Investigation of the Accompaniment of Calcium During Active Calcium Transport from Human Erythrocyte Ghosts

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ABSTRACT To determine whether a cell metabolite was involved in active calcium transport, the cell contents of human erythrocytes were subjected to high dilutions and the resultant ghosts were checked for their ability to actively transport calcium. It was found that the diluted erythrocyte ghosts did retain their capacity to actively transport calcium and that the characteristics of this transport process appeared to be unaltered by the high dilutions. Calcium analysis of the cell membrane and cell supernatant indicated that almost all of the calcium was lost from the cell solution rather than the cell membrane as active calcium transport proceeded. Therefore it appeared that calcium was able to cross the cell membrane without the aid of a cell metabolite. Investigations with layered erythrocytes indicated that the active transport of calcium was not assisted by centrifugation. Neither inorganic phosphate, pyrophosphate, nor an adenine nucleotide appeared to accompany calcium across the membrane as indicated by total phosphate and inorganic phosphate analysis and 260-nm readings of the deproteinized supernatant.

When either calcium or strontium is infused into erythrocytes they can be transported from the resulting erythrocyte ghosts against a concentration gradient (Schatzmann, 1966; Schatzmann and Vincenzi, 1969; Olson and Cazort, 1969; Lee and Shin, 1969). Wins and Schoffeniels (1966) found that the erythrocyte membrane contains an ATPase which is activated by calcium or strontium and magnesium. Substances which inhibit this ATPase also inhibit calcium transport (Vincenzi and Schatzmann, 1967) and phosphate liberation from ATP occurs as active calcium transport proceeds (Schatzmann and Vincenzi, 1969). Furthermore calcium uptake by erythrocyte membrane fragments was found to be directly related to this ATPase activity (Cha et al., 1971). For these reasons this ATPase is considered to be involved in the active transport of calcium and strontium from erythrocyte ghosts.

While ATP hydrolysis undoubtedly is the energy source for active calcium transport, the transported calcium ion may also require a chelating anion to enable it to cross the membrane. This anion may be a cell metabolite such as 2,3 diphosphoglycerate, or a product of ATP hydrolysis such as inorganic phosphate, pyrophosphate, or adenosine diphosphate. While the diffusion of such a compound would not drive calcium out of the cells it may be needed to get the transported calcium past binding sites on the cell membrane. This paper deals with this possibility. In this regard it has been noted that aspartic acid accompanies the transport of sodium from crab nerve when it is immersed in a potassium-free medium (Baker, 1964); aspartate does not, of course, chelate Na^+ and is presumably an accompanying anion rather than a chelating anion.

METHODS

Infusion of Erythrocytes with Calcium or Strontium

Human erythrocytes were collected from heparinized blood and washed several times with a solution of 0.13 M NaCl and 0.02 M Tris buffered at pH 7.4. The washed erythrocytes were infused with either calcium or strontium by a modification of Schatzmann's method (1966). Aliquots of the washed erythrocytes were added to a specified amount of a solution which contained 1.5 mM of $CaCl_2$ and/or $SrCl_2$, 1.0 mM of $MgCl_2$, and 2.0 mM of Tris buffered at pH 7.4. When present the concentration of adenosine triphosphate was 3.3 mM. After 4 min the ghosts were brought back to isotonicity with 2.0 M KCl which was added over a period of 4 min. The resulting erythrocyte ghosts were separated from the hemolysate by centrifugation at 8,000 *g* for 20 min and suspended in approximately 4 vol of either the sodium or potassium incubation solution which contained 180 mM of NaCl or KCl, 20 mM of Tris buffer at pH 7.4, 1.5 mM of $CaCl_2$ and/or $SrCl_2$, 1.0 mM of $MgCl_2$, and 1 g/liter of bovine serum albumin. The suspension was centrifuged for 10 min at 8,000 *g* and the supernatant was discarded. This procedure was repeated at least twice with 2 vol of the incubation solution. If the supernatants from a wash were colorless the ghosts were ready for incubation at 37°C. All steps were conducted at 0°C.

Suspended Incubation of the Infused Ghosts

The washed erythrocyte ghosts were suspended in an equal volume of either the sodium or the potassium incubation solution described above. The suspension was incubated at 37°C for a prescribed period of time and then brought back to 0°C by immersion in ice and water for 5 min. Erythrocyte ghosts incubated at 0°C in the same solution for the same period of time served as controls. The incubated suspension was centrifuged at 8,000 *g* for 10 min at 0°C; the resulting supernatant was separated from the cells.

