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Calcium Ion Transport By Pig Erythrocyte Membrane Vesicles

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Preincubating pig erythrocyte membranes with ATP enhances their ability to accumulate Ca^{2+} against a concentration gradient. The extent of this increase is dependent on preincubation time over the period 0–60 min. As the accessibility of outside membrane markers is decreased by preincubation and as accumulated Ca^{2+} is not removed by EGTA [ethanedioxybis(ethylamine)tetra-acetate], it is suggested that ATP causes the formation of sealed inside-out vesicles which can transport Ca^{2+} inward. The transport system requires ATP and Mg^{2+} and exhibits an apparent dissociation constant for Ca^{2+} of approx. 100 μM. Since the dissociation constant for Ca^{2+}-sensitive ATPase (adenosine triphosphatase) in these preparations is similar, it is concluded that this ATPase is responsible for Ca^{2+} transport. Polyphosphoinositide concentrations are also increased during incubation with ATP; however, there is no change in their rate of synthesis or breakdown during Ca^{2+} transport.

It has been known for some time that Ca^{2+} is actively extruded from the human erythrocyte (Schatzmann, 1966; Schatzmann & Vincenzi, 1969) and indirect evidence suggests that Ca^{2+} transport is associated with Ca^{2+}-specific ATP hydrolysis in the erythrocyte (Cha *et al.*, 1971). Incubation of pig erythrocyte membrane preparations with ATP in the presence of Mg^{2+} results in an increase in the concentrations of polyphosphoinositides (Schneider & Kirschner, 1970), paralleled by an increase in the amount of Ca^{2+} that will bind to the membrane (Buckley & Hawthorne, 1972). Preincubation with ATP also leads to an increase in the rate of Ca^{2+}-sensitive ATP hydrolysis by pig erythrocyte membranes (Buckley & Hawthorne, 1972); however, no correlation has been made between erythrocyte polyphosphoinositide concentrations and the rate of Ca^{2+} transport.

Human erythrocytes may be haemolysed and resealed so that they retain their ability to transport Ca^{2+} (Olson & Cazort, 1969). Unfortunately, adequate resealing usually requires an incubation period at 37°C, which leads to difficulties in comparing transport and Ca^{2+}-sensitive enzyme activity. However, Cha *et al.* (1971) have reported uptake of Ca^{2+} by erythrocyte-membrane fragments prepared directly from human erythrocytes by haemolysis and washing. In the present paper, Ca^{2+} uptake by pig erythrocyte membrane fragments is measured and the relation of Ca^{2+} uptake to preincubation with ATP, Ca^{2+}-sensitive ATPase* activity and polyphosphoinositide turnover is described.

* Abbreviation: ATPase, adenosine triphosphatase.

Materials and Methods

Materials

All chemicals and reagents were the purest available. [^{32}P]P_i and $^{45}CaCl_2$ (10–25 mCi/mg of Ca) were obtained from Amersham–Searle Corp. (Arlington Heights, Ill., U.S.A.)

Methods

Preparation of erythrocyte membranes. The method of 'ghost' preparation has been described (Buckley & Hawthorne, 1972). Pig erythrocytes were haemolysed in 1.0mM-Tris–HCl–1.44mM-EDTA, pH 7.4, and washed twice with 1.0mM-Tris–HCl–1.44mM-EDTA–17mM-NaCl, pH 7.4. A concentrated suspension was stored frozen at −20°C. Before incubation, the suspension was thawed and washed twice with 20mM-Tris–HCl, pH 7.4. The white 'ghosts' were resuspended in 20mM-Tris–HCl, pH 7.4, to approximately one-fifth the volume of the original erythrocyte suspension.

Preincubation of erythrocyte membranes. 'Ghosts' were incubated at 37°C and pH 7.4 in 50mM-Tris–HCl containing 20mM-$MgCl_2$, in the presence or absence of 5mM-ATP: preincubation time was normally 30 min. After incubation, the suspension was centrifuged and the membranes were washed twice with 20mM-Tris–HCl, pH 7.4. The membranes were resuspended to a concentration of 10mg of membrane protein/ml in the Tris–HCl and either used immediately or stored at −20°C.

