

# Role of Calcium in Volume Regulation by Dog Red Blood Cells

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**ABSTRACT** Dog red blood cells (RBC) are shown to regulate their volume in anisosmotic media. Extrusion of water from osmotically swollen cells requires external calcium and is associated with net outward sodium movement. Accumulation of water by osmotically shrunken cells is not calcium dependent and is associated with net sodium uptake. Net movements of calcium are influenced by several variables including cell volume, pH, medium sodium concentration, and cellular sodium concentration. Osmotic swelling of cells increases calcium permeability, and this effect is diminished at acid pH. Net calcium flux in either direction between cells and medium is facilitated when the sodium concentration is low in the compartment from which calcium moves and/or high in the compartment to which calcium moves. The hypothesis is advanced that energy for active sodium extrusion in dog RBC comes from passive, inward flow of calcium through a countertransport mechanism.

## INTRODUCTION

Mature red blood cells (RBC) from adult dogs lack an ATP-dependent, ouabain-sensitive sodium-potassium pump (3, 5, 6, 10, 15). The process by which these cells control their volume appears to involve calcium-induced active sodium transport (16). This report shows the volume regulatory behavior of dog RBC in anisosmotic solutions. In addition, certain relationships between calcium and sodium movements are described which raise the possibility that the cells have a calcium-sodium exchange mechanism similar to that found in excitable tissues. The role of this system in cell volume regulation is discussed. Some of the findings have been presented in abstract form (17).

## METHODS

Blood from healthy, unmedicated, adult, mongrel dogs was drawn into heparin-rinsed syringes, and all experiments were begun within 15 min of venipuncture. Before all incubations plasma and buffy coat were removed, and the RBC were washed four times with isosmotic NaCl buffered with glycylglycine 5 mM (pH 7.4). Detailed proto-

cols are given with the figure legends. All RBC incubations were at 37°C in suspensions having a cell/medium ratio of  $\frac{1}{10}$  to  $\frac{1}{20}$ . At each sampling time some of the suspension was removed, and in preparation for sodium, potassium, and water measurements the cells were washed four times by repeated centrifugation at room temperature in solutions which contained glycylglycine 5 mM (pH 7.4) plus sufficient NaCl to bring the freezing point of the wash solution to that of the medium in which the cells had been incubated. Methods for cell sodium, potassium, dry weight (DW), and water have been previously reported from this laboratory (13-16).

Cells were prepared for calcium determinations by washing four times at room temperature with a solution containing (mM): NaCl 140, glycylglycine 5, pH 7.35. Sufficient wash solution was then added to the cells to make a suspension with a packed cell volume of 20-30% (determined by microhematocrit). One volume of this suspension was mixed with 4 vol of ultrapure, 0.2 M H<sub>2</sub>SO<sub>4</sub> containing 0.5% Triton X-100. Two microliters of this mixture were then injected into the heated graphite atomizer (HGA 2000) attachment of a Perkin-Elmer 403 Atomic Absorption Spectrophotometer (Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.). The sample was dried for 20 s at 250°C, charred for 20 s at 1,400°C, and atomized for 25 s at 2,500°C. Calcium was read at a wavelength of 422.7 nm. The suitability of this method was established by showing that serial dilutions of RBC gave linear results for calcium and that 103-109% of calcium added to RBC suspensions or hemolysates was recovered.

Table I shows the effect of repeated washes on the calcium content of fresh and calcium-loaded cells. In the subsequent figures all values for calcium were determined after four washes. Cell washing was begun promptly after sampling, and all batches of cells from each time point were treated identically and simultaneously.

## RESULTS

Prolonged incubation of dog RBC in calcium-containing, anisosmotic sodium solutions gave evidence for volume regulation (Fig. 1). In both hyper- and hyposmotic circumstances the values for cell water converge, while sodium

TABLE I  
EFFECT OF REPEATED WASHING ON CELL CALCIUM CONTENT

Number of washes	Calcium	
	Fresh RBC	Calcium-loaded RBC
	<i>mmol/liter cells</i>	
2	0.033	0.640
3	0.027	0.525
4	0.021	0.432
6	0.018	0.400

Fresh RBC compared with cells loaded with calcium by brief alkaline incubation as explained in Fig. 13. Cells washed each time in 10 vol of solution containing (Mm): NaCl 140, glycylglycine 5, pH 7.35.

