Shrinkage-induced activation of Na\(^+/\)H\(^+\) exchange in rat renal mesangial cells

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1Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520; 2Institut für Neurobiologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; and 3Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

Bevensee, Mark O., Esther Bashi, Wolf-Rüdiger Schlue, Gregory Boyarsky, and Walter F. Boron. Shrinkage-induced activation of Na\(^+/\)H\(^+\) exchange in rat renal mesangial cells. Am. J. Physiol. 276 (Cell Physiol. 45): C674–C683, 1999.—Using the pH-sensitive dye 2',7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein (BCECF), we examined the effect of hyperosmolar solutions, which presumably caused cell shrinkage, on intracellular pH (pH\(_i\)) regulation in mesangial cells (single cells or populations) cultured from the rat kidney. The calibration of BCECF is identical in shrunken and unshrunken mesangial cells if the extracellular K\(^+\) concentration ([K\(^+\)]) is adjusted to match the predicted intracellular [K\(^+\)]. For pH\(_i\) values between ~6.7 and ~7.4, the intrinsic buffering power in shrunken cells (600 mosmol/kgH\(_2\)O) is threefold larger than in unshrunken cells (~300 mosmol/kgH\(_2\)O). In the nominal absence of CO\(_2\)/HCO\(_3\), exposing cell populations to a HEPES-buffered solution supplemented with ~300 mM mannitol (600 mosmol/kgH\(_2\)O) causes steady-state pH\(_i\) to increase by ~0.4. The pH\(_i\) increase is due to activation of Na\(^+/\)H\(^+\) exchange because, in single cells, it is blocked in the absence of external Na\(^+\) or in the presence of 50 \(\mu\)M ethylisopropylamiloride (EIPA). Preincubating cells in a Cl\(^-\)-free solution for at least 14 min inhibits the shrinkage-induced pH\(_i\) increase by 80%. We calculated the pH\(_i\) dependence of the Na\(^+/\)H\(^+\) exchange rate in cell populations under normosmolar and hyperosmolar conditions by summing 1) the pH dependence of the total acid-extrusion rate and 2) the pH\(_i\) dependence of the EIPA-insensitive acid-loading rate. Shrinkage alkali shifts the pH\(_i\) dependence of Na\(^+/\)H\(^+\) exchange by ~0.7 pH units.

Acute responses of a cell to volume disturbances involve the immediate stimulation of ion transport across plasma membranes. For example, cell swelling can stimulate the efflux of ions and thus H\(_2\)O out of cells, a process termed volume regulatory decrease (2, 9, 15, 25, 27, 39). Conversely, cell shrinkage stimulates the influx of ions and thus H\(_2\)O in cells. The subsequent increase in cell volume, a volume regulatory increase, can be caused by stimulation of 1) Na-K-Cl cotransport and 2) stimulation of Na\(^+/\)H\(^+\) exchange, which together with Cl-HCO\(_3\) exchange has the net effect of accumulating NaCl (see Refs. 10, 18, 20, 22, 28).

Several groups have studied the shrinkage-induced activation of Na\(^+/\)H\(^+\) exchange. Of course, decreased intracellular pH (pH\(_i\)) can also activate Na\(^+/\)H\(^+\) exchange independently of shrinkage. Both shrinkage and low pH\(_i\) activate Na\(^+/\)H\(^+\) exchange in human lymphocytes (17), C\(_5\) rat glial cells (24, 37), dog erythrocytes (32), barnacle muscle fibers (14), rabbit alveolar macrophages (21), and rat mandibular salivary glands (36). In barnacle muscle fibers, a G protein appears to be an intermediate in the transduction of the shrinkage signal to the activation of the Na\(^+/\)H\(^+\) exchanger (13, 23). The presence of intracellular Cl\(^-\) also appears to be necessary for the shrinkage-induced activation of the transporter in both dog erythrocytes and barnacle muscle fibers. In the dog erythrocytes, the activation can be inhibited by removing external Cl\(^-\), which presumably depletes intracellular Cl\(^-\) (31, 33). Indeed, in the barnacle muscle fiber, shrinkage-induced activation of the Na\(^+/\)H\(^+\) exchanger requires intracellular Cl\(^-\) (14, 23) at a step at, or before, activation of a heterotrimeric G protein (23).

In experiments on the pH\(_i\) dependence of the Na\(^+/\)H\(^+\) exchanger in barnacle muscle fibers (14), the shrinkage-induced activation of the transporter is approximately
threefold greater at relatively acidic values (pH$_i$ ~ 6.8) than at relatively alkaline values (pH$_i$ ~ 7.6). In this barnacle study, the authors defined the Na$^+/H^+$ exchange rate as the amidolide-sensitive acid-extrusion rate in experiments in which they measured pH$_i$ with glass microelectrodes. Also, several groups have presented data consistent with the idea that shrinkage activates the Na$^+/H^+$ exchanger in mammalian cells by shifting the flux vs. pH$_i$ profile of the exchanger in the alkaline direction (19, 21, 24, 36, 37).

In the present study, we performed experiments specifically designed to isolate the pH$_i$ dependence of the Na$^+/H^+$ exchange rate in cultured rat mesangial cells under both normosmolar and hypertonic conditions. We found that exposing cells to a hypertonic solution, which presumably causes cell shrinkage, alkali shifts the pH$_i$ dependence of the transporter by ~0.7 pH units. This shift explains why cell shrinkage causes steady-state pH$_i$ to increase.

