Extracellular Cl\(^{-}\) modulates shrinkage-induced activation of Na\(^{+}\)/H\(^{+}\) exchanger in rat mesangial cells

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The sodium/hydrogen exchanger (NHE) is a plasma membrane transport protein found in a broad range of biological systems, including glomerular mesangial cells (MCs) (4, 42). In MCs, entry of Na\(^{+}\) into cells in exchange for an intracellular H\(^{+}\) is the main effect of NHE, which therefore is involved in the regulation of intracellular pH (pHi) and initiation of cell growth and proliferation (12, 13).

Cell volume regulation generally involves the movement of ions and organic osmolytes across the surface membrane and is often mediated by pH\(_{i}\)-regulating transporters (reviewed in Refs. 6 and 19). In a variety of cells (8, 10, 15, 16, 20, 30–32, 37, 38), one such transporter, the NHE, is activated by shrinkage in a hyperosmotic solution, resulting in Na\(^{+}\) entry into the cell. Osmotic uptake of water due to this Na\(^{+}\) entry leads to cell swelling, a process termed regulatory volume increase. Activation of NHE also results in H\(^{+}\) extrusion, which causes cell alkalization. However, it is not yet known whether hyperosmolality activates NHE in MCs.

MCs are rich in contractile fibers and smooth muscle-like cells (1, 21). They are located in the intercapillary space of the glomerulus, and may regulate, through its contraction, the intraglomerular hemodynamics and thus the glomerular filtration rate (GFR) (reviewed in Ref. 23). Some vasoactive peptides, angiotensin II (ANG II) and arginine vasopressin (AVP), cause MC contraction through an increase in intracellular Ca\(^{2+}\) and consequently reduce glomerular ultrafiltration (23).

Because the sensitivity of contractile proteins to Ca\(^{2+}\) is reduced at low pH, in skeletal and cardiac muscles (11), pH change might influence MC contractility, and subsequently GFR. Cultured MCs have also been shown to change morphologically, altering their growth characteristics and metabolic activity during periods of cyclic stretching-relaxation (18). Therefore, the ability to regulate cell volume is especially important for MCs, in which cell volume changes can alter glomerular hemodynamics.

In dog erythrocytes (31, 32) and in barnacle muscle fibers (10), it has been demonstrated that hyperosmolality-induced activation of NHE is inhibited by removing Cl\(^{-}\) from the extracellular fluid. In the apical membrane vesicle of rat colonic crypt cells, NHE activity under isosmotic conditions has been reported to be dependent on extracellular Cl\(^{-}\) (33). In addition, both increase in intracellular Ca\(^{2+}\) concentration and MC contraction induced by ANG II and AVP are attenuated when the extracellular Cl\(^{-}\) concentration is reduced (28). It is also well known that Cl\(^{-}\) is an important mediator of tubuloglomerular feedback (TGF) regulation of glomerular filtration by the distal tubular flow (35). These reports suggest the possibility that MC NHE activity may be modified by extracellular Cl\(^{-}\} under iso- and/or hyperosmotic conditions.

Therefore, we isolated and cultured MCs from rat glomeruli to address the following issues: 1) whether hyperosmolality activates NHE activity, and 2) whether...
Table 1. Composition of solutions

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All individual concentrations are in mM. NMDG, N-methyl-D-glucamine.

and how NHE activity under iso- or hyperosmotic conditions is modulated by extracellular Cl\(^-\).

METHODS

Solutions. The composition of the solutions is given in Table 1. All solutions were nominally CO\(_2\)/HCO\(_3\) free and were adjusted to pH 7.4 at 37°C. The osmolality of all solutions was measured before the experiment, and was verified to be within a range of 300 ± 5 mosmol/kg H\(_2\)O. The nigerin calibrating solutions were titrated to different pH values at 37°C with either HCl or N-methyl-D-glucamine (NMDG). For the Na\(^+\)-free solutions (solutions 2 and 3, Table 1), Na\(^+\) was replaced with NMDG titrated with the appropriate acid. In Cl\(^-\)-free solutions 3 and 4, Table 1) and low-Cl\(^-\) solutions 5–8, Table 1) solutions, Cl\(^-\) was replaced with gluconate. Because of the chelation of Ca\(^{2+}\) in gluconate-containing solutions, we compensated by increasing total extracellular Ca\(^{2+}\) concentrations, as shown in Table 1. 2, 7-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) was prepared as a 10 mM stock solution and diluted 1:1,000 to a final concentration of 10 µM. Ethylisopropylamiloride (EIPA) was prepared as a 100 mM stock solution in methanol and was diluted 1:1,000 to a final concentration of 100 µM. Nigericin was prepared as a 10 mM stock solution in methanol and was diluted 1:1,000 into solution 13 Table 1 to a final concentration of 10 µM.

MC culture. Glomerular MCs were obtained by the methods previously described in our laboratory (27). In brief, kidneys were removed from 150- to 200-g male Sprague-Dawley rats. All subsequent steps were performed with sterile conditions under a laminar flow hood. Renal cortices from two to three rats were pooled and minced with a sterile razor blade. The tissue was collected and suspended in PBS. The sediments were gently poured onto a series of stainless steel sieves of decreasing pore sizes (250, 150, and 75 µm; NBC Kogyo, Tokyo, Japan). The filtrates were collected and resuspended in PBS. The sediments comprised a large number of glomeruli that were generally free of capsular and tubular debris. The glomerular suspension was passed through successive sieves and was resuspended in PBS in a culture tube. After centrifugation at 1,000 rpm for 5 min, the supernatant was discarded and the glomeruli were then plated onto 100-mm culture dishes (Becton Dickinson, Rutherford, N.J.). The cells were cultured in RPMI 1640, buffered with 10 mM HEPES at pH 7.4, and supplemented with 20% fetal bovine serum (FBS), 2 mg/ml NaHCO\(_3\), 100 U/ml penicillin, 100 µg/ml streptomycin, and 15.5 µg/ml insulin-transferrin-selenium in a 5% CO\(_2\) incubator at 37°C. Glomerular attachment was 20–30% after 48 h. Under these conditions, epithelial cells started to grow from the glomeruli within 2–3 days. MCs started to grow rapidly after 7–10 days, but the number of epithelial cells continuously decreased. MCs grew to confluence after 21–28 days. By this time, the cultures were virtually free of epithelial cells and showed positive staining for α-actin, myosin, and desmin, and showed negative staining for an endothelial cell marker factor VIII. ANG II–induced contraction of MCs was also detected by phase-contrast microscopy.

