THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 22, Issue of August 5, pp. 16369-16377, 1993
Printed in U.S.A.

Heterologous Expression and Functional Properties of Amiloride High Affinity (NHE-1) and Low Affinity (NHE-3) Isoforms of the Rat Na/H Exchanger*

(Received for publication, March 23, 1993)

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Recent molecular cloning studies have identified four distinct members (NHE-1,-2,-3,-4) of the Na/H exchanger gene family that are expressed in a tissue-specific pattern. To examine some of their functional properties, full-length cDNAs for two of these isoforms, rat NHE-1 and NHE-3, were stably transfected in Na/H exchanger-deficient Chinese hamster ovary cells (AP-1) and assayed for transport activity by measuring amiloride-inhibitable H^+-activated $^{22}Na^+$ influx. Pharmacological analyses revealed that the activity of NHE-1 was substantially more sensitive to inhibition by amiloride and its analogues than NHE-3. Similarly, both isoforms were differentially sensitive to inhibition by cimetidine, clonidine, and harmaline; agents also known to inhibit the activity of the Na/H exchanger. Their rank order of potency for NHE-1 was cimetidine > harmaline ≥ clonidine whereas the reverse order was observed for NHE-3.

The isoforms were also distinguished by their kinetic properties. While the extracellular Na^+ (Na^+_o) dependence of both isoforms showed simple, saturating Michaelis-Menten kinetics, NHE-1 exhibited a 2-fold lower affinity for Na^+_o than NHE-3, with apparent K_{Na} values of 10.0 ± 1.4 and 4.7 ± 0.6 mM, respectively. In contrast to Na^+_o, intracellular H^+ (H^+_i) activated both isoforms by a positive cooperative mechanism. However, NHE-1 had a 2-fold higher apparent affinity for H^+_i compared to NHE-3, with half-maximal activation values of pK 6.75 ± 0.05 and 6.45 ± 0.08, respectively. Other external cations also interacted with both exchangers. Li^+_o and H^+_o inhibited $^{22}Na^+$ influx by both isoforms with similar kinetics. In contrast, K^+_o inhibited $^{22}Na^+$ influx by NHE-1, but had no effect on NHE-3. Thus NHE-1 and -3 exhibited diverse functional properties when expressed in the same cell type. Furthermore, the functional properties associated with NHE-3 closely mimicked those described for the apical membrane Na/H exchanger of renal proximal tubules, suggesting that these proteins are molecularly identical.

* This research was supported by Grant MT-11221 from the Medical Research Council (MRC) of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The Na/H exchanger (NHE)[1] is an integral plasma membrane protein that is expressed in all mammalian cells. Substantial biochemical and pharmacological evidence supports the existence of at least two forms of the Na/H exchanger in a variety of tissues, including ileum (1–3), colon (4), kidney (5, 6), heart (7), and hippocampal neurons (8). These putative isoforms exhibit differences with respect to plasma membrane targeting in polarized epithelial cells (apical or basolateral domains) (1, 5), sensitivity to inhibition by amiloride and its derivatives (5, 6, 9), ion specificity (7), activation by intracellular protons (4, 8), and molecular mass (6, 10–13).

Recent molecular cloning studies have confirmed the existence of multiple isoforms of the Na/H exchanger. The primary structure of the human growth factor-activatable Na/H exchanger (NHE-1) was first described by Sardet *et al.* (14). Subsequent cloning studies have extended these earlier results by identifying four discrete members of this gene family in rat (15, 16). Similarly, three isoforms have been identified in rabbit to date (17–19). These isoforms share approximately 40–60% amino acid identity and are expressed in different tissues in an isoform-specific pattern (15–19). The diverse physiological roles for these multiple isoforms, as well as their distinct biochemical and pharmacological properties, remain to be elucidated. At present, only human NHE-1 has been characterized more extensively at the molecular level. In addition to being expressed in all tissues examined to date (20), this isoform is highly sensitive to inhibition by amiloride analogues (20, 21) and is activated by phosphorylation of serine residues in the carboxyl-terminal region of the protein in response to growth factor and mitogen stimulation (11, 21–23). An essential feature of NHE-1 is its allosteric activation by intracellular H^+ (23). While the precise molecular mechanism has not been defined, intracellular protons are presumed to interact at a "modifier" site that is separate from the sites involved in Na^+ and H^+ transport. Binding of H^+ is believed to cause a conformational change that results in increased transport activity (23, 24). These properties correspond most closely to those described for the ubiquitous or "housekeeping" form of the Na/H exchanger whose primary responsibilities are to maintain intracellular pH homeostasis and regulate cell volume (reviewed in Refs. 25–27). In polarized epithelial cells, this isoform generally resides in the basolateral membrane (17, 28), although exceptions have been reported (9, 29). In addition to its high sensitivity to amiloride analogues, this isoform is also inhibited by other pharmacological agents such as cimetidine, clonidine, and harmaline, with cimetidine being the most effective (9).

[1] The abbreviations used are: NHE, Na/H exchanger; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; DMA, 5-(*N*,*N*-dimethyl)amiloride; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; CMV, cytomegalovirus; MES, 4-morpholineethanesulfonic acid.

At present, the molecular identities of the more "specialized" Na/H exchangers detected in microvillus or apical membranes of a variety of polarized epithelial cells, most notably in kidney and intestine (reviewed in Refs. 27), have yet to be defined. The physiological functions of these exchangers also differ from NHE-1, participating primarily in the vectorial transport of electrolytes across the epithelium. In renal proximal tubule cells, the apical membrane Na/H exchanger plays a significant role in facilitating Na^+ reabsorption as well as the luminal secretion of H^+ that is necessary for HCO_3^- reabsorption (30, 31). This isoform is also distinguished from NHE-1 by its low affinity for a number of pharmacological compounds that inhibit the Na/H exchanger, most notably amiloride and its analogues (5, 6, 9, 32). Hence, it is often referred to as the amiloride-insensitive form; or more preferably, the amiloride low affinity form since it can be inhibited by high concentrations of these compounds. It is also less sensitive to inhibition by cimetidine, clonidine, and harmaline, although the order of potency for these compounds is reversed relative to NHE-1 (9). However, similar to NHE-1, this isoform contains an allosteric "modifier" site that is sensitive to the intracellular H^+ concentration (33). Furthermore, it is regulated by hormones such as angiotensin II (34, 35) and parathyroid hormone (36, 37), suggesting that it is also a target for phosphorylation by serine/threonine protein kinases.