Portions of this work have been published in abstract form (35).

**METHODS**

**Solutions**

All experiments were performed in the nominal absence of CO$_2$/HCO$_3$$. The standard HEPES-buffered solution (~305 mosmol/kgH$_2$O) contained (in mM) 125 NaCl, 5 KCl, 1 CaCl$_2$, 1.2 MgSO$_4$, 2 NaH$_2$PO$_4$, 32 HEPES, and 10.5 glucose and was titrated to 7.4 at 37°C with NaOH. Hyperosmolar solutions (600 mosmol/kgH$_2$O) were made by adding ~300 mM mannitol to a HEPES-buffered solution, thereby increasing the volume by ~3%. In the Na$^+$-free solutions, the Na$^+$ substitute was N-methyl-D-glucammonium (NMDG$^+$). In the Cl$^-$-free solutions, the Cl$^-$ substitute was gluconate. In some of the Cl$^-$-free solutions, the Ca$^{2+}$ concentration was increased to 3 mM to compensate for Ca$^{2+}$ chelation by the gluconate. The inhibitory effect of removing external Cl$^-$ on the shrinkage-induced alkalization (see results) was the same whether or not we compensated for Ca$^{2+}$ chelation by gluconate. In NH$_4$/NH$_3$$_2$$_2$ solutions, NaCl (or NMDG-Cl) was replaced by an equal concentration of NH$_4$Cl. Both the normosmolar and hypertonic dye-calibration solutions, which were based on the corresponding HEPES-buffered solutions, contained 10 mM nigericin. In addition, in the normosmolar calibration solution, we replaced Na$^+$ with 100 mM K$^+$ [final K$^+$ concentration ([K$^+$]) = 105 mM] and NMDG$^+$. In the hyperosmolar calibration solution, we replaced the Na$^+$ with 205 mM KCl (final [K$^+$] = 210 mM) and NMDG$^+$ and adjusted the osmolality to 600 mosmol/kgH$_2$O by adding ~120 mM mannitol. We measured the osmolality of all solutions with a vapor-pressure osmometer (model 5100C; Wescor, Logan, UT). Ethylisopropylamiloride (EIPA) was diluted 1:1,000 from a 50 µM stock solution in distilled H$_2$O and then ethanol and then H$_2$O. These washing protocols substantially reduce nigericin contamination from one experiment to the next (Bevensee, Bash, and Boron, unpublished observations). Moreover, because we report the Na$^+/H^+$ exchange rate as the difference between the total and EIPA-insensitive acid-extrusion rates, our Na$^+/H^+$ exchanger data should be immune to any residual nigericin contamination (Bevensee, Bash, and Boron, unpublished observations).

**Cell Preparation**

Rat mesangial cells were prepared as previously described (6, 29). Briefly, cells from passages 3–7 were plated on glass coverslips and were grown at 37°C in Dulbecco’s modified Eagle’s medium ( Gibco-BRL, Life Technologies, Gaithersburg, MD) exposed to an atmosphere of 5% CO$_2$/balance air. The medium was supplemented with 100 U/ml penicillin plus streptomycin (Gibco-BRL), 5 µg/ml of insulin plus transferrin plus selenious acid (Becton-Dickinson Labware, Bedford, MA), and 10% fetal calf serum (Gemini Bioproducts, Calabasas, CA or Gibco-BRL). To render cells quiescent at the time of experimentation, the cells were incubated for 16–18 h in a medium containing only 0.5% fetal calf serum. Experiments were performed on cells at least 80% confluent. A coverslip with attached cells was exposed to 10 µM BCECF-AM in the standard HEPES-buffered solution in an air incubator at 37°C for 20 min. The coverslip was then transferred to a temperature-regulated, flow-through cuvette and was perfused with the standard HEPES-buffered solution, prewarmed to 37°C, for ~5 min before the start of the experiment to wash nonhydrolyzed BCECF-AM from the cells.

**Measurement of pH$_i$**

Our technique for measuring pH$_i$ in a population of cells has been previously described (26), and it will only be briefly summarized here. The flow-through cuvette containing a coverslip was placed in the excitation light path of a SPEX Fluorolog-2 spectrofluorometer (model CMIT10E; SPEX Industries, Edison, NJ). Cells loaded with BCECF were alternately excited with light at the pH-sensitive wavelength of 502 nm (I$_{502}$) and the relatively pH-insensitive wavelength of 440 nm (I$_{440}$). The emission wavelength was 526 nm. The sample integration time was 1 s, and the sample frequency was once every 3 s. The fluorescence-excitation ratio (I$_{502}$/I$_{440}$) was normalized and converted to a pH$_i$ value using the high-K$^+$/nigericin technique (38), as modified for a single-point calibration (6). Background I$_{502}$ and I$_{440}$ signals from cells without dye were subtracted from total I$_{502}$ and I$_{440}$ signals. An evaluation of shrinkage on the calibration of BCECF in mesangial cells is presented in RESULTS.

After each experiment in which a dye calibration was performed, the perfusion lines were washed with either ethanol followed by distilled H$_2$O or albumin followed by ethanol and then H$_2$O. These washing protocols substantially reduce nigericin contamination from one experiment to the next (Bevensee, Bash, and Boron, unpublished observations). Moreover, because we report the Na$^+/H^+$ exchange rate as the difference between the total and EIPA-insensitive acid-extrusion rates, our Na$^+/H^+$ exchanger data should be immune to any residual nigericin contamination (Bevensee, Bash, and Boron, unpublished observations).