Ca^{2+}-transport measurements. Unless otherwise specified, the erythrocyte membranes (usually 1 mg of

protein) were incubated in 120mM-KCl–15mM-NaCl–20mM-Tris–HCl–50μM-$^{45}CaCl_2$, containing 2mM-$MgCl_2$ and 2mM-sodium ATP in a total volume of 1ml, at pH7.4, 37°C. At the end of the incubation period, uptake was stopped by adding 3vol. of ice-cold 120mM-KCl – 15mM-NaCl – 20mM-Tris – HCl, pH7.4. The contents of the Pyrex tubes were mixed and centrifuged at 10000g for 10min. A 1ml portion of the clear aqueous supernatant was taken and counted for radioactivity. Ca^{2+} uptake was calculated by difference from unincubated controls. At the ionic strength and Ca^{2+} concentration used, the amount of Ca^{2+} bound to the 'ghosts' (as opposed to transported), was less than 0.5nmol/mg of membrane protein.

Ca^{2+}-sensitive ATPase activity. Incubation conditions were similar to those described above for Ca^{2+}-transport measurements. Ca^{2+} concentration was varied over the range 10–500μM in the medium. Incubation was stopped after 20min by adding 2ml of cold 15% (w/v) trichloroacetic acid, and after centrifugation for 10min at 10000g, 2ml of the supernatant was taken for inorganic phosphorus determination (King, 1932). The incubation medium always contained 0.1mM-ouabain and ATP hydrolysis was corrected for Mg^{2+}-sensitive ATPase by using 1mM-EDTA to remove Ca^{2+}.

Measurement of 'ghost' 'sidedness'. Acetylcholinesterase (EC 3.1.1.7) and sialic acid content were measured by using the methods described by Steck & Kant (1973). The marker enzymes NADH–cytochrome *c* oxidoreductase (EC 1.6.99.3) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) could not be detected in these preparations. It was found that 1% Triton X-100 was necessary to render acetylcholinesterase and sialic acid completely accessible.

Incorporation of [^{32}P]P_i into intact erythrocytes. Whole washed cells were incubated at 37°C in an equal volume of 120mM-NaCl–20mM-Tris–HCl–10mM-KCl–2mM-$MgCl_2$, containing 1mg of adenosine/ml, 1mg of glucose/ml and 2μCi of carrier-free [^{32}P]P_i/ml at pH7.4. Where indicated 1mM-$CaCl_2$ was added. At the time-intervals shown, 4ml samples were taken and haemolysed as described above to prepare membranes. Extraction and isolation procedures for polyphosphoinositides have been described (Buckley & Hawthorne, 1972).

Analytical procedures. Aqueous solutions containing ^{45}Ca were counted for radioactivity after addition of 10vol. of toluene containing 6g of PPO (2,5-diphenyloxazole)/litre and 0.12g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre, mixed 2:1 (v/v) with Triton X-100. Lipid ^{32}P was measured in toluene containing 5g of PPO/litre and 0.3g of POPOP/litre. Efficiency in a Picker–Nuclear/Liquimat 220 was approx. 95% in each case. When low concentrations of ^{45}Ca were to be measured, additional non-radioactive $CaCl_2$ was added to lessen wall effects. Protein was measured by using the method of Lowry *et al.* (1951). All other analytical procedures have been described (Buckley & Hawthorne, 1972).

Results

Preincubation of pig erythrocyte membranes in the absence of ATP had little or no effect on their ability to accumulate Ca^{2+}. In contrast, preincubation with ATP resulted in a marked increase in Ca^{2+} uptake from the medium over a period of at least 60min (Fig. 1). This increased capacity to accumulate Ca^{2+} was retained after several freezing and thawing cycles, and after storage for at least 1 week at −20°C. The increased Ca^{2+} accumulation after incubation with ATP was not observed when equivalent concentrations of EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], EDTA, ADP or P_i replaced ATP or when 5mM-NaF was present with ATP in the pre-incubation medium (not shown here). Thus it appears that ATP does not simply trap Ca^{2+} inside vesicles sealed during incubation.

