

Activation of Na^+/H^+ Exchange in Lymphocytes by Osmotically Induced Volume Changes and by Cytoplasmic Acidification

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ABSTRACT After swelling in hypotonic solutions, peripheral blood mononuclear cells (PBM) shrink toward their original volumes. Upon restoration of isotonicity, the cells initially shrink but then regain near-normal size again. This regulatory volume increase (RVI) is abolished by removal of Na_o^+ or Cl_o^- or by addition of amiloride. RVI is unaffected by removal of K_o^+ or by ouabain and is only partially inhibited by 1 mM furosemide. As a result of increased influx, the cells gain both Na^+ and K^+ during reswelling. In contrast, only Na^+ content increases in the presence of ouabain. Amiloride largely eliminates the changes in the content of both cations. Using diS-C₃(5), no significant membrane potential changes were detected during RVI, which suggests that the fluxes are electroneutral. The cytoplasmic pH of volume-static cells was measured with 5,6-dicarboxyfluorescein. After acid loading, the addition of extracellular Na^+ induced an amiloride-inhibitable alkalization, which is consistent with Na^+/H^+ exchange. Cytoplasmic pH was not affected by cell shrinkage itself, but an internal alkalization, which was also amiloride sensitive and Na^+ dependent, developed during reswelling. In isotonic lightly buffered solutions without HCO_3^- , an amiloride-sensitive acidification of the medium was measurable when Na^+ was added to shrunken PBM. K^+ was unable to mimic this effect. The observations are compatible with the model proposed by Cala (*J. Gen. Physiol.* 1980. 76:683–708), whereby an electroneutral $\text{Na}_o^+/\text{H}_i^+$ exchange is activated by osmotic shrinking. Cellular volume gain occurs as Cl_o^- simultaneously exchanges for either HCO_3^- or OH_i^- . Na_i^+ is secondarily replaced by K^+ through the pump, but this step is not essential for RVI.

INTRODUCTION

Many cell types regulate their volumes in anisotonic media (MacKnight and Leaf, 1977; Kregenow, 1981; Cala, 1983). When suspended in hypotonic solutions, peripheral blood mononuclear cells (PBM) rapidly swell as water

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enters the cytoplasm, but this initial phase is immediately followed by a regulatory volume decrease (RVD), whereby the cells return to near-normal size within minutes (Ben-Sasson et al., 1975; Bui and Wiley, 1981; Cheung et al., 1982; Deutsch et al., 1982). This regulatory shrinking is largely attributable to a loss of KCl and osmotically obliged water. The efflux of K^+ and Cl^- occurs as a result of independent increases in their permeabilities, which return to basal levels when RVD is terminated (Grinstein et al., 1982a, c).

Some cell types can also regulate their volumes after being shrunk in hypertonic solutions. Nucleated avian and amphibian red cells swell back to their original size within hours (Kregenow, 1974; Schmidt and McManus, 1977; Cala, 1980), whereas a few minutes suffices in the case of epithelial cells (Spring and Ericson, 1982). Two types of mechanisms have been proposed, based on the ionic requirements and inhibitor sensitivity of the regulatory volume increase (RVI). A K^+ - Na^+ - Cl^- co-transport system has been found to operate in avian erythrocytes (see Kregenow, 1981, for review). This mechanism functions only when all three electrolytes are present in the medium and is inhibited by furosemide and other 3-amino-benzoic acid derivatives. In contrast, RVI in *Amphiuma* red cells and in *Necturus* gallbladder cells is inhibited by amiloride (Siebens and Kregenow, 1978; Ericson and Spring, 1982), and, at least in the latter case, bumetanide has no significant effect. In these systems, a countertransport of Na^+/H^+ is thought to be activated upon shrinking. Volume gain occurs as a result of this mechanism and the parallel exchange of extracellular Cl^- for internal OH^- or HCO_3^- .

Under the conditions studied to date, lymphoid cells shrunk in hypertonic media fail to regain their original volumes (Roti-Roti and Rothstein, 1973; Hempling et al., 1978). However, cultured lymphoblasts pretreated hypotonically display a response resembling RVI after isotonicity is restored. The hypotonically equilibrated cells, which have undergone RVD, shrink rapidly when transferred to iso-osmotic solutions, but then reswell toward their original volumes (Roti-Roti and Rothstein, 1973). A similar response has been reported recently in Ehrlich ascites cells (Hoffman et al., 1981, 1983), which are also unresponsive when exposed directly to hypertonic media.

As discussed elsewhere (Grinstein et al., 1982b), PBM are a convenient model for studies of volume regulation in nontransformed mammalian cells, as they are readily available and are amenable to electronic sizing. The present communication describes the behavior of PBM when shrunk directly in hypertonic media or after restoration of isotonicity after RVD. The ionic basis of the observed volume changes was investigated by means of ion substitution experiments and determinations of ion fluxes, pH, and membrane potential. Inhibitors were used to establish analogies with the systems described in the literature.

MATERIALS AND METHODS

Nigericin was purchased from Calbiochem-Behring Corp., San Diego, CA. Monensin, gramicidin, quinine, and ouabain were from Sigma Chemical Co., St. Louis, MO.

Amiloride was the gift of Merck Sharp & Dohme, Montreal, Canada. Furosemide was a gift from Hoechst Canada Inc., Montreal. Trifluoperazine was a gift from Smith, Kline and French, Canada Ltd., Toronto. 5,6-Dicarboxyfluorescein acetoxymethylester was the kind gift of Dr. T. J. Rink, University of Cambridge. 3,3'-Dipropylthiadicarbocyanine [diS-C₃-(5)] was the kind gift of Dr. A. Waggoner, Amherst College. RPMI 1640, fetal bovine serum, and trypan blue were from Grand Island Biological Co., Grand Island, NY. ³H₂O, [¹⁴C]- and [³H]polyethylene glycol, and Aquasol II were from New England Nuclear, Boston, MA. ⁸⁶Rb and ²⁴Na⁺ were from Amersham Corp., Arlington Heights, IL.

The standard isotonic Na⁺ solution contained: 103 mM NaCl, 5.4 mM KCl, 23.8 mM NaHCO₃, 5.6 mM Na₂HPO₄, 0.4 mM MgSO₄, 0.04 mM CaCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.2 at 37°C). In some experiments, K⁺ or Ca²⁺ was replaced by equimolar Na⁺. In Na⁺-free choline solution, choline chloride and choline bicarbonate replaced NaCl and NaHCO₃. In Na⁺-free K⁺ solution, K⁺ salts were used as substitute for Na⁺. Cl⁻-free solutions were prepared by replacement with gluconate, nitrate, isethionate, or SO₄²⁻. HCO₃⁻-free medium was made by replacement with Cl⁻. Unless otherwise indicated, hypotonic medium refers to 50%-dilute RPMI 1640.

