

Mechanism of Osmotic Activation of Na⁺/H⁺ Exchange in Rat Thymic Lymphocytes

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ABSTRACT The activity of the Na⁺/H⁺ exchange system of rat thymic lymphocytes was determined by means of intracellular (pH_i) and extracellular pH (pH_o) measurements. In isotonic media, the antiport is virtually quiescent at physiological pH_i (7.0–7.1), but is greatly activated by cytoplasmic acidification. At normal pH_i, the antiport can also be activated by osmotic shrinking. Osmotic activation occurs after a delay of 20–30 s and is reversed several minutes after iso-osmolarity is restored. The mechanism of activation was analyzed by comparing the kinetic parameters of transport in resting (isotonic) and hyperosmotically stressed cells. The affinities of the external substrate site for Na⁺ and H⁺ are not altered in shrunken cells. In contrast, the H⁺ sensitivity of the antiport (which is largely dictated by an allosteric modifier site) was increased, which accounted for the activation. The concentration of free cytoplasmic Ca²⁺ ([Ca²⁺]_i) increased after osmotic shrinking. This increase was dependent on the presence of extracellular Ca²⁺ and Na⁺ and was blocked by inhibitors of Na⁺/H⁺ exchange, which suggests that it is a consequence, rather than the cause, of the activation of the antiport. It is concluded that the shift in the pH_i dependence of the modifier site of the Na⁺/H⁺ antiport is the primary event underlying the regulatory volume increase that follows osmotic shrinkage.

INTRODUCTION

Under certain conditions, human peripheral blood lymphocytes regain near-normal volume after shrinking in hypertonic solutions (Grinstein et al., 1983). This regulatory volume increase (RVI) occurs only in the presence of extracellular Na⁺, is associated with an increased rate of ²²Na⁺ uptake, and is dependent on the presence of HCO₃⁻. In the nominal absence of HCO₃⁻, a cytoplasmic alkalinization can be recorded after hypertonic stress, which is consistent with extrusion of cytoplasmic H⁺. Because the phenomenon appears to be electroneutral and sensitive to amiloride, and is analogous to other systems such as *Amphiuma* and dog erythrocytes (Cala, 1980, 1983; Parker, 1983; Parker and Castranova, 1984), it was suggested that RVI in blood lymphocytes is mediated by activation of a Na⁺/H⁺ antiport, which operates in parallel to a Cl⁻/HCO₃⁻ exchanger,

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allowing salt uptake to occur (Grinstein et al., 1983). The mechanism of activation of the Na^+/H^+ antiport in blood lymphocytes has not, however, been defined.

An electroneutral Na^+/H^+ exchange system has also been identified in rat thymic lymphocytes (Grinstein et al., 1984*a, b*). This antiport is virtually quiescent at normal cytoplasmic pH (pH_i), but is greatly activated in acid-loaded cells by a mechanism involving a "modifier" site at the cytoplasmic side of the membrane. In the present report, we demonstrate that the antiport of thymocytes can also be stimulated by a hypertonic challenge, but this activation is not a consequence of cytoplasmic acidification. Experiments were therefore designed to analyze the mechanism of osmotic activation, by comparing the kinetic parameters of countertransport in resting (isotonic) and hypertonically stressed cells. Measurable rates of transport under isotonic conditions were obtained by manipulation of pH_i . The results indicate that volume-induced activation of Na^+/H^+ exchange is brought about largely by a shift in the pH_i sensitivity of the "modifier" site that controls the activity of the antiport. In addition, the role of cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) in the activation process was also considered. Changes in $[\text{Ca}^{2+}]_i$, although present during RVI, are not essential to the response and appear to be a consequence, rather than the cause, of the activation of the Na^+/H^+ antiport.

MATERIALS AND METHODS

Reagents

Nigericin was purchased from Calbiochem-Behring Corp., San Diego, CA; 2-[*N*-morpholino]ethanesulfonic acid (MES), Trizma base, and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO; *N*-methyl-D-glucamine was from Aldrich Chemical Co., Milwaukee, WI; quin-2-acetoxymethyl ester was the kind gift of Dr. T. J. Rink, University of Cambridge, England; bis(carboxyethyl)carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from the Hospital for Sick Children Research Development Corp., Toronto, courtesy of Dr. M. Ramjeesingh; ionomycin was the gift of Squibb, Princeton, NJ; amiloride and 5-*N*-ethyl-*N*-propyl amiloride were the gift of Merck, Sharp & Dohme, West Point, PA, and Montreal, Canada; 10-fold-concentrated solution RPMI 1640 (without HCO_3^-) was from Gibco Laboratories, Grand Island, NY.

Solutions

Stock solutions of ionomycin, quin-2 acetoxymethyl ester, BCECF acetoxymethyl ester, and 5-*N*-ethyl-*N*-propyl amiloride were prepared in dimethylsulfoxide. A nigericin stock was made in ethanol. A 1-M aqueous stock of *N*-methyl-D-glucamine chloride was prepared by titration of the base with HCl to pH 7.3.

N-methyl-D-glucamine⁺ solution contained (in mM): 140 *N*-methyl-D-glucamine chloride, 1 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 glucose, and 20 mM Tris-MES, pH 7.2. Choline⁺, Na⁺, and K⁺ solutions were prepared by iso-osmotic replacement of *N*-methyl-D-glucamine⁺ by choline⁺, Na⁺, or K⁺, respectively, but were otherwise identical. Unless otherwise noted, all the media were nominally HCO_3^- free and their osmolarity was adjusted to 290 ± 5 mosM with the major salt, using an Osmette A osmometer (Precision Systems Inc., Natick MA). Where indicated, the media were made hypertonic by addition of NaCl or *N*-methyl-D-glucamine chloride.

Cell Isolation and Characterization

Thymocytes were isolated from male Wistar rats weighing 200–250 g as previously described (Grinstein et al., 1984*a*). Viability, determined by exclusion of Trypan blue,

was >95% throughout the experimental period. Cell counting and sizing were performed with a Coulter ZM-Channelyzer combination (Coulter Electronics, Hialeah, FL), using the shape-deformability factor determined by Segel et al. (1981) for human peripheral lymphocytes.

Cytoplasmic pH (pH_i) Determination and Manipulation

For pH_i determination, the cells ($5 \times 10^7/\text{ml}$) were loaded with BCECF (Rink et al., 1982) by incubation with $3 \mu\text{g}/\text{ml}$ of the parent acetoxyethyl ester for 30 min at 37°C . The cells were then sedimented, washed once, and resuspended in HEPES-buffered RPMI 1640 at 10^8 cells/ml. Aliquots of this suspension were used for the fluorimetric determination of pH_i as described previously (Grinstein et al., 1984a), using a Perkin-Elmer Corp. (Norwalk, CT) 650-40 or LS5 fluorescence spectrophotometer. Calibration of fluorescence vs. pH_i was made using nigericin in K^+ solution (Thomas et al., 1979). Using the value for the buffering power (β) determined earlier (25 mmol/liter cells $\cdot \text{pH}$; Grinstein et al., 1984a), the rate of change of pH_i ($\Delta\text{pH}/t$) can be converted to an equivalent H^+ flux (J_{H^+}) by the following calculation: $J_{\text{H}^+} = \beta \cdot \Delta\text{pH}/t$.

In some experiments, cells were acid-loaded with nigericin essentially as described (Grinstein et al., 1984a). Briefly, the cells ($3 \times 10^6/\text{ml}$) were suspended in *N*-methyl-D-glucamine $^+$ solution and $0.3 \mu\text{g}/\text{ml}$ nigericin was added while monitoring pH_i with BCECF as described above. The ionophore catalyzes the exchange of intracellular K^+ (and to a lesser extent Na^+) for external H^+ , which results in cytoplasmic acidification. When the desired pH_i was attained, the acid-loading was terminated by scavenging nigericin by addition of concentrated albumin (5 mg/ml final). If necessary, the cells were then rapidly sedimented and resuspended in the indicated medium. As predicted from the value of the buffering power (see above), only minor (<10%) changes in cell volume are observed when pH_i is varied within one unit of the physiological level. This behavior differs from that described for red cells, which shrink substantially during alkalization. This shrinking of red cells is due to titration of hemoglobin and subsequent loss of Cl^- , which is distributed according to a Donnan equilibrium.

