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Inhibition of Active Strontium Transport from Erythrocyte Ghosts by Internal Calcium: Evidence for a Specificity Controlling Site

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Summary. The inhibition of strontium transport from erythrocyte ghosts by internal calcium was investigated. When active strontium transport was measured in the presence of increasing levels of internal calcium it was found that the inhibition of strontium transport started at an internal calcium level of 0.3 mM and was virtually complete when this concentration reached 1.0 mM. It was also noted that calcium transport was virtually constant between concentrations of 0.3 and 1.0 mM. This experiment indicated that calcium did not inhibit strontium transport by competing for the active site of the transport system. This inhibition was partially reversed by increasing the internal magnesium concentration from 1 to 4 mM. A higher level of magnesium at the time of lysis and during incubation enhanced strontium transport. However, the inhibition remained noncompetitive with respect to calcium. Manganese was also found to support calcium and strontium transport. However, it could not reverse the inhibition of strontium transport by internal calcium at any concentration tested. In fact, manganese restored the inhibition of strontium transport by calcium in ghosts that were prepared and incubated in solutions that had high magnesium levels.

Erythrocyte ghosts which contain calcium, magnesium, and ATP can transport calcium into a surrounding medium against a concentration gradient (Schatzmann, 1966; Schatzmann & Vincenzi, 1969; Olson & Cazort, 1969). Further investigation indicated that this transport involves the passage of calcium through the cell membrane rather than release from it (Olson & Cazort, 1974). It was also noted that no calcium binding metabolite or inorganic phosphate accompanied the transported calcium into the surrounding medium (Olson & Cazort, 1974).

One mole of inorganic phosphate is liberated from ATP for every mole of calcium transported (Schatzmann, 1973), and an ATPase, which is activated by calcium and magnesium, was found in the erythrocyte membrane (Wins & Schoeffeniels, 1966). When oxalate-stimulated calcium storage in erythrocyte membrane inside-out vesicles and erythrocyte membrane ATPase activity were plotted against increasing magnesium

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concentrations, a striking parallelism could be observed (Cha, Shin & Lee, 1971). Therefore, it is considered that this membrane-bound ATPase is involved in active calcium transport.

Strontium is also transported from erythrocyte ghosts which contain ATP and magnesium (Schatzmann, 1969; Olson & Cazort, 1969). However, while calcium and strontium appear to have the same affinity for the membrane-bound ATPase (Wins & Schoeffeniels, 1966), calcium is transported almost exclusively when calcium and strontium are present in the erythrocyte ghosts at the same time (Schatzmann & Vincenzi, 1969). Therefore, active strontium transport and its inhibition by intracellular calcium was investigated to uncover additional features of this transport system.

Materials and Methods

Materials

The Na salt of ATP was obtained from ICN (Cleveland, O.) and bovine serum albumin (BSA) was obtained from Metric Pharmaceuticals (Chicago, Ill.). All experiments were performed with erythrocytes from freshly drawn heparinized blood and confirmed with erythrocytes from outdated blood from the blood bank.

Preparation of Erythrocyte Ghosts

The erythrocytes were washed several times at room temperature with a solution of 130 mM of NaCl and 20 mM of Tris buffered at room temperature at pH 7.4. Without precooling, the washed erythrocytes were hemolyzed at 0° with either 5 or 40 vol of a solution containing 3.3 mM of ATP, 2 mM of Tris buffered at pH 7.4, and, except where otherwise specified, 1.0 mM of $MgCl_2$, 1.5 mM of $CaCl_2$ and/or 1.5 mM of $SrCl_2$. After 4 min the resulting erythrocyte ghosts were restored to isotonicity by the addition of 2 M KCl. The ghosts were then collected by centrifugation at $10{,}000 \times g$ for 20 min at 0 °C. The centrifuged ghosts were suspended at 0 °C in a medium which contained 180 mM of KCl, 20 mM of tris buffered at pH 7.4, 1 mg/ml of BSA and, except where otherwise specified, the concentrations of $MgCl_2$, $SrCl_2$ and $CaCl_2$ that were present in the hypotonic solution that was used to hemolyze the erythrocytes. The suspended ghosts were then centrifuged at $10{,}000 \times g$ for 10 min at 0 °C. This washing procedure was repeated either two or three times before the ghosts were ready to be incubated.

Incubation of the Erythrocyte Ghosts

Except where otherwise specified, the washed erythrocyte ghosts were suspended at 37 °C for specified periods of time in an equal volume of the solution that was used to wash the erythrocyte ghosts (hematocrit 50%). After prescribed time intervals the incubated ghost suspensions were placed in an ice-water slurry for 5 min before being centrifuged at 0 °C for 10 min at $10{,}000 \times g$. After centrifugation the incubated medium was separated from the ghosts so that both could be subjected to the appropriate analyses.

Preparation of Erythrocyte Ghost Fractions

Ghost supernatants and membranes were prepared by sonication as previously described (Olson & Cazort, 1974).

Analysis of Calcium, Strontium, Magnesium and Manganese

The analysis of calcium, strontium, and magnesium in the incubated media were conducted by the method of Trudeau & Frier (1967), as was the analysis of manganese in the supernatants from sonicated ghosts. The supernatant solution and membranes from sonicated ghosts were analyzed for calcium and strontium by atomic absorption spectrophotometry as previously described (Olson & Cazort, 1974).

