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Calcium Ion-Dependent *p*-Nitrophenyl Phosphate Phosphatase Activity and Calcium Ion-Dependent Adenosine Triphosphatase Activity from Human Erythrocyte Membranes

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In the presence of ATP and of Mg^{2+}, human erythrocyte membranes show a phosphatase activity towards *p*-nitrophenyl phosphate which is activated by low concentrations of Ca^{2+}. The effect of Ca^{2+} is strongly enhanced if either K^+ or Na^+ is also present. Activation of the *p*-nitrophenyl phosphate phosphatase by Ca^{2+} reaches a half-maximum at about 8 μM-Ca^{2+} and is apparent only when the ion has access to the inner surface of the cell membrane. Ca^{2+}-dependent phosphatase activity can only be observed if ATP is at the inner surface of the cell membrane, and the presence of ATP seems to be absolutely necessary, since either its removal or its replacement by other nucleoside triphosphates abolishes the activating effect of Ca^{2+}. The properties of the (ATP+Ca^{2+})-dependent phosphatase are very similar to those of the Ca^{2+}-dependent ATPase (adenosine triphosphatase), also present in erythrocyte membranes, which probably is involved in Ca^{2+} transport in erythrocytes. The similarities suggest that both activities may be properties of the same molecular system. This view is further supported by the fact that *p*-nitrophenyl phosphate inhibits to a similar extent Ca^{2+}-dependent ATPase activity and ATP-dependent Ca^{2+} extrusion from erythrocytes.

It now seems well established that the very low intracellular concentration of calcium in human erythrocytes (Harrison & Long, 1968), is maintained by active Ca^{2+} extrusion (Schatzmann & Vincenzi, 1969). The energy for this movement comes from the hydrolysis of ATP at the inner surface of the cell membrane (Schatzmann & Vincenzi, 1969) and hence, in isolated erythrocyte membranes, the Ca^{2+}-transport system survives as a Ca^{2+}-dependent ATPase* activity (Dunham & Glynn, 1961; Wins & Schoffeniels, 1966; Bond & Green, 1971; Schatzmann & Rossi, 1971).

We have reported elsewhere (Pouchan *et al.*, 1969; Garrahan *et al.*, 1970) that, provided that ATP is present, isolated erythrocyte membranes show a phosphatase activity which is activated by Ca^{2+}. This phosphatase activity requires Mg^{2+} and is enhanced by K^+, the activating effect of K^+ being insensitive to cardiac glycosides such as ouabain. These properties, together with the similarity between the plots of velocity versus Ca^{2+} concentration of the phosphatase and the ATPase activities (Garrahan *et al.*, 1970), suggested that both activities may be related.

In the present paper we report experiments designed to characterize in detail the properties of the phosphatase by using *p*-nitrophenyl phosphate as substrate and to compare them with those of the ATPase. A preliminary account of some of the experiments reported here has been published (Rega *et al.*, 1972).

* Abbreviation: ATPase, adenosine triphosphatase.

Experimental

Preparation of erythrocyte membranes

Erythrocyte membranes were prepared from freshly drawn human blood by the procedure described by Garrahan *et al.* (1969). The membranes were fragmented by freezing and thawing and kept in 15mM-Tris–HCl buffer (pH 7.2) at 5°C. The storage time at low temperature was never more than 60min.

Preparation of reconstituted 'ghosts'

Reconstituted 'ghosts' were prepared by a modification of the procedure of Schatzmann & Vincenzi (1969). For the flux experiments the 'ghosts' were prepared by lysing 1vol. of packed erythrocytes in 50vol. of an ice-cold solution containing (mM): $CaCl_2$, 2; $MgCl_2$, 8; ATP, 0 or 3; Tris–HCl, 10; (pH 7.4 at 25°C). The haemolysate was left for 2min, and then a sufficient volume of a concentrated choline chloride solution was added to restore the osmoticity of the haemolysate to about 300 milliosomolar. After this step, the 'ghosts' were washed with 2×40vol. of

an ice-cold solution containing (mM): choline chloride, 130; Tris–HCl, 30; (pH 7.4 at 25°C).

Two kinds of 'ghosts' were used during the asymmetry experiments. Singly haemolysed reconstituted 'ghosts' were prepared by lysing 1 vol. of packed erythrocytes in 800 vol. of an ice-cold solution containing (mM): $MgCl_2$, 5; ATP, 0.3; EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], 0.5; Tris–HCl, 40; (pH 7.2 at 25°C). Doubly haemolysed 'ghosts' were prepared by lysing 1 vol. of singly haemolysed 'ghosts' in 40 vol. of an ice-cold solution containing (mM): $CaCl_2$, 0 or 1; $MgCl_2$, 5; ATP, 2; Tris–HCl, 10; (pH 7.2 at 25°C). For both kinds of 'ghosts' the haemolysate was left for 10 min. After this step the 'ghosts' were washed with about 3 × 40 vol. of a solution containing (mM): $MgCl_2$, 5; Tris–HCl, 150 (pH 7.4 at 25°C). The second haemolysis was necessary because 'ghosts' lysed in Ca^{2+}-containing solutions retain a significant amount of the soluble phosphatase normally present in erythrocytes. The fraction of 'ghosts' that had regained their low permeability to Ca^{2+} and ATP was measured by the procedure of Rega *et al.* (1970). About 50% (v/v) of the singly haemolysed 'ghosts' and about 20% (v/v) of the doubly haemolysed 'ghosts' were impermeable to ATP and Ca^{2+}.