Recent tissue distribution studies of the different isoform mRNAs of the rat Na/H exchanger revealed that NHE-3 is the most abundant isoform expressed in kidney (15, 16). Thus, it is reasonable to postulate that NHE-3 is a likely candidate for the amiloride low affinity form of the Na/H exchanger that is present at significant levels in the apical membrane of renal proximal tubule cells. Delineating the intrinsic biochemical and pharmacological properties of each isoform under controlled conditions would clarify this issue. To accomplish this goal, a heterologous mammalian expression system was used to examine the individual rat Na/H exchanger isoforms. In this report, rat NHE-1 and -3 have been stably transfected and expressed in Chinese hamster ovary cells (AP-1) that are deficient in endogenous Na/H exchange activity. The results revealed that NHE-3 is highly insensitive to inhibition by amiloride compounds relative to NHE-1 and exhibits several other properties previously ascribed to the Na/H exchanger present in apical membranes of renal proximal tubules.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free $^{22}NaCl$ (radioactivity, 5 mCi/ml) was obtained from NEN Research Products (Du Pont Canada Inc., Mississauga, Ontario). Amiloride and the amiloride derivatives 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), 5-(*N*,*N*-dimethyl)amiloride (DMA), and benzamil were kindly provided by Dr. D. Pong (Merck Sharp & Dohme, Kirkland, Québec). Cimetidine, clonidine, harmaline, ouabain, and bumetanide were purchased from Sigma. α-Minimal essential medium, fetal bovine serum, kanamycin sulfate, geneticin (G418 sulfate), and trysin-EDTA were purchased from GIBCO BRL (Burlington, Ontario). Cell culture dishes and flasks were purchased from Becton Dickinson and Co. (Fisher Scientific, Montréal, Québec). All other other chemicals and reagents used in these experiments were purchased from BDH Inc. (St. Laurent, Québec) or Fisher Scientific and were of the highest grade available.

Preparation of Pharmacological Reagents—Stock solutions of the following compounds were prepared in dimethyl sulfoxide at the indicated concentrations: amiloride (0.5 M), EIPA (0.1 M), DMA (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.

Cell Culture—The Na/H exchanger-deficient Chinese hamster ovary cells (AP-1) (38), generously provided by Dr. S. Grinstein (Hospital for Sick Children, Toronto, Ontario), 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 an humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

Stable Transfection and Expression of the Rat NHE-1 and NHE-3 cDNAs in Na/H Exchanger-deficient Chinese Hamster Ovary Cells—To facilitate subcloning and expression of the NHE isoform cDNAs, a hybrid mammalian expression vector (called pCMV) was constructed from plasmid DNA fragments derived from pBluescript II KS^+ (Stratagene) and the eukaryotic expression vector pRc/CMV (Invitrogen Corp.). The main advantages of this reconstructed plasmid relative to other commercially available eukaryotic expression vectors are its small size (3260 base pairs), thereby allowing for easy insertion of large DNA fragments, and its multiple cloning region that contains 13 convenient unique restriction enzyme sites which facilitate unidirectional subcloning. As illustrated in Fig. 1, construction of the new pCMV vector involved the isolation of a DNA fragment (fragment A) from pBluescript (*Puv*II 977 to *Sca*I 2526, 1549 base pairs in length) containing the 5′ half of the ampicillin-resistance β-lactamase gene (AMP) and the ColE1 origin of replication for selection and maintenance in *Escherichia coli*. This was ligated to a DNA fragment (fragment D) isolated from pRc/CMV (*Sca*I 5001 to *Puv*II 1266, 1711 base pairs in length) containing the enhancer/promoter region from the immediate early gene of human cytomegalovirus (CMV), an extensive multiple cloning region flanked by the T7 and SP6 RNA promoter sequences, the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene, and the remaining 3′-terminal sequence of the β-lactamase gene that is necessary to reconstitute it into a functional gene.

To express the rat NHE-1 cDNA, a *Not*I-*Nsi*I fragment from clone RHNHE7-1 (15) that contains the complete coding region of NHE-1 as well as the 5′- and 3′-untranslated sequences (nucleotides −761 to 3820) was isolated. Similarly, to express the rat NHE-3 cDNA, a *Kpn*I-*Sma*I fragment from RKNHE2-1 (nucleotides −44 to 4228) (15) that also contains the entire coding region of NHE-3 and parts of the 5′- and 3′-untranslated regions was isolated. Both fragments were separately ligated into the multiple cloning region of pCMV and called pCMV/NHE-1 and pCMV/NHE-3.

To select for stable expression of NHE-1 and NHE-3, AP-1 cells deficient in Na/H exchange activity were cotransfected with either pCMV/NHE-1 or pCMV/NHE-3 (18 μg each) and pRc/RSV (Invitrogen) (2 μg) plasmids by the calcium phosphate-DNA coprecipitation technique of Chen and Okayama (39). The pRc/RSV plasmid contains the aminoglycoside phosphotransferase 3′ gene under the transcriptional control of the SV40 promoter and confers resistance to the antibiotic geneticin (G418 sulfate). Starting 48 h after transfection, the AP-1 cells were selected for growth in culture media containing G418 (400 μg/ml) for a 1–2-week period. In order to select for those cells that also express Na/H exchange activity, the G418-resistant colonies were further selected for survival in response to an acute acid load essentially as described (40, 41). Briefly, this procedure was performed as follows: cell culture medium was aspirated and replaced with isotonic NH_4Cl medium (50 mM NH_4Cl, 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). The cells were incubated in this media for 30 min at 37 °C in a nominally CO_2-free atmosphere. Following this acid-load treatment, the cells were rapidly washed twice with isotonic saline solution (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-NaOH, pH 7.4) to remove extracellular NH^+_4 and then incubated in the same medium for 60 min at 37 °C in a nominally CO_2-free atmosphere. Following this incubation period, the saline solution was aspirated and replaced with regular culture medium and atmospheric conditions. This acid-load selection was repeated three to five times over a 1–2-week period and discriminates between Na/H exchanger positive and negative transformants. It is also quite feasible to select for NHE isoform transformants using the acid-load technique alone. However, the double selection scheme employed (resistance to G418 and an acid load) greatly minimizes selection of AP-1 cells that may have undergone a spontaneous reversion of the original mutation that abolished the endogenous Na/H exchanger activity.

$^{22}Na^+$ Influx Measurements—The cells were grown to confluence in 6- or 24-well plates. Na/H exchanger activity was determined by first preloading the cells with H^+ using the NH_4Cl technique, followed by measurements of the initial rates of $^{22}Na^+$ influx essentially as described (40, 41). Briefly, the cell culture medium was aspirated and replaced by isotonic NH_4Cl medium (50 mM NH_4Cl, 70 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM

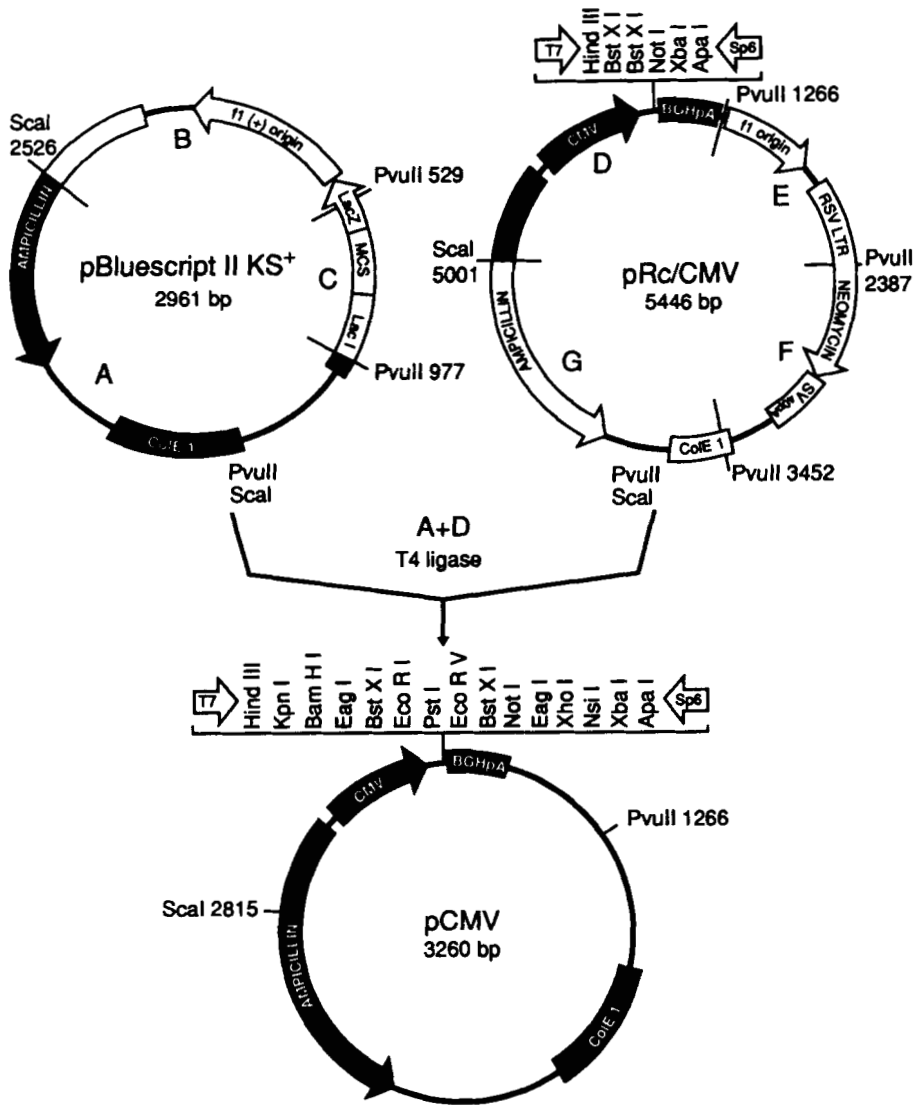

FIG. 1. **Scheme for construction of the mammalian expression vector pCMV.** A hybrid mammalian expression vector (*pCMV*) was constructed from plasmid DNA fragments derived from pBluescript II KS$^+$ and the eukaryotic expression vector pRc/CMV.

HEPES-Tris, pH 7.4). The cells were incubated in this media for 30 min at 37 °C in a nominally CO_2-free atmosphere. Following preloading with H^+, the cell monolayers were rapidly washed twice with isotonic 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 cells in isotonic choline chloride solution containing 1 mM ouabain and 1 μCi of ^{22}NaCl (carrier-free)/ml. The assay medium was K^+-free and included ouabain as measures to prevent the transport of $^{22}Na^+$ catalyzed by Na-K-Cl cotransporter and Na,K-ATPase. For experiments designed to examine the effect of extracellular K^+ on $^{22}Na^+$ influx, the assay medium was further supplemented with 0.1 mM bumetanide to inhibit the Na-K-Cl cotransporter. Under the conditions of H^+ loading, uptake of $^{22}Na^+$ was linear with time for 8–10 min at low Na^+ concentrations at 22 °C (data not shown). Therefore, a time course of 5 min was selected for most studies with the following exceptions.

In studies examining the kinetics of Na/H exchanger activity as a function of extracellular Na^+ (Na^+_o) concentration, $^{22}Na^+$ influx was linear with time for approximately 4 min when Na^+_o was increased to 40 mM. Hence, for these studies, an uptake time of 1 min was chosen. The extracellular Na^+ concentration ranged from 1.25 to 40 mM.

In studies examining Na/H exchanger activity as a function of intracellular pH (pH_i), $^{22}Na^+$ influx was linear for 4 min at pH 7.4. An uptake time of 2 min was selected for these studies. The pH_i was set over the range of 6.0 to 8.0 using the potassium-nigericin method (42). Briefly, the cell culture media was removed and the cells washed twice with isotonic choline chloride solution. The sodium-free solution was then aspirated, and the cells were incubated for 5 min at 22 °C with 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 μM 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 aspirating the K^+-nigericin solutions followed by two washes of 1 ml of isotonic choline chloride solution containing 5 mg/ml bovine serum albumin. The albumin essentially scavenges the ionophore from the plasma membrane (43). The $^{22}Na^+$ influx measurements were identical to that described above with the exception that albumin (5 mg/ml final concentration) 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 and expressed as amiloride-inhibitable $^{22}Na^+$ influx. In general, the background levels of $^{22}Na^+$ influx that were not attributable to the Na/H exchanger were < 5%. Influx of $^{22}Na^+$ was terminated by rapidly washing the cell monolayers four 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 cell monolayers were solubilized in 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 added to scintillation vials, and radioactivity was assayed by liquid scintillation spectroscopy. Protein content was determined using the Bio-Rad *DC* protein assay procedure. All experiments represent the average of two experiments, each performed in duplicate or quadruplicate.

RESULTS

Stable Transfection and Expression of Rat NHE-1 and -3 in Na/H Exchanger-deficient Chinese Hamster Ovary Cells—To

demonstrate that the rat NHE-1 and -3 cDNAs we had recently cloned (15) encoded functional Na/H exchangers, both cDNAs were subcloned into the mammalian expression vector pCMV (see "Experimental Procedures"). Both expression plasmids, pCMV/NHE-1 and pCMV/NHE-3, were transfected into AP-1 cells, and colonies were selected that survived treatment with both the antibiotic G418 and exposure to acute acid-loading. Fig. 2 shows the initial rates of $^{22}Na^+$ influx in response to acid-loading in AP-1 cells alone, or in isolated AP-1 cell colonies that had been transfected with NHE-1 (AP-1^{NHE-1}) and NHE-3 (AP-1^{NHE-3}). Untransfected AP-1 cells showed negligible uptake of $^{22}Na^+$, whereas AP-1 cells transfected with either NHE-1 or -3 demonstrated significant H^+_i-activated $^{22}Na^+$ influx. In AP-1^{NHE-1} cells (colonies C1 and C5), this H^+_i-activated $^{22}Na^+$ influx was completely abolished by the amiloride analogue EIPA (50 μM), a highly selective inhibitor of Na/H exchange activity. Similarly, EIPA inhibited H^+_i-activated $^{22}Na^+$ influx in AP-1^{NHE-3} cells (colonies C1 and C2) by approximately 92%. Thus, the results confirmed that both isoform cDNAs encoded functional proteins that exhibited amiloride-inhibitable Na/H exchange activity. Colonies AP-$1^{NHE-1/C1}$ and AP-$1^{NHE-3/C1}$ were selected for further study.

