J. Membrane Biol. 41, 361–376 (1978)

On the Red Blood Cell Ca^{2+}-Pump: An Estimate of Stoichiometry

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Received 5 December 1977

Summary. Efflux of Ca^{2+} from reversibly hemolyzed human red blood cell ghosts was determined by a Ca^{2+} selective electrode, by atomic absorption spectroscopy, and by the use of ^{45}Ca. Hydrolysis of ATP was determined by measurement of inorganic phosphate (P_i). At 25 °C, ghosts loaded with $CaCl_2$, $MgCl_2$, Na_2ATP, and Tris buffer (pH 7.4) extruded Ca^{2+}, with mean rates ranging from 58.8 ± 3.5 (SD) to 74.7 ± 8.2 (SD) μmoles·liter ghosts^{-1}·min^{-1} depending on the method of Ca^{2+} determination. The ratio of Ca^{2+} transported to P_i released in the presence of ouabain without correction for background ATP splitting was 0.83, 0.83, and 0.80, respectively, for the three methods of Ca^{2+} determination. Correction for the ATPase activity not associated with Ca^{2+} transport resulted in a ratio of 0.91:1. In other experiments, the use of La^{3+} to inhibit the Ca^{2+}-pump allowed an estimate of the ATPase activity associated with Ca^{2+} extrusion. In the presence of various concentrations of La^{3+}, the ratio of Ca^{2+} pumped to P_i liberated was 0.86 or 1.02, depending on the method of Ca^{2+} determination. It is concluded that the stoichiometry of the Ca^{2+}-pump of the RBC plasma membrane is one Ca^{2+} pumped per ATP hydrolyzed.

Red blood cell (RBC) ghosts loaded with Ca^{2+}, Mg^{2+}, and ATP extrude Ca^{2+} against an electrochemical gradient (Schatzmann, 1966; Lee & Shin, 1969; Olson & Cazort, 1969; Schatzmann & Vincenzi, 1969). This active transport of Ca^{2+} is associated with the release of inorganic phosphate (P_i) via a Ca^{2+}-activated, Mg^{2+}-dependent ATPase (($Ca^{2+}+Mg^{2+}$)-ATPase; Schatzmann & Vincenzi, 1969; Schatzmann, 1973, 1975). Attempts to determine the number of Ca^{2+} pumped per ATP hydrolyzed have yielded conflicting reports. Schatzmann and Vincenzi (1969) and Schatzmann (1973) suggested a Ca^{2+} to P_i ratio of 1, whereas Quist and Roufogalis (1975*a*) and Sarkadi, Szász, Gerlóczy and Gárdos (1977) reported a stoichiometry estimate of 2:1. Both the latter groups used La^{3+} to inhibit the Ca^{2+}-pump and showed maximum inhibition of Ca^{2+} transport by La^{3+} was associated with only a 50% inhibition of ATPase activity. The remaining ATPase activity which was not associated with Ca^{2+} extrusion was subtracted from the total ATPase

0022-2631/78/0041-0361 $03.20
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activity in the absence of La^{3+}, yielding an estimated Ca^{2+} to P_i ratio for the pump of 2:1.

The present paper describes an ion-selective electrode method for rapid, continuous assessment of Ca^{2+} efflux from resealed RBC ghosts. In addition, atomic absorption spectroscopy and ^{45}Ca were used to monitor Ca^{2+} transport. Comparison of Ca^{2+} transport rates estimated by these methods with ATP hydrolysis, both in the presence and absence of La^{3+}, resulted in an apparent stoichiometry for the plasma membrane pump of one Ca^{2+} pumped per ATP hydrolyzed.

Materials and Methods

Techniques for preparing resealed RBC ghosts were adapted from previously described methods (Schatzmann & Vincenzi, 1969). Recently outdated RBCs obtained from the blood bank were washed twice in normal saline to remove the buffy coat and once in transporting solution (*see below*). Approximately one-third of the top layer of cells was discarded during each wash. An aliquot of the remaining packed cells was injected rapidly into a tenfold volume of a 0–4 °C hypotonic solution which contained Tris (10 mM; pH 7.4), $MgCl_2$ (4 mM), ouabain (0.1 mM), $CaCl_2$ and Na_2ATP at various concentrations and, when required, ^{45}Ca (final specific activity, sp act $= 9.25 \times 10^5$ dpm/µmole Ca^{2+}). After 135 sec, resealing was initiated by adding a sufficient volume of 3 M KCl to bring the suspension to isotonicity. The suspension was either left on ice for 1/2 hr or warmed rapidly to 25 °C in a 50 °C water bath and kept at 25 °C for 2 min before chilling on ice. Ghosts were then centrifuged at $12{,}000 \times g$ in a Sorvall RC-5 centrifuge for 10 min. Ghosts were washed twice in transporting solution (*see below*), and left on ice from 1 to 3 hr until transport experiments were begun.

Transport experiments were initiated by rapidly warming 2 ml of packed ghosts to 25 °C in a 50 °C water bath and injecting the ghosts into 6 ml of constantly stirred transporting solution. This solution contained NaCl (140 mM), Tris (20 mM; pH 7.4), $MgCl_2$ (4 mM), $CaCl_2$ (1 mM) and ouabain (0.1 mM). Unless otherwise stated, all experiments were conducted at 25 °C. Microhematocrit values were obtained in all experiments by spinning samples 10 min in a Clay Adams Autocrit Centrifuge. The number of ghosts present per ml of suspension was determined using a Coulter Counter model ZB_I.