For some experiments, we measured pH$_i$ in single mesangial cells using an inverted Zeiss IM-35 microscope equipped for epi-illumination. Our technique for measuring pH$_i$ in single cells is described in detail in Ref. 6.

**Calculation of Acid-Extrusion and Acid-Loading Rates**

Acid-extrusion and acid-loading rates are defined as the product of the rate of change in pH$_i$ (dpH$_i$/dt) and the total intracellular buffering power (β$_{Total}$). Because it is difficult to measure the ratio of volume to surface area in many cells, it has become customary to report acid-extrusion and acid-loading rates in units of moles per liter of cell volume per second (i.e., µM/s), instead of the classical flux units of moles per unit area per unit time (i.e., µmol·cm$^{-2}$·s$^{-1}$). We use the symbol ψ to refer to these acid-extrusion and acid-loading rates, or “pseudofluxes.”
Calculation of Intrinsic Buffering Power

In the nominal absence of \(\text{CO}_2/\text{HCO}_3\), \(B_{\text{real}}\) is equivalent to the intrinsic buffering power of the cell (\(\beta_i\)). Cell shrinkage will concentrate intracellular \(\text{H}^+\) buffers and likely increase \(\beta_i\). Therefore, we used the approach introduced by Boyarsky et al. (6) to calculate the \(\text{pH}_i\) dependence of \(\beta_i\) in mesangial cells exposed to both normo- and hyperosmolar conditions. \(\beta_i\) experiments were performed in the absence of external \(\text{Na}^+\) to eliminate \(\text{pH}_i\) changes caused by \(\text{Na}^+/-\text{H}^+\) exchange. After initially exposing the cells to a \(\text{Na}^+\)-free solution containing 40 mM \(\text{NH}_3/\text{NH}_4^+\), we recorded step changes in \(\text{pH}_i\) elicited by progressively lowering extracellular \(\text{NH}_3/\text{NH}_4^+\); \(\beta_i\) is defined as the change in intracellular \(\text{NH}_3^+\) per unit change in \(\text{pH}_i\).

In the experiment shown in Fig. 1A, mesangial cells in the nominally \(\text{CO}_2/\text{HCO}_3\)-free, standard HEPES-buffered solution had a \(\text{pH}_i\) of \(-7.1\) before point a. Exposing the cells to the HEPES-buffered solution supplemented with \(-300\) mM mannitol (total osmolality \(= 600\) mosmol/kgH\(_2\)O) elicited an initial decrease in \(\text{pH}_i\) (segment ab), followed by a larger decrease to \(-7.5\) (segment bc). These osmolality-induced changes in \(\text{pH}_i\) are described in more detail in RESULTS. For the remainder of the experiment shown in Fig. 1A (segment ck), the cells were exposed to hyperosmolar solutions. Removing external \(\text{Na}^+\) caused \(\text{pH}_i\) to decrease to \(-6.7\) (segment cd). In the continued absence of external \(\text{Na}^+\), switching the cells to a solution containing \(40\) mM \(\text{NH}_3/\text{NH}_4^+\) elicited a rapid increase in \(\text{pH}_i\) (segment de). Subsequently, exposing the cells to solutions containing progressively lower concentrations of \(\text{NH}_3/\text{NH}_4^+\) caused stepwise decreases in \(\text{pH}_i\) (segments ef, fg, gh, hi, ij, and jk). In some experiments (not shown), the \(\text{pH}_i\) continued to decrease slowly after the initial step changes. For these experiments, we back-extrapolated the \(\text{pH}_i\) vs. time record to determine more accurately the initial \(\text{pH}_i\) changes elicited by the \(\text{NH}_3/\text{NH}_4^+\) solutions (1, 3, 12).

Using data from experiments similar to that shown in Fig. 1A, we plotted the \(\text{pH}_i\) dependence of \(\beta_i\) for mesangial cells under hyperosmolar conditions (Fig. 1B). In other experiments, we used the same \(\text{NH}_3/\text{NH}_4^+\) step technique to determine the \(\text{pH}_i\) dependence of \(\beta_i\) for mesangial cells under normosmolar conditions (Fig. 1B). As shown by the best-fit lines to the data, \(\beta_i\) in shrunken cells is threefold greater than in unshrunken cells for \(\text{pH}_i\) values between \(-6.7\) and \(-7.4\).

Statistics

Data are reported as means \(\pm\) SE. Levels of significance were assessed using the unpaired Student’s \(t\)-test. A \(P\) value \(< 0.05\) was considered significant. The \(\text{pH}_i\) dependencies of \(\beta_i\) were fitted by straight lines using a least-squares method. The \(\text{dpH}_i/\text{dt}\) values were fitted by a third-order polynomial using a least-squares method.

RESULTS

Calibration of BCECF in Mesangial Cells Under Normosmolar and Hyperosmolar Conditions

For maintaining a stable \(\text{pH}_i\), in nigericin calibration solutions, doubling the osmolality requires doubling the extracellular \([	ext{K}^+]_o\). As described in METHODS, we calibrated the \(\text{pH}\)-sensitive dye BCECF using the high-\(\text{K}^+/-\text{nigericin}\) technique. In our first series of experiments, we evaluated the effect of shrinkage on the calibration of the dye. If the cells were perfect osmometers, then increasing the extracellular osmolality from \(-300\) to \(-600\) mosmol/kgH\(_2\)O should decrease cell volume by one-half and thereby double the concentration of intracellular constituents (e.g., \(\text{K}^+\)), assuming no change in membrane permeability. Accordingly, the dye calibration solutions used for such shrunken cells should have two times the nominal \([\text{K}^+]_o\) (210 vs. 105 mM) to satisfy the calibration requirement that extracellular \(\text{K}^+\) concentration \((K_{o})\) equals the intracellular \(\text{K}^+\) concentration \((K_i)\).