Analysis of Inorganic Phosphate and Total Phosphate

The ghost supernatants and incubation media were analyzed for inorganic phosphate by the method of Fiske & Subbarow (1925) after being deprotenized at 0 °C with an equal volume of 10% trichloroacetic acid. The incubation media was also analyzed for total phosphate by the method of Fiske & Subbarow (1925) after being hydrolyzed with hydrochloric acid as previously described (Olson & Cazort, 1974). The recoveries of added inorganic phosphate from the various ghost supernatants were all over 90% as were the recoveries of inorganic phosphate added to incubated media and hydrolysates of incubated media.

Nucleotide Analysis of Incubated Media

Nucleotide analysis of the incubated media were performed as previously described (Olson & Cazort, 1974).

Dry Weight Analysis

Dry weight analysis of erythrocyte ghosts were performed as previously described (Olson & Cazort, 1969).

Results

Phosphate Liberation from ATP

It was initially suspected that, when present in erythrocyte ghosts, strontium ions occupied the active sites of the transport ATPase and catalyzed the hydrolysis of ATP molecules before being transported. It was also suspected that when calcium ions were also present in the ghosts they would displace strontium on the transport system and be transported instead. Therefore when both ions were present in ghosts,

active calcium transport would take precedence over active strontium transport.

To determine if this was so, the ghost supernatants and incubation media were analyzed for inorganic phosphate as calcium or strontium were transported from the ghosts. The results of these experiments are presented in Table 1. These data indicate that active transport of calcium and strontium proceeded with the same apparent stoichiometry. Furthermore, it may also be noted that the apparent stoichiometry of this process was not significantly reduced when calcium was transported from ghosts which contained both calcium and strontium. Therefore, it does not seem likely that calcium inhibits strontium transport by uncoupling it

Table 1. Stoichiometry of active calcium and active strontium transport[a]

Time (min)	Concentrations					
	Ca^{2+}		Sr^{2+}		Inorg. phosphate	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series A: Ca^{2+} in ghosts						
0	1.53	1.28	–	–	0.05	0.10
15	2.20	0.68	–	–	0.15	0.75
Differences	0.67	0.60			0.10	0.65
Ratio $\frac{Ca^{2+}\text{ transported}}{\text{inorganic phosphate liberated}}$	$=0.91$ SD ± 0.09					
Series B: Sr^{2+} in ghosts						
0	–	–	1.50	1.43	0.08	0.16
15	–	–	2.05	0.83	0.20	0.65
Differences			0.55	0.50	0.12	0.49
Ratio $\frac{Sr^{2+}\text{ transported}}{\text{inorganic phosphate liberated}}$	$=0.90$ SD ± 0.11					
Series C: Ca^{2+} and Sr^{2+} in ghosts						
0	1.49	1.25	1.48	1.05	0.03	0.18
15	2.01	0.65	0.68	0.85	0.08	1.04
Differences	0.52	0.60	0.20	0.20	0.05	0.86
Ratio $\frac{Ca^{2+}+Sr^{2+}\text{ transported}}{\text{inorganic phosphate liberated}}$	$+0.80$ SD ± 0.17					

[a] Ghosts were prepared by diluting erythrocytes with 40 vol hypotonic solution. After washing in a potassium medium containing 1 mM magnesium, the ghosts were incubated in the same solution in which they were washed.

[b] Ratios were determined by five experiments.

from ATP hydrolysis. It may also be noted from this table that the accumulation of inorganic phosphate in the incubation media amounted to only a small fraction of the calcium or strontium transported from the ghosts and neither nucleotides or other phosphate compounds accumulated in the incubated media as the transport of these ions proceeded. Both calcium transport in the presence or absence of strontium and strontium transport proceeded from the solution inside the ghosts rather than the ghost membrane.

The Rate of Active Calcium Transport from Erythrocyte Ghosts which Contain Either Calcium Alone or Calcium and Strontium

To determine if calcium transport from ghosts was inhibited by the presence of strontium in the ghosts, the rate of calcium transport was measured from ghosts that contained either calcium alone or calcium and strontium. As can be observed from Table 2, the presence of strontium in the ghosts did not affect the rate of active calcium transport

Table 2. Comparison of the rates of active calcium transport in the presence or absence of intracellular strontium[a]

Time (min)	Dry wt (%)	Concentrations			
		Ca^{2+}		Sr^{2+}	
		Medium[a] (mM)	Ghosts (mM)	Medium[a] (mM)	Ghosts (mM)
Series A: Ca^{2+} in ghosts					
0	6.3	1.40	1.80	—	—
8	6.4	1.70	1.50	—	—
15	6.7	1.95	1.20	—	—
Differences		0.55	0.60		
Series B: Ca^{2+} and Sr^{2+} in ghosts					
0	6.7	1.40	1.65	1.45	1.30
8	7.6	1.70	1.50	1.50	1.30
15	6.8	1.95	1.00	1.55	1.20
Differences		0.55	0.65	0.60	0.10

[a] Ghosts were prepared by diluting erythrocytes with five volumes of hypotonic solution. After washing in a potassium medium containing 1 mM magnesium, the ghosts were incubated in the same solution in which they were washed.

from the ghosts. Increasing the amount of hypotonic solution that was used to make the ghosts from 5 to 40 volumes did not alter these experimental results.

