

Active Calcium Ion Uptake by Inside-Out and Right Side-Out Vesicles of Red Blood Cell Membranes

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ABSTRACT The relationship between active extrusion of Ca^{++} from red cell ghosts and active uptake of Ca^{++} by isolated red cell membrane fragments was investigated by studying the Ca^{++} uptake activities of inside-out and right side-out vesicles. Preparations A and B which had mainly inside-out and right side-out vesicles, respectively, were isolated from red cell membranes and were compared with respect to Ca^{++} adenosine triphosphatase (ATPase) and ATP-dependent Ca^{++} uptake activities. Preparation A had nearly eight times more inside-out vesicles and took up eight times more ^{45}Ca in the presence of ATP compared to preparation B. Separation of the ^{45}Ca -labeled membrane vesicles by density gradient centrifugation showed that the ^{45}Ca label was localized to the inside-out vesicle fraction. In addition, the ^{45}Ca taken up in the presence of ATP was lost during a subsequent incubation in the absence of ATP. The rate of ^{45}Ca loss was not influenced by the presence of EGTA, but was slowed in the presence of La^{+3} (0.1 mM) in the efflux medium. The results presented here support the thesis that the active uptake of Ca^{++} by red cell membrane fragments is due to the active transport of Ca^{++} into inside-out vesicles.

INTRODUCTION

It has been shown previously that the red blood cell membrane has an active Ca^{++} transport mechanism and that adenosine triphosphate (ATP) is hydrolyzed at the inner surface of the cytoplasmic membrane by a Ca^{++} -stimulated Mg^{++} ATPase (Ca^{++} ATPase) during the transport process (Schatzmann and Vincenzi, 1969; Lee and Shin, 1969). Thus, it was suggested that Ca^{++} ATPase is intimately associated with the Ca^{++} transport mechanism. Recently Cha et al. (1971) reported that fragmented red blood cell membranes take up ^{45}Ca in the presence of ATP and Mg^{++} . Since the ATP-dependent Ca^{++} uptake and the Ca^{++} ATPase activities of the red

blood cell membrane fragments (RBCMF) were closely correlated under a variety of experimental conditions, Cha et al. (1971) proposed that the active Ca⁺⁺ uptake phenomenon is intimately related to the outward Ca⁺⁺ transport mechanism as observed in ghost cells. Two possibilities can be posed to explain the mechanism of this uptake phenomenon: (a) the active binding of Ca⁺⁺ to membrane components or (b) the transport of Ca⁺⁺ into membrane vesicles which are morphologically inside out. These alternatives were investigated in the present study by comparing Ca⁺⁺ uptake and Ca⁺⁺ ATPase activities of RBCMF which contain two morphologically different types of vesicles, namely inside-out and right side-out vesicles. The data presented here strongly support the proposal that active Ca⁺⁺ uptake by RBCMF is due to transport of Ca⁺⁺ into inside-out vesicles.

METHODS

Preparation of RBCMF Three different methods were employed to prepare RBCMF: those of Steck et al. (1970) to produce mainly inside-out or right side-out vesicles, and that of Cha et al. (1971). These three types of RBCMF preparations will be termed preparations A, B, and C, respectively. All procedures were performed at 0°–4°C on human red blood cells stored in acid-citrate-dextrose for 3 wk. The methods of Steck et al. (1970) were modified slightly and are as follows. Red cells were washed three times in 10 times their volume of 0.15 M NaCl containing 5 mM Na₂HPO₄ buffer, pH 8.0. After each wash the cells were centrifuged at 5000 g for 10 min and the overlying layer of white cells was carefully removed by suction. The washed red cells were lysed in 10 times their volume of 5 mM Na₂HPO₄ buffer, pH 8.0, for 15 min, then centrifuged at 20,000 g for 10 min. The ghost membranes were washed twice more in the 5 mM Na₂HPO₄ buffer solution in the same way. The washed membranes were then suspended in 10 times their volume of either 0.5 mM Na₂HPO₄ buffer, pH 8.0 (preparation A), or 0.5 mM Na₂HPO₄ buffer containing 1 × 10⁻⁴ M MgSO₄ (preparation B), and stirred occasionally over a 1 hr period. The suspensions were then centrifuged in a Spinco Model L Ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) at 24,000 rpm for 1 hr. The resulting pellets were suspended by vortexing in 0.5 mM Na₂HPO₄ buffer, pH 8.0, (preparation A) or 0.5 mM Na₂HPO₄ buffer containing 1 × 10⁻⁴ M MgSO₄ (preparation B) to a final concentration of 7–10 mg protein/ml. Preparations A and B were always prepared simultaneously from the same unit of blood. The RBCMF preparations were stored at –20°C for not more than 3 wk, and were gently homogenized by hand with a Teflon homogenizer immediately before use. The protein concentration of the RBCMF suspensions was determined by the Biuret method.