To test this hypothesis, we first calibrated BCECF in mesangial cells under normosmolar conditions using our standard 105 mM \(\text{K}^+/-\text{nigericin}\) calibration solution and then monitored \(\text{pH}_i\) as we shrank the cells in hyperosmolar (600 mosmol/kgH\(_2\)O) calibration solu-
tions containing either 105 or 210 mM K+. Shrinking cells in the presence of only 105 mM K+ would be expected to lead to a decrease in pH, because [K+]i (i.e., 105 mM) will be less than the predicted [K+]i (i.e., 210 mM), thereby promoting the nigericin-mediated exchange of internal K+ for external H+. On the other hand, shrinking the cells in a calibration solution containing 210 mM K+ should have no effect on nigericin-mediated pH changes because [K+]i will match [K+]o.

During the initial portions of the recordings shown in Fig. 2 (prior to point a), both groups of mesangial cells were exposed to our standard 105 mM K+/nigericin solution (pH 7.0). Thus, by definition, the pH before point a is 7.0. Shrinking the cells in the standard calibration solution supplemented with ~300 mM mannitol elicited a pH decrease of ~0.25 (segment ab). This acidification was likely caused by a nigericin-mediated exchange of internal K+ for external H+, due to the shrinkage-induced increase of [K+]o. As indicated by the broken line, the decrease in pH was relatively constant for >40 min. Returning the cells to the normosmolar calibration solution caused pH to increase (segment bc) to near the initial value at the onset of the experiment (prior to point a). In 10 experiments similar to that shown in Fig. 2, shrinking mesangial cells with a 105 mM K+/~300 mM mannitol calibration solution (pH 7.0) caused pH to decrease from 7.0 to 6.76 ± 0.01 (P < 0.001). Clearly then, BCECF in shrunken cells cannot be adequately calibrated using the standard calibration solution containing 105 mM K+.

In contrast to what we observed with the 105 mM K+/~300 mM mannitol calibration solution (600 mosmol/kgH2O), shrinking cells in a calibration solution containing 210 mM K+/~120 mM mannitol (600 mosmol/kgH2O) elicited only a slight decrease in pH, ~0.04 (segment ab'). This small acidification was reversed by returning the cells to the normosmolar calibration solution (segment b’c’). In 16 experiments similar to that shown in Fig. 2, shrinking mesangial cells with a 210 mM K+~120 mM mannitol calibration solution caused pH to decrease from 7.0 to 6.98 ± 0.01 (P = 0.03). Thus, at a pH of 7.0, the calibration of BCECF in mesangial cells shrunken in a 600 mosmol/kgH2O solution containing 210 mM K+/nigericin solution is equivalent to the calibration of the dye in unshrunken cells incubated in an ~300 mosmol/kgH2O solution containing 105 mM K+/nigericin. In similar experiments on BCECF-loaded rat thymic lymphocytes, Grinstein et al. (19) demonstrated that the fluorescence ratio of the dye remained constant when the cells were exposed to nigericin-containing solutions (pH 7.0) of different osmolalities but a constant [K+]i-[Na+]i ratio.

BCECF calibration is identical in shrunken and unshrunk mesangial cells, provided [K+]o is adjusted to match the predicted [K+]i. Using an approach similar to that shown in Fig. 2, we extended our analysis of the effect of shrinkage and [K+]o on the intracellular calibration of BCECF for pH values between ~6 and ~8.2. In Fig. 3, we plot the normalized fluorescence excitation ratio for the BCECF vs. pH relationship for cells calibrated either under normosmolar conditions (~300 mosmol/kgH2O) at a [K+]o of 105 mM or under hyperosmolar conditions (600 mosmol/kgH2O) with an external [K+] of 210 mM. The best-fit curves are the result of a nonlinear least-squares curve fit (see legend for Fig. 3). The two curves are virtually identical to one another. In other words, a single BCECF calibration can be used in experiments in which the mesangial cells undergo volume changes.