Because in some experiments pH_i was determined in osmotically shrunken cells, it was essential to demonstrate that the fluorescence determinations were not affected by changing cell volume. For this purpose, the effect of volume changes on fluorescence was determined in cells at constant pH_i . The constancy of pH_i during the course of these measurements was ensured by clamping with nigericin. The procedure is based on the premise that, in the presence of an excess of nigericin, pH_i will reach a steady state when $[\text{K}^+ + \Omega\text{Na}^+]_i/[\text{K}^+ + \Omega\text{Na}^+]_o = [\text{H}^+]_i/[\text{H}^+]_o$, where Ω is the relative rate of Na^+/K^+ transport through the ionophore. Practically, pH_i clamping was accomplished by suspending the cells in a medium containing 122.5 mM K^+ and 17.5 mM Na^+ , which is similar to the intracellular K^+/Na^+ ratio of $\sim 7:1$ determined earlier for thymocytes by flame photometry (Grinstein et al., 1984a). After the addition of nigericin ($1 \mu\text{g}/\text{ml}$), both cellular volume and BCECF fluorescence were monitored in parallel as the osmolarity of the medium was increased stepwise by addition of aliquots of a concentrated solution of the chloride salts of K^+ and Na^+ (7:1 ratio). Fig. 1A shows a typical fluorescence recording of cells challenged with progressively increasing concentrations of K^+/Na^+ (7:1) in the presence of nigericin. No significant change in fluorescence was observed in these pH_i -clamped cells, which indicates that BCECF fluorescence is unaffected by cell volume under these conditions. That cell volume was variable during these experiments was demonstrated by electronic cell sizing (Fig. 1B). Because RVI in these conditions is precluded by the low $[\text{Na}^+]_o$ and by the virtual absence of HCO_3^- , the osmotic behavior of the cells approximates the van't Hoff prediction.

Measurements of Acid Extrusion as ΔpH_o

2×10^8 cells were sedimented and resuspended in 2 ml of unbuffered Na^+ solution (buffered only by the Hepes carried over with the pellet). The pH of this suspension was measured under magnetic stirring with an Orion Research Inc. (Cambridge, MA) 601A

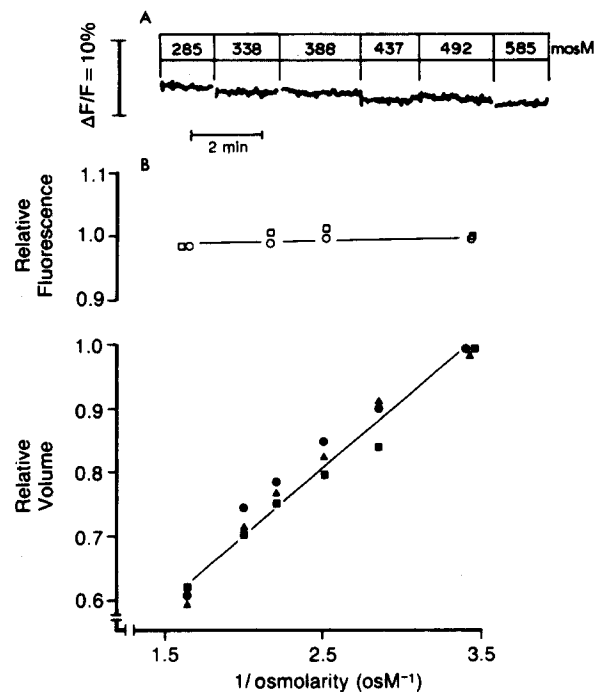


FIGURE 1. Insensitivity of BCECF fluorescence to changes in cellular volume. (A) Typical fluorescence recording (left to right) from a suspension of BCECF-loaded, pH_i -clamped thymocytes. The cells were initially suspended in an isotonic medium (285 mosM) containing a 7:1 ratio of K^+/Na^+ , plus $1 \mu\text{g}/\text{ml}$ of nigericin to stabilize pH_i . The osmolarity of the medium was then increased stepwise as indicated, while keeping the K^+/Na^+ ratio constant. The small ($<3\%$) dilution artifacts were corrected. The numbers indicate the final medium osmolarity. $\Delta F/F$ is the relative fluorescence change, where F is the total initial fluorescence of the isotonic cell suspension. Representative of three similar experiments. (B) Changes in BCECF fluorescence (top, open symbols) and in cellular volume (bottom, solid symbols) as a function of medium osmolarity. Osmolarity was changed while keeping the K^+/Na^+ ratio constant, as above. Fluorescence changes were determined as in A; the median cell volume was measured electronically using the Coulter-Channelyzer combination. Different symbols were used for individual experiments. Notice that under these conditions, and over the range of osmolarities studied, the cells obey the van't Hoff relationship.

digital Ionalyzer attached to an X vs. time flatbed recorder (model PE 100, Perkin-Elmer Corp.). Where indicated, concentrated NaCl, KCl, or choline chloride was added to increase the medium osmolarity. Extracellular pH was maintained between 7.3 and 7.0 during the course of the measurements by manual addition of small aliquots of KOH.

The buffering power of the medium was determined at the end of the experiment by titration with KOH and HCl.

Other Methods

The cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i) was determined using quin-2 by the method of Tsien et al. (1982). Thymocyte suspensions in HEPES-buffered RPMI 1640 (5 × 10⁷ cells/ml) were loaded for 30 min at 37°C with 10 μM quin-2 acetoxymethyl ester. The cells were then washed and resuspended in the required medium at 3–6 × 10⁶ cells/ml. Fluorescence was measured with excitation at 339 nm, using a 3-nm slit to minimize photolysis of the probe, and emission at 495 nm with a 15-nm slit. Calibration was made with ionomycin and Mn²⁺ (Rink et al., 1983).

All the experiments were carried out at room temperature (20–22°C). The results are presented as representative traces or as the means ± SE of the number of experiments indicated. Straight lines were fitted by least squares and statistical comparisons were made using Student's *t* test for unpaired samples.

RESULTS

Effect of Medium Osmolarity on Na⁺/H⁺ Exchange

In this study, the activity of the Na⁺/H⁺ antiport was determined in most cases by measurement of pH_i. In order to maximize the changes in pH_i, all the experiments were carried out in nominally HCO₃⁻-free solutions, thereby minimizing the regulatory volume changes (see Cala, 1980, for a discussion of the role of HCO₃⁻ in RVI). The pH_i of thymocytes at room temperature in isotonic (290 ± 5 mosM) Na⁺ solution ranges between 7.0 and 7.1 (Grinstein et al., 1984a, b; Fig. 2A). Increasing the medium osmolarity to 550 mosM with *N*-methyl-D-glucamine chloride while keeping external Na⁺ ([Na⁺]_o) constant at 140 mM results in a pronounced cytoplasmic alkalinization (Fig. 2A). The change in pH_i becomes apparent after a lag of ~20 s, and within 5–7 min reaches a maximum that is 0.26 ± 0.04 (*n* = 8) units more alkaline than the isotonic control. As shown in Fig. 2B, this cytoplasmic alkalinization is accompanied by the appearance of proton equivalents in the external medium, measurable as an increased rate of medium acidification in a poorly buffered Na⁺ solution. For these experiments, Na⁺ or choline⁺ (a quaternary ammonium ion) were substituted for *N*-methyl-D-glucamine⁺ (a secondary amine) because the buffering power of the latter obscured the changes of external pH (pH_o). As in the case of changes in pH_i (Fig. 2A), the increased rate of external acidification is only apparent ~30 s after the change in osmolarity, and maximal rates are attained after 2–3 min. These data suggest that the osmotically induced cytoplasmic alkalinization results from the outward transmembrane transport of internal proton (H⁺) equivalents. This notion is further supported by the similarity between the amount of acid appearing in the medium and that leaving the cells. In the experiment of Fig. 2, the amount of acid appearing externally, calculated as the area under the peak in Fig. 2B, was ~0.67 nmol/10⁶ cells. By comparison, the acid extruded from the cells, estimated as the product of the osmotically induced ΔpH and the cellular buffering capacity (25 mmol·liter⁻¹·pH⁻¹, from Grinstein et al., 1984a), averaged 0.74 nmol/10⁶ cells (calculated from the mean

ΔpH reported above and using a volume of $114 \mu\text{m}^3/\text{cell}$, determined by electronic sizing).