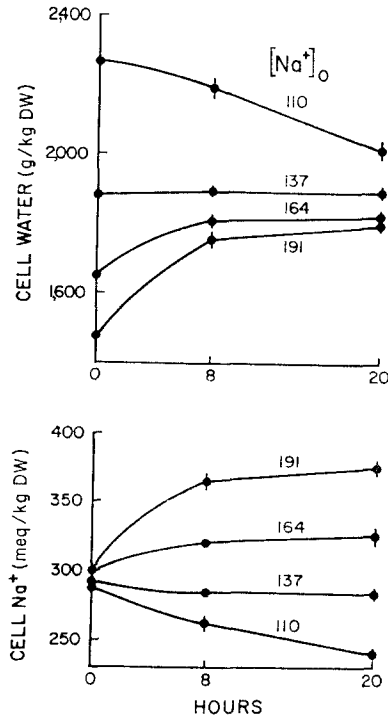


FIGURE 1

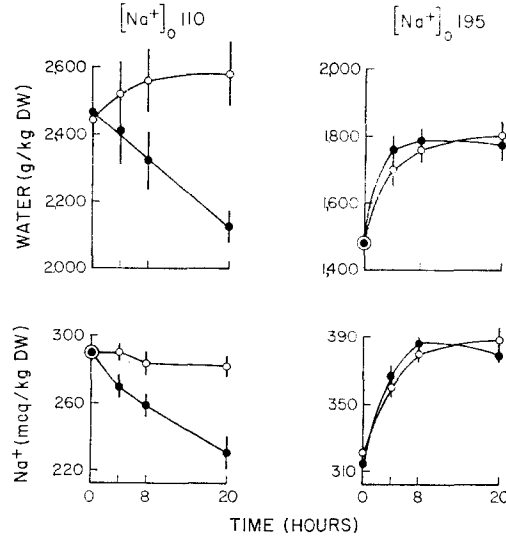


FIGURE 2

FIGURE 1. Cell water and sodium content as a function of incubation time at 37°C. Media contained sodium in the concentrations (millimolar) shown with each line, plus (mM): bicarbonate 25, glucose 10, calcium 5, chloride = anion remainder. All media contained bovine serum albumin 1 g/100 ml, and all suspensions were gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. pH was 7.3-7.4 at 37°C. Mean  $\pm$  SEM for four studies.

FIGURE 2. Cell water and sodium content as a function of incubation time at 37°C, in the presence (solid circles) and absence (clear circles) of external calcium 5 mM. Left-hand panel: hypoosmotic medium (NaCl 110 mM); right-hand panel: hyperosmotic medium (NaCl 195 mM). Other medium constituents as noted in Fig. 1, with or without calcium as noted. Mean  $\pm$  SEM for four studies.

is either lost or gained. Potassium movements amount to less than 5 meq/kg DW, and are not shown.

Fig. 2 shows that while calcium is necessary for the extrusion of sodium and water from osmotically swollen cells, the adjustments to hyperosmotic saline take place equally well whether or not calcium is present.

The studies in Figs. 3 and 4 test whether calcium-induced ion extrusion from swollen cells is specific for sodium. It has been previously reported that millimolar concentrations of external ATP can induce a marked increase of sodium and potassium permeability in dog RBC (14). Because of its reversibility the ATP effect provides a convenient way to alter cell cation composition. Fig. 3 shows that the cells gain sodium and water on incubation for 1 or 2 h in