Active Strontium Transport from Ghosts which Contain Varying Amounts of Calcium

I then sought to determine what concentrations of calcium in ghosts are necessary to inhibit active strontium transport. The results of these experiments are shown in Table 3. It can be seen from this table that while there may have been some inhibition of active strontium transport when the hypotonic solution, which was used to make the ghosts, contained 0.3 mM $CaCl_2$, significant inhibition was observed when this concentration in the hypotonic solution was raised to 0.6 mM. When the calcium concentration in the hypotonic solution was raised to 0.9 mM the inhibition of strontium transport was virtually complete. It may also be observed that active calcium transport was observed at all the calcium concentrations that were used. This transport was relatively constant in spite of the fact that strontium transport varied several-fold.

If under these experimental conditions calcium inhibited strontium transport by simply replacing strontium at the active site of the transport system, then fewer transport units will be occupied by strontium and more transport units will be occupied by calcium as increasing amounts of calcium are added to the media. Therefore, as the inhibition of strontium increases, the transport of calcium should increase. On this basis if 0.2 mM of calcium transport is inhibited 28% then one would expect to see 0.6 mM of calcium transported when strontium transport is inhibited 85%. When such calculations were made for individual experiments, it was found that at the 0.9 mM level, values of calcium were significantly lower than the values that would be predicted on the basis of competitive inhibition ($0.52 \pm 0.26 > 0.23 \pm 0.08$; $P < 0.05$ by student's t test).

These results were not altered by increasing the amount of hypotonic solution used to make the ghosts from 5 to 40 vol.

Investigation of Active Strontium Transport from Ghosts which Contain Calcium and Varying Amounts of Magnesium

Since the concentrations of magnesium that Wins and Schoeffeniels used in their ATPase experiments (Wins & Schoeffeniels, 1966) were

Table 3. Active strontium transport in the presence of calcium[a]

Time (min)	Concentrations			
	Ca^{2+}		Sr^{2+}	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series A: 0.0 mM Ca^{2+} in hypotonic solution				
0	–	–	1.50[e,f]	1.55
8	–	–	2.20[e,f]	0.95
Differences			0.70	0.60
Series B: 0.3 mM Ca^{2+} in hypotonic solution				
0	0.30[b]	0.45	1.45	1.60
8	0.50[b]	0.30	1.95	1.05
Differences	0.20	0.15	0.50	0.55
Series C: 0.6 mM Ca^{2+} in hypotonic solution				
0	0.60[c]	0.75	1.50[e]	1.70
8	0.85[c]	0.45	1.80[e]	1.35
Differences	0.25	0.30	0.30	0.35
Series D: 0.9 mM Ca^{2+} in hypotonic solution				
0	0.90[d]	1.10	1.45[f]	1.45
8	1.15[d]	0.90	1.55[f]	1.40
Differences	0.25	0.20	0.10	0.05

[a] Ghosts were prepared by diluting erythrocytes with 5 vol hypotonic solution. After washing in a potassium medium containing 1 mM magnesium, the ghosts were incubated in the same solution in which they were washed.

[b] Averages of six separate experiments. t_8 Series B SD $\pm 0.08 > t_0$ Series B SD ± 0.05. Significance by student's t test; $P < 0.01$.

[c] Averages of six separate experiments. t_8 Series C SD $\pm 0.10 > t_0$ Series C SD 0.035. Significance by student's t test; $P < 0.001$.

[d] Averages of six separate experiments. t_8 Series D SD $\pm 0.11 > t_0$ Series D SD ± 0.035. Significance by student's t test; $P < 0.001$.

[e] Averages of six separate experiments. $t_8 - t_0$ Series A SD $\pm 0.31 > t_8 - t_0$ Series C SD ± 0.125. Significance by student's t test; $P < 0.05$.

[f] Averages of six separate experiments. $t_8 - t_0$ Series A SD $\pm 0.31 > t_8 - t_0$ Series D SD ± 0.10. Significance by student's t test; $P < 0.001$.

higher than those in Schatzmann and Vincenzi's experiments with ghosts that contained calcium and strontium (Schatzmann & Vincenzi 1969), the effect of magnesium on active strontium transport in the presence of calcium was investigated. It was found that increasing the magnesium concentration of the ghosts and external media reduced the inhibition

of active strontium transport by calcium (Table 4). It is also noted that the higher concentrations of magnesium enhance transport generally. However, since calcium transport was the same during the first 8 min of the Series *B* and Series *C* incubations (Table 4), the enhanced strontium transport observed in Series *C* cannot be attributed to the depletion of calcium from the ghosts.

Table 4. The effect of magnesium on active strontium transport in the presence of calcium[a]

Time (min)	Concentrations			
	Ca^{2+}		Sr^{2+}	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series A: 1.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.40	1.55	1.50[b,c,d]	1.50
8	1.70	1.45	1.50[d]	1.50
15	1.90	1.15	1.57[b,c]	1.30
Differences	0.50	0.40	0.07	0.20
Series B: 2.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.40	1.10	1.45[b,e]	1.30
8	1.90	0.80	1.50[e]	1.10
15	2.40	0.50	1.95[b]	0.80
Differences	1.00	0.60	0.50	0.50
Series C: 4.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.40	1.40	1.45[c,d,e]	1.45
8	1.90	0.70	1.80[d,e]	1.15
15	2.40	0.60	2.05[c]	0.75
Differences	1.00	0.80	0.60	0.70

[a] Ghosts were prepared by diluting erythrocytes with 5 vol hypotonic solution. After washing in a potassium medium, the ghosts were incubated in the solution in which they were washed.