Ca^{2+} efflux was monitored with the following methods: (i) a Ca^{2+}-selective electrode; (ii) atomic absorption spectrophotometry and (iii) ^{45}Ca measurements. The output of a Radiometer Ca^{2+}-selective electrode (F2112Ca) was connected to a model PHM 64 Radiometer digital pH meter equipped to read pCa directly; adapter A3701. The electrode was calibrated by standards and by standard additions to the transporting solution. The output of the meter was recorded on a Heath Servorecorder. This technique was adapted from a method described by Madeira (1975). Ca^{2+} efflux was calculated from the change in pCa values and the total volume of ghosts determined by the hematocrit. Aliquots of the incubation suspension were removed at predetermined time intervals for additional Ca^{2+} determinations using either an atomic absorption spectrophotometer (IL 251) or a liquid scintillation spectrometer and to measure $[P_i]$. P_i was measured by the method of Fiske and SubbaRow (1925).

Aliquots for determination of Ca^{2+} using atomic absorption spectroscopy were placed in equal volumes of ice-cold transporting solution, centrifuged at $12{,}000 \times g$ for 10 min

and were washed once in ice-cold 0.9% saline containing 0.1 mM EGTA and Tris buffer (10 mM, pH 7.4). Aliquots of washed, packed ghosts were then hemolyzed in distilled water and diluted with 1% $LaCl_3$ to a final 0.4% [La]. Standard additions of $CaCl_2$ were added to each sample to assess [Ca]. All samples were prepared in duplicate.

Aliquots for determining ^{45}Ca efflux were placed in cold (−2 °C) transporting solution (1:2.5 dilution), centrifuged at $12{,}000 \times g$ for 10 min, and washed in the same solution (0–4 °C). Packed ghosts were precipitated in perchloric acid, centrifuged, and aliquots of supernate counted in a Beckman LS 9000 scintillation counter. In experiments designed to assess Ca^{2+} leak, ^{45}Ca (sp act $= 2.4987 \times 10^6$ dpm/µmole Ca^{2+}) and ^3H-inulin (sp act $= 7.948 \times 10^5$ dpm/µg) were added to the incubation suspension 2 min after the addition of ghosts. Aliquots of this ^{45}Ca containing suspension were removed 30 sec, 3 min, and 6 min later and were centrifuged in a Beckman 152 centrifuge for 3 min at 14,000 rpm. Aliquots of packed ghosts and supernate were precipitated in perchloric acid and centrifuged. Aliquots of the supernate were counted for ^3H and ^{45}Ca activity. The ^3H-inulin count was taken as an estimate of the external fluid trapped in the ghost pellet.

In some experiments, red cells were depleted of endogenous ATP prior to hemolysis. This involved preincubation at 37 °C for 15 hr in a solution containing 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM Tris (pH 7.4) and 1 mg/100 ml of chloramphenicol. Other experiments utilized the Ca^{2+} ionophore, A23187. The ionophore was dissolved in 95% ethanol and a final concentration of 2 µM ionophore was used in each instance.

Chemicals: disodium ATP (Sigma), ouabain (Sigma), perchloric acid (Allied), ethylene glycol-bis-(β-amino-ethyl ether) N,N′-tetraacetic acid (EGTA; Sigma), and various salts were of reagent grade. ^{45}Ca and ^3H-inulin were from ICN. Scintillation cocktail (Aquasol) was from New England Nuclear. Ionophore A23187 was a gift from Eli Lilly and Company.

Results

Electrode Determination of Ca^{2+} Efflux and an Estimate of Pump Stoichiometry

The response of the Ca^{2+} selective electrode to the addition of resealed ghosts to the incubation chamber is shown in Fig. 1. The upper tracing shows the response when 2 ml of packed loaded ghosts were added to 6 ml of transporting solution. The ghosts were loaded in medium containing 1 mM $CaCl_2$ and 1 mM ATP, as well as 10 mM Tris (pH 7.4), 4 mM $MgCl_2$, 0.1 mM ouabain, and were resealed with 150 mM KCl. The lower tracing shows the response of the electrode to the addition of ghosts prepared from ATP depleted RBC loaded in the same manner but excluding ATP. The artifacts present in each tracing correspond to the addition of ghosts to the reaction chamber (first artifact) and to the removal of aliquots for P_i determination at time zero (T_0) and 8 min (T_1) respectively. As shown, the electrode stabilizes rapidly after the addition of ghosts. It was necessary to prewarm the ghosts, as noted in *Methods*, to the approximate temperature of the transporting solution