The osmotically induced change in pH_i was dependent on the presence of high $[\text{Na}^+]_o$. Shrinking had no significant effect on pH_i at 5 mM Na^+ (Fig. 2A) and

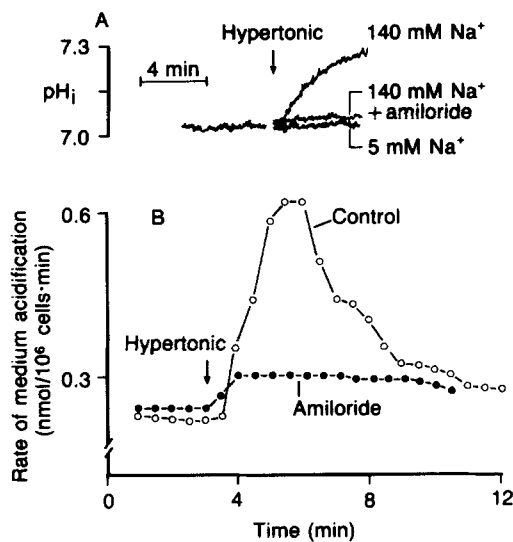


FIGURE 2. Effect of osmotic shrinking on cytoplasmic pH (pH_i). BCECF-loaded thymocytes (3×10^6 cells/ml) were suspended either in isotonic K^+ solution containing 5 mM Na^+ or in isotonic Na^+ solution (140 mM Na^+) with or without 100 μM amiloride. Where indicated, these solutions were made hypertonic (550 mosM) by addition of concentrated *N*-methyl-D-glucamine chloride, the dilution artifact was compensated, and recording was resumed. Similar results were obtained when the cells were sedimented and resuspended in the hypertonic solution, in which case the dilution correlation was not necessary. The figure is a composite of traces that are representative of at least four similar experiments. The pH_i values were calculated by calibration with nigericin (Thomas et al., 1979). (B) Osmotically induced extracellular acidification. Thymocytes ($10^8/\text{ml}$) were suspended in unbuffered isotonic Na^+ solution with or without 200 μM amiloride. The pH of this suspension (buffered only by the Heps carried over with the pellet) was measured under constant magnetic stirring as described under Methods. Where indicated, the solution was made hypertonic (550 mosM) by addition of concentrated NaCl. The buffering capacity of the medium, used to estimate the rate of medium acidification (ordinate), was determined at the end of each experiment by titration with KOH and HCl. Extracellular pH was maintained between 7.3 and 7.0 during the course of the experiment by addition of KOH. Representative of three experiments.

occasionally produced a slight acidification in Na^+ -free medium (not shown). Similarly, the acidification of the external medium was absent if Na^+ was omitted from the solution. As shown in Fig. 2, both responses were also blocked by 100 μM amiloride, an inhibitor of Na^+/H^+ exchange in thymocytes (Grinstein et al.,

1984a) and other cells (see Benos, 1982, for a review). Taken together, these results indicate that osmotic shrinking activates the Na^+/H^+ antiport of rat thymocytes.

Activation of Exchange as a Function of Medium Osmolarity

The maximal rate of amiloride-sensitive H^+ efflux (measured as an alkalization in BCECF-loaded cells and calculated as described in Methods) was studied as a function of medium osmolarity (Fig. 3). In these experiments, $[\text{Na}^+]_o$ was main-

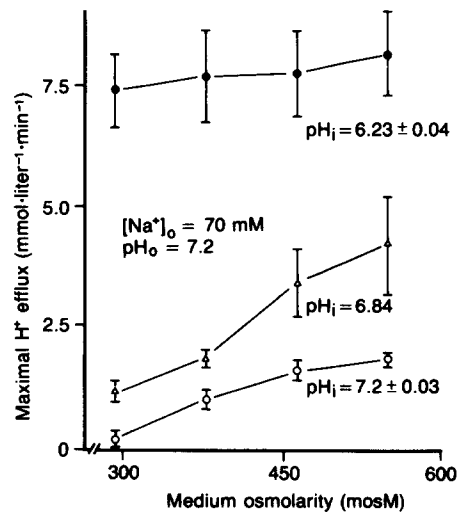


FIGURE 3. Relationship between medium osmolarity (abscissa, in mosmolar) and the maximal rate of H^+ efflux (ordinate, in $\text{mmol} \cdot \text{liter}^{-1} \cdot \text{min}^{-1}$). The maximal rate of H^+ efflux was calculated from the rate of ΔpH_i , measured fluorimetrically in BCECF-loaded cells, as described under Methods. The osmolarity was adjusted with *N*-methyl-D-glucamine chloride while keeping $[\text{Na}^+]_o$ constant at 70 mM. The cells were either suspended directly in the media with the indicated osmolarity (open circles), in which case the initial pH_i was unaltered (7.2 ± 0.03 , $n = 3$), or previously acid-loaded in isotonic media to the extent indicated beside the curves (triangles and solid circles). Acid-loading was accomplished using nigericin in *N*-methyl-D-glucamine⁺ solution as described (see Methods). Circles: means \pm SE of three experiments. Triangles: means \pm range of two experiments. External pH (pH_o) was constant at 7.2.

tained constant at 70 mM, while the osmolarity was varied with *N*-methyl-D-glucamine⁺. As shown in Fig. 3, H^+ efflux was negligible in otherwise untreated cells ($\text{pH}_i = 7.2 \pm 0.03$; open circles) suspended in isotonic medium, but increased substantially with osmolarity up to 460 mosM. Only a small further increase was recorded between 460 and 550 mosM.

As described earlier, Na^+/H^+ exchange in thymocytes can also be activated by cytoplasmic acidification. It was therefore important to determine the relationship between the two modes of activation. For this purpose, cells were acid-loaded to varying degrees with nigericin in Na^+ -free solution and then suspended

in media of different osmolarities containing 70 mM Na⁺. As expected, amiloride-sensitive H⁺ efflux was detectable in acid-loaded cells even at normal osmolarity (Fig. 3). In moderately acidified cells (pH_i = 6.84; triangles), transport was further increased by raising the osmolarity of the medium. In contrast, only a statistically insignificant increase was noted when severely acidified cells (pH_i = 6.23 ± 0.04; solid circles) were subjected to hypertonicity, which suggests that a maximal transport rate had been attained (see below).

[Na⁺]_o Dependence

What is the mechanism underlying the osmotic activation of Na⁺/H⁺ exchange? This question was addressed by comparing the kinetic parameters of transport in cells suspended in isotonic and hypertonic media. In order to obtain measurable transport rates in the isotonic media, their pH_i was manipulated as described above.

One conceivable mechanism of activation is an increased extracellular affinity for Na⁺. This possibility was analyzed in the experiment illustrated in Fig. 4. The rate of alkalization was measured in BCECF-loaded cells as a function of [Na⁺]_o while keeping the osmolarity constant at either 550 (for cells at normal pH_i) or 285 mosM (for acid-loaded cells). As reported earlier (Grinstein et al., 1984a), the activation of H⁺ efflux in isotonic acid-loaded cells follows a rectangular hyperbola compatible with Michaelis-Menten-type kinetics. This relationship was confirmed in the present report using the same batches of cells tested in hypertonic solutions (Fig. 4A). Although under the conditions used the absolute rates of transport differ (notice different ordinates), the relative pattern of [Na⁺]_o dependence is essentially identical for pH_i and osmotically activated cells (Fig. 4A). The data could be linearized according to Lineweaver-Burk, as shown in Fig. 4B, where the means ± SE for each Na⁺ concentration were used: the apparent K_m for Na_o⁺ in shrunken cells, calculated from the intercept of this line on the abscissa, was 56 mM. This number is not significantly different from

FIGURE 4. (*opposite*) Comparison of the Na_o⁺ concentration dependence of Na⁺/H⁺ exchange (amiloride-sensitive H⁺ efflux) in isotonic and hypertonically shrunken cells. (A) Dependence of the maximal H⁺ extrusion rates on [Na⁺]_o in isotonic acid-loaded cells (285 mosM, initial pH_i = 6.37; open circles) and in hypertonically treated cells (550 mosM, initial pH_i = 7.14 ± 0.02, n = 3; solid circles). In one case (triangles), the acid-loaded cells were suspended in moderately hypertonic medium. In all cases, the osmolarity was balanced with N-methyl-D-glucamine chloride. H⁺ efflux was calculated from the maximal rate of ΔpH_i as in Fig. 3. Notice that the left-hand ordinate applies to the solid symbols and the right-hand one to the open symbols. pH_o was constant at 7.2. (B) Lineweaver-Burk linearization of the data for hypertonically treated cells in A. For simplicity, data were averaged for individual Na⁺ concentrations and the means ± SE are illustrated. Error bars were omitted when they were smaller than the symbol. The line was calculated by least squares and had a correlation coefficient of 0.997. For comparison, the line calculated previously for isotonic, acid-loaded cells is included (dashed line; from Fig. 5 in Grinstein et al., 1984a). The intercepts of the two lines in the abscissa (1/K_m) are not significantly different.

that obtained earlier for isotonic acid-loaded cells by the fluorescence method (59 mM; see dotted line in Fig. 4B), or by the measurement of unidirectional Na^+ uptake (51 mM; Grinstein et al., 1984c).