Confluent cultures were passaged through trypsin-EDTA and seeded at a 1:3 ratio in 100-mm culture dishes (Becton Dickinson). The passages were performed at 5- to 7-day intervals after confluence of the cells. Because major phenotypic changes (e.g., failure to contract in response to AVP) occur in MCs after multiple passages (e.g., Ref. 21), we only studied cells passages 3–5.

Measurement of pH. For pH measurements, MCs were plated on 35-mm petri dishes containing a glass coverslip bottom (MatTec, Ashland, MA) and were used 5–7 days later. Cells were incubated in 0.5% FBS-containing RPMI 1640 for 24 h before use. Before each experiment, the cells grown to subconfluence were incubated for 20 min in 0.5% FBS-containing RPMI 1640 treated with 10 µM BCECF-AM at 37°C. The petri dish was then placed on the stage of an inverted epifluorescence microscope (IMT-2; Olympus, Tokyo, Japan).
respectively. We fitted calibration data to the above equation, from experiments in which MCs were exposed to 10 µM total of the curve. The values of $pK_b$ can be calculated from the observed pH $i$. $pK_a$ is the distance between the upper and lower asymptote of the curve. The values of $pK_a$ and $b$ were determined from experiments in which MCs were exposed to 10 µM nigericin-containing solutions at different pH $i$, always including pH 7.0. We fitted calibration data to the above equation, using a nonlinear least-squares method, and obtained best-fit values for $b$ and $pK_a$ of $1.54 \pm 0.02$ (SE) and $7.12 \pm 0.02$ (SE), respectively. Determination of intracellular buffering power. Intrinsic buffering power ($\beta_i$) of MCs was determined using the method of Boyarsky et al. (4). As shown in Fig. 1A, acid-loaded MCs were exposed to a series of nominally Na$^+$-free solutions (solution 2) that contained 20, 10, 5, 2.5, 1, 0.5, and 0 mM total ammonium (NH$_2$NH$_3$). Total NH$_2$NH$_3$-containing solutions were prepared by adding NH$_4$Cl with replacement of NMDG in Na$^+$-free solution. With each stepwise decrease in [NH$_2$NH$_3$]$_o$, the amount of protons delivered to the cytoplasm ($\Delta$[acid]) was considered equal to the resultant change in [NH$_3$]. If it is assumed that [NH$_3$]$_i$ equals [NH$_3$]$_o$, and that the pH$_i$ governing the NH$_3$/NH$_2$NH$_3$ equilibrium (8.9 at 37°C) is the same in the cytoplasm as in the extracellular fluid, [NH$_3$]$_i$ can be calculated from the observed pH$_i$. $\Delta$pH was taken as the change in pH$_i$ produced by the stepwise decrease in [NH$_2$NH$_3$]$_o$. $\beta_i$ was then calculated as $-\Delta$[acid]/$\Delta$pH (34). $\beta_i$ was assigned to the mean of the two pH$_i$ values used for its calculation. Figure 1B shows the pH$_i$ dependence of $\beta_i$. The buffering power data were grouped into pH intervals of 0.2. Each closed circle represents the mean buffering power of 0.2 pH interval, and the line represents the least-squares fit to all 56 individual data points. We used this straight line to determine $\beta_i$ as a function of pH$_i$ in buffering power calculations in the present study. The equation of the best-fit line is $\beta_i = 147.2 - 19.7 \times$ pH$_i$ ($r = 0.997$) at a range of physiological pH$_i$. Computation of net acid extrusion rates. Net acid extrusion rates ($J_{i\text{H}^+}$) in MCs were calculated from rates of pH$_i$ increase (dpH$_i$/dt) and $\beta_i$ in two kinds of experiments. First, Na$^+$ was removed from the extracellular solution, causing pH$_i$ to decrease. We analyzed the pH$_i$ increase elicited by readdition of Na$^+$. Second, we analyzed pH$_i$ increase when, at a steady-state pH$_i$, MCs were exposed to hyperosmotic solutions containing mannitol, sucrose, or urea. For both experiments, we fitted a third or a fourth-degree polynomial to the pH$_i$ increase using a least-squares method, as described previously (7). At each pH$_i$, data from four or more experiments were averaged to produce a plot of mean net acid extrusion rates vs. pH$_i$ (4, 34).

Drugs and chemicals. All chemicals were obtained from Wako (Osaka, Japan) unless noted as follows: RPMI 1640, penicillin, streptomycin, insulin-transferrin-selenium, and FBS were obtained from Gibco BRL (Rockville, MD); HEPES and BCECF-AM were from Dojindo (Kumamoto, Japan); EIPA and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were from Research Biochemicals International (Natick, MA); and NMDG, nigericin, DIDS, colchicine, and cytochalasin B were from Sigma (St. Louis, MO).