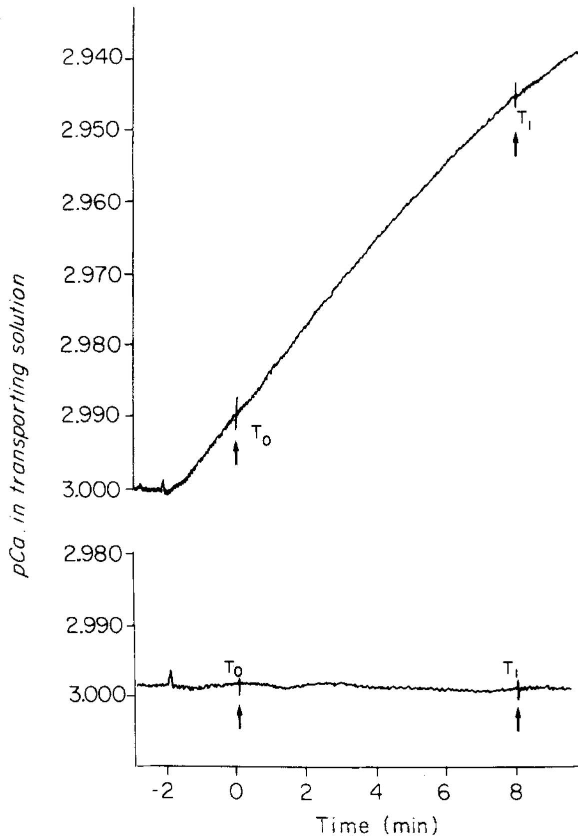

Fig. 1. Ion selective electrode determination of Ca^{2+} efflux from loaded ghosts. The pCa change in the transporting solution was recorded as a function of time. Two ml of prewarmed ghosts loaded with either 10 mM Tris (pH 7.4), 4 mM $MgCl_2$, 1 mM ATP and 1 mM $CaCl_2$ (upper tracing) or the same solution without ATP (lower tracing) were injected into 6 ml of transporting solution containing (in mM) 140 NaCl, 20 Tris (pH 7.4), 4 $MgCl_2$, 1 $CaCl_2$, and 0.1 ouabain at 25 °C with a final hematocrit of approximately 24%. The first artifact was caused by addition of ghosts. T_0 and T_1 are artifacts caused by removal of aliquots for P_i determinations

or a serious temperature artifact resulted. This figure shows clearly the dependence of this process on an energy source such as ATP.

Figure 2 presents data from 25 experiments at 25 °C. The mean amount of Ca^{2+} pumped per liter of ghosts is plotted as a function of time after removal of the first aliquot (T_0). These data and the data shown in Table 1 were obtained from Ca^{2+} loaded ghosts prepared from both ATP depleted and nondepleted, outdated RBC. Ghosts represented in Fig. 2 were resealed entirely in the cold or with an additional warming step (*see Methods*). Since the pump rates and P_i liberation rates for

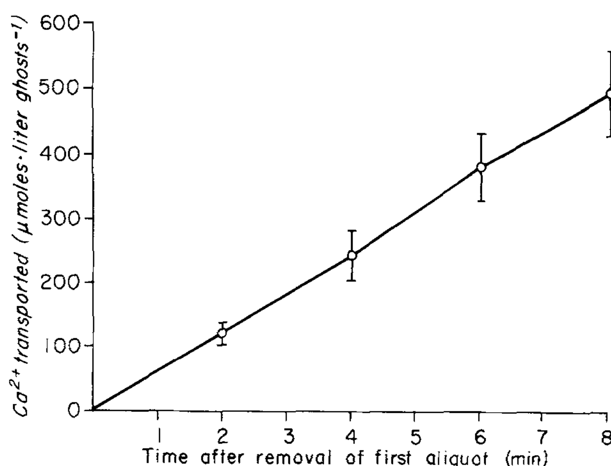

Fig. 2. Time course of Ca^{2+} transport from resealed RBC ghosts. The quantity of Ca^{2+} pumped (μmoles·liter ghosts^{-1}) as determined by the ion selective electrode plotted as a function of time after removal of first aliquot (T_0; *see Methods*). Resealed RBC ghosts were prepared as described in Fig. 1. Results represent data from 25 experiments. Vertical bars represent the standard deviation

Table 1. Ca^{2+} transport and ATPase activities in RBC resealed ghosts

Ghosts loaded with:	N	(μmoles·liter ghosts^{-1}·min^{-1})		Ca^{2+}/P_i
		Ca^{2+} transported	P_i liberated	
1 mM ATP 11 mM Ca^{2+}	25	62.8±8.1[b]	76.5±3.5	0.83±0.04[a]
1 mM ATP no Ca^{2+}	4	–	2.6±1.0	–
no ATP 1 mM Ca^{2+}	4	0	0	–

Values represent mean ±SD. All measurements are at 25 °C.

[a] Value not corrected for "background" P_i liberation.

[b] Determined by Ca^{2+}-selective electrode.

resealed ghosts prepared by these different methods were not significantly different ($P>0.10$), the data were pooled. As shown in Fig. 2, the rate of Ca^{2+} transport for ghosts at 25 °C was linear for at least 8 min. Therefore, P_i determinations were made on samples taken during this interval. Also, maximum pump rates were observed during this interval with ghosts containing 1 mM Ca^{2+} and 1 mM ATP. Further increases in $[Ca^{2+}]_{in}$ or $[ATP]_{in}$, or both, increased only the length of time that

the transport rates remained linear (not shown). Ghosts incubated at 37 °C pumped approximately 2.8 times as rapidly as ghosts at 25 °C (not shown). An Arrhenius plot of pump rates at 25, 30 and 37 °C resulted in a calculated energy of activation (*Ea*) of 15.5 $Kcal \cdot mole^{-1}$.