Cell Volume and Viability Determinations

Human PBM were isolated on Ficoll-Isopaque as described previously (Cheung et al., 1982) and resuspended at ~10⁷ cells/ml in RPMI 1640 containing 10% serum. Aliquots of this suspension were used for all the determinations described below. Cells were sized in a Coulter Counter (model ZB1 or ZM) adapted with a Coulter Channelyzer (all from Coulter Electronics Inc., Hialeah, FL). In some experiments, cell volume was estimated from the water content, determined by isotope dilution using ³H₂O and [¹⁴C]polyethylene glycol as described (Grinstein et al., 1982c). Viability was determined by trypan blue exclusion.

Protocol for RVI Experiments

Although the cell numbers varied for the different determinations (i.e., smaller numbers for fluorescence or isotopic determinations than for ion content or medium pH measurements), similar protocols were used in all cases to induce RVI. Typically, cells suspended in RPMI 1640 were hypototically stressed by addition of 1 vol of distilled water. The cells were then allowed to regulate their volumes, with monitoring at regular intervals by electronic sizing. After 20 min, the cells were sedimented at 200 *g* for 10 min and resuspended in a small volume (usually 50 μl) of hypotonic medium. They were then mixed into a relatively large volume (10 ml) of isotonic solution to induce osmotic shrinking. In some experiments, the cells were resuspended in hypotonic medium and isotonicity was subsequently restored by addition of a small volume of 2 M NaCl or of sixfold-concentrated Na⁺ solution. Unless otherwise indicated, all the measurements were carried out at 37°C.

Ion Flux and Content Determinations

Uptake of ⁸⁶Rb (20–30 μCi/ml) or ²⁴Na (30–50 μCi/ml) was measured by resuspending 10–15 × 10⁶ PBM/ml of the appropriate buffer containing the radioactive cation plus [³H]polyethylene glycol. The mixtures were incubated at 37°C for the indicated time, at which point 50-μl aliquots were diluted into 1 ml of nonradioactive buffer, sedimented through oil, and counted as reported (Grinstein et al., 1982c).

Intracellular contents of Na⁺ and K⁺ were determined by flame photometry (model

443 Photometer; Instrumentation Laboratory, Inc., Lexington, MA), using Li^+ as the internal standard essentially as described previously (Cheung et al., 1982). Trapped extracellular space was measured with [^3H]polyethylene glycol.

Membrane Potential and pH Determinations

The membrane potential was estimated fluorimetrically as described (Grinstein et al., 1982a) using $0.5 \mu\text{M}$ diS-C₅(5). Intracellular pH was measured with 5,6-dicarboxyfluorescein in a spectrofluorimeter (model 650-40; Perkin-Elmer Corp., Japan) using 5-nm slits and recorded on a y vs. t flatbed plotter (model 2210; LKB Bromma, Sweden). Excitation and emission wavelengths were 495 and 525 nm, respectively. The cells ($2\text{--}3 \times 10^7/\text{ml}$) were loaded with the dye by incubation with $1 \mu\text{M}$ of the permeant 5,6-dicarboxyfluorescein acetoxymethyl ester in RPMI 1640 for 45 min at 37°C . The poorly permeant 5,6-dicarboxyfluorescein moiety is formed inside the cells upon hydrolysis of the parent compound by cytoplasmic esterases (Rink et al., 1982). The loaded cells were washed and treated as indicated in the text. Immediately before each measurement, the cells were sedimented to remove any extracellular dye. Each determination was followed by disruption of the cells with Triton X-100 and calibration of the fluorescent signal. A correction to offset the red shift of fluorescence of the dye inside intact cells was applied to all the measurements. The correction factor was calculated by determining the fluorescence of cells before and after lysis under conditions where the intracellular and external pH are identical (i.e., in high- K^+ media containing $1 \mu\text{g}/\text{ml}$ nigericin; see Thomas et al., 1979).

The intracellular buffering capacity was calculated as $\Delta[\text{NH}_4^+]_i/\Delta\text{pH}_i$. The pH of hypotonically pretreated cells was measured in isotonic media containing amiloride before and immediately after the addition of 5 mM NH_4^+ to the bathing solution. The internal NH_4^+ concentration was calculated using a pK of 9.21 and assuming that NH_3 is in equilibrium across the membrane. Penetration of the protonated base was corrected for by back-extrapolation of the slow ΔpH relaxation that occurs after addition of NH_4^+ (Roos and Boron, 1981).

Measurements of ΔpH with 9-amino acridine were performed fluorimetrically using excitation and emission wavelengths of 400 and 450 nm, respectively. Typically, $1 \mu\text{M}$ of the dye and $2\text{--}3 \times 10^6$ cells/ml were used for each measurement.

Extracellular pH was recorded with a 601A meter (Orion Research Inc., Cambridge, MA) connected to a 7044A recorder (Hewlett-Packard Co., Palo Alto, CA). The cells ($1\text{--}2 \times 10^7/\text{ml}$) were suspended in the indicated medium in a magnetically stirred, water-jacketed reservoir maintained at 37°C . The basal rate of H^+ production was monitored while the pH was maintained in the 7.1–7.3 range by pipetting manually aliquots of 20 mM KOH. Then, NaCl, KCl, amiloride, or ionophores were added and recording continued.

RESULTS

As reported previously (Hempling et al., 1978), PBM exposed to hypertonic media rapidly shrink as predicted by the Boyle-Van't Hoff relationship. The cells retain the shrunken volume for at least 45 min when the solution is made hypertonic with sucrose, but frequently show a very modest, transient volume recovery in NaCl (Fig. 1A). Similar results are obtained for both solutes in the hypertonic range 150–300 mosmol above isotonicity.

As mentioned in the introduction, PBM swollen in hypotonic media return to near-normal volume within minutes, attaining a new steady state (Fig. 1B). If isotonicity is restored, these cells shrink within seconds. However, unlike

PBM suspended directly in hypertonic media, the cells in this case reswell toward their original volumes. Maximal reswelling rates approximate $5 \mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{min}^{-1}$ and volume recovery is complete by ~ 25 min. Because of its simplicity and accuracy, electronic sizing was used for most of the measure-