Statistical analysis. The data are expressed as means ± SE. Comparisons were performed by paired or nonpaired Student’s t-test or one-way ANOVA where appropriate. P values <0.05 were considered significant.
RESULTS

Steady-state pH_i. When MCs were perfused with the HEPES-buffered solution (solution 1, Table 1) at a rate of 4.5–5.5 ml/min at 37°C, their pH_i values were gradually decreased and sustained at a steady-state level. In MCs, mean steady-state pH_i values were 6.96 ± 0.01 (n = 227).

pH_i regulation of MCs under isosmotic conditions. At first we observed pH_i recovery after removal of extracellular Na^+. Cells were first bathed in the Na^+-containing HEPES-buffered solution (solution 1, Table 1) and were then incubated in the Na^+-free solution (solution 2, Table 1). The removal of extracellular Na^+ caused a substantial and sustained decrease in pH_i from 7.01 ± 0.07 to 6.48 ± 0.02 (P < 0.001, n = 5). Subsequent addition of Na^+ to the extracellular solution caused pH_i to rapidly increase to similar values of the initial steady-state pH_i (7.00 ± 0.06, n = 5). When a specific inhibitor of NHE, EIPA (100 µM), was added in the continued absence of external Na^+, pH_i was not changed at all (Fig. 2B). Readdition of Na^+ to the extracellular solution in the continued presence of EIPA also caused no effect on pH_i (Fig. 2B).

Fig. 2. Evidence for NHE in mesangial cells (MCs). A: MCs were first bathed in the Na^+-containing HEPES-buffered solution (300 mosmol/kg H_2O) and were then incubated in the Na^+-free solution. The removal of external Na^+ caused a substantial and sustained decrease in pH_i. Readdition of external Na^+ caused pH_i to rapidly increase to similar values of the initial steady-state pH_i. B: readdition of external Na^+ in the presence of ethylisopropylamiloride (EIPA) (100 µM) completely inhibited the Na^+-dependent pH_i increase. C: when MCs were treated with EIPA (100 µM) after readdition of external Na^+, no further increase in pH_i was observed. D: removing extracellular Cl^- by replacement with gluconate caused no effect on Na^+-dependent pH_i increase after acid load.

Fig. 3. Cell shrinkage causes an EIPA-sensitive cell alkalinization in MCs. A: exposure of MCs to hyperosmotic HEPES solution (500 mosmol/kg H_2O) containing 200 mM mannitol caused cell alkalinization. B: when MCs were treated with EIPA (100 µM) in the continued presence of hyperosmotic mannitol solution, no further increase in pH_i was observed. C: addition of EIPA (100 µM) to the MCs caused pH_i to decrease. In the continued presence of EIPA, exposure of MCs to hyperosmotic mannitol solution did not elicit a pH_i increase at all.

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specific inhibitor of NHE, EIPA (100 µM), was added in the continued absence of external Na^+, pH_i was not changed at all (Fig. 2B). Readdition of Na^+ to the extracellular solution in the continued presence of EIPA also caused no effect on pH_i (Fig. 2B). When cells were treated with EIPA after readdition of Na^+ to the extracellular solution, no further increase in pH_i was observed (Fig. 2C). These results indicate that MCs possess NHE and that these cells exclusively regulate their pH_i through a NHE mechanism in the nominal absence of CO_2/HCO_3^-.

pH_i regulation of MCs under hyperosmotic conditions. We next examined whether hyperosmolality affects pH_i in MCs. MCs were incubated in HEPES-buffered Na^+-containing solution (solution 1, 300 mosmol/kg H_2O) and were then exposed to the HEPES-buffered solution treated with 200 mM mannitol (500 mosmol/kg H_2O). As shown in Fig. 3A, pH_i significantly increased from 6.86 ± 0.03 to 7.26 ± 0.02 (P < 0.001, n = 11). When MCs were treated with EIPA (100 µM) in the continued presence of hyperosmotic mannitol solution, no further increase in pH_i was observed (Fig. 3B).
When MCs were treated with EIPA (100 µM) in the absence of hyperosmotic mannitol solution, pH_i significantly decreased from 6.99 ± 0.06 to 6.91 ± 0.06 (P < 0.001, n = 5; Fig. 3C), indicating that in the nominal absence of CO_2/HCO_3, the NHE must be active in the normal steady-state pH_i to balance a substantial rate of intracellular acid loading. In the continued presence of EIPA, exposure of MCs to the hyperosmotic mannitol solution did not elicit a pH_i increase at all (Fig. 3C). We next examined whether cell alkalinization induced by hyperosmotic mannitol is dependent on external Na^+. Exposure of the cells to hyperosmotic mannitol solution in the continued absence of external Na^+ caused no increase in pH_i (Fig. 4A). However, when external Na^+ was readded in the presence of hyperosmotic mannitol, pH_i recovery was substantially faster, and the steady-state pH_i values (7.35 ± 0.06, P < 0.01, n = 7) were greater than in its absence (6.97 ± 0.07, n = 7; see Figs. 2A and 4A). These findings are consistent with the notion that hyperosmotic mannitol activates NHE to cause cell alkalinization in MCs. From the Na^+-dependent pH_i recovery rate and β, we calculated the relationship between Na^+-dependent acid extrusion rate (J_H) and pH_i under iso- and hyperosmotic conditions (Fig. 5). Under the two conditions, Na^+-dependent J_H decreased as pH_i increased. However, the cells exposed to the hyperosmotic mannitol solution had a significantly greater J_H than those exposed to the isosmotic solution at comparable pH_i values. Furthermore, hyperosmolality shifted the J_H vs. pH_i by 0.15-0.3 pH units in the alkaline direction.