For comparison, acid-loaded cells in hypertonic media were also studied to

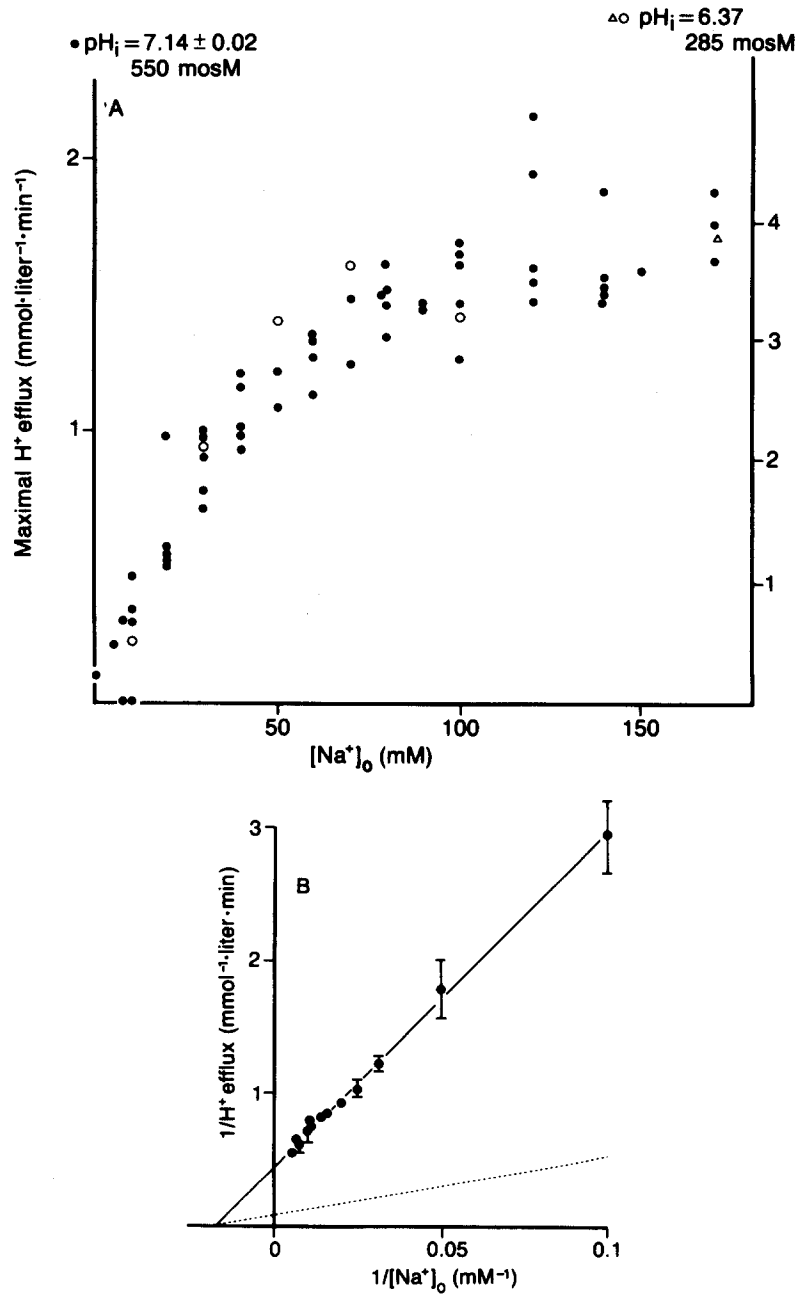


FIGURE 4.

rule out pH_i -induced differences. Four similar experiments were performed using acid-loaded cells (mean $\text{pH}_i = 6.5$) suspended in hyperosmotic (550 mosM) medium at varying $[\text{Na}^+]_o$ (not illustrated). The apparent affinity for Na^+ was not significantly different from that in iso-osmotic cells or in hyperosmotic cells at normal pH_i . In summary, a change in the K_m for external Na^+ cannot account for the osmotic activation of the antiport.

External pH (pH_o) Dependence

Na^+/H^+ exchange in a variety of cells, including thymocytes, is inhibited by H_o^+ (Rindler and Saier, 1981; Aronson, 1983; Aronson et al., 1983; Grinstein et al., 1984a), at least partly by competition with Na^+ for the externally facing binding site. Thus, a change in the inhibitory potency of H_o^+ could conceivably result in activation of forward (Na^+/H^+) exchange. This was investigated in the experiments of Fig. 5, where the maximal rate of H^+ efflux was measured as a function of pH_o in iso- and hyperosmotic cells at constant $[\text{Na}^+]_o$. The reported inhibitory effect of H_o^+ in acid-loaded cells ($\text{pH}_i = 6.33$) was confirmed using cells from the same batch used for osmotic activation (Fig. 5). Maximal exchange rates were found at pH_o 7.7–7.8 (Grinstein et al., 1984a; Fig. 5) and complete inhibition of net H^+ extrusion was obtained at ≈ 6.2 . As pointed out above, although the absolute rates of exchange in shrunken cells (550 mosM, $\text{pH}_i = 7.16 \pm 0.08$, $n = 3$) are lower, a valid comparison can be made if the data are normalized by using two ordinates (Fig. 5). The pattern obtained in three experiments is indistinguishable from that of iso-osmotic cells: saturation above pH_o 7.8 (not shown), approximate linearity below 7.6, and total suppression at ≈ 6.2 . In two experiments using acid-loaded cells (pH_i 6.2 and 6.45) in hyperosmotic medium (550 mosM), the results were virtually identical (not illustrated). In conclusion, a change in the inhibitory potency of H_o^+ is not involved in the osmotic activation of the Na^+/H^+ antiport.

Amiloride Sensitivity

In thymocytes (Grinstein et al., 1984a) as well as in other cells (Kinsella and Aronson, 1981; Paris and Pouyssegur, 1983), amiloride inhibits exchange by a competitive interaction with Na^+ on the external substrate site. We reasoned that a comparison of the inhibitory potency of the diuretic in normal and shrunken cells would further rule out substantive changes in the external cation- (and inhibitor-) binding site. A summary of these experiments is presented in Fig. 6. Using 70 mM Na_o^+ and $\text{pH}_o = 7.2$, the alkalizing response in hypertonic cells (550 mosM) with normal starting pH_i (7.14 ± 0.05 , $n = 3$) was inhibited by amiloride with an apparent K_i of 13.6 μM (solid circles). Under similar conditions, amiloride inhibited exchange in acid-loaded isotonic cells from the same batch with an apparent K_i of 7.35 μM (open circles), a lower value. Because the interaction between amiloride and Na^+ is competitive, the actual K_i can be calculated from the relationship

$$K_{i,\text{app}} = K_i(1 + [\text{Na}^+]_o/K_{m,\text{Na}^+})$$

where K_{m,Na^+} is the Michaelis constant for Na^+ (i.e., 59 mM). Using this equation,

we obtain a true K_i of $3.36 \mu\text{M}$ for isotonic cells, which is very similar to the value of $2.5 \mu\text{M}$ reported earlier (Grinstein et al., 1984a). The true K_i for shrunken cells is $6.24 \mu\text{M}$.