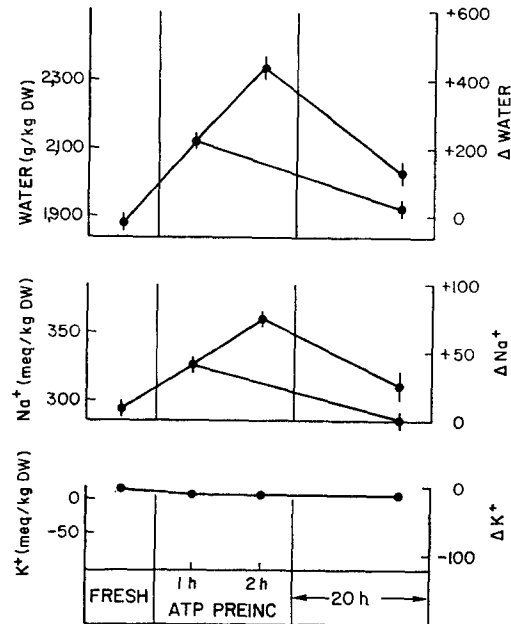


FIGURE 3. Cell water, sodium, and potassium as a function of incubation time in various media. Cells washed and incubated at 37°C in solution containing (mM): ATP 0.7, NaCl 145, glucose 10, glycylglycine 15, pH 7.4. After 1 or 2 h in ATP medium cells washed in NaCl 140, glycylglycine 5, pH 7.4 and then reincubated for 20 h in medium containing NaCl 105, NaHCO<sub>3</sub> 25, glucose 10, CaCl<sub>2</sub> 5, plus albumin 1 g/100 ml. Gassed as in Fig. 1. Mean  $\pm$  SEM for four studies.

isosmotic NaCl with ATP. When the ATP is washed off, and the cells are incubated in a calcium-containing NaCl medium for 20 h, they extrude the ions and water which had entered during ATP treatment. Fig. 4 shows the same procedures done with potassium salts. ATP causes the cells to fill with potassium and water, while sodium is lost to the medium. However, when potassium is the principal cation in both cells and medium, calcium-induced recovery from cell swelling is not seen.

The remaining figures deal with interactions between calcium and sodium ions in dog RBC. To minimize the possibility of extracellular effects due to calcium precipitation or binding, albumin was omitted from all solutions, and various organic buffers were used in place of bicarbonate. Dog RBC become fragile on prolonged incubation when their calcium content is appreciably raised. In all the present studies conditions of pH (25), calcium concentration, and incubation time were selected which resulted in less than 3% cell loss.

Fig. 5 shows that movements of sodium and water in anisomotic media are accompanied by net transfers of calcium between medium and cells. Swollen cells gain calcium at 5 h and then discharge some of it by 20 h. In contrast,

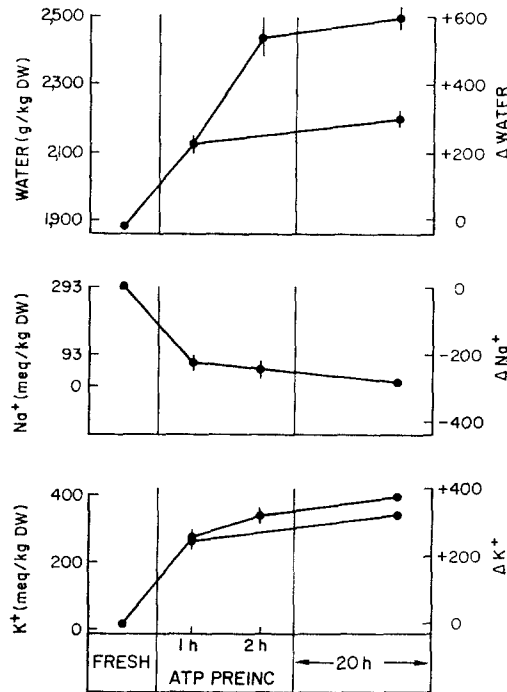


FIGURE 4. Same procedure as for Fig. 3, except that in all incubation and wash media potassium salts were used in place of sodium. Mean  $\pm$  SEM for four studies.

shrunken cells lose a small amount of calcium into calcium-containing media. The observation that calcium accumulation is stimulated by low medium osmolality is confirmed in Fig. 6. The stimulatory effect of hypoosmotic sodium media on calcium gain is sensitive to pH values in the range from 7.0 to 7.5 (Fig. 7), but the pH influence is not seen in isosmotic circumstances. A somewhat similar pH dependence for calcium entry was reported in depleted human RBC (20). Fig. 8 shows that the influence of a low-sodium medium can be diminished by a number of nonsodium solutes. Thus, one effect of lowering the extracellular sodium concentration on calcium accumulation can be ascribed to the associated alteration in osmolality.