Table 1 presents a summary of transport experiments using the electrode method to determine Ca^{2+} transport. Ghosts loaded with ATP, $MgCl_2$, $CaCl_2$, and Tris (pH 7.4) pumped an average 62.8 µmoles $Ca^{2+} \cdot$ liter $ghosts^{-1} \cdot min^{-1}$. This corresponds to a rate of 3.86×10^6 Ca^{2+} ions per single ghost per min with a mean ghost volume of 102 fl (calculated from microhematocrit and count). These same ghosts liberate 76.5 µmoles $P_i \cdot$ liter $ghosts^{-1} \cdot min^{-1}$. This gives a Ca^{2+} to P_i ratio of 0.83:1. Ghosts loaded with ATP and 0.1 mM EGTA but no $CaCl_2$ (Table 1) liberated 2.6 µmoles $P_i \cdot$ liter $ghosts^{-1} \cdot min^{-1}$. We take this rate as an estimate of the ouabain-insensitive Mg^{2+} activated ATPase (Mg^{2+}-ATPase) activity not associated with Ca^{2+} pumping (ouabain present in all experiments). When this estimate of the Mg^{2+}-ATPase activity is subtracted from the total ATPase activity shown in Table 1, the ratio of Ca^{2+} transported to P_i released is 0.85:1. It should be noted that this estimate of Mg^{2+}-ATPase activity does not include any ($Ca^{2+} + Mg^{2+}$)-ATPase activity not associated with the pump. It also does not include a possibly elevated ATPase activity due to Ca^{2+} back leak into the ghosts. Although previous workers (e.g., Schatzmann & Vincenzi, 1969) showed that resealed RBC ghosts are tight to Ca^{2+}, an additional estimate of background Mg^{2+}-ATPase activity plus ATPase activity due to Ca^{2+} back leak was made. Ghosts loaded with 1 mM $CaCl_2$, 2 mM ATP, 4 mM $MgCl_2$ and 10 mM Tris (pH 7.4) were incubated at 25 °C for 20 min. At this time, transport was complete, as indicated by the fact that the electrode output had reached a stable plateau. The rate of P_i liberation during this post-transport plateau reflects the Mg^{2+}-ATPase activity plus any ($Ca^{2+} + Mg^{2+}$)-ATPase activity due to Ca^{2+} leak. A rate of 7.8 ± 1.6 (SD) µmoles $P_i \cdot$ liter $ghosts^{-1} \cdot min^{-1}$ was found. Subtracting this value from the total ATPase activity (76.5 µmoles $\cdot$ liter $ghosts^{-1} \cdot min^{-1}$; Table 1) yields a P_i liberation rate of 68.7 µmoles $\cdot$ liter $ghosts^{-1} \cdot min^{-1}$. When compared with a Ca^{2+} transport rate of 62.8 µmoles $\cdot$ liter $ghosts^{-1} \cdot min^{-1}$ produces a Ca^{2+} to P_i ratio of 0.91. A low ATPase activity during the post-transport plateau implies that the ghosts were impermeable to Ca^{2+}. This is further supported by the fact that the addition of ionophore A23187 (2 µM) during post-transport plateau resulted in a rapid increase in P_i liberation (40.0 µmoles $P_i \cdot$ liter $ghosts^{-1} \cdot min^{-1}$ over a 6 min period).

The possibility remained that these ghosts were leaky to Ca^{2+} initially and became tight during incubation at 25 °C. Thus, a stoichiometry of 2 Ca^{2+}/ATP could resemble 1 Ca^{2+}/ATP if a sufficient amount of the Ca^{2+} pumped out leaked back in. To test this, resealed ghosts were loaded with 2×10^{-5} M $CaCl_2$ as an aid to resealing (Schatzmann, 1973), 1 mM ATP, 4 mM $MgCl_2$ and 10 mM Tris (pH 7.4) and were suspended in the transporting solution with or without 1 mM $CaCl_2$. Due to the activation of the ($Ca^{2+}+Mg^{2+}$)-ATPase, ghosts leaky to Ca^{2+} and suspended in the Ca^{2+} solution would split more P_i than ghosts suspended in the Ca^{2+} free solution. This increased P_i liberation rate should be proportional to the Ca^{2+} influx rate. Ghosts incubated in 1 mM $CaCl_2$ liberated 15.6 ± 2.9 (SD) µmoles $P_i \cdot$liter ghosts$^{-1} \cdot$min^{-1}, whereas ghosts incubated in Ca^{2+} free medium liberated 8.9 ± 1.3 µmoles $P_i \cdot$liter ghosts$^{-1} \cdot$min^{-1}. The difference between these two groups (6.7 µmoles·liter ghosts$^{-1} \cdot$min^{-1}) is presumed to be the ATP splitting due to Ca^{2+} leak. This P_i liberation rate is approximately 10% of the total ATPase activity shown in Table 1 and is indicative of the fact that resealed RBC ghosts are somewhat leaky to Ca^{2+}. If the stoichiometry is corrected for this leak, assuming 1 Ca^{2+} pumped per ATP split, a Ca^{2+} to P_i ratio of 0.93 is obtained.

Further experiments designed to test the leakiness of ghosts to Ca^{2+} were conducted using ^{45}Ca. Ghosts resealed both in the cold and with the additional warming step (*see Methods*) were used. Washed, outdated red cells were also used in some experiments. Ghosts were prepared and treated as before with a small volume of ^{3}H-inulin and ^{45}Ca solution added 2 min after addition of ghosts to the chamber. This corresponds to time T_0 when the first aliquot was normally removed. Aliquots of the tracer containing suspension were then removed 30 sec, 3 min, and 6 min later (*see Methods*). As shown in Fig. 3, washed cells took up virtually no ^{45}Ca. Resealed ghosts initially took up only slightly more. The addition of ionophore A23187 (2 µM) caused a rapid uptake of ^{45}Ca in resealed ghosts. These data suggest that a small percentage of ghosts were leaky to Ca^{2+} but not inulin with rapid Ca^{2+} equilibration taking place. The majority of ghosts appeared impermeable to both Ca^{2+} and inulin. Comparison of the apparent volume of distribution for ^{3}H-inulin in the ghost pellet with that for ^{45}Ca allowed an estimate of the percentage of ghosts that were permeable to ^{45}Ca but not ^{3}H-inulin. This value was calculated to be 9.1%, assuming that the ghosts permeable to ^{45}Ca were highly permeable and the remainder impermeable or, at least, immeasurably permeable.