Fig. 5. pH_i dependence of the Na^+-dependent acid extrusion rates (J_H) under iso- and hyperosmotic conditions in the presence and absence of extracellular Cl^−. The plots were computed from experiments such as illustrated in Figs. 2, A and D, and 4, A and B. Data represent means ± SE. Isosmo, isosmolality; hyperosmo, hyperosmolality. * P < 0.05, ** P < 0.005 compared with isosmolality in the presence of Cl^− at comparable pH_i values. † P < 0.05, †† P < 0.005 with hyperosmolality in the presence of Cl^− at comparable pH_i values. Note that under hyperosmotic conditions, the J_H is not only pH_i dependent but also Cl^− dependent.

To determine the effects of different osmolytes on pH_i, we used other osmolytes, sucrose and urea. For this purpose, MCs were incubated in HEPES-buffered solution (solution 1, 300 mosmol/kgH_2O, Table 1), and were then exposed to the HEPES-buffered solution treated with 200 mM sucrose or urea (500 mosmol/kgH_2O). As shown in Fig. 6A, hyperosmotic sucrose solution caused steady-state pH_i to increase from 6.93 ± 0.03 to 7.29 ± 0.04 (P < 0.001, n = 6). EIPA (100 µM) caused pH_i to decrease from 7.11 ± 0.12 to 7.05 ± 0.13 (P < 0.001, n = 4), but completely inhibited the hyperosmotic sucrose-induced cell alkalinization (Fig. 6B). By sharp contrast, hyperosmotic urea solution had no effect on pH_i (6.86 ± 0.06 to 6.86 ± 0.06, n = 8; Fig. 6C). Hyperosmotic mannitol and sucrose solutions showed significant initial rates of cell alkalinization (initial dpH_i/dt), but hyperosmotic urea solution failed to detect the initial dpH_i/dt (Fig. 6D).

Glomerular MCs require Cl^− for the development of a variety of metabolic and functional properties (22, 28, 29). Also, in canine erythrocytes (31, 32) and barnacle muscle fibers (10), hyperosmolality-induced NHE activation has been reported to require extracellular Cl^−. Therefore, we next examined whether NHE activity in MCs under iso- or hyperosmotic conditions is dependent on extracellular Cl^−. For this purpose, extracellular Cl^− was replaced with a nonpermeant anion, gluconate (solutions 3 and 4, Table 1). Removal of extracellular Cl^− by replacement with gluconate did not influence pH_i (Fig. 7A). When hyperosmotic mannitol solution was added in the continued absence of extracellular Cl^−, pH_i was not changed at all (Fig. 7A). However, when extracellular Cl^− was readded in the continued presence of hyperosmotic mannitol, pH_i significantly increased from 6.92 ± 0.03 to 7.30 ± 0.02 (P < 0.001, n = 12; Fig. 7A and Table 2). EIPA (100 µM) alone caused the steady-state pH_i to decrease by 0.08. When extracellular Cl^− was readded to the MCs treated
with hyperosmotic mannitol in the continued presence of EIPA, the Cl⁻-dependent cell alkalinization was completely inhibited (Fig. 7B). We next examined whether removal of extracellular Cl⁻ affects the Na⁺-dependent pHi recovery in the presence of hyperosmotic mannitol. As shown in Fig. 4B, the Na⁺-dependent pHi recovery rate in the absence of extracellular Cl⁻ (56.1 ± 6.4 pH units/s × 10⁴, P < 0.005, n = 9) was significantly smaller than that in its presence (92.7 ± 9.1 pH units/s × 10⁴, n = 7). The relationship between Na⁺-dependent JH and pHi under hyperosmotic conditions in the absence of extracellular Cl⁻ is shown in Fig. 5. Removing extracellular Cl⁻ under hyperosmotic conditions caused a significant decrease in JH at comparable pHi values. By sharp contrast, when extracellular Cl⁻ was removed under isosmotic conditions, either pHi, Na⁺-dependent pHi recovery rate (Fig. 2D), or Na⁺-dependent JH at different pHi values (Fig. 5) were not affected.

To examine the dose-dependent effect of Cl⁻ on the shrinkage-induced NHE activation, MCs were exposed to hyperosmotic mannitol solution (500 mosmol/kgH₂O) with different extracellular Cl⁻ concentrations (0, 13, 36, 64, 93, and 132 mM) by replacement with gluconate (solutions 1, 4, and 5–8, Table 1). Figure 8 shows the relationship between extracellular Cl⁻ concentrations and the rate of cell alkalinization (dpH/dt) induced by the hyperosmotic mannitol at pHi of 6.85. The dpH/dt decreased in a sigmoidal fashion as extracellular Cl⁻ concentrations decreased. The apparent 50% inhibitory concentration of extracellular Cl⁻ for the dpH/dt induced by hyperosmotic mannitol was 69.2 mM.

We next examined whether the Cl⁻-dependent NHE activation induced by hyperosmotic mannitol occurs through Cl⁻-dependent transport processes, including Cl⁻ channel and Cl⁻/base exchange. For this purpose, we added NPPB (100 µM), a Cl⁻ channel inhibitor, or DIDS (100 µM), an inhibitor of Cl⁻/base exchange, to the MCs, and then observed the Cl⁻-dependent cell alkalinization induced by hyperosmotic mannitol. NPPB or DIDS alone caused no effects on pHi values (Fig. 7C and D). When MCs were treated with hyperosmotic mannitol in the presence of NPPB, pHi significantly increased from 6.94 ± 0.03 to 7.25 ± 0.07 (P < 0.001,
Table 2. Effect of various inhibitors on Cl\(^{-}\)-dependent initial rate of pH\(_{i}\) increase after hyperosmotic mannitol solution