Measurements of enzyme activities

Phosphatase activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate as previously described (Garrahan *et al.*, 1969). ATPase activity was assayed by measuring the amount of P_i (Fiske & Subba Row, 1925) in the supernatant of the reaction mixture after deproteinization with trichloroacetic acid (final concentration 5%, w/v). When ATPase activity was measured in media containing *p*-nitrophenyl phosphate, the amount of P_i released from ATP was calculated as the difference between total P_i and P_i released from *p*-nitrophenyl phosphate calculated from the *p*-nitrophenol concentration at the end of the incubation time. The composition of the incubation media differed in the different experiments but the total molarity was always 160 mM and the pH was kept at 7.8 (at 37°C). The quantity of either membranes or 'ghosts' in the reaction mixture was that which gave an haematocrit of 5% calculated on the original volume of the cells. All incubations were carried out at 37°C for a 20–30 min period.

Calculation of 'contaminating' calcium

The apparent affinity of the membrane phosphatase and the membrane ATPase for Ca^{2+} is high enough to allow traces of Ca^{2+} present in the nominally Ca^{2+}-free media to elicit significant Ca^{2+}-dependent enzymic activities. Therefore, when the effects of low Ca^{2+} concentration were studied, it was necessary to determine the amount of Ca^{2+} present as contaminant. This was done by the following procedure. Ca^{2+}-dependent activity (v) was taken as the difference between the activities in the absence and in the presence of 0.5 mM-EGTA. Since total Ca^{2+} in the medium is equal to the Ca^{2+} added (C) plus a constant amount of contaminating Ca^{2+} (c), if activation by Ca^{2+} follows Michaelis–Menten kinetics we may write:

$$v = \frac{V}{\left[1 + \frac{K}{(C+c)}\right]} \quad (1)$$

where V is the Ca^{2+}-dependent activity at non-limiting Ca^{2+} concentration and K is the total Ca^{2+} concentration giving half-maximal activation.

Eqn. (1) can be rearranged to give:

$$v/(V-v) = C/K + c/K \quad (2)$$

Eqn. (2) shows that when the ratio $v/(V-v)$ is plotted against the concentration of added Ca^{2+} a straight line will be obtained cutting the abscissa at a point equal to $-c$.

Eqn. (2) was used to calculate the concentration of contaminating Ca^{2+} in the experiments of Figs. 1 and 2. To do this V was assumed to be equal to the Ca^{2+}-dependent activity at 250 μM-Ca^{2+}. In all cases the experimental results yielded a straight line. The calculated amount of contaminating Ca^{2+} was about 4 μmol/litre.

Measurement of Ca^{2+} efflux

Ca^{2+} containing reconstituted 'ghosts' were suspended in an ice-cold solution containing (mM): choline chloride, 130; Tris–HCl, 50; (pH 7.8 at 37°C). The haematocrit of the 'ghost' suspension was 20% in terms of original cells. When present *p*-nitrophenyl phosphate replaced an equimolar amount of Tris–HCl. When the effects of K^+ were tested, 30 mM-KCl replaced an equimolar amount of choline chloride. Suitable volumes of the 'ghost' suspension were added to a series of tubes immersed in an ice bath. The tubes were transferred to a bath at 37°C, and individual tubes were removed at 5 min intervals during 20 min, returned to the ice bath and then centrifuged for 5 min at 12000*g* at 2°C. The concentration of Ca^{2+} in the supernatants was measured. Ca^{2+} efflux was calculated from the initial slope of the plot of external Ca^{2+} concentration versus time. All effluxes were related to the volume of 'ghosts'; to do this the relative volume of 'ghosts' in the 'ghost' suspensions was measured by the capillary microhaematocrit technique.

Measurement of calcium

The concentration of calcium in ghosts and in incubation media was measured by atomic-absorp-

tion spectrophotometry in an EEL instrument by using an acetylene–air flame. All solutions were deproteinized with trichloroacetic acid (final concn. 5%, w/v). Before measurement 1 vol. of a 17.4% (w/v) solution of $LaCl_3,7H_2O$ in 0.2M-HNO_3 was added to 10 vol. of each sample.

Determination of intracellular P_i and p-nitrophenol

These were measured by the procedure of Garrahan & Rega (1972).

Sources of materials

p-Nitrophenyl phosphate was always used as its Tris salt and was prepared from 'Sigma 104 Phosphatase Substrate' (Sigma Chemical Co., St. Louis, Mo., U.S.A.). *p*-Nitrophenol was crystalline *p*-nitrophenol 'Spectrophotometer Grade' (Sigma). ATP, ITP CTP, GTP and UTP were obtained from Sigma. Before use, nucleotides were converted into their Tris salt by the procedure of Rega *et al.* (1970). $CaCl_2$ solutions were prepared from AnalaR $CaCO_3$ (BDH Chemicals Ltd., Poole, Dorset, U.K.). All other salts and reagents were A.R. grade. The solutions were prepared in double-glass-distilled water.