To examine whether there was a more specific effect of sodium concentration on calcium movements it was necessary to replace sodium with some other solute. There are not many good cation substitutes for sodium in dog RBC. Unless the alternative cation is placed in both cells and medium, as in Fig. 4, incubation of dog RBC in most isosmotic, sodium-free solutions is attended by prompt sodium loss, cell dehydration, and some lysis (4). This is because sodium is so much more permeant than, for example, potassium, Tris, choline, tetraethylammonium, and divalent cations. However, when sodium is replaced by equimolar amounts of lithium, cell water is unchanged and

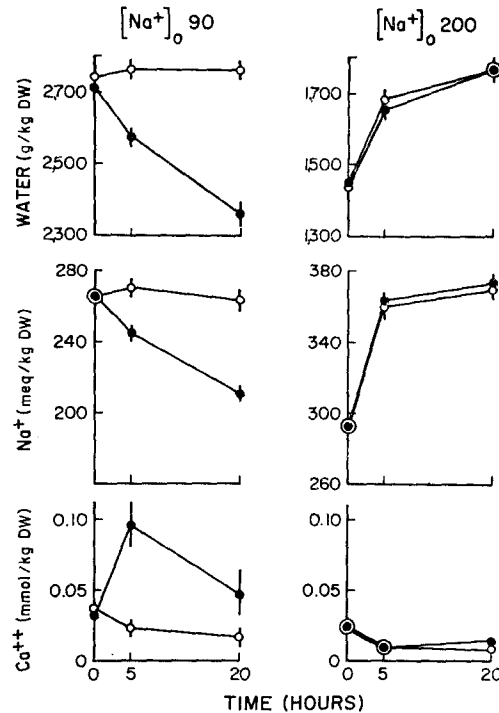


FIGURE 5. Cell water, sodium, and calcium as a function of incubation time at 37°C in the presence (solid circles) and absence (clear circles) of external calcium 5 mM. Left-hand panel: hypoosmotic medium (NaCl 90 mM); right-hand panel: hyperosmotic medium (NaCl 200 mM). Other medium constituents included (mM): KCl 5, HEPES 10, glucose 10, with or without calcium as noted. pH 7.4. Mean  $\pm$  SEM for four studies.

stable: Sodium leaves the cells presumably in exchange for lithium, at a rate of 6%/h in swollen or normal cells and 17%/h in shrunken cells (Fig. 9). Lithium is thus an appropriate replacement ion for sodium where cell volume is an important variable. Fig. 10 shows that in media of the same osmolality, replacement of sodium by lithium salts causes calcium accumulation.

The experiments in Figs. 11 and 12 are concerned with “*trans*” effects of sodium on calcium movements, i.e., the influences of external sodium on calcium loss and of internal sodium on calcium gain. The cells in Fig. 11 were loaded with calcium by incubation in a zero-sodium high-lithium medium. On reincubation in calcium-free solutions, cellular calcium is released more rapidly when the medium contains sodium than when lithium is the principal extracellular cation. Fig. 12 presents studies of calcium accumulation in cells whose sodium content has been altered by preincubation in ATP solutions, as explained in connection with Figs. 3 and 4. Low-sodium cells take up less calcium than their high-sodium counterparts. The effect is particularly striking when the medium is sodium free.

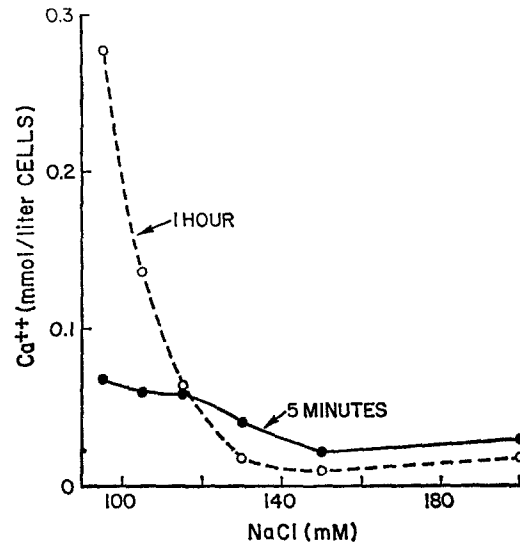


FIGURE 6. Cell calcium after 5-min (solid circles, solid line) and 1-h (clear circles, dashed line) incubations in solutions of various NaCl concentrations, noted on the abscissa. Other medium constituents included (mM): KCl 5, CaCl<sub>2</sub> 5, HEPES 10, Tris 10, glucose 10, pH 7.45. Representative of three studies.