Density Gradient Centrifugation Gradients of Dextran-110 (Pharmacia Fine Chemicals, Uppsala, Sweden), containing 0.5 mM Na₂HPO₄ buffer, pH 8.0, and 1 × 10⁻⁴ M MgSO₄, were prepared on a Hitachi, Ltd. of Japan density gradienter. RBCMF homogenates (0.2 mg protein) were carefully layered on the gradient. The gradient tubes were centrifuged to equilibrium at 100,000 g for 16 hr in a Spinco SW-39 rotor. After centrifugation the contents of each tube were carefully separated

into nine 500- μ l fractions with an Oxford automatic pipette (Oxford Instrument Corp., Annapolis, Md.). The density of each fraction was measured after centrifugation with a Zeiss refractometer. The protein concentration of the Dextran-110 fractions was determined by the fluorometric method of Udenfriend (1962), which provides a simple, sensitive determination of protein in the concentration range of 20–200 μ g/ml. Samples and bovine serum albumin standards were read on an Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.) with an excitation wavelength of 280 m μ and an emission wavelength of 340 m μ . Blanks of each Dextran-110 fraction were read along with the corresponding gradient fractions containing protein samples. The transmission of the Dextran-110 blanks was always less than 10% and was subtracted from sample values. Dilutions of RBCMF suspensions of known protein concentration (from the Biuret method) were also run and compared to the bovine serum albumin standards for correction of light scattering due to membrane particulate matter. However, at the low concentrations of protein analyzed by this method, light scattering was negligible.

Measurement of ATPase Activities The composition of standard reaction mixtures for ATPase activities is shown in Table I. The procedure for measuring ATPase activities was identical to that of Cha et al. (1971). Inorganic phosphate was measured by the Fiske and Subbarow method (1929).

Measurement of ^{45}Ca Uptake of RBCMF The reaction mixture for ^{45}Ca uptake was the same as that for the measurement of Ca^{++} ATPase as shown in Table I with the addition of ^{45}Ca at a concentration of 0.08 $\mu\text{Ci/ml}$. ^{45}Ca uptake was measured by the method of Cha et al. (1971). Labeled RBCMF were washed in a solution containing 250 mM sucrose, 15 mM histidine-imidazole buffer, pH 7.0, and 5 mM MgCl_2 (sucrose wash solution). When various media, including reaction media (as in Table I), buffered isotonic KCl or NaCl, or sucrose wash solution, were tested as wash solutions, there was no difference in the amount of ^{45}Ca retained by the membranes after different wash solutions had been used. The sucrose wash solution was chosen because its nonionic nature was thought to be least likely to introduce exchange with ^{45}Ca retained by the RBCMF.

TABLE I
COMPOSITION OF THE STANDARD REACTION MIXTURES FOR MEASUREMENT
OF ATPase ACTIVITIES

	$\text{Mg}^{++}\text{ATPase}$	$\text{Ca}^{++}\text{ATPase}$	$\text{Na}^+ + \text{K}^+\text{ATPase}$
	<i>mM</i>	<i>mM</i>	<i>mM</i>
NaCl	100	—	100
KCl	20	120	20
CaCl_2	—	0.2	—
EGTA	0.5	—	0.5
Histidine-imidazole buffer, pH 7.0	30	30	30
MgCl_2	5	5	5
ATP	1	1	1
Ouabain	0.05	—	—

Measurement of ⁴⁵Ca Efflux from RBCMF RBCMF were incubated for 1 hr at 37°C in standard incubation medium for ⁴⁵Ca uptake, and then centrifuged at 20,000 *g* for 15 min. The resulting pellet was resuspended in its sucrose wash solution. This suspension was divided equally into a number of tubes. These tubes were centrifuged at 20,000 *g* for 15 min. Before the start of the efflux experiment, the sucrose supernatant was removed by suction pipette and was replaced by an equal volume of reaction mixture containing the appropriate concentrations of all test substances. The pellets were immediately resuspended in the fresh reaction mixtures by vortexing and the timing of the efflux experiments was begun. One tube, designated as the zero-time control, was resuspended in the sucrose supernatant of its last wash and otherwise treated identically to the other samples. This treatment of the zero-time control allowed accurate estimation of the amount of ⁴⁵Ca label associated with the RBCMF before exchange of isotope with ions in the efflux medium could occur. At proper time intervals, aliquots were removed from the suspensions with an Oxford automatic pipette and filtered through 5- μ pore width Millipore filters (Millipore Corp., Bedford, Mass.) which had been prewashed with 5 ml of 1 M KCl. This size filter allowed filtration of the mixture at a fast rate while retaining all the RBCMF on the filter. The RBCMF on the filter were washed with 1 ml of sucrose wash solution before counting. A control filter which did not contain RBCMF was treated with radioactive solution and rinsed in the same way as the samples. The counts of this filter were very low and were subtracted from the sample counts. The counts of the zero-time control were assumed to represent 100% of the radioactivity in the RBCMF. The filters were air dried and placed on planchettes for counting in a Nuclear Chicago Gas-Flow counter (Nuclear-Chicago, Des Plaines, Ill.).