The reason for the increase in the apparent K_i in shrunken cells with normal

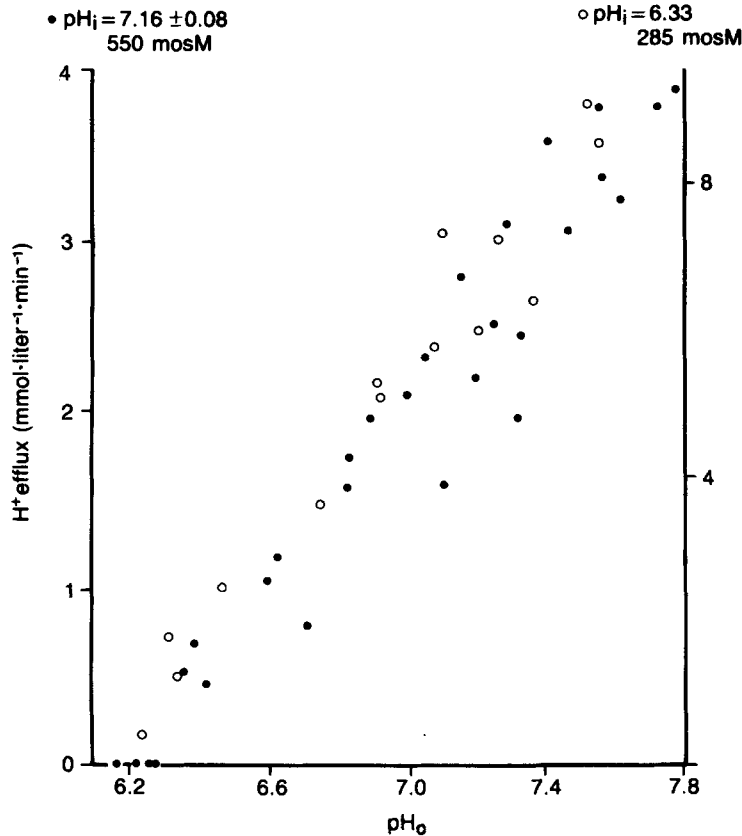


FIGURE 5. Comparison of the extracellular pH (pH_o) dependence of H^+ efflux in isotonic acid-loaded cells (285 mosM, $\text{pH}_i = 6.33$, $n = 2$; open circles) and hypertonically stressed cells (550 mosM, $\text{pH}_i = 7.16 \pm 0.08$, $n = 3$; solid circles). Maximal rates of H^+ extrusion were estimated from the rate of ΔpH_i , measured fluorimetrically. The medium pH was varied by changing the ratio of Tris to MES, while keeping their sum constant at 20 mM. The media contained 100 mM Na^+ and were osmotically balanced with *N*-methyl-D-glucamine $^+$. Data from three hypertonic experiments (left-hand ordinate) and two isotonic experiments (right-hand ordinate) are presented.

pH_i is not completely understood, but it may be related to the difference in pH_i (see Discussion). This is suggested by the finding that in two experiments using acid-loaded cells (pH_i 6.15 and 6.3) in hypertonic medium (550 mosM), the apparent K_i for inhibition was $9.8 \mu\text{M}$, which is lower than the values for cells with normal starting pH_i . The true K_i for these cells was $4.5 \mu\text{M}$.

pH_i Dependence

One of the most important determinants of the rate of Na⁺/H⁺ exchange is the internal pH (Aronson et al., 1982; Moolenaar et al., 1983; Grinstein et al., 1984a). It seems evident that the antiport has at least two separate types of H⁺-binding sites: the substrate and the modifier site(s) (Aronson et al., 1982; Grinstein et al., 1984c), both of which are affected by [H⁺]_i. Hence, this parameter plays an important role in the regulation of cation countertransport (see Discussion). For this reason, we compared the pH_i dependence of the rate of H⁺ extrusion in normal and osmotically shrunken cells. In these experiments, pH_o and [Na⁺]_o were maintained constant at 7.2 and 70 mM, respectively. Internal

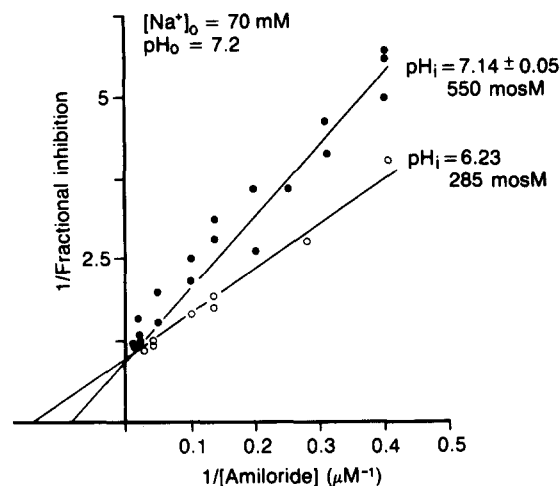


FIGURE 6. Comparison of the inhibitory effects of amiloride on H⁺ efflux in iso- and hyperosmotic cells. The concentration dependence of the inhibitory effect of amiloride was studied in isotonic, acid-loaded cells (285 mosM, pH_i = 6.23, *n* = 2; open circles) and in hypertonically stressed cells (550 mosM, pH_i = 7.14 ± 0.05, *n* = 3; solid circles). The rate of H⁺ extrusion was determined from ΔpH_i as above. Extracellular Na⁺ was constant at 70 mM. External pH (pH_o) was 7.2. Abscissa: inverse amiloride concentration. Ordinate: inverse fractional inhibition.

pH was pre-set using nigericin in isotonic *N*-methyl-D-glucamine⁺ solution while monitoring pH_i with BCECF. The ionophore was then scavenged with albumin, and the cells were sedimented and finally resuspended in the appropriate medium for the fluorimetric determination of H⁺ extrusion rates (see Methods for more details). The results of four experiments are summarized in Fig. 7. As reported earlier for thymocytes and other cells in isotonic media (Moolenaar et al., 1983; Grinstein et al., 1984a; Paris and Pouyssegur, 1984), the rate of Na⁺/H⁺ exchange is almost negligible at normal pH_i (≈7.1), but increases sharply as the cytoplasm is acidified, following an approximately linear relationship. In the case of hypertonic cells, the pH_i dependence is shifted to higher values. Transport is significant at pH_i ≥ 7.1 and becomes quiescent only at ~7.3, which is consistent

with the final pH_i attained after equilibration in hypertonic solution (e.g., Figs. 1A and 9). However, the form of the relationship is similar, with an approximately parallel linear portion. In the pH_i range studied, osmotic shrinking appears to cause a 0.2–0.3-unit alkaline shift of the pH_i dependence curve of the antiport, which accounts for the observed activation in hyperosmotic solutions.

The effect of shrinking appears to be to change the “set point” of exchange from $\text{pH}_i \approx 7.0$ to $\text{pH}_i \approx 7.3$, rather than to induce an alkalization per se relative to the isotonic level. This conclusion is supported by experiments in

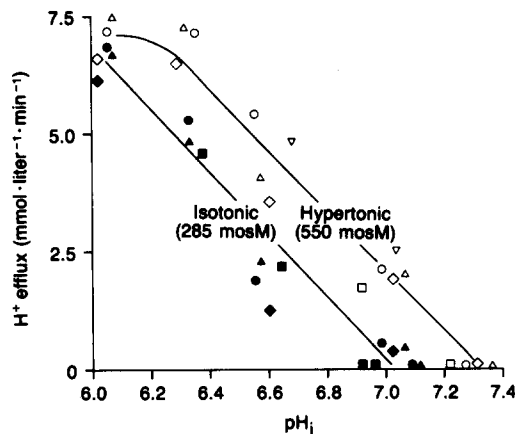


FIGURE 7. Comparison of the cytoplasmic pH (pH_i) dependence of the rate of H^+ efflux in isotonic and hypertonically treated cells. Cells stained with BCECF were acid-loaded to the pH_i levels indicated in the abscissa by incubation in *N*-methyl-D-glucamine⁺ solution with nigericin (0.2 $\mu\text{g}/\text{ml}$ final). Acid-loading was terminated by addition of albumin (5 mg/ml) followed by centrifugation. The cells were then resuspended into either isotonic (285 mosM; solid symbols) or hypertonic (550 mosM; open symbols) solution containing 70 mM Na^+ , pH_o 7.2, and osmotically balanced with *N*-methyl-D-glucamine⁺. H^+ extrusion rates were calculated as the product of the rate of ΔpH_i , measured over the first minute after resuspension in the Na^+ -containing medium, and the buffering power. Each type of symbol identifies an individual experiment. The lines were drawn by eye.

which the pH_i of isotonic cells was initially driven to 7.6 by incubation in K^+ solution of pH_o 7.6 in the presence of nigericin (0.3 $\mu\text{g}/\text{ml}$ final). The ionophore was then removed and the cells were suspended in hypertonic (550 mosM) Na^+ -containing (140 mM) solution at pH_o 7.2. Under these conditions, pH_i slowly drifted downward toward 7.3 (not illustrated), i.e., an acidification relative to the starting isotonic pH_i .