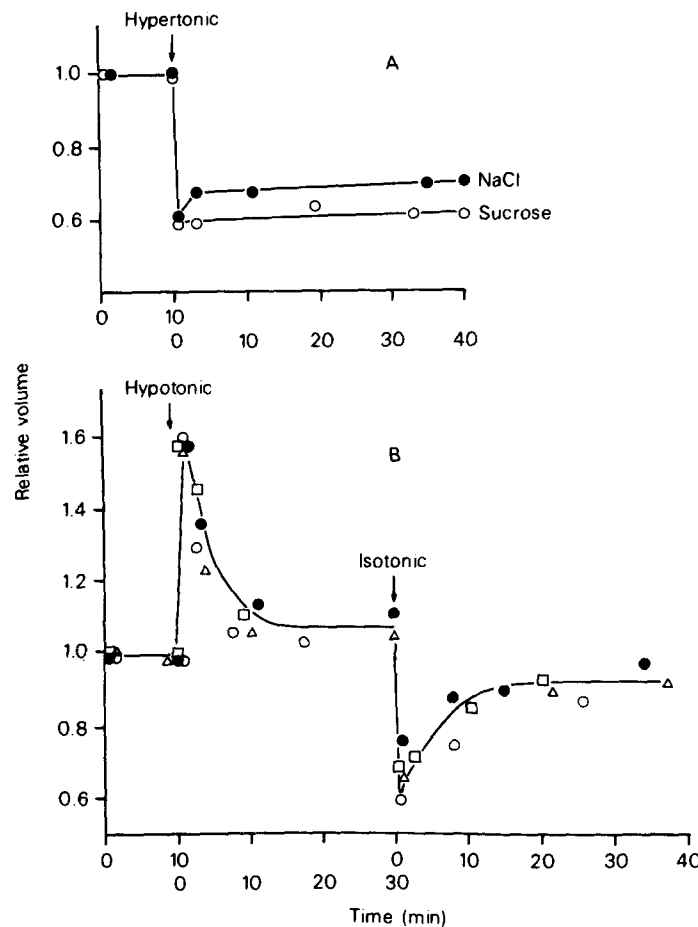


FIGURE 1. (A) Effect of hypertonic solutions on the volume of PBM. The volume of cells incubated in isotonic Na^+ solution was measured for 10 min. Where indicated, 300 mosmol of sucrose (O) or NaCl (●) was added to the medium. Volume was measured electronically with the Coulter Counter and is given relative to the isotonic control. The points are representative of four similar experiments. Temperature = 37°C . (B) Volume regulation of swollen and shrunken PBM. Cells suspended in isotonic RPMI 1640 were stressed hypotonically ($0.5\times$ isotonic) and allowed to equilibrate for 30 min. During the last 10 min of this period, the cells were sedimented at $200g$ and then resuspended in isotonic Na^+ solution at 37°C , where indicated. Each type of symbol indicates a separate experiment. The four experiments illustrated are representative of over 40 such experiments. Equivalent changes in the tonicity and conductivity of the medium did not significantly affect measurements of the volume of latex beads.

ments. However, to rule out the contribution of shape or deformability changes to the measurements, a number of volume determinations were made by isotope dilution using $^3\text{H}_2\text{O}$. Reswelling of the hypotonically treated cells in isotonic media was also reproducibly measurable by this method and the results were quantitatively similar to those obtained by electronic determinations.

Effect of Temperature and Ion Substitution

Unless otherwise specified, the experiments described hereafter refer to cells pre-equilibrated in hypotonic media that were subsequently shrunk by resuspension in isotonic solutions, as in Fig. 1B. Reswelling of these cells was markedly temperature dependent (Fig. 2A), with no significant swelling at 4°C and an initial rate of $2 \mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{min}^{-1}$ at 22°C (60% less than the 37°C rate).

Reswelling was sensitive to the nature of the major monovalent ions in the medium. It was observed regularly in Na^+ -based media in the presence or absence of K^+ . In contrast, total substitution of K^+ or choline for Na^+ entirely abolished volume restoration (Fig. 2A). Similarly, isotonic replacement of Cl^- and HCO_3^- by gluconate or isethionate eliminated reswelling. Only a modest volume gain was noted in SO_4^{2-} or NO_3^- media (Fig. 2B). Intermediate and somewhat variable swelling rates were observed in HCO_3^- -free, Cl^- -based media. The reduced responsiveness was more pronounced and consistent if HCO_3^- was also absent during the hypotonic pretreatment (not illustrated).

Effects of Inhibitors

A number of agents were tested as potential inhibitors of the volume change. As shown in Table I, amiloride produced the largest inhibition. The effect was concentration dependent and $>80\%$ inhibition was obtained at 0.5 mM. At this and higher concentrations, only a brief reswelling, resembling that in Fig. 1A (NaCl , solid circles), was recorded, and no further volume change was noticeable for up to 40 min. Consistent inhibitions were also obtained with 10 μM trifluoperazine, a phenothiazine commonly used as a calmodulin antagonist. Higher concentrations of this drug affected cell viability significantly and are therefore not reported. Only partial inhibition was obtained with 0.1 mM quinine, a concentration that completely eliminates RVD and Ca^{2+} -induced K^+ fluxes in PBM and other cell types (Grinstein et al., 1982c; and unpublished observations). Concentrations of furosemide that totally block the co-transport systems involved in volume regulation in duck erythrocytes (Kregenow, 1981) and Ehrlich ascites cells (Hoffmann et al., 1981) had only a partial inhibitory effect (29%) in the case of PBM. Ouabain and DNDS had only marginal effects on reswelling.

Changes in Cation Content

To gain understanding of the ionic basis of reswelling, the Na^+ and K^+ contents of PBM were analyzed before and after changing the tonicity of the medium. The results are summarized in Table II; as reported (Bui and Wiley,

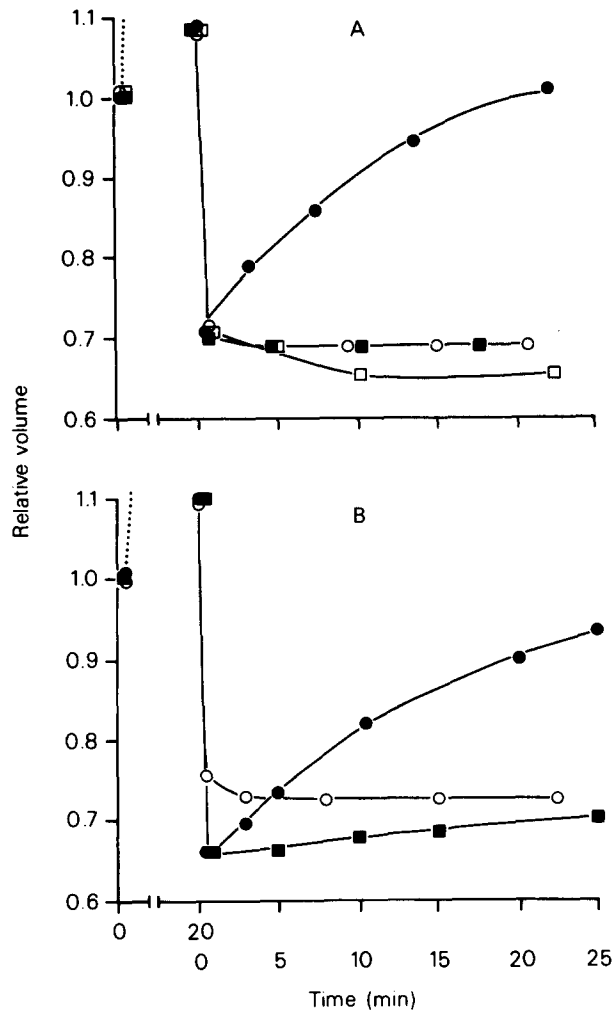


FIGURE 2. (A) Effect of temperature and cation composition on RVI. PBM were pretreated with hypotonic RPMI 1640 (0.5× isotonic, dashed line) and allowed 20 min for equilibration. Where indicated by the new time scale, the cells were resuspended in one of the following media: Na^+ solution at 37°C (●); Na^+ solution at 4°C (○); K^+ solution at 37°C (■); choline solution at 37°C (□). Data are representative of at least three experiments. (B) Effect of anion substitution on RVI. Cells were pretreated hypototically as in A and then resuspended in Na^+ -based media of various anion compositions: Cl^- and HCO_3^- (normal medium, ●), gluconate medium (○), or NO_3^- medium (■). Temperature during RVI was 37°C. Data are representative of at least three experiments. The experiments with solid circles in A and B are equivalent and illustrate the range of individual variability among donors. Volume was measured electronically as in Fig. 1 and is expressed relative to the isotonic control.