Layered Incubation of Ghosts

Aliquots of the ghosts were placed in a test tube and an equal volume of the appropriate incubation solution was layered over it. The tubes were then incubated at 37°C

for a prescribed period of time and cooled to 0°C as previously described. After cooling the erythrocyte ghosts were slowly stirred so that the solution in the interstices of the cells mixed with the incubation solution without disaggregating the cells. The erythrocyte ghosts were allowed to stand at 0°C for at least an hour so that the cells in the supernatant would settle from it. The supernatant was separated from the cell mass and centrifuged at 2,000 *g* for 20 min to remove any ghosts that remained. The layered ghosts were also centrifuged for 30 min at 1,000 *g* and the resulting supernatant solution was discarded. Both centrifugations were conducted at 0°C.

Fractionation of Ghosts

The incubated ghosts were diluted with 4 vol of water and either homogenized with a glass Teflon homogenizer for 30 s or sonicated for 45 s at 0°C. The resulting suspension was centrifuged at 20,000 *g* for 40 min. The clear supernatant was separated from the membrane fragments which were grey in color.

Hemoglobin Reading of Erythrocytes and Erythrocyte Ghosts

Cell supernatant fractions were prepared as described in the previous section and read at 550 nm.

Dry Weight Analysis

Dry weight analysis was performed as previously described (Olson and Cazort, 1969).

Calcium, Strontium, and Magnesium Analysis of Supernatants

These analyses were performed by the method of Trudeau and Frier (1967).

Calcium and Strontium Analysis of Ghosts and Ghost Fractions

Calcium and strontium analysis of ghosts and ghosts' membranes were conducted as previously described (Olson and Cazort, 1969). Calcium analysis of ghost supernatants was performed using atomic absorption spectroscopy after diluting the sample of supernatant with three-fifths of its volume of acetic acid and two-fifths of its volume of 0.1% $LaCl_2$.

Analysis of Total Phosphate in Supernatant

Total phosphate analysis was performed by the method of Fiske and Subbarow (1925) after the supernatant was hydrolyzed overnight in a sealed tube at 100°C with an equal volume of 1.0 M hydrochloric acid.

Inorganic Phosphate Analysis of the Supernatant

Inorganic phosphate analysis was performed by the method of Lowry and Lopez (1946). Standards were prepared by adding phosphate to aliquots of the incubation solution before deproteinization with 10% (wt/vol) trichloroacetic acid at 0°C.

Nucleotide Analysis of the Supernatant

Specified volumes of the supernatant were diluted to 0.4 ml and cooled to 0°C and were deproteinized by the addition of 0.1 ml of 4.0 M perchloric acid and centrifuged. The centrifugates were diluted to 3.0 ml and read at 260 nm. These readings were

related to standards prepared by adding known amounts of adenosine diphosphate to the incubation solution before deproteinization.

Extraction of 260-nm Absorbing Component with Ion Exchange Resin

Supernatant aliquots of 0.3 ml were diluted to 0.4 ml with water and deproteinized with 0.1 ml of 4.0 M perchloric acid. After centrifugation the resultant supernatant was added to 2.0 ml of 0.4 M potassium acetate. The supernatant was separated from the potassium perchlorate precipitates and diluted to 3.0 ml before being read at 260 nm. This solution was shaken with 1.2 g of Ag 1 × 4 anion exchange resin for 1 min and read again. The readings obtained from the incubated supernatants were compared to the readings obtained when known amounts of adenine, adenosine, adenylic acid, and adenosine diphosphate were added to the nonincubated sodium reaction solution which contained magnesium.

Chemicals

Adenosine triphosphate was obtained as its sodium salt from Nutritional Biochemicals Corp., Cleveland, Ohio. Bovine serum albumin was obtained from Armour Inc., Chicago, Ill.

RESULTS

Effectiveness of the Dilution Technique

To determine whether a cell metabolite is involved in active calcium transport, erythrocytes were diluted with either 40 or 125 vol of the calcium infusion solution. The resulting erythrocyte ghosts were incubated at 37°C for the investigation of active calcium transport. To determine how effective the increased volumes of the infusion solutions were in diluting the cell contents, hemoglobin readings of the erythrocytes and erythrocyte ghosts were determined as described in Methods. From Table I it can be seen that the hemoglobin readings are roughly inversely proportional to the volume of the hypotonic infusion solution used. Thus it appeared that the larger volumes of the infusion solution did dilute the contents of the cell.

TABLE I

EFFECTIVENESS OF THE DILUTION TECHNIQUE

Samples	OD readings
	550 nm
Erythrocytes	0.940*
Ghosts from erythrocytes diluted 1:5	0.160*
Ghosts from erythrocytes diluted 1:125	0.006‡

* 0.05-ml aliquots of supernatant diluted with 2.95 ml of water before reading.

