Kinetic Dissection of Two Distinct Proton Binding Sites in Na\(^+\)/H\(^+\) Exchangers by Measurement of Reverse Mode Reaction*  

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We examined the effect of intracellular acidification on the reverse mode of Na\(^+\)/H\(^+\) exchange by measuring \(^{22}\text{Na}^\text{+}\) efflux from \(^{22}\text{Na}^\text{+}\)-loaded PS120 cells expressing the Na\(^+\)/H\(^+\) exchanger (NHE) isoforms NHE1, NHE2, and NHE3. The 5-(\text{N}-ethyl-N-isopropyl)amiloride (EIPA)- or amiloride-sensitive fraction of \(^{22}\text{Na}^\text{+}\) efflux was dramatically accelerated by cytosolic acidification as opposed to thermodynamic prediction, supporting the concept that these NHE isoforms are activated by protonation of an internal binding site(s) distinct from the H\(^+\) transport site. Intracellular pH (pHi) dependence of \(^{22}\text{Na}^\text{+}\) efflux roughly exhibited a bell-shaped profile; mild acidification from pHi 7.5 to 7 dramatically accelerated \(^{22}\text{Na}^\text{+}\) efflux, whereas acidification from pHi 6.6 gradually decreased it. Alkalization above pHi 7.5 completely suppressed EIPA-sensitive \(^{22}\text{Na}^\text{+}\) efflux. Cell ATP depletion and mutation of NHE1 at Arg\(^{440}\) (R440D) caused a large acidic shift of the pH profile for \(^{22}\text{Na}^\text{+}\) efflux, whereas mutation at Gly\(^{455}\) (G455Q) caused a significant alkaline shift. Because these mutations and ATP depletion cause correspondingly similar effects on the forward mode of Na\(^+\)/H\(^+\) exchange, it is most likely that they alter exchange activity by modulating affinity of the internal modifier site for protons. The data provide substantial evidence that a proton modifier site(s) distinct from the transport site controls activities of at least three NHE isoforms through cooperative interaction with multiple protons.

The Na\(^+\)/H\(^+\) exchangers (NHEs) belong to one of the secondary active transporter families that catalyze the transport of ions or solutes using a driving force generated by active ion pumps. NHEs are involved in various cellular functions such as pH homeostasis, cell volume regulation, and transepithelial Na\(^+\) absorption (1–4), and at least eight isoforms differing in tissue and subcellular localization have been identified. The activities of NHEs are controlled by various extrinsic factors including hormones, growth factors, pharmacological agents, and mechanical stimuli (1–4). Many of these stimuli modulate the activity by changing the apparent affinity for intracellular H\(^+\) (Refs. 5–8; see Refs. 1–4 for reviews).

Physiologically, NHE catalyzes an electroneutral exchange of extracellular Na\(^+\) for intracellular H\(^+\), i.e. a forward mode of exchange with the aid of a constant driving force provided by a Na\(^+\) pump. However, NHE is also able to catalyze the exchange of intracellular Na\(^+\) for extracellular H\(^+\), i.e. a reverse mode of exchange under certain conditions. In cells, NHE is inactivated usually at an intracellular pH (pHi) of ~7.2, a value much lower than that (>8) predicted from the thermodynamic equilibrium between intracellular and extracellular Na\(^+\) and H\(^+\) ions. This “set point” behavior has been attributed to the existence of an allosteric regulatory site(s) called the “H\(^+\) modifier” site or “pH sensor” in NHE. Twenty years ago, Aronson et al. (9, 10) elegantly presented evidence for such a site based on analysis of the kinetics of ion exchange in renal brush border membrane vesicles; they found that \(^{22}\text{Na}^\text{+}\) uptake into Na\(^+\)-loaded vesicles (Na\(^+\)/Na\(^+\) exchange) is stimulated by intravesicular H\(^+\) and that \(^{22}\text{Na}^\text{+}\) efflux is stimulated by intravesicular H\(^+\), a finding opposite to the expected competitive interaction of H\(^+\) with Na\(^+\). These findings led to the idea that NHE becomes active only when the internal H\(^+\) modifier site is occupied by a proton. In many subsequent studies using native or NHE-transfected cells, exchange activity was reported to exhibit a complex, cooperative dependence on the internal H\(^+\) concentration despite its hyperbolic dependence on external Na\(^+\) or H\(^+\) concentration (11–18), suggesting the involvement of at least two binding sites for internal H\(^+\). However, because only the forward mode of exchange was measured in these studies and because very accurate measurement of pHi is sometimes difficult, it still remains difficult to clearly distinguish the H\(^+\) modifier and H\(^+\) transport sites. Furthermore, it is not clear that the H\(^+\) modifier site exists in various NHE isoforms, because detailed analysis has not yet been performed using cells expressing each NHE isoform.