Effect of Shrinkage on Steady-State pH

Shrinkage elicits an increase in steady-state pH. We evaluated the effect of shrinkage on the steady-state pH of mesangial cells by exposing them to our standard HEPES-buffered solution supplemented with ~300 mM mannitol (600 mosmol/kgH2O). Before point a in Fig. 4, the cells had an average pH of ~7.05. Exposing the
cells to a hyperosmolar solution containing ~300 mM mannitol consistently elicited a small, transient decrease in pH \textit{i} (segment ab), presumably due to the effect of concentrating and then reequilibrating H\textsuperscript{+} and intracellular buffers. This transient pH \textit{i} decrease was followed by a robust increase in pH \textit{i} (segment bc) to a value ~0.7 pH units higher than the initial pH \textit{i} (prior to point a). The hyperosmolar-induced pH \textit{i} increase was at least partially reversed by returning to the normosmolar solution (segment cd). In a total of 54 experiments similar to that shown in Fig. 4, ~300 mM mannitol caused an increase in pH \textit{i} from 7.17 ± 0.02 to 7.56 ± 0.03 (P < 0.0001). The average pH \textit{i} increase of ~0.4 was not dependent on the initial steady-state pH \textit{i} before exposing the cells to the hyperosmolar solution (data not shown). Mesangial cells with an initial steady-state pH \textit{i} of ~7.0 were just as likely to alkalinize to the same extent as cells with an initial steady-state pH \textit{i} of ~7.4. Shrinkage-induced increase in pH \textit{i} is blocked by removing external Na\textsuperscript{+} or by applying 50 µM EIPA. Using an inverted microscope equipped for epi-illumination, we also performed experiments on single mesangial cells. Exposing single cells (not shown) to a HEPES-buffered solution supplemented with ~300 mM mannitol (600 mosmol/kgH\textsubscript{2}O) elicited a series of pH \textit{i} changes very similar to those in Fig. 4; a transient decrease in pH \textit{i}, followed by a rapid and reversible increase to a value ~0.6 pH units greater, on average, than the pH \textit{i} at the onset of the experiments. In the experiment shown in Fig. 5A on a single mesangial cell, removing external Na\textsuperscript{+} caused pH \textit{i} to decrease from ~7.2 to ~6.7 (segment ab), probably due to reversal of Na\textsuperscript{+}/H\textsuperscript{+} exchange and/or unmasking of background acid loading. In the continued absence of external Na\textsuperscript{+}, exposing the cell to the HEPES-buffered solution supplemented with ~300 mM mannitol did not elicit a pH \textit{i} increase (segment cd). In other experiments similar to that shown in Fig. 5B, exposing single cells to 50 µM EIPA caused pH \textit{i} to decrease from ~7.15 to ~6.75 (segment ab), due to unmasking of background acid loading that is usually balanced by Na\textsuperscript{+}/H\textsuperscript{+} exchange at steady-state pH \textit{i}. In the continued presence of EIPA, exposing the cell to a solution supplemented with ~300 mM mannitol did not elicit a pH \textit{i} increase (segment bc). In summary, the pH \textit{i} increase elicited by hyperosmolarity can be inhibited by either removing external Na\textsuperscript{+} or by applying 50 µM EIPA. Therefore, shrinkage appears to increase the steady-state pH \textit{i} of mesangial cells by activating the Na\textsuperscript{+}/H\textsuperscript{+} exchanger.

Shrinkage-induced increase in pH \textit{i} is reduced by incubating the cells for 14 or 90 min in a Cl\textsuperscript{−}-free medium. As noted in the introduction, Cl\textsuperscript{−} is required for the shrinkage-induced activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange in dog erythrocytes and barnacle muscle fibers. We therefore tested if the shrinkage-induced pH \textit{i} increase in mesangial cells could be reduced by exposing the cells to a Cl\textsuperscript{−}-free solution. In Fig. 6, we show the results from two experiments in which cells were exposed to the HEPES-buffered solution supplemented with ~300 mM mannitol either in the absence or presence of external Cl\textsuperscript{−}. In both experiments, the
Fig. 6. The pH increase elicited by shrinkage can be reduced by incubating cells in a Cl⁻-free medium for 14 or 90 min. In both experiments, the cells were exposed to a HEPES-buffered solution supplemented with ~300 mM mannitol (600 mosmol/kg H₂O). The pHᵢ increase elicited by the mannitol solution was 0.36 units greater in the control cells (segment abc) than in the cells preincubated in a Cl⁻-free solution for 90 min before the experiment (segment ab). In a total of 5 experiments, the cells were preincubated in the Cl⁻-free solution for either 14 min (n = 3) or 90 min (n = 2).

mannotl solution elicited a small decrease in pHᵢ (segment ab), similar to segment ab in Fig. 4. However, the subsequent pHᵢ increase was 0.36 pH units greater in the control cells (segment bc) than in those exposed to the Cl⁻-free medium for ~90 min before the experiment (segment bc). In five experiments, we found that the mean pHᵢ increase elicited by hyperosmolarity was 0.09 ± 0.05 in the absence of Cl⁻. In seven, week-matched control experiments conducted in the presence of Cl⁻, the pHᵢ increase averaged 0.45 ± 0.07. Thus Cl⁻ removal reduced the hyperosmolar-induced pHᵢ increase by 80%.

Effect of Shrinkage on pHᵢ Dependence of Na⁺/H⁺ Exchange

Shrinkage increases the rate of pHᵢ recovery from an acid load. To determine the effect of shrinkage on the pHᵢ dependence of Na⁺/H⁺ exchange in mesangial cells, we used the approach introduced by Boyarsky et al. (5). For cells subjected to either normosmolar or hyperosmolar conditions, we determined 1) the pHᵢ dependence of “total acid extrusion” (ψₑ) during the pHᵢ recovery from an acid load and 2) the pHᵢ dependence of “EIPA-insensitive acid loading” (ψₑₑ). We obtained the pHᵢ dependence of the Na⁺/H⁺ exchange rate by adding ψₑₑ to ψₑ for identical pHᵢ values.

The results from two experiments, performed on unshrunken and unshrunken mesangial cells, are shown in Fig. 7. In both cases, we used the NH₃⁻-prepulse technique to acid load the cells (4) and then monitored the pHᵢ recovery from the acid load. For the hyperosmolar experiment, cells in the standard HEPES-buffered solution had an average pHᵢ of ~7.3. Shrinkage in the solution supplemented with ~300 mM mannitol elicited the usual transient decrease and rapid increase in pHᵢ (segment abc). Using the NH₃⁻-prepulse technique (4), we then acid loaded the cells (segment cdef). After the acid load, pHᵢ recovered rapidly (segment fg). After the pHᵢ recovery was complete, applying 50 µM EIPA elicited a decrease in pHᵢ (segment gh) that represents an unmasking of background acid loading that is normally balanced by acid extrusion mediated by Na⁺/H⁺ exchange.

