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Stimulation of calcium-sodium exchange in dog red blood cells by hemolysis and resealing

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Osmotic hemolysis and resealing greatly increase calcium influx in dog red blood cells. The resealed ghosts show a saturable calcium entry pathway with complex kinetics. As expected for a calcium-sodium exchanger, calcium uptake is stimulated by internal sodium and inhibited by external sodium. Compared to fresh, intact red cells the resealed ghost calcium-sodium exchanger is less responsive to quinidine and to alterations in medium tonicity. The differences in calcium uptake rate among cells from different donors are minimized in the ghost preparation. There are several ways to stimulate sodium-dependent calcium movements in these cells, of which hemolysis-resealing is the most potent. The results of these and previous studies suggest that dog red blood cells have a latent capacity for calcium-sodium exchange.

Introduction

The ability to study a transport system in resealed ghosts present several potential advantages for the red blood cell investigator [1]. Substrates and modulators that do not penetrate the membrane of intact cells can be incorporated into the ghost interior. The dilution of normal cytoplasmic contents during hemolysis may disclose previously unsuspected physiological relationships [2,3]. Questions of membrane asymmetry and sidedness can be addressed [4].

The experiments reported here were undertaken to develop a resealed ghost preparation in which to study the calcium-sodium exchanger in dog red blood cells, which lack Na^+/K^+ pumps and have a high cytoplasmic sodium concentration. Because the maintenance of normal solute and water content in these cells depends on external calcium, a role for calcium-sodium exchange in volume regulation has been postulated. An unexpected finding of the present investigation was that the process of hemolysis and resealing by itself resulted in stimulation of the transporter, suggesting that the mechanism normally functions under restraint [5], and/or that a latent capacity for calcium-sodium exchange exists in these cells. Evidence for the latter hypothesis has been presented elsewhere [6].

Calcium-sodium antiport was demonstrated by Ortiz and Sjodin in a membrane preparation from dog red blood cells [7], but the inside-out vesicles used in that study do not lend themselves to a quantitative comparison of the transport activity in membranes versus intact cells.

Methods

Preparation of resealed ghosts. Ghosts were made by a modification of the method of Schwoch and Passow [8]. Venous blood was drawn from mongrel

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dogs into heparin-rinsed syringes. After centrifugation at 5000 × *g* the plasma and buffy coat were discarded and the cells were washed three times with an ice-cold, unbuffered 165 mM NaCl solution. In some studies the 165 mM NaCl wash was preceded by a wash with a room temperature solution containing 100 mM LiCl, 10 mM Hepes, 5 mM glucose, and 0.1 mM EGTA (pH 7.4 at 37 °C), but this made no difference in the final results. The cells were finally suspended at a cell/medium ratio of about 2:1; 3.0 ml portions of this cell suspension were placed in syringes on ice in preparation for lysis and resealing. Hemolysis was accomplished by injecting 3 ml of the concentrated, ice-cold cell suspension into 200 ml of a 0 °C, constantly stirred hemolyzing medium containing 4.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, and either 1.3 mM acetic acid alone, or (for ATP-fortified ghosts) a combination of 1 mM Na_2ATP and 0.2 mM acetic acid. The pH of the 0 °C hemolysate rose to 5.8–6.2 within 2–3 minutes of adding the cell suspension. After 5 min swirling at 0 °C the hemolysis mixture was supplemented with 37 ml of ice-cold reconstituting solution, which contained 900 mM (sodium, Tris, choline, or *N*-methyl-D-glucamine) chloride plus 3.96 mM Tris base. The pH of the 0 °C hemolysis mixture rose at this point to 7.0–7.3. After five minutes' further swirling at 0 °C the mixture was transferred to a 37 °C water bath-shaker and incubated 30 min, during which resealing took place [9]. The ghosts were pelleted by centrifugation at 40000 × *g* and then washed three times in an ice-cold medium containing 150 mM LiCl, 10 mM Hepes, and 0.1 mM EGTA (pH 7.4 at 37 °C) in preparation for studies of calcium uptake or of ATP content.

In one series of experiments the volumes of hemolyzing medium and reconstituting solution were altered so as to vary the degree of dilution of cell contents during the ghosting process. The precise hemolysis conditions for this study are presented with the results.