Results

Effects of Ca^{2+} on phosphatase and ATPase activities

Fig. 1 shows the results of an experiment in which the effects of increasing Ca^{2+} concentrations on the rate of *p*-nitrophenyl phosphate hydrolysis by the erythrocyte-membrane phosphatase were assayed in media containing ATP and K^+. Ca^{2+} raises the rate of *p*-nitrophenyl phosphate hydrolysis along a curve which levels off at about 60μM-Ca^{2+}. When the reciprocal of the Ca^{2+}-dependent phosphatase activity is plotted against the reciprocal of Ca^{2+} concentration, the experimental points can be fitted to a straight line (inset in Fig. 1). Half-maximal activation of the phosphatase by Ca^{2+} is reached at 8.3μM-Ca^{2+}. Results in Fig. 1 also show that when K^+ is omitted from the incubation media most of the activating effect of Ca^{2+} disappears. The small effect of Ca^{2+} in the absence of K^+ is maximal at about 10μM-Ca^{2+}.

In the presence of K^+ the response of the phosphatase to low concentrations of Ca^{2+} resembles very much that of the erythrocyte membrane ATPase, as reported by Bond & Green (1971). This is further confirmed by the experiment in Fig. 2, in which the effects of Ca^{2+} on erythrocyte membrane ATPase activity were assayed on the same preparation and under conditions identical with those used in the experiment of Fig. 1. Ca^{2+} increases ATP hydrolysis along hyperbolic curves, half-maximal activity recurring at 9.3μM-Ca^{2+}, i.e. just about the concentration of Ca^{2+} required in the medium for half-maximal activity of the phosphatase in the presence of K^+. In contrast with the large dependence on K^+ of the phosphatase, removal of K^+ decreases by only 30% the maximum effect of Ca^{2+} on the ATPase, leaving unaltered the shape of the Ca^{2+}-activation curve.

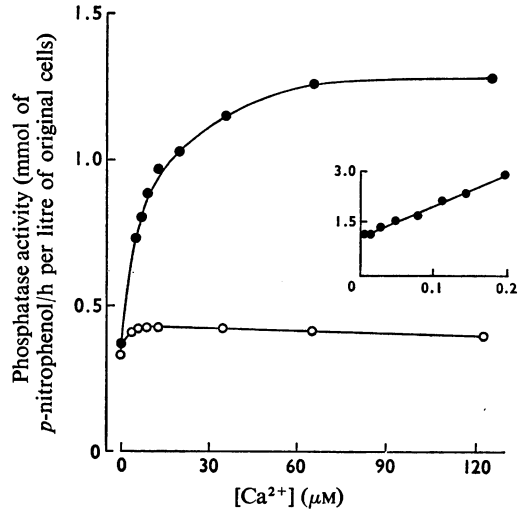

Fig. 1. *Effect of Ca^{2+} on p-nitrophenyl phosphate hydrolysis by fragmented erythrocyte membranes in the presence* (●) *and absence* (○) *of K^+*

Membranes were incubated in a medium containing (mM): $MgCl_2$, 5; KCl, 100; Tris–HCl, 40; *p*-nitrophenyl phosphate, 5; ATP, 0.5; (pH 7.8 at 37°C). In the K^+-free media K^+ was replaced by 100mM-Tris–HCl. Ca^{2+} was added as $CaCl_2$. The total Ca^{2+} concentration in the media was calculated by taking into account the concentration of contaminating Ca^{2+} by the procedure outlined in the Experimental section. The zero-Ca^{2+} points are the activities measured in media containing 0.5mM-EGTA. The inset shows a reciprocal plot of the concentration of Ca^{2+} (μM^{-1}) versus phosphatase activity [(mmol of *p*-nitrophenol/h per litre of original cells)$^{-1}$] in K^+-containing medium.

Requirement of ATP for activation by Ca^{2+}

Ca^{2+}-dependent phosphatase activity is only apparent when ATP is present in the incubation media. Other nucleoside triphosphatases seem to be without effect (Garrahan *et al.*, 1970). On the other hand Watson *et al.* (1971) have shown that nucleoside triphosphates other than ATP are almost ineffective as substrates of the Ca^{2+}-dependent ATPase from

erythrocyte membranes. These observations suggest that both enzymes may have the same nucleotide requirements. A more direct analysis of this point was undertaken in the experiment shown in Table 1, in which erythrocyte membranes were assayed for Ca^{2+}-dependent phosphatase and ATPase activities in media containing different nucleoside triphosphates. Results show that: (i) even at concentrations four times that of ATP which allows the maximal effect of Ca^{2+}, CTP, GTP, ITP or UTP do not replace ATP in promoting activation by Ca^{2+} of the phosphatase, since the inhibitory effect of Ca^{2+} in the absence of ATP persists when other nucleotides are added, (ii) the inability of nucleoside triphosphates other than ATP in promoting activation by Ca^{2+} of the phosphatase is associated to their ineffectiveness as substrates of the Ca^{2+}-dependent ATPase; (iii) a threefold excess of either ITP or UTP over ATP leaves unaltered the effect of ATP on Ca^{2+}-dependent phosphatase, suggesting that the lack of effectiveness of nucleoside triphosphates other than ATP is due to their inability to bind to the site for ATP.