[b] Averages of four separate experiments. $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *B* SD $\pm 0.25 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *A* SD ± 0.10. Significance by student's *t* test; $P<0.05$.

[c] Averages of four separate experiments. $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *C* SD $\pm 0.06 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *A* SD ± 0.10. Significance by student's *t* test; $P<0.001$.

[d] Averages of four separate experiments. t_8-t_0 Sr^{2+}/t_8-t_0 Ca^{2+} Series *C* SD $\pm 0.006 > t_8-t_0$ Sr^{2+}/t_8-t_0 Ca^{2+} Series *A* SD ± 0.10. Significance by student's *t* test; $P<0.001$.

[e] Average of four separate experiments. t_8-t_0 $Sr^{2+}/t_8-t_0 Ca^{2+}$ Series *C* SD $\pm 0.06 > t_8-t_0$ Sr^{2+}/t_8-t_0 Ca^{2+} Series *B* SD ± 0.10. Significance by student's *t* test; $P<0.05$.

Increasing the amount of hypotonic solution that was used to make the ghosts from 5 to 40 vol did not alter these results. No significant accumulations of phosphate or nucleotides could be detected when the more dilute ghosts were incubated.

The Effect of Increasing the Strontium Concentrations of High and Low Magnesium Ghosts on the Inhibition of Strontium Transport by Calcium

It was suspected that the higher levels of magnesium reversed calcium's inhibition of strontium transport by enabling strontium to compete with calcium for access to the transport system. To check this possibility, we incubated high magnesium and low magnesium ghosts containing either 0.75 or 2.25 mM strontium with or without internal calcium in the appropriate potassium media.

The data shown in Table 5 indicate that at both levels of magnesium, the percentage of inhibition was not altered by raising the level of strontium in the ghosts from 0.75 to 2.25 mM. Therefore, this possibility was discounted.

Table 5. Inhibition of strontium transport by calcium in the presence of 1.0 or 4 mM magnesium[a]

Time (min)	Concentrations			
	Sr^{2+}		Ca^{+}	
	Medium	Ghosts	Medium	Ghosts
0.75 mM Sr^{2+} and 1.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	0.70	0.70	—	—
8	1.05	0.33	—	—
Differences	0.35	0.37		
0.75 mM Sr 0.5 mM Ca^{2+} and 1.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	0.70	0.70	0.52	0.60
8	0.82	0.60	0.72	0.43
Differences	0.12	0.10	0.20	0.17

Ratio $\frac{Sr^{2+} \text{ transported with } Ca^{2+}}{Sr^{2+} \text{ transported without } Ca^{2+}} = 0.35 \text{ SD} \pm 0.13$

Table 5 (continued)

Time (min)	Concentrations			
	Sr^{2+}		Ca^{+}	
	Medium	Ghosts	Medium	Ghosts
2.25 mM Sr^{2+} and 1.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	2.20	2.20	–	–
8	2.55	2.00	–	–
Differences	0.35	0.20		
2.25 mM Sr^{2+} 0.50 mM Ca and 1.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	2.15	2.25	0.55	0.70
8	2.28	2.15	0.75	0.53
Differences	0.13	0.10	0.20	0.15
Ratio $\frac{Sr^{2+} \text{ transported with } Ca^{2+}}{Sr^{2+} \text{ transported without } Ca^{2+}}$	$=0.35$ SD ± 0.07			
0.75 mM Sr^{2+} and 4.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	0.72	0.80	–	–
8	1.15	0.45	–	–
Differences	0.43	0.35		
0.75 mM Sr^{2+} 0.75 mM Ca^{2+} and 4.0 mM Mg in hypotonic and incubation solutions				
0	0.72	0.75	0.72	0.73
8	0.90	0.55	1.07	0.46
Differences	0.18	0.20	0.35	0.46
Ratio $\frac{Sr^{2+} \text{ transported with } Ca^{2+}}{Sr^{2+} \text{ transported without } Ca^{2+}}$	$=0.42$ SD ± 0.22			
2.25 mM Sr^{2+} and 4.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	2.18	2.15	–	–
8	2.90	1.45	–	–
Differences	0.72	0.70		
2.25 mM Sr 0.75 mM Ca^{2+} and 4.0 mM Mg^{2+} in hypotonic and incubation solutions				
0	2.20	2.05	0.75	0.80
8	2.50	1.85	1.05	0.45
Differences	0.30	0.20	0.30	0.35
Ratio $\frac{Sr^{2+} \text{ transported with } Ca^{2+}}{Sr^{2+} \text{ transported without } Ca^{2+}}$	$=0.40$ SD ± 0.12			

[a] Ghosts were prepared using 5 vol appropriate hypotonic media. Calculations were based on supernatant analysis.