To obtain insight into the modifier role of intracellular H\(^+\), in this study we measured pHi dependence of \(^{22}\text{Na}^\text{+}\) efflux from cells expressing NHE1, NHE2, or NHE3. We found that \(^{22}\text{Na}^\text{+}\) efflux is dramatically stimulated by intracellular acidification but almost completely inhibited by modest alkalization (pHi ~7.5), which provides a strong piece of evidence for the existence of an intracellular H\(^+\) modifier site in these NHE isoforms. We show that pHi dependence of the Na\(^+\)/H\(^+\) exchange can be explained by assuming the interaction of multiple protons with the regulatory site(s) and the interaction of a single proton with the transport site.

EXPERIMENTAL PROCEDURES

Materials—The amiloride derivative 5-(\text{N}-ethyl-N-isopropyl)amiloride (EIPA) was a gift from New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). \(^{22}\text{NaCl}\) was purchased from PerkinElmer Life Sciences. All other chemicals were of the highest purity available.
Cell Culture and Stable Expression—A Na+/H+ exchanger-deficient cell line (PS120) (19) and corresponding transfectants were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) as described (16). Plasmid transfection and selection of cell populations expressing NHE variants were performed as described (16).

Construction of Na+/H+ Exchanger Mutants—A plasmid carrying cDNA coding for the Na+/H+ exchanger isoforms NHE1–3 cloned into the mammalian expression vector pECE was described previously (16). The construction of two mutants, G455Q and R440D, was also described previously (20). Gly455 and Arg440 were reported to be located within the mammalian expression vector pECE was described previously (16).

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RESULTS

To measure 22Na+ efflux, we first preincubated PS120 cells or their NHE transfectants for 30 min in a radioactive solution containing 1 mM 22NaCl, the K+/H+ ionophore nigericin, and either 48 or 140 mM KCl. This preincubation allowed cells to be pH-i-clamped at 7 or 7.5 and, at the same time, to be loaded with 22Na+. 22Na+ was rapidly taken up by cells and reached nearly to the plateau after the preincubation for 20 min under both conditions (Fig. 1B), suggesting that the radioactivity of 22Na+ was equilibrated in the extra- and intracellular medium. The intracellular 22Na+ concentration at 30 min was 1–2 mM by assuming a value of 5 μl of cell water per milligram of protein. After removal of the preincubation solution, cells were placed in a Na+-free, non-radioactive solution (pH 7.4) to start 22Na+ efflux. By this procedure, we were able to measure 22Na+ efflux in the presence of an outwardly directed H+ gradient (at pH 7) or in the absence of the H+ gradient (at pH 7.5) (Fig. 1A). We expected that NHE-independent background 22Na+ leakage would be minimal, because the efflux medium contained the Na+-pump inhibitor ouabain and the Na+/K+/Cl– cotransporter inhibitor bumetanide but not HCO3−, a substrate for both Na+/HCO3− cotransporter and Na+–dependent Cl–/HCO3− exchanger. In fact, we observed only slow 22Na+ efflux in exchange-deficient PS120 cells and their NHE1 transfectants in the presence of the NHE-specific inhibitor EIPA (Fig. 1, C–E).