Pharmacological Properties of Rat NHE-1 and -3—To characterize the amiloride sensitivity of each isoform in greater detail, concentration-response experiments were conducted with amiloride and three of its analogues, EIPA, DMA, and benzamil. The inhibitor concentration profiles are shown in Fig. 3, and the values for apparent half-maximal inhibition ($K_{0.5}$) of H^+_i-activated $^{22}Na^+$ influx are summarized in Table I. With the exception of benzamil, the other three amiloride compounds inhibited H^+_i-activated $^{22}Na^+$ influx of AP-1^{NHE-1} cells to a significantly greater extent than AP-1^{NHE-3} cells, with the range of potency varying between 62- and 608-fold

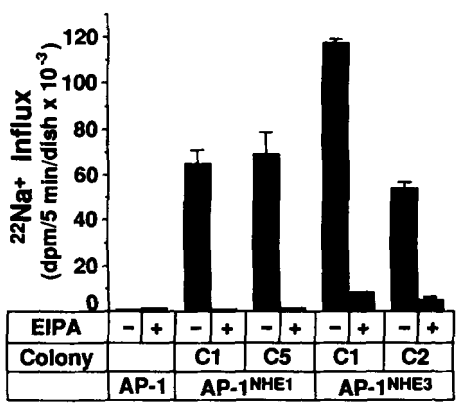

FIG. 2. **Stable expression of rat NHE-1 and -3 cDNAs in Na/H exchanger-deficient Chinese hamster ovary cells.** Rat NHE-1 and -3 cDNAs were subcloned into the mammalian expression plasmid pCMV and cotransfected with the plasmid pRc/RSV containing the neomycin resistance gene into Na/H exchanger-deficient Chinese hamster ovary cells (*AP-1*). Transfected AP-1 cells that had stably integrated both plasmids into their genome were selected for their resistance to G418 and repeated acute acid loads. Isolated cell colonies were picked and assayed for Na/H exchanger activity in 6-well plates. Prior to $^{22}Na^+$ influx measurements, the cells were loaded with H^+ using the NH_4Cl prepulse technique. The cells were washed with Na^+-free isotonic choline chloride solution and then incubated in assay medium containing carrier-free $^{22}NaCl$ (1 μCi/ml) (for details, see "Experimental Procedures"). Initial rates of H^+-activated $^{22}Na^+$ influx were measured in untransfected AP-1 cells, cells transfected with NHE-1 (AP-1^{NHE-1}, *colonies C1* and *C5*), and NHE-3 (AP-1^{NHE-3}, *colonies C1* and *C2*) in the absence or presence of EIPA (50 μM), a highly specific inhibitor of Na/H exchanger activity. Values represent the mean ± S.D. of three to six determinations.

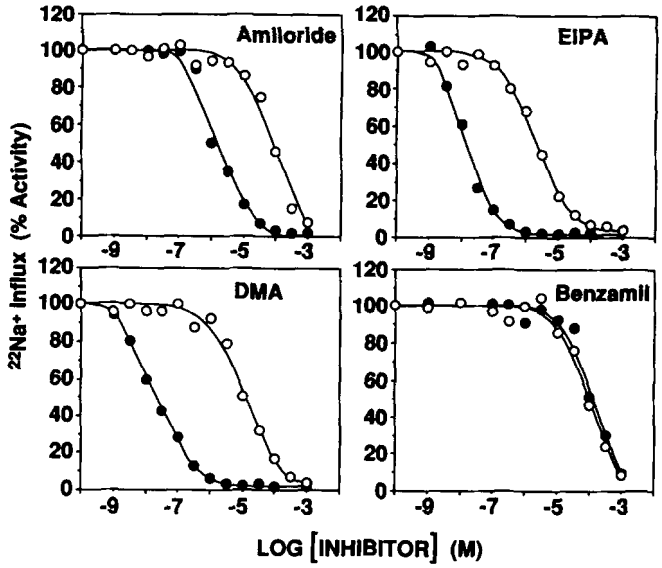

FIG. 3. **Concentration-response profiles for inhibition of rat NHE-1 and -3 transport activity in AP-1 cells by amiloride and its analogues.** AP-1 cells expressing rat NHE-1 (AP-$1^{NHE-1/C1}$) and NHE-3 (AP-$1^{NHE-3/C1}$) were grown to confluence in 24-well plates. Prior to $^{22}Na^+$ influx measurements, the cells were loaded with H^+ using the NH_4Cl prepulse technique. Initial rates of H^+-activated $^{22}Na^+$ influx for NHE-1 (*closed circles*) and NHE-3 (*open circles*) were measured in the presence of increasing concentrations (10^{-9} to 10^{-3} M) of amiloride and its analogues (for details, see "Experimental Procedures"). 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 duplicate.

TABLE I

Comparison of the inhibition constants of the NHE-1 and -3 isoforms of the rat Na/H exchanger for amiloride and nonamiloride compounds

Inhibitor	Inhibition Constants ($K_{0.5}$)	
	NHE-1	NHE-3
	M	
Amiloride compounds		
Amiloride	$1.6 \pm 0.1 \times 10^{-6}$	$1.0 \pm 0.1 \times 10^{-4}$
EIPA	$1.5 \pm 0.2 \times 10^{-8}$	$2.4 \pm 0.2 \times 10^{-6}$
DMA	$2.3 \pm 0.1 \times 10^{-8}$	$1.4 \pm 0.1 \times 10^{-5}$
Benzamil	$1.2 \pm 0.1 \times 10^{-4}$	$1.0 \pm 0.1 \times 10^{-4}$
Nonamiloride compounds		
Cimetidine	$2.6 \pm 0.2 \times 10^{-5}$	$6.2 \pm 0.4 \times 10^{-3}$
Clonidine	$2.1 \pm 0.2 \times 10^{-4}$	$6.2 \pm 0.5 \times 10^{-4}$
Harmaline	$1.4 \pm 0.2 \times 10^{-4}$	$1.0 \pm 0.1 \times 10^{-3}$

depending on the analogue. However, their order of potency was similar for both isoforms:

NHE-1 EIPA ≥ DMA ≫ amiloride ≫ benzamil (Eq. 1)
NHE-3 EIPA > DMA > amiloride ≈ benzamil

A variety of other pharmacological agents have also been tested as to their effects on the transport activity of the Na/H exchanger. Transport inhibitors such as acetazolamide, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, SITS, furosemide, and bumetanide have previously been shown to have no effect (43, 45). However, other compounds such as cimetidine (9, 46), clonidine (9, 47), and harmaline (9, 44, 48), significantly inhibited the exchanger. In order to determine the individual effects of these three compounds on NHE-1 and -3, similar concentration-response experiments were performed. The inhibitor concentration profiles are presented in

Fig. 4 and the values for apparent half-maximal inhibition ($K_{0.5}$) of H^+_i-activated $^{22}Na^+$ influx are summarized in Table I. Similar to the amiloride compounds, all three agents inhibited the transport activity of NHE-1 to a greater extent than NHE-3. The degree of potency ranged from 3- to 238-fold. Unlike the amiloride series, however, the order of potency of these compounds for NHE-1 was the converse of that found for NHE-3:

NHE-1	cimetidine > harmaline ≥ clonidine	(Eq. 2)
NHE-3	clonidine ≥ harmaline > cimetidine	