The Stoichiometry of Calcium and Strontium Transport in High Magnesium Ghosts

It was also suspected that the higher levels of magnesium in the ghosts permitted strontium to be transported with calcium without the

Table 6. Stoichiometry of calcium and strontium transport from high magnesium ghosts[a]

Time (min)	Concentrations					
	Ca^{2+}		Sr^{2+}		Inorg. phosphate	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)	Medium[b] (mM)	Ghosts[b] (mM)
1.5 mM Ca^{2+}, 15 mM Sr^{2+} and 8 mM Mg^{2+} in hypotonic and incubation solutions						
0	1.50	1.00	1.55	1.00	0.06	0.36
10	2.28	0.60	1.90	0.70	0.09	1.46
Differences	0.78	0.40	0.35	0.30	0.03	1.10
Ratio[c] $\frac{Ca^{2+}+Sr^{2+}\text{ transported}}{\text{inorganic phosphate liberated}}$	$=1.01$ SD ± 0.260					
Ratio[c] $\frac{Ca^{2+}+Sr^{2+}\text{ transported}}{Ca^{2+}\text{ transported}}$	$=1.53$ SD ± 0.15					
1.5 mM Ca^{2+}, 1.5 mM Sr^{2+} and 8 mM Mg^{2+} in hypotonic solution 1.0 mM Ca^{2+}, 1.0 mM Ca^{2+}, 1.0 mM Sr^{2+} and 8 mM Mg^{2+} in incubation solution						
0	0.97	0.85	1.00	1.25	0.00	0.08
10	1.53	0.55	1.40	1.00	0.00	1.26
Differences	0.56	0.30	0.40	0.25	0.00	1.18
Ratio[d] $\frac{Ca^{2+}+Sr^{2+}\text{ transported}}{\text{inorganic phosphate liberated}}$	$=0.85$ SD ± 0.28					
Ratio[d] $\frac{Ca^{2+}+Sr^{2+}\text{ transported}}{Ca^{2+}\text{ transported}}$	$=1.72$ SD ± 0.16					

[a] Ghosts/were prepared by diluting erythrocytes with 40 vol hypotonic solution before being restored to isotonicity with 2 M KCl. Ghosts/were then washed and incubated in potassium incubation media. Calculations were based on supernatant analysis.

[b] Corrected for inorganic phosphate liberated from ghosts prepared with 40 vol of a hypotonic solution containing 8 mM magnesium and 3.3 mM of ATP but no calcium. These ghosts were washed and incubated in potassium incubation media which contained 8 mM magnesium but no calcium or magnesium.

[c] Averages of six separate experiments. $Ca^{2+}+Sr^{2+}$ transported/Ca^{2+} > transported $Ca^{2+}+Sr^{2+}$ transported/inorganic phosphate liberated. Significance by student's t test; $P<0.01$.

[d] Averages of three separate experiments. $Ca^{2+}+Sr^{2+}$ transported/Ca^{2+} transported $Ca^{2+}+Sr^{2+}$ transported/inorganic phosphate liberated. Significance by student's t test; $P<0.01$.

liberation of additional phosphate from ATP. We therefore incubated high magnesium ghosts which contained calcium and strontium in isotonic potassium media that contained either 1.5 or 1.0 mM of calcium and strontium and 8.0 mM of magnesium. It can be seen from Table 6 that strontium is transported from high magnesium ghosts that also contain calcium with the apparent liberation of phosphate, making this possibility seem unlikely.

Investigation of the Effect of Magnesium in the Hypotonic and Incubation Solutions on Active Strontium Transport in the Presence of Intracellular Calcium

To determine whether the enhanced strontium transport in the presence of intracellular calcium was due to the presence of high magnesium concentrations in the ghosts at the time of incubation or the time of hemolysis, the experiments described in Table 7 were performed. It is evident from these experiments that a high magnesium concentration at the time of incubation is necessary for enhanced strontium transport from ghosts which also contain calcium. It may also be noted that the presence of a high magnesium concentration at the time of hemolysis enhanced strontium transport further. Since the nucleotide content of the ghosts was not affected by the magnesium concentration of the hemolyzing solution, this enhanced strontium transport could not be explained on this basis.

To determine if the higher magnesium concentrations stimulated strontium transport generally, the same experiment was performed with ghosts that contained only strontium. However, neither higher magnesium concentrations at the time of hemolysis nor at the time of incubation stimulated active strontium transport from erythrocyte ghosts. When these experiments were performed with ghosts that contained only calcium, it was found that the transport of calcium was stimulated only by higher magnesium concentrations at the time of incubation.

