role of histidine 95 on the pH gating of the cardiac gap junction protein connexin43. *Circ.Res.* 74, 1058-1064.
- Escobales, N. and Canessa, M. (1985). Ca^{2+} -activated Na^+ fluxes in human red cells. Amiloride sensitivity. *J Biol Chem* 260, 11914-11923.
- Fafournoux, P., Noël, J., and Pouysségur, J. (1994). Evidence that Na^+/H^+ exchanger isoforms NHE1 and NHE3 exist as stable dimers in membranes with a high degree of specificity for homodimers. *J.Biol.Chem.* 269, 2589-2596.

- Fliegel, L. and Fröhlich, O. (1993). The Na⁺/H⁺ exchanger: An update on structure, regulation and cardiac physiology. *Biochem.J.* 296, 273-285.
- Fliegel, L., Haworth, R.S., and Dyck, J.R.B. (1993). Characterization of the placental brush border membrane Na⁺/H⁺ exchanger: Identification of thiol-dependent transitions in apparent molecular size. *Biochem.J.* 289, 101-107.
- Franchi, A., Cragoe, E.J., Jr., and Pouyssegur, J. (1986). Isolation and properties of fibroblast mutants overexpressing an altered Na⁺/H⁺ antiporter. *J.Biol.Chem.* 261, 14614-14620.
- Garlid, K.D. and Beavis, A.D. (1985). Swelling and contraction of the mitochondrial matrix. II. Quantitative application of the light scattering technique to solute transport across the inner membrane. *J.Biol.Chem.* 260, 13434-13441.
- Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S., and Padan, E. (1993). Histidine-226 is part of the pH sensor of NhaA, a Na⁺/H⁺ antiporter in *Escherichia coli*. *Proc.Natl.Acad.Sci.USA* 90, 1212-1216.
- Giovannini, P., Seydoux, J., and Girardier, L. (1988). Evidence for a modulating effect of Na⁺/H⁺ exchange on the metabolic response of rat brown adipose tissue. *Pflugers Arch* 411, 273-277.
- Goss, G.G., Woodside, M., Wakabayashi, S., Pouyssegur, J., Waddell, T., Downey, G.P., and Grinstein, S. (1994). ATP dependence of NHE-1, the ubiquitous isoform of the Na⁺/H⁺ antiporter. Analysis of phosphorylation and subcellular localization. *J.Biol.Chem.* 269, 8741-8748.
- Green, J., Foellmer, O., Kleeman, C.R., and Basic, M.M. (1993). Acute phosphate depletion inhibits the Na⁺/H⁺ antiporter in a cultured renal cell line. *Am.J.Physiol.* 265, F440-F448
- Grillo, F.G. and Aronson, P.S. (1986). Inactivation of the renal microvillus membrane Na⁺-H⁺ exchanger by histidine-specific reagents. *J Biol Chem* 261, 1120-1125.
- Grinstein, S., Cohen, S., and Rothstein, A. (1984). Cytoplasmic pH regulation in thymic lymphocytes by an amiloride- sensitive Na⁺/H⁺ antiport. *J.Gen.Physiol.* 83, 341-369.
- Grinstein, S., Furuya, W., and Rotstein, O.D. (1988). Intracellular pH regulation in neutrophils: properties and distribution of the Na⁺/H⁺ antiporter. *Soc.Gen.Physiol.Ser.* 43, 303-314.
- Grinstein, S. and Rothstein, A. (1986). Mechanisms of regulation of the Na⁺/H⁺ exchanger. *J.Membr.Biol.* 90, 1-12.
- Grinstein, S., Rothstein, A., and Cohen, S. (1985). Mechanism of osmotic activation of Na⁺/H⁺ exchange in rat thymic lymphocytes. *J Gen Physiol* 85, 765-787.

- Grinstein, S., Rothstein, A., Sarkadi, B., and Gelfand, E.W. (1984). Responses of lymphocytes to anisotonic media: volume-regulating behavior. *Am J Physiol* 246, C204-C215
- Grinstein, S., Rotin, D., and Mason, M.J. (1989). Na/H exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim.Biophys.Acta* 988, 73-97.
- Grinstein, S., Woodside, M., Sardet, C., Pouysségur, J., and Rotin, D. (1992). Activation of the Na⁺/H⁺ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *J.Biol.Chem.* 267, 23823-23828.
- Gupta, A., Schwiening, C.J., and Boron, W.F. (1994). Effects of CGRP, forskolin, PMA, and ionomycin on pH_i dependence of Na-H exchange in UMR-106 cells. *Am.J.Physiol.* 266, C1083-C1092
- Gurich, R.W. and Warnock, D.G. (1986). Electrically neutral Na⁺-H⁺ exchange in endosomes obtained from rabbit renal cortex. *Am.J.Physiol.* 251, F702-F709
- Harold, R. S. and Papineau, D. Cation transport and electrogenesis by *Streptococcus faecalis*. *Journal of Membrane Biology* 8, 45-62. 1972. (GENERIC)
Ref Type: Generic
- Hoffmann, E.K. and Simonsen, L.O. (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol Rev* 69, 315-382.
- Huang, C.L., Cogan, M.G., Cragoe, E.J.J., and Ives, H.E. (1987). Thrombin activation of the Na⁺/H⁺ exchanger in vascular smooth muscle cells. Evidence for a kinase C-independent pathway which is Ca²⁺-dependent and pertussis toxin-sensitive. *J Biol Chem* 262, 14134-14140.
- Ikeda, T., Schmitt, B., Pouysségur, J., Wakabayashi, S., and Shigekawa, M. (1997). Identification of cytoplasmic subdomains that control pH-sensing of the Na⁺/H⁺ exchanger (NHE1): pH-maintenance, ATP-Sensitive, and flexible loop domains. *J.Biochem.(Tokyo)* 121, 295-303.
- Jennings, M.L. (1989). Structure and function of the red blood cell anion exchange protein. *Prog Clin Biol Res* 292, 327-338.
- Kaback, H.R. (1988). Site-directed mutagenesis and ion-gradient driven active transport: on the path of the proton. *Annu Rev Physiol* 50, 243-256.
- Kandasamy, R.A., Yu, F.H., Harris, R., Boucher, A., Hanrahan, J.W., and Orlowski, J. (1995). Plasma membrane Na⁺/H⁺ exchanger isoforms (NHE-1,-2, and -3) are differentially responsive to second messenger agonists of the protein kinase A and C pathways. *J.Biol.Chem.* 270, 29209-29216.

- Kapus, A., Grinstein, S., Wasan, S., Kandasamy, R.A., and Orlowski, J. (1994). Functional characterization of three isoforms of the Na^+/H^+ exchanger stably expressed in Chinese hamster ovary cells: ATP dependence, osmotic sensitivity and role in cell proliferation. *J.Biol.Chem.* 269, 23544-23552.
- Klanke, C.A., Su, Y.R., Callen, D.F., Wang, Z., Meneton, P., Baird, N., Kandasamy, R.A., Orlowski, J., Otterud, B.E., Leppert, M., Shull, G.E., and Menon, A.G. (1995). Molecular cloning, physical and genetic mapping of a novel human Na^+/H^+ exchanger (NHE5/SLC9A5) to chromosome 16q22.1. *Genomics* 25, 615-622.
- Kong, S.K., Choy, Y.M., Fung, K.P., and Lee, C.Y. (1989). cAMP activates Na^+/H^+ antiporter in murine macrophages. *Biochem.Biophys.Res.Commun.* 165, 131-137.
- Kopito, R.R., Lee, B.S., Simmons, D.M., Lindsey, A.E., Morgans, C.W., and Schneider, K. (1989). Regulation of intracellular pH by a neuronal homolog of the erythrocyte anion exchanger. *Cell* 59, 927-937.
- Kulanthaivel, P., Furesz, T.C., Moe, A.J., Smith, C.H., Mahesh, V.B., Leibach, F.H., and Ganapathy, V. (1992). Human placental syncytiotrophoblast expresses two pharmacologically distinguishable types of Na^+/H^+ exchangers, NHE-1 in the maternal-facing (brush border) membrane and NHE-2 in the fetal-facing (basal) membrane. *Biochem.J.* 284, 33-38.
- Kurashima, K., Yu, F.H., Cabado, A.G., Szabó, E.Z., Grinstein, S., and Orlowski, J. (1997). Identification of sites required for down-regulation of Na^+/H^+ exchanger NHE3 activity by cAMP-dependent protein kinase. Phosphorylation-dependent and -independent mechanisms. *J.Biol.Chem.* 272, 28672-28679.
- Levine, S.A., Montrose, M.H., Tse, C.M., and Donowitz, M. (1993). Kinetics and regulation of three cloned mammalian Na^+/H^+ exchangers stably expressed in a fibroblast cell line. *J.Biol.Chem.* 268, 25527-25535.
- Lin, X. and Barber, D.L. (1996). A calcineurin homologous protein inhibits GTPase-stimulated Na-H exchange. *Proc.Natl.Acad.Sci.USA* 93, 12631-12636.
- Little, P.J., Weissberg, P.L., Cragoe, E.J., Jr., and Bobik, A. (1988). Dependence of Na^+/H^+ antiport activation in cultured rat aortic smooth muscle on calmodulin, calcium, and ATP. Evidence for the involvement of calmodulin-dependent kinases. *J.Biol.Chem.* 263, 16780-16786.
- Lowe, J.H., Huang, C.L., and Ives, H.E. (1990). Sphingosine differentially inhibits activation of the Na^+/H^+ exchanger by phorbol esters and growth factors. *J.Biol.Chem.* 265, 7188-7194.
- Macknight, A.D. and Leaf, A. (1977). Regulation of cellular volume. *Physiol Rev* 57, 510-573.

- Mahnensmith, R.L. and Aronson, P.S. (1985). The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. *Circ.Res.* 56, 773-788.
- Manganel, M. and Turner, R.J. (1989). Agonist-induced activation of Na⁺/H⁺ exchange in rat parotid acinar cells. *J.Membr.Biol.* 111, 191-198.
- Meech, R.W. and Thomas, R.C. (1977). The effect of calcium injection on the intracellular sodium and pH of snail neurones. *J Physiol (Lond)* 265, 867-879.
- Mitchell, P. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature Lond.* 191, 144-146. 1961. (GENERIC)
Ref Type: Generic
- Mitchell, P. and Moyle, J. (1969). Translocation of some anions, cations and acids in rat liver mitochondria. *Eur.J.Biochem.* 9, 149-155.
- Moolenaar, W.H. (1986). Effects of growth factors on intracellular pH regulation. *Annu.Rev.Physiol.* 48, 363-376.
- Moolenaar, W.H., Tertoolen, L.G., and De Laat, S.W. (1984). Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. *Nature* 312, 371-374.
- Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T., and De Laat, S.W. (1983). Na⁺/H⁺ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature* 304, 645-648.
- Morley, G.E., Taffet, S.M., and Delmar, M. (1996). Intramolecular interactions mediate pH regulation of connexin43 channels. *Biophys J* 70, 1294-1302.
- Moseley, R.H., Meier, P.J., Aronson, P.S., and Boyer, J.L. (1986). Na-H exchange in rat liver basolateral but not canalicular plasma membrane vesicles. *Am.J.Physiol.* 250, G35-G43
- Moule, S.K. and McGivan, J.D. (1990). Epidermal growth factor and cyclic AMP stimulate Na⁺/H⁺ exchange in isolated rat hepatocytes. *Eur.J.Biochem.* 187, 677-682.
- Murer, H., Hopfer, U., and Kinne, R. (1976). Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochem J* 154, 597-604.
- Nakhoul, N.L. and Boron, W.F. (1988). Acetate transport in the S3 segment of the rabbit proximal tubule and its effect on intracellular pH. *J Gen Physiol* 92, 395-412.

- Niggli, V., Sigel, E., and Carafoli, E. (1982). The purified Ca²⁺ pump of human erythrocyte membranes catalyzes an electroneutral Ca²⁺-H⁺ exchange in reconstituted liposomal systems. *J Biol Chem* 257, 2350-2356.
- Numata, M., Petrecca, K., Lake, N., and Orłowski, J. (1998). Identification of a mitochondrial Na⁺/H⁺ exchanger. *J.Biol.Chem.* 273, 6951-6959.
- Orłowski, J. (1993). Heterologous expression and functional properties of the amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger. *J.Biol.Chem.* 268, 16369-16377.
- Orłowski, J. and Grinstein, S. (1997). Na⁺/H⁺ exchangers in mammalian cells. *J.Biol.Chem.* 272, 22373-22376.
- Orłowski, J. and Kandasamy, R.A. (1996). Delineation of transmembrane domains of the Na⁺/H⁺ exchanger that confer sensitivity to pharmacological antagonists. *J.Biol.Chem.* 271, 19922-19927.
- Orłowski, J., Kandasamy, R.A., and Shull, G.E. (1992). Molecular cloning of putative members of the Na/H exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat Na/H exchanger NHE- 1 and two structurally related proteins. *J.Biol.Chem.* 267, 9331-9339.
- Owen, N.E. and Villereal, M.L. (1982). Evidence for a role of calmodulin in serum stimulation of Na⁺ influx in human fibroblasts. *Proc Natl Acad Sci U S A* 79, 3537-3541.
- Paris, S. and Pouyssegur, J. (1983). Biochemical characterization of the amiloride-sensitive Na⁺/H⁺ antiport in Chinese hamster lung fibroblasts. *J.Biol.Chem.* 258, 3503-3508.
- Paris, S. and Pouyssegur, J. (1984). Growth factors activate the Na⁺/H⁺ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H⁺. *J.Biol.Chem.* 259, 10989-10994.
- Pouyssegur, J., Franchi, A., L'Allemain, G., Magnaldo, I., Paris, S., and Sardet, C. (1987). Genetic approach to structure, function, and regulation of the Na⁺/H⁺ antiporter. *Kidney Int.Suppl.* 23, S144-S155
- Rao, G.N., Sardet, C., Pouyssegur, J., and Berk, B.C. (1992). Na⁺/H⁺ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J.Cell.Physiol.* 151, 361-366.
- Rimon, A., Gerchman, Y., Olami, Y., Schuldiner, S., and Padan, E. (1995). Replacements of histidine 226 of NhaA-Na⁺/H⁺ antiporter of *Escherichia coli* - Cysteine (H226C) or serine (H226S) retain both normal activity and pH sensitivity, aspartate (H226D) shifts the pH profile toward basic pH, and alanine (H226A) inactivates the carrier at all pH values. *J.Biol.Chem.* 270, 26813-26817.

- Rotin, D. and Grinstein, S. (1989). Impaired cell volume regulation in Na⁺-H⁺ exchange-deficient mutants. *Am.J.Physiol.* 257, C1158-C1165
- Sardet, C., Counillon, L., Franchi, A., and Pouysségur, J. (1990). Growth factors induce phosphorylation of the Na/H antiporter, a glycoprotein of 110 kD. *Science* 247, 723-725.
- Sardet, C., Fafournoux, P., and Pouysségur, J. (1991). α -Thrombin, epidermal growth factor, and okadaic acid activate the Na⁺/H⁺ exchanger, NHE-1, by phosphorylating a set of common sites. *J.Biol.Chem.* 266, 19166-19171.
- Sardet, C., Franchi, A., and Pouysségur, J. (1989). Molecular cloning, primary structure, and expression of the human growth factor-activatable Na/H antiporter. *Cell* 56, 271-280.
- Seifter, J.L. and Aronson, P.S. (1986). Properties and physiologic roles of the plasma membrane sodium-hydrogen exchanger. *J Clin Invest* 78, 859-864.
- Shigekawa, M., Iwamoto, T., and Wakabayashi, S. (1996). Phosphorylation and modulation of the Na(+)-Ca²⁺ exchanger in vascular smooth muscle cells. *Ann N Y Acad Sci* 779, 249-257.
- Shrode, L.D., Gan, B.S., D'Souza, S.J., Orlowski, J., and Grinstein, S. (1998b). Topological analysis of NHE1, the ubiquitous Na⁺/H⁺ exchanger using chymotryptic cleavage. *Am.J.Physiol.Cell Physiol.* 275, C431-C439
- Shrode, L.D., Tapper, H., and Grinstein, S. (1997a). Role of intracellular pH in proliferation, transformation, and apoptosis. *J.Bioenerg.Biomembr.* 29, 393-399.
- Spray, D.C. and Bennett, M.V. (1985). Physiology and pharmacology of gap junctions. [Review]. *Annu.Rev.Physiol.* 47, 281-303.
- Sun, A.M., Liu, Y., Centracchio, J., and Dworkin, L.D. (1998). Expression of Na⁺/H⁺ exchanger isoforms in inner segment of inner medullary collecting duct (IMCD₃). *J.Membr.Biol.* 164, 293-300.
- Tse, C.-M., Brant, S.R., Walker, M.S., Pouysségur, J., and Donowitz, M. (1992). Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na⁺/H⁺ exchanger isoform (NHE-3). *J.Biol.Chem.* 267, 9340-9346.
- Tse, C.-M., Levine, S.A., Yun, C.H.C., Brant, S.R., Pouysségur, J., Montrose, M.H., and Donowitz, M. (1993b). Functional characteristics of a cloned epithelial Na⁺/H⁺ exchanger (NHE3): Resistance to amiloride and inhibition by protein kinase C. *Proc.Natl.Acad.Sci.USA* 90, 9110-9114.
- Tse, C.-M., Levine, S.A., Yun, C.H.C., Khurana, S., and Donowitz, M. (1994). Na⁺/H⁺ exchanger-2 is an O-linked but not an N-linked sialoglycoprotein. *Biochemistry* 33, 12954-12961.

- Tse, C.-M., Levine, S.A., Yun, C.H.C., Montrose, M.H., Little, P.J., Pouysségur, J., and Donowitz, M. (1993a). Cloning and expression of a rabbit cDNA encoding a serum-activated ethylisopropylamiloride-resistant epithelial Na^+/H^+ exchanger isoform (NHE-2). *J.Biol.Chem.* 268, 11917-11924.
- Tse, C.-M., Ma, A.I., Yang, V.W., Watson, A.J.M., Levine, S., Montrose, M.H., Potter, J., Sardet, C., Pouysségur, J., and Donowitz, M. (1991). Molecular cloning and expression of a cDNA encoding the rabbit ileal villus cell basolateral membrane Na^+/H^+ exchanger. *EMBO J.* 10, 1957-1967.
- Vicentini, L.M. and Villereal, M.L. (1985). Activation of Na^+/H^+ exchange in cultured fibroblasts: synergism and antagonism between phorbol ester, Ca^{2+} ionophore, and growth factors. *Proc.Natl.Acad.Sci.USA* 82, 8053-8056.
- Vigne, P., Frelin, C., and Lazdunski, M. (1985). The Na^+/H^+ antiport is activated by serum and phorbol esters in proliferating myoblasts but not in differentiated myotubes. Properties of the activation process. *J.Biol.Chem.* 260, 8008-8013.
- Villereal, M.L. (1981). Sodium fluxes in human fibroblasts: kinetics of serum-dependent and serum-independent pathways. *J Cell Physiol* 108, 251-259.
- Wakabayashi, S., Bertrand, B., Ikeda, T., Pouysségur, J., and Shigekawa, M. (1994b). Mutation of calmodulin-binding site renders the Na^+/H^+ exchanger (NHE1) highly H^+ -sensitive and Ca^{2+} regulation-defective. *J.Biol.Chem.* 269, 13710-13715.
- Wakabayashi, S., Bertrand, B., Shigekawa, M., Fournoux, P., and Pouysségur, J. (1994a). Growth factor activation and " H^+ -sensing" of the Na^+/H^+ exchanger Isoform 1 (NHE1). Evidence for an additional mechanism not requiring direct phosphorylation. *J.Biol.Chem.* 269, 5583-5588.
- Wakabayashi, S., Fournoux, P., Sardet, C., and Pouysségur, J. (1992a). The Na^+/H^+ antiporter cytoplasmic domain mediates growth factor signals and controls " H^+ -sensing". *Proc.Natl.Acad.Sci.USA* 89, 2424-2428.
- Wakabayashi, S., Ikeda, T., Iwamoto, T., Pouysségur, J., and Shigekawa, M. (1997). Calmodulin-binding autoinhibitory domain controls " pH -sensing" in the Na^+/H^+ exchanger NHE1 through sequence-specific interaction. *Biochemistry* 36, 12854-12861.
- Wakabayashi, S., Ikeda, T., Noël, J., Schmitt, B., Orlowski, J., Pouysségur, J., and Shigekawa, M. (1995). Cytoplasmic domain of the ubiquitous Na^+/H^+ exchanger NHE1 can confer Ca^{2+} responsiveness to the apical isoform NHE3. *J.Biol.Chem.* 270, 26460-26465.
- Wakabayashi, S., Pang, T., Su, X., and Shigekawa, M. (2000). A novel topology model of the human $\text{Na}(+)/\text{H}(+)$ exchanger isoform 1. *J Biol Chem* 275, 7942-7949.

- Wakabayashi, S., Sardet, C., Fafournoux, P., Counillon, L., Meloche, S., Pages, G., and Pouyssegur, J. (1992b). Structure function of the growth factor-activatable Na⁺/H⁺ exchanger (NHE1). *Rev Physiol Biochem Pharmacol* 119, 157-186.
- Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. (1997). Molecular physiology of vertebrate Na⁺/H⁺ exchangers. *Physiol.Rev.* 77, 51-74.
- Wang, D., Balkovetz, D.F., and Warnock, D.G. (1995). Mutational analysis of transmembrane histidines in the amiloride- sensitive Na⁺/H⁺ exchanger. *Am.J.Physiol.* 269, C392-C402
- Wang, Z., Orłowski, J., and Shull, G.E. (1993). Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na/H exchanger. *J.Biol.Chem.* 268, 11925-11928.
- Weinman, E.J., Minkoff, C., and Shenolikar, S. (2000). Signal complex regulation of renal transport proteins: NHERF and regulation of NHE3 by PKA. *Am J Physiol Renal Physiol* 279, F393-F399
- Weissberg, P.L., Little, P.J., Cragoe, E.J., Jr., and Bobik, A. (1989). The pH of spontaneously beating cultured rat heart cells is regulated by an ATP-calmodulin-dependent Na⁺/H⁺ antiport. *Circ.Res.* 64, 676-685.
- Williams, K.A., Geldmacher-Kaufer, U., Padan, E., Schuldiner, S., and Kühlbrandt, W. (1999). Projection structure of NhaA, a secondary transporter from *Escherichia coli*, at 4.0 Å resolution. *EMBO J.* 18, 3558-3563.
- Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.L., and Lerner, R.A. (1984). The structure of an antigenic determinant in a protein. *Cell* 37, 767-778.
- Winkel, G.K., Sardet, C., Pouyssegur, J., and Ives, H.E. (1993). Role of cytoplasmic domain of the Na⁺/H⁺ exchanger in hormonal activation. *J.Biol.Chem.* 268, 3396-3400.
- Yu, F.H., Shull, G.E., and Orłowski, J. (1993). Functional properties of the rat Na/H exchanger NHE-2 isoform expressed in Na/H exchanger-deficient Chinese hamster ovary cells. *J.Biol.Chem.* 268, 25536-25541.
- Yun, C.H.C., Little, P.J., Nath, S.K., Levine, S.A., Pouyssegur, J., Tse, C.M., and Donowitz, M. (1993). Leu143 in the putative fourth membrane spanning domain is critical for amiloride inhibition of an epithelial Na⁺/H⁺ exchanger isoform (NHE-2). *Biochem.Biophys.Res.Commun.* 193, 532-539.
- Yun, C.H.C., Tse, C.M., and Donowitz, M. (1995). Chimeric Na⁺/H⁺ exchangers: An epithelial membrane- bound N- terminal domain requires an epithelial cytoplasmic C-terminal domain for regulation by protein kinases. *Proc.Natl.Acad.Sci.USA* 92, 10723-10727.