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ORIGINAL ARTICLE

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Evidence for the presence of a Na^+-H^+ exchanger in the endolymphatic sac epithelium of guinea-pigs

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Abstract The intracellular pH (pH_i) of epithelial cells from the endolymphatic sac (ES) of the guinea-pig was measured microfluorometrically with the pH-sensitive fluorescent dye, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to examine the presence of a Na^+-H^+ exchanger (NHE) in the ES epithelial cells. pH_i recovery from acid loading with an NH_4^+-prepulse in a nominally HCO_3-free solution was dependent on extracellular Na^+ ($[Na^+]_o$) and was inhibited by amiloride and its analogue ethylisopropylamiloride (EIPA), suggesting that a decreased pH_i induced by an acute acid load may be equilibrated by a NHE. In the steady-state, amiloride had no effect on pH_i, indicating that the NHE activity is low at the resting pH_i. However, the intracellular acidification induced by the removal of $[Na^+]_o$ was inhibited by the simultaneous application of amiloride. H^+-efflux rate (J_H, mean activity of NHE), which was calculated as the product of the recovery rate (dpH_i/dt) from the acid loading and the intrinsic buffering capacity (β_i) at the corresponding pH_i, was decreased as pH_i was increased. The concentration/response curve for the inhibition of initial J_H by EIPA revealed an apparent 50% inhibitory constant (K_i) of 0.85 μM. Kinetic analysis of initial J_H as a function of $[Na^+]_o$ revealed a Michaelis-Menten constant (K_m) of 24.14 mM for Na^+-dependent H^+ efflux. The results indicate that NHE in the ES epithelium belongs to an amiloride-sensitive subtype.

Key words Amiloride · BCECF · Ethylisopropylamiloride (EIPA) · H^+-efflux rate · Intracellular pH

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Introduction

The endolymphatic sac (ES) epithelium of the mammalian inner ear is presumed to absorb endolymphatic fluid generated from the stria vascularis in the cochlea and the dark cells in the vestibule organ, to regulate endolymph volume and to maintain homeostasis of the endolymphatic system [2, 9, 12]. It is reported that the cation concentrations in the ES endolymph are close to those in the perilymph, rather than those in the cochlea and vestibule [14, 15]. An in vivo experiment [14] suggests the presence of an active Na^+ uptake mechanism across the apical membrane of the ES epithelial cells from the endolymph towards the blood. Na^+ absorption is achieved by the entry of Na^+ into cells across their apical membrane, through an amiloride-sensitive Na^+ channel, and then exit across the basolateral membrane in exchange for K^+ via the Na^+-K^+-ATPase. K^+ is recycled across the basolateral membrane through K^+ channels or it leaves the cell via K^+ channels in the apical membrane. Recent investigations of ion transport in the ES epithelium have located a Na^+-K^+-ATPase at the basolateral membrane [13, 21, 27], a K^+ conductance at the basolateral and apical membranes [26] and an amiloride-sensitive Na^+ conductance at the apical membrane [16, 20]. These results suggest that Na^+ transport may play a crucial role in the absorption of endolymph in the ES.

Na^+ absorption via epithelial cells is pH sensitive, because apical Na^+-H^+ exchange is coupled to Cl^--HCO_3^- exchange [10]. The Na^+-H^+ exchanger (NHE) in the mammal is an electroneutral transporter protein, which moves extracellular Na^+ into cells in exchange for intracellular H^+ with a stoichiometry of 1:1. NHE, which is inhibitable by the diuretic amiloride, functions in the control of intracellular pH (pH_i) and cellular volume, it exerts Na^+ absorption and participates in cellular proliferation and division [29]. Four subtypes, NHE-1, NHE-2, NHE-3 and NHE-4, have been cloned in the mammal [17, 29]. A pharmacological distinction can be made between the "amiloride-sensitive" NHE-1 and NHE-2, and the "amiloride-insensitive" NHE-3, based on their affini-

ty for amiloride and amiloride analogues, such as ethylisopropylamiloride (EIPA) [28].