The studies of sodium efflux were done on ghosts made by a slight variation of the procedure above, in the interest of conserving radioactive isotope. After the reconstituting solution had been added, and after the 5 min incubation at 0 °C, the hemolysis mixture was centrifuged at 0 °C – a temperature at which the ghosts would remain unsealed. Most of the supernatant medium was removed from the unsealed ghost pellet, and then 10 μCi of $^{22}NaCl$ (Amersham) was added to the ghosts prior to the 37 °C resealing incubation. Calcium uptake studies performed in ghosts made according to this slight variation in the method gave results entirely comparable to those in which resealing was carried out in the larger volume.

Preparation of energy-depleted intact red blood cells. Fresh red cells were washed four times at room temperature in a medium containing 150 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.1 mM EGTA, 5 mM 2-deoxyglucose, and 0.4 g% bovine serum albumin (pH 7.4 at 37 °C). The cells were resuspended in ten times their volume of this same solution, and incubated at 37 °C for about 20 h.

Calcium uptake studies. 1 ml packed ghosts was suspended in 8 ml of flux medium, containing 150 mM (sodium + lithium) chloride, 10 mM Hepes, 0.1 mM EGTA (pH 7.4 at 37 °C), supplemented with various calcium concentrations. At zero time 10 μCi of $^{45}CaCl$ (Amersham, Arlington Heights, IL)) was added to the suspension. At various time points afterwards (indicated with the results) one volume of the ghost suspension was plunged into 3 volumes of ice-cold stopping solution, containing 150 mM LiCl, 5 mM Hepes, and 5 mM EGTA (pH 7.6 at 25 °C). At the end of the flux period the ghosts were pelleted and washed twice with a solution containing 150 mM LiCl, 10 mM Hepes, and 0.1 mM EGTA (pH 7.4 at 37 °C). A portion of the ghost pellet was diluted 100000-fold for enumeration by particle counting (Coulter Electronics, Hialeah, FL). Another portion of ghosts was added to 11 volumes of 12% perchloric acid and the mixture centrifuged. One ml of the clear supernatant was added to 9 ml of Optifluor scintillation fluid (Packard Instruments, Downers Grove, IL) for beta counting, using an internal standard for quench correction. Calcium uptake (amol per ghost per two min) was calculated as the difference in ghost radioactivity between zero and 2 minutes, divided by the specific activity of the calcium in the external medium. This value was multiplied by 0.03 to give the results in fmol/ghost per h. The same procedure was followed for calcium uptake studies in intact red blood cells, but the time over which calcium uptake was measured was 1 h instead of 2 min.

Sodium efflux studies. One-half ml of packed resealed ghosts containing ^{22}Na were injected into 30 ml 37°C efflux media. At 2 and 4 min 5 ml aliquots of ghost suspension were removed, centrifuged at 0°C, 40000 × *g*, and the supernatants removed for counting. The supernatant radioactivity was compared with that of a detergent-lysed sample of ghost suspension, and *f*, the fraction of isotope released from the ghosts, was calculated. The natural log of $(1 - f)$ was a linear function of incubation time, with a slope equal to the rate constant for sodium efflux. The flux itself (fmol/ghost per h) was calculated by multiplying the rate constant times the average amount of sodium in a ghost. This latter quantity was calculated from the sodium concentration of the final hemolysis mixture during the preparation of the ghosts (about 140 fmol/pl) times the average volume of a resealed ghost (0.06 pl).

Assay of ATP in ghosts and intact red blood cells. Aliquots of packed red blood cells or ghosts were prepared as described above for particle counting and sizing. To one volume of these same pellets was added 4 volumes of 10% ice-cold perchloric acid. After centrifugation the supernatant was removed and neutralized with solid K_2CO_3, using phenol red as an indicator. ATP was assayed by enzyme-coupled conversion of NADP to NADPH as described by Lowry and Passonneau [10]. Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Sigma, and NADPH was quantitated with a filter fluorometer (Farrand Optical, New York, NY). ATP content was calculated per ghost or cell and also per liter of packed ghosts or cells, using 60 fl/cell or ghost as a conversion factor.