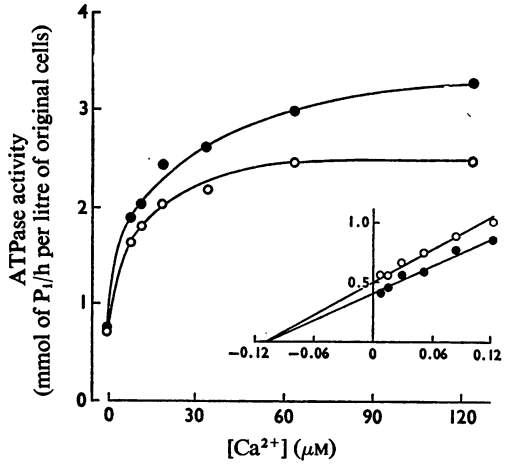

Fig. 2. *Effect of Ca^{2+} on ATP hydrolysis by fragmented erythrocyte membranes in the presence* (●) *and absence* (○) *of K^+*

The composition of the incubation media was similar to that used in the experiment of Fig. 1 except that *p*-nitrophenyl phosphate was replaced by Tris–HCl. Other conditions are given in Fig. 1. The inset shows a reciprocal plot of the concentration of Ca^{2+} (μM^{-1}) versus ATPase activity [(mmol of P_i/h per litre of original cells)$^{-1}$].

The K_m for ATP of the Ca^{2+}-dependent ATPase is 60 μM (A. F. Rega, D. E. Richards & P. J. Garrahan, unpublished work). In Fig. 3 Ca^{2+}-dependent ATPase activity, measured at four different ATP concentrations, is plotted against the ratio of Ca^{2+}-dependent to Ca^{2+}-independent phosphatase activities. The experimental points fit reasonably well a straight line with zero intercept, suggesting that Ca^{2+}-dependent phosphatase and ATPase activities not only share the same nucleotide specificity but also have a similar apparent affinity for ATP.

Effects of univalent cations

Schatzmann & Rossi (1971) have shown that maximal activation by Ca^{2+} of the erythrocyte mem-

Table 1. *Specific requirement of ATP for Ca^{2+} activation of the phosphatase and the ATPase activities from erythrocyte membranes*

The activities were measured simultaneously and on the same batch of membranes. The incubation media had similar salt composition to the K^+-containing media employed in the experiments of Figs. 1 and 2.

Additions (mM)	(Activity with 100 μM-Ca^{2+}) − (Activity without Ca^{2+})	
	Phosphatase (mmol of *p*-nitrophenol/h per litre of original cells)	ATPase (mmol of P_i/h per litre of original cells)
None	−0.14	—
ATP (0.25)	0.62	0.80
ITP (0.25)	−0.15	0.00
(1.00)	−0.11	−0.02
CTP (0.25)	−0.13	0.06
(1.00)	−0.14	0.00
GTP (0.25)	−0.12	0.00
(1.00)	−0.12	0.07
UTP (0.25)	−0.14	0.02
(1.00)	−0.10	0.00
ATP (0.25)+UTP (0.75)	0.61	—
ATP (0.25)+ITP (0.75)	0.57	—

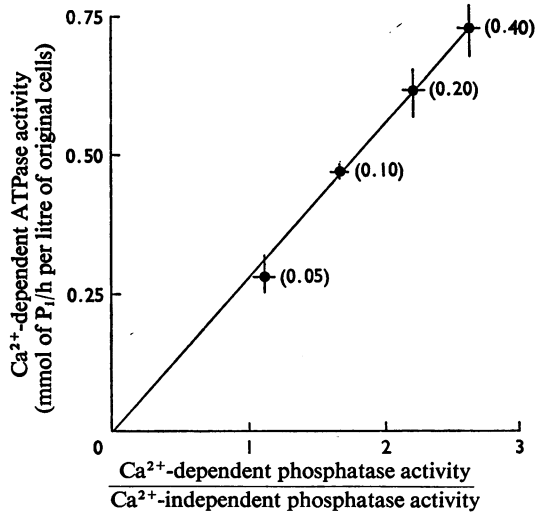

Fig. 3. *Comparison of the effects of four different concentrations of ATP on Ca^{2+}-dependent ATPase and ($ATP+Ca^{2+}$)-dependent phosphatase activity from erythrocyte membranes*

Ca^{2+}-dependent enzymic activities are the difference between the activities in media containing 0.10mM-$CaCl_2$ and in Ca^{2+}-free media containing 0.5mM-EGTA. Phosphatase activity is expressed as the ratio of Ca^{2+}-dependent to Ca^{2+}-independent activities to cancel the effect of ATP on the K_m of the phosphatase (Garrahan *et al.*, 1970). This mode of expression is justified since the K_m of the phosphatase is independent of the concentration of Ca^{2+} (Garrahan *et al.*, 1970). The activities were measured in K^+-containing media of similar salt composition to those used in the experiments of Figs. 1 and 2. The numbers in parentheses represent the concentration of ATP (mM). The vertical and horizontal bars are the ranges. Each experimental point represents the mean of three determinations.