The investigation of NHE characteristics in ES epithelium may improve the understanding of transepithelial Na^+ transport in the ES. We monitored pH_i in ES epithelium of the guinea-pig using a pH-sensitive fluorescent dye, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), and characterized NHE in the ES epithelium of guinea-pig by: (1) examining Na^+-dependent pH_i recovery from intracellular acidification with the preparation bathed in a nominally HCO_3^--free solution, (2) examining the sensitivity of NHE to amiloride and EIPA, (3) examining the kinetics of pH_i recovery with the preparation bathed in solutions of various Na^+ concentrations, and (4) measuring the sensitivity of NHE to pH_i.

Materials and methods

Chemicals and solutions

BCECF acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, Ore., USA). Amiloride hydrochloride dihydrate, nigericin and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) were obtained from Sigma (St. Louis, Mo., USA). EIPA was obtained from RBI Research Biochemicals (Natick, Mass., USA). Cell-Tak was obtained from Collaborative Research (Bedford, Mass., USA). All other chemicals were of analytical grade and were obtained from Wake Pure Chemicals (Tokyo, Japan). Stock solutions of BCECF-AM (4 mM in dimethylsulphoxide), amiloride (1 M in dimethylsulphoxide), EIPA (50 mM in dimethylsulphoxide) and nigericin (10 mM in ethanol) were stored at –20°C.

A nominally HCO_3^--free perfusion solution buffered with HEPES had the following composition (in mM): 140 NaCl, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 1.13 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and 5 D-glucose. The Na^+-free solution was prepared by replacing NaCl with *N*-methyl-D-glucamine chloride (NMDG-Cl). The NH_4Cl solution was prepared by substituting 20 mM NH_4Cl for 20 mM NaCl when no intrinsic buffer capacities (β_i)were determined. The high-K^+/nigericin calibration solution, whose pH was adjusted to between 6.4 and 8.0, consisted of (in mM): 140 KCl, 1.13 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and 5 D-glucose. Nigercin was added to the high-K^+ solution to a final concentration of 10 µM. All solutions were equilibrated with 100% O_2 and the pH was adjusted to 7.4 at 37°C.

Tissue preparation and loading with BCECF-AM

Healthy albino guinea-pigs (300–400 g weight, Preyer reflex positive) of either sex were anaesthetized by inhalation of diethyl ether and decapitated. The temporal bones were quickly removed and placed in the extracellular solution containing (in mM): 140 NaCl, 5.4 KCl, 1.13 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 5 D-glucose; pH was adjusted to 7.4 with NaOH at 24°C. The bony shell between the ES and the endolymphatic duct was peeled off carefully, using a microelectric drill, and then the ES and the endolymphatic duct were picked up with fine forceps with the aid of a stereomicroscope. Thereafter the intermediate portion of the ES was separated by cutting the endolymphatic duct, and was suspended in the extracellular solution containing 4 µM of the AM form of BCECF and incubated for 60 min at room temperature. The AM form of BCECF readily enters the cells where the ester is cleaved by nonspecific esterases, yielding the impermeant, fluorescent form of the dye [8, 22]. After the loading period, the tissues were washed twice in the dye-free extracellular solution for 30 min at 24°C to remove the extracellular dye. A patch of ES epithelium was dissociated carefully from the connective tissue for pH_i measurement.

This research was approved by the Animal Care and Use Committee of Kagawa Medical University under the heading, “Electrophysiological and Morphological Study of the Endolymphatic Sac in the Guinea-pig”, and carried out in accordance with the Declaration of Helsinki.