Kinetic Properties of NHE-1 and -3 in AP-1 Cells—To establish the kinetic parameters of Na^+ and H^+ exchange for the NHE-1 and -3 isoforms, the initial rates of H^+_i-activated $^{22}Na^+$ influx as a function of the extracellular Na^+ concentration were examined in AP-1^{NHE-1} and AP-1^{NHE-3} cells. As illustrated in Fig. 5, the velocity of amiloride-inhibitable $^{22}Na^+$ influx gradually approached saturation with increasing Na^+_o concentration for both isoforms and conformed to simple Michaelis-Menten kinetics. Analysis of the data using the algorithm of Eadie-Hofstee (V *versus* V/[S]) (Fig. 5, *inset*) yielded linear lines, consistent with the presence of single binding sites. Determination of the values of the negative slopes gave apparent Na^+ affinity constants (K_{Na}) of 10.0 ± 1.4 and 4.7 ± 0.6 mM for NHE-1 and -3, respectively. NHE-1

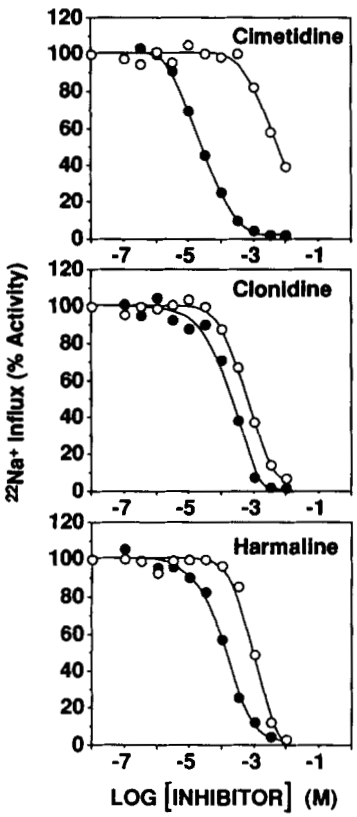

FIG. 4. **Concentration-response profiles for inhibition of rat NHE-1 and -3 transport activity in AP-1 cells by cimetidine, clonidine, and harmaline.** AP-1 cells expressing rat NHE-1 (AP-$1^{NHE-1/C1}$) and NHE-3 (AP-$1^{NHE-3/C1}$) were grown to confluence in 24-well plates. Prior to $^{22}Na^+$ influx measurements, the cells were loaded with H^+ using the NH_4Cl prepulse technique. Initial rates of H^+-activated $^{22}Na^+$ influx for NHE-1 (*closed circles*) and NHE-3 (*open circles*) were measured in the presence of increasing concentrations (10^{-7}-10^{-2} M) of cimetidine, clonidine, and harmaline. 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 duplicate.

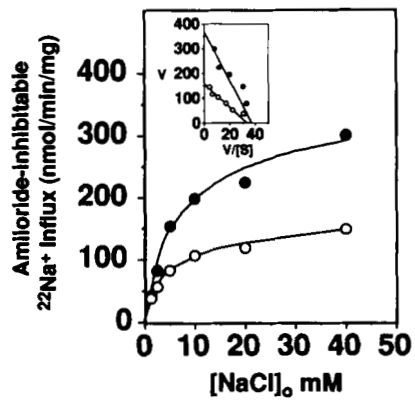

FIG. 5. **Transport activity of rat NHE-1 and -3 in AP-1 cells as a function of the extracellular Na^+ concentration.** AP-$1^{NHE-1/C1}$ and AP-$1^{NHE-3/C1}$ cells were preloaded with H^+ using the NH_4Cl technique. Initial rates of H^+-activated $^{22}Na^+$ influx by NHE-1 (*closed circles*) and NHE-3 (*open circles*) was measured at increasing concentrations of extracellular Na^+. Isoosmolarity was maintained by adjusting the choline chloride concentration. Low levels of background $^{22}Na^+$ influx that were not inhibitable by amiloride (1 mM for NHE-1) or EIPA (0.1 mM for NHE-3) (<20% of total) were subtracted from the total influx. Na/H exchanger activity was expressed as amiloride-inhibitable $^{22}Na^+$ influx (nmol/min/mg protein). Apparent affinity constants (K_{Na}) for Na^+_o were calculated from the linear transformation of the data (*inset*) in Fig. 5 according to the algorithm of Eadie-Hofstee. Values represent the average of two experiments, each performed in duplicate.

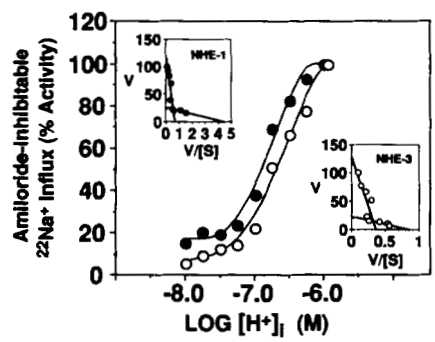

FIG. 6. **Transport activity of rat NHE-1 and -3 in AP-1 cells as a function of the intracellular H^+ concentration.** The initial rates of amiloride-inhibitable $^{22}Na^+$ influx by AP-$1^{NHE-1/C1}$ (*closed circles*) and AP-$1^{NHE-3/C1}$ (*open circles*) cells were measured as a function of the intracellular H^+ concentration over the range of pH_i 6.0–8.0. The intracellular H^+ concentration was adjusted by the K^+-nigericin method as described under "Experimental Procedures." Data were normalized as a percentage of the maximal rate of $^{22}Na^+$ influx at pH 6.0. Apparent affinity constants (K_H) for H^+_i for the high capacity site were calculated from the linear transformation of the data (*insets*) in Fig. 6 according to the algorithm of Eadie-Hofstee. Values represent the average of two experiments, each performed in quadruplicate.

and -3 exchange activity was also measured as a function of intracellular pH over the range of pH_i 6.0–8.0. Nigericin, an ionophore that couples the K^+ and H^+ gradients across the plasma membrane, was used to impose different pH_i values in the transfected AP-1 cells. The Na/H exchanger activity was assessed again by measuring amiloride-inhibitable $^{22}Na^+$ influx. The results are presented in Fig. 6 and expressed as a percentage of the value obtained at pH 6.0, which was assigned a value of 100%. The absolute rates of $^{22}Na^+$ influx at pH 6.0 for both isoforms were very similar to those obtained following an acute acid load by 50 mM NH_4Cl treatment. Each isoform displayed a similar pH_i dependence profile, although the H^+_i-activation curve for NHE-3 was shifted toward the acidic

direction relative to NHE-1. As illustrated, both plots showed minimal change in the rates of exchange between pH_i 8.0 and 7.0 and then rose significantly as the H^+_i concentration increased from pH_i 7.0 to 6.0. Although the true maximum rates of velocity for NHE-1 and -3 were not reached at pH 6.0, almost complete activation of the exchangers occurred over 1–1.25 pH units, consistent with the concept that intracellular H^+ acts by a positive cooperative mechanism. Transformation of the data by the algorithm of Eadie-Hofstee yielded nonlinear lines for NHE-1 and -3 (Fig. 6, *insets*). This nonlinearity was also suggestive of the existence of two binding sites for intracellular H^+. The half-maximal H^+_i activation values for the high capacity site of NHE-1 and -3 were pK 6.75 ± 0.05 and 6.45 ± 0.08, respectively.