As expected, EIPA-sensitive 22Na+ efflux was not detectable in exchange-deficient PS120 cells whose pH-i was maintained at 7 (Fig. 1C) or 7.5 (not shown). In NHE1 transfectants, however, rapid EIPA-sensitive 22Na+ efflux was observed at pH 7 (Fig. 1D), although little EIPA-sensitive 22Na+ efflux was observed at pH 7.5 (Fig. 1E). Thus, 22Na+ efflux via NHE1 is dramatically stimulated by intracellular acidification, whereas it is completely inhibited by alkalization. If we assume that NHE1 catalyzes a counter transport reaction only involving the transport site, the outwardly directed H+ gradient should lead to inhibition of 22Na+ efflux. The data support the view that activity of NHE1 is regulated by protonation/deprotonation of the H+ modifier site(s), which is different from the H+ transport site.

To examine pH dependence of 22Na+ efflux, we clamped pH-i at various values by incubating NHE1-expressing cells for 30 min in solutions containing nigericin and different concentrations of KCl. Because 22Na+ was taken up by cells via the exchanger during pH-i clamping, 22Na+ loading was greater in cells clamped at lower pH-i than in cells clamped at higher pH-i. To minimize such differences in 22Na+ loading, we used a pH-i clamp solution containing different concentrations (0.2–1.2 mM) of 22NaCl. As shown in Fig. 2A, the level of 22Na+ loading varied from 10 to 16 nmol/mg (corresponding to intracellular concentrations of 2–3 mM), indicating that the outwardly directed Na+ gradient produced under the conditions used was not very different. Fig. 2A also shows levels of 22Na+ remaining in cells 3 min after the addition of the efflux solution with or without EIPA. We plotted the EIPA-sensitive fraction of 22Na+ efflux (Fig. 2B, open circles), and the values normalized to the initial level of 22Na+ loading as a function of pH-i (Fig. 2C). The 22Na+ efflux increased steeply with decreasing pH-i from 7.5 to 7.2, reached the maximum at pH 6.6, and then decreased with decreasing pH-i from 6.6 to 5.6. The bell-shaped pH-i profile of 22Na+ efflux suggests that there are at least two intracellular proton-binding sites, each involved in the stimulation and inhibition of NHE1 function, respectively.

We examined the effect of cell ATP depletion on the pH-i,
dependence of $^{22}\text{Na}^+$ efflux. We treated cells for 30 min with metabolic inhibitors 2-deoxyglucose (5 mM) and oligomycin (2 μg/ml) during pHj clamping. Such treatment decreased cell ATP to less than 5% of that in normal cells (16). In these cells, the initial $^{22}\text{Na}^+$ loading was 6–9 nmol/mg (corresponding to intracellular concentrations of 1.2–1.8 mM) at pHj values from 6.6 to 7.5, although $^{22}\text{Na}^+$ was loaded to high levels (20–30 nmol/mg) at pHj 6.2 or 5.6. $^{22}\text{Na}^+$ efflux and its normalized values were plotted against pHj. As shown in Fig. 2, B and C, $^{22}\text{Na}^+$ efflux was almost completely inhibited by ATP depletion at least in the pHj range from 6.6 to 7.5, consistent with the previous finding (16, 21–23) that ATP depletion greatly shifts the pHj dependence of the forward mode of Na+/H+ exchange to an acidic side, as shown in Fig. 2D. It is also noted that, similar to the forward mode, the inhibitory effect of ATP-depletion on $^{22}\text{Na}^+$ efflux is alleviated by acidification.