Determination of Calcium Contents of RBCMF Samples of RBCMF (2–4 mg protein) which had been incubated under different conditions and washed once in sucrose wash solution were suspended in 1 ml of deionized water. The suspension was transferred quantitatively to a Pyrex test tube. 1 ml of concentrated nitric-perchloric acid solution (1:1 v/v) was added. The tube was placed in a drying oven at 82°C overnight. The resulting ash was dissolved in 200 μ l of 0.1 N HCl, and the reagents for the fluorometric method of calcium determination of von Hattingberg et al. (1966) were added. All glassware was acid-washed in a concentrated sulfuric acid-potassium permanganate mixture and rinsed sequentially with 10⁻⁴ M ethylenediaminetetraacetic acid (EDTA), tap water, and deionized water.

RESULTS

Characterization of RBCMF

Preparations A and B, when subjected to density gradient centrifugation, separated into two visible density zones: one located between 1.028 and 1.035 g/cc and one between 1.055 and 1.067 g/cc. The former density zone has been shown to contain the inside-out vesicles and the latter zone has been shown to contain the right side-out vesicles (Steck et al., 1970). The quantitative distribution of membrane protein on the density gradient is shown in Table II. Preparation A had similar amounts of inside-out and

right side-out vesicles, 42.6 and 43.8% of the total protein, respectively, whereas 70.7% of the total protein of preparation B was present as right side-out vesicles and only 6.2% as inside-out vesicles. The protein distribution of preparation C on the density gradient occurred over a more widespread density range than that of preparations A and B; however, 31.2% of the total protein was located in the 1.017–1.035 g/cc zone (inside-out vesicle zone) and 26.7% in the 1.050–1.067 g/cc zone (right side-out vesicle zone).

TABLE II
PROTEIN DISTRIBUTION OF RBCMF ON A DEXTRAN-110 GRADIENT

Fraction No., top to bottom	Average density*	% of total protein in the fraction for RBCMF preparations†		
		A	B	C
	<i>g/ml</i>			
1	1.017	0.9	2.1	0.5
2	1.028	0.4	2.1	8.8
3	1.035	41.3	2.0	21.9
4	1.042	5.0	1.1	17.8
5	1.048	3.8	9.5	16.8
6	1.050	1.7	36.4	13.5
7	1.055	30.6	17.6	6.3
8	1.067	11.5	16.7	6.9
9	1.080	5.0	12.6	7.3

* The densities were determined by refractometry on each fraction after centrifugation. Protein concentration of each fraction was determined by the fluorometric method. The total protein concentration in each tube was 0.2 mg.

† The per cent of total protein in fractions 1–3 and 6–8 are pooled, and taken to represent the zones containing inside-out and right side-out vesicles, respectively.

Comparison of Ca⁺⁺ ATPase and Ca⁺⁺ Uptake Activities of Preparations A, B, and C

The data shown in Table III indicate that the total calcium contents of preparations A, B, and C were greater after a 60 min incubation in the presence of ATP (1.0 mM) than after the same incubation in the absence of ATP. However, the absolute amount of calcium taken up by preparations A and C in the presence of ATP was much greater than that of preparation B. The increase in total calcium content of the RBCMF after incubation with ATP indicates that the Ca⁺⁺ uptake phenomenon is not simply due to an increase in exchange of membrane-bound Ca⁺⁺ with tracer in the medium when ATP is present. Ca⁺⁺ ATPase activities were similar in all three preparations, whereas Mg⁺⁺ ATPase activity was somewhat greater in preparation C than in preparations A and B.

The time-courses for preparations A and B of Ca⁺⁺ ATPase and ⁴⁵Ca

uptake activities were measured simultaneously and are shown in Fig. 1. In both preparations A and B, Ca⁺⁺ ATPase activity increased with time and reached a similar level of activity after 120 min of incubation. ATP-dependent ⁴⁵Ca uptake also increased with time of incubation, but the absolute amount of ⁴⁵Ca taken up by preparation A was much greater than that of preparation B. ⁴⁵Ca uptake was paralleled in all instances by a proportional, time-dependent increase in total calcium content of the RBCMF.

Density Gradient Separation of the ⁴⁵Ca-Labeled RBCMF

RBCMF previously labeled with ⁴⁵Ca in the presence of ATP were centrifuged on a Dextran-110 gradient and the distribution of the ⁴⁵Ca label on the gradi-

TABLE III
COMPARISON OF Ca⁺⁺ ATPase AND Ca⁺⁺ UPTAKE ACTIVITIES OF RBCMF PREPARATIONS A, B, AND C

Activity*	Membrane preparations		
	A	B	C
Ca ⁺⁺ ATPase ($\mu\text{mole } P_i/\text{mg protein per hr}$)	1.86±0.42 (5)	1.77±0.31 (5)	1.63±0.24 (5)
Mg ⁺⁺ ATPase ($\mu\text{mole}/P_i \text{ per mg protein per hr}$)	0.04±0.02 (7)	0.05±0.02 (7)	0.26±0.03 (7)
ATP-dependent Ca ⁺⁺ uptake ($\text{nmole } Ca^{++}/\text{mg protein}$)	35.9±2.1 (10)	10.1±1.0 (10)	32.8±0.8 (5)
Ca ⁺⁺ uptake in the absence of ATP ($\text{nmole } Ca^{++}/\text{mg protein}$)	4.6±1.0 (5)	3.2±0.2 (6)	4.5±1.2 (5)