Active Calcium and Strontium Transport in the Presence of Manganese. Active calcium and strontium transport from erythrocyte ghosts which contained manganese instead of magnesium occurred when the original erythrocytes were lysed with 40 vol of a hypotonic solution which contained calcium or strontium, ATP, and manganese (Table 8). This transport, which proceeded more slowly than magnesium supported transport, was not accompanied by either inorganic phosphate or nucleotides nor

Table 7. The effect of magnesium in the hypotonic medium and incubation medium on active strontium transport in the presence of intracellular calcium

Time (min)	Concentrations					
	Ca^{2+}		Sr^{2+}		Mg^{2+}	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series A: 4 mM Mg^{2+} in hypotonic solution and incubation solution						
0	1.45	1.55	1.40[b,c]	1.45	3.75	4.10
8	2.00	1.00	1.60	1.25	3.75	4.00
15	2.45	0.75	1.95[b,c]	0.95	3.75	4.00
Differences	1.00	0.80	0.55	0.50	0.00	0.00
Series B: 4 mM Mg^{2+} in hypotonic solution and 1 mM Mg^{2+} in incubation solution[a]						
0	1.45	1.65	1.45[b,e]	1.50	1.00	1.40
8	1.80	1.45	1.55	1.50	0.95	1.45
15	2.50	1.20	1.65[b,e]	1.45	0.95	1.40
Differences	0.50	0.45	0.20	0.05	0.05	0.00
Series C: 1 mM Mg^{2+} in hypotonic solution and 4 mM Mg^{2+} in incubation solution[a]						
0	1.45	1.55	1.40[c,d,e]	1.50	3.50	3.90
8	1.85	1.20	1.50	1.30	3.40	3.75
15	2.20	0.95	1.70[c,d,e]	1.25	3.60	3.95
Differences	0.75	0.60	0.30	0.25	0.10	0.05
Series D: 1 mM Mg^{2+} in hypotonic solution and incubation solution						
0	1.35	1.70	1.40[d]	1.50	1.00	1.20
8	1.60	1.45	1.45	1.45	1.00	1.25
15	1.85	1.30	1.50[d]	1.45	1.00	1.30
Differences	0.50	0.40	0.10	0.05	0.00	0.10

[a] Ghosts were prepared by diluting erythrocytes with five volumes of hypotonic solution. Ghosts, were washed and incubated in potassium medium. Ghosts of Series *B* were depleted of magnesium before incubation by being suspended in a magnesium-free solution before being washed and incubated in the potassium incubation solution which contained 1 mM magnesium. The magnesium concentration of the ghosts used in Series *C* was increased by being suspended three times in the potassium incubation solution which contained 4 mM magnesium before being incubated in the same solution.

[b] Averages of four separate experiments. $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *A* SD $\pm 0.06 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *B* SD ± 0.07. Significance by student's *t* test; $P<0.01$.

[c] Averages of four separate experiments. $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *A* SD $\pm 0.06 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *C* SD ± 0.025. Significance by student's *t* test; $P<0.001$.

[d] Averages of four separate experiments. $t_{15}-t_0$ $Sr^{2+}/t_{16}-t_0$ Ca^{2+} Series *C* SD $\pm 0.025 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *D* SD ± 0.055. Significance by student's *t* test; $P<0.01$.

[e] Averages of four separate experiments. $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *C* SD $\pm 0.025 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series *B* SD ± 0.07. Significance by student's *t* test; $P<0.05$.

Table 8. Active calcium and strontium transport in the presence of manganese[a]

Time (min)	Dry wt (%)	Concentrations			
		Ca^{2+}		Sr^{2+}	
		Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series A: No Mg^{2+} or Mn^{2+} in hypotonic solution or sodium incubation solution					
0	2.7	1.50[b]	1.60	–	–
15	3.0	1.50[b]	1.75	–	–
Differences		0.00	0.15		
Series B: 2 mM Mn in hypotonic solution and sodium incubation solution					
0	2.8	1.40[b]	1.40	–	–
15	3.1	1.85[b]	1.10	–	–
Differences		0.45	0.30		
Series C: No Mg^{2+} or Mn^{2+} in hypotonic solution or potassium incubation solution					
0	2.6	1.40[c]	1.55	–	–
15	2.9	1.50[c]	1.70	–	–
Differences		0.10	0.15		
Series D: 2 mM Mn^{2+} in hypotonic solution and potassium incubation solution					
0	3.0	1.40[c]	1.50	–	–
15	3.2	1.85[c]	1.15	–	–
Differences		0.45	0.35		
Series E: No Mg^{2+} or Mn^{2+} in hypotonic solution or sodium incubation solution					
0	3.4	–	–	1.45[d]	1.60
15	3.1	–	–	1.40[d]	1.75
Differences				0.05	0.15
Series F: 2 mM Mn^{2+} in hypotonic solution and sodium incubation solution					
0	3.1	–	–	1.45[d]	1.30
15	3.4	–	–	1.70[d]	0.90
Differences				0.25	0.40
Series G: No Mg^{2+} or Mn^{2+} in hypotonic solution or potassium incubation solution					
0	2.9	–	–	1.45[e]	1.65
15	3.1	–	–	1.50[e]	1.50
Differences				0.05	0.15

Table 8 (continued)

Time (min)	Dry wt (%)	Concentrations			
		Ca^{2+}		Sr^{2+}	
		Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series H: 2 mM Mn^{2+} in hypotonic solution and potassium incubation solution					
0	3.1	–	–	1.50[e]	1.25
15	3.1	–	–	1.70[e]	0.95
Differences				0.20	0.30

[a] Ghosts prepared by diluting erythrocytes with 40 vol hypotonic solution. In Series *A*, *B*, *E* and *F* erythrocyte ghosts were washed in a potassium medium before being incubated in a sodium medium. Sodium medium is like potassium medium except that 180 mM KCl is replaced with 180 mM NaCl.