Time Course of Activation of Na^+/H^+ Exchange

As discussed above, the activation of Na^+/H^+ exchange upon osmotic shrinking of thymocytes is detectable only after a delay of 20–30 s (see Fig. 2). The delay is detectable when H^+ extrusion is measured either as changes in intracellular or

extracellular pH (Fig. 2, A and B), which would rule out the possibility that activation of exchange is instantaneous, and that the buffering power of the cell is not constant over this pH_i range. An experiment designed to define more clearly the nature of the time dependence is illustrated in Fig. 8. Cells were either challenged with hypertonic solution in the presence of Na^+ or, alternatively, challenged osmotically in the absence of Na^+ , followed after 5 min by addition of Na^+ to the medium (while keeping the hypertonicity constant). As shown in Fig. 8, the lag time observed in the former case is not observed when the cells are pre-incubated in hypertonic solution before the addition of Na^+ . Thus, extracellular Na^+ is not required for the activating process. Presumably, activation was fully developed after 5 min, when transport was initiated by addition of extracellular substrate. The time displacement of the curves in Fig. 8 is just under 60 s. Thus, the activation process is reflected in a measurable flux increase in 20–30 s and is maximal in ~ 60 s. In addition, experiments like that of Fig. 8 allow the calculation of the true maximal rates of exchange in osmotically

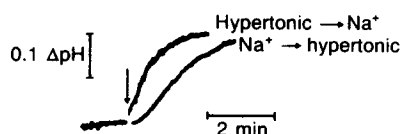


FIGURE 8. Time course of the osmotically induced cytoplasmic alkalization. Lower trace: BCECF-loaded cells were suspended in isotonic Na^+ solution and, where indicated by the arrow, the medium was made hypertonic (550 mosM) by addition of *N*-methyl-D-glucamine chloride. Upper trace: cells were suspended in hypertonic (550 mosM) *N*-methyl-D-glucamine⁺ (Na^+ -free) solution. Where indicated by the arrow, the cells were transferred to an equally hypertonic solution containing 140 mM Na^+ . A composite trace, representative of at least four experiments, is illustrated.

activated cells. In three similar experiments using 140 mM Na^+ , 550 mosM, and pH_o 7.2, the maximal rate of Na^+ -induced alkalization averaged 2.67 ± 0.16 mmol H^+ · liter⁻¹ · min⁻¹.

An increased Na^+ uptake rate of cells shrunken before the addition of Na^+ was previously reported for *Amphiuma* red cells by Siebens and Kregenow (1982). These authors reported a similar increase when the cells were shrunken in the presence of amiloride, followed by initiation of the flux upon removal of the inhibitor.

Reversibility of the Osmotic Activation

Experiments like the one illustrated in Fig. 9 were performed to investigate whether the osmotically induced stimulation of Na^+/H^+ exchange is reversible. Briefly, cells were activated by increasing the osmolarity from 285 to 550 mosM, keeping $[\text{Na}^+]_o$ constant at 140 mM. After a new steady pH_i was obtained, the cells were sedimented (indicated by the dotted line in Fig. 9) and resuspended in normal Na^+ solution (285 mosM). Restoration of the normal osmolarity led to a gradual decline of pH_i toward the original level. The half-time for pH_i recovery was typically 6–7 min.

The mechanism responsible for the recovery of the original pH_i is presently unclear, but leakage of H^+ down its electrochemical gradient and generation of metabolic H^+ are likely possibilities. A role for the Na^+/H^+ exchanger can be ruled out on thermodynamic grounds, since the combined Na^+ and H^+ gradients would drive the antiport forward (i.e., exchanging Na_o^+ for H_i^+) and because recovery is amiloride-insensitive.

Role of Ca^{2+} in the Activation Process

It has been suggested that activation of the antiport by serum factors in other cell types (Owen and Villereal, 1982a, b) is mediated by an increase in $[\text{Ca}^{2+}]_i$. We performed a number of experiments to determine whether $[\text{Ca}^{2+}]_i$ is altered during osmotic shrinking and whether the observed changes play a role in the activation of Na^+/H^+ countertransport. $[\text{Ca}^{2+}]_i$ was determined using the fluorescent indicator quin-2 (Tsien et al., 1982). Typical results are shown in Fig. 10A. The resting $[\text{Ca}^{2+}]_i$ in thymocytes suspended in isotonic Na^+ -containing solutions was 107 ± 5.4 nM ($n = 9$), in agreement with earlier determinations in

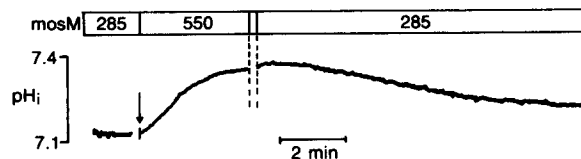


FIGURE 9. Reversibility of the osmotically induced cytoplasmic alkalization. BCECF-loaded cells were suspended in isotonic Na^+ solution. Where indicated by the arrow, the medium was made hypertonic (550 mosM) by addition of concentrated *N*-methyl-D-glucamine chloride. After several minutes, the cells were sedimented (dotted lines) and resuspended in isotonic Na^+ solution. The osmolarity of the medium in the different stages is indicated. Representative of three similar experiments.

lymphocytes from different sources and species (Tsien et al., 1982; Rink et al., 1983; Hesketh et al., 1983). After hypertonic stress (550 mosM at 140 mM Na^+) and a delay of almost 1 min, $[\text{Ca}^{2+}]_i$ gradually increased, reaching a maximum after 6–8 min. In eight experiments, the maximum $[\text{Ca}^{2+}]_i$ averaged 214 ± 26 nM. A further increase was observed upon addition of ionomycin, a nonfluorescent Ca^{2+} ionophore.

Also shown in Fig. 10A is the external Ca^{2+} dependence of the osmotically induced change in $[\text{Ca}^{2+}]_i$. As reported earlier for thymocytes from other species (Tsien et al., 1982), $[\text{Ca}^{2+}]_i$ gradually falls when the cells are suspended in nominally Ca^{2+} -free medium. The imposition of a hypertonic stress under these conditions failed to increase $[\text{Ca}^{2+}]_i$ significantly, and addition of ionomycin produced only a small and transitory increase, which is indicative of release from intracellular stores. The subsequent addition of extracellular Ca^{2+} resulted in the expected rapid increase of $[\text{Ca}^{2+}]_i$, because of the prior incorporation of the ionophore into the plasma membrane. These data suggest that osmotic shrinking results in increased Ca^{2+} influx.

The absence of an increase in $[\text{Ca}^{2+}]_i$ in cells shrunken in nominally Ca^{2+} -free

medium enabled us to test whether changes in the concentration of this divalent cation are essential for the activation of Na^+/H^+ exchange. For this purpose, pH_i was measured in BCECF-loaded cells that were osmotically shrunken in nominally

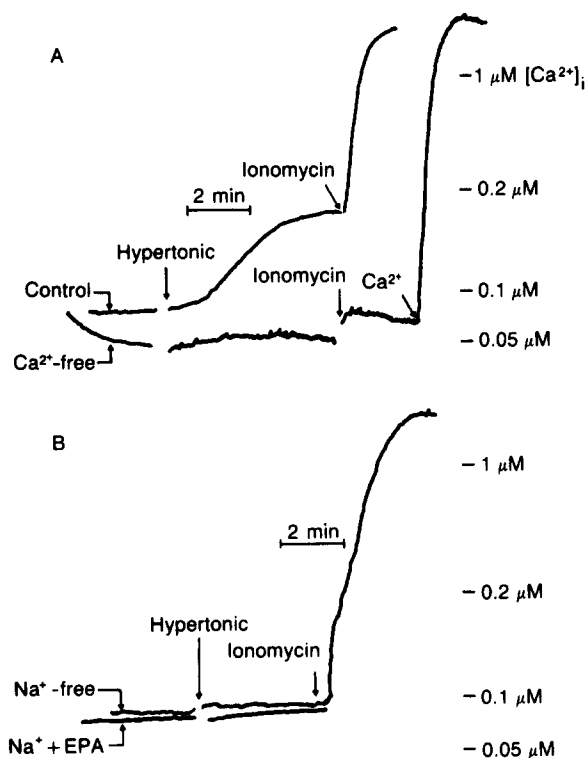


FIGURE 10. Changes in cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) after osmotic shrinking of thymocytes. Cells were loaded with the fluorescent $[\text{Ca}^{2+}]_i$ indicator quin-2 by incubation with the parent acetoxymethyl ester. (A) Top trace: cells were suspended in Ca^{2+} -containing Na^+ solution; where indicated, the medium was made hypertonic (550 mosM) by addition of *N*-methyl-D-glucamine chloride. Finally, $0.5 \mu\text{M}$ ionomycin was added. Lower trace: cells were suspended in nominally Ca^{2+} -free Na^+ solution; where indicated, the medium was made hypertonic as above and then ionomycin ($0.5 \mu\text{M}$) was added. Finally, 1 mM Ca^{2+} was added as CaCl_2 . (B) Upper trace: cells were suspended in Na^+ -free *N*-methyl-D-glucamine $^+$ solution (with Ca^{2+}); where indicated, the medium was made hypertonic with additional *N*-methyl-D-glucamine chloride. Finally, ionomycin ($0.5 \mu\text{M}$) was added. Lower trace: cells were suspended in Na^+ solution (with Ca^{2+}) containing $1 \mu\text{M}$ of 5-*N*-ethyl-*N*-propyl amiloride (EPA); where indicated, the medium was made hypertonic as above. In all cases, calibration was made by determining autofluorescence after quenching of quin-2 with 2 mM Mn^{2+} . Representative of three similar experiments.