1981; Cheung et al., 1982) PBM lose K^+ and H_2O during RVD, whereas the Na^+ content remains essentially unaltered (compare the first two lines of Table II). Restoration of isotonicity, which brings about rapid shrinking followed by gradual reswelling, produces a gain of both Na^+ and K^+ . If movement of the cations is accompanied by Cl^- , as suggested by the substitution experiments, the tonicity of the solution taken up by the cells can be calculated. For a volume gain of $50\text{--}60 \mu\text{m}^3 \cdot \text{cell}^{-1}$, measured over the 20-min interval allowed for the cation determinations, an osmolarity of 270–324 mosmol can be calculated. Thus, the movement of water during reswelling appears to occur isotonicity.

As mentioned above, reswelling proceeds normally in the presence of ouabain at concentrations that fully inhibit Na^+K^+ pumping. Accordingly, a net gain of cations was measured ($7.1 \text{ nmol} \cdot 10^6 \text{ cells}^{-1}$), which is not significantly different from that observed in the absence of the glycoside ($8.1 \text{ nmol} \cdot$

TABLE I
Effect of Inhibitors on RVI

Inhibitor	Concentration	Inhibition
	<i>mM</i>	%
Amiloride	0.01	21±3
Amiloride	0.05	41±9
Amiloride	0.1	53±11
Amiloride	0.5	83±3
Trifluoperazine	0.01	55±6
Quinine	0.1	43±5
Furosemide	1.0	29±6
Ouabain	0.1	8±3
DNDS	0.1	2±0.2

PBM were pre-equilibrated for 30 min in hypotonic (50%) Na^+ medium, sedimented, and resuspended in isotonic Na^+ medium containing the indicated concentration of inhibitor. The volume recovery was measured at 10 and 20 min, and the effect of the drugs is expressed as the fractional inhibition of the control response. Data are means \pm SE of four determinations, except for DNDS, where $n = 2$.

10^6 cells^{-1}). However, the gain in this case was entirely attributable to Na^+ and in fact a further loss of K^+ was noted. This suggests that a gain of Na^+ is the primary event underlying RVI. A subsequent Na^+K^+ exchange through the pump, not essential to the volume gain, apparently occurs as a consequence of the increased Na^+ concentration.

Amiloride (0.5 mM), which substantially inhibits the volume gain, also largely prevented the uptake of cations. Only a small increase in Na^+ content was detectable (Table II), which is consistent with the small residual swelling (Table I).

Isotopic Cation Flux Determinations

Further insight on the nature of the ion pathways involved in RVI was gained using $^{24}Na^+$ and ^{86}Rb (as a K^+ analogue). Fig. 3 illustrates the time course of $^{24}Na^+$ uptake by PBM under volume-static and reswelling conditions. Both

sets of measurements were performed in identical isotonic media, but the cells in one case had been pre-equilibrated in hypotonic medium. All the measurements were performed in the presence of maximally inhibitory concentrations of ouabain, to minimize the secondary Na^+ back-flux. PBM in steady state display a relatively rapid Na^+ uptake, which probably represents Na^+/Na^+ exchange. Amiloride produced only a small, statistically insignificant inhibition of this flux. As shown in Fig. 3, pretreatment in hypotonic medium markedly stimulated $^{24}\text{Na}^+$ uptake when the cells were returned to isotonicity. Importantly, >65% of this stimulation was prevented by 0.5 mM amiloride. Inasmuch as this concentration of the diuretic almost entirely blocked RVI, the residual flux may represent activated Na^+/Na^+ exchange.

The uptake of ^{86}Rb was measured in the presence and absence of either ouabain (Fig. 4A) or amiloride (Fig. 4B). All other conditions were as in Fig. 3. As expected, >70% of the uptake in control cells was ouabain sensitive. When measured during the reswelling phase, ^{86}Rb influx was activated three- to fourfold and most of this stimulation was inhibitable by ouabain (Fig. 4A).

TABLE II
Changes in Monovalent Cation Content During RVI

First incubation	Second incubation	Na^+	K^+	<i>n</i>
<i>nmol/10⁶ cells</i>				
Isotonic	—	7.5±1.3	26.8±4.2	6
Hypotonic	—	7.1±0.6	13.5±2.2	14
Hypotonic	Isotonic	11.1±1.0	17.6±1.3	14
Hypotonic	Isotonic + ouabain	17.1±1.5	10.6±1.9	10
Hypotonic	Isotonic + amiloride	8.3±1.3	13.8±1.3	12

Isotonic Na^+ medium and 50%-dilute Na^+ medium (hypotonic) were used in all the experiments. Ouabain and amiloride concentrations were 0.1 and 0.5 mM, respectively. The first incubation was for 30 min; the second was for 20 min. Data are the mean ± SE of the number of experiments (*n*) indicated.

The nature of the ouabain-insensitive volume-activated flux remains obscure. As shown in Fig. 4B, amiloride had no effect on the control rate of K^+ (^{86}Rb) uptake, but it significantly reduced the flux in reswelling cells. Thus, most of the uptake of K^+ during the reswelling phase apparently occurs via the Na^+-K^+ pump, which is stimulated, at least in part, by an amiloride-sensitive mechanism, most likely the increased Na^+ influx (Fig. 3).

Membrane Potential Determinations

Transport of ions through conductive pathways should be reflected by changes in transmembrane voltage. Therefore, potential determinations were performed in PBM during the reswelling phase. Details on the use and validation of this method with PBM have been published before (Grinstein et al., 1982a). The cells, suspended in hypotonic medium, were equilibrated with the potential indicator dye diS-C₃-(5) while they were in the steady state after completion of RVD. Once a stable fluorescence reading was obtained, the osmolarity of the medium was raised back to isotonicity by addition of a

small volume of 10-fold-concentrated solution, and recording continued. Finally, the channel-former gramicidin was added to confirm the sensitivity of the recording setup. The results of a typical experiment are illustrated in Fig. 5. Upon restoration of isotonicity, a sharp, step-like drop in fluorescence