[b] $t_{15}-t_0$ Series *B* SD $\pm 0.15 > t_{15}-t_0$ Series *A* SD ± 0.14. Significance by student's *t* test; $P<0.01$.

[c] $t_{15}-t_0$ Series *D* SD $\pm 0.17 > t_{15}-t_0$ Series *C* SD ± 0.07. Significance by student's *t* test; $P<0.01$.

[d] $t_{15}-t_0$ Series *F* SD $\pm 0.06 > t_{15}-t_0$ Series *E* SD ± 0.07. Significance by student's *t* test; $P<0.01$.

[e] $t_{15}-t_0$ Series *H* SD $\pm 0.06 > t_{15}-t_0$ Series *G* SD ± 0.06. Significance by student's *t* test; $P<0.01$.

Table 9. Calcium and strontium transport from ghost containing calcium and strontium in the presence of magnesium and manganese[a]

Time (min)	Concentrations			
	Ca^{2+}		Sr^{2+}	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series A: 2 mM Mn^{2+} in hypotonic and incubation solutions				
0	1.50[b]	1.30	1.50[b]	1.45
8	1.80	0.90	1.55	1.30
15	1.95[b]	0.75	1.60[b]	1.35
Differences	0.45	0.55	0.10	0.10
Series B: 2 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.40[b]	1.50	1.45[b]	1.50
8	1.85	0.95	1.45	1.35
15	2.05[b]	0.75	1.70[b]	1.25
Differences	0.55	0.75	0.25	0.25

Table 9 (continued)

Time (min)	Concentrations			
	Ca^{2+}		Sr^{2+}	
	Medium (mM)	Ghosts (mM)	Medium (mM)	Ghosts (mM)
Series C: 4 mM Mn^{2+} in hypotonic and incubation solutions				
0	1.50[c]	1.20	1.50[c]	1.25
8	1.75	0.95	1.50	1.30
15	1.95[c]	0.80	1.60[c]	1.20
Differences	0.45	0.40	0.10	0.05
Series D: 4 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.45[c]	1.30	1.45[c]	1.15
8	2.00[c]	0.95	1.70[c]	0.95
15	2.20	0.70	1.90	0.80
Differences	0.75	0.60	0.45	0.35
Series E: 8 mM Mn^{2+} in hypotonic and incubation solutions				
0	1.50[d]	1.20	1.50[d]	1.20
8	1.65	1.10	1.50	1.15
15	1.80[d]	1.00	1.55[d]	1.15
Differences	0.30	0.10	0.05	0.05
Series F: 8 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.50[d]	1.15	1.50[d]	1.60
8	2.15[d]	0.70	1.75[d]	1.50
15	2.25	0.60	2.05	1.20
Differences	0.75	0.55	0.55	0.40
Serie G: 4 mM Mn^{2+} and 4 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.50[e]	1.30	1.50[e]	1.00
8	1.70	1.05	1.55	1.00
15	1.90[e]	0.90	1.60[e]	0.95
Differences	0.40	0.40	0.10	0.05
Series H: 4 mM Mg^{2+} in hypotonic and incubation solutions				
0	1.50[e]	1.40	1.50[e]	1.10
8	1.95	0.90	1.65	1.05
15	2.25[e]	0.65	1.95[e]	0.80
Differences	0.75	0.75	0.45	0.70

[a] Ghosts were prepared by diluting erythrocytes with 40 vol hypotonic solution. The ghosts were then washed and incubated in a potassium medium.

[b] $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series B SD $\pm 0.07 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series A SD ± 0.05. Significance by student's t test; $P<0.05$.

[c] t_8-t_0 Sr^{2+}/t_8-t_0 Ca^{2+} Series D SD $\pm 0.09 > t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series C SD ± 0.08. Significance by student's t test; $P<0.01$.

[d] $t_8-t_0\,Sr^{2+}/t_8-t_0\,Ca^{2+}$ Series F SD $\pm 0.10 > t_{15}-t_0\,Sr^{2+}/t_{15}-t_0\,Ca^{2+}$ Series E SD ± 0.14. Significance by student's t test; $P<0.01$.

[e] $t_{15}-t_0$ $Sr^{2+}/t_{15}-t_0$ Ca^{2+} Series H SD $\pm 0.10 > t_{15}-t_0$ $St^{2+}/t_{15}-t_0$ Ca^{2+} Series G SD ± 0.10. Significance by student's t test; $P<0.01$.

manganese exchange. When ghosts contained 1.5 mM of calcium and 1.5 mM of strontium as well as manganese, calcium transport occurred almost exclusively at all manganese concentrations tested (Table 9). The specific reduction of strontium transport which occurred when magnesium was replaced by manganese in the ghosts and incubation medium was in evidence even when the calcium depletion from the magnesium and manganese containing ghosts was the same (Table 9). It may also be noted from this table that the presence of manganese in ghosts which also contained magnesium inhibited transport generally and strontium transport particularly.

Discussion

When one considers the inhibition of strontium transport by calcium in the ghosts, it might be suspected that calcium increases the permeability of the ghost membranes to strontium so that the backflow of strontium from the medium equals the rate of strontium transport. However, if this were true one would expect that the apparent stoichiometry of calcium transport would be significantly reduced when strontium is also present in the ghosts. However, since this is not the case, this possibility seems unlikely.

