Cytosolic Protein Concentration Is the Primary Volume Signal in Dog Red Cells

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ABSTRACT It is not known whether the activation of Na/H exchange by shrinkage in dog red cells is due to the packing of cell contents or a change in cell configuration. To make this distinction we prepared resealed ghosts that resembled intact cells in hemoglobin concentration and surface area, but had one-third their volume. A shrinkage-induced, amiloride-sensitive Na flux in the ghosts was activated at a much smaller volume in the ghosts than in the intact cells, but at the same concentration (by weight) of dry solids in both preparations. Na/H exchange in ghosts containing a mixture of 40% albumin and 60% hemoglobin (weight/weight) was activated by osmotic shrinkage at a dry solid concentration similar to that of intact cells or of ghosts containing only hemoglobin. We conclude that the process of Na/H exchange activation by cell shrinkage originates with an increase in the concentration of intracellular protein and not with a change in membrane configuration or tension. The macromolecular crowding that accompanies the reduction in cell volume probably alters the activities of key enzymes that in turn modulate the Na/H exchanger.

INTRODUCTION

Most animal cells respond to volume perturbation by activating transporters or enzymes that enable them to accumulate or jettison solute (Siebens, 1985; Chamberlin and Strange, 1989; Hoffmann and Simonson, 1989) and thereby return toward their original volume. Although the responses of cells to swelling and shrinking have been well described, virtually nothing is known about how cell volume is perceived. There is some evidence that cytoskeletal proteins are involved and that changes in cell volume (usually swelling) activate membrane stretch receptors (Pierce and Politis, 1990; Watson, 1991).

Cell preparations differ in their suitability for studies of volume regulation. Among the most thoroughly investigated are lymphocytes, Ehrlich ascites tumor cells, and certain epithelia (Chamberlin and Strange, 1989; Hoffmann and Simonson, 1989). Red cells of duck (McManus, Haas, Starke, and Lytle, 1985), Amphiuma (Cala, 1980; Siebens and Kregenow, 1985), sheep (Lauf, 1988), and rabbit (Jennings and Al-Rohil, 1990) all show volume regulatory behavior during brief incubations in vitro, via a
diversity of pathways including Na/H exchange, K/H exchange, [Na + K + 2Cl] cotransport, and [K-Cl] cotransport.

Dog red cells are particularly useful for studying volume transduction because they have easily measurable transport systems that are activated by swelling and shrinking (Parker and Hoffman, 1965, 1976; Parker, McManus, Starke, and Gitelman, 1990), and they can be resealed after hemolysis (Parker, 1979, 1988).

Whenever cells swell or shrink, two changes occur: (a) there is distortion of the cell (a shape change), and (b) the impermeant contents of the cell become diluted or concentrated. Our objective was to distinguish which of these two general consequences of cell volume change initiates the signal that results in the activation of a membrane transporter. Our strategy was to make resealed ghosts that had the same concentration of cytoplasmic protein as intact cells, but a greatly reduced volume, and to use amiloride-sensitive Na/H exchange as an indicator of volume-sensitive transport.

The results have been presented in abstract form (Colclasure and Parker, 1991).

METHODS

Preparation of Resealed Ghosts Unsupplemented with Albumin

Venous blood was collected from mongrel dogs into heparinized syringes within 30 min of processing. After discarding the plasma and buffy coat the cells were washed three times in 10 vol of ice-cold 0.16 M NaCl and pelleted at 20,000 g for 1 min at 0°C. Ghosts were made by a modification of methods reviewed by Schwoch and Passow (1973) and Wood and Passow (1981). Concentrations of acetic acid and other reagents were adjusted for the lower ratio of hemolysis medium to cells. A small amount of calcium was added to the hemolysis medium because we found it tightened up the ghosts without activating calcium-dependent K channels, as described by Colombe and Macey (1974). Minor deviations from the pH optima described by Schwoch and Passow (1973) were found to give a better yield of tight ghosts. The cells were drawn into a chilled syringe and injected into four times their volume of a swirling, 0–0.5°C solution that contained 10 μM CaCl₂ and 13 mM acetic acid. The hemolysate was stirred at 0–0.5°C for 1 min, after which its pH was ~6.5. The hemolysate was supplemented with (final added concentrations) 150 μM ATP, 325 μM MgCl₂ and 500 μM glutathione. These additions increased the fluid volume of the hemolysate by <1%. Crystalline Tris base was added to a final concentration of ~1.5 mM, at which point the hemolysate pH was 6.8. Note that no salt was added before the rescaling phase. After these additions the hemolysis mixture had an osmolality of 50–60 mosM as determined by freezing point depression (Advanced Instruments, Inc., Needham Heights, MA). After a further 5 min of stirring at 0–0.5°C the hemolysis mixture was placed in a 37°C water bath and incubated for 30 min to promote rescaling. Previous work on the course of rescaling in dog red cell ghosts showed that Na permeability was restored after 10 min at 37°C (Parker, 1979). The ghosts were then centrifuged, washed three times at room temperature with 0.1 M NaCl, and transferred to other media for the experiments described.