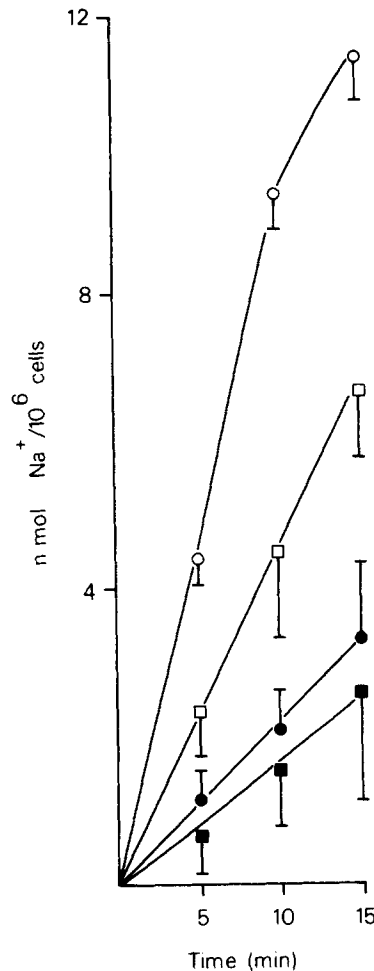


FIGURE 3. Uptake of Na^+ during RVI. $^{24}\text{Na}^+$ influx was measured in isotonic Na^+ solution in osmotically undisturbed cells (solid symbols) and in cells that had been preequilibrated for 30 min in 0.5 \times isotonic medium (open symbols). The determinations were performed at 37°C in the absence (circles) or presence (squares) of 0.5 mM amiloride. All the uptake media contained 0.1 mM ouabain. Data are the mean \pm SE of four experiments, each with duplicate determinations.

was observed that was caused by the dilution of the cell suspension. A comparable step is obtained when control cells (in isotonic Na^+ medium) are diluted with an equivalent volume of iso-osmolar medium. No significant potential change was detected during the reswelling phase of hypotonically

pretreated cells (Fig. 5 is representative of eight similar experiments). In contrast, addition of 500 nM gramicidin resulted in a rapid fluorescence increase (depolarization), which was absent if Tris was substituted for Na^+ ; this is consistent with changes expected in membranes made permeable to Na^+ and K^+ by the ionophore. The results indicate that the cells maintain a substantial membrane potential (inside negative) after RVD and that no

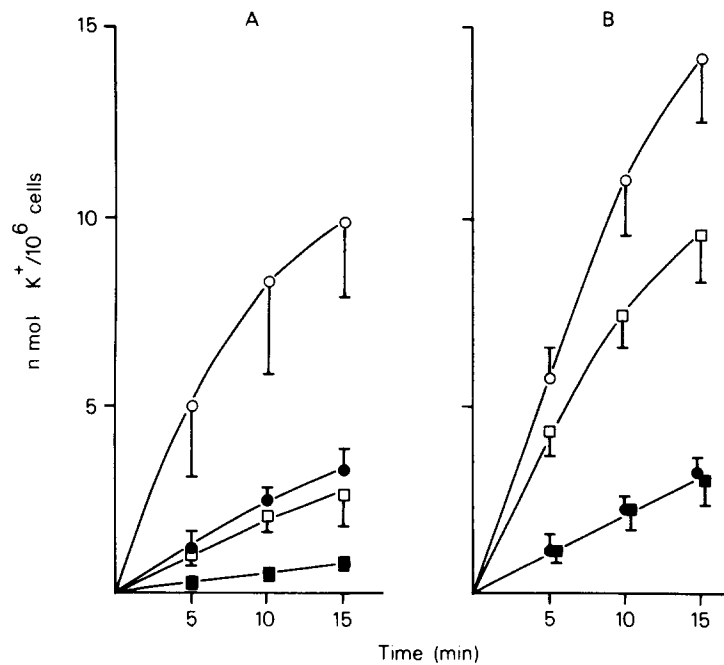


FIGURE 4. Uptake of K^+ (^{86}Rb) during RVI. (A) Influx of ^{86}Rb was measured in isotonic Na^+ solution in osmotically undisturbed cells (solid symbols) and in cells that had been pre-equilibrated for 30 min in 0.5 \times isotonic medium as described in Materials and Methods (open symbols). The determinations were performed at 37°C in the absence (circles) or presence (squares) of 0.1 mM ouabain, a concentration that completely inhibits the Na^+/K^+ pump in human PBM. The uptake rates of K^+ and $^{86}\text{Rb}^+$ were assumed to be identical. Data are means \pm SE of four experiments, each with duplicate determinations. Where not indicated, error bars are smaller than the symbol. (B) Effect of amiloride on RVI-induced ^{86}Rb uptake. PBM were treated as in A, but the influx of ^{86}Rb was measured in the presence (squares) or absence (circles) of 0.5 mM amiloride. Data are means \pm SE of four experiments with duplicate determinations.

significant voltage changes occur during reswelling. Similar negative results were obtained in Cl^- -free media, so it is unlikely that equivalent anion and cation conductance increases cancel each other.

Internal pH Determinations

Regulatory volume increase in hypertonically shrunken *Amphiuma* red cells

has been shown to be inhibited by amiloride (Siebens and Kregenow, 1978; Cala, 1980). The suggested mechanism was a coupled Na^+/H^+ exchange operating in parallel with $\text{Cl}^-/\text{HCO}_3^-$ or Cl^-/OH^- countertransport through the anion exchange system (Cala, 1980). The activity of the latter system is known to be high in the membrane before the osmotic stress, whereas the amiloride-sensitive cation exchange system is thought to be induced or activated upon shrinking. Cala (1980) suggested that a cytoplasmic acidification consequent to the shrinking could be the step that activates Na^+/H^+

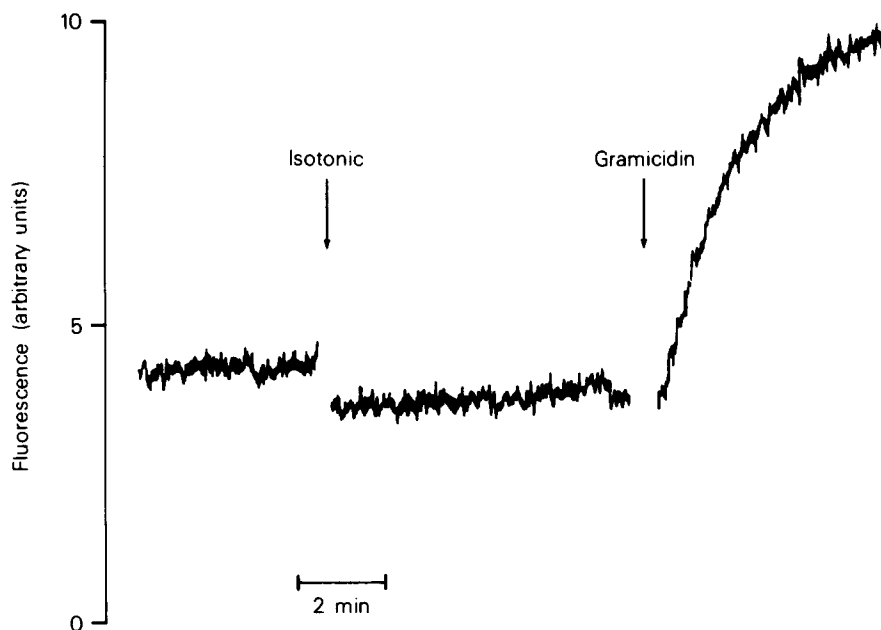


FIGURE 5. Measurement of membrane potential during RVI. PBM were hypotonically stressed and allowed 50 min to undergo RVD. The potential indicator diS-C₃(5) (0.5 μM) was then added and fluorescence was recorded as described in Materials and Methods. The initial part of the trace reflects the equilibrium distribution of the dye with cells in hypotonic Na^+ solution. Where indicated, isotonicity was restored by addition of $1/10$ volume of a sixfold-concentrated Na^+ solution. Finally, 0.5 μM gramicidin was added. Ordinate: fluorescence in arbitrary units. The trace is representative of eight similar experiments.

countertransport. Because of the common amiloride sensitivity of the PBM and *Amphiuma* systems, cytoplasmic pH (pH_i) determinations were undertaken to define: (a) whether a Na^+/H^+ countertransport system exists or can be induced in the PBM membrane and (b) whether an acid shift is observed when PBM are shrunk upon reintroduction to isotonic medium.