For the normosmolar experiment, we only show the record for the recovery of pHᵢ from the acid load (segment f'g'). The decrease in pHᵢ from the subsequent application of EIPA (segment g'h').

It is important to compare the data from these two experiments at comparable pHᵢ values, because acid-base transport rates can vary markedly with changes in pHᵢ. In comparing the segment fg and segment f'g' pHᵢ recoveries in shrunken and unshrunken cells, respectively, we see that there is only a narrow pHᵢ range (near ~7.2) in which the two pHᵢ recoveries overlap. At this single pHᵢ of 7.2, the pHᵢ recovery rate is substantially greater in shrunken cells (point f) than in unshrunken cells (point g').

Shrinkage alkali shifts the pHᵢ dependence of the Na⁺/H⁺ exchange rate by ~0.7 pH units. From segment fg and segment f'g' pHᵢ trajectories similar to those shown in Fig. 7, we computed the pHᵢ dependence of total acid extrusion (ψₑ) for both shrunken and unshrunken cells (Fig. 8A). We also used the EIPA-unmasked acidifications similar to those in segments gh and g'h' in Fig. 7 to obtain the pHᵢ dependence of EIPA-insensitive acid loading (ψₑₑ) for both shrunken and unshrunken cells (Fig. 8B). Some of the normosmolar ψₑₑ data points at the lower pHᵢ values in Fig. 8B were obtained by monitoring the recovery of pHᵢ from an acid load (analogous to segments fg and f'g' in Fig. 7) in the presence of EIPA (data not shown). In principle,
we would have obtained normosmolar $\varphi_{\text{EIPA}}$ data for $p_{\text{H}_i}$ values between 6.9 and 6.6 if either EIPA had caused $p_{\text{H}_i}$ to fall below pH 6.9 or if pH had recovered from an acid load in the presence of EIPA above pH 6.6. In Fig. 8C, we plot the $p_{\text{H}_i}$ dependence of the $\text{Na}^+/\text{H}^+$ exchange rate ($\varphi_{\text{Na-H}}$) for both shrunken cells and unshrunken cells. We obtained the $\varphi_{\text{Na-H}}$ plots in Fig. 8C by adding the respective $\varphi_E$ and $\varphi_{\text{EIPA}}$ data sets for norm- and hyperosmolar conditions. For both shrunken and unshrunken cells, $\varphi_{\text{Na-H}}$ decreases linearly with increasing values of $p_{\text{H}_i}$. However, the plot of $\varphi_{\text{Na-H}}$ vs. $p_{\text{H}_i}$ for the shrunken cells is alkaline shifted by $\sim 0.7$ pH units compared with the same plot for the unshrunken cells.

**DISCUSSION**

$\text{Na}^+/\text{H}^+$ Exchange in Shrunken Cells

Shrinkage activates the $\text{Na}^+/\text{H}^+$ exchanger in mesangial cells. In the present study, we demonstrate that shrinking mesangial cells in a hyperosmolar solution containing $\sim 300$ mM mannitol (600 mosmol/kg H$_2$O) elicits a sustained increase in steady-state $p_{\text{H}_i}$ of $\sim 0.4$ pH units. The increase is caused by stimulation of $\text{Na}^+/\text{H}^+$ exchange because it can be inhibited by removing external $\text{Na}^+$ or by applying 50 µM EIPA. The $\sim 0.4$ pH unit increase is substantially larger than the maximal $\sim 0.15$ pH unit increase observed when mesangial cells are stimulated by the growth factor arginine vasopressin in the nominal absence of CO$_2$/HCO$_3$ (16). Therefore, shrinkage is a potent stimulator of $\text{Na}^+/\text{H}^+$ exchange in mesangial cells. In other experiments in which we monitored the $p_{\text{H}_i}$ recovery from acid loads in the presence or absence of EIPA, the $p_{\text{H}_i}$ dependence of $\varphi_{\text{Na-H}}$ is alkali shifted by $\sim 0.7$ pH units in shrunken vs. unshrunken mesangial cells. As discussed in the Appendix, there is no reason to expect that this $\sim 0.7$ pH unit shift should be of the same magnitude as the shrinkage-induced increase in steady-state $p_{\text{H}_i}$.