Influence of Other Extracellular Cations on Rat NHE-1 and -3 Exchange Activity—The presence of other extracellular monovalent cations, including H^+, Li^+, and NH_4^+, has been shown to inhibit H^+_i-activated $^{22}Na^+$ influx in a competitive manner (43, 45, 49–54). However, conflicting results have been obtained with cations such as K^+ and Cs^+, with some studies reporting inhibition (44) while others showing no effect (43, 45, 54). Thus, it was of interest to determine whether the rates of Na^+ transport for NHE-1 and -3 are differentially sensitive to the presence of other extracellular cations. Fig. 7 shows that H^+_o inhibited the rates of H^+_i-activated $^{22}Na^+$ influx of AP-1^{NHE-1} and AP-1^{NHE-3} cells equally, with apparent pK values of 7.0 ± 0.1. Similarly (Fig. 8), Li^+_o inhibited $^{22}Na^+$ influx with an apparent $K_{0.5}$ of 3.4 ± 0.3 mM for AP-1^{NHE-1} cells and 2.6 ± 0.6 mM for AP-1^{NHE-3} cells. Transformation of these data by the algorithm of Dixon (1/V *versus* [I]) yielded straight lines, implying that external H^+ and Li^+ inhibited by interacting at a single binding site for both isoforms (data not shown).

In contrast to H^+_o and Li^+_o, K^+_o (Fig. 9) had a differential effect on Na/H exchange activity in AP-1^{NHE-1} and AP-1^{NHE-3} cells. Increasing concentrations of K^+_o inhibited H^+_i-activated $^{22}Na^+$ influx with an apparent $K_{0.5}$ of 19.5 ± 1.3 mM in AP-1^{NHE-1} cells, whereas no effect was observed in AP-1^{NHE-3} cells. To characterize the mechanism underlying K^+_o inhibition of NHE-1 transport activity in greater detail, the initial rates of

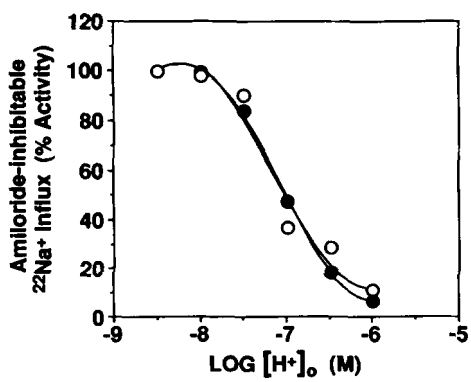

FIG. 7. **Influence of extracellular H^+ on amiloride-inhibitable $^{22}Na^+$ influx in AP-1 cells expressing rat NHE-1 and -3.** AP-$1^{NHE-1/C1}$ and AP-$1^{NHE-3/C1}$ cells were preloaded with H^+ using the NH_4Cl technique. Initial rates of $^{22}Na^+$ influx by NHE-1 (*closed circles*) and NHE-3 (*open circles*) were measured in the presence of increasing concentrations of extracellular H^+ (pH_o 6.0–8.5). The $^{22}Na^+$ influx medium containing carrier-free $^{22}NaCl$ (1 μCi/ml) was buffered with 30 mM MES-Tris, pH 6.0 and 6.5, 30 mM MOPS-Tris, pH 7.0, 30 mM HEPES-Tris, pH 7.5–8.5. Low levels of background $^{22}Na^+$ influx that were not inhibitable by amiloride (1 mM) were subtracted from the total influx. Data were normalized as a percentage of the maximal rate of $^{22}Na^+$ influx at pH 8.5. Values represent the average of two experiments, each performed in duplicate.

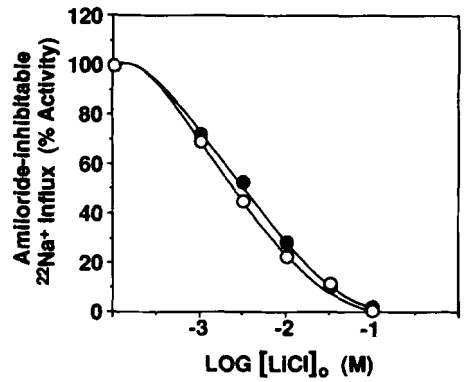

FIG. 8. **Influence of extracellular Li^+ on amiloride-inhibitable $^{22}Na^+$ influx in AP-1 cells expressing rat NHE-1 and -3.** AP-$1^{NHE-1/C1}$ and AP-$1^{NHE-3/C1}$ cells were preloaded with H^+ using the NH_4Cl technique. Initial rates of amiloride-inhibitable $^{22}Na^+$ influx by NHE-1 (*closed circles*) and NHE-3 (*open circles*) were measured in the presence of increasing concentrations of extracellular Li^+. 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^+_o. Values represent the average of two experiments, each performed in duplicate.

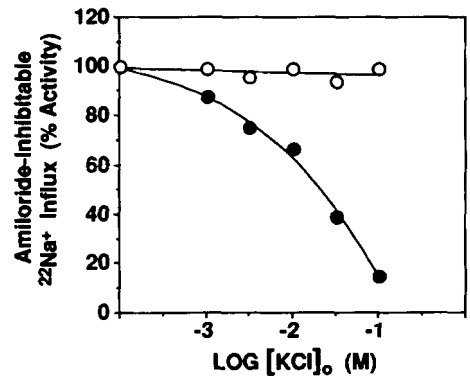

FIG. 9. **Influence of extracellular K^+ on amiloride-inhibitable $^{22}Na^+$ influx in AP-1 cells expressing rat NHE-1 and -3.** AP-$1^{NHE-1/C1}$ and AP-$1^{NHE-3/C1}$ cells were preloaded with H^+ using the NH_4Cl technique. Initial rates of amiloride-inhibitable $^{22}Na^+$ influx by NHE-1 (*closed circles*) and NHE-3 (*open circles*) were measured in the presence of increasing concentrations of extracellular K^+. Isoosmolarity was maintained by adjusting the choline chloride concentration. The assay medium, which contained carrier-free $^{22}NaCl$ (1 μCi/ml) and 1 mM ouabain, was further supplemented with 0.1 mM bumetanide to inhibit the Na-K-Cl cotransporter. Data were normalized as a percentage of the maximal rate of $^{22}Na^+$ influx in the absence of K^+_o.

1 and 10 mM $^{22}Na^+$ influx were measured as a function of the K^+_o concentration. As illustrated in Fig. 10, the presence of an increasing concentration of Na^+_o attenuated K^+_o inhibition of NHE-1 transport activity. Further analysis of the data by Dixon plot (Fig. 10, *inset*) revealed that the apparent half-maximal inhibition constant (K_i) for K^+_o was approximately 180 ± 23 mM. The linearity of the plots and decreased slope of the line in the presence of increased Na^+_o levels is consistent with K^+_o acting as a competitive inhibitor of H^+_i-activated $^{22}Na^+$ influx at a single site. Thus, this implies that their binding is mutually exclusive.