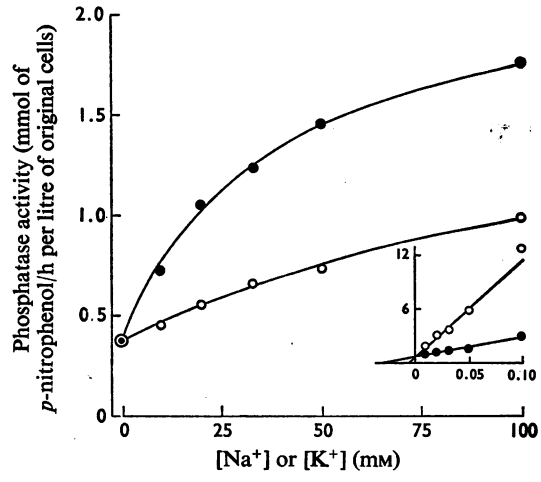

Fig. 4. *Effects of Na^+ (○) or K^+ (●) on phosphatase activity of erythrocyte membranes*

The media contained (mM): $MgCl_2$, 5; Tris–HCl, 150; $CaCl_2$, 0.1; ATP, 0.5; *p*-nitrophenyl phosphate, 10; (pH7.4 at 37°C). For other details see the Experimental section. When present NaCl or KCl replaced an equimolar amount of Tris–HCl. The inset shows reciprocal plots of the concentration of Na^+ and K^+ (mM^{-1}) versus phosphatase activity [(mmol of *p*-nitrophenol/h per litre of original cells)$^{-1}$].

brane ATPase requires either K^+ or Na^+, the effect of K^+ being half-maximal at 5.8mM-K^+ and that of Na^+ at 33mM-Na^+. In view of these results it was decided to see whether Na^+ could replace K^+ as an activator of the phosphatase. Fig. 4 shows the results of an experiment in which the effects of increasing concentrations of either Na^+ or K^+ were tested on phosphatase activity in the presence of ATP and Ca^{2+}. Both cations activate the phosphatase though, within the range of concentrations studied, K^+ is considerably more effective than Na^+. However, reciprocal plots of the activation curves (inset in Fig. 4) yield straight lines that intersect on the ordinate, suggesting that at non-limiting concentrations Na^+ and K^+ are equally effective in activating the phosphatase. The effect of K^+ is half-maximal at 35mM-K^+ and that of Na^+ at 190mM-Na^+. Although the apparent affinity for Na^+ and K^+ of the phosphatase is considerably lower than that reported by Schatzmann & Rossi (1971) for the ATPase, the ratio of the apparent affinity for K^+ and for Na^+ is the same for both enzymes.

Effect of storage of membrane suspensions on the response of phosphatase and ATPase activities to Ca^{2+}

In the experiment of Fig. 5, Ca^{2+}-dependent and Ca^{2+}-independent phosphatase and ATPase activities were measured in erythrocyte membranes that had been previously stored in 15mM-Tris–HCl (pH7.8) at 6°C during 0, 4.5 and 22h. Storage induces a progressive and parallel decrease in Ca^{2+}-dependent ATPase and in ($ATP+Ca^{2+}$)-dependent phosphatase, having practically no effect on Ca^{2+}-independent ATPase and phosphatase activities. Although loss of Ca^{2+}-dependent activities on storage was always observed, the rate and extent of this phenomenon varied between different preparations.

The conditions of storage used in the experiment of Fig. 5 are not a requisite for the loss in Ca^{2+}-dependent enzymic activities, since this effect is also

apparent when membranes are stored in media of physiological ionic strength or when buffers other than Tris–HCl are used. Results in Fig. 5 also show that the concentrations of Ca^{2+} and ATP in packed membranes are not very different from those in the suspending media and remain constant during storage. The disappearance of the effect of Ca^{2+} cannot be attributed, therefore, to the loss in accessibility of the sites for Ca^{2+} owing to 'sealing' (see Bramley *et al.*, 1971) of the membranes during storage. Further, the effect of storage does not seem to be caused by solubilization of the Ca^{2+}-dependent enzymes (see Rosenthal *et al.*, 1970), since no Ca^{2+}-dependent phosphatase or ATPase activities could be detected in high speed (12000*g* for 15min) supernatants of membrane suspensions after storage.

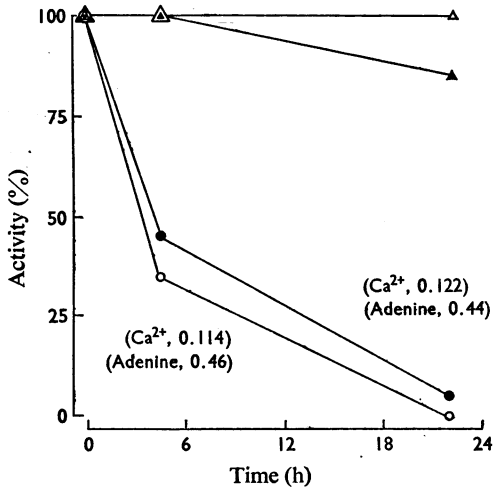

Fig. 5. *Ca^{2+}-dependent* (●) *and Ca^{2+}-independent* (▲) *phosphatase activity and Ca^{2+}-dependent* (○) and *Ca^{2+}-independent* (△) *ATPase activity as a function of the duration of storage in* 15mM-*Tris–HCl* (*pH*7.4 *at* 5°*C*) *at* 6°*C*

Ca^{2+}-dependent activities were measured as indicated in Fig. 3. Activities were assayed simultaneously on the same preparation of membranes. Final incubation media had the same composition as the K^+-containing media used in the experiments of Figs. 1 and 2. The values in parentheses are the concentrations of Ca^{2+} and of adenine (mmol/l) in high-speed pellets of the membrane suspension at 4.5 and 22h. Adenine was estimated from the absorbance at 260nm of the pellets after deproteinization with $HClO_4$ (final concn. 3%, w/v).