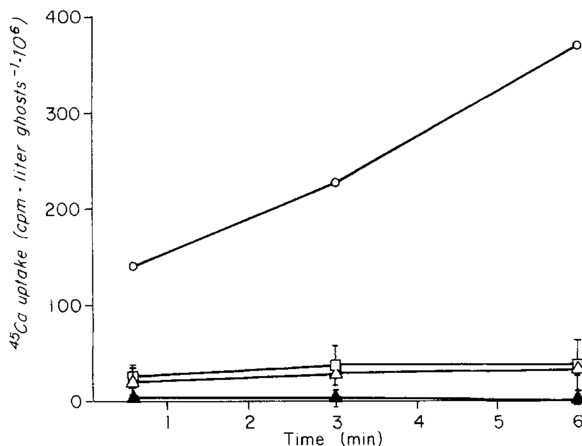

Fig. 3. Time course of ^{45}Ca uptake in RBC's and RBC ghosts. ^{45}Ca uptake was determined in washed red blood cells (▲), ghosts resealed with warming step (△; *see Methods*), ghosts resealed at 0–4 °C and incubated in the absence (□) and presence (○) of 2 μM A23187. ^{3}H-inulin was taken as a zero uptake reference. Ghosts were loaded and suspended as described in Fig. 1. Two minutes after addition of ghosts to the incubation chamber, a small volume of ^{45}Ca (sp act=2.4987 × 10^{6} dpm/μmole Ca^{2+}; 96% efficiency) and ^{3}H-inulin (sp act=7.948 × 10^{5} dpm/μg) was added. Aliquots were removed at 30 sec, 3 min, and 6 min later and centrifuged. Packed ghosts were precipitated in perchloric acid, centrifuged, and aliquots of supernate counted. Vertical bars represent the SD from 3 experiments

Lanthanum Inhibition of Ca^{2+} Extrusion

Lanthanum was tested and found to be an effective inhibitor of the Ca^{2+}-pump as previously reported (Quist & Roufogalis, 1975*a*; Larsen & Vincenzi, 1977; Sarkadi *et al.*, 1977). Lanthanum interfered with

Table 2. A comparison of Ca^{2+} transport stoichiometry estimates in RBC resealed ghosts using three methods of Ca^{2+} determination

	(μmoles · liter ghosts^{-1} · min^{-1})		
	Atomic absorption (n=4)	Ca^{2+} electrode (n=25)	^{45}Ca efflux (n=4)
Ca^{2+} pumped	74.7 ±8.2	62.8 ±8.1	58.8 ±3.5
P_i liberated	95.8 ±6.2	76.5 ±3.5	73.5 ±5.0
Ca^{2+}/P_i[a]	0.83±0.10	0.83±0.05	0.80±0.05

Values represent mean ±SD. All measurements are at 25 °C.

[a] Not corrected for background P_i liberation.

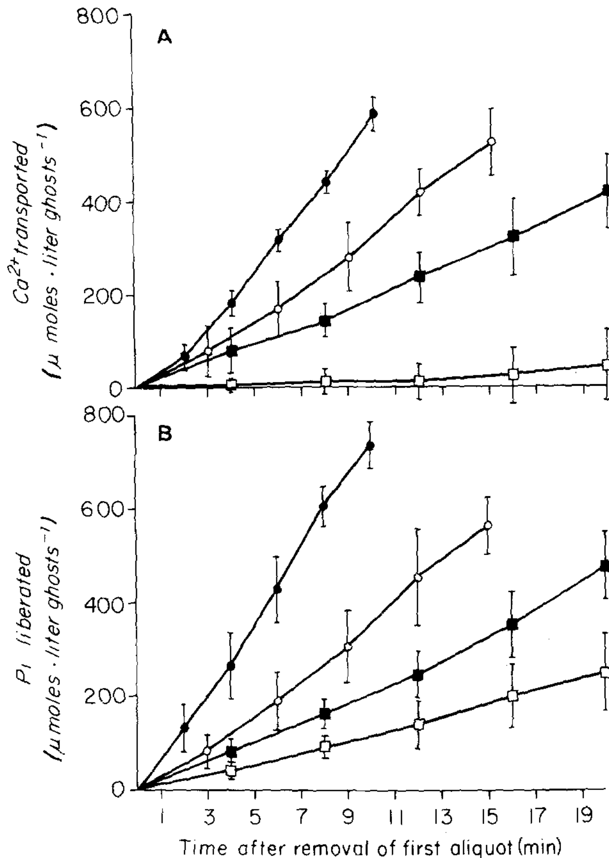

Fig. 4. Ca^{2+} transport and P_i liberation in the presence of various concentrations of $LaCl_3$. Amounts of Ca^{2+} pumped (*A*) and P_i split (*B*) per liter of ghosts are plotted as a function of time after removal of the first aliquot (T_0). Ca^{2+} determinations were made by measuring ^{45}Ca flux. Ghosts loaded with (in mM) 10 Tris (pH 7.4), 4 $MgCl_2$, 2 ATP, 2 $CaCl_2$ and ^{45}Ca were resealed with KCl and the additional 25 °C warming step (*see Methods*). Ghosts were incubated at 25 °C in transporting solution that contained no La^{3+} (●), 50 μM La^{3+} (○), 100 μM La^{3+} (■) and 250 μM La^{3+} (□). Aliquots for Ca^{2+} and P_i determinations were removed at times indicated by the points and were placed into a large volume of cold (−2 °C) transporting solution (1:2.5 dilution). The suspension was centrifuged and washed with cold transporting solution and re-centrifuged. Aliquots of packed ghosts were vigorously shaken in perchloric acid, centrifuged, and samples of supernate were tested for ^{45}Ca and P_i. Each point is the mean of four experiments. Vertical bars represent the SD