Experiments were performed to detect the operation of the putative Na^+/H^+ exchanger by the appearance of a Na^+ -induced intracellular alkalinization. Because in epithelial and excitable cells, Na^+/H^+ exchange is greatly activated by lowering pH_i , these experiments were carried out in acid-loaded cells.

Acid loading was achieved by exchange of intracellular K^+ for external H^+ using the ionophore nigericin, a cation exchanger. Changes of pH_i were measured fluorimetrically using 5,6-dicarboxyfluorescein. A typical experiment is illustrated in Fig. 6. PBM loaded with the pH indicator were suspended in Na^+ -free medium and acid-loaded with nigericin. The ionophore was then removed by addition of albumin (this procedure, to be described in detail elsewhere, largely removes nigericin from the cells), stabilizing pH_i between 6.2 and 6.3. Addition of 40 mM Na^+ to the external medium resulted in a rapid intracellular alkalinization. As shown in Fig. 6,

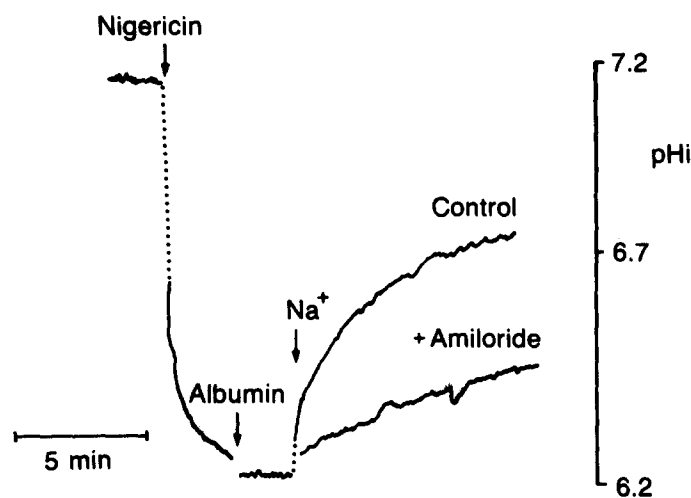


FIGURE 6. Evidence for the existence of a Na^+/H^+ antiport in PBM. Cells loaded with 5,6-dicarboxyfluorescein were suspended in 1.2 ml of a medium containing 140 mM choline chloride, 1 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES-Tris (pH 7.1), with or without 20 μM amiloride, and fluorescence was recorded as described in Materials and Methods. Where indicated, 0.5 $\mu\text{g}/\text{ml}$ nigericin was added. The acid loading was terminated by addition of concentrated albumin (5 mg/ml final), which quenches the ionophore. Finally, 40 mM NaCl (final) was added with minimal dilution and recording continued. The experiment was terminated by disruption of the cells with Triton X-100 and calibration of fluorescence vs. pH (not illustrated). A predetermined factor was used to correct for the red shift of the intracellular dye (see Materials and Methods). The trace is representative of six similar experiments. Ordinate: cytoplasmic pH (pH_i).

this Na^+ -induced change in pH_i was inhibited >60% by 200 μM amiloride. These data support the existence of an amiloride-sensitive Na^+/H^+ antiport system in the plasma membrane of PBM,¹ which can be activated by acidification of the cytoplasm.

¹ A manuscript providing a detailed description of the Na^+/H^+ exchanger in lymphocytes is in preparation. Briefly, the results show that alkalinization is accompanied by Na^+ uptake and that the antiporter is activated by Na^+ or Li^+ , but not by other monovalent cations. The exchange is electrically silent and fully inhibitable by amiloride but is not affected by furosemide or bumetanide.

The average pH_i of PBM suspended in isotonic Na^+ medium was 7.05 ± 0.06 (mean \pm SE of five determinations). The value of pH_i was not significantly different in hypotonically equilibrated cells ($pH_i = 7.03 \pm 0.04$, $n = 12$). As shown in Fig. 7A, restoration of isotonicity by addition of concentrated NaCl had no immediate effect on pH_i . However, a slow alkalization developed over the following minutes that usually reached a steady value 6–8 min after addition of NaCl. In eight experiments, the ΔpH observed during this period averaged 0.14 ± 0.03 units. Fig. 7B illustrates typical results obtained when isotonicity was restored in hypotonically treated cells pre-equilibrated with $200 \mu M$ amiloride. The diuretic did not significantly alter the resting pH of these cells, but largely abolished the increase associated with RVI. In the presence of amiloride, ΔpH was only 0.03 ± 0.01 ($n = 7$).

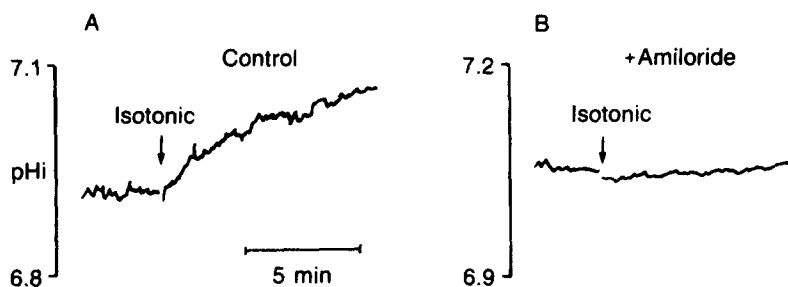


FIGURE 7. Changes in intracellular pH during RVI. Cells loaded with the fluorescent indicator were hypotonically treated and equilibrated as described in Materials and Methods. They were then suspended in hypotonic Na^+ medium without (A) or with (B) $200 \mu M$ amiloride and used for fluorescence recording as in Fig. 6. Where indicated, concentrated NaCl was added to restore the osmolarity to the original (isotonic) value. The small (3%) dilution artifact caused by the addition of NaCl has been corrected for. Calibration was as in Fig. 6. The traces are representative of eight similar pairs of experiments. Ordinate: cytoplasmic pH (pH_i).

Alkalinization of the cytoplasm was also observed when isotonicity was restored with concentrated Na^+ solution and in cells suspended in Cl^- - and HCO_3^- -free media, using concentrated Na-gluconate or Na_2SO_4 to increase the osmolarity. In contrast, no ΔpH could be induced in Na^+ -free media, a finding that parallels the Na^+ dependence of RVI.