* Ca⁺⁺ ATPase and ATP-dependent Ca⁺⁺ uptake activities were measured after 1 hr incubation at 37°C in standard incubation medium containing ATP (1 mM) and CaCl₂ (0.2 mM) + ⁴⁵Ca. Ca⁺⁺ uptake in the absence of ATP was measured under identical conditions. Values represent the mean ± the standard error of *n* determinations, where *n* is indicated in parentheses.

ent was determined. The results are shown in Table IV. In both preparations A and B the 1.017–1.035 g/cc band (inside-out vesicle zone) contained most of the radioactivity: 84.7 and 72.6% of the total radioactivity, respectively. This result shows that the ATP-dependent ⁴⁵Ca uptake by these preparations occurs almost exclusively in the inside-out vesicle fraction. When the same experiment was performed employing preparation C, the same association of radioactivity with the 1.017–1.035 g/cc zone was observed.

ATP-dependent Ca⁺⁺ uptake could be due to passive diffusion of external Ca⁺⁺ into the inside-out vesicles, or to sequestration of Ca⁺⁺ against a concentration gradient by an active process. This question was investigated by

determining the ratio of the $[Ca^{++}]_m$ of the membrane to that of the supernatant after maximal ATP-dependent Ca^{++} uptake had occurred. The data in Table V indicate that in the presence of ATP the uptake of calcium results in a higher concentration of calcium in the membrane compared to that in the external solution with a $[Ca^{++}]_m/[Ca^{++}]_s$ ratio of 3.62 at 0.5 mM external $CaCl_2$. In the absence of ATP the calcium concentration in the RBCMF

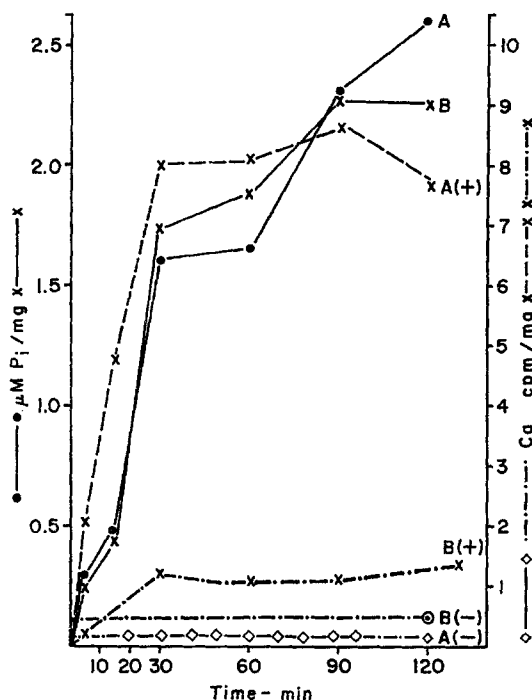


FIGURE 1. Ca^{++} ATPase and ^{45}Ca uptake activities of preparations A and B during incubation at $37^\circ C$. RBCMF were incubated under standard conditions in the absence (-) or presence (+) of ATP (1.0 mM). Preparation A (A) and preparation B (B) were isolated at the same time from the same unit of blood. Each point represents the average of three determinations from the same preparation.

was lower than that of the external solution, with a $[Ca^{++}]_m/[Ca^{++}]_s$ ratio of 0.15 at 0.5 mM $CaCl_2$.

Effect of ATP and $CaCl_2$ on ^{45}Ca Efflux from "Preloaded" RBCMF

The effects of ATP, cations, and ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) on the efflux of ^{45}Ca and ^{40}Ca from RBCMF previously incubated with calcium in the presence of ATP ("pre-loaded") were studied. In these experiments preparations A and C were employed and gave similar results.

The results depicted in Fig. 2 (curves A and B) show the efflux of ^{45}Ca

TABLE IV
THE DISTRIBUTION OF ⁴⁵Ca OF ⁴⁵Ca-LABELED MEMBRANE FRAGMENTS ON A DEXTRAN-110 GRADIENT

Fraction No., top to bottom	Average density* <i>g/ml</i>	% of total radioactivity in the fraction for membrane preparations†		
		A	B	C
1	1.017	55.3	26.7	34.6
2	1.028	26.7	29.8	32.3
3	1.035	2.7	16.1	14.0
4	1.042	4.3	2.5	6.3
5	1.048	4.1	6.2	3.9
6	1.050	1.0	2.2	5.6
7	1.055	1.6	3.7	0.2
8	1.064	4.0	6.8	2.0
9	1.080	0.3	6.0	0.6

* The densities were determined by refractometry on each 500 μ l fraction after centrifugation.
 † Membrane fragments were incubated under standard incubation conditions for ⁴⁵Ca uptake in the presence of 1.0 mM ATP at 37°C for 30 min (no CaCl₂ was added with the ⁴⁵Ca). A 0.1 ml aliquot of the washed, ⁴⁵Ca-labeled membranes containing about 0.2 mg protein was carefully placed on the top of the gradient. Each tube contained approximately 3000 cpm. The gradient solution contained 2 mM ATP in addition to the Dextran-110 solutions. The radioactivity in fractions 1-3 and 6-8 is pooled and indicated to the right of each column.