Ca^{2+} uptake was gradually enhanced as the first incubation period was extended, up to at least 60min (Fig. 2). This would suggest that the effect of ATP was not to cause some rapid physical alteration in the membrane fragments, leading to an immediate change in their transport abilities. The time of the first incubation had no effect in the absence of ATP.

It has been suggested that a soluble protein will activate human Ca^{2+}-sensitive ATPase (Bond & Clough, 1973). It is therefore possible that during the first incubation ATP prevented the removal of a protein necessary for activation of uptake in the second incubation. The results in Table 1 show that this is not the case. Membranes preincubated without ATP

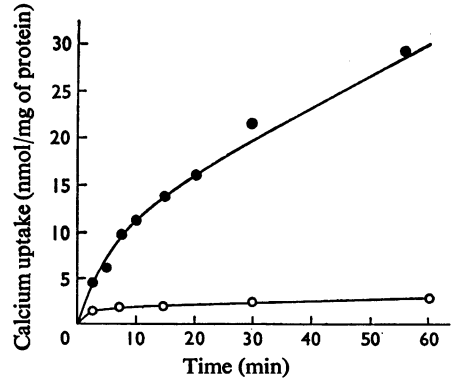

Fig. 1. *Ca^{2+} uptake by pig erythrocyte membranes*

The membranes were preincubated for 45min with (●) or without (○) 5mM-ATP. Ca^{2+} uptake was then measured as described in the Materials and Methods section.

can be activated by a second incubation with ATP, in the presence or absence of the original supernatant.

No Ca^{2+} is lost from the vesicles when they are incubated at 0°C with EGTA. However, EGTA prevents further Ca^{2+} uptake, and incubation at 37°C results in a small loss from the vesicles, likely owing to passive Ca^{2+} efflux (Porzig, 1970) into the medium (Table 2). Hence it can be concluded that the Ca^{2+} associated with the membranes is not simply bound as described under different conditions by Buckley & Hawthorne (1972), but that it is sequestered and inaccessible from the outside. This may mean that sealed inverted vesicles similar to those described by Kant & Steck (1972) are formed during preincubation with ATP.

Further evidence of inversion and sealing is provided by the data in Table 3. Student's *t* test showed that in the membrane preparations examined, the accessibility of acetylcholinesterase ($P<0.01$) and sialic acid ($P<0.02$) were both significantly decreased by preincubation with ATP. These markers are normally located on the outside of the erythrocyte membrane.

The ability of the treated membranes to establish a Ca^{2+} concentration gradient is demonstrated by the data in Table 4. Because the internal volume was simply taken as the volume of the packed membranes, it should be emphasized that the inside/outside ratios represent lower limits. Nevertheless, the ratios are higher than those reported by Weiner & Lee (1972) at similar Ca^{2+} concentrations.

The Mg^{2+} dependence of Ca^{2+} transport (Fig. 3) is similar to that reported by Cha *et al.* (1971) for Ca^{2+} accumulation by human membrane fragments. Uptake was maximal at 4mM-Mg^{2+}.

The effects of alterations to the medium on Ca^{2+} transport are reported in Table 5. Ca^{2+} uptake by preincubated ghosts appears to require ATP and Mg^{2+}. No uptake was recorded when ADP replaced ATP, although Mn^{2+} seems able to replace Mg^{2+}. High concentrations of Ruthenium Red lowered Ca^{2+} accumulation by the membranes. Uptake was completely inhibited by 1mM-EGTA. In other experiments, not shown here, it was observed that 0.1mM-ouabain or 20mM-phosphate in the medium had no effect on Ca^{2+} uptake, nor did the presence of either 50μM-cyclic AMP or 50μM-dibutyryl cyclic AMP. Finally, replacing the univalent cations in the normal incubation medium by 120mM-KCl, 120mM-NaCl or 120mM-choline chloride did not influence uptake. Schatzmann & Vincenzi (1969) have reported that Ca^{2+} efflux from resealed human erythrocytes is not affected by the nature of the univalent ions.