Fig. 3 shows the results of similar $^{22}\text{Na}^+$ efflux measurements using cells expressing G455Q or R440D in which the pHj dependence of the forward mode of exchange markedly shifts to an acidic and alkaline sides, respectively (Fig. 4B; see also Ref. 20). As in the case with the wild type NHE1, $^{22}\text{Na}^+$ efflux in G455Q-expressing cells was much faster at pHj 7 than at pHj 7.5 (Fig. 3B, C and D). Interestingly, a significant level of EIPA-sensitive $^{22}\text{Na}^+$ efflux was still observed in these cells even at pHj 7.5 (Fig. 3B, C and D), unlike the case with the wild-type NHE1. In contrast, a significant level of EIPA-sensitive $^{22}\text{Na}^+$ efflux was not observed in R440D-expressing cells at pHj 7 (Fig. 3C) or 7.5 (Fig. 3D), although low levels of $^{22}\text{Na}^+$ efflux were observed in a more acidic range of pHj (Fig. 4A). Fig. 4 shows pHj profiles of $^{22}\text{Na}^+$ efflux in cells expressing G455Q or R440D. The pHj profile was significantly shifted to an alkaline side in G455Q-expressing cells compared with cells expressing the wild-type NHE1 (Fig. 4, A and B). In contrast, the pHj profile was markedly shifted to an acidic side in cells expressing R440D.

Finally, we examined whether intracellular acidification activates $^{22}\text{Na}^+$ efflux in cells expressing NHE2 or NHE3 (Fig. 5). In these experiments, we used a high concentration (5 mM) of amiloride in place of EIPA as an inhibitor, because these isoforms are relatively less sensitive to the amiloride analogue. As in the case of NHE1, modest intracellular acidification (pHj 7) significantly accelerated amiloride-sensitive $^{22}\text{Na}^+$ efflux from cells expressing NHE2 or NHE3, although $^{22}\text{Na}^+$ efflux from
NHE2 transfectants was relatively slow (Fig. 5). The slow efflux in NHE2 transfectants may be due to lower expression of the exchanger in the plasma membrane, because exchange activity of these transfectants was 20–30% of that of NHE1 or NHE3 transfectants.

In this work we tried to kinetically dissect proton-binding sites in the Na+/H+ exchanger by measuring EIPA-sensitive $^{22}$Na$^+$ efflux from cells expressing different NHE isoforms. We loaded cells with $^{22}$Na$^+$ while clamping pH at various values using a K+/nigericin technique and measured $^{22}$Na$^+$ efflux at a constant extracellular pH (7.4) in the nominal absence of extracellular Na$^+$. To avoid possible interaction of cytosolic Na$^+$ with the H$^+$ modifier site as suggested previously (14, 25), we limited the extent of $^{22}$Na$^+$ loading to low levels (2–3 mM). To our knowledge, $^{22}$Na$^+$ efflux has never been measured using cultured cells under such defined conditions.

$^{22}$Na$^+$ efflux from cells expressing NHE1, NHE2, or NHE3 was markedly stimulated by cytoplasmic acidification, contrary to the prediction based on reduced H$^+$ concentration gradient. As proposed previously (9, 10), the results can be interpreted as indicating that these NHE isoforms possess the H$^+$ modifier site and that occupancy of the latter by a proton(s) results in activation of exchange activity. We observed that the NHE1-mediated $^{22}$Na$^+$ efflux exhibited roughly bell-shaped pH dependence. Modest cytosolic alkalization (pH 7.5) abolished $^{22}$Na$^+$ efflux (Figs. 1D, 2B, and 4), whereas modest cytosolic acidification from pH 7.5 to 7–7.2 dramatically enhanced it (Figs. 1C, 2B, and 4). The increase in $^{22}$Na$^+$ efflux in the latter pH range was very steep, suggesting the binding of two or more protons to the modifier site. In a near-neutral pH range, cell ATP depletion or mutations of NHE1 (R440D and G455Q) caused marked shifts in the pH dependence of $^{22}$Na$^+$ efflux (Figs. 2B, 3B, and 4). Because they also caused similar large

**FIG. 5.** Time courses of $^{22}$Na$^+$ efflux in cells expressing NHE2 or NHE3. Cells expressing NHE2 or NHE3 were pH-clamped and $^{22}$Na$^+$-loaded as described under “Experimental Procedures”. A and B, time courses of $^{22}$Na$^+$ efflux in cells expressing NHE2 were measured in the absence (○) and presence (●) of 5 mM amiloride at pH 7.5 and 7, respectively. C and D, time courses of $^{22}$Na$^+$ efflux in cells expressing NHE3 were measured in the absence (○) and presence (●) of 5 mM amiloride at pH 7.5 and 7, respectively. Data represent means ± S.D. of three determinations.