Results

The basic observation is shown in Fig. 1, where the time course of calcium uptake in resealed ghosts is compared with that of fresh and energy-depleted intact red blood cells. The uptake medium consisted in all cases of an isotonic LiCl solution containing 0.5 mM free calcium. The rate for ghosts (about 10 amol/ghost per min or 0.6 fmol/ghost per h) is several times higher than even the fastest rate in depleted intact cells (1.6 amol/ cell per min or 0.1 fmol/cell per h). In

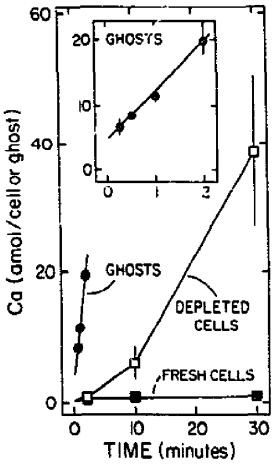

Fig. 1. Time course of calcium entry in high-Na resealed ghosts, energy-depleted intact cells, and fresh intact cells. Fluxes measured in a 150 mM LiCl, 10 mM Hepes medium, containing EGTA 0.1 mM, $CaCl_2$ 0.6 mM, adjusted to pH 7.4 at 37°C. Free calcium of this medium is 0.5 mM. Inset shows replot of data from resealed ghosts on expanded time scale. Mean and range for studies on blood from two dogs.

fresh cells the calcium uptake rate is virtually undetectable over a 30 minute time course. The volume of both resealed ghosts and intact dog red blood cells, as determined on the particle counter, was 60–63 fl, a value that agrees with previous determinations in this species [11]. Thus, calcium uptake rates in this paper can be converted to mmol per liter of cells or ghosts per hour by the following formula:

Uptake (fmol per cell or ghost per h) × 16.6
= Uptake (mmol/l of cells or ghosts per h)

The uptake rate for resealed ghosts in Fig. 1 (0.6 fmol/ghost per h) is therefore equivalent to a value of 10 mmol/liter ghosts per h. This number should be compared with values for fresh, intact, swollen dog red cells incubated in sodium-free, 0.5 mM calcium media of 0.1–0.3 mmol/liter cells per h [6,12].

The kinetics of calcium uptake by resealed ghosts are complex, as shown in Fig. 2. Plots of calcium uptake as a function of external calcium consistently show a point of inflection at around 1 mM $[Ca^{2+}]_o$. An Eadie-Hofstee plot [13] is shown in the inset to Fig. 2, where the data appear to resolve into two components, suggesting that there

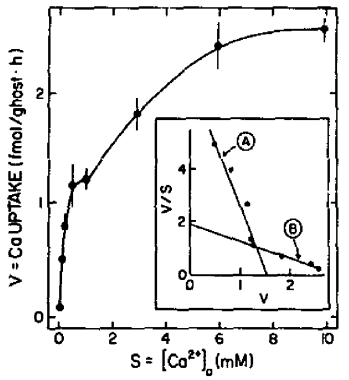

Fig. 2. Calcium uptake as a function of external free calcium in high-Na resealed ghosts. The flux media were Hepes-buffered 150 mM LiCl as noted in the legend to Fig. 1. Inset shows an Eadie-Hofstee plot [13] of the mean values for the data; the lines were computed by linear regression analysis. Values for r of lines A and B are 0.966 and 0.980, respectively. K_m and V_{max} values are given in the text. Points represent mean ± S.E. for five studies at external Ca^{2+} of 0–1 mM and three studies at external Ca^{2+} 2–10 mM.

may be parallel pathways for calcium uptake, one (line A in Fig. 2) with apparent K_m 0.2 mM, V_{max} 1.5 fmol/ghost per h and the other (line B in Fig. 2) with apparent K_m 1.5 mM, V_{max} 2.9 fmol/ghost per h. There may be other explanations for the shape of the curves in Fig. 2, but the relationships described there suggested that interactions between calcium and sodium be surveyed at an external calcium concentration of 0.5 mM.

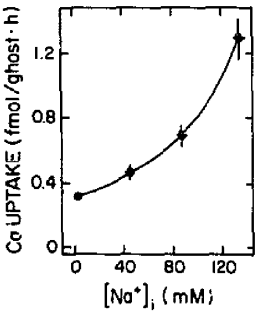

Fig. 3. Calcium uptake as a function of internal Na^+ concentration, the latter measured on the whole hemolysate after addition of the reconstituting medium (see Methods). The flux medium was Hepes-buffered 150 mM LiCl as noted in the legend to Fig. 1, containing 0.5 mM free calcium. $Tris^+$ was used as the non-Na^+ cation substitute in the studies shown, but similar results were obtained with choline$^+$ and *n*-methyl-D-glucamine$^+$ salts. Mean ± S.E. for six studies.

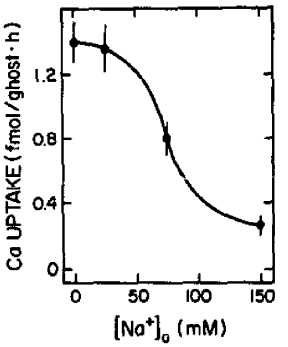

Fig. 4. Calcium uptake as a function of external Na^+ concentration in high-Na^+ resealed ghosts. Lithium was the non-Na^+ cation substitute in the studies shown, but similar results were obtained with $Tris^+$ and choline$^+$ salts. External free calcium was 0.5 mM. Mean ± S.E. for four studies.