Measurement of pH_i

pH_i in ES epithelium was monitored using the pH-sensitive fluorescent dye BCECF by the method similar to that previously described [22]. A BCECF- loaded epithelium containing 30–40 cells, which was pretreated with Cell-Tak for 20 min, was attached to the glass coverslip. The coverslip was then attached to the base of a 120 µl Perspex perfusion chamber mounted on the stage of an inverted microscope (TMD-EF, Nikon, Tokyo, Japan). Thereafter, the epithelium was continuously superfused at 37°C with the solution perfusion at a rate of 2 ml/min. Complete exchange occurred within several seconds. During the experiment the epithelium was illuminated alternately at excitation wavelengths of 490 and 450 nm, which were selected by the bandpass filters (band width=10 nm) mounted on a rotating wheel. Excitation light source was provided by a 100-W mercury arc lamp. A neutral density filter was inserted in the excitation light path to decrease photobleaching. The excitation light was directed to the objective (Nikon 40×, oil immersion, numerical aperture: 1.30) by a dichroic mirror centered at 510 nm. The fluorescence emitted from epithelial cells was collected by the above-mentioned objective, filtered by a 530-nm barrier filter, and monitored by a low-light, silicon-intensified target (SIT) camera (C-2400–7, Hamamatsu Photonics KK, Japan). The fluorescence images for each excitation wavelength were acquired at 10-s intervals. After subtraction of the background fluorescence, the ratio values of fluorescence image were analysed by dividing the value for the 490 nm image by that for the 450 nm image (490/450 nm) with an image processor (Argus-50, Hamamatsu Photonics).

Calculation of pH_i recovery rate and H^+-efflux rate

To quantify the rate of pH_i recovery after intracellular acidification, the traces were fitted to an exponential function of the form:

$$pH_t = pH_\infty - (pH_\infty - pH_{initial})\exp^{-kt} \quad (1)$$

where pH_t is pH_i at time t, pH_∞ is steady-state pH_i, $pH_{initial}$ is pH_i at the beginning of recovery, and k is the rate coefficient determined from the slope of a least-squares regression plot against time. Initial rate of pH_i recovery from an acid load was derived from the expression k ($pH_\infty - pH_{initial}$). Because the pH_i change after intracellular acidification is dependent on pH_i, the rate of change in pH_i, dpH_i/dt, at a given pH_t was calculated as:

$$dpH_i/dt = k\,(pH_\infty - pH_{initial})\exp^{-kt} \quad (2)$$

H^+-efflux rate, J_H, was calculated at a given $pH_i = pH_t$

$$J_H = (dpH_i/dt)(\beta_{it}) \quad (3)$$

where β_{it} represents the intrinsic buffering capacity at a given pH_t, as described by Wenzl et al. [25].

Statistical analysis

Data are presented as means ± SE in the text and SE is indicated by a vertical bar in figures. Comparison between mean values was made by Student’s *t*-test for unpaired and paired data. $P<0.05$ was considered significantly different.

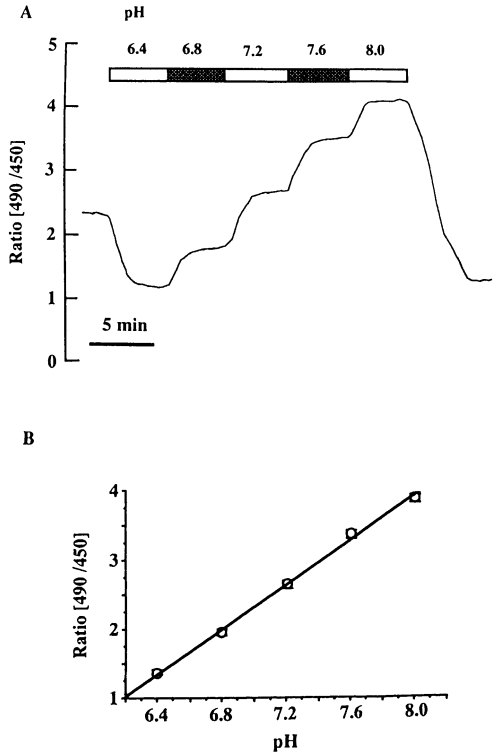

Fig. 1A, B In situ calibration of BCECF fluorescence ratio for measurement of intracellular pH (pH_i) in endolymphatic sac (*ES*) epithelium. **A** Calibration was performed in situ by preincubating the epithelium with high-K^+/nigericin solutions. The epithelium was exposed to superfusates of various pH for periods indicated by *horizontal frame bars*. The BCECF fluorescence ratio was recorded for the excitation wavelengths of 490 and 450 nm at the emission wavelength of 530 nm. **B** The relationship between the measured BCECF fluorescence ratio in the epithelium and the pH of the perfusate. Data are means ± SE (n=18) and the line was fitted with a linear regression equation