‡ 0.30-ml aliquots of supernatant diluted with 2.70 ml of water before reading. Reading obtained divided by 6.

Basic Characteristics of Calcium and Strontium Transport from Diluted Erythrocyte Ghosts

The initial investigations of calcium transport from ghosts derived from diluted erythrocytes are presented in Table II. The results of these investigations indicated that the basic characteristics of the active calcium transport system were not altered by the use of higher dilutions of the calcium infusion solution. The active calcium transport that was observed did require adenosine triphosphate and occurred in either sodium or potassium solutions (Schatzmann, 1966). As in earlier investigations (Schatzmann, 1967; Olson and Cazort, 1969) no magnesium exchange was observed nor was there any observed change in the dry weights of the erythrocyte ghosts during the incubations. Strontium transport also occurred, and in keeping with the findings of Schatzmann and Vincenzi (1969), calcium transport occurred exclusively when calcium was infused into the cells with strontium.

Table II describes active calcium transport into a sodium medium. Calcium transport without magnesium exchange also occurred when the diluted cells were incubated in a potassium medium.

The dilution of the parent erythrocytes with 250 vol of the calcium infusion solution did not appear to impair active calcium transport from the erythrocyte ghosts.

Ghost Fractionation Experiments

The result of the fractionation experiments are presented in Table III. It appeared that most of the infused calcium was associated with the liquid part of the ghosts and that the observed distribution was essentially the same for sonication and homogenization. It also was apparent that the amount of calcium transported was accounted for by a decline in the amount of calcium in the ghost supernatant. Therefore the process of active calcium transport appeared to involve calcium ions crossing the membrane rather than calcium ions being released from the membrane.

Calcium Transport from Layered Erythrocyte Ghosts

Although calcium apparently crossed the membrane during active calcium transport calcium could be pumped into the membrane by the hydrolysis of ATP during the incubation period and then removed from the membrane when the ghosts were centrifuged from the medium. To check this possibility layered incubations were performed so that the necessity of collecting the supernatant by centrfugation was eliminated. In the layered experiments the residua that were centrifuged from the supernatants accounted for no more than $1/10$ of the supernatant volume. These experiments indicated that the

TABLE II

EFFECT OF HIGHER VOLUMES OF INFUSING SOLUTION OF THE ACTIVE CALCIUM TRANSPORT SYSTEM

Red cell sample	Time	Dry weight	Concentrations Ca^{2+} Supernatant	Ca^{2+} Ghosts	Sr^{2+} Supernatant	Sr^{2+} Ghosts	Mg^{2+} Supernatant
	min	%	*mM*		*mM*		*mM*
1:125 Dilution, sodium medium							
None	—	—	1.50	—	—	—	1.00
Control	0	3.3	1.60*	1.25	—	—	1.05
Control	20	3.1	1.60*	1.35	—	—	1.00
None	—	—	1.50	—	—	—	1.00
ATP	0	2.6	1.60*	1.20	—	—	1.15
ATP	20	2.9	2.10*	0.85	—	—	1.00
None	—	—	—	—	1.50	—	1.00
ATP	0	3.2	—	—	1.65‡	1.70	1.05
ATP	20	3.3	—	—	2.10‡	1.15	1.05
None	—	—	1.50	—	1.50	—	1.00
ATP	0	3.7	1.65	1.65	1.65‡	1.45	1.10
ATP	20	3.9	2.20	1.35	1.70‡	1.40	1.10

* Average for difference $t_{20} - t_0$ for three separate experiments. For ATP 0.50 SD ± 0.05, for control 0.0 SD ± 0.05. Significance by Student *t*-test $P < 0.01$.
‡ Average difference $t_{20} - t_0$ for three separate experiments. For Sr^{2+} alone 0.65 SD ± 0.2 for $Sr^{2+} + Ca^{2+}$ 0.05 SD ± 0.05. Significance by Student *t*-test $P < 0.05$.