The studies shown in Figs. 1 and 2 employed ghosts that were resealed with a high internal Na^+ concentration. Fig. 3 shows that more than a 4-fold decrease in calcium influx is observed when the intra-ghost Na^+ is reduced from 135 to 3 mM.

The studies shown in Figs. 1–3 all employed nominally Na^+-free media. The effect of raising external Na is shown in Figs. 4 and 5. At a constant extracellular free calcium of 0.5 mM, external Na^+ inhibits calcium uptake by 50% at a concentration of about 80 mM (Fig. 4). The effect of external sodium is more pronounced at low than at high calcium concentrations (Fig. 5), but

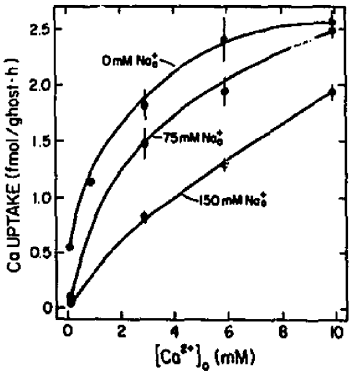

Fig. 5. Calcium uptake in high-Na^+ resealed ghosts, as a function of external free calcium at three different external Na^+ concentrations noted on the graph. Lithium was the non-Na^+ cation substitute. Mean and range for studies from two dogs.

the complexity of the kinetics and the variation in results from day to day preclude a formal analysis of the nature of the inhibitory action of Na in terms of competitive versus non-competitive effects for each component of the system shown in Fig. 2. In two studies at 20 mM external calcium there was no difference in the values for calcium uptake performed in solutions with 0 and 120 mM sodium (data not shown). Taken together, these results suggest that the action of external Na is to reduce the affinity of the mechanism for external calcium, rather than to diminish the maximum turnover rate of the exchanger.

The effect of incorporating ATP into the resealed ghosts was to cut the rate of calcium uptake by about half (Table I). Addition of 1 mM ATP to the hemolyzing medium results in a measured ATP concentration of 10–40 μmol per liter of ghosts, a result consistent with the kinetics of ATP disappearance during resealing in human red blood cell ghosts [14]. It is important to point out that the ATP-supplemented resealed dog ghosts have a final ATP concentration greater than that of energy-depleted intact dog red blood cells, and that therefore the effect of hemolysis-resealing on calcium uptake is not explained by dilution of cytoplasmic ATP.

TABLE I

COMPARISON OF CALCIUM UPTAKE AND ATP CONTENT IN CONTROL GHOSTS, ATP-LOADED GHOSTS, AND ATP-DEPLETED INTACT RED BLOOD CELLS

High-Na ghosts and energy-depleted intact cells were prepared as noted in Methods. Fluxes were done in Hepes-buffered 150 mM LiCl containing 0.5 mM Ca^{2+}. Blood from two different dogs was used. The value of ATP for fresh cells is about 300 μmol/l cells [26].

	Calcium uptake (fmol/ghost or cell per h)	ATP content (μmol/l ghosts or cells)
Dog 503		
Ghosts	0.62	0.4
Ghosts + ATP	0.32	15.2
Depleted cells	0.05	3.1
Dog 787		
Ghosts	1.72	0.5
Ghosts + ATP	0.72	41.3
Depleted cells	0.08	1.6

TABLE II

RESULTS OF USING DIFFERENT HEMOLYSIS RATIOS

High-Na ghosts were prepared at different hemolytic ratios by altering the procedure in Methods. Volumes of cells, hemolyzing medium, and reconstituting solution are noted in the table. The '3:240' ghosts were made exactly as described in Methods. NaCl was added to the hemolyzing media and the reconstituting solutions in such proportions that the NaCl concentration just after hemolysis was in all cases 20–30 mM. After adding the reconstituting solutions all preparations had a NaCl concentration of 137–141 mM. Calcium uptakes were done in Hepes-buffered 150 mM LiCl containing 0.5 mM Ca^{2+}. Blood from two different dogs was used.