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Control</th>
<th>NPPB</th>
<th>DIDS</th>
<th>Colchicine</th>
<th>Cytochalasin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial dpH(_{i})/dt, pH units/s (\times 10^4)</td>
<td>73.3 ± 8.0</td>
<td>41.4 ± 6.8(\uparrow)</td>
<td>51.8 ± 7.1(\uparrow)</td>
<td>32.7 ± 9.5(\uparrow)</td>
<td>65.4 ± 5.8</td>
</tr>
<tr>
<td>Initial pH(_{i})</td>
<td>6.92 ± 0.03</td>
<td>6.94 ± 0.03</td>
<td>6.95 ± 0.02</td>
<td>6.95 ± 0.02</td>
<td>6.97 ± 0.02</td>
</tr>
<tr>
<td>Final pH(_{i})</td>
<td>7.30 ± 0.02</td>
<td>7.25 ± 0.07(\uparrow)</td>
<td>7.36 ± 0.05</td>
<td>7.12 ± 0.02(\uparrow)</td>
<td>7.27 ± 0.03</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

Data represent means ± SE; n, no. of observations. *P < 0.05, †P < 0.005, and ‡P < 0.001 compared with control. Note that initial pH\(_{i}\) values in absence and presence of various inhibitors were not significantly different. Initial dpH\(_{i}\)/dt, initial rate of pH\(_{i}\) increase; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.

n = 8). However, the final pH\(_{i}\) values (7.25 ± 0.07, P < 0.05, n = 8) in the presence of NPPB were significantly smaller than those in its absence (7.30 ± 0.02, n = 12; Fig. 7, A and C, Table 2). Furthermore, in the presence of NPPB, the Cl\(^{-}\)-dependent initial alkalinization rate induced by hyperosmotic mannitol (initial dpH\(_{i}\)/dt; 41.4 ± 6.8 pH units/s \(\times 10^4\), P < 0.005, n = 8) was significantly smaller than that in its absence (73.3 ± 8.0 pH units/s \(\times 10^4\), n = 12; Table 2). When MCs were treated with hyperosmotic mannitol in the presence of DIDS, pH\(_{i}\) significantly increased from 6.95 ± 0.02 to 7.36 ± 0.05 (P < 0.001, n = 8; Fig. 7D and Table 2). However, in the presence of DIDS, the initial dpH\(_{i}\)/dt under hyperosmotic conditions (51.8 ± 7.1 pH units/s \(\times 10^4\), P < 0.05, n = 8) was significantly smaller than that in its absence, although the final pH\(_{i}\) values (7.36 ± 0.05, n = 8) in the presence of DIDS were not significantly different from those in its absence (Table 2). It has been reported that the interactions between the cytoskeleton and the plasma membrane regulate the activity of many ion channels and transport proteins, including NHE (8, 42, 43). Thus we examined whether the cytoskeletal elements are involved in the Cl\(^{-}\)-dependent NHE activation induced by hyperosmotic mannitol. For this purpose, we added colchicine (100 µM), a disruptor of microtubules, or cytochalasin B (100 µM), a disruptor of filamentous actin, to the MCs, and then observed the Cl\(^{-}\)-dependent cell alkalinization induced by hyperosmotic mannitol. Colchicine or cytochalasin B alone had no effects on pH\(_{i}\) values (Fig. 7, E and F, Table 2). When MCs were treated with hyperosmotic mannitol in the presence of colchicine, pH\(_{i}\) significantly increased from 6.95 ± 0.02 to 7.12 ± 0.02 (P < 0.001, n = 7). However, the final pH\(_{i}\) values (7.12 ± 0.02, P < 0.001, n = 7) in the presence of colchicine were significantly smaller than those in its absence (Fig. 7, A and E, Table 2). Furthermore, in the presence of colchicine, the initial dpH\(_{i}\)/dt under hyperosmotic conditions (32.7 ± 9.5 pH units/s \(\times 10^4\), P < 0.001, n = 7) was significantly smaller than that in its absence (Table 2). In sharp contrast, pretreatment with cytochalasin B caused no effect on Cl\(^{-}\)-dependent pH\(_{i}\) increase induced by hyperosmotic mannitol (Fig. 7F and Table 2).

To further examine the specificity of extracellular Cl\(^{-}\) for the shrinkage-induced NHE activation, 125 mM Cl\(^{-}\) in the solution was substituted with equimolar gluconate, I\(^{-}\), Br\(^{-}\), SCN\(^{-}\), or F\(^{-}\) (solutions 5 and 9–12, Table 1). Effects of substitution of extracellular Cl\(^{-}\) with I\(^{-}\), Br\(^{-}\), SCN\(^{-}\), or F\(^{-}\) on pH\(_{i}\) under iso- and hyperosmotic conditions are illustrated in Fig. 9, A-D, respectively. Substitution of Cl\(^{-}\) with I\(^{-}\), Br\(^{-}\), or SCN\(^{-}\) under isosmotic conditions had no effect on pH\(_{i}\). However, when the cells were exposed to hyperosmotic mannitol (500 mosmol/kgH\(_{2}\)O) in the presence of I\(^{-}\), Br\(^{-}\), and SCN\(^{-}\), the pH\(_{i}\) significantly increased from 6.82 ± 0.01 to 7.35 ± 0.03 (P < 0.001, n = 9), from 6.82 ± 0.04 to 7.26 ± 0.02 (P < 0.001, n = 6), and from 6.79 ± 0.02 to 6.95 ± 0.05 (P < 0.001, n = 6), respectively. Pretreatment with EIPA (100 µM) completely inhibited the pH\(_{i}\) increase induced by hyperosmotic mannitol in the presence of I\(^{-}\), Br\(^{-}\), and SCN\(^{-}\) (Fig. 9, E-G, respectively). By sharp contrast, substitution of Cl\(^{-}\) with F\(^{-}\) under isosmotic conditions caused a significant decrease in pH\(_{i}\) from 6.83 ± 0.02 to 6.61 ± 0.03 (P < 0.001, n = 9). When hyperosmotic mannitol was added in the presence of F\(^{-}\), pH\(_{i}\) was increased to 6.81 ± 0.02 (P < 0.001, n = 9). However, these pH\(_{i}\) values (6.81 ± 0.02) were not significantly different from basal pH\(_{i}\) values (6.83 ± 0.02). When MCs were treated with EIPA under isosmotic conditions, pH\(_{i}\) significantly decreased from 6.89 ± 0.03 to 6.75 ± 0.02 (P < 0.001, n = 5; Fig. 9H). When MCs were exposed to the solution containing F\(^{-}\) in the continued presence of EIPA, pH\(_{i}\) further decreased to 6.62 ± 0.02 (P < 0.001, n = 5). However, the EIPA-sensitive pH\(_{i}\) decrease in the presence of F\(^{-}\) (ΔpH\(_{i}\): 0.13 ± 0.02, P < 0.05, n = 5) was significantly smaller than that in its absence (ΔpH\(_{i}\):...
0.22 ± 0.03, n = 9). Pretreatment with EIPA completely inhibited the pH increase induced by hyperosmotic mannitol in the presence of F⁻ (Fig. 9H). We calculated acid extrusion rates (initial $J_{H^+}$) at the initial point of cell alkalinization induced by hyperosmotic mannitol solution with different anions. As shown in Fig. 10, these studies yielded an apparent specificity of $Cl^-$ > $Br^-$ > $I^-$ > SCN⁻ > F⁻ = gluconate for the initial $J_{H^+}$ induced by hyperosmolality. When 125 mM $Cl^-$ in the solution was substituted with equimolar cyclamate, the initial $J_{H^+}$ induced by hyperosmolality were similar to those observed in the gluconate solution (data not shown).