Since it had been previously shown that H^+, Li^+, and NH_4^+ can replace Na^+ in amiloride-inhibitable Na^+-H^+ exchange (reviewed in Ref. 24), it was of interest to determine whether extracellular K^+ could also replace Na^+ and couple its influx to the efflux of intracellular H^+. To test this supposition, a transport assay was conducted that was identical to that used to measure $^{22}Na^+$ influx, except that $^{22}Na^+$ was replaced with

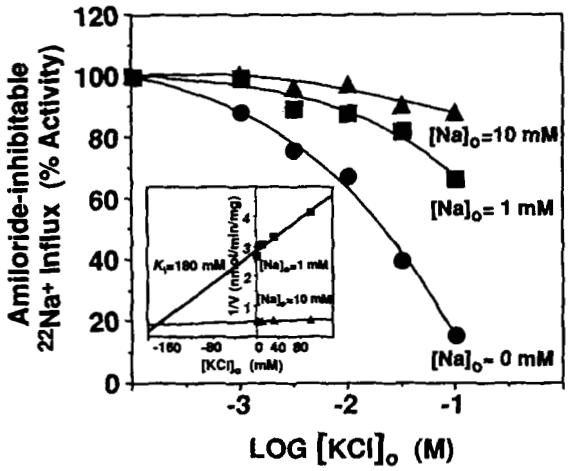

FIG. 10. **Interaction between K^+_o and Na^+_o on the initial rate of amiloride-inhibitable $^{22}Na^+$ influx in AP-1 cells expressing rat NHE-1.** AP-$1^{NHE\text{-}1/Cl}$ cells were preloaded with H^+ using the NH_4Cl technique. Initial rates of amiloride-inhibitable $^{22}Na^+$ influx by NHE-1 at concentrations of 1 mM (*closed squares*) and 10 mM (*closed triangles*) $^{22}NaCl$ were measured over a 2-min period in the presence of increasing concentrations of extracellular K^+. Data from Fig. 9 showing the initial rates of amiloride-inhibitable $^{22}Na^+$ influx of NHE-1 under carrier-free conditions ($[Na]_o \approx 0$ mM) (*closed circles*) was included for comparison. The assay medium was further supplemented with 0.1 mM bumetanide to inhibit the Na-K-Cl cotransporter. Data were normalized as a percentage of the maximal rate of $^{22}Na^+$ influx in the absence of K^+_o. Determination of the apparent K_i for K^+_o was calculated by linearization of the data according to the algorithm of Dixon (*inset*).

the radioactive K^+ congener, $^{86}Rb^+$ (carrier-free $^{86}Rb^+$, 1 μCi/ml). The results showed that AP-$1^{NHE\text{-}1}$ cells did not exhibit H^+-activated $^{86}Rb^+$ influx (data not shown). Thus, although K^+ most likely competes with Na^+ for the same binding site on the exchanger, it does not appear to substitute for Na^+ as a transportable cation.

DISCUSSION

The objective of this study was to characterize and compare the functional properties of two different isoforms (NHE-1 and NHE-3) of the rat Na/H exchanger gene family. In order to accomplish this, a heterologous expression system was utilized that is analogous to the one ingeniously developed by Pouysségur and colleagues (55) involving Na/H exchanger-deficient fibroblasts. In this study, chemically mutagenized Chinese hamster ovary cells that were also deficient in Na/H exchanger activity (AP-1 cells) were used as previously described (38). The advantages of this experimental approach include a common cellular background and absence of endogenous Na/H exchanger activity, thereby permitting an unambiguous characterization of the enzymatic properties of individual Na/H exchanger isoforms. The data presented in this paper show that rat NHE-1 and NHE-3 exhibited intrinsic differences with respect to their inhibition by amiloride analogues and other pharmacological agents, their binding affinities for extracellular Na^+ and intracellular H^+, and their differential sensitivity to extracellular K^+.

The Na/H exchanger has long been known to have different affinities for amiloride and its derivatives depending on the tissue or cell type examined (reviewed in Refs. 27, 56). The apparent inhibitory constant ($K_{0.5}$) for amiloride of the Na/H exchanger isoform present in nonepithelial cells or in the basolateral membrane of polarized epithelial cells (*i.e.* NHE-1) has been reported to be in the low micromolar range. In contrast, this value is one to two orders of magnitude greater for the Na/H exchanger on the apical membrane of polarized epithlial cells from kidney and intestine. However, identification of the NHE isoforms expressed in these specific cell types has not been clearly established. Using the heterologous expression system presented in this study, the NHE-1 isoform exhibited a higher affinity for amiloride than NHE-3, with apparent $K_{0.5}$ values of 1.6 and 100 μM, respectively. Similarly, NHE-1 was found to be substantially more sensitive to the amiloride analogues EIPA and DMA than NHE-3, with differences in sensitivity of approximately 160- and 608-fold, respectively. In contrast, benzamil was found to have low, but similar, affinities for NHE-1 and -3. Since benzamil is a very potent inhibitor of the epithelial Na^+ channel (56, 57), it is evident that this channel does not contribute to the observed H^+-activated $^{22}Na^+$ influx in AP-1 cells. Overall, the order of potency of the different amiloride compounds was similar for both isoforms. Thus, the values obtained for amiloride analogue inhibition of rat NHE-1 activity expressed in AP-1 cells are concordant with values obtained for the amiloride sensitive or high affinity Na/H exchanger found in most cells from a variety of species. In comparison, the data for NHE-3 suggested that this isoform could represent the amiloride-insensitive or low affinity isoform that is present on the apical membrane of various polarized epithelial cells. Because of its predominant expression in rat kidney relative to the other three isoforms (15, 16), it is likely that NHE-3 is the amiloride low affinity form of the Na/H exchanger present at high abundance in the apical membrane of renal proximal tubule cells. Although the amiloride sensitivities of rat NHE-2 and -4 have yet to be reported, preliminary data[2] for rat NHE-2 revealed that it has intermediate amiloride sensitivity relative to NHE-1 and -3. The mechanism of amiloride's interaction with NHE-1 and -3 was not examined in this study. However, numerous other studies have demonstrated that this interaction involves simple competitive inhibition at the external Na^+ transport site (32, 50, 54, 58), although mixed inhibition has also been observed (49). These differences appear to reflect variations in the chloride content of the external buffer (59). Furthermore, the extracellular Na^+- and amiloride-binding sites can be altered independently of each other using genetic selection techniques (52). Overall, these data have been interpreted as indicating that, in addition to the transport site, amiloride may also bind to other regions of the exchanger.