Ghosts	Packed cell volume (ml)	Hemolysis medium (ml)	Reconstituting solution (ml)	Calcium uptake (fmol/ghost per h)	
				Dog 63	Dog 787
'3:240'	3	200	37	1.01	1.72
'3:126'	3	100	23	0.92	1.47
'3:68'	3	60	5	1.10	1.60
'3:33'	3	25	5	1.07	1.96

Measurements of sodium efflux in the resealed ghost preparation gave single rate constants and reproducible data, but the values were more than 50-times higher than the values for calcium uptake, and there was no hint of stimulation by external calcium (data not shown). *Trans* stimulation of sodium efflux by external calcium, easily shown in intact dog red blood cells [12,15], is likely obscured in the resealed ghost preparation by much higher background fluxes which can be reduced by various transport inhibitors (e.g., quinidine, phloretin), but not brought down to values that would allow stoichiometric measurements of calcium-sodium counterflow.

Table II shows that the stimulation of calcium-sodium exchange incident to hemolysis and resealing is not critically influenced by varying the volume ratio of ghosts to hemolyzing-reconstituting media over a range from 3:33 to 3:240. Therefore, if the stimulation of calcium-sodium exchange by ghosting is due to a dilution of some intracellular restraining substance, an 80-fold dilution of the latter (in the '3:340' ghosts) gives no more of an effect than does an 11-fold dilution.

Table III compares calcium uptake in fresh red blood cells and resealed ghosts from three dogs, in hypo- and hypertonic media, and in the presence

TABLE III

COMPARISON OF FRESH RED CELLS AND RESEALED GHOSTS WITH RESPECT TO DONOR, MEDIUM TONICITY, AND QUINIDINE

Fresh intact cells and high-Na ghosts prepared according to Methods were incubated in Hepes-buffered LiCl media (either 85 or 200 mM), containing 0.5 mM Ca^{2+}, in the presence and absence of quinidine. Incubation time for the cells was 60 min and for the ghosts 2 min. The three donor dogs were selected because they have differing levels of calcium uptake in intact cells (see text).

	Calcium uptake (fmol/cell or ghost per h)			
	intact cells		resealed ghosts	
	control	quinidine	control	quinidine
Dog No. 787				
LiCl 85 mM (swollen)	0.092	0.007	1.40	0.86
LiCl 200 mM (shrunken)	0.004	0.003	1.29	1.07
Dog No. 503				
LiCl 85 mM (swollen)	0.004	0.001	0.58	0.55
LiCl 200 mM (shrunken)	< 0.001	< 0.001	0.61	0.53
Dog No. 63				
LiCl 85 mM (swollen)	0.017	0.001	1.06	0.87
LiCl 200 mM (shrunken)	< 0.001	< 0.001	0.95	1.10

and absence of quinidine. Calcium uptake in ghosts lacks two features that are seen in the calcium-sodium exchanger of intact red blood cells, namely, a strong inhibition by quinidine [9,12,15], and a responsiveness to alterations in cell (ghost) volume [6,12,15]. A further point addressed in Table III relates to the difference among dogs in the degree of calcium uptake in intact red cells. Dog 503 has almost no quinidine- or shrinkage-suppressible calcium uptake in fresh cells, similar to results obtained in one of our previous donors [16], whereas the other two dogs' cells show a more typical set of responses. The resealed ghost preparation, however, shows much smaller differences among the three dogs.

Discussion

Because dog red blood cells have a calcium pump [9,17], the amount of calcium in the cell at any moment is likely a resultant of the combined activities of passive uptake and active extrusion, although the kinetic relationships of undirectional influx and efflux have been extremely difficult to approach due to the lack of a specific calcium pump inhibitor. In intact dog red blood cells, energy depletion causes an increase in net calcium uptake (Fig. 1), in part because of calcium pump failure [9]. Does the increased initial rate of calcium uptake by resealed dog ghosts come about simply because of reduced pump activity due to dilution of cell ATP at hemolysis? The data in Table I suggest that this is not the explanation. Although addition of ATP to the hemolysate causes some fall in calcium uptake, ATP-repleted ghosts still accumulate calcium at a 5-fold greater rate than energy-depleted intact cells.