TABLE III

DEPLETION OF GHOST FRACTIONS DURING ACTIVE CALCIUM TRANSPORT

Time	Dry weight	Concentrations Ca^{2+} Supernatant	Ca^{2+} Ghosts supernatant	Ca^{2+} Ghosts membrane	Mg^{2+} Supernatant
min	%	*mM*	*mM*		*mM*
Series A	1:125 Dilution, sodium medium, sonication				
—	—	1.50	—	—	1.00
0	2.7	1.55	1.20	0.13	1.00
3	2.5	1.70	0.90	0.11	0.95
15	3.1	2.10	0.85	0.11	0.95
Series B	1:125 Dilution, sodium medium, homogenization				
—	—	1.50	—	—	1.00
0	3.8	1.50	1.00	0.16	1.00
3	3.4	1.70	0.85	0.15	1.00
15	3.8	2.25	0.55	0.12	1.00

TABLE IV
ACTIVE CALCIUM TRANSPORT FROM LAYERED ERYTHROCYTE GHOSTS

Time	Dry weight	Concentrations: Ca^{2+} Supernatant	Concentrations: Ca^{2+} Ghosts	Concentrations: Mg^{2+} Supernatant
min	%	*mM*		*mM*
Series A	1:125 Dilution, sodium medium			
—	—	1.50	—	1.00
0	3.0	1.55*	1.35	1.05
20	3.2	1.90*	1.15	0.95
Series B	1:125 Dilution, potassium medium			
—	—	1.50	—	1.00
0	3.1	1.60‡	1.10	1.10
20	3.3	2.15‡	0.75	0.90

* Average of six separate experiments, SD, $t_0 \pm 0.15$, $t_{20} \pm 0.25$. Significance by Student t-test $P < 0.025$.

‡ Average of four separate experiments, SD, $t_0 \pm 0.05$, $t_{20} \pm 0.05$. Significance by Student t-test $P < 0.025$.

observed increase in the amount of calcium in the supernatant exceeded the amount of calcium that could be accounted for by such residua (Table IV.

Supernatant Analysis after Active Calcium Transport

While the possibility that a cell metabolite is involved in active calcium transport may be considered unlikely, the possibility that inorganic phosphate, pyrophosphate, or an adenine nucleotide was involved in the transport of calcium through the membrane still existed. To check these possibilities the incubated supernatants were analyzed for total phosphate, inorganic phosphate, and 260-nm absorption. When these analyses were performed no change in the amounts of total phosphate or 260-nm absorption could be observed as the active transport of calcium proceeded (Table V). In one experiment an increase in the concentration of inorganic phosphate was observed as the incubation proceeded but these increased amounts of phosphate in the supernatant were only a fraction of the amounts of calcium transported. The substitution of potassium for sodium in the incubation solution did not alter these results.

When the erythrocyte ghosts were suspended in the reaction mixture at 0°C and centrifuged immediately, the total phosphate readings were at least two-thirds of the t_0 readings presented in Table V, which actually represent 20-min incubation at 0°C. Most of the total phosphate initially present in the t_0 supernatants is considered to be due to the rapid leakage of phosphate compounds, including adenine nucleotides, from damaged erythrocyte

TABLE V

ANALYSIS OF SUPERNATANTS AFTER ACTIVE CALCIUM TRANSPORT

		Concentrations				
		Ca^{2+}		Supernatant phosphate		Supernatant
Time	Dry weight	Supernatant	Ghosts	Total*	Inorganic‡	Nucleotides§, ‖
min	%	*mM*		*mM*		*mM*
Series A	1:40 Dilution, sodium incubation medium¶					
—	—	1.50	—	—	—	—
0	4.2	1.55	1.50	0.30	0.14	0.15
10	4.3	1.95	1.00	0.40	0.18	0.15
20	4.6	2.30	0.80	0.50	0.36	0.14
Series B	1:40 Dilution, potassium reaction medium¶					
—	—	1.50	—	—	—	—
0	4.5	1.55	1.40	0.46	0.00	0.18
10	4.4	2.10	1.05	0.41	0.07	0.25
20	4.5	2.40	0.65	0.40	0.07	0.14
Series C	1:125 Sodium Reaction Medium¶					
0	3.3	1.50‖	1.80	0.42	0.15	0.09
15	3.3	2.10‖	1.15	0.44	0.20	0.09
Series D	1:125 Potassium reaction medium¶					
0	3.3	1.50	1.40	0.30	0.05	0.20
15	3.3	2.00	1.05	0.27	0.04	0.20

* Recoveries: From sodium reaction medium initially containing 1.0 mM Mg^{2+} 0.084 μmol total phosphate + 0.05 μmol ADP, predicted phosphate 0.184 μmol observed 0.168 μmol, observed recovery 84%. From potassium reaction medium initially containing 1.0 mM Mg^{2+} 0.125 μmol total phosphate + 0.100 μmol ADP, predicted phosphate 0.325 μmol, observed 0.30 μmol, observed recovery 87.5%. 0.130 μmol total phosphate + .200 μmol added phosphate, predicted phosphate 0.330 μmol observed 0.310 μmol, observed recovery 90%.