Figure 9, D and H, showed that, under isosmotic conditions, F⁻ caused pH$_i$ to decrease and that the decreased pH$_i$ induced by F⁻ was partially inhibited by EIPA. These data suggest the possibility that, under isosmotic conditions, F⁻ may partially inhibit NHE activity. To demonstrate this possibility, we examined Na⁺-dependent pH$_i$ increase after acid load in the presence of F⁻. When MCs were first bathed in the Na⁺-containing HEPES solution (control, solution 1, Table 1) and were then incubated in the Na⁺-free solution (solution 2, Table 1), pH$_i$ was decreased to ~6.50. Thereafter, MCs were exposed to the Na⁺-containing control solution (solution 1, Table 1) or the Na⁺- and F⁻-containing solution (solution 12, Table 1). As shown in Fig. 11A, subsequent addition of Na⁺ to the extracellular solution caused a rapid increase in pH$_i$ in MCs exposed to both control and F⁻-containing solutions, but the rise in pH$_i$ in the presence of F⁻ (19.2 ± 4.7 pH units/s * 10$^4$, P < 0.005, n = 9) was significantly smaller than that in its absence (56.2 ±

Fig. 10. Initial $J_{H^+}$ at the initial point of cell alkalinization induced by hyperosmotic mannitol solution with different anions. Data represent means ± SE; number of experiments in parentheses.
11.1 pH units/s $\times 10^4$, $n = 11$). Furthermore, the Na$^+$-dependent $J_H$ at pH of 6.60 after acid load in the presence of F$^-$ (36.6 $\pm$ 9.7 mM/s, $P < 0.05$, $n = 9$) was significantly smaller than that in its absence (110.6 $\pm$ 22.6 mM/s, $n = 11$; Fig. 11B). In MCs exposed to both control and F$^-$-containing solutions, the Na$^+$-dependent $pHi$ increase after acid load was completely inhibited by EIPA (100 µM; Figs. 2B and 11A, respectively). The control solution contained 132 mM Cl$^-$, whereas the F$^-$-containing solution contained 13 mM Cl$^-$ (Table 1). Because removing extracellular Cl$^-$ had no effect on NHE activity under isosmotic conditions (Fig. 5), it is unlikely that the decreased $J_H$ in the presence of F$^-$ is due to decreased Cl$^-$ concentration of the solution. Rather, the above data indicate that F$^-$ decreased the Na$^+$-dependent $J_H$ via an NHE process.

**DISCUSSION**

The present study was designed to determine whether hyperosmolality activates NHE in MCs and, if so, to determine the underlying mechanisms, especially the role of extracellular Cl$^-$. We demonstrate that hyperosmotic mannitol and sucrose activate NHE to cause cell alkalinization, but hyperosmotic urea causes no effect. The acid extrusion rate via a NHE process is greater under hyperosmotic conditions than under isosmotic conditions. Furthermore, NHE activation under hyperosmotic conditions requires the presence of extracellular Cl$^-$, whereas NHE activity under isosmotic conditions is independent of extracellular Cl$^-$. The Cl$^-$-dependent NHE activation under hyperosmotic conditions occurs, at least in part, via Cl$^-$ channel- and microtubule-dependent processes. Substitution of extracellular Cl$^-$ with different anions modulates NHE activation by hyperosmolality.

**Evidence for NHE in MCs**

The present study demonstrates that, in the nominal absence of CO$_3^-/HCO_3^-$, $pHi$ recovery rate from an acid load was dependent on external Na$^+$ and was completely inhibited by a specific NHE inhibitor (EIPA; see Fig. 2, A-C). Thus MCs exclusively regulate their $pHi$ through a NHE mechanism in the nominal absence of CO$_3^-/HCO_3^-$. These findings confirm previous studies (4, 13).