What is the evidence that calcium uptake in these resealed ghosts is calcium-sodium exchange? The complex kinetic picture represented by the data in Fig. 2 raises the possibility that more than one calcium transporter is stimulated by hemolysis, or that there are two populations of cells. Of the two saturable pathways suggested by the Eadie-Hofstee plot of Fig. 2, the one that most closely resembles the calcium-sodium exchanger is the one with the higher affinity for external calcium. Its apparent K_m of 0.2 mM is close to the value seen in intact cells [6,15]. When measured at low extracellular calcium concentrations (< 1 mM), calcium uptake by ghosts has two features that are characteristic of a calcium-sodium exchanger: the uptake rate is stimulated by sodium in the ghost interior (Fig. 3), and it is inhibited by

sodium in the external medium (Figs. 4 and 5). Calcium uptake through a calcium-sodium exchanger should, of course, be linked to a $[Ca^{2+}]_o$-dependent sodium efflux. Because of a high baseline Na^+ efflux, it was not possible in the ghost preparation to demonstrate calcium-activated Na^+ extrusion, although this phenomenon can be measured in intact dog red blood cells [6,12,15].

The data in Table III suggest that in the intact red blood cell the calcium-sodium exchanger is activated or regulated by some component of the cell that is distinct from the transporter itself. Calcium uptake through the exchanger of intact red cells is sensitive to cell volume: swelling activates it and shrinking inhibits it [6,12,15] (Table II). Moreover, quinidine inhibits calcium-sodium exchange in intact cells [9,12,15] (Table III). But in resealed ghosts the calcium uptake is not influenced by hyper- or hypotonicity, and the action of quinidine is greatly decreased. A second point in Table III relates to the variation in calcium-sodium exchange activity among blood samples from different dogs. Although most intact dog red cells will show a calcium uptake in hypotonic LiCl of 50–200 μmol/liter cells per h [6,12,16], some dogs' cells have rates much faster than this [15], and an occasional dog will show little or no calcium uptake under optimal circumstances for observing calcium-sodium exchange [16]. Red cells from dog 503, with very little calcium entry in the intact state, showed vigorous calcium uptake once they were ghosted (Table III). Ghosts from this animal showed typical *trans* activation and *cis* inhibition of calcium uptake by sodium (data not shown). It is as if the process of ghosting released the calcium-sodium exchanger from the influence of a regulatory factor that mediates the cell volume and quinidine responses and that accounts for the variability of calcium-sodium exchange activity in intact red blood cells from different dogs.

The calcium-sodium exchanger in dog red blood cells can be stimulated by other agents besides ghosting and cell swelling. Additional activating influences include high pH [15], energy depletion [9], treatment with the mild oxidizing agent, diamide [6], replacement of chloride by nitrate or thiocyanate [12], and incubation of cells with the cardiotonic agent, amrinone [18]. These observations provide additional evidence for the notion that dog red blood cells have a large, latent capacity for calcium-sodium exchange. There is recent evidence that the calcium-sodium antiporter is a multimer held together by disulfide bonds [19]. This, plus the finding that in dog red blood cells a sulfhydryl group on a 35 kDa membrane polypeptide is involved in activation of calcium-sodium exchange [20], suggest that some of the stimulatory influences mentioned above may operate through the assembly of functional transporters from subunits, perhaps by the forging of disulfide bridges [19]. Indeed, some of the striking increase in calcium-sodium exchange seen with energy depletion in dog red blood cells [9] may be due to depletion of reduced glutathione stores with consequent thiol crosslinking. The possibility that hemolysis and resealing of human red blood cells results in oxidation of membrane thiol groups was raised by Dunham and Logue [21], who showed that this procedure causes a severalfold stimulation of the potassium-chloride cotransporter. Their ghosting effect was abolished if dithiothreitol was included in the hemolysate. In the present study dithiothreitol had no effect on calcium uptake, either in intact cells or resealed ghosts (data not shown).

Another transport system affected by hemolysis and resealing is the band 3-mediated anion exchanger, with effects thought due to proteolysis, altered interactions between integral and skeletal membrane components, or influences on the lipid components of the membrane [22].

In other tissues capable of calcium-sodium exchange, a variety of perturbations–phospholipase treatment, exposure to certain fatty acids, limited protein digestion, incubation with redox compounds–stimulate the activity of the transporter [23,24]. These observations, plus data indicating that cytoplasmic ATP can modulate calcium-sodium counterflow [25], suggest that the transport protein is subject to regulation by physiologically important agents.

There are a number of unresolved questions about calcium-sodium exchange in dog red blood cells, such as its stoichiometry, its relationship to the metabolic state of the cell, and the mechanism of its volume-dependence and quinidine sensitivity. The resealed ghost model may be useful for

studying some of these problems, but the high baseline sodium movements represent an important limitation of this preparation. On the other hand, the large calcium fluxes and the ease of preparing the ghosts make them ideal for assaying agents (e.g., antibodies, impermeant metabolites) that might affect the transporter at its cytoplasmic face.

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