A double-reciprocal plot of Ca^{2+} concentration in the medium against uptake velocity was linear over Ca^{2+} concentrations from 10 to 250μM (Fig. 4). The apparent dissociation constant for uptake ranged between 80 and 100μM in three separate experiments.

Preincubation of pig erythrocyte membrane frag-

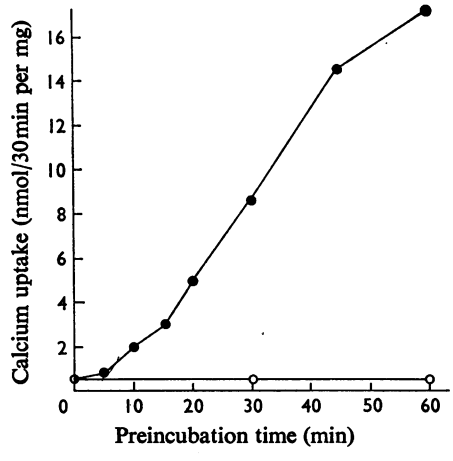

Fig. 2. *Effect of preincubation time on Ca^{2+} uptake*

Membrane preparations were incubated with (●) or without (○) 5mM-ATP for the time-intervals indicated and Ca^{2+} uptake was then measured. See the text for details.

Table 1. *Influence of incubation conditions on Ca^{2+} uptake by erythrocyte membranes*

Erythrocyte membranes were first incubated for 45min in the presence or absence of 5mM-ATP as described in the Materials and Methods section. Then, where indicated, ATP was added or the medium was replaced with fresh buffer containing 5mM-ATP and the 'ghosts' were incubated for an additional 45min. After these incubations, the membranes were washed with 20mM-Tris–HCl and samples were taken to measure Ca^{2+} uptake.

First incubation	Second incubation	Ca^{2+} uptake (nmol/20min per mg of protein)
+5mM-ATP	—	7.2
No ATP	—	1.8
No ATP	Same medium+5mM-ATP	7.1
No ATP	New medium+5mM-ATP	7.2

Table 2. *Effect of EGTA on Ca^{2+} retention*

Membrane preparations preincubated with ATP were used. All samples were first incubated for 30min with 50μM-$^{45}Ca^{2+}$ as described in the text. The amount of Ca^{2+} accumulated was measured and EGTA (1mM final concn.) was then added where indicated. After a second incubation at 0°C or 37°C, the amount of Ca^{2+} retained by the membranes was measured. The results are the mean of duplicate determinations and are typical of three such experiments.

Conditions of second incubation	Ca^{2+} retained (nmol/mg)
No second incubation	17.8
30min, 37°C	25.1
30min, +EGTA, 0°C	17.7
10min, +EGTA, 37°C	16.9
30min, +EGTA, 37°C	13.4

Table 3. *Accessibility of acetylcholinesterase and sialic acid in preincubated membranes*

Accessible acetylcholinesterase and sialic acid were determined on membranes preincubated with (+ATP) or without (−ATP) 5mM-ATP. Total membrane acetylcholinesterase and sialic acid were determined in the presence of Triton X-100. See the text for details. Each value is the mean of four determinations on different membrane preparations, ±S.E.M.

	Membranes (+ATP)	Membranes (−ATP)
Acetylcholinesterase (μmol of product/min per mg of protein)		
Accessible activity	0.91±0.01	1.24±0.17
Total activity	1.35±0.03	1.36±0.03
% Accessible	68±0.8	92±5.8
Sialic acid (expressed as nmol of *N*-acetylneuraminic acid/mg of protein)		
Accessible	21±1.6	28±0.5
Total	29±1.5	29±1.5
% Accessible	73±4.2	94±4.3

ments with ATP results in an increase in Ca^{2+}-sensitive ATPase activity (Buckley & Hawthorne, 1972). A double-reciprocal plot of calcium concentration in the medium against the rate of ATP hydrolysis was also linear over the concentration range 30–250μM (Fig. 5). It is not clear whether deviations from linearity below 30μM were due to contamination by Ca^{2+} in the reagents or to a biphasic response of the ATPase, as has been reported for human erythrocytes (Schatzmann & Rossi, 1971). Over the entire concentration range, 0.1mM-Ruthenium Red had little or no effect on ATP hydrolysis. In contrast, Watson *et al.* (1971) have reported pronounced inhibition of human