**pHi Regulation of MCs Under Iso- and Hyperosmotic Conditions**

The $pHi$ dependence of NHE. Our results show that cell alkalinization induced by hyperosmotic mannitol is due to activation of NHE because it is dependent on external Na$^+$ and is inhibited by EIPA (see Figs. 3 and 4A). As shown in Fig. 5, under both iso- and hyperosmotic conditions, Na$^+$-dependent $J_H$ through a NHE process decreased as $pHi$ increased from the acid to the normal range. However, the cells exposed to the hyperosmotic mannitol solution had a significantly greater $J_H$ than those exposed to the isosmotic solution at comparable $pHi$ values. In other words, hyperosmolality shifted the $J_H$ vs. $pHi$ by 0.15–0.3 pH units in the alkaline direction. Similarly, in Na$^+$-depleted glial cells, Jean et al. (20) reported that shrinkage shifted the flux vs. $pHi$ profile by 0.3–0.4 pH units in the alkaline direction. In addition, Grinstein et al. (15) studied the Na$^+$-dependent component of a $pHi$ recovery from an acid load in lymphocytes, finding that shrinkage shifts the flux vs. $pHi$ relationship by 0.2–0.3 pH units in the alkaline direction. Similar findings have been reported in barnacle muscle fibers exposed to hyperosmolality (10) and in C$_6$ glioma cells treated with hyperosmotic mannitol solution (37). Because the $pHi$ sensitivity of the exchange system appears to be largely determined by an allosteric modifier site (2) located on the cytoplasmic face of the membrane, the mechanisms for the shrinkage-induced NHE activation seem to be a shift in the $pHi$ dependence of the antiport.

**Effects of different osmolytes.** In the present study, hyperosmotic mannitol and sucrose solutions showed
significant initial rate of cell alkalinization, whereas hyperosmotic urea solution failed to detect it (see Figs. 3 and 6). In addition, the cell alkalinization induced by hyperosmotic mannitol and sucrose was completely inhibited by pretreatment with EIPA (see Figs. 3C and 6B). Therefore, these findings indicate that, at steady-state pHᵢ, both hyperosmotic mannitol and sucrose activate NHE, but hyperosmotic urea does not. Because mannitol and sucrose are relatively impermeant, it is most likely that they make the solution hypertonic. In contrast, urea is freely permeant and raises the osmolality but does not affect the solution toxicity (40). Accordingly, solution toxicity rather than absolute osmolality is the important factor for the NHE activation at steady-state pHᵢ. Similarly, Muto et al. (24) reported that, in vascular smooth muscle cells, hyperosmotic glucose or mannitol media stimulated Na⁺-K⁺-ATPase α1- and β1-mRNA accumulation, α1- and β1-subunit protein accumulation, and Na⁺-K⁺-ATPase activity, whereas hyperosmotic urea medium caused no effect. Similar actions of hyperosmotic NaCl, raffinose, or urea on accumulation of organic osmolytes have been reported in Madin-Darby canine kidney cells (25, 26): NaCl- or raffinose-induced hyperosmotic stress resulted in the accumulation of betaine, glycerophosphorylcholine, and myo-inositol, but urea-induced hyperosmotic stress failed to induce osmolyte accumulation.

Effects of removing extracellular Cl⁻. NHE activity in the apical membrane of the colonic crypt cell under isosmotic conditions is dependent on extracellular Cl⁻ (33). In dog erythrocytes (31, 32) and in barnacle muscle fibers (10), NHE activation under hyperosmotic conditions is inhibited by removing extracellular Cl⁻. Therefore, the present study examined whether the MC NHE activity under iso- or hyperosmotic conditions is dependent on extracellular Cl⁻. The MCs exposed to the hyperosmotic mannitol solution in the absence of extracellular Cl⁻ had a significantly smaller jᵢ than that in its presence at comparable pHᵢ values (see Fig. 5). Furthermore, extracellular Cl⁻ modulates the shrinkage-induced NHE activation in a dose-dependent manner (Figs. 2 and 9), whereas, under isosmotic conditions, it causes no effect on NHE activity (Fig. 2, A and D, and Fig. 5). Therefore, the NHE activation under hyperosmotic conditions is not only pHᵢ dependent but also Cl⁻ dependent. Also, NHE in MCs is distinct from that of the apical membrane in the colonic crypt cell.

The mechanisms by which extracellular Cl⁻ affects the shrinkage-induced NHE activation are not clear presently. The effects of extracellular Cl⁻ may be through changes in intracellular Cl⁻, as reported in barnacle muscle fibers treated with hyperosmotic mannitol solution (10). Several mechanisms would be proposed for the Cl⁻-dependent NHE activation induced by hyperosmolality. Removal of extracellular Cl⁻ by replacement with gluconate did not influence NHE activity under isosmotic conditions (see Fig. 2, A and D, and Fig. 5), whereas it inhibited NHE activity under hyperosmotic conditions (Figs. 4 and 5). On the contrary, when external Cl⁻ was readded in the continued presence of hyperosmotic mannitol, NHE activation occurred (Fig. 7A). Therefore, it is unlikely that this inhibition was due to the introduction of gluconate as the extracellular Cl⁻ replacement. Rather, Cl⁻ might be required for some aspects of the shrinkage-induced activation process, the sensor or signal transduction system, as Davis et al. (10) suggested. Regarding the latter, the activation of G proteins has been reported to be involved in the shrinkage-induced activation of NHE in barnacle muscle fibers (9). Regarding the former, we found that the Cl⁻-dependent NHE activation induced by hyperosmolality was partially inhibited by pretreatment with NPPB (the Cl⁻ channel inhibitor; see Fig. 7, A and C, Table 2). These results indicate that the shrinkage-induced NHE activation partly occurs through Cl⁻ channel activation. Pretreatment with DIDS (the inhibitor of Cl⁻/base exchange) also partially inhibited the Cl⁻-dependent NHE activation induced by hyperosmolality (see Fig. 7, A and D, Table 2). In the nominal absence of CO₂/HCO₃⁻, removing extracellular Cl⁻ caused no increase in pHᵢ, as shown in Fig. 7A. Thus, under these conditions, the Cl⁻/base exchange process is indeed inoperative, although, in the presence of CO₂/HCO₃⁻, the MC anion exchanger has been reported to transport base (5). Rather, in the nominal absence of CO₂/HCO₃⁻, DIDS may act on MCs as a Cl⁻ channel inhibitor. In other cell systems, DIDS has been reported to be one of the Cl⁻ channel inhibitors (17, 36). As shown in Fig. 7, E and F, and Table 2, colchicine, but not cytochalasin B, partially inhibited the Cl⁻-dependent NHE activation induced by hyperosmolality. These findings indicate that microtubule, but not filamentous actin, is involved in the Cl⁻-dependent NHE activation induced by hyperosmolality, and that under these conditions, a colchicine-sensitive process may be activated, and microtubule may regulate the exocytic insertion of the NHE protein-containing vesicles into the plasma membrane. Similarly, Wagner et al. (41) reported in rat proximal tubules that ANG II stimulated H⁺-ATPase activity and that this stimulation was inhibited by removing external Cl⁻, NPPB, and colchicine. Their report suggests that ANG II stimulates proton extrusion via H⁺-ATPase by a Cl⁻-dependent process involving brush-border insertion of vesicles. The mechanisms by which the Cl⁻-dependent NHE activation induced by hyperosmolality occurs via a colchicine-sensitive process are still unclear.