Ca^{2+} -free solution. As shown in Fig. 11, a cytoplasmic alkalization was observed that was similar in magnitude and time course to that observed in Ca^{2+} -containing medium (compare with Figs. 2A and 8). These results indicate that the osmotic activation of the antiport is not mediated by changes in $[\text{Ca}^{2+}]_i$.

Fig. 10B presents evidence indicating that the presence $[\text{Ca}^{2+}]_i$ is secondary to the activation of Na^+/H^+ exchange. This conclusion was reached by studying the effects of osmotic stress under conditions where forward Na^+/H^+ exchange was prevented by means of ion substitution or by use of inhibitors. In the upper trace, cells were suspended in Na^+ -free *N*-methyl-D-glucamine⁺ solution (with Ca^{2+}). Where indicated, the solution was made hypertonic (550 mosM) by addition of extra *N*-methyl-D-glucamine chloride. No significant change in $[\text{Ca}^{2+}]_i$ was noted for several minutes. It was demonstrated with ionomycin that the fluorescent probe is still responsive to Ca^{2+} under these conditions. The ionophore produced a rapid and extensive change in fluorescence (Fig. 10B). The bottom trace in Fig. 10B illustrates the effects of 5-*N*-ethyl-*N*-propyl amiloride ($1 \mu\text{M}$)¹ on the osmotically induced changes of $[\text{Ca}^{2+}]_i$. This blocker of the Na^+/H^+ antiport was used because, in contrast to amiloride, the concentrations required for complete inhibition of exchange do not interfere with quin-2 fluorescence recording. The cells were suspended in isotonic Na^+ solution (with Ca^{2+}) containing the inhibitor and, where indicated, the medium was made

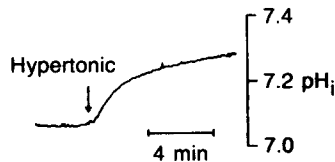


FIGURE 11. Volume-induced activation of Na^+/H^+ exchange in Ca^{2+} -free medium. BCECF-loaded thymocytes were suspended in nominally Ca^{2+} -free isotonic Na^+ solution. Where indicated by the arrow, the medium was made hypertonic (550 mosM) by addition of concentrated *N*-methyl-D-glucamine chloride. The pH_i values were calibrated as described for Fig. 2A. Representative of three similar experiments.

hypertonic with *N*-methyl-D-glucamine chloride. Under these conditions, the osmotic challenge failed to increase $[\text{Ca}^{2+}]_i$. As before, addition of ionomycin rapidly increased $[\text{Ca}^{2+}]_i$ (not illustrated). Taken together, these results indicate that the presence of Ca_o^{2+} and a functioning Na^+/H^+ antiport are required for the observed increase in $[\text{Ca}^{2+}]_i$, and that the increased $[\text{Ca}^{2+}]_i$ is not a factor in the activation of the antiport (see Discussion).

DISCUSSION

Mechanism of Osmotic Activation

Acidification of the cytoplasm in thymocytes results in activation of the Na^+/H^+ antiport. However, activation by osmotic shrinking does not involve cytoplasmic acidification (Fig. 2). In fact, in the presence of Na_o^+ , the pH_i becomes more alkaline. Therefore, an analysis of the kinetic properties of the exchanger in

¹ The dose dependence of the inhibitory effect of 5-*N*-ethyl-*N*-propyl amiloride was determined in independent experiments. Cells were acid-loaded with nigericin in *N*-methyl-D-glucamine⁺ solution and then Na^+/H^+ exchange was detected as the Na^+ (70 mM)-induced alkalization. In three experiments, the apparent K_i averaged 35 nM. Thus, complete inhibition is expected at $1 \mu\text{M}$.

shrunk cells was undertaken to define the mechanism of activation. A comparison of iso-osmotic (acid-loaded) and osmotically activated cells (with either normal or acidic pH_i) indicated that the apparent affinities of the antiport for extracellular Na^+ and H^+ are not significantly affected upon shrinking (Figs. 4 and 5). Therefore, the osmotic activation of countertransport cannot be accounted for by a change in these parameters. Indeed, because the apparent K_m for Na_o^+ in iso-osmotic cells is ~ 51 mM (Grinstein et al., 1984c), it is theoretically unlikely that osmotic activation in Na^+ solution (which contains 140 mM Na^+) is due to an increased affinity for this cation.

Amiloride is thought to inhibit exchange by competitively interacting with the extracellular transport site of the antiport (Kinsella and Aronson, 1981; Paris and Pouyssegur, 1983; Grinstein et al., 1984a). Even though the properties of this site, judged by its interaction with cations, were not noticeably affected upon cell shrinking, the affinity for amiloride seemed to be somewhat lower (Fig. 6). However, parallel experiments using shrunk acid-loaded cells suggested that this decrease may reflect a difference in the experimental conditions used (i.e., the internal pH), rather than a property of the antiport. Two explanations can be envisaged: because in Fig. 6 the pH_i was lower in iso-osmotic cells (a necessary condition for measurements of exchange), it is conceivable that they contained a higher intracellular concentration of amiloride, a permeant weak base (Benos, 1982). Although inhibition has been shown to occur externally (it is competitive with Na_o^+ ; Kinsella and Aronson, 1981; Paris and Pouyssegur, 1983; Grinstein et al., 1984a), binding from the cytoplasmic side has not been ruled out. Alternatively, rapid diffusion of internal H^+ from acid-loaded cells might have reduced the local pH in the external surface, thereby increasing the fraction of amiloride in the biologically active protonated form (Benos, 1982). The small apparent change in amiloride affinity affords no explanation of the osmotic activation of the exchanger.

Data obtained in isolated membrane vesicles (Aronson et al., 1982; Aronson, 1983), as well as in intact cells (Moolenaar et al., 1983; Grinstein et al., 1984a, c; Paris and Pouyssegur, 1984), indicate that, under physiological conditions, pH_i is the primary determinant of the rate of Na^+/H^+ exchange. Several findings indicate that the control by pH_i is exerted allosterically. (a) The pH_i dependence of transport is markedly sigmoidal, showing a higher than first-order relationship, which is unexpected for a one-to-one exchanger that displays Michaelian behavior with respect to extracellular substrate (Aronson et al., 1982; Grinstein et al., 1984a, c). (b) Increasing $[\text{H}^+]_i$ failed to inhibit Na_i^+ efflux (Aronson et al., 1982; Grinstein et al., 1984c), as expected from a purely competitive interaction at the transport site. In fact, a stimulation was reported in the case of renal brush border vesicles (Aronson et al., 1982). (c) The antiport in intact iso-osmotic cells at normal pH_i is virtually quiescent (Moolenaar et al., 1983, 1984; Grinstein et al., 1984a, c; Paris and Pouyssegur, 1984), even though, under these conditions, Na^+ and H^+ are not at thermodynamic equilibrium (Grinstein et al., 1984a, c). Thus, the antiporter is virtually inactive despite the presence of a substantial driving force. A marked activation is obtained by lowering pH_i .

The above observations are compatible with the existence of one or more inwardly facing titratable groups that bind H_i^+ and thereby increase transport

activity (Aronson et al., 1982; Aronson, 1983). These groups constitute the internal "modifier" site (Aronson et al., 1982).

As shown in Fig. 8, the mechanism underlying the osmotic activation of Na⁺/H⁺ exchange in thymocytes seems to be an alkaline shift in the pH_i dependence of the antiport. This probably reflects an altered behavior of the "modifier" site, inasmuch as this site largely determines the pH_i sensitivity of the exchanger. According to this model, the "set point" of the "modifier," which normally prevents transport at pH_i ≥ 7.1, is adjusted upward. As a result, the nearly quiescent exchanger is activated, but the activation persists only until pH_i attains a value of ~7.3, the new "set point." It is noteworthy that even at this elevated pH_i, a one-to-one electroneutral exchange is not at electrochemical equilibrium (calculated to occur at pH_i ≈ 8.2, given the intra- and extracellular Na⁺ concentrations and a pH_o of 7.2), which indicates that the allosteric control of transport has not been lost but has only shifted to a more alkaline level.