Preparation of Resealed Ghosts Supplemented with Albumin

The procedures for albumin-supplemented ghosts were in general the same as those outlined above, with the following exceptions: Fatty acid–free bovine serum albumin that had been dialyzed against distilled water and lyophilized was dissolved in the hemolysis solution at a concentration of 43 mg/ml before the addition of packed cells. The hemolysis solution contained 5.7 mM acetic acid and 10 μM CaCl₂. 1 vol of cells was added to 8.5 vol (instead of 4 vol as noted above) of hemolysis solution. Solid NaCl (final concentration 15 mM) was added to
the medium 6 min after hemolysis, along with ATP, Mg, and glutathione as noted above. After
titrated to pH 7.2 with crystalline Tris base the hemolysate had an osmolality of 50–60 mosM.
It was incubated for 20 min at 0°C with stirring and 20 min in an ice bath without stirring
before being transferred to the 37°C bath for resealing.

Measurement of Cell and Ghost Dry Solid Content

Cells/ghosts were washed twice in the appropriate media containing 0.5% bovine serum
albumin. Dialyzed $^{125}$I-labeled albumin (0.1 μCi/ml) was added to the second wash, and the
cells/ghosts were pelleted by centrifugation at 28,000 g. The pellet was weighed before and
drying for 20 h at 95°C, and the result was corrected for trapped medium using the
radioactive albumin as an extracellular marker.

Measurement of Na Influx

Ghosts/cells were suspended in 10 times their volume of incubation media, which covered a
range of tonicities and consisted of 100 mM NaCl, 0–120 mM N-methyl-d-glucamine hydro-
chloride, 10 mM HEPES buffer (pH 7.7 at 25°C), 5 mM glucose, and 0.5 g/dl bovine serum
albumin, with or without 0.5 mM amiloride. The flux was begun by adding $^{22}$Na (1 μCi/ml) to
each suspension and sampling for 30 min at 37°C. The influx was calculated as the number of
counts taken up by the cells/ghosts in 30 min, divided by the specific activity of extracellular
Na, and normalized to the number of cells/ghosts per suspension as determined with a model
B Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Measurement of Ghost/Cell Volumes and Measurement of Osmotic Properties of the
Ghosts and Cells

Ghost and cell volumes were measured by determining the hematocrit of a suspension
centrifuged at 20,000 g (corrected for trapped medium) and the number of ghosts or cells per
unit volume of the same suspension as measured on a Coulter counter.

The plots of ghost/cell volume as functions of suspending medium osmolality were compared
with values predicted by Ponder's equation. Ponder (1933) noted that when a cell is transferred
from one solution to another with a different tonicity, the measured volume change is less than
what one would expect if cells were perfect osmometers. He expressed the discrepancy between
observed and calculated cell osmotic behavior as a ratio: $R = \frac{(V_p - V_o)/(V_i - V_o)}{\text{change in volume if the cell were a perfect osmometer}}$, or

$$R = \frac{V_p - V_o}{V_i - V_o}$$

where $V_p$ is the measured cell volume (in femtoliters) after the change in medium osmolality, $V_i$
is the cell volume (in femtoliters) based on perfect osmometric behavior, and $V_o$ is the original

cell volume (in femtoliters). To generate a curve for $V_p$ as a function of medium osmolality, $C$,
one can express the Ponder equation as follows:

$V_w$, the reference cell volume in femtoliters

$V_i$, the ideal cell volume in femtoliters, after transfer of cells to the new medium, without

correction for Ponder's $R$

$V_p$, the cell volume in femtoliters, after transfer of cells to the new medium, with correction

for Ponder's $R$

$V_w$, the water volume of the cell (in femtoliters) at the reference volume

$V_w$, the water volume of the cell (in femtoliters) in the new medium

$C_r$, the reference osmolality in milliosmolar

$C$, the new medium's osmolality in milliosmolar

$D_w$, the reference dry ghost weight fraction (dry solids/wet weight)

$B$, the nonwater volume in femtoliters
Using the hematocrit and Coulter counter we measured \( V_0 \), the volume of ghosts and intact cells in isotonic medium, and with an osmometer we measured \( C_0 \) and \( C_0 \). \( D_0 \) was measured by weighing cells before and after drying and correcting for trapped medium. The weight of a cell at the reference volume is \( V_0 \), multiplied by the cell density, which at isotonicity is 1.100. \( B \), the volume of the dry cell contents, is equal to the weight of the dry cell contents \((V_0 \times 1.100 \times D_0)\) divided by the density of the dry cell contents (mostly hemoglobin), which has been measured by Kwant and Seeman (1970) at 1.17 g/ml.

From Eq. 1 we get

\[
V_p = V_0 + R(V_0 - V_0) \tag{2}
\]

\( V_0 \) is measured, we assign a value to \( R \) based on the literature (see below), and we solve for \( V_i \) from the following relationship for a perfect osmometer:

\[
V_0 \times C_0 = V_i \times C \tag{3}
\]

Since \( V_0 = V_0 - B \), and \( V_i = V_i - B \), Eq. 3 can be written as follows:

\[
V_i = C_0(V_0 - B)/(C)^{-1} + B \tag{4}
\]

Substituting the right-hand side of Eq. 4 for the term \( V_i \) in Eq. 2, we get

\[
V_p = V_0 + R\left[\frac{C_0(V_0 - B)/(C)^{-1} + B - V_0}{V_0}\right] \tag{5}
\]

\( V_p \) is calculated from Eq. 5 and plotted as a function of \( C \). Following the suggestion of Sachs, Knauf, and Dunham (1975), we assigned two values for \( R \): 0.9 for hypotonic solutions and 0.8 for hypertonic ones.

The curves in Fig. 1 B are plots of measured cell/ghost volumes. Superimposed on each plot are curves for two versions of Eq. 5, one with an \( R \) of 0.9, and one with an \( R \) of 0.8, using the volume, and dry solid content of cells suspended in isotonic Coulter diluent as \( V_0 \) and \( D_0 \).

**Determination of Albumin and Hemoglobin Content of Resealed Ghosts by Scanning Densitometry**

Scanning densitometry (LKB Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD) of Coomassie blue-stained SDS-PAGE gels was used to estimate the relative concentrations of hemoglobin and albumin in resealed ghosts and the resealing medium.

**RESULTS**

We use the term dry solids to mean “100 – the cell water (% wet weight)” or “(1 – (wet weight – dry weight)) (wet weight)^{-1} × 100.” More than 95% of the dry solids of mature mammalian red cells is hemoglobin (Harris and Kellermeyer, 1970). While it would have been valid to express the ghost contents in terms of cell water, the convention we adopted highlights the relationship between sodium transport and the concentration of protein in the cells.

The properties of resealed ghosts depend on the circumstances of hemolysis, on the resealing conditions, and on the osmolality of the medium in which the ghosts are suspended after they have sealed. Ghost hemoglobin content depends on the cell/medium volume ratio during resealing. Ghost volume depends on the osmolality of the resealing suspension and, once the ghosts have sealed, the osmolality of the extracellular medium (Hoffman, 1958; Hoffman, Eden, Barr, and Bedell, 1958; Kwant and Seeman, 1970; Schwoch and Passow, 1973; Funder and Wieth, 1976;
Wood and Passow, 1981). The results reported in Figs. 1–4 were obtained on ghosts prepared by hemolyzing and resealing at a cell/medium ratio of 1:4. Once resealed, the ghosts contained about one-fifth the hemoglobin and one-fifth the salt of intact cells. When resuspended in a medium of physiologic tonicity, the resealed ghosts shrank until their hemoglobin concentration approached that of intact cells.

Fig. 1 shows the osmometric behavior of cells and ghosts. Fig. 1A plots the dry solids vs. the osmolality of the suspending medium. At any osmolality the resealed ghosts contained less dry solid (more water) than the intact cells. Although there were no cells with < 30% dry solids and no ghosts with > 35% dry solids, there were both ghosts and cells that had dry solid values between 30 and 35%.

Fig. 1B shows that the ghost volume was about one-third that of the intact cells. The changes in volume of both cells and ghosts could be accurately predicted from

![Graph A](image1)

**Figure 1.** Cell/ghost dry solids (A) and cell/ghost volume (B) as a function of the osmolality of the suspending medium. Values for intact cells are shown by open symbols and for ghosts by filled symbols. In B the points represent measurements of the mean cell volume of ghosts and cells, as computed from the packed cell volume and cell count of a ghost/cell suspension. The solid lines and dashed lines in B represent plots of Eq. 2 with Ponder's R values of 0.9 and 0.8, respectively.

Ponder's equation, whether the R value was set at 0.8 or 0.9. Comparison of A and B of Fig. 1 shows that over the range of medium osmolalities tested, there were ghosts and cells with comparable dry solids, but there was no overlap between the ghost volumes and the cell volumes.

Fig. 2 shows osmotic fragility profiles for both ghosts and cells. When suspended in progressively hypotonic media the resealed ghosts became 50% rehemolyzed at 28 mosM, whereas intact cells became 50% hemolyzed at 138 mosM. This result indicates that, when suspended in media of the same tonicity, the surface/volume ratio of the ghosts was very much greater than that of the cells (Hoffman et al., 1958).

Fig. 3 shows that a shrinkage-induced, amiloride-sensitive Na influx can be seen in
both intact cells and resealed ghosts. Grinstein and Smith (1987) showed that amiloride-sensitive Na/H exchange could be activated in resealed ghosts from dog red cells by acidifying the cytosol, but they did not report volume-activated Na/H exchange in the ghost preparation. Their ghosts were lysed at a cell/lysate volume ratio of ~ 1:20, then resealed under physiologically isotonic conditions, so that when suspended in normal saline they would have a hemoglobin concentration 1/20th that of intact red cells. Attempts in our laboratory to activate this transport pathway by shrinkage in resealed dog red cell ghosts with dilute hemoglobin concentrations were unsuccessful until we modified the method of hemolysis and resealing to achieve a high cytoplasmic protein concentration.

Fig. 4 shows the results of duplicate studies in which amiloride-sensitive Na influx was assayed as a function of cell volume in ghosts and cells suspended in media of varying osmolality. The volume, and therefore the volume/surface ratio, at which

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**Figure 2.** Osmotic lysis of cells (open circles, dashed line) and ghosts (filled circles, solid line). The cells/ghosts were suspended in media of varying osmolality (abscissa), incubated at room temperature for 30 min, and the suspensions centrifuged. The hemoglobin concentration (OD$_{540}$) of the supernatant at zero osmolality for each preparation was taken as 100% lysis. Values for 50% lysis are indicated on the abscissa by arrows.

**Figure 3.** Na influx in intact cells (left) and resealed ghosts (right) as a function of cell/ghost dry solids. Control flux values are shown by filled circles and solid lines. Flux values in the presence of 0.5 mM amiloride are shown by the open circles and dashed lines.

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amiloride-sensitive Na transport is activated in ghosts is very much smaller than that of intact cells.

In Fig. 5 the results of the studies shown in Fig. 4 are plotted as a function of dry solids. The dry solids value at which amiloride-sensitive Na transport is activated in both cells and ghosts is \( \sim 30\% \). Comparison of Figs. 4 and 5 indicates that it is the solids content of the ghosts/cells, not their volume, that correlates with the increase in Na/H exchange.

Fig. 6 shows a study in which ghosts containing hemoglobin only were compared with ghosts in which the hemoglobin content was reduced by half, but with the addition of albumin, to give the two ghost preparations roughly the same content by weight of total protein. In preparing the albumin-supplemented ghosts the cells were
hemolyzed in twice the volume used for ghosts unsupplemented with albumin. Before resealing, sufficient NaCl was added to keep the ratio between the mass of total protein and the molar quantity of salt equal to that of the control ghosts and intact red cells. Na/H exchange in the albumin-loaded ghosts was activated at a level of dry solids comparable to the controls. As shown in Fig. 7, electrophoresis of the resealed ghosts reported in Fig. 6 indicated that their albumin/hemoglobin ratio as determined by scanning densitometry (40:60) was close to that of the resealing medium (50:50).

Figure 6. Na influx as a function of dry solids in ghosts containing only hemoglobin (open circles, dashed lines) and a mixture of ~40% albumin and 60% hemoglobin (filled circles, solid lines).

Figure 7. Polyacrylamide gel stained with Coomassie blue showing the albumin (upper band; molecular mass, 68 kD) and hemoglobin monomer (lower band; molecular mass, 16 kD) content of the resealing solution (left lane) and the washed ghost preparation (right lane). The same amount of hemoglobin was loaded in each lane.
**DISCUSSION**

As cells shrink in response to an increase in medium tonicity, all their contents become concentrated, including salts. However, it is known from previous work that activation of Na/H exchange by shrinkage in dog red cells is independent of internal salt concentration and osmolality (Parker, 1983). Thus, if volume is perceived by dint of a change in the chemical activity of some substance in the cell, that substance cannot be salt and must be some other, probably impermeant, cell constituent. To study the influence of impermeant cell contents on the activation of Na/H exchange, we prepared resealed ghosts that at a given osmolality were smaller in volume than intact cells but had roughly the same protein concentration by weight.

The results showed that shrinkage-induced activation of Na/H exchange was not due to decrease in cell volume per se, but rather to an increase in the concentration of the cell contents. All the cell contents were equally diluted during the hemolytic stage of ghost preparation, and all were equally reconcentrated as the resealed ghosts were shrunken by suspension in the incubation media. One can therefore ask which cell components are responsible for the primary signal that ultimately activated Na/H exchange. Some insight into this question can be gained from the observation that Na/H exchange was induced in the albumin-loaded ghosts at the same concentration of dry solids, but at half the concentration of hemoglobin and other cell contents, as in the control ghosts or intact cells (Figs. 5 and 6). Perception of the volume stimulus by the albumin-loaded ghosts was therefore not limited by the concentration of an indigenous cytoplasmic component.

Free magnesium ions have large effects on both Na/H exchange and [K-Cl] cotransport, and it has been suggested that the concentration of free magnesium could mediate volume perception (Starke and McManus, 1990). However, in previous work with dilute ghosts made from dog red cells according to the methods outlined by Schwoch and Passow (1973) at a ghost/hemolyzing volume ratio of ~1:10, no effects on Na transport could be found when the magnesium concentration in the hemolyzing medium was varied from 0.1 to 5 mM (Parker, J. C., and J. O. Wieth, unpublished observations). In intact dog red cells brisk volume-sensitive Na flux was observed after preequilibration of cells with A23187 and magnesium concentrations ranging from 0.01 to 3.0 mM (Parker et al., 1990). Thus we think it is unlikely that perturbations of volume in dog red cells are perceived as changes in the cytosolic free magnesium concentration. Activation of the Na/H exchanger is in our view more likely to be due to the shrinkage-induced increase in cytosolic protein concentration.

A theoretical basis for the effect of protein concentration on membrane transport activation is provided by the concept of macromolecular crowding (Minton, 1983). According to this view, the thermodynamic properties of enzymatic reactions can be altered by high concentrations of inert macromolecules. Minton (1983) showed experimentally that the specific activity of an enzyme (e.g., glyceraldehyde-3-phosphate dehydrogenase) could be greatly influenced by small changes in concentration of various inert proteins within the range seen in living cells (Fulton, 1982). Whether these effects can be explained simply by volume occupancy, or whether they are due to local variations in molecular surface charge or pH is not known, but the
phenomenon has been observed in many systems (Zimmerman and Harrison, 1987; Jarvis, Ring, Daube, and von Hippel, 1990; Sherwin and Winzor, 1990).

If macromolecular crowding can affect the activity of enzymes, and if volume activation of transport involves enzymes such as phosphatases and kinases, as recently suggested (Jennings and Al-Rohil, 1990; Jennings and Schulz, 1991; Parker, Colclasure, and McManus, 1991), then it seems plausible that changes in the specific activities of cytoplasmic or membrane-associated phosphatases and/or kinases might be brought about by concentration and dilution of hemoglobin associated with the shrinking and swelling of red cells. Given the crowded state of the cytoplasm in most cell types (Fulton, 1982), these observations may apply to other examples of volume regulatory behavior.

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