The loss in Ca^{2+}-dependent phosphatase and ATPase activities can be fully prevented if 0.5mM-EDTA is present during storage (Table 2). EDTA not only protects against inactivation but is also able to reverse the effect of storage since, if added after a 24h storage, 75% of the original (ATP+Ca^{2+})-dependent phosphatase and 81% of the Ca^{2+}-dependent ATPase activities are recovered.

These results seem to indicate that the decrease in Ca^{2+}-dependent activities with time is the expression of the reversible inhibition of these activities by an EDTA-chelatable substance, present as an impurity in the media in which membranes were stored. In an attempt to identify this hypothetical inhibitor, the effects of Cu^{2+}, Fe^{3+}, Ni^{2+}, Mn^{2+}, Co^{2+} and Cd^{2+} on ATPase and phosphatase activities were tested. None of these cations was able to mimic the effects of storage, since in concentrations ranging from 25 to 100μM they had either no effect or induced a generalized and unspecific loss of phosphatase and ATPase activities.

Asymmetrical effects of Ca^{2+} and ATP

The experiments reported in this section were designed to test at which surface of the erythrocyte membrane ATP and Ca^{2+} are effective as activators

Table 2. *Effects of EDTA on the time-dependent inactivation of Ca^{2+}-dependent phosphatase and ATPase activities from erythrocyte membranes*

Membranes were stored at 6°C and then washed with 50vol. of 15mM-Tris–HCl to remove most of the EGTA; the amount of membranes present was that which gave a haematocrit of 10% calculated on the original volume of cells. Both activities were measured at the same time on the same batch of membranes. All final incubation media contained 0.5mM-ATP and were otherwise identical with those used in the experiment of Fig. 3.

Conditions of storage	Ca^{2+}-dependent activity after storage / Ca^{2+}-dependent activity before storage	
	ATPase	Phosphatase
24h in 15mM-Tris–HCl	0.11	−0.09
24h in 15mM-Tris–HCl+0.5mM-EDTA	1.00	0.99
24h in15mM-Tris–HCl followed by 20min in 15mM-Tris–HCl+0.5mM-EDTA	0.81	0.75

Table 3. *K^+-dependent p-nitrophenyl phosphate hydrolysis in reconstituted 'ghosts' containing either ATP or ATP+Ca^{2+}*

Doubly haemolysed ghosts were used in expt. no. 1 and singly haemolysed ghosts were used in expt. no. 2. The composition of the media in which 'ghosts' were prepared is given in the Experimental section. In expt. no. 1 'ghosts' were suspended in media containing (mM): Tris–HCl, 100; KCl, 50; *p*-nitrophenyl phosphate, 10; EGTA, 0.5; EDTA, 0.5; (pH 7.8 at 37°C). In expt. no. 2 'ghosts' were suspended in media containing (mM): $MgCl_2$, 5; Tris–HCl, 100; KCl, 50; *p*-nitrophenyl phosphate, 10; EGTA, 0.5; (pH 7.8 at 37°C). Where indicated 0.75 mM-$CaCl_2$ and 0.5 mM-ATP were present. K^+-dependent phosphatase activity is the difference between the activities in the above-mentioned media and the activities in media in which KCl was replaced by an equimolar amount of Tris–HCl. 'Ghosts' were disrupted by freezing and thawing.

	Additions to the suspending media	K^+-dependent phosphatase activity (mmol of *p*-nitrophenol/h per litre of original cells)
Expt. no. 1		
'Ghosts' containing ATP	None	0.24
'Ghosts' containing ATP+Ca^{2+}	None	0.81
Expt. no. 2		
'Ghosts' containing ATP	None	0.30
'Ghosts' containing ATP	Ca^{2+}	0.31
'Ghosts' containing ATP	Ca^{2+}+ATP	0.46
Disrupted 'ghosts'	Ca^{2+}+ATP	0.79

of the phosphatase. For this purpose, phosphatase activity was measured in reconstituted 'ghosts'. K^+-dependent phosphatase activity was measured in 'ghosts' containing either ATP or (ATP+Ca^{2+}) (Table 3, expt. no. 1). To prevent K^+-dependent phosphatase activity by the membranes which did not regain their low permeability to ATP and Ca^{2+}, in this experiment the 'ghosts' were suspended in Mg^{2+}-free media containing 0.5 mM-EDTA. Addition of intracellular Ca^{2+} to ATP-containing 'ghosts' leads to a more than threefold increase in K^+-dependent phosphatase activity. Although this result shows that internal Ca^{2+} and ATP are sufficient for phosphatase activation, they do not rule out the possibility that Ca^{2+} and ATP are effective from the outer surface of the cell membrane. To test this point ATP-containing reconstituted 'ghosts' were assayed in media with and without Ca^{2+} (Table 3, expt. no. 2). It is clear that: (i) addition of Ca^{2+} to the suspending media of ATP-containing ghosts has almost no activating effect on membrane phosphatase; (ii) if external Ca^{2+} is added together with ATP, phosphatase activity is increased, but this increase is trebled after disruption of the permeability barrier of the 'ghosts', which allows Ca^{2+} to have access to both surfaces of the membrane. The activation observed when ATP and Ca^{2+} are present in the external medium can therefore be fully accounted for by the penetration of ATP and Ca^{2+} into the population of 'ghosts' which do not regain their low permeability after haemolysis.