Effects of substitution of Cl⁻ with different anions. The present study further examined the Cl⁻ specificity for the shrinkage-induced NHE activation. When extracellular Cl⁻ under isosmotic conditions was replaced with Br⁻, I⁻, or SCN⁻, basal pHᵢ values were not affected (see Fig. 9, A-C). Substitution of extracellular Cl⁻ with different anions yielded an apparent specificity of Cl⁻ ≥ Br⁻ ≥ I⁻ > SCN⁻ > gluconate for the shrinkage-induced acid extrusion rates through a NHE process (Fig. 10). The hyperosmolality-induced cell alkalinization in the presence of Cl⁻, Br⁻, I⁻, SCN⁻, and gluconate was completely inhibited by pretreatment with EIPA (Figs. 3C and 9, E-G). These results
indicate that both Br\(^-\) and I\(^-\), like Cl\(^-\), modulate the shrinkage-induced NHE activation. Therefore, these three anions may share common metabolic and/or transport pathway(s), as discussed above. On the other hand, when Cl\(^-\) was replaced with SCN\(^-\), the acid extrusion rate induced by hyperosmolality was 38.9% of that of Cl\(^-\). These findings indicate that SCN\(^-\) partially inhibits the Cl\(^-\)-dependent NHE activation induced by hyperosmolality. When external Cl\(^-\) under isosmotic conditions was substituted with F\(^-\), basal pH\(_i\) values were significantly decreased (Fig. 9D). Pretreatment with EIPA partially inhibits the F\(^-\)-induced cell acidification (Fig. 9H). Furthermore, as shown in Fig. 11, the Na\(^+\)-dependent J\(_{H+}\) at pH\(_i\) of 6.60 after acid load in the presence of F\(^-\) was significantly smaller than that in its absence, and the Na\(^+\)-dependent pH\(_i\) recovery was completely inhibited by EIPA. Thus, at steady-state pH\(_i\), F\(^-\) inhibits NHE activity, and consequently causes cell acidification, although the possibility that the F\(^-\)-induced cell acidification may occur via mechanisms other than inhibition of the NHE cannot be excluded. NaF (more specifically AlF\(_4^+\)) at a concentration of 10 mM is a well-known pharmacological probe for establishing the significance of G protein activation in cellular systems (14, 30). The concentration of NaF used in the present study was 125 mM. It is not known whether this concentration of NaF actually acts on MC NHE as a G protein activator. We found that the hyperosmolality-induced cell alkalinization in the presence of F\(^-\) was completely inhibited by pretreatment with EIPA (Fig. 9H) and that the initial J\(_{H+}\) in the hyperosmotic F\(^-\) solution was similar to that in the hyperosmotic gluconate solution (Fig. 10). These findings indicate that the hyperosmolality-induced cell alkalinization in the presence of F\(^-\) is due to NHE activation and that F\(^-\) does not influence the Cl\(^-\)-dependent NHE activation induced by hyperosmolality. In contrast to MCs, in vascular smooth muscle cells, NaF has been shown to stimulate NHE activity under both iso- and hyperosmotic conditions (30). From our present findings, different anions modulate Cl\(^-\)-dependent NHE activation induced by hyperosmolality. However, the underlying mechanisms will be required to be elucidated.

In conclusion, the present study clearly demonstrates that, at steady-state pH\(_i\), hyperosmolality by the poorly permeating solutes (mannitol and sucrose) activates NHE to cause cell alkalinization, whereas the rapidly permeating solute (urea) has no effect, and shrinkage-induced NHE activation requires extracellular Cl\(^-\) and is modulated by substitution of Cl\(^-\) with different anions. The Cl\(^-\)-dependent NHE activation induced by hyperosmolality partly occurs via Cl\(^-\)-channel- and microtubule-dependent processes. The Cl\(^-\)-dependent NHE activation under hyperosmotic conditions may be important for cell volume regulation, intraglomerular hemodynamics, and/or TGF mechanisms.

We thank H. Kasakura for expert secretarial assistance in preparing the manuscript.

This work was supported in part by a grant from the Japanese Kidney Foundation (J Inkenkyukai), by a grant from the Salt Science Foundation, and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan. A portion of this work was presented at the 1998 Annual Meeting of the American Society of Nephrology in Philadelphia, PA, and has been published in abstract form (23a).

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Received 25 February 1999; accepted in final form 7 January 2000.

REFERENCES