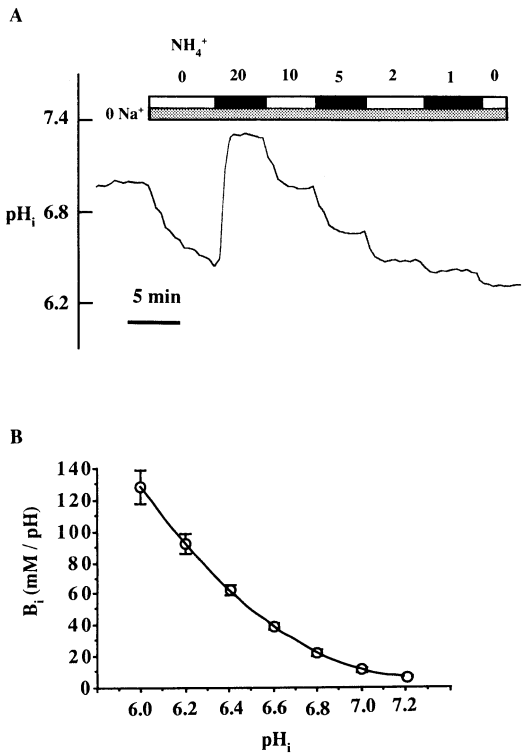

Fig. 2A, B Measurement of intrinsic buffering capacity (β_i). **A** One experiment to measure β_i from a patch of epithelium in Na^+-free solution with various concentrations of NH_4^+ indicated by *horizontal frame bars*. A decrease in bath NH_4^+ concentration induced a stepwise reduction in pH_i. **B** The relationship between pH_i and β_i. Data gathered into intervals of 0.2 pH units are shown as mean ± SE (n=9). The *continuous line* was fitted with a second-order polynomial function

Results

Calibration

Calibration was performed in situ by permeabilizing the epithelium with 10 µM nigericin in high-K^+ solutions [18, 22] at the end of each experiment. The fluorescence ratio signals (490/450 nm) were then determined by varying the pH of the perfusate (Fig. 1A). Figure 1B shows the relationship between the measured fluorescence ratios and pH of the perfusate. A plot of fluorescence ratio against pH over a pH range of 6.4–8.0 was linear, and then the fluorescence ratio signals (490/450 nm) were converted to pH_i. Recently, it has been reported that the high-K^+/nigericin technique for calibrating intracellular BCECF produces systematic errors in estimating steady-state pH_i [5], and that the correction to the nigericin-calibrated pH_i increases from zero at ≅6.0 to ≅0.2 at steady-state pH_i and to ≅0.3 at alkaline pH_i [6]. Although these errors must be considered in measurements of pH_i, the value of pH_i in the present study was not corrected for errors produced by the nigericin calibration curve.

Intrinsic buffer capacity

To allow calculation of J_H, β_i was measured with the preparation bathed in Na^+-free solutions in which Na^+ was replaced by $NMDG^+$. This procedure reduces the steady-state pH_i values by blocking Na^+-dependent proton extrusion. NH_4Cl was then added to the perfusion solution at an initial concentration of 20 mM by substituting equimolar NMDG-Cl. This induced pH_i enhancement by approximately 1.0 unit. The concentration of NH_4Cl was stepwise reduced to 10, 5, 2, 1 and 0 mM as shown in Fig. 2A. β_i was calculated at each mid-point of the resulting step changes in pH_i (dpH_i) as $\beta_i=\Delta[NH_4^+]_i/dpH_i$, where $\Delta[NH_4^+]_i$ is the change in the intracellular NH_4^+ concentration. Assuming that $[NH_3]_i$ is equal to the extracellular NH_3 concentration, and that pK_a of NH_4Cl is 8.9 at 37°C [19], $[NH_4^+]_i$ was calculated from the known pH_i using the Henderson-Hasselbalch equation [4]. The data for β_i against pH_i were fitted with a second-order polynomial expression: $\beta_i=78.502(pH_i)^2-1137.492(pH_i)+4127.061$ (Fig. 2B). The relationship between pH_i and β_i indicates that β_i increases with decreasing pH_i. Similar