TABLE V
THE EFFECT OF ATP ON THE RATIO OF [Ca⁺⁺]_m/[Ca⁺⁺]_s

Concentration in the incubation medium*		Ratio, [Ca ⁺⁺] _m /[Ca ⁺⁺] _s	
CaCl ₂	ATP	⁴⁵ Ca data	⁴⁰ Ca data
<i>mM</i>	<i>mM</i>		
0.1	0	0.30	0.30
0.1	1.0	16.60	12.00
0.5	0	0.18	0.15
0.5	1.0	3.68	3.62

* RBCMF (preparation C) were incubated for 1 hr at 37°C, with the CaCl₂ and ATP concentrations indicated above, then centrifuged. ⁴⁵Ca and ⁴⁰Ca measurements were made on the resulting supernatants and the precipitates which had been washed once to remove extraneous calcium. The data were calculated on the basis of these determinations and the volumes of supernatants and membrane precipitates. Each value indicated above is the mean of three determinations. *m* refers to membrane and *s* refers to supernatant.

from preloaded RBCMF in medium not containing ATP. The efflux of ⁴⁵Ca was paralleled by a decrease in the total Ca⁺⁺ content of the RBCMF from 31.4 nmole Ca⁺⁺/mg protein at the start of the efflux to 7.6 nmole Ca⁺⁺/mg protein (curve A) and 10.4 nmole Ca⁺⁺/mg protein (curve B) after 120 min in the absence of ATP. Addition of CaCl₂ (0.2 mM) to the efflux medium did not significantly affect the loss of calcium from the RBCMF.

On the other hand, when ATP was present in the efflux medium, the calcium contents of the RBCMF after 120 min for the experiments illustrated in curves C and D of Fig. 2 were 33.6 and 36.4 nmole Ca^{++} /mg protein, respectively. Thus, none of the calcium gained during the preloading period was lost during the efflux period when ATP was present. When CaCl_2 and ATP were both present in the efflux medium, the efflux of ^{45}Ca from the

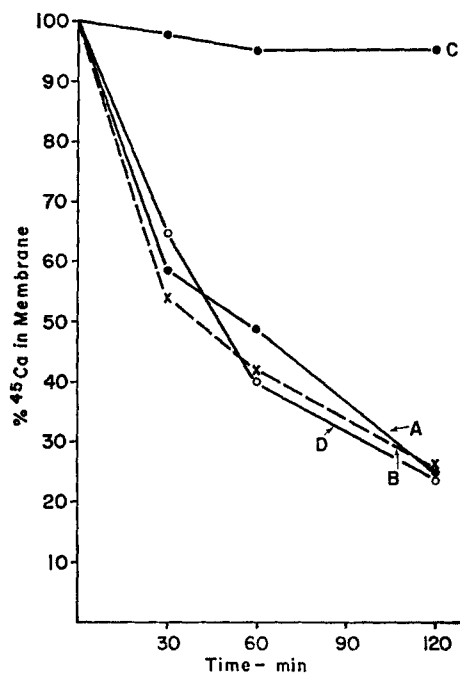


FIGURE 2. Effects of ATP and CaCl_2 on the efflux of ^{45}Ca from preloaded RBCMF. RBCMF were preloaded at 37°C as described in the Methods section. The efflux media used for all experiments contained 120 mM KCl, 5 mM MgCl_2 , and 30 mM histidine-imidazole buffer, pH 7.0, as common constituents. In addition, the efflux media for the experiments indicated above contained the following: 0.2 mM CaCl_2 (curve B), 1.0 mM ATP (curve C), 0.2 mM CaCl_2 and 1.0 mM ATP (curve D). The efflux medium of the experiment indicated in curve A had no further additions. All experiments were carried out at 37°C . Each point represents the mean of four experiments.

RBCMF proves that ^{45}Ca - ^{40}Ca exchange occurred. Addition of ATP to the efflux medium at 15 or 60 min after the start of the efflux period resulted in the reuptake of about 74% of the ^{45}Ca lost before the addition of ATP, as shown in Fig. 3. These results clearly demonstrate the importance of ATP in maintaining the calcium content of the RBCMF.

Effects of LaCl_3 and EGTA on ^{45}Ca Efflux from Preloaded RBCMF

The effects of LaCl_3 and EGTA on ^{45}Ca efflux in the absence of ATP are illustrated in Fig. 4. The presence of LaCl_2 (0.1 mM) in the efflux medium

(curve I) greatly slowed the rate of ⁴⁵Ca loss compared to the control (curve H), whereas the presence of EGTA (curve J) did not affect the rate of ⁴⁵Ca loss. However, the presence of EGTA had a profound effect on ⁴⁵Ca efflux when ATP was present. When EGTA and ATP were both present in the