Ca^{2+} determinations by the Ca^{2+}-selective electrode, and Ca^{2+} efflux was therefore determined in the presence of La^{3+} by atomic absorption spectroscopy or ^{45}Ca. Table 2 lists the Ca^{2+} transport rates (obtained with three methods of Ca^{2+} determination), the corresponding ATPase activities (based upon a single method of determination) and apparent stoichiometries. The rates are not identical but are comparable.

Figure 4*A* shows Ca^{2+} transport at various concentrations of La^{3+} plotted as a function of time after removal of the first aliquot. These experiments used ghosts loaded with 2 mM $CaCl_2$, 2 mM ATP, 4 mM $MgCl_2$, and 10 mM Tris (pH 7.4) and resealed with KCl and the additional warming step (*see Methods*). As shown, ghosts pumped Ca^{2+} at all La^{3+} concentrations except at 250 µM, where almost complete inhibition was noted. Corresponding ATP hydrolysis is shown in Figure 4*B*. ATPase inhibition closely paralleled the inhibition of Ca^{2+} transport at all La^{3+} concentrations tested. At 250 µM, La^{3+} produced 95% inhibition of the pump, whereas ATPase activity was inhibited approximately 80%. The relationship between Ca^{2+} pumped and ATP split in the presence of various concentrations of La^{3+} is presented in Fig. 5. Each data point represents a single transport determination. The error bars represent the means (intercept) and 95% confidence intervals (*CI*) for data obtained at each La^{3+} concentration. When plotted in this fashion, the slope of the regression line reflects the Ca^{2+} to P_i ratio for the pump and the intercept reflects the La^{3+}-insensitive ATPase activity. A slope of

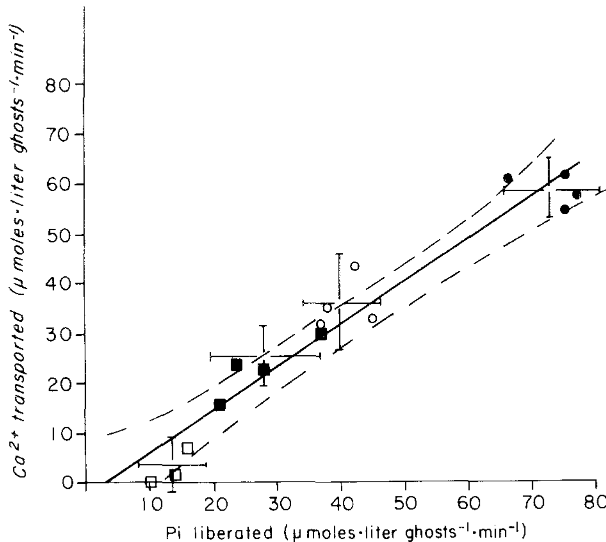

Fig. 5. Correlation between Ca^{2+} transport and P_i liberation in resealed RBC ghosts incubated in the presence of various concentrations of $LaCl_3$. In each experiment, the initial Ca^{2+} transport rate and the initial P_i liberation rate was determined. Each point in this figure represents the two values determined in a single experiment. Means of four experiments are presented as a function of time in Fig. 4. The symbols represent $LaCl_3$ concentrations as in Fig. 4. The error bars represent the means (intercept) and 95% confidence intervals for the data obtained at each La^{3+} concentration. The slope of the line is 0.86 and is indicative of the apparent stoichiometry. The abscissa intercept represents the La^{3+}-insensitive ATP splitting rate

0.86 ± 0.14 (95% *CI*) with a La^{3+}-insensitive ATP splitting rate of 4 µmoles $P_i \cdot$liter ghosts$^{-1} \cdot$min^{-1} was obtained in Figure 5. In four similar experiments using atomic absorption spectroscopy to determine Ca^{2+} transport in the presence of La^{3+} a slope of 1.02 ± 0.09 (95% *CI*) was found.

Discussion

The present results demonstrate the usefulness of a Ca^{2+} selective electrode for determining Ca^{2+} flux from resealed RBC ghosts. Pump rates determined by the electrode and by atomic absorption spectroscopy or ^{45}Ca measurements were in good agreement. A major advantage of the electrode method is the continuous recording of the change in free Ca^{2+} activity in the extracellular medium. A drawback to this method is that certain ions, such as La^{3+}, interfere with the measurement of Ca^{2+}.