Results from previous studies are consistent with shrinkage alkali shifting the $p_{\text{H}_i}$ dependence of $\varphi_{\text{Na-H}}$. As mentioned in the Introduction, several groups have provided evidence consistent with the idea that cell shrinkage alkali shifts the $p_{\text{H}_i}$ dependence of $\varphi_{\text{Na-H}}$ in mammalian cells. For example, in acid-loaded human lymphocytes, shrinkage alkali shifted the $\text{Na}^+$-dependent acid-extrusion vs. $p_{\text{H}_i}$ relationship by $0.2$–$0.3$ pH units (19). However, the $\text{Na}^+$-dependent acid-extrusion rate includes the contributions of all $\text{Na}^+$-dependent acid-base transporters, including the nigericin used to acid load the cells (Beversee, Bash, and Boron, unpublished observations and Ref. 34). In $\text{Na}^+$-depleted C6 rat glioma cells, shrinkage alkali shifted the $^{22}\text{Na}^+$ flux vs. $p_{\text{H}_i}$ relationship by $0.3$–$0.4$ pH units (24). However, the total, unidirectional $^{22}\text{Na}^+$ influx includes the fluxes of all $\text{Na}^+$ transport processes and cannot distinguish $\text{Na}^+$/Na$^+$ exchange from $\text{Na}^+$/$\text{H}^+$ exchange. In another study on acid-loaded rabbit alveolar macrophages, shrinkage alkali shifted the bafilomycin-resistant acid extrusion vs. $p_{\text{H}_i}$ relationship by $\sim 0.2$ pH units (21). However, the bafilomycin-resistant acid-extrusion rate represents the net effect of all acid-loading and acid-
extruding processes other than the vacuolar H⁺ pump. Finally, in acid-loaded rat mandibular salivary glands, shrinkage alkali shifted the total acid-extrusion rate vs. pH relationship by 0.15 pH units (36). However, the total acid-extrusion rate represents the algebraically summed contributions of all acid-loading and acid-extruding mechanisms. Thus, although the above four studies show that shrinkage causes an alkali shift in the pH dependence of some combination of acid-base parameters, the studies were not specifically designed to isolate the pH dependence of other acid-extrusion and acid-loading mechanisms.

Evidence has been presented that cell shrinkage alkali shifts the pH dependence of \( \varphi_{\text{Na-H}} \) in rat C6 glioma cells (37). However, the activity of the Na⁺/H⁺ exchanger at alkaline pH values was underestimated because there was no compensation for background acid loading unmasked by amiloride at the high pH values reached during cell shrinkage.

Issues to Consider When Measuring Changes in Acid-Extrusion and Acid-Loading Rates Elicited by Changes in Cell Volume

Shrinking mesangial cells does not alter the BCECF calibration curve. Because shrinkage will increase [K⁺], we calibrated the intracellular dye by exposing the cells to an extracellular solution with [K⁺] chosen to match the predicted, shrinkage-increased [K⁺]. We found that simultaneously doubling both osmolality (from ~300 to 600 mosmol/kgH₂O) and [K⁺], (from 105 to 210 mM) had little effect on the pH of cells already clamped with nigericin to 7.0 (segment ab' in Fig. 2). Thus the doubling of osmolality must have doubled [K⁺], so that we can conclude that the cells behave as near-perfect osmometers.

At least under the conditions of our calibration experiments (i.e., neither Na⁻ nor CO₂/HCO₃ were present), shrunken mesangial cells did not exhibit volume recoveries for at least 40 min after cell shrinkage. The evidence is that pH failed to increase during segment ab in Fig. 2. On the other hand, cell volume may have recovered during our other experiments (e.g., conducted in the presence of Na⁺). If so, then our postexperimenat pH calibrations would have been erroneously high because [K⁺] would have been lower than we thought. However, the size of this error is expected to be very small because the likely degree of volume recovery is very low. For example, when we exposed cells to ~300 mM mannitol in Fig. 4, Na⁺/H⁺ exchange caused pH to increase by ~0.7. This alkalinization represents the extrusion of ~14 mM H⁺ (and thus the uptake of 14 mM Na⁺), assuming a buffering power of 20 mM (Fig. 1). The complete recovery of cell volume would have required the uptake of ~300 mM Na⁺. Thus the computed uptake of 14 mM represents less than a 5% volume recovery. A 5% volume recovery would lead to a 5% error in the estimated [H⁺], which translates to an overestimation of 0.02 pH units. Thus it is unlikely that volume recovery seriously affected either our calibration or its application to physiological experiments.

In extending our analysis to other pH values, we deduced that the calibration of intracellular BCECF is the same in unshrunken and shrunken cells (600 mosmol/kgH₂O) provided that the appropriate [K⁺], is used. Therefore, at least in mesangial cells, the calibration of intracellular BCECF is insensitive to cell-volume changes per se. Apparently, the pH sensitivity of intracellular BCECF in mesangial cells is unaffected by shrinkage-induced increases in ionic strength and concentrations of intracellular constituents (excluding K⁺).

Shrinking mesangial cells increases β₁. We had anticipated that a doubling of osmolality, and a subsequent halving of cell volume, would have doubled the intracellular concentrations of proton buffers and thus have increased β₁ by twofold. However, over the pH range of 6.7–7.4, the apparent β₁ was threefold greater in shrunken (600 mosmol/kgH₂O) vs. unshrunken cells. One possible explanation for the larger-than-expected increase in β₁, is cytosolic crowding of proton buffers. Macromolecular crowding can cause changes in protein-protein interactions that are disproportionately larger than the increase in protein concentration (30). If macromolecular crowding were to cause the pK values of buffers to shift closer to the physiological pH, the effect would be an increase in buffering power. Regardless of the mechanism for the increase in β₁, given identical pH recovery rates, the calculated acid-extrusion rate will be twofold greater in shrunken vs. unshrunken mesangial cells. Our β₁ results are at least qualitatively consistent with those previously reported in barnacle muscle fibers (14) and rat C6 glioma cells (37).

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If the volume had increased, [K⁺] should have fallen, and nigericin should have mediated an influx of K⁺ and an efflux of H⁺ (i.e., produced a pH increase).
Shrinkage can lead to overestimates in calculating acid-extrusion and acid-loading rates. One can report acid-base transport rates either in units of moles per surface area per time ($J$) or in units of moles per volume per time ($v$). Shrunken cells have less volume but presumably the same plasma membrane surface area as unshrunken cells. Therefore, shrinkage will have different effects on acid-base transport rates reported in terms of $J$ vs. $v$.

As discussed in METHODS, it has become customary to report acid-base fluxes in terms of $v$ for small mammalian cells in which it is difficult to compute the volume-to-surface ratio. In these pseudoflux units, the acid-base transport rate would be higher for the smaller of the two cells, even though the $J$ values for the two cells would be identical. Ideally, one would correct for cell volume changes when reporting acid-base fluxes in terms of $v$. However, it is extremely difficult to measure volume changes accurately in most small cells. In at least one example though, Seo et al. (36) used proton nuclear magnetic resonance spectroscopy to measure cell-volume changes and then corrected acid-extrusion rates in perfused rat mandibular salivary glands under hyperosmolar conditions.

According to the above discussion, acid-extrusion and acid-loading rates reported in terms of $v$, as in the present study, should be greater in shrunken (600 mosmol/kgH$_2$O) vs. unshrunken (300 mosmol/kgH$_2$O) mesangial cells, even if shrinkage had no effect on acid-base transport per se (i.e., even if shrinkage had no effect on $J$). Thus the $v$-$J$ effect would predict that mesangial cells shrunken to one-half their initial volume would display a doubling of their acid-extrusion and acid-loading rates when expressed in terms of $v$ but no change when expressed in terms of $J$. This doubling arises because of the effect of shrinkage on surface-to-volume ratio; any effects on $v$ are automatically incorporated in the calculated $v$. However, we found that shrinkage produced a 5- to 12-fold increase in $v$Na/H exchange, 2.5- to 6-fold greater than the doubling expected because of the $v$-$J$ effect. Thus we conclude that shrinkage increases the Na$^+$/H$^+$ exchange rate by a factor of at least 2.5–6.

Two other arguments support our conclusion that shrinkage does indeed stimulate Na$^+$/H$^+$ exchange, independent of a $v$-$J$ effect. First, shrinkage leads to an increase in steady-state pH$_i$ that is blocked by removing Na$^+$ or applying EIPA. Second, shrinkage produces a substantial alkali shift of the $v$Na/H vs. pH$_i$ relationship, without substantially changing its slope. A $v$-$J$ effect could affect the magnitude of the transport parameter at any given pH, but could not produce such a shift.

In conclusion, shrinkage of mesangial cells leads to a substantial elevation in steady-state pH$_i$ by stimulating the Na$^+$/H$^+$ exchanger. The pH$_i$ increase requires external Na$^+$ and can be inhibited by EIPA. The kinetic basis of the Na$^+$/H$^+$ exchange stimulation is a large alkali shift in the $v$Na/H vs. pH$_i$ relationship.

**APPENDIX**

Relationship Between a Shift in the pH$_i$ Dependence of an Acid-Base Transporter and the Resultant Change in Steady-State pH$_i$

pH$_i$ is controlled by the sum of fluxes or other processes that extrude acid from the cell ($J_E$) and those that load acid into the cell ($J_L$). As shown in Fig. 9, $J_E$ tends to decrease with increasing pH$_i$, whereas $J_L$ tends to increase. Imagine that a mesangial cell has only one acid loader (e.g., H$^+$-influx) and one acid extruder (e.g., Na$^+$/H$^+$ exchange). $J_E$ describes the kinetics of the Na$^+$/H$^+$ exchanger under control conditions, and $J_L$ describes the kinetics of acid loading. The steady-state pH$_i$ of the cell is 7.1, determined by the point where $J_E = J_L$ (point a). We now switch to a hyperosmolar solution, which alkali shifts the pH$_i$ dependence of the Na$^+$/H$^+$ exchanger by $-0.7$ pH units (point $J_E-1-J_L$). Will this 0.7 pH unit alkali shift of $J_E$ elicit a 0.7 pH unit increase in steady-state pH$_i$? In the unlikely event that the plot of $J_L$ vs. pH$_i$ is horizontal ($J_L$), then $J_E - J_L$ will indeed increase steady-state pH$_i$ by 0.7 pH units, from 7.1 to 7.8 (point a $\rightarrow$ b). However, in the equally unlikely event that the plot of $J_E$ vs. pH$_i$ is vertical ($J_E$), then $J_E - J_L$ will not increase steady-state pH$_i$ at all (point a $\rightarrow$ c). In all likelihood, the plot of $J_L$ vs. pH$_i$ will slope upward, as indicated by $J_L$ in Fig. 9. Therefore, the shrinkage stimulus that elicits a 0.7 pH unit alkali shift of $J_E$ will result in a smaller shift of the steady-state pH$_i$ (point a $\rightarrow$ d). Thus the steeper the slope of $J_L$, the smaller the change in steady-state pH$_i$. Similarly, the steeper the slope of $J_E$, the smaller the effect on steady-state pH$_i$ of shifting the pH$_i$ profile of an acid loader.

If the cell has multiple acid extruders, then, all things being equal, an alkali shift of the Na$^+$/H$^+$ exchanger will elicit an even smaller alkali shift in steady-state pH$_i$, inasmuch as the Na$^+$/H$^+$ exchanger makes a smaller contribution to the overall acid-extrusion rate. One should generally expect the shift in steady-state pH$_i$ to be smaller than the instigating shift in the pH$_i$ profile of either an acid loader or acid extruder.

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