Our data suggests that calcium inhibits strontium transport noncompetitively and in so doing inhibits strontium transport by occupying a site other than the active site. This site may be called a specificity site. It has been noted that calcium increases the susceptibility of the calcium plus magnesium ATPase to proteolytic attack, *n* ethyl maleimide and heat (Bond, 1972). One might, therefore, suspect that calcium prevents the binding of strontium to this enzyme by altering its configuration. Kinetic studies with the membrane calcium plus magnesium ATPase also suggest that calcium ions cooperate to enhance the activity of this enzyme (Scharff, 1978).

At present no definite statement can be made as to why higher levels of magnesium promote strontium transport in the presence of internal calcium.

The fact that high magnesium concentrations at the time of lysis enhanced strontium transport from calcium containing ghosts beyond that observed when high magnesium concentrations were present only at the time of incubation suggests that high magnesium concentrations at the time of lysis induce structural changes in the transport system.

In this regard it is perhaps noteworthy that other proteins appear to be associated with the calcium plus magnesium ATPase (Bond & Clough, 1973; Quist & Roufogalis, 1975). One of them increases the affinity of the calcium plus magnesium ATPase for calcium (Quist and Roufogalis, 1975). There is also evidence that magnesium can induce conformational changes in erythrocyte membrane proteins (Graham & Wallach, 1971).

It has also been noted that calcium can alter the state of aggregation of membrane proteins (Triplett, Wingate & Carraway, 1972). Perhaps magnesium reverses calcium's inhibition of strontium transport by liberating calcium from ATP so that calcium can change the state of protein aggregation in the membrane. In this regard it may be noted that in the low magnesium ghosts the sum of the concentrations of calcium, strontium, and magnesium are approximately equal to the concentration of ATP, while in high magnesium ghosts the sum of these concentrations exceeds the concentration of ATP. However, even in high magnesium ghosts some inhibition of strontium transport by internal calcium can be observed. This inhibition was not overcome by increasing the concentration of strontium in the ghosts and medium.

It is possible that the higher magnesium levels activate a separate transport system which can accommodate strontium more readily. However, the fact that high magnesium concentrations at the time of incubation or lysis failed to stimulate strontium transport from ghosts that contained only strontium makes this possibility seem unlikely. In contrast, it may be noted that calcium transport from ghosts that contained only calcium was stimulated by the presence of higher levels of magnesium at the time of incubation. This finding is in keeping with the observation that the ATPase activity of ghosts was maximal when the magnesium to calcium ratio was approximately five (Dunham & Glynn, 1961).

It has been observed that both calcium and higher levels of magnesium can induce the phosphorylation of erythrocyte membrane proteins by ATP (Knauf, Proverbio & Hoffman, 1974; Rega & Garrahan, 1975; Cha & Lee, 1976) and both types of phosphate bonds are abolished by hydroxylamine. It has also been noted that the phosphoprotein formed in the presence of calcium was dephosphorylated by magnesium (Rega & Garrahan, 1975) while the phosphoprotein formed in the presence of magnesium was dephosphorylated by calcium (Cha & Lee, 1976). One might speculate that the transport of calcium from erythrocyte ghosts may proceed by two mechanisms. In low magnesium ghosts calcium may catalyze the phosphorylation of the transport ATPase by ATP and

magnesium catalyzes the dephosphorylation of the ATPase, while in high magnesium ghosts magnesium at least in part catalyzes the phosphorylation of the transport ATPase by ATP and calcium catalyzes the dephosphorylation of the ATPase. One might speculate further that strontium can only catalyze the dephosphorylation of the transport ATPase and therefore cannot be transported from low magnesium ghosts which also contain 1 mM or more of calcium. The proposed specificity site may be the site that determines which mechanism will be followed by the transport system. If such a site is occupied by calcium, calcium will catalyze the phosphorylation of the transport ATPase; while if the site is occupied by magnesium, calcium or strontium will catalyze the dephosphorylation of the ATPase. It has been noted that the inclusion of 5 mM magnesium in a reaction mixture will enhance the phosphorylation of ghost membranes when calcium is also present in the medium, while the addition of ATP to a suspension of ghost in a solution which contains 1.5 mM magnesium but no calcium will enhance dephosphorylation (Garrahan & Rega, 1978).

When both calcium and strontium are present in the ghosts it is also possible that the presence of magnesium at this site may direct calcium to phosphorylate the transport system and strontium to dephosphorylate it. This latter mechanism could account for the fact that as the calcium concentration in the ghosts declines, calcium transport remains relatively constant while strontium transport increases several-fold (Table 3).

Finally, one might speculate about manganese. Although manganese supports active calcium and strontium transport, it may not be able to support the strontium catalyzed dephosphorylation of the transport system when calcium is also present in the ghosts.

Since manganese which is 0.91 Å in in diameter supports the transport of calcium which is 0.95 Å in diameter, it does not seem likely that magnesium acts as a supporting ion for calcium transport because it is smaller than calcium (0.78 Å *vs.* 0.95 Å in diameter). It is, of course, possible that manganese supports a separate transport system. However, this possibility seems unlikely.

In view of these results and those of others (Scharff, 1978), the possibility that the calcium transport system has a single calcium sight seems unlikely.

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