Table 4. *Effect of initial Ca^{2+} concentrations on ratios of inside/outside Ca^{2+} concentration after uptake*

Initial Ca^{2+} concentrations were varied in the incubation medium described in the Materials and Methods section. At each Ca^{2+} concentration less than 1% of the Ca^{2+} associated with the membranes after 30min incubation was found to be bound at zero time. Inside concentrations after incubation were calculated from the amount of Ca^{2+} removed from the medium by using the 'ghost' pellet size as a measure of inside volume.

Initial concn. of Ca^{2+} (zero time) (μM)	Uptake ratio [inside]/[outside] (30min)
450	10
180	23
90	38
45	72
22.5	164
9	237
4.5	263

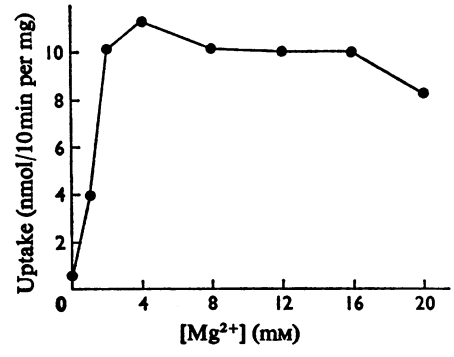

Fig. 3. *Mg^{2+} requirement for Ca^{2+} transport*

The concentration of Mg^{2+} in the medium was varied between 1 and 20mM. For further details see the text.

Table 5. *Effect of alterations to the medium on Ca^{2+} uptake*

The medium described in the Materials and Methods section was used with the modifications shown in the Table. All concentrations are final concentrations in the medium.

Alteration	Uptake (% control)
−ATP	12
−ATP, 2mM-ADP	9
$-Mg^{2+}$, 2mM-$MnCl_2$	85
Ruthenium Red (0.05mM)	70
(0.5mM)	62
EGTA (1mM)	0

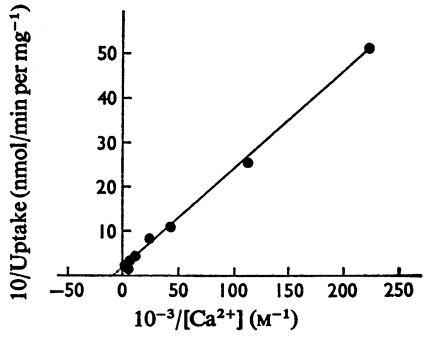

Fig. 4. *Double-reciprocal plot of Ca^{2+} uptake against concentration*

Initial Ca^{2+} concentration in the medium was varied between 4 and 500 μM and Ca^{2+} uptake at each concentration was measured over a period of 10 min.

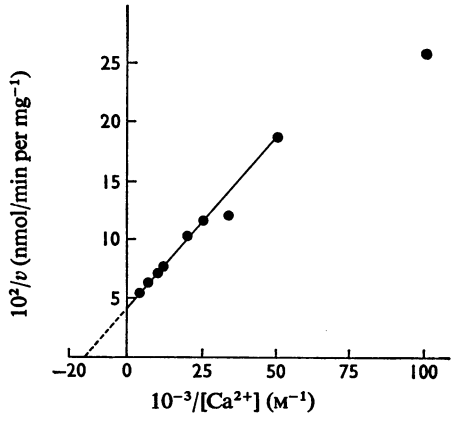

Fig. 5. *Double-reciprocal plot of Ca^{2+}-ATPase activity against Ca^{2+} concentration*

The procedure used for the measurement of Ca^{2+}-ATPase activity is described in the Materials and Methods section.