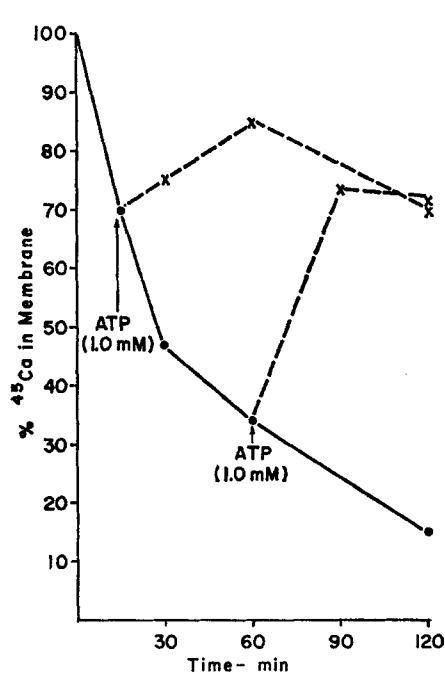


FIGURE 3

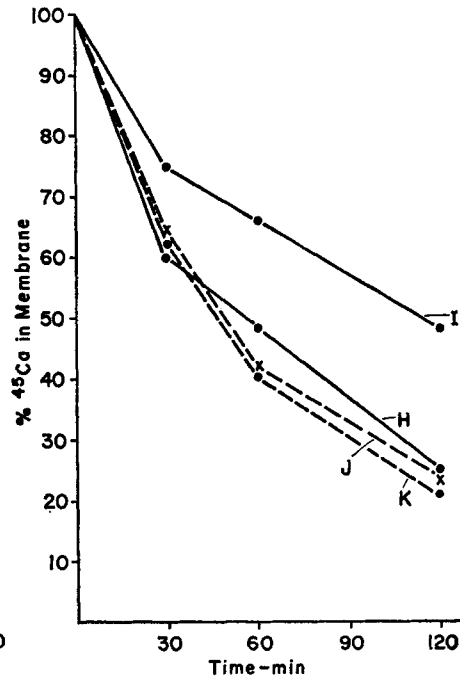


FIGURE 4

FIGURE 3. Effect of ATP addition on ⁴⁵Ca efflux. Preloaded RBCMF were incubated at 37°C in efflux medium containing 120 mM KCl, 5 mM MgCl₂, and 30 mM histidine-imidazole buffer, pH 7.0. ATP was added at 15 or 60 min after the start of efflux at a concentration of 1.0 mM. The efflux experiments were carried out at 37°C.

FIGURE 4. Effects of LaCl₃ and EGTA on the efflux of ⁴⁵Ca from preloaded RBCMF. RBCMF were preloaded at 37°C as described in the Methods section. The efflux media used for all experiments contained 120 mM KCl, 5 mM MgCl₂, and 30 mM histidine-imidazole buffer, pH 7.0, as common constituents. In addition the efflux media for the experiments depicted above contained the following: 0.1 mM LaCl₃ (curve I), 0.1 mM EGTA (curve J), 0.1 mM EGTA and 1.0 mM ATP (curve K). The efflux medium of curve H contained no further addition. All of the above efflux experiments were carried out at 37°C. Each point represents the mean of four experiments.

efflux medium, ⁴⁵Ca efflux occurred at the same rate as that observed when no ATP was present (curve K). These results imply that any Ca⁺⁺ that leaks out of the membrane vesicles in the presence of ATP is chelated by EGTA and therefore not available for reuptake.

Effect of LaCl₃ Pretreatment on ATPase Activities

Since lanthanum has been shown to have a high affinity for calcium binding sites, the effect of LaCl₃ pretreatment on the Ca⁺⁺ ATPase activity was investigated and is shown in Table VI. Pretreatment of RBCMF with low concentrations of LaCl₃ selectively inhibited Ca⁺⁺ ATPase activity without affecting Mg⁺⁺ ATPase or Na⁺ + K⁺ ATPase activities.

DISCUSSION

The elucidation of the mechanism of ATP-dependent Ca⁺⁺ uptake was approached in two ways: (a) by comparing the Ca⁺⁺ uptake and Ca⁺⁺ ATPase activities of preparations A and B, and (b) by studying the effects of ATP, EGTA, and cations on the efflux of ⁴⁵Ca from preloaded RBCMF.

RBCMF were characterized for the presence of inside-out and right side-out vesicles by the density gradient centrifugation technique of Steck et al. (1970). Using freeze-cleave electron microscopy these investigators identified the membrane material isolated from the 1.01–1.035 g/cc and 1.05–1.065 g/cc density fractions as containing inside-out and right side-out vesicles, respectively. When we prepared membrane vesicles exactly according to their procedure we were able to duplicate their data for the density gradient separation of membrane protein; however, the resulting membranes had very low Ca⁺⁺ ATPase activity. Therefore, we modified their procedure by decreasing the lysing ratio to prevent excess protein solubilization. Our preparations A and B gave similar protein distribution to those of Steck et al. (1970) on the Dextran-110 gradients (Table II). We feel that the membrane material was fairly accurately characterized by this method.¹