**FIG. 6.** Schemes explaining the pH dependence of the Na$^+/H^+$ exchange. We assumed that multiple protons (n molecules) cooperatively bind to the H$^+$ modifier sites (circular space), whereas a single proton binds to the transport site (rectangular space) on the cytosolic side of the exchanger. In addition, we assumed that these two types of H$^+$ binding sites are independent, i.e. H$^+$ binding at one site does not affect the H$^+$ affinity for the other. In the forward mode of exchange ($^{22}$Na$^+$ uptake) (A), only the exchanger occupied by protons at both sites would be able to participate in the exchange reaction, whereas in the reverse mode of exchange ($^{22}$Na$^+$ efflux) (B), the exchanger having protons at the modifier site but $^{22}$Na$^+$ at the transport site would become active. In the reverse mode, H$^+$ would competitively inhibit Na$^+$ binding at the transport site. Based on these assumptions, we simulated the pH dependences of $^{22}$Na$^+$ uptake (C) and $^{22}$Na$^+$ efflux reactions (D). We used the following steady-state equations that were developed assuming a rapid equilibrium of H$^+$ and Na$^+$ binding (37): $\nu\nu = \frac{[\text{Na}^+]K_K(1 + \frac{[\text{H}^+]K_H}{[\text{H}^+]})}{V_{\text{max}}}$ for the relative rate of $^{22}$Na$^+$ uptake, and $\nu\nu = \frac{[\text{Na}^+]K_K(1 + \frac{[\text{H}^+]K_H}{[\text{H}^+]})}{V_{\text{max}}}$ for the relative rate of $^{22}$Na$^+$ efflux, where $K_K$ and $K_K$ are the intrinsic H$^+$ dissociation constants for the transport and modifier sites, respectively, and $K_K$ is the Na$^+$ dissociation constant for the transport site. For simulation of $^{22}$Na$^+$ efflux, $K_K$ and the intracellular Na$^+$ concentration were assumed to be 10 and 2 mM, respectively. The values for other parameters obtained by means of manual fitting trials are given in panels C and D.
shifts in pH dependence of the forward mode of Na\(^+\)/H\(^+\) exchange under corresponding conditions (20), it is most likely that modulation of Na\(^+\)/H\(^+\) exchange by these procedures is attributable to altered interaction of the H\(^+\) modifier site with activating protons. On the other hand, acidosis (<pH 6.2) caused an extensive inhibition of 22Na\(^+\) efflux (Figs. 2B and 4). This inhibition appears to result from competition between Na\(^+\) and H\(^+\) for the intracellular transport site. Our measurement of the descending and ascending slopes of pH dependence of 22Na\(^+\) efflux thus allowed us to observe the binding of protons to the H\(^+\) modifier and H\(^+\) transport sites, respectively. The effect of pH on 22Na\(^+\) efflux from cells was measured previously using thymic lymphocytes (13) in which a significant difference in 22Na\(^+\) efflux was not observed at the two pH values tested (7.2 and 6.3). A possible explanation for such data is that the two pH values used for thymic lymphocytes may not be optimal for observing a large pH-dependent change in 22Na\(^+\) efflux.