The results obtained with 5,6-dicarboxyfluorescein were confirmed using 9-amino acridine, an indicator that partitions across the membrane in response to a change in ΔpH . As expected, the dye was taken up by PBM in both isotonic or hypotonic media and accumulated further (consistent with cytoplasmic acidification) in the presence of nigericin. The dye was partially released (cytoplasmic alkalization) by the addition of either NH_4^+ or monensin (an ionophore that promotes Na^+/H^+ exchange) in Na^+ solution. An alkalization was also detected when the cells were undergoing RVI. The time course of this pH change was virtually identical to that recorded with the fluorescein derivative and it was similarly Na^+ specific and observable in the absence of Cl^- and HCO_3^- .

External pH Determinations

As pointed out by Cala (1980), if net Na^+ uptake is coupled to H^+ efflux, then, to the extent that the rate of this exchange exceeds Cl^- /base countertransport, medium pH changes should be measurable during reswelling. To reduce anion countertransport and to improve detectability by reducing the buffering capacity, these experiments were carried out in HCO_3^- -free solutions, buffered only with 0.5 mM HEPES. PBM were hypotonically treated, sedimented, and resuspended in an isotonic Na^+ -free medium. The results are shown in Table III. The pH of the suspension declined spontaneously after addition of the cells, presumably as a result of release of metabolic acid. This decline was equivalent to $\sim 0.3 \text{ nmol } \text{H}^+ \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ and was not affected by amiloride. After a stable rate of H^+ production was established, Na^+ or K^+ was added to the medium as recording continued. Because of the

TABLE III
 Na^+ -induced Medium Acidification During RVI

Condition	H^+ efflux
	<i>nmol/10⁶ cells · 3 min</i>
Choline	0.9±0.3
Choline → Na^+	2.7±0.9
Choline + amiloride → Na^+	1.6±0.6
Choline → K^+	1.2±0.3

PBM were hypotonically treated, sedimented, and resuspended in an isotonic medium containing 140 mM choline chloride, 3 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM glucose, and 0.5 mM HEPES- K^+ , pH 7.3, at 37°C. External pH was measured as described in Materials and Methods. Where indicated, NaCl or KCl (30 mM final) with or without amiloride (0.5 mM final) was added and ΔpH was measured over a 3-min period. The buffering capacity of the solution in the pH range studied, which varied somewhat between experiments because of the addition of amiloride or buffer carried over with the cells, was determined after each experiment by titration with KOH. The results are given as nanomoles of H^+ produced per 10^6 cells over the 3-min period following the addition of the alkali cation, and are the mean \pm SE of three to four determinations.

imperfect selectivity of the pH electrode, the addition of the concentrated solutions of both cations produced a transient artifactual acidification lasting a fraction of a minute, but this did not affect the determinations of H^+ efflux that were calculated over a 3-min period from ΔpH and the buffering capacity of the medium in the pH 7.1–7.3 range. Na^+ induced an acceleration of acid production that was maximal during the first 2–3 min and declined rapidly thereafter. In contrast, K^+ did not significantly alter H^+ efflux. The Na^+ -induced acidification of the medium was largely blocked by amiloride, as was the case for the corresponding cytoplasmic alkalization (Fig. 7). These results suggest that transmembrane H^+ efflux (or the equivalent OH^- uptake) occurs during RVI.

Role of Ca^{2+} in RVI

Ca^{2+} , an important intracellular messenger in a variety of transduction systems, has been suggested to participate in volume regulation in PBM and

epithelial cells (Grinstein et al., 1982c; Spring and Ericson, 1982). The role of extracellular Ca^{2+} in the amiloride-sensitive reswelling of PBM was investigated by suspending hypotonically treated cells in isotonic Na^+ solution devoid of Ca^{2+} , with or without 100 μM EGTA. In both cases volume restoration proceeded at normal rates.

DISCUSSION

During the RVD that followed hypotonic exposure, the permeabilities of the PBM membrane to K^+ and Cl^- increase independently (Grinstein et al., 1982a). These pathways, which appear to be conductive, are not likely to participate in RVI when the cells are reintroduced into isotonic media for several reasons: (a) The RVD permeability increases are transient; restoration of ^{86}Rb permeability and of Cl^- conductance to the original low values occurs within 10–15 min (B. Sarkadi, personal communication). Over 20 min was allowed between hypotonic stress and the measurement of RVI. (b) No reswelling was observed in KCl medium. (c) The ion selectivities of the two phenomena are different, e.g., NO_3^- permeates the hypotonically induced pathway but does not support RVI. (d) Concentrations of inhibitors such as quinine and trifluoperazine, which entirely eliminate RVD, have much smaller effects on RVI.

The salient features of the reswelling process are Na^+ and Cl^- dependence, electroneutrality, and associated pH changes. The external Na^+ requirement was associated with increased $^{24}\text{Na}^+$ influx and net gain of Na^+ and K^+ by the cells. Amiloride, which largely prevented RVI, also inhibited the Na^+ and K^+ content increases and partially inhibited the stimulated isotopic fluxes. The K^+ accumulation, which can be accounted for by an increased influx (Fig. 4), is inhibited by ouabain, which suggests that it is mediated by the Na^+ pump. The glycoside, however, does not prevent reswelling (Table I), which indicates that Na^+/K^+ exchange is not essential to RVI and is probably secondary to the increase in cytoplasmic Na^+ concentration. The failure of amiloride to block the isotopic fluxes completely while preventing reswelling implies that other pathways, not necessarily involved in RVI, are also activated. In this regard, an external, Na^+ -independent activation of the Na^+/K^+ pump has been reported to occur upon hypotonic stress (Cheung et al., 1982). In addition, amiloride-insensitive Na^+/Na^+ exchange may also be involved.

No changes in membrane potential were recorded during RVI (Fig. 5), which suggests that the fluxes involved are electroneutral. This is supported by the fact that NO_3^- , which has a larger conductive permeability than Cl^- in most cells, including hypotonically stressed PBM (Grinstein et al., 1982a), failed to support RVI.

A cytoplasmic alkalization was found to occur during RVI. A concomitant medium acidification could be demonstrated in media with low buffering capacity, which implies that transmembrane movement of H^+ or OH^- has taken place. These pH changes were Na^+ dependent and amiloride inhibitable. Taken together, the above results conform to the model originally proposed by Cala (1980), whereby Na^+/H^+ countertransport is activated

during RVI. As a result, external Na⁺ is rapidly exchanged for cytoplasmic H⁺ in an electroneutral process. Volume gain results only when external Cl⁻ is exchanged in parallel for internal HCO₃⁻ or OH⁻, which brings about a net osmotic gain. The inhibitory effect of preincubation in HCO₃⁻-free media supports this model.

A minimum estimate of the contribution of Na⁺/H⁺ exchange to the total cation gain during RVI can be calculated from the observed ΔpH if the intracellular buffering capacity is known. The latter was determined in the pH 6.9–7.4 range by titration of intact cells with NH₄⁺, as described in Materials and Methods. In eight determinations, the mean buffering capacity of cells shrunken in isotonic medium was 52 ± 12 mmol·pH⁻¹·liter⁻¹. This value is similar to that reported for pig mesenteric lymphocytes by Rink et al. (1982). Therefore, 7 mmol Na⁺·liter cells⁻¹ must have entered the cell to raise pH_i by 0.14 unit. If the mean cellular volume under these conditions is considered, then at least 1.5 nmol Na⁺·10⁶ cells⁻¹ are taken up via the amiloride sensitive antiport during the initial 6–8 min. This is ~20–25% of the total unidirectional ²⁴Na influx and ~50% of the amiloride-sensitive fraction of this flux. The remainder of the Na⁺/H⁺ exchange is probably not manifested as a pH change because of the simultaneous operation of the Cl⁻/HCO₃⁻ or Cl⁻/OH⁻ exchange, which in turn enables the cell to regain its volume. Therefore, even though the operation of other mechanisms cannot be ruled out, it is clear that a substantial fraction of the RVI occurs via Na⁺/H⁺ exchange.