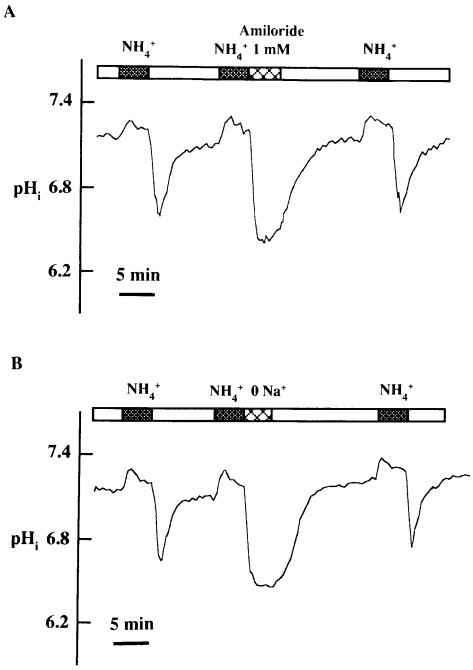

Fig. 3A, B Amiloride-sensitive and Na^+-dependent recovery from an NH_4^+-induced acute acid load. **A** After pH_i recovered to the initial value following the first control NH_4^+-induced acute acid load, addition of amiloride at 1 mM concentration to the standard perfusion solution following the second NH_4^+ prepulse increased intracellular acidification and inhibited pH_i recovery. **B** After pH_i recovered to the initial value following the first control NH_4^+-induced acute acid load, removal of extracellular Na^+ after the second NH_4^+ prepulse increased intracellular acidification and almost completely inhibited pH_i recovery, which rapidly recovered to a steady-state level by re-addition of extracellular Na^+

observations have been reported in studies of other cells [4, 11, 23, 24].

pH_i recovery from an NH_4^+-induced acute acid load

This series of experiments were performed to test the presence of NHE in the ES epithelium. If it is present, pH_i recovery from an acid load should be inhibited by amiloride and dependent on $[Na^+]_o$. pH_i recovery from an acid load in the absence of inhibitor was monitored by using the NH_4^+ prepulse technique as shown in Fig. 3 (also shown in Fig. 5A). Application of 20 mM NH_4Cl in the perfusion chamber induced a prompt pH_i rise from an average value of 7.12±0.02 pH units, ranging from 7.04 to 7.26 pH units (n=19) due to the rapid influx of NH_3, followed by a slow decline in pH_i resulting from the slow influx of NH_4^+ and other acid-loading processes. Withdrawal of NH_4Cl elicited pH_i to decrease quickly below the initial pH_i because intracellular NH_4^+ is dissociated into NH_3 and H^+, NH_3 abruptly leaves the cells, and H^+ is trapped inside [3]. pH_i rapidly recovered from this acid load to the initial level.

Figure 3A shows the effect of amiloride on pH_i recovery from an acid load in an ES epithelium. When pH_i recovered to the initial value from the first control NH_4^+-induced acute acid load, addition of amiloride at 1 mM concentration to the standard perfusion solution following the second NH_4^+ prepulse increased the degree of intracellular acidification and inhibited the rate of pH_i recovery. However, the removal of amiloride from the bath solution rapidly induced pH_i to return to its initial level. The average value of initial pH_i was 7.12±0.02 pH units in ten ES epithelia. The initial acidification under the control condition was 0.49±0.02 pH units, which was increased to 0.65±0.03 pH in the presence of amiloride. The average initial J_H in control condition was 16.07±1.11 mM/min when pH_i was 6.63±0.02 pH units. The average initial J_H in the presence of amiloride was 3.54±0.39 mM/min when pH_i was 6.47±0.02 pH units.