These results strongly suggest that the sites for ATP and Ca^{2+} of the (ATP+Ca^{2+})-dependent phosphatase are only accessible from the inner surface of the cell membrane. The asymmetrical requirements of the phosphatase for ATP and Ca^{2+} are therefore identical with those reported by Schatzmann & Vincenzi (1969) for the Ca^{2+}-dependent ATPase.

Effects of p-nitrophenyl phosphate on the efflux of Ca^{2+} from reconstituted 'ghosts'

The experiments reported so far show that the (ATP+Ca^{2+})-dependent phosphatase and the Ca^{2+}-dependent ATPase from human erythrocyte membranes share sufficient common features as to make it likely that both are properties of a single system. Since Ca^{2+}-dependent ATPase and active Ca^{2+} transport in erythrocytes seem to be intimately connected (Schatzmann & Vincenzi, 1969), it was decided to investigate the effect of *p*-nitrophenyl phosphate on Ca^{2+} transport. For this purpose, the effects of *p*-nitrophenyl phosphate were studied on the Ca^{2+} efflux from reconstituted 'ghosts' (Table 4). ATP-dependent Ca^{2+} efflux was inhibited by 28% by 20 mM-*p*-nitrophenyl phosphate. Results also show that inclusion of K^+ in the suspending media does not affect the degree of inhibition by *p*-nitrophenyl phosphate.

Results in Table 5 make clear that more than two-thirds of the inhibition of Ca^{2+} efflux observed on addition of *p*-nitrophenyl phosphate disappears when reconstituted 'ghosts', with intracellular composition similar to that found at the end of the experiment of Table 4, are incubated in a *p*-nitrophenyl

Table 4. *Effect of p-nitrophenyl phosphate on the efflux of Ca^{2+} from reconstituted 'ghosts'*

For details see the Experimental section.

Additions to the suspending media (mM)	Ca^{2+} efflux (mmol/h per litre of 'ghosts') from ATP-containing ghosts	from ATP-free ghosts	ATP-dependent efflux
None	6.95	0.09	6.86
20mM-*p*-nitrophenyl phosphate	5.15	0.18	4.97
30mM-KCl	6.50	0.24	6.26
30mM-KCl+20mM-*p*-nitrophenyl phosphate	4.80	0.36	4.44

Table 5. *Effect of preincubation of reconstituted 'ghosts' in p-nitrophenyl phosphate-containing media on the efflux of Ca^{2+} into p-nitrophenyl phosphate-free media*

'Ghosts' were 'sealed' in a medium containing (mM): $CaCl_2$, 5; ATP, 6; $MgCl_2$, 11; choline chloride, 130; Tris–HCl, 10 (pH 7.8 at 37°C). One portion of the 'ghosts' was incubated at 37°C for 20min in the medium used in the efflux experiments and the rest was incubated under similar conditions in the presence of 20mM-*p*-nitrophenyl phosphate. At the end of the incubation *p*-nitrophenyl phosphate concentration was 0.29mM. After incubation the 'ghosts' were washed twice in the cold with about 30vol. of a 150mM-choline chloride solution. In view of the permeability properties of the erythrocyte membrane it can be expected that most of the intracellular P_i will remain whereas most of the intracellular *p*-nitrophenyl phosphate and *p*-nitrophenol will be removed by the washing (Garrahan & Rega, 1972). A sample of each kind of 'ghost' was taken for measuring intracellular P_i and the rest was used to measure Ca^{2+} efflux. The medium in which the 'ghosts' pretreated with *p*-nitrophenyl phosphate were suspended to measure Ca^{2+} efflux contained 0.4mM-*p*-nitrophenol.

Additions to the preincubation media	Intracellular P_i (mmol per litre of 'ghosts')	Ca^{2+} efflux (mmol/h per litre of 'ghosts')
None	1.99	3.60
20mM-*p*-nitrophenyl phosphate	3.50	3.30

phosphate-free medium. It seems therefore that inhibition of Ca^{2+} efflux observed in cells incubated in media containing *p*-nitrophenyl phosphate is mainly due to *p*-nitrophenyl phosphate rather than to the products of its hydrolysis.

In the experiment shown in Fig. 6 the effects of increasing concentrations of *p*-nitrophenyl phosphate on both Ca^{2+} efflux and Ca^{2+}-dependent ATPase activity are compared. Results show that (i) inhibition of Ca^{2+} efflux increases as *p*-nitrophenyl phosphate concentration raises, and (ii) inhibition of Ca^{2+} efflux by *p*-nitrophenyl phosphate is paralleled by a decline in Ca^{2+}-dependent ATPase activity, suggesting that both phenomena are inhibited by *p*-nitrophenyl phosphate with equal effectiveness.

Discussion

Results presented in this paper show that the (ATP+Ca^{2+})-dependent phosphatase and the Ca^{2+}-dependent ATPase activities from erythrocyte membranes share a significant number of common features, as follows:

(i) The concentration for half-maximal activation by Ca^{2+} is 8.3μM for the phosphatase and 9.3μM for the ATPase. (ii) Both enzymes require Ca^{2+} at the inner surface of the cell membrane. (iii) Activation by Ca^{2+} of the phosphatase is dependent on the presence of ATP; CTP, ITP, GTP or UTP cannot replace ATP in this respect. Likewise, only ATP is hydrolysed by the Ca^{2+}-dependent ATPase. (iv) For both enzymes ATP is only effective when present at the inner surface of the cell membrane. (v) The relation between ATP concentration and (ATP+Ca^{2+})-dependent phosphatase activity is similar to the relation between ATP concentration and Ca^{2+}-dependent ATPase activity. (vi) When erythrocyte membranes are stored, (ATP+Ca^{2+})-dependent phosphatase and Ca^{2+}-dependent ATPase activities decay following the same time-course. For both activities the effect of storage is prevented or reversed by EDTA.