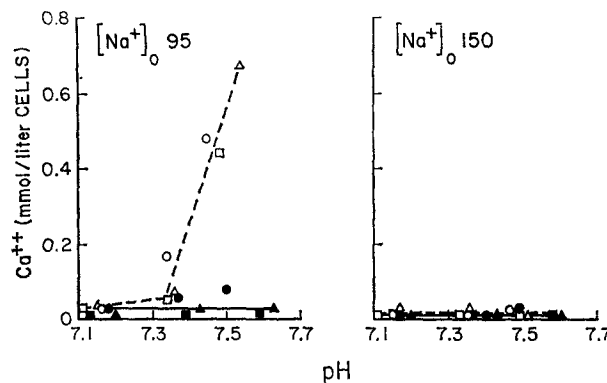


FIGURE 7. Cell calcium after 5-min (solid symbols, solid lines) and 1-h (clear symbols, dashed lines) incubations in suspensions of differing pH values noted on the abscissa. pH measured at each sample time at 37°C. Medium NaCl in left-hand panel was 95 mM; in right-hand panel 150 mM. All other ingredients as in Fig. 6. Data from three experiments (circles, squares, triangles) plotted together.

Figs. 13 and 14 deal with movements of calcium in cells which had been loaded with this cation by brief preincubation in a hypotonic, alkaline medium containing CaCl<sub>2</sub> 5 mM. After a calcium-free wash at physiological pH the loaded cells were reincubated in the absence (Fig. 13) and presence (Fig. 14) of extracellular calcium under a variety of conditions. Medium osmolality had

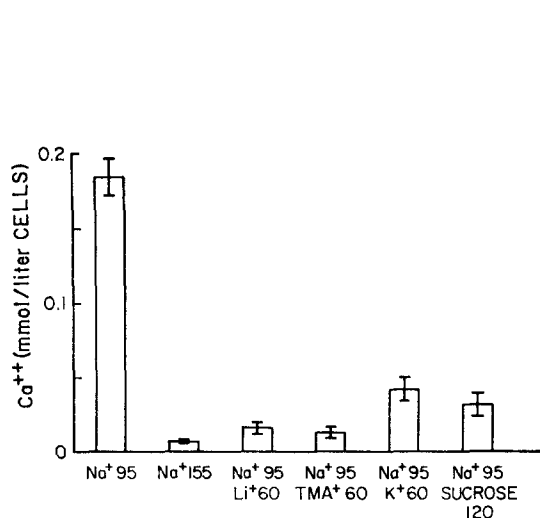


FIGURE 8

FIGURE 8. Cell calcium after 1-h incubation at 37°C in various solutions with compositions noted along the abscissa, all cations as chloride salts. Other medium ingredients as noted in Fig. 6. Mean  $\pm$  SEM for four studies.

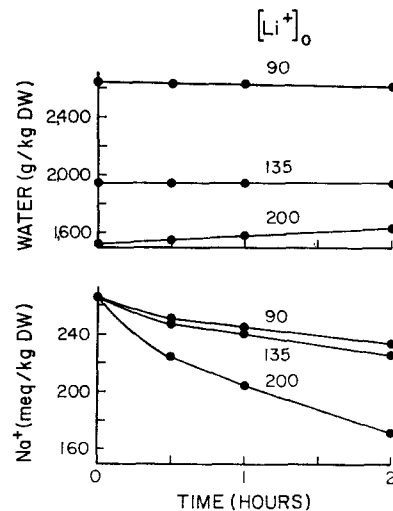


FIGURE 9

FIGURE 9. Water and sodium content of cells incubated in media containing lithium as principal cation in concentrations (millimolar) noted with each line, as a function of time at 37°C. Other medium constituents included (mM): chloride = lithium, KCl 5, HEPES 10, Tris 10, glucose 10, pH 7.4. Representative of three studies.

little effect on calcium efflux into a calcium-free medium (Fig. 13). If anything, the calcium exited faster in a hypoosmotic solution. In the presence of 1.5 mM extracellular calcium, however, hypoosmotic conditions were associated with a slow or negligible calcium efflux (Fig. 14). When the osmolality of the medium was raised with lithium, and to a greater extent when the external sodium concentration was increased, calcium extrusion was stimulated.