The maximum Ca^{2+} extrusion rate found in our preparation (65 µmoles ·liter ghosts$^{-1} \cdot$min^{-1} at 25 °C) was somewhat higher than previous reports for incubations at 25 °C (Table 3). Sarkadi, Szász and Gárdos (1976) reported a rate of 85 µmoles·liter cells$^{-1} \cdot$min^{-1} at 37 °C for red blood cells loaded with Ca^{2+} by ionophore A23187. In their follow-up study, Sardaki *et al.* (1977) reported an *Ea* of 15.2 Kcal·mole^{-1} and a transport rate of approximately 30 µmoles Ca^{2+}·liter ghosts$^{-1} \cdot$min^{-1} at 25 °C. Data of Quist and Roufogalis (1975*a*), as calculated by Sarkadi *et al.* (1976), gave a rate of 40 µmoles Ca^{2+}·liter ghosts$^{-1} \cdot$min^{-1} at 37 °C, and a rate of 16 µmoles·liter ghosts$^{-1} \cdot$min^{-1} at 25 °C (Table3). In our hands, resealed RBC ghosts pump approximately 170 µmoles

Table 3. A comparison of Ca^{2+} transport rates and *Ea* values for the RBC plasma membrane as determined by various authors

	Quist & Roufogalis (1975^{a-b})	Sarkadi et al. (1977)	Schatzmann (1973)	Present study
Ea[a]	14.4	15.2	25.03	15.5
Transport 25 °C[b]	16[c]	31[c]	97[c]	65
Transport 37 °C[b]	40[c]	85	502[c]	171

[a] kcal·mole^{-1}.
[b] µmoles Ca^{2+}·liter ghosts or cells$^{-1} \cdot$min^{-1}.
[c] Calculated from their data.

Ca^{2+}·liter ghosts^{-1}·min^{-1} at 37 °C and were calculated to have an *Ea* of 15.5 kcal·mole^{-1} (Table 3). This *Ea* agrees with the 15.2 kcal ·mole^{-1} determined by Sarkadi *et al.* (1977) and the 14.6 kcal·mole^{-1} calculated by Quist and Roufogalis (1975*b*). However, the above pump rates are considerably less than the rates reported by Schatzmann (1973). In ghosts resealed at 25 °C and incubated at 28 °C, he observed a Ca^{2+} efflux rate of 148 μmoles·liter ghosts^{-1}·min^{-1}. Considering an *Ea* of 25.03 (Schatzmann & Vincenzi, 1969), this corresponds to a rate of 502 μmoles·liter ghosts^{-1}·min^{-1} when calculated for 37 °C (Table 3).

Reasons for these quantitative discrepancies in rate are not known, but differences in preparations are likely. Quist and Roufogalis (1975*a*) subjected red cells to repeated hemolysis prior to resealing. Our experience with their procedure suggests that as many as 60% of the ghosts do not completely reseal (*unpublished observations*). This could explain why their rates were low. However, we prepared ghosts using their procedure from recently outdated cells, whereas they used fresh cells.

It would seem that the most physiological preparation would be that described by Sarkadi *et al.* (1976). These authors loaded Ca^{2+} into fresh red blood cells by treatment with the Ca^{2+} ionophore, A23187. The ionophore was subsequently removed by washing with albumin to which A23187 binds. They were able to achieve total $[Ca]_{in}$ of up to 3 mM with reportedly no detectable membrane permeability to ^{45}Ca after removal of the ionophore. However, their preparation was admittedly complex and required the addition of iodoacetamide and Na^{+}-tetrathionate to inhibit metabolic pathways. Since sulfydryl reagents are known to inhibit Ca^{2+} efflux (Schatzmann, 1975; Sarkadi *et al.*, 1977), the addition of these agents could inhibit Ca^{2+} extrusion. These authors apparently did the necessary controls and reported that at concentrations used these compounds had no effect on Ca^{2+} efflux. However, they did not describe the conditions of the control experiments.

Differences in internal pH might explain the disparity in Ca^{2+} transport rates reported. We used outdated cells that were reversibly hemolyzed in 10 mM Tris (pH 7.4), whereas Sarkadi *et el.* (1977) used fresh, intact cells. Differences in membrane potential and/or hemoglobin concentration would result in a different internal pH. Sarkadi *et al.* (1977) showed increasing pH produced higher pump rates.

The $[Ca^{2+}]_{in}$ necessary to achieve maximal activation of the Ca^{2+}-pump is somewhat controversial. Using ghosts loaded with Ca^{2+}-EGTA buffers, Schatzmann (1973) reported that maximum activation occurred at 10^{-5} M Ca^{2+} or lower. Sarkadi *et al.* (1977) reported maximum activa-

tion required 1 mM internal Ca^{2+}. Sarkadi *et al.* (1977) suggested that this finding supported the hypothesis of Quist and Roufogalis (1975*b*) that the low affinity ATPase (also described by Wolf, 1972) is the Ca^{2+}-pump. However, Sarkadi *et al.* (1976) showed results with maximum activation still present at a $[Ca^{2+}]_{in}$ of 0.2 mM. Our own results suggest that maximum activation of the pump is still present at $[Ca^{2+}]_{in}$ of 10^{-4} M. However, neither we nor Sarkadi *et al.* (1977) used Ca^{2+}-EGTA buffers and the actual $[Ca^{2+}]_{in}$ necessary to achieve maximal activation of the pump could be much lower.