The above-mentioned similarities rather strongly suggest that the (ATP+Ca^{2+})-dependent phosphatase and the Ca^{2+}-dependent ATPase activities are properties of the same enzymic system. If this were the case, it seems reasonable to suppose that the (ATP+Ca^{2+})-dependent phosphatase and the Ca^{2+}-dependent ATPase share the same site for Ca^{2+} and

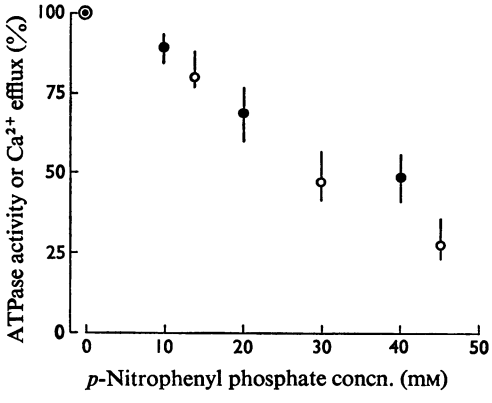

Fig. 6. *Effect of increasing concentrations of p-nitrophenyl phosphate on Ca^{2+} efflux (●) from reconstituted 'ghosts' and on Ca^{2+}-dependent ATPase activity (○) from fragmented erythrocyte membranes*

Ca^{2+} efflux was measured in K^+-containing media (see the Experimental section). ATPase was measured in a medium containing (mM): $MgCl_2$, 5; Tris–HCl, 120; KCl, 30; ATP, 0.5; $CaCl_2$, 0.1; (pH7.8 at 37°C) Ca^{2+}-dependent ATPase activity is the difference between the activities in the above-mentioned media and in media lacking $CaCl_2$ and containing 0.5mM-EGTA. *p*-Nitrophenyl phosphate replaced an equimolar amount of Tris–HCl. The points are the mean of three determinations; vertical bars are ranges. For other details see the Experimental section.

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that the site at which ATP has to combine to promote activation by Ca^{2+} of the phosphatase is the active centre of the ATPase. The similarities in specificity, apparent affinity and asymmetry for Ca^{2+} and ATP of both enzymic activities lend experimental support to this hypothesis.

Schatzmann & Rossi (1971) have shown that when erythrocyte membranes are assayed for Ca^{2+}-dependent ATPase activity at least two different components are detectable, one insensitive to univalent cations and the other requiring Na^+ or K^+. The Na^+- or K^+-dependent component needs 175μM-Ca^{2+} for half-maximal activation, and according to Schatzmann & Rossi (1971) does not pertain to the Ca^{2+} transport in erythrocytes. In view of its high dependence on K^+, it would seem reasonable to ascribe the (ATP+Ca^{2+})-dependent phosphatase to that part of the Ca^{2+}-dependent ATPase that requires Na^+ or K^+. Our experimental results, however, are against this possibility since they clearly show that in the presence of K^+ (ATP+Ca^{2+})-dependent phosphatase is half-maximally activated by 8.3μM-Ca^{2+}, a value not significantly different from that required for half-maximal activation of the univalent-cation-independent ATPase. Moreover, we have shown in the present paper that *p*-nitrophenyl phosphate is able to interact directly with the system responsible for Ca^{2+} transport since, in concentrations similar to those required to elicit phosphatase activity, *p*-nitrophenyl phosphate decreases both ATP-dependent Ca^{2+} extrusion and Ca^{2+}-dependent ATPase activity. It seems economical to assume that the interaction of *p*-nitrophenyl phosphate with the cell membrane that leads to inhibition of Ca^{2+} transport is the same that results in (ATP+Ca^{2+})-dependent phosphatase activity. If this hypothesis is correct, lack of effect of K^+ on the degree of inhibition of Ca^{2+} transport by *p*-nitrophenyl phosphate, would indicate that K^+ is required for the hydrolysis but not for the binding of *p*-nitrophenyl phosphate to the active centre of the membrane phosphatase.

Two alternative explanations may account for the mechanism of the inhibitory effect of *p*-nitrophenyl phosphate on ATP-dependent Ca^{2+} extrusion, i.e.: (i) combination of *p*-nitrophenyl phosphate with the system responsible for Ca^{2+} transport completely blocks this phenomenon, implying that (ATP+Ca^{2+})-dependent *p*-nitrophenyl phosphate hydrolysis is unable to drive Ca^{2+} transport; (ii) (ATP+Ca^{2+})-dependent *p*-nitrophenyl phosphate hydrolysis is able to replace ATP hydrolysis in driving Ca^{2+} movements, but the *p*-nitrophenyl phosphate-dependent Ca^{2+} extrusion proceeds at a slower rate than the ATP-dependent Ca^{2+} extrusion. Our experimental results, showing that ATP-dependent Ca^{2+} efflux was inhibited by increasing concentrations of *p*-nitrophenyl phosphