The primary structure and functional expression of the rat Na/H exchanger (NHE) NHE-2 isoform has recently been reported (Wang, Z., Orłowski, J., and Shull, G. E. (1993) J. Biol. Chem. 268, 11925-11928). To further characterize some of its functional properties, biochemical and pharmacological analyses were performed on exchanger-deficient Chinese hamster ovary cells (AP-1) that had been stably transfected with a full-length NHE-2 cDNA. Transport activity for NHE-2 was assayed by measuring amiloride-inhibitable $^{22}$Na$^+$ influx following an acute intracellular acid load. Pharmacological analyses revealed that NHE-2 had a relatively high affinity for amiloride among some of its analogues. The most potent analogue was 5-(N-ethyl-N-isopropyl)amiloride (EIPA) ($K_{o} = 79$ nM), followed by 5-(N,N-dimethylamiloride (DMA) ($K_{o} = 250$ nM), amiloride ($K_{o} = 1.4$ $\mu$M), and benzamil ($K_{o} = 320$ $\mu$M). Nonamiloride compounds known to inhibit the activity of other Na/H exchanger isoforms also inhibited NHE-2 with the following order of potency: clonidine ($K_{o} = 42$ $\mu$M) > harmaline ($K_{o} = 330$ $\mu$M) ≈ cimetidine ($K_{o} = 330$ $\mu$M). Biochemical analyses showed that the extracellular Na$^+$ dependence of NHE-2 followed simple, saturating Michaelis-Menten kinetics with an apparent affinity constant for Na$^+$ ($K_{Na}$) of 50 mM. In contrast, intracellular H$^+$ appeared to activate NHE-2 by a positive cooperative mechanism with an apparent half-maximal activation value of $pK_a 6.90$. Other cations, such as extracellular Li$^+$ and H$^+$, acted as competitive inhibitors of $^{22}$Na$^+$ influx by NHE-2, with apparent $K_i$ values of 3.0 mM and 10 nM, respectively. In contrast, extracellular K$^+$ had no effect on the transport activity of NHE-2. These results indicated that the rat NHE-2 cDNA encodes a functional Na/H exchanger isoform with distinct properties compared to rat NHE-1 and -3.

All mammalian cells contain an integral plasma membrane glycoprotein that mediates the electroneutral transport of extracellular Na$^+$ in exchange for intracellular H$. This Na/H exchanger (NHE) participates in a number of important cellular processes, including the regulation of intracellular pH and maintenance of cell volume as well as the facilitation of cell growth and proliferation in response to growth factor and mitogen stimulation. Furthermore, it is also involved in more specialized functions, such as the transepithelial absorption and secretion of electrolytes in kidney and intestine (reviewed in Refs. 1-3).

The diverse physiological roles fulfilled by the plasma membrane Na/H exchanger can be attributed to the existence of multiple isoforms. Prior studies have provided evidence for at least two forms of this cation transporter (4-8). More recently, molecular cloning studies have confirmed and extended these earlier observations by identifying four distinct members (NHE-1, -2, -3, -4) of this gene family (9-15). The deduced amino acid sequences of these isoforms exhibit approximately 40-60% amino acid identity to each other. Northern blot analyses of rat tissues have revealed that the NHE-1 mRNA is present in all tissues while the other isoforms are expressed in a more restricted range of tissues (10, 11). The NHE-3 transcripts are predominantly expressed in the small intestine and colon with significant levels also present in the kidney and stomach. The NHE-2 and -4 transcripts are most abundant in the gastrointestinal tract, especially the stomach, but are also present in other tissues at lower abundance.

The functional features that distinguish these isoforms have yet to be fully elucidated. Current efforts to characterize some of their biochemical and pharmacological properties have involved the use of Na/H exchanger-deficient cells stably transfected with the NHE-1 (9, 16-19) and NHE-3 (18) isoforms. Based on this and other evidence (20-22), NHE-1 is the amiloride-sensitive, growth factor-activatable exchanger that is expressed in most cells and regulates cytoplasmic pH and cell volume. In polarized epithelial cells, this isoform is generally localized to the basolateral membrane (13, 23). In comparison, NHE-3 has a substantially lower affinity for amiloride and exhibits many of the molecular features attributed to the Na/H exchanger present in apical membranes of renal proximal tubule epithelia (16). As such, this isoform most likely participates in the renal transepithelial reabsorption of Na$^+$ and the luminal secretion of H$^+$ which is necessary for HCO$_3^-$ reabsorption.

In contrast to NHE-1 and -3, the biochemical and pharmacological properties of the remaining two isoforms have not yet been examined in detail. We have recently shown that the rat NHE-2 cDNA encodes a functional transporter capable of mediating the influx of $^{22}$Na$^+$ following an acute intracellular acid load (11). The present study was initiated to

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†The abbreviations used are: NHE, Na/H exchanger; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; DMA, 5-(N,N-dimethyl)amiloride; MES, 4-morpholinopanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.
further define the functional properties of this isoform with respect to its affinity for various pharmacological agents as well as extra- and intracellular cations that are known to influence Na/H exchanger activity. Here, we demonstrate that NHE-2 exhibits distinct pharmacological and kinetic properties in comparison to rat NHE-1 and -3.

**EXPERIMENTAL PROCEDURES**

Materials—Carrier-free $^{22}$NaCl (5 mCi/ml) was obtained from NEN Research Products (Du Pont Canada Inc., Mississauga, Ontario). The following compounds were from Sigma. α-Minimal essential medium, fetal bovine serum, kanamycin sulfate, and trypsin/EDTA were from Gibco-BRL (Burlington, Ontario). Cell culture dishes and flasks were purchased from Becton Dickinson and Co. (Fisher Scientific, Montréal, Québec). All other chemicals and reagents were from BDH Inc. (St. Laurent, Québec) or Fisher Scientific, and were of the highest grade available.

Preparation of Na/H Exchanger Isoforms—Stock solutions of the following compounds were prepared in dimethyl sulfoxide at the indicated concentrations: amiloride (0.5 M), EIPA (0.1 M), DCA (0.1 M), benzamil (0.1 M), bumetanide (0.1 M), cimetidine (1 M), clonidine (0.1 M), and harmaline (1 M). Ouabain was prepared as an aqueous stock at a concentration of 10 mM and nigericin was prepared as a 1 M stock in ethanol.

Cell Culture—A Chinese hamster ovary cell line (AP-1; a generous gift from Dr. S. Grinstein of Hospital for Sick Children, Toronto, Ontario) that is deficient in endogenous Na/H exchanger activity was stably transfected with a full-length rat NHE-2 cDNA (AP-1NHE-2) (11). This cell line was used between passages 3 and 17. The cells were maintained in complete α-minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml kanamycin sulfate, and 25 mM NaHCO$_3$, pH 7.4, and incubated in a humidified atmosphere of 5% CO$_2$ and 95% air.

**RESULTS**

Pharmacological Properties of Rat NHE-2—Na/H exchanger isoforms are known to exhibit differential sensitivities to amiloride-based compounds. To define the amiloride analogue sensitivity of rat NHE-2, the rate of H$^+$-activated $^{22}$Na$^+$ influx was measured as a function of the concentration of amiloride, EIPA, DCA, and benzamil. The results are presented in Fig. 1, and the values for apparent half-maximal inhibition (K$_{50}$) are summarized in Table I. The order of potency of these compounds was EIPA > DCA > amiloride >> benzamil with

![Fig. 1. Concentration-response profiles for inhibition of rat NHE-2 transport activity in AP-1 cells by amiloride and its analogues. AP-1 cells expressing rat NHE-2 (AP-1NHE-2) were grown to confluency in 24-well plates. Prior to $^{22}$Na$^+$ influx measurements, the cells were incubated with isonicotic NH$_4$Cl medium (50 mM NH$_4$Cl, 70 mM choline chloride, 5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) for 30 min at 37 °C in a nominally CO$_2$-free atmosphere. Following preloading with $^+$, the cell monolayers were rapidly washed twice with isonicotic choline chloride solution (125 mM choline chloride, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). $^{22}$Na$^+$ influx assays were initiated by incubating the cells in 0.25 ml of isonicotic choline chloride solution containing 1 mM ouabain and 1 μCi of $^{22}$NaCl (carrier-free)/ml. The assay medium was replaced with the transport ($^{22}$Na$^+$ catalyzed by the Na-K-Cl cotransporter and Na-ATPase.) In experiments designed to examine the effect of extracellular K$^+$ on $^{22}$Na$^+$ influx, the assay medium was further supplemented with 0.1 M bumetanide to inhibit the Na-K-Cl cotransporter. Under the conditions of H$^+$ loading, the time course of $^{22}$Na$^+$ influx was linear up to 10 min at low Na$^+$ concentrations at 22 °C (data not shown). Therefore, a time course of 5 min was chosen for most studies with the following exceptions.

In studies examining the kinetics of Na/H exchanger activity as a function of extracellular Na$^+$ concentration, the uptake of $^{22}$Na$^+$ was linear for only 2 min when Na$^+$ concentration was increased to 100 mM. Hence, for these studies, an uptake time of 1 min was selected.

In studies examining Na/H exchanger activity as a function of intracellular pH (pHi), $^{22}$Na$^+$ influx was linear for approximately 2 min when the pH was set over the range of 6.0 to 8.0 using the K$^+$-nigericin method as detailed by others (24). Briefly, the cells were washed twice with two volumes of isonicotic choline chloride solution. The cells were then incubated for 5 min at 22 °C with 0.5 ml of KCl solutions (130 mM KCl, 5 mM choline chloride, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, and 10 mM nigericin) that were adjusted from pH 6.0 to 8.0 at increments of 0.25 units. Under these conditions, the intracellular pH approaches that of the extracellular medium. The acid loading was terminated by two washes of 1 ml of isonicotic choline chloride solution containing 5 mg/ml bovine serum albumin to scavange H$^+$ ions from the plasma membrane (25). The $^{22}$Na$^+$ uptake assay was essentially as described above with the exception that 5 mg/ml bovine serum albumin was also present.

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 and presence of 1 mM amiloride or 100 μM EIPA and expressed as amiloride- or EIPA-inhibitable $^{22}$Na$^+$ influx.

The influx of $^{22}$Na$^+$ was terminated by washing the cells three times with four volumes of ice-cold isotonic saline solution (130 mM NaCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 20 mM HEPES-NaOH, pH 7.4). The washed monolayers were solubilized with 0.5 ml of 0.5 N NaOH, and the wells were washed with 0.5 ml of 0.5 N HCl. Both the solubilized cell extract and wash solutions were combined, and radioactivity was determined by liquid scintillation spectroscopy. Protein content was determined using the Bio-Rad DC protein assay kit as per the manufacturer's protocol. Each data point represents the average of at least two experiments, each performed in quadruplicate.

![Table I. Comparison of the inhibition constants of the NHE-1, -2, and -3 isoforms of the rat Na/H exchanger for amiloride and nonamiloride compounds](https://example.com/table.png)

**Figure 1**. Concentration-response profiles for inhibition of rat NHE-2 transport activity in AP-1 cells by amiloride and its analogues. AP-1 cells expressing rat NHE-2 (AP-1NHE-2) were grown to confluence in 24-well plates. Prior to $^{22}$Na$^+$ influx measurements, the cells were loaded with H$^+$ using the NH$_4$Cl prepulse technique. Initial rates of H$^+$-activated $^{22}$Na$^+$ influx were measured in the presence of increasing concentrations (10$^{-9}$ to 10$^{-5}$ M) of amiloride (closed square), EIPA (closed triangle), DCA (open circle), and benzamil (open diamond) as detailed under "Experimental Procedures." Data were normalized as a percentage of the maximal rate (100%) to facilitate comparison of the maximal rates of rat NHE-2 transport activity in the presence of H$^+$ and H$^+$-activated $^{22}$Na$^+$ influx in the absence of inhibitor. Values represent the average of at least two experiments, each performed in quadruplicate.
apparent $K_{0.5}$ values of 79 nM, 250 nM, 1.4 µM, and 320 µM, respectively. Comparison of rat NHE-1, -2, and -3 revealed that NHE-2 had intermediate sensitivity to EIPA and DMA relative to NHE-1 and -3. Interestingly, NHE-2 had the same affinity for amiloride as NHE-1. Hence, amiloride cannot be used to distinguish these two isoforms.

A number of nonamiloride compounds, including cimetidine (16, 26), clonidine (16, 26), and harmaline (16, 26, 27), have also been reported to have significant inhibitory effects on Na/H exchanger activity. In order to assess their effects on NHE-2, similar concentration-response experiments were performed as shown in Fig. 2, the order of potency of these compounds was cimetidine $\cong$ harmaline $\cong$ clonidine, with values for apparent half-maximal inhibition of 42, 330, and 330 µM, respectively. These values were generally intermediate to those found for NHE-1 and -3 with the notable exception of clonidine which showed a 5-fold greater affinity for NHE-2 than NHE-1.

Kinetic Properties of Rat NHE-2—To determine the extracellular Na$^+$ (Na$^+$) affinity of NHE-2, the initial rates of H$^+$-activated $^{22}$Na$^+$ influx as a function of the Na$^+$ concentration were examined in AP-NHE-2/C12 cells. As illustrated in Fig. 3, the rate of EIPA-inhibitable $^{22}$Na$^+$ influx was a saturable process that conformed to simple Michaelis-Menten kinetics. Analysis of the data using the algorithm of Eadie-Hofstee (V versus V/[S]; Fig. 3, inset) showed a linear relationship, consistent with Na$^+$ interacting at a single site. Calculation of the value of the negative slope yielded an apparent affinity constant for Na$^+$ ($K_{Na}$) of 50.0 ± 5.8 nM. The transport activity of NHE-2 was also measured as a function of intracellular pH (pHi). Nigicin, an ionophore that couples the transmembrane K$^+$ and H$^+$ gradients, was used to set the pHi over the range of 6.0 to 8.0. The results are presented in Fig. 4 and expressed as a percentage of amiloride-inhibitable $^{22}$Na$^+$ influx at pH 6.0. The rates of Na/H exchange were minimally responsive to a decrease in pH, from 8.0 to 7.5, but increased markedly thereafter as pH was further decreased to 6.0. An Eadie-Hofstee plot of the data is shown in Fig. 4 (inset). The analysis revealed a nonlinear distribution of the data and suggested that NHE-2 might also possess a H$^+$-modifier site in addition to the H$^+$-transport site. However, this nonlinearity was not as well defined as that observed for NHE-1 and NHE-3 (16), and hence the presence of a H$^+$-modifier site is less apparent. The half-maximal H$^+$ activation value for the high capacity site of NHE-2 was estimated to be pK 6.90 ± 0.3

![Fig. 2. Concentration-response profiles for inhibition of rat NHE-2 transport activity in AP-1 cells by cimetidine, clonidine, and harmaline. AP-1 cells expressing rat NHE-2 (AP-1NHE-2/C12) were grown to confluence in 24-well plates. Prior to $^{22}$Na$^+$ influx measurements, the cells were loaded with H$^+$ using the NH$_4$Cl prepulse technique. Initial rates of H$^+$-activated $^{22}$Na$^+$ influx were measured in the presence of increasing concentrations (10$^{-8}$ to 20$^{-4}$ M) of clonidine (open triangle), cimetidine (open circle), and harmaline (closed square). Data were normalized as a percentage of the maximal rate of H$^+$-activated $^{22}$Na$^+$ influx in the absence of inhibitor. Values represent the average of two experiments, each performed in quadruplicate.](image-url)
The presence of certain extracellular monovalent cations, including H\(^+\), Li\(^+\), and NH\(_3\)\(^+\), has been shown to competitively inhibit the influx of \(^{22}\)Na\(^+\) by Na/H exchangers (16, 25, 28-34). However, disparate results have been reported for the influence of other monovalent cations such as K\(^+\), and Cs\(^+\), with some studies reporting inhibition (35) while others showing no effect (25, 28, 34). Our recent study indicated that K\(^+\) can inhibit rat NHE-1 activity, but not that of NHE-3 (16). Thus, it was of interest to determine the influence of H\(^+\), Li\(^+\), and K\(^+\) on the transport activity of NHE-2. As shown in Fig. 5A, the rate of H\(^+\)-activated \(^{22}\)Na\(^+\) influx of AP-1(NHE-2/CIC) cells was markedly inhibited by increasing concentrations of H\(^+\), with an apparent half-maximal inhibition value of pK 7.9 ± 0.1. To further define the molecular mechanism underlying H\(^+\)-inhibition of NHE-2 activity, the initial rates of 1 and 10 mM \(^{22}\)Na\(^+\) influx were measured as a function of the H\(^+\) concentration. Analysis of the data by Dixon plot (1/V versus [H\(^+\)]) yielded straight lines, with the slope of the line decreasing in the presence of increased Na\(^+\) levels. This result indicated that H\(^+\) was acting as a competitive inhibitor of \(^{22}\)Na\(^+\) influx at a single site. Determination of the value for the intercept of the two lines yielded an inhibition constant (pK\(_i\)) of 8.00 ± 0.12. To confirm this observation, the influence of different H\(^+\) concentrations (pH 7.4, 7.9, and 8.4) on the rate of \(^{22}\)Na\(^+\) influx was also examined as a function of increasing Na\(^+\) concentration. Transformation of the data by the algorithm of Eadie-Hofstee showed that the negative slope of the line increased as the H\(^+\) concentration increased, indicating an increased apparent K\(_a\) (i.e. a decreased apparent affinity) for Na\(^+\) with no significant change in the maximal velocity (Fig. 5B). These results are again consistent with a competitive mechanism of inhibition where H\(^+\), and Na\(^+\) compete for the same extracellular binding site.

Similar conclusions were also drawn for Li\(^+\) inhibition of H\(^+\)-activated \(^{22}\)Na\(^+\) influx. As presented in Fig. 6A, Li\(^+\) inhibited \(^{22}\)Na\(^+\) influx with an apparent K\(_a\) of 2.2 ± 0.4 mM. To characterize the nature of this inhibition in greater detail, the initial rates of 1 and 10 mM \(^{22}\)Na\(^+\) influx were measured as a function of the Li\(^+\) concentration. Analysis of the data by Dixon plot (Fig. 6A, inset) yielded straight lines, with the slope of the line decreasing in the presence of increased Na\(^+\) levels. This suggested that these cations also interacted in a competitive manner with an apparent K\(_i\) of 3.8 ± 0.7 mM. To further examine this mechanism, the effect of different Li\(^+\) concentrations (0.1, 1, and 2.5 mM) on the rate of \(^{22}\)Na\(^+\) influx was measured as a function of the Na\(^+\) concentration. As shown in Fig. 6B, increasing concentrations of Li\(^+\) increased the apparent K\(_a\) without affecting the maximal velocity, consistent with a mechanism involving competitive inhibition. Thus, the data indicated that extracellular H\(^+\), and Li\(^+\) inhibited \(^{22}\)Na\(^+\) influx by interacting at a single binding site. In contrast to H\(^+\), and Li\(^+\), concentrations of K\(^+\), ranging from 1 to 100 mM had no significant effect on the initial

**Fig. 5. Influence of extracellular H\(^+\) on amiloride-inhibitable \(^{22}\)Na\(^+\) influx in AP-1 cells expressing rat NHE-2.** A. AP-1(NHE-2/CIC) cells were preloaded with H\(^+\) using the NH\(_4\)Cl prepulse technique. Initial rates of amiloride-inhibitable \(^{22}\)Na\(^+\) influx were measured as a function of increasing extracellular H\(^+\) (pH 6.0-9.5). The \(^{22}\)Na\(^+\) influx medium containing carrier-free \(^{22}\)NaCl (1 μCi/ml) was buffered with 30 mM MOPS-Tris (pH 6.0-6.5), 30 mM MOPS-Tris (pH 7.0), 30 mM HEPES-Tris (pH 7.5-9.5). Data were normalized as a percentage of the maximal rate of \(^{22}\)Na\(^+\) influx at pH 9.5. The apparent K\(_i\) for H\(^+\) was determined from linearity of the rate of 1 mM (closed square) and 10 mM (closed triangle) \(^{22}\)Na\(^+\) influx (inset) according to algorithm of Dixon. B, inhibition kinetics of H\(^+\), were determined for the initial rates of H\(^+\)-activated \(^{22}\)Na\(^+\) influx over a Na\(^+\) concentration range of 1.25 to 100 mM. The initial rates of \(^{22}\)Na\(^+\) influx at pH 7.4 (open triangle), 7.9 (open circle), 8.4 (open square) were transformed by the algorithm of Eadie-Hofstee. Values represent the average of two experiments, each performed in quadruplicate.

**Fig. 6. Influence of extracellular Li\(^+\) on amiloride-inhibitable \(^{22}\)Na\(^+\) influx in AP-1 cells expressing rat NHE-2.** A. AP-1(NHE-2/CIC) cells were preloaded with H\(^+\) using the NH\(_4\)Cl prepulse technique. Initial rates of \(^{22}\)Na\(^+\) influx were measured as a function of increasing concentrations of Li\(^+\) (10\(^{-5}\) to 10\(^{-1}\) M). Isoosmolarity was maintained by adjusting the choline chloride concentration. Data were normalized as a percentage of the maximal rate of \(^{22}\)Na\(^+\) influx in the absence of Li\(^+\). The apparent K\(_i\) for Li\(^+\) was determined from linearity of the rate of 1 mM (closed square) and 10 mM (closed triangle) \(^{22}\)Na\(^+\) influx (inset) according to algorithm of Dixon. B, inhibition kinetics of Li\(^+\), were determined for the initial rates of H\(^+\)-activated \(^{22}\)Na\(^+\) influx over a Na\(^+\) concentration range of 1.25 to 100 mM. The initial rates of \(^{22}\)Na\(^+\) influx in the absence of (open triangle) or presence of 1 mM (open circle) and 2.5 mM (open square) Li\(^+\), were transformed by the algorithm of Eadie-Hofstee. Values represent the average of two experiments, each performed in quadruplicate.
transport rates of $\text{H}^+$-activated $^{22}\text{Na}^+$ influx by AP-1$^{\text{NHE-2/C12}}$ cells (Fig. 7).

**DISCUSSION**

In this study, the pharmacological and kinetic properties of rat NHE-2 were characterized in a heterologous expression system utilizing AP-1 cells, a mutant Chinese hamster ovary cell line devoid of endogenous Na/H exchange activity. The results revealed that the general functional properties of NHE-2 are similar to those of other Na/H exchanger isoforms. However, significant differences in the affinity of NHE-2 for amiloride analogues and nonamiloride compounds as well as Na$^+$, H$^+$, and H$^-$ clearly distinguish this isoform from NHE-1 and -3.

The present study demonstrated that $\text{H}^+$-activated $^{22}\text{Na}^+$ influx of AP-1$^{\text{NHE-2/C12}}$ cells was inhibited by amiloride and its analogues. The value for apparent half-maximal inhibition ($K_{0.5}$) by amiloride was 1.4 $\mu$M and is virtually identical to that of rat NHE-1 (16). In accordance with other observations for NHE-1 and -3 (16, 26, 36–39), the 5'-alkyl substituted analogues, EIPA and DMA, were more potent inhibitors of NHE-2 than amiloride itself. Lastly benzamil, a potent inhibitor of epithelial Na$^+$ channels (40, 41) was a poor inhibitor of NHE-2 activity, similar to that observed for NHE-1 and -3. Comparison of the half-maximal inhibition constants for both EIPA ($K_{0.5} = 79$ nM) and DMA ($K_{0.5} = 250$ nM) indicated that rat NHE-2 had intermediate affinities for these compounds relative to rat NHE-1 and NHE-3 (16). Thus, these two analogues provide a pharmacological means of distinguishing these three isoforms. Although the mechanism of amiloride inhibition of NHE-2 was not investigated, available evidence for other Na/H exchangers strongly supports the view that amiloride competes with extracellular Na$^+$ at the same, or a closely associated, binding site (29–31, 34–36).

Other pharmacological agents are also known to inhibit the Na/H exchanger. Clonidine, an $\alpha_2$-adrenergic receptor agonist (26), was a more potent inhibitor of NHE-2 than cimetidine, a histamine H$_2$-receptor antagonist (26), or harmaline, a hallucinogenic drug known to inhibit amine oxidase and antagonize other Na$^+$-dependent transport proteins (27, 35). This order of potency was similar to that found for NHE-3 but the reverse of that for NHE-1 (16). It was of interest to note that while most of the pharmacological agents tested tended to display a higher affinity for NHE-1 relative to NHE-2 and -3, only clonidine exhibited a significantly greater affinity for NHE-2 compared to NHE-1 and -3 (16). Overall, the pharmacological properties of rat NHE-2 (Table I) clearly distinguish it from the other isoforms and provide a functional basis for its identification.

In addition to the inhibitory effects of these pharmacological agents, a number of monovalent cations are also known to antagonize the influx of $^{22}\text{Na}^+$ via the Na/H exchanger. External H$^+$, Li$^+$, and NH$^+$ have been shown to interact competitively at the Na$^+$-binding site. Furthermore, these cations can stimulate a net H$^+$ influx by serving as alternative substrates. Thus, the Na/H exchanger is capable of functioning in multiple exchange modes (reviewed in Ref. 1). As shown in the present study, the apparent half-maximal inhibitory constant of Li$^+$ ($K_i = 3.0 \pm 0.7$ mM) for NHE-2 was similar to that determined for rat NHE-1 and NHE-3 (16). Additional mechanistic studies demonstrated that this inhibition was competitive (Fig. 6A, inset, B). Extracellular H$^+$ also competed with Na$^+$ for binding at a single site on NHE-2 (Fig. 5B). Interestingly, the affinity of H$^+$, for NHE-2 was an order of magnitude greater than that for NHE-1 and NHE-3, with an apparent $K_i$ estimated to be 10 nM ($pK_i = 8.00 \pm 0.12$). In contrast to Li$^+$, and H$^+$, K$^+$ had no effect on $H^+$-activated $^{22}\text{Na}^+$ influx mediated by NHE-2. This result was similar to that found for NHE-3 but opposite to that of NHE-1 where K$^+$ acted as a weak competitive inhibitor (16).

The Na$^+$, and H$^+$, dependence of $^{22}\text{Na}^+$ influx were also characterized in AP-1$^{\text{NHE-2/C12}}$ cells. Na/H exchanger activity has generally been found to be proportional to the extracellular concentration of Na$^+$. The rate of activation of NHE-2 by Na$^+$ followed a rectangular hyperbola, consistent with simple, saturating Michaelis-Menten kinetics. Half-maximal stimulation by Na$^+$ was attained at 50 mM, within the range of $K_m$ values (5–50 mM) characterized for the Na/H exchanger in various other systems (4, 16, 25, 27, 31, 38, 39). In comparison to rat NHE-1 and -3 expressed in AP-1 cells (16), NHE-2 exhibited a 5- and 10-fold lower affinity for Na$^+$, respectively. In contrast to Na$^+$, the H$^+$, dependence of NHE-2 did not appear to follow simple Michaelis-Menten kinetics. Eadie-Hofstee transformation of the data revealed a nonlinear plot composed of two components, although this nonlinearity was not as readily apparent as those detected for rat NHE-1 and NHE-3 (16). As suggested by Aronson et al. (8), this characteristic of the Na/H exchanger could be accounted for by the existence of a H$^+$-sensitive modifier site in addition to the transport site. This apparent allosteric activation by H$^+$, can be interpreted most simply by assuming the presence of one or more ionizable groups that, upon protonation, alter the conformation of the protein and cause activation. Additional evidence supporting this paradigm was recently provided by Wakabayashi et al. (19) who, using deletion mutagenesis, reported that the NH$_2$-terminal transmembrane region of human NHE-1 most likely contains the H$^+$-modifier site while the COOH-terminal cytoplasmic domain modulates the pH, set point value. Furthermore, in a related study, site-directed mutagenesis was used to identify a histidine residue (His$^{299}$) as a component of the pH sensor of the Nha A isoform of the *Escherichia coli* Na/H antiporter (42). Presumably an analogous residue(s) in the mammalian Na/H exchanger fulfills a similar role. The apparent half-maximal H$^+$, activation value for the high capacity site of NHE-2 was pK 6.90. Thus, this isoform appears to be slightly more sensitive to intracellular pH than NHE-1 (pK 6.75) and NHE-3 (pK 6.45) when expressed in AP-1 cells.

Comparison of the biochemical and pharmacological properties of rat NHE-2 in AP-1 cells with other mammalian systems reveals a high degree of functional similarity with the endogenous Na/H exchanger present in rat thymic lymphocytes (25). This exchanger is highly sensitive to inhibition by...
amiloride (apparent $K_i = 2.5 \mu M$) and $H^+$ (apparent $pK_i \approx 7.6$) and has a low affinity for $Na^+$ (apparent $K_i = 59 \mu M$). Thus, it is conceivable that rat thymic lymphocytes express NHE-2 or an NHE-2-like isoform, although this remains to be verified. As well, the Na/H exchanger systems characterized in primary cultures of rat skeletal myotubes (38) and a rat smooth muscle cell line (39) exhibit affinities for amiloride analogues, $H^+$, and $Na^+$ that are intermediate to those determined for AP-I NHE-1 and AP-I NHE-2 cells, suggesting that these muscle cell types may express both NHE-1 and -2. In keeping with this suggestion, RNA blot analyses of adult rat hind leg skeletal muscle and uterus have revealed moderate levels of expression of both NHE-1 and -2 mRNAs, as well as very low amounts of NHE-4 (10, 11). The physiological significance of NHE-2 in muscle tissue is unclear. However, the slightly higher affinity of NHE-2 for intracellular $H^+$ may possibly be important for a precise regulation of $H^+$ in muscle tissues since several studies have demonstrated that increased intracellular $H^+$ concentrations reduce force development in cardiac and skeletal muscles by decreasing the affinity of myofilaments for Ca$^{2+}$ (43, 44), and by reducing the actomyosin ATPase activity in smooth (45) and skeletal (46) muscles. However, while comparisons of rat NHE-2 functional properties with Na/H exchanger activities in other tissues are interesting, they should be interpreted judiciously since it has been suggested that host cell regulatory influences may modulate the kinetic properties of the exchanger, particularly the set-point of the $H^+$ modifier site (47).

In summary, the transport dynamics of NHE-2 expressed in AP-1 cells are similar to that of other Na/H exchanger isoforms, although it is distinguished by its different affinities for various pharmacological agents and extracellular and intracellular cations. The physiological function of this particular isoform remains to be established.

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