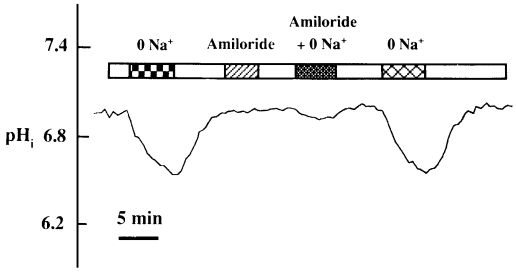

Fig. 4 Effects of $[Na^+]_o$ and amiloride on the steady-state pH_i. Intracellular acidification induced by switching from the standard external solution to Na^+-free solution was inhibited by the addition of 1 mM amiloride

Figure 3B shows Na^+-dependent pH_i recovery from an acid load in an ES epithelium. When pH_i was recovered to the initial value from the first control NH_4^+-induced acute acid load, removal of $[Na^+]_o$ following the second NH_4^+ prepulse increased the degree of intracellular acidification and almost completely inhibited the rate of pH_i recovery. Re-addition of $[Na^+]_o$ rapidly led pH_i to return to the initial level. The average value of initial pH_i was 7.13±0.03 pH units in nine ES epithelia. The initial acidification under control conditions was 0.46±0.02 pH units, which was increased to 0.62±0.04 pH under Na^+-free conditions. The average initial J_H under control conditions was 16.54±1.20 mM/min when pH_i was 6.62±0.02 pH units. The average initial J_H in Na^+-free solution was 0.75±0.16 mM/min when pH_i was 6.48±0.02 pH units.

Influences on the steady-state pH_i of removing extracellular Na^+ and applying amiloride

Figure 4 shows that removal of $[Na^+]_o$ under steady-state conditions induced a pH_i decrease from 7.10±0.05 to 6.59±0.04 pH units (n=6), and that the addition of amiloride to the perfusate induced no change in pH_i. pH_i slightly decreased from the control value to 7.03±0.05 (n=6) in Na^+-free solution in the presence of 1 mM amiloride. The addition of amiloride to the preparation when bathed in Na^+-free solution significantly changed pH_i (P<0.05).

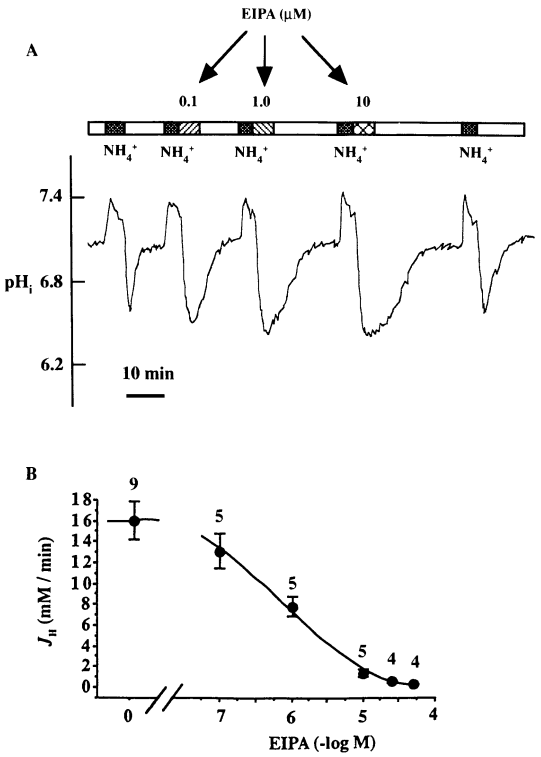

Fig. 5A, B Effect of ethylisopropylamiloride (*EIPA*) on pH_i recovery from an NH_4^+-induced acute acid load. **A** The typical responses of pH_i recovery from an NH_4^+-induced acute acid load in presence of 0, 0.1, 1.0 and 10 μM EIPA. **B** Concentration/response curve was fitted with a modified Michaelis-Menten equation: $J_H=J_{Hmax}/(1+EIPA/K_i)$. The apparent 50% inhibitory constant (K_i) obtained from this fit was 0.85 μM