We attempted to reproduce by simulation the pH dependence of the forward and reverse modes of Na\(^+\)/H\(^+\) exchange and their modulation by ATP depletion or the exchanger mutations using a simplified reaction model and the assumption that three protons (n = 3) cooperatively interact with the modifier site and a single proton interacts with the transport site (Figs. 6, A and B). We were able to at least qualitatively reproduce complex pH profiles of the forward and reverse modes of exchange and their modulation (Fig. 6, C and D). For example, ATP depletion or mutation of Arg440 inhibited the uptake and efflux activities more strongly in the neutral pH range (6.6–7.5). In addition, pH dependence between 6.6 and 5.6 was very apparent affinities for extracellular substrates Na\(^+\)/H\(^+\) for the intracellular transport site. Our measurement of pH dependence of 22Na\(^+\) efflux permitted us to analyze kinetic properties of the H\(^+\) modifier site interacting with activating protons. The observed properties are compatible with the predicted roles of the exchanger in cell pH regulation, i.e. protection of cells from excessive alkalosis, acceleration of recovery of cells from acidosis, and modulation of exchange activity due to alteration in H\(^+\) affinity of the modifier site, in particular in the near-neutral pH range.

Mutation of Arg440 shifts pH dependence of the forward mode of Na\(^+\)/H\(^+\) exchange toward an acidic side, whereas that of Gly455 shifts it toward an alkaline side without changing apparent affinities for extracellular substrates Na\(^+\) and H\(^+\) and the inhibitor EIPA (20) (see Fig. 4B). Thus, the present data reinforce our previous conclusion that the region encompassing the intracellular loop II5 and the transmembrane domain TM11 plays a crucial role in the proper functioning of the H\(^+\) modifier site (20). Although the structure of the H\(^+\) modifier site is not known, we suggested previously that pH sensing of NHE1 may be controlled by a substructure consisting of intracellular loop II5 and the juxtamembrane subdomain I of the cytoplasmic domain (amino acids 503–595) with a tightly bound calcineurin B homologous protein (20, 26). In general, a histidine residue has been thought to be a good candidate for a residue involved in pH sensing, because its imidazole moiety is the only side chain that ionizes in solution within a physiological pH range. Indeed, His259 and His297 have been identified as important residues for pH sensing of the Na\(^+\)/H\(^+\) antiporters of Escherichia coli (NhaA) (27, 28) and Schizosaccharomyces pombe (Sod2) (29, 30), respectively. However, little change was observed in pH sensitivity when histidine residues at positions 76, 81, 250, 285, 325, 373, 376, 407, 408, and 473 were substituted by cysteine in NHE1, although a recent study (31) reported that mutations at His479 and His499 in the juxtamembrane cytoplasmic domain of rabbit NHE3 shifted the pH profile to an acidic side. Other histidine residues of NHE1, i.e. His60, His120 and His149 in the membrane-spanning segments (32) and those in the histidine cluster (HYHHHH) in the cytoplasmic domain (30), do not appear to directly influence exchange activity.

Although the present results suggest the existence of a H\(^+\) modifier site in NHEs, it should be noted that complex kinetic effects of protons on exchanger activation have been reported as follows. (i) Transient kinetic studies of the exchanger using kidney brush border membrane vesicles revealed that the exchanger exhibits cooperativity with respect to the external Na\(^+\) concentration when vesicles are acid-loaded (33), suggesting that protonation of the modifier site may change the oligomeric interaction. (ii) Exchange activity of NHE3 (34, 35) or NHE1 (36) is slowly (3–5 min) activated by intracellular acidification, suggesting that a slow conformational change or phosphorylation-dependent event may be involved in the expression of exchanger activity. (iii) Intracellular Na\(^+\) is able to activate the exchanger (14, 25), suggesting a possible interaction of Na\(^+\) with the H\(^+\) modifier site. However, the structural basis for these properties of the H\(^+\) modifier site is not known.

In summary, using cultured cells expressing different NHE isoforms, we obtained evidence for the existence of the H\(^+\) modifier site(s) distinguishable from the H\(^+\) transport site. Our results suggest that interaction of multiple protons with the modifier site results in a dramatic activation of Na\(^+\)/H\(^+\) exchange in response to modest acidification. Clearly, further work is required to clarify the structure and function of the H\(^+\) modifier site, which is a hallmark of NHE regulation.

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