Our results are compatible with a stoichiometry for the RBC Ca^{2+}-pump of 1 Ca^{2+} pumped per ATP hydrolyzed. This differs from the reports of Quist and Roufogalis (1975*a*) and Sarkadi *et al.* (1977). In each case, La^{3+} was used to inhibit the Ca^{2+}-pump. Under maximal pump inhibition by La^{3+}, Quist and Roufogalis (1975*a*) and Sarkadi *et al.* (1977) observed 50% inhibition of the total ATPase activity observed in the absence of La^{3+}. The La^{3+} insensitive ATPase activity was subtracted from the total ATPase activity in the absence of La^{3+}, yielding a Ca^{2+} to P_i ratio for the pump of 2:1. We were unable to duplicate these results. In our hands, maximal inhibition of the pump resulted in 80–90% inhibition of the ATPase activity. We estimated, in the manner of Quist and Roufogalis (1975*a*), that this La^{3+} insensitive ATPase activity was about 10 µmoles $P_i \cdot$liter ghosts$^{-1} \cdot$min^{-1}. If subtracted from the total ATPase activity in the absence of La^{3+}, as shown in Table 1, we obtained a Ca^{2+} to P_i ratio of 0.96:1. Though this method may be valid, we suggest that a better method for determining the relationship between Ca^{2+} pumped and P_i split in the presence and absence of La^{3+} is shown in Fig. 5. This approach does not rely on a single concentration of La^{3+}, but determines the relationship between Ca^{2+} pumped and ATP split at several concentrations of La^{3+}. The slope of the regression line (0.86 ± 0.14, 95% *CI*) is the apparent stoichiometry. The possibility that the data in Fig. 5 reflect a nonlinear relationship, indicative of a variable stoichiometry, is not ruled out by these experiments. Though r^2 is somewhat less if the points are fitted with a second degree polynomial ($r^2 = 0.95$) than with a straight line ($r^2 = 0.93$), only 7% of the total variability in *Y* is unaccounted for after linear regression. This is most likely due to experimental error.

At this time, we do not know why a discrepancy exists between our results and those of Quist and Roufogalis (1975*a*) and Sarkadi *et al.* (1977). The leakiness of resealed ghosts prepared by us from outdated red blood cells using the method of Quist and Roufogalis (1975*a*) suggests

one possible explanation. Perhaps La^{3+} did not completely inhibit the Ca^{2+}-pump in their experiments, but merely inhibited enough pump sites so that net efflux equalled influx. The Ca^{2+} leaking in would be pumped out and ATP split. This ATPase activity in the absence of a detectable Ca^{2+} efflux would be interpreted as a $(Ca^{2+}+Mg^{2+})$-ATPase not associated with the pump. This idea appears even more plausible if one considers that only 100 μM La^{3+} was required to maximally inhibit the Ca^{2+}-pump in the experiments by Quist and Roufogalis (1975*a*), whereas 250 μM was required to inhibit the pump in our experiments as well as those of Sarkadi *et al.* (1977).

We believe that the method of Sarkadi *et al.* (1977) may be too complex to adequately study the relationship between Ca^{2+} pumped and energy utilized. For example, the glyceraldehyde 3-phosphate dehydrogenase enzyme in the Embden-Meyerhof pathway uses P_i to phosphorylate glyceraldehyde 3-phosphate. Any attempt to assess ATP hydrolysis in whole cells through the measurement of P_i must control for this reaction, a problem of much less magnitude in reversibly hemolyzed RBC ghosts due to dilution of this enzyme (and others) during hemolysis. A decrease in $[P_i]$ would result in an underestimation of ATP utilized. To inhibit glyceraldehyde 3-phosphate dehydrogenase, Sarkadi *et al.* (1977) added iodoacetamide to the reaction mixture. These authors also added Na-tetrathionate to the same mixture. It has been suggested (Duhn, Deuticke & Gerlach, 1968) that Na-tetrathionate inhibits 2,3-diphosphoglycerate phosphate. Under physiological conditions, one molecule of tetrathionate is rapidly reduced by a number of thiols (including cysteine and glutathione) to 2 molecules of thiosulfate (Gilman *et al.*, 1946). The thiosulfate thus formed could react with either the iodoacetamide to form acetamide 2-thiosulfate (Trudinger, 1965) or with a number of proteins since thiosulfate is a good reducing agent (Roy & Trudinger, 1970). Furthermore, under slightly acidic conditions, thiosulfate is known to spontaneously decompose to sulphite with the sulphite in turn being removed by rapid conversion to bisulfite (Roy & Trudinger, 1970). Duhn *et al.* (1968) showed that bisulfite and sulphite are both good activators of 2,3-diphosphoglycerate phosphatase, the same enzyme which tetrathionate supposedly inhibits.

Sarkadi *et al.* (1977) also suggested that the high $[Ca^{2+}]_{in}$ would inhibit certain kinases of the Embden-Meyerhof pathway effectively inhibiting glycolysis. However, at the $[Ca^{2+}]_{in}$ which they reported only 50% inhibition of these kinases occurs, and this is readily reversed by 2.8 mM Mg^{2+} (Bygrave, 1966). This concentration of Mg^{2+} is only

slightly higher than what Sarkadi *et al.* (1977) reportedly found in their RBC. Since Sarkadi *et al.* (1977) measured 2,3-diphosphoglycerate rather than lactate, glycolysis may have been functioning to a considerable extent. This would lead to the removal of P_i from the reaction mixture by glyceraldehyde 3-phosphate dehydrogenase with an underestimation of the ATP utilized.

In summary, Quist and Roufogalis (1975*a*) and Sarkadi *et al.* (1977) inhibited the Ca^{2+}-pump with La^{3+} and found a large portion of the total ATPase activity not associated with Ca^{2+} extrusion. Our results do not support these observations. We conclude, therefore, that the stoichiometry of the plasma membrane Ca^{2+}-pump is one Ca^{2+} transported per ATP hydrolyzed as Schatzmann and Vincenzi (1969) originally suggested.

This work was supported by NIH Grants AM 16436, GM 00109 and by a grant from the Muscular Dystrophy Association. We are grateful to Dr. H.J. Schatzmann for his useful comments and review.

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