‡ Recoveries: From sodium reaction medium initially containing 1.0 mM Mg^{2+} 0.050 μmol phosphate + 0.100 μmol phosphate added before TCA, predicted phosphate 0.150 μmol, observed 0.150 μmol, observed recovery 100%. From potassium reaction medium initially containing 1.0 mM Mg^{2+} 0.047 μmol phosphate + 0.100 μmol phosphate added before TCA, predicted phosphate 0.147 μmol, observed 0.150, observed recovery 103%.

§ Recoveries: From sodium reactions mediums initially containing 1.0 mM Mg^{2+} 0.0085 μmol ADP + 0.0500 μmol ADP added before perchlorate, predicted ADP 0.0585 μmol, observed 0.0585 μmol, recovery 100%. From potassium reaction medium initially containing 1.0 mM Mg^{2+} 0.0215 μmol ADP + 0.0500 μmol ADP added before perchlorate, predicted ADP 0.0715 μmol, observed 0.0675 μmol, observed recovery 92%.

‖ Data based on ADP standards.

¶ Containing 1.0 mM Mg^{2+}.

ghosts. The presence of phosphate compounds in the supernatant was observed after layered incubations as well as suspended incubations and, therefore, did not appear to be an artifact due to centrifugation.

When deproteinized supernatants were neutralized and shaken with AG-1 × 4 anion exchange resin the absorption peak at 260 nm, was virtually eliminated. Since 90% of adenosine diphosphate and only 50% of the ade-

nine or adenosine were extracted under the same conditions the absorption in the supernates which were incubated at 37° was considered to be due to adenosine diphosphate rather than to adenosine or adenine.

From the observations it would appear that the total phosphate found in the supernatant can be accounted for by adenosine diphosphate and inorganic phosphate. Therefore it does not seem likely that phospholipids disengage from the membrane during the process of active calcium transport. It was also noted that the absorption spectrum of the incubated supernatants between 220 and 280 nm was almost identical to the absorption spectrum of adenine diphosphate in this region.

DISCUSSION

It seems unlikely that an unidentified metabolite is involved in active calcium transport since the transport system functions in ghosts formed by exposure of the parent erythrocytes to 125 or 250 vol of the hypotonic calcium-loading solution. Furthermore analysis of the medium into which calcium was transported indicated that no specific product of adenosine triphosphate was involved in active calcium transport. Neither inorganic phosphate, pyrophosphate, nor any adenine nucleotide accumulated in the medium in amounts that were comparable to the amount of calcium transported. Since the total phosphate observed in the supernatants could be accounted for by inorganic phosphate or nucleotides it was concluded that a phosphate containing membrane component was not released as the active transport of calcium proceeds. Loading experiments had ruled out the possibility that the diffusion of ATP or its hydrolytic productions drive calcium out of the cell (Schatzmann and Vincenzi, 1969).

The low quantity of phosphate found in the medium after incubation indicated that ATP was hydrolyzed on the inside of the membrane. This finding was consistent with the observation that only inside-out vesicles from erythrocyte membranes take up calcium in the presence of ATP (Weiner and Lee, 1972), and with the observation that external calcium did not stimulate the ATPase activity of erythrocyte ghosts (Schatzmann and Vincenzi, 1969). Inhibition experiments also indicated that the ($Na^+ + K^+$)-activated ATPase was located on the inside of the erythrocyte membrane. (Rummel et al., 1963).

While the possibility that calcium was accompanied by a specific metabolite or breakdown product of adenosine triphosphate as it traversed the membrane during active calcium transport seemed unlikely, the possibility that actively transported calcium was accompanied by an internal membrane carrier remains. It has been observed that erythrocyte membranes with elevated polyphosphoinositide contents also have elevated Ca-activated ATPase levels (Buckley and Hawthorne, 1972). Calcium also could traverse

the membrane accompanied by a protein carrier. Calcium-binding proteins have been isolated from the sarcoplasmic reticulum (Bertrand et al., 1971; MacLennan and Wong, 1971), and most of the calcium in erythrocyte ghost membranes was protein bound (Forstner and Mannery, 1971; Long and Mouat, 1971).

When calcium crosses the membrane it must be accompanied by an anion or exchanged for a cation. Calcium extrusion from heart muscle proceeded most rapidly in the presence of external sodium (Reuter and Seitz, 1968; Romero and Whittam, 1971), while the extrusion of calcium from erythrocyte ghosts was not sodium dependent (Schatzmann, 1966). The present authors believe that active calcium transport probably was accompanied by a nonspecific anion movement.

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