The results illustrated in Table IV demonstrate that the ATP-dependent ⁴⁵Ca uptake by RBCMF occurred almost exclusively in the inside-out vesicle fractions (fractions 1, 2, and 3) of all three membrane preparations. However, there appears to be a discrepancy among the three inside-out vesicle fractions with respect to ⁴⁵Ca uptake activity; namely, the fractions with the highest protein content had the lowest ⁴⁵Ca content (compare Tables II and IV). Since Steck et al. (1970) demonstrated that 98% of the membrane material isolated from the single 1.01–1.035 g/cc density zone was present as inside-out vesicles, it is possible that our finer resolution of their single fraction into three fractions has revealed a biochemical heterogeneity among the inside-out vesicles. Further work is needed to clarify this point. Nevertheless, the rather striking localization of the ⁴⁵Ca label to the inside-out vesicle zones suggests that the difference in the ATP-dependent Ca⁺⁺ uptake activities of

¹ Preliminary work by Dr. Weiner indicates that the membrane material in the 1.01–1.035 g/cc and 1.05–1.067 g/cc zones is present as inside-out and right side-out vesicles, respectively, by the criterion of per cent of the total sialic acid released by sialidase (for details of this biochemical test see Steck et al., 1970.)

preparations A and B is due to the different amounts of inside-out vesicles which they contain.

From this close association of the ATP-dependent Ca⁺⁺ uptake with the inside-out vesicles, it is tempting to explain this uptake phenomenon as a reversal of the outward Ca⁺⁺ transport mechanism, namely the transport of Ca⁺⁺ into inside-out vesicles. The time-course of the ATP-dependent Ca⁺⁺ uptake is suggestive of a transport process. The progressive increase in both ⁴⁵Ca and total calcium contents from 0 to 30 min is similar to the time-course of active calcium extrusion from red cell ghosts (Schatzmann and Vincenzi, 1969; Lee and Shin, 1969). This is in contrast to the extremely rapid ATP-dependent Ca⁺⁺ binding by skeletal and cardiac sarcoplasmic reticulum (Ohnishi and Ebashi, 1963; Harigaya and Schwartz, 1969).

TABLE VI
THE EFFECT OF LANTHANUM PRETREATMENT ON ATPase ACTIVITIES

Additions to preincubation medium*		ATPase activity (μ mole inorganic phosphate/mg protein per hr)		
		Mg ⁺⁺ ATPase	Ca ⁺⁺ ATPase	Na ⁺ + K ⁺ ATPase
No addition		0.14	0.65	0.33
10 ⁻⁶ M	LaCl ₃	0.14	0.65	0.30
4 × 10 ⁻⁸ M	LaCl ₃	0.14	0.44	0.34
10 ⁻⁴ M	LaCl ₃	0.14	0.20	0.34
10 ⁻⁸ M	LaCl ₃	0.06	0.06	0.03

* RBCMF of preparation C were preincubated in 0.5 mM histidine-imidazole buffer, pH 7.0, for 15 min at 20°C. The RBCMF were then centrifuged at 20,000 *g* for 15 min and the supernatant was removed. Measurement of ATPase activities was started by addition of the appropriate incubation media as indicated in Table I, to the membrane precipitates. The incubation period for ATPase measurement was 30 min at 37°C. Each value is the mean of three experiments.

The active sequestration of calcium by the RBCMF is indicated by the observation that the calcium content of the membranes was greater than that of the external solution after a 1 hr incubation in the presence of ATP (Table V). The values for the $[Ca^{++}]_m/[Ca^{++}]_e$ ratio in Table V represent a minimal estimation of the ability of the inside-out vesicles to accumulate calcium since no attempt was made to subtract the radioactive solution trapped within the membrane precipitate.

The data obtained in the Ca⁺⁺ efflux experiments demonstrates that the calcium content of the RBCMF was subsequently lost from the membranes if ATP was removed from the efflux medium. The importance of ATP in maintaining the calcium content of the RBCMF is illustrated by the results in Figs. 2 and 3. Therefore, in contrast to the ATP-dependent Ca⁺⁺ binding by skeletal sarcoplasmic reticulum, which is not lost upon removal of ATP (Carvalho and Muto, 1971), the ATP-dependent Ca⁺⁺ uptake by inside-out vesicles is lost when ATP is removed.

The role of binding in the calcium uptake process was investigated by comparing the loss of ^{45}Ca from preloaded RBCMF in Ca^{++} -free medium in the presence of La^{+3} , or EGTA. The effects of lanthanum in a variety of biological systems indicate that lanthanum can displace calcium from superficial binding sites, but that it cannot penetrate across the cell membrane under normal conditions (Revel and Karnovsky, 1964; van Breeman and De Weer, 1970; see Weiner, 1971; Weiss and Goodman, 1969). If it is assumed that ATP-dependent Ca^{++} uptake is due to superficial binding of calcium to membrane components, then lanthanum would be expected to displace this calcium and to increase the rate of ^{45}Ca efflux in the absence of ATP. However, addition of La^{+3} to the efflux medium greatly slowed the rate of ^{45}Ca loss (Fig. 4). This effect of La^{+3} could be due to the blocking of the transmembrane movement of calcium out of the vesicles by the binding of La^{+3} to membrane sites involved in a calcium-carrier system. This suggestion is supported by the finding that pretreatment of the RBCMF with low concentrations of La^{+3} resulted in a selective inhibition of Ca^{++} ATPase (Table VI).