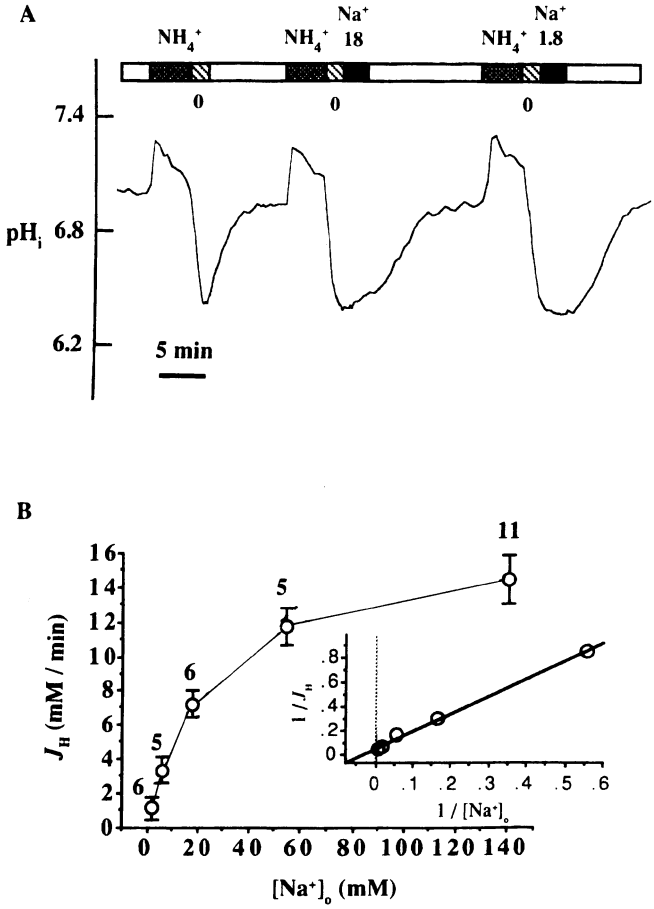

Fig. 6A, B Kinetics of Na^+-dependent recovery from an NH_4^+-induced acute acid load. **A** Responses of pH_i recovery from an NH_4^+-induced acute acid load in the presence of 140, 18, and 1.8 mM Na^+ following a short Na^+-free period. **B** Initial J_H versus $[Na^+]_o$ following an NH_4^+-induced acute acid load. Lineweaver-Burk plot revealed apparent K_m of 24.14 mM and J_{Hmax} of 16. 95 mM/min shown in *inset* of Fig. 6B

NHE inhibited by EIPA

As described in the Introduction, two types of NHEs – one sensitive to amiloride, the other not – can be distinguished by their sensitivity to amiloride and its analogue, EIPA [28]. Figure 3A shows that amiloride at 1.0 mM concentration inhibited pH_i recovery from an acid load. Further distinction was performed by inhibition using EIPA at different concentrations. The effect of different EIPA concentrations on pH_i recoveries from an NH_4^+-induced acute acid load was examined in the presence of 0, 0.1, 1.0, 10, 25 and 50 μM EIPA during recovery phase (Fig. 5A). A dose/response curve for inhibition of initial J_H by EIPA following an NH_4^+-induced acute acid load is shown in Fig. 5B. The apparent K_i for EIPA obtained from this curve fit was 0.85 μM.

Kinetics of NHE

The kinetics of Na^+-dependent recovery from an acute acid load were examined by changing $[Na^+]_o$ from 140 to 54, 18, 6 and finally 1.8 mM after a short Na^+-free period as indicated (Fig. 6A). The initial dpH_i/dt can be calculated at the pH_i after the acid load. J_H as a function of $[Na^+]_o$ displayed simple saturation kinetics (Fig. 6B). An apparent K_m of 24.14 mM for Na^+ and a $J_{H\ max}$ of 16.95 mM/min were obtained from the Lineweaver-Burk plot (inset, Fig. 6B).

Dependence of J_H on pH_i

Since Na^+-H^+ exchange is activated at lower pH_i and is inactive near physiological pH_i, J_H following an acid load can be considered to be a reflection of NHE activity. To quantitatively analyse the relationship between NHE activity and pH_i in ES epithelium, J_H was measured as a function of pH_i during the recovery phase after an NH_4^+-prepulse-induced acid load under control conditions, as illustrated in Fig. 3 (n=19). Recovery rates of pH_i from an acid load were determined at intervals of 0.05 pH units by Eq. 2. J_H was then calculated as a product of dpH_i/dt and β_{it} obtained from a second-order polynomial expression shown in Fig. 2B. Figure 7 shows that J_H decreased with a pH_i increase, and that it was near zero when pH_i was above 7.2 pH units.