#### DISCUSSION

Dog RBC appear to regulate their water content by losing or gaining sodium. Passive movements of sodium in dog RBC are strongly influenced by cell volume: Sodium flux in cells with a low water content is 10–25 times greater than in swollen cells (6, 12, 15, 21). The slow gain in water which occurs in hyperosmotic sodium media (Figs. 1, 2, 5) can be explained by the high sodium permeability of shrunken cells. This process is not influenced by calcium.

In contrast, cells with a high water content regulate their volume by a calcium-dependent process in which sodium is extruded, against an electro-



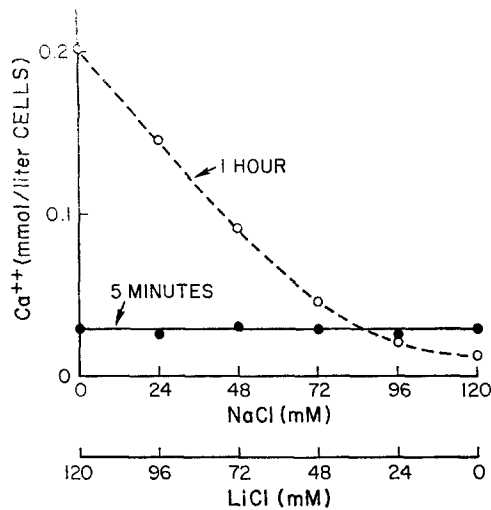


FIGURE 10

FIGURE 10. Cell calcium after 5-min (solid circles, solid line) and 1-h (clear circles, dashed line) incubations in equimolar solutions containing different proportions of NaCl and LiCl as noted on the abscissa. Other ingredients as in Fig. 6. Representative of three studies.

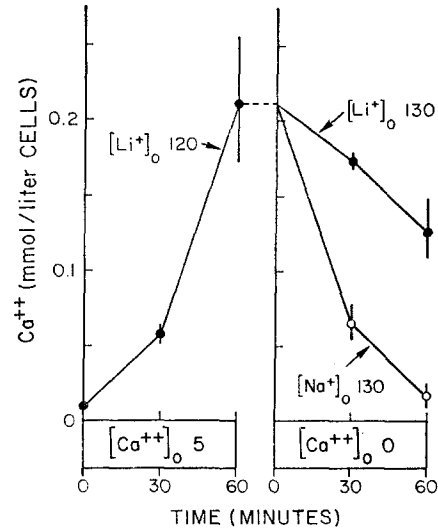


FIGURE 11

FIGURE 11. Cell calcium as a function of time at 37°C. All media had constituents noted in the figure as chloride salts (millimolar), plus KCl, HEPES, Tris, and glucose as noted in Fig. 6. After 1-h incubation in the calcium-lithium solution cells washed and reincubated a second hour period in either lithium or sodium media containing no calcium. Mean and range for three studies.

chemical gradient (16). In this circumstance calcium accumulation is observed (Figs. 5-8).

There are two ways in which calcium might activate sodium transport: (*a*) A mechanism responsive in a scalar sense to calcium concentration might be involved. For example, there might be a contractile protein in the cell membrane which, when activated by calcium at some intracellular or extracellular site, can cause water and sodium to be squeezed out of the cell (7). Or an ATP-dependent sodium pump might exist which requires calcium as a non-transported cofactor. (*b*) Sodium transport might be related in a vector sense to calcium movements. Specifically, passive calcium entry across the membrane might be linked through a countertransport system to active sodium extrusion.

Regarding the first possibility, a contractile mechanism for volume control, dependent on calcium, has been postulated for various tissues, including myometrium (19). If such a system existed in dog RBC it might be expected that, as in myometrium, calcium would work as well in a potassium as in a sodium system. The studies in Figs. 3 and 4, however, show specificity for sodium. The ionophore A23187 promotes contraction in smooth muscle, presumably by