Studies on the rate of ^{45}Ca efflux in the presence of EGTA provide additional support for the proposed mechanism of active calcium uptake as a transport process. Since EGTA is known to chelate calcium ion from the incubation medium and from superficial membrane sites (Weiss and Goodman, 1969), it is expected that addition of EGTA to the efflux medium would increase the rate of ^{45}Ca efflux if the ^{45}Ca were superficially bound. The results in Fig. 4 show that the rate of ^{45}Ca loss from the inside-out vesicles in the absence of ATP was not affected by the presence of EGTA. In a similar study of passive calcium movements in resealed red cell ghosts, Porzig (1970) found that EDTA did not increase the rate of loss of ^{45}Ca from ghosts containing ^{45}Ca sealed inside by reversible hemolysis over that observed in Ca^{++} -free medium not containing EDTA. From the results in Fig. 4, indicating the effects of La^{+3} and EGTA on ^{45}Ca efflux, we conclude that the Ca^{++} taken up by the RBCMF in the presence of ATP is not bound to superficial membrane components, but is transported across the membrane barrier.

The influence of ATP on the calcium movements observed with the inside-out vesicles appears to be identical to that observed in the intact red cell ghost, only reversed in direction. The outwardly directed transport system of the red blood cell maintains a low intracellular calcium concentration at the expense of metabolic energy (Schatzmann and Vincenzi, 1969; Lee and Shin, 1969). Depletion of cellular ATP results in a dramatic rise in the erythrocyte calcium content of up to 400% and is accompanied by deleterious effects on cellular rigidity and deformability (Weed et al., 1969). Regeneration of cellular ATP in these depleted cells by incubation with adenosine was found to reduce significantly the calcium content, and reverse the observed me-

chanical changes. These observations suggest that calcium accumulated during metabolic depletion is pumped out of the cell by the active transport system when ATP is restored. In a similar manner, calcium taken up by the inside-out vesicles in the presence of ATP was lost when ATP was removed, and was taken up again when ATP was readded (Fig. 3). The inside-out vesicles appear to retain the biochemical properties of the Ca⁺⁺ transport system of the intact red blood cell and can serve as useful models to study the asymmetric nature of the membrane.

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REFERENCES

- BREEMAN, C. VAN, and P. DE WEER. 1970. Lanthanum inhibition of Ca⁴⁵ efflux from squid giant axon. *Nature (London)*. **226**:760.
- CARVALHO, A. P., and A. M. MUTO. 1971. The role of ATP and of a bound phosphoryl group acceptor on Ca⁺⁺ binding and exchangeability in sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **142**:201.
- CHA, Y. N., B. C. SHIN, and K. S. LEE. 1971. Active uptake of Ca⁺⁺ and Ca⁺⁺-activated Mg⁺⁺ ATPase in red cell membrane fragments. *J. Gen. Physiol.* **57**:202.
- FISKE, C. H., and Y. SUBBAROW. 1929. Phosphocreatine. *J. Biol. Chem.* **81**:629.
- HARIGAYA, S., and A. SCHWARTZ. 1969. Rate of calcium binding and uptake in normal animal and failing human cardiac muscle vesicles (relaxing system) and mitochondria. *Circ. Res.* **25**:781.
- HATTINGBERG, H. M. VON, W. KLAUS, H. LULLMAN, and S. ZEPP. 1966. Fluorometrische Bestimmung von mikromengen Calcium in Muskelgewebe. *Experientia (Basel)*. **22**:553.
- LEE, K. S., and B. C. SHIN. 1969. Studies on the active transport of calcium in human red cells. *J. Gen. Physiol.* **54**:713.
- OHNISHI, T., and S. EBASHI. 1963. Spectrophotometrical measurement of instantaneous calcium binding of the relaxing factor of muscle. *J. Biochem.* **54**:506.
- PORZIG, H. 1970. Calcium efflux from human erythrocyte ghosts. *J. Membrane Biol.* **2**:324.
- REVEL, J. P., and M. J. KARNOVSKY. 1964. Hexagonal array of subunit in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**:C7.
- SCHATZMANN, H. J., and F. F. VINGENZI. 1969. Calcium movements across the membrane of human red cells. *J. Physiol. (London)*. **201**:369.
- STECK, T. L., R. S. WEINSTEIN, J. H. STRAUS, and D. F. H. WALLACH. 1970. Inside-out red cell membrane vesicles: preparation and purification. *Science (Washington)*. **168**:255.
- UDENFRIEND, S. 1962. Fluorescence Assay in Biology and Medicine. Academic Press, Inc., New York. 206.
- WEED, R. I., P. L. LACELLE, and E. W. MERRILL. 1969. Metabolic dependence of red cell deformability. *J. Clin. Invest.* **48**:795.
- WEINER, M. L. 1971. The mechanism of ATP-dependent calcium uptake in red cell membrane fragments. Ph.D. Thesis. State University of New York, Downstate Medical Center, Brooklyn, New York.
- WEISS, G. B., and F. R. GOODMAN. 1969. Effects of lanthanum on contraction, calcium distribution and Ca⁴⁵ movements in intestinal smooth muscle. *J. Pharmacol. Exp. Ther.* **169**:46.