erythrocyte Ca^{2+}-sensitive ATPase at similar concentrations of Ruthenium Red. Extrapolation of the linear portion of the curve in Fig. 5 led to an apparent dissociation constant for ATP hydrolysis of 70–90 μM in three experiments, in close agreement with the value for Ca^{2+} transport and the value for the low-affinity Ca^{2+}-sensitive ATPase of the human erythrocyte (Schatzmann & Rossi, 1971); Schatzmann & Vincenzi (1969) have reported a ratio of about one terminal pyrophosphate bond of ATP hydrolysed/molecule of calcium transported by resealed human erythrocytes. As might be expected, this ratio was much higher in the membrane preparations. The rate of Ca^{2+}-sensitive ATP hydrolysis was usually at least ten times higher than the rate of calcium transport.

Polyphosphoinositide concentrations are also increased during incubation of erythrocyte membranes with ATP (Buckley & Hawthorne, 1972). To test for dynamic involvement of these inositides in Ca^{2+} transport, the rate of $[^{32}P]P_i$ incorporation into the di- and tri-phosphoinositides of intact cells was measured in the presence and absence of Ca^{2+} in the medium. There was no significant change in incorporation rate.

Finally, the monoesterified phosphate groups of membrane inositides were labelled during preincubation with [^{32}P]ATP and the effect of Ca^{2+} uptake on polyphosphoinositide breakdown was measured in a second incubation. Ca^{2+} accumulation did not lead to a detectable loss of polyphosphoinositides.

Discussion

The results presented here provide clear evidence that erythrocyte membrane vesicles accumulate Ca^{2+} by a process of active transport. Both ATP (Table 5) and Mg^{2+} (Fig. 3) are required for Ca^{2+} uptake, in agreement with the observations of Duffy & Schwarz (1973). The apparent dissociation constant for Ca^{2+} transport (Fig. 4) compares favourably with the dissociation constant for Ca^{2+}-sensitive ATPase, suggesting that this enzyme is responsible for uptake. It is unlikely that the observed accumulation is due to the sequestration of Ca^{2+} inside sealed vesicles, bound to ATP. If this were the case both ATP and Mg^{2+} outside the vesicles would be expected to lower uptake. Further, EGTA, which will discriminate between Mg^{2+} and Ca^{2+} in the presence of ATP, will not replace ATP in the preincubation medium. Finally, NaF prevents the stimulation of uptake ability by ATP.

Ca^{2+} transport by the erythrocyte is normally from the inside to the outside (Schatzmann, 1966); however, the direction of Ca^{2+} movement is reversed in inside-out resealed human erythrocytes (Weiner & Lee, 1972). The results presented here suggest that ATP causes inversion and sealing of pig erythrocyte membranes, leading to a marked increase in their ability to transport Ca^{2+}. Penniston & Green (1968) have demonstrated an energized change in the shape of pig erythrocyte 'ghosts' during incubation with ATP, resulting in pinocytosis and the formation of inverted membrane vesicles. It appears as if such a change is occurring under the conditions described here and that the vesicles which are formed are responsible for the uptake of Ca^{2+}. Since the increase is gradual (Fig. 2) and permanent, it is unlikely that it is due to a conformational change in Ca^{2+}-sensitive ATPase induced by Mg^{2+}-ATP as described by Bond (1972). Such a change would be expected to be both rapid and transitory.

The mechanism by which ATP alters the erythrocyte membrane is unknown. Resealing of erythrocyte

'ghosts' is dependent on the charge of ionizable groups in the membrane (Lepke & Passow, 1971); however, it is doubtful whether phosphorylation of membrane proteins by ATP could significantly alter the overall membrane charge. As incubation of pig erythrocyte membranes with ATP results in a significant increase in their polyphosphoinositide concentrations, it is tempting to speculate that these acidic lipids play a role in vesiculation and resealing of the membrane. Certainly, however, the polyphosphoinositides are not dynamically involved in the transport of Ca^{2+}.

Because the preincubated membranes actively transport Ca^{2+} before or after freezing and thawing, they would appear to provide an excellent system for the continued study of Ca^{2+} transport.

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