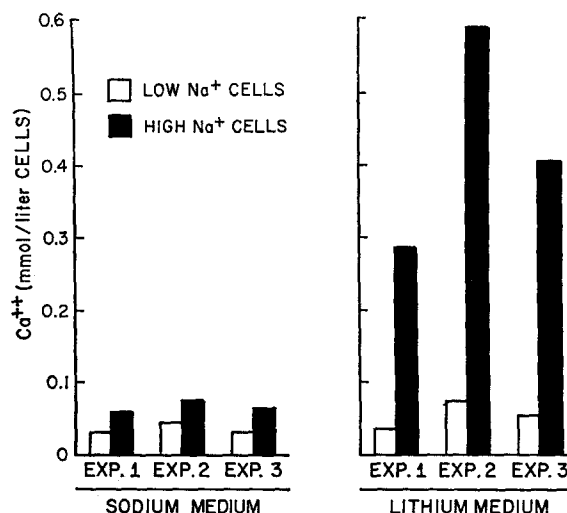


FIGURE 12. Cell calcium after 1-h incubation in calcium-containing media with sodium (left-hand panel) or lithium (right-hand panel) as the principal cation. Cells were pretreated with ATP to alter their sodium content as in Figs. 3 and 4. *Pretreatment procedure:* Cells washed and incubated 1 h at 37°C in calcium-free sodium or lithium buffer with ATP (mM): NaCl or LiCl 145, glycylglycine 15, glucose 10, ATP 0.7, pH 7.4. This produced low-sodium cells (Na 90–96 meq/kg DW), denoted by clear bars, and high-sodium cells (Na 294–300 meq/kg DW), denoted by solid bars. *Incubation with calcium:* Each group of cells was washed and incubated at 37°C in either sodium or lithium media containing (mM): NaCl or LiCl 120, CaCl<sub>2</sub> 5, HEPES 10, Tris 10, glucose 10, pH 7.4. Cell calcium determined after 1 h. Data from three studies plotted together.

facilitating calcium movement into the cell (18). This agent caused prompt swelling and lysis in dog RBC, provided calcium was in the medium, and thus gave no evidence in favor of a contractile process (Parker, unpublished data). The possibility of a calcium-requiring, ATP-dependent sodium pump is not ruled out by the current studies, but we are not aware that such a system has been described.

The alternative explanation, that calcium-sodium countertransport occurs in dog RBC, is supported by observations relating net calcium movements to sodium concentrations in cells and medium: Accumulation of calcium by dog RBC is increased in low-sodium media (Figs. 10, 11, 12), as was also found by Omachi et al. (11). Conversely, the release of calcium into a calcium-free medium is retarded when extracellular sodium is replaced by lithium (Fig. 11). Intracellular sodium also determines calcium movements: high-sodium cells gain calcium faster than do low-sodium cells (Fig. 12). These interactions between calcium movements and sodium concentration are entirely similar to phenomena described in squid axon (1, 2), barnacle muscle (22), and myocardium (9). Calcium movements in either direction across the membrane are faster when sodium is (a) low in the compartment from which calcium is

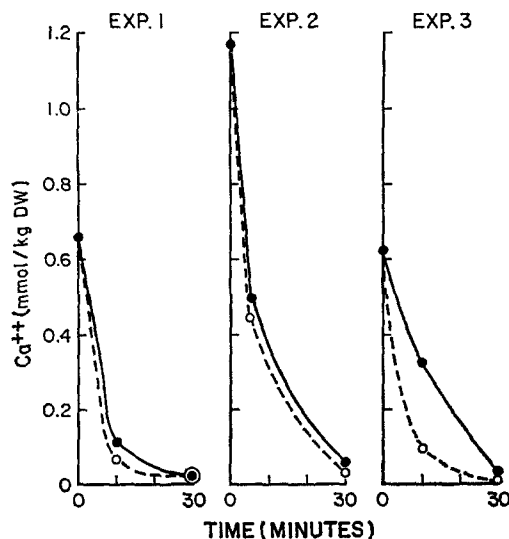


FIGURE 13. Efflux of calcium from calcium-loaded cells into a calcium-free medium at 37°C under hyperosmotic (solid circles, solid lines) and hypoosmotic (clear circles, dashed lines) circumstances. *Calcium loading*: Fresh cells washed twice and incubated 10 min at 37°C in pH 8.4 solution containing (mM): NaCl 125, KCl 5, Tris 5, glucose 10, CaCl<sub>2</sub> 5. To stop calcium loading suspension volume was doubled by addition of a pH 5.0 solution containing NaCl 125, KCl 5, glycylglycine 10, glucose 10. Portion of cells washed for zero time calcium point. *Efflux studies*: Calcium-loaded cells were washed and incubated in calcium-free, hyperosmotic (NaCl 180 mM) or hypoosmotic (NaCl 90 mM) media containing KCl 5, HEPES 10, glucose 10, pH 7.4. Sample times noted on graph; three separate studies.