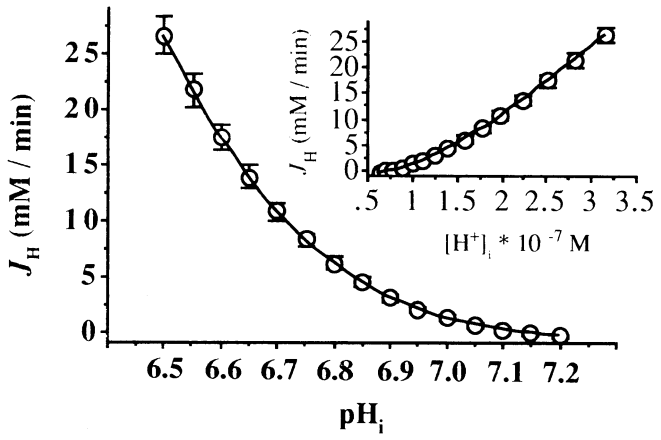

Fig. 7 pH_i sensitivity of J_H. J_H versus pH_i was determined during the recovery phase following an NH_4^+-prepulse-induced acid load under control conditions (from Fig. 3, n=19). *Inset:* the same data plotted against intracellular H^+ ($[H^+]_i$)

Discussion

The present study demonstrates that the pH_i recovery in ES epithelium following intracellular acidification, which is entirely dependent on $[Na^+]_o$, is inhibited by amiloride and its analogue, EIPA, under nominally HCO_3^--free conditions, suggesting that a decrease in pH_i induced by an acute acid load may be equilibrated by a NHE.

In the steady-state, pH_i was not changed by the application of amiloride at 1 mM concentration, indicating that NHE activity is very small or has a tendency to be inactive at steady-state. The intracellular acidification induced by removal of $[Na^+]_o$, however, was almost completely inhibited by amiloride, suggesting that the former may be due to blockade of H^+ efflux into cells by the reversal of a NHE.

The J_H following an acid load shows that NHE is inactive when pH_i is greater than 7.2. The result supports the hypothesis that NHE, which has an intracellular modifier site that is allosterically sensitive to pH_i, may automatically increase in activity as pH_i decreases or decrease as pH_i increases [1]. In addition, we found that the plot of J_H as a function of intracellular H^+ concentration displays a non-linear pattern (Fig. 7, inset), indicating that more than one H^+ ion may interact at the intracellular surface with the exchanger [23]. The effect of different $[Na^+]_o$ on J_H shows that the NHE in the ES epithelium has simple saturation kinetics. The K_m of 24.14 mM for Na^+ is similar to that reported in studies of several other cells that contain an amiloride-sensitive NHE [7].

In epithelial cells four subtypes of NHE isoform, NHE1, NHE2, NHE3 and NHE4, have been cloned to date [29]. NHE1 is assumed to regulate cell pH_i and cell volume, and NHE2, NHE3 and NHE4 are thought to be involved in transepithelial transport of Na^+ [17]. The sensitivity of NHE to amiloride and its analogues decreases in the order of NHE1>NHE2≫NHE3 [17]. Amiloride-sensitive and -insensitive NHE can be distinguished pharmacologically by K_i analysis of amiloride and its analogue such as EIPA. K_i for EIPA is about 1 μM as an operational criterion for these two types of NHEs [7]. NHE1 and NHE2 belongs to the amiloride-sensitive category, whereas NHE3 is amiloride insensitive [17]. The sensitivity of the NHE in ES epithelium to EIPA (K_i=0.85 μM) suggests that it is of the amiloride-sensitive type. The results of the present experiments, performed in the absence of HCO_3^-, do not allow adequate clarification of the in vivo functional role of NHE in the ES. Further investigations are required to characterize the NHE in ES epithelium in terms of subtype, localization and function.

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