Modest but measurable volume changes can be recorded in the nominal absence of HCO₃⁻. Shrinking will occur if nigericin is added to cells in HCO₃⁻-free, low-K⁺ media with a marked concomitant acidification of the cytoplasm (e.g., Fig. 6). Resuspension of these acid-loaded cells in Na⁺-containing media rapidly restores the normal intracellular pH, while the volume increases back to its original value (unpublished observations).

As discussed by Kregenow (1981), the pH changes observed during volume regulation in red cells could be secondary to alterations in the Cl⁻ distribution. Thus, the Cl⁻ taken up during RVI would tend to exit from the cell in exchange for OH⁻, since the Donnan ratio remains unaltered, thereby bringing about cytoplasmic alkalinization. In PBM, the observed ΔpH is unlikely to be secondary to changes in Cl⁻ distribution inasmuch as (a) it is blocked by amiloride, a rather specific Na⁺/H⁺ exchange inhibitor, and (b) an alkalinization was also observed when isotonicity was restored by addition of Na⁺ in Cl⁻-free media. In these cases ΔpH was not significantly greater than in Cl⁻-containing solutions, which suggests that alkalinization of the cytoplasm limits Na⁺/H⁺ exchange and that anion exchange is considerably slower.

Anion exchange is the predominant transport function in volume-static red blood cells, so that induction of cation countertransport suffices to produce RVI. The existence of a similar anion exchanger in PBM has not been directly demonstrated; however, its presence can be inferred from the comparison of Cl⁻ permeability (measured isotopically) and Cl⁻ conductance

(determined from volume changes in the presence of cationophores). The latter is at least 25-fold lower, which indicates the presence of an electrically silent exchanger (Grinstein et al., 1982a). The existence of an independent anion exchanger is also suggested by the following experiment: PBM were shrunk by addition of 200 mM sucrose to the Na^+ medium. As indicated above, these cells fail to reswell. However, addition of the ionophore monensin, which produces a transient cytoplasmic alkalization through Na^+/H^+ exchange, resulted in a substantial ($>15 \mu\text{m}^3 \cdot \text{cell}^{-1}$) volume gain. This experiment suggests that coupled anion exchange occurred in parallel with ionophore-mediated transport.

On the other hand, a number of observations indicate that the anion exchangers of PBM and red cells are not identical. First, 100 μM DNDS, a powerful inhibitor of anion fluxes in red cells, failed to block RVI (Table I) as well as ^{36}Cl exchange in PBM. Similarly, addition of 100 μM DIDS up to 20 min before shrinking also failed to inhibit RVI (unpublished observations). Second, NO_3^- , which is readily accepted by the red cell exchanger, did not support RVI in PBM. The latter, however, could be explained by a direct effect of the anion on Na^+/H^+ exchange: Parker (1983) and P. M. Cala (personal communication) have found that NO_3^- and other anions have profound inhibitory effects on the cation antiporter. Thus, the participation of an anion exchanger in RVI remains somewhat inferential and determination of the extent of coupling with the volume-induced cation exchanger must await further characterization of the anion transport in volume-static PBM.

Changes in intracellular pH and $[\text{Ca}^{2+}]$ have been proposed to trigger the activation of transport pathways during volume regulation (Cala, 1980; Spring and Ericson, 1982). An acidification of the cytoplasm was suggested to activate Na^+/H^+ exchange in *Amphiuma* erythrocytes after hypertonic shrinking. Indeed, stimulation of Na^+/H^+ countertransport by lowering the internal pH has been reported in a number of cell types (Aronson et al., 1982; Moolenaar et al., 1982; Boron and Boulpaep, 1983) and is also observed in blood and thymic lymphocytes (Fig. 6 and unpublished observations). Nevertheless, direct measurements failed to show a significant acid shift when PBM were shrunk, which suggests that Na^+/H^+ exchange is activated by an alternative mechanism. RVI was unaffected by external Ca^{2+} removal. In addition, preliminary experiments using the Ca^{2+} indicator Quin 2 failed to show significant changes in the cytoplasmic Ca^{2+} concentration. Therefore, changes in the intracellular concentration of this cation are also unlikely to mediate RVI.²

In addition to Cala's (1980) countertransport model, anion-cation co-transport systems have been suggested to participate in RVI in certain cells.

² It is important to bear in mind that high (millimolar) concentrations of Quin 2 are required for adequate measurements. This confers on the cytoplasm a considerable Ca^{2+} -buffering capacity that can obliterate Ca^{2+} transients. Therefore, the release of a comparatively small amount of Ca^{2+} from internal stores, sufficient to alter the free cytoplasmic level in the absence of the dye, may remain undetected.

Avian erythrocytes reswell in hypertonic solutions by activating a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ symport mechanism that is sensitive to furosemide and its analogues (Kregenow, 1981). Ehrlich ascites cells pre-equilibrated in hypotonic media will shrink and then reswell when isotonicity is restored (Hoffmann et al., 1983), as described here for PBM. Hoffmann et al. attribute reswelling to a bumetanide- and furosemide-sensitive co-transport of Na^+ and Cl^- , which is active even at low external K^+ . The PBM system differs from those above in its K^+ independence and relative insensitivity to furosemide. However, as pointed out by Kregenow (1981), the co-transport systems may involve two or more coupled antiports, e.g., NaCl co-transport would be composed of Na^+/H^+ and Cl^-/OH^- exchanging subunits. Thus, Kregenow's co-transport system would differ from Cala's antiporter mechanism only in the degree of coupling of the exchangers. At the molecular level, this may in turn be manifested as differences in inhibitor sensitivity.

The failure of the cells to reswell when placed directly into hypertonic solutions remains unexplained. One attractive hypothesis is that the depletion of intracellular ions, particularly Cl^- , that occurs during RVD is essential to provide an inward gradient for NaCl uptake during RVI. Alternatively, Na^+/H^+ exchange may be activated only if swelling occurs before shrinking. In summary, an amiloride-sensitive Na^+/H^+ antiport can be activated by osmotic shrinking of hypotonically pretreated PBM. The cells gain volume as Cl^- is taken up in exchange for HCO_3^- or OH^- . Although an anion exchanger seems to operate in volume-static cells, the extent of its coupling with the volume-induced cation exchanger remains undefined; the countertransport of external Cl^- for internal HCO_3^- or OH^- might be stimulated by the depletion of internal Cl^- , which occurs during RVD, and/or by the cytoplasmic alkalization prompted by Na^+/H^+ exchange.

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