In addition to amiloride analogues, other pharmacological agents are known to inhibit the exchanger. These include cimetidine, a histamine H_2-receptor antagonist (9, 46), clonidine, an α_2-adrenergic receptor agonist (9, 47), harmaline, a hallucinogenic drug known to inhibit amine oxidase and antagonize some Na^+-dependent transport systems (9, 44, 48), loperamide, an opiate receptor agonist (60), and various guanidinium derivatives (61). While these compounds are chemically unrelated to amiloride, they generally possess either an imidazoline or guanidinium moiety and hence bear some structural similarity to amiloride. Results in the present study showed that cimetidine, clonidine, and harmaline inhibited both NHE-1 and -3, with the former isoform being more sensitive to these compounds. A comparison of their relative affinities for NHE-1 revealed that cimetidine was the most potent inhibitor, while clonidine was the least effective. In contrast, the order of potency of cimetidine and clonidine for NHE-3 was reversed compared to NHE-1. These results are very similar to those of a previous study (9) that compared the effects of these agents on the human placental, amiloride high affinity Na/H exchanger and the rabbit renal, amiloride

[2] F. Yu and J. Orlowski, unpublished observations.

low affinity Na/H exchanger. Thus, these results are again consistent with the hypothesis that NHE-3 is the renal proximal tubule Na/H exchanger. More definitive proof as to the identity of the Na/H exchanger on the apical membrane of renal proximal tubule cells, as well as other epithelial cells, awaits cellular localization studies using *in situ* hybridization and immunohistochemical analyses.

Several studies have established that the Na/H exchanger can function in multiple exchange modes involving Na^+, H^+, Li^+, and NH_4^+ (reviewed in Refs. 24). Inward gradients of these cations can effectively inhibit the influx of $^{22}Na^+$ as well as stimulate net H^+ efflux. In general, these interactions appear to follow simple Michaelis-Menten kinetics and are suggestive of a single binding site. External Li^+ has been shown to bind to amiloride high affinity (52, 62) and low affinity (49–51, 53) Na/H exchangers with similar affinities, with K_{Li} values ranging from 1 to 10 mM. The data presented in this study are concordant with these observations, with half-maximal inhibition of $^{22}Na^+$ influx by Li^+ being attained at 3.4 ± 0.3 and 2.6 ± 0.6 mM for NHE-1 and -3, respectively. Likewise, the affinity of external H^+ for amiloride high affinity (52, 63) and low affinity (53) Na/H exchangers has been shown to be similar, with apparent K_H values ranging from 32 to 112 nM. Again, the results for rat NHE-1 and -3 are within this range. Half-maximal inhibition of $^{22}Na^+$ influx by H^+_o for both NHE-1 and -3 was observed at a concentration of 100 nM (pK 7.0). In contrast to Li^+_o and H^+_o, K^+_o competitively inhibited H^+_i-activated $^{22}Na^+$ influx by NHE-1, but had no effect on NHE-3. While the low affinity ($K_i \approx 180$ mM) of K^+_o for NHE-1 indicated that its inhibition of $^{22}Na^+$ influx was not physiologically relevant, it did suggest that the extracellular cation binding sites for NHE-1 and -3 are structurally different.

Comparison of the extracellular cation binding specificities of rat NHE-1 and -3 demonstrated that their apparent cation selectivity is $H^+ >> Li^+ > Na^+ >> K^+$. This is in accord with results from other studies (reviewed in Ref. 24). Interestingly, this sequence differs from the known sequence for external cation transport following binding to the Na/H exchanger, which is $Na^+ \approx NH_4^+ > H^+ > Li^+$ (24). This implies that rate-limiting steps involved in cation translocation and intracellular release are faster for Na^+ than other cations.

The rate of Na/H exchange has generally been found to have a first order dependence on the external Na^+ concentration and exhibits simple, saturating Michaelis-Menten kinetics. A similar Na^+ dependence was also obtained for both rat NHE-1 and -3 expressed in AP-1 cells. The affinity constants characterizing the Na^+ dependence of the Na/H exchanger have been determined in several systems and range from 5 to 59 mM (43, 46, 48, 51, 52, 54, 62, 64–66). This wide variation may be attributed to several factors, including expression of one or more Na/H exchanger isoforms in specific cell types, species differences, as well as variations in experimental conditions (*i.e.* membrane vesicles *versus* whole cells and measurements of $^{22}Na^+$ influx *versus* fluorescence dye measurements of H^+ efflux). In general, however, the apparent K_{Na} values for the amiloride high affinity Na/H exchanger (*i.e.* presumably the NHE-1 isoform) from rat brain synaptosomes (62), Chinese hamster lung fibroblasts (54), and human placenta (46) ranged from approximately 10 to 13 mM and compared favorably with the value obtained for rat NHE-1 (K_{Na} = 10.0 ± 1.4 mM) under our experimental conditions. While the published K_{Na} values for amiloride low affinity Na/H exchanger(s) varied more widely depending on the tissue examined, reported values for rat (64, 65) and rabbit (48, 51) renal tissue ranged from 5 to 10 mM and are concordant with the value determined for rat NHE-3 (K_{Na} = 4.7 ± 0.6 mM). The higher Na^+ affinity of NHE-3 compared to NHE-1 suggests that it is better suited to mediating Na^+ uptake at low extracellular Na^+ concentrations, consistent with its presumed role as the apical Na/H exchanger responsible for reabsorbing Na^+ from the lumen of renal proximal tubules.

One of the most interesting kinetic features of the Na/H exchanger is its asymmetric interaction with H^+ on the extracellular and intracellular membrane. As mentioned above, extracellular H^+ interacts with the Na/H exchanger with simple Michaelis-Menten kinetics at a single binding site. However, intracellular H^+ has been shown to interact with both the amiloride high affinity (23, 43, 62, 67) and low affinity (33, 64, 65, 68) Na/H exchangers with a greater than first order dependence, implying the existence of more than a single binding site for intracellular H^+. The apparent allosteric H^+ activation of rat NHE-1 and -3 are concordant with these observations, although the precise molecular mechanism for this effect has yet to be defined. Since it is known that acidic and basic groups participate in enzymatic catalysis, this type of activation curve 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. This paradigm was first supported by Aronson *et al.* (33) as a likely mechanism to account for their findings that increased H^+_i stimulates Na^+-Na^+ exchange as well as the net efflux of Na^+ in exchange for the influx of H^+.

Comparison of the two isoforms reveals that NHE-1 is more sensitive to changes in the intracellular H^+ concentration than NHE-3, with apparent pK values of 6.75 and 6.45, respectively. These two affinity constants closely agree with corresponding values reported for the amiloride high affinity Na/H exchanger (~pK 6.65) in rat brain synaptosomes (62), Chinese hamster lung fibroblasts (67), and human NHE-1 expressed in mouse fibroblasts (23), and the amiloride low affinity Na/H exchanger (pK 6.36–6.55) in rat renal microvillus membranes (64, 65). The higher H^+_i sensitivity of the ubiquitous, amiloride high affinity Na/H exchanger (NHE-1 isoform) is also consistent with its physiological role in maintaining intracellular pH.

In summary, the NHE-1 and -3 isoforms of the rat Na/H exchanger exhibit distinct biochemical and pharmacological properties when expressed in the same cell type. Comparison of their respective kinetic properties indicates that NHE-1, with its high affinity for intracellular H^+, is well suited for maintaining intracellular pH homeostasis. In contrast, the higher affinity of NHE-3 for extracellular Na^+ suggests that its structure is more appropriately designed for the reabsorption of Na^+ across polarized epithelial cells of kidney as well as other tissues such as the gastrointestinal tract. The heterologous expression system used in the present study will be useful for investigating other structural, functional, and regulatory aspects of plasma membrane Na/H exchangers.

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