In some tissues, activation of the antiport can also be accomplished in isotonic cells at normal pH_i by treatment with serum (Owen and Villereal, 1982*a, b*; Moolenaar et al., 1983), defined growth factors (Burns and Rozengurt, 1983; Rothenberg et al., 1983; L'Allemain et al., 1981), or phorbol esters (Burns and Rozengurt, 1983; Besterman and Cuatrecasas, 1984; Grinstein et al., 1985). In fibroblasts, fetal calf serum (Moolenaar et al., 1983) or a mixture of alpha-thrombin and insulin (Paris and Pouyssegur, 1984) stimulated exchange by ostensibly increasing the pH_i sensitivity of the antiport. In the case of lymphocytes activated by 12-*O*-tetradecanoylphorbol-13-acetate, the pH_i activation curve is displaced to the alkaline side (Grinstein et al., 1985) in a manner very similar to that found in osmotically shrunken cells (Fig. 7). The primary, and perhaps the sole, receptor for this phorbol ester is thought to be the Ca²⁺- and phospholipid-dependent protein kinase C (Nishizuka, 1984). Similarly, the receptors for insulin and other growth factors are reported to display protein (tyrosine) kinase activity upon ligand binding (Rees-Jones et al., 1984). This has prompted the speculation that the increased pH_i sensitivity, i.e., the shift in the "set point" of the "modifier," is brought about by phosphorylation of the exchanger or of an ancillary protein (Grinstein et al., 1985). By analogy, it is conceivable that protein kinases are also stimulated during osmotic challenge, mediating a similar activation of Na⁺/H⁺ exchange. Indeed, preliminary experiments indicate that phosphorylation of thymocyte proteins is increased upon osmotic shrinking (Goetz, J. D., unpublished observations).

Control through a chemical process such as phosphorylation is also consistent with the time course of activation. After shrinkage, there is a delay of ~20–30 s before activation of exchange is detectable (Figs. 2 and 8). A similar delay was reported for activation by phorbol esters (Grinstein et al., 1985) and by growth factors (Moolenaar et al., 1983; Rothenberg et al., 1983). This delay could be attributed to the time required for stimulation of the kinase and phosphorylation of the exchanger.

Role of Ca²⁺ in the Activation of Na⁺/H⁺ Exchange

Owen and Villereal (1982*a, b*) have suggested that activation of the exchanger by serum in fibroblasts is mediated by an increase in [Ca²⁺]_i and the formation

of Ca^{2+} -calmodulin complexes. This conclusion was based on the following observations: (a) the divalent cation ionophore A23187 can mimic the effects of serum on amiloride-sensitive Na^+ uptake (Villereal, 1981); (b) the intracellular Ca^{2+} antagonist TMB-8 inhibits serum-induced but not A23187-induced Na^+ influx (Owen and Villereal, 1982b); (c) a number of psychoactive drugs that bind to calmodulin inhibit both serum- and A23187-stimulated Na^+ uptake (Owen and Villereal, 1982a).

We used the fluorescent indicator quin-2 to determine whether an increase in $[\text{Ca}^{2+}]_i$ also mediates the osmotic activation of the antiport. $[\text{Ca}^{2+}]_i$ was found to increase significantly after shrinking (Fig. 10), and the effect was dependent on the presence of extracellular Ca^{2+} . However, two lines of evidence indicate that the elevated $[\text{Ca}^{2+}]_i$ is not causally related to the activation of Na^+/H^+ exchange. First, the monovalent cation exchanger appears to be activated before any changes in $[\text{Ca}^{2+}]_i$ are measurable (compare Figs. 2 and 10).² Second, the omission of extracellular Ca^{2+} , which prevented the elevation of $[\text{Ca}^{2+}]_i$, failed to inhibit the activation of the Na^+/H^+ antiport (Fig. 11). Indeed, it is likely that the increased uptake of Ca^{2+} is a consequence, rather than the cause, of the activation of Na^+/H^+ exchange. This is suggested by the finding that inhibition of $\text{Na}_o^+/\text{H}_i^+$ countertransport either by omission of Na_o^+ or by 5-*N*-ethyl-*N*-propyl amiloride precluded the rise in $[\text{Ca}^{2+}]_i$ (Fig. 10B). The fact that the change in $[\text{Ca}^{2+}]_i$ is secondary to the stimulation of the antiport is also consistent with the somewhat slower kinetics of the former.

Activation of $^+/\text{H}^+$ Exchange and RVI

What is the physiological purpose of the activation of Na^+/H^+ exchange in osmotically shrunken cells? As discussed in detail by Cala (1980, 1983), this mechanism could underlie RVI. When regulatory processes are prevented, thymocyte volumes follow the van't Hoff relationship, which suggests that they are in osmotic equilibrium with the medium. Therefore, reswelling during RVI is indicative of an increased number of osmotically active particles in the cytoplasm. In the absence of HCO_3^- , cellular swelling could result from activation of exchange because the protons extruded by the antiport are replaced from the intracellular buffers, resulting in a net osmotic (Na^+) gain. In the presence of HCO_3^- , the eventual alkalization produces accumulation of cytoplasmic HCO_3^- , which drives Cl^- into the cell through the anion exchange system. This is tantamount to increasing the dynamic buffering power of the cells.

The potential significance of the activation of the antiport for RVI can be calculated in the case of thymocytes, where the magnitude of the ΔpH_i and the buffering power are known. In nominally HCO_3^- -free solutions, the buffering power has been determined to be $\approx 25 \text{ mmol} \cdot \text{liter}^{-1} \cdot \text{pH}^{-1}$ (Grinstein et al., 1984a). Therefore, $\sim 6.5 \text{ mmol Na}^+/\text{liter}$ cells must be taken up to produce a ΔpH of 0.26 units (the observed shift of the "set point"). This would produce only an insignificant swelling ($< 2\%$ of the cell volume). In fact, reswelling of

² The $[\text{Ca}^{2+}]_i$ response may have been spuriously slowed by the Ca^{2+} -buffering properties of the indicator quin-2.

lymphocytes in the absence of HCO₃⁻ is minimal (Grinstein et al., 1983). In the presence of extracellular HCO₃⁻, a further osmotic gain is expected. For a medium with 20 mM HCO₃⁻ and pH_o 7.4, assuming that P_{CO₂} and the solubility of CO₂ are equal on both sides of the membrane, a ΔpH_i of 0.26 would increase the internal HCO₃⁻ concentration by ~8 mmol/liter cells, which would account only for a small fraction of the RVI.

With the data available at present, the contribution of Cl⁻ to the reswelling can only be approximated, with variations depending on the assumptions made. Maximal effects are expected if the distribution of Cl⁻ is determined mainly by an electroneutral anion exchanger, so that Cl_i⁻/Cl_o⁻ = HCO_{3i}⁻/HCO_{3o}⁻ at all times.³ For the conditions listed above (pH_o = 7.4, ΔpH_i = 0.26, and starting pH_i = 7.1) and with [Cl⁻]_o = 140 mM, a change of up to 50 mmol/liter cells can be expected. When combined, the net gains of Na⁺, HCO₃⁻, and Cl⁻ (in excess of 60 mmol/liter) and the osmotically obliged water should result in a significant volume increase. However, incomplete reswelling would be expected for severely shrunken cells (e.g., at 550 mosM), which is consistent with reported observations in peripheral blood lymphocytes (Grinstein et al., 1983). In addition, the above model would partially explain why reswelling is more extensive in cells that have undergone a cycle of regulatory volume decrease (RVD) in hypotonic medium, followed by shrinking upon re-introduction into iso-osmotic solution (Hoffmann et al., 1983; Grinstein et al., 1983). The net osmotic gain is determined by the ΔpH_i and the extracellular Cl⁻ and HCO₃⁻ concentrations, but is independent of the initial osmotic content of the cells. If the former parameters and the buffering power remain constant while the osmotic content of the cells is reduced during RVD, the relative swelling will be significantly higher, as observed experimentally.

As shown in Fig. 3, the rate of change of pH_i is related to the magnitude of the imposed osmotic stress. Because of technical limitations, we were unable to determine the precise relationship between medium osmolarity and the final pH_i, particularly when moderate hypertonicity was used, in which case equilibration is very slow. However, measurements at higher osmolarities showed that the maximal ΔpH_i was practically constant between 360 and 550 mosM (not illustrated). This implies that the extent of reswelling is not proportional to the extent of shrinking, which is a monotonic function of the osmolarity (e.g., Fig. 1B). If this is the case, the Na⁺/H⁺ exchanger, although activated by osmotic shrinking, would be inadequate for maintaining volume homeostasis.

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³ The contribution of Cl⁻ to reswelling will vary with the relative influence of Cl⁻/HCO₃⁻ exchange and Cl⁻ conductance to the distribution of Cl⁻. Smaller effects are expected if the latter predominates (assuming that the membrane potential remains constant during RVI, as reported: Cala, 1980; Grinstein et al., 1983; Parker and Castranova, 1984).

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