moving and/or (b) high in the compartment to which calcium is moving. Measurements of unidirectional ion flux in nerve (2) have given rise to the notion that there is a carrier in the cell membrane with sites which are competed for by sodium and calcium. Loading of calcium onto the carrier should be facilitated in the absence of sodium, and unloading of calcium on the opposite surface should be hastened by having sodium there.

If passive calcium entry fuels active sodium extrusion through a counter-transport process, then there must be an independent mechanism whereby RBC use energy to keep intracellular calcium at a low level. The results in Figs. 5 and 14 show that dog RBC can eject calcium into a calcium-containing medium. Inasmuch as the present studies offer no insight into the physical state of calcium associated with dog RBC, it is hazardous to interpret the results in terms of pumps, leaks, and carriers in a two-compartment system. Nevertheless, if all the calcium in the cell were in free solution in the cytoplasm, the net outward movements of calcium shown in Figs. 5 and 14 would be against an electrochemical gradient, assuming the cell interior is electrically negative with respect to the medium. It is possible that some of the energy for active calcium extrusion into sodium solutions (Fig. 14) was de-

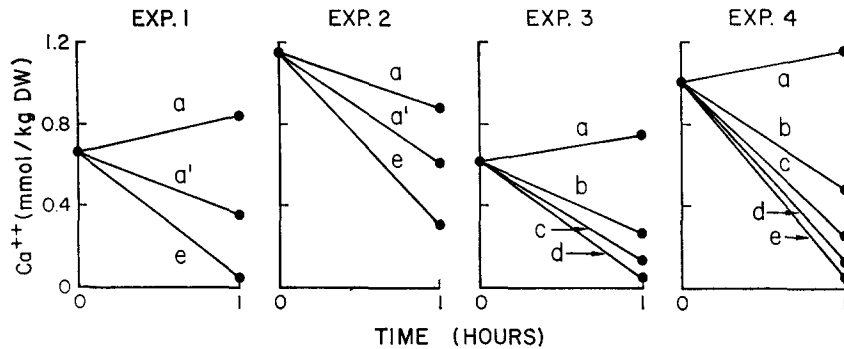


FIGURE 14. Movements of calcium between calcium-loaded cells and a 1.5 mM calcium medium under various conditions. Cells loaded with calcium as described in Fig. 13, then transferred to media containing (mM): (letters refer to lines on graph) (a) NaCl 90, (a') NaCl 90 plus LiCl 90, (b) NaCl 120, (c) NaCl 140, (d) NaCl 160, (e) NaCl 180. All media contained KCl 5, HEPES 10,  $\text{CaCl}_2$  1.5, glucose 10. pH 7.4, four separate experiments.

rived from calcium-sodium exchange, since there is an electrochemical gradient favoring sodium entry (16, 21). The experiments in which LiCl was added to raise the osmolality of a 90 mM NaCl medium are addressed to this point (Fig. 14, experiments 1 and 2, lines *a* and *a'*). With added lithium in the medium the cells shrink (Fig. 9), and the cell sodium concentration must therefore rise to a higher level than in cells exposed to 90 mM NaCl alone. The gradient for sodium entry is thus reduced, and yet net calcium extrusion is stimulated. Therefore, not all of the uphill calcium transport in Fig. 14 is fueled by passive sodium entry. Presumably there is a metabolism-dependent calcium pump (23, 24).

The hypothesis that energy for active sodium transport in dog RBC is derived from passive calcium movements would be strengthened if some kind of stoichiometry could be shown for calcium-sodium exchange. This would require measurements of unidirectional fluxes, which for reasons previously alluded to (16) may be difficult to interpret. There are multiple kinetic compartments for sodium in dog RBC (8), and preliminary experiments with calcium-45 indicate that movements of this isotope are also complex. Until these problems are resolved, it is still not possible to exclude the hypothesis that it is the calcium concentration at some critical site, rather than the movement of calcium across the membrane, which is the important determinant of active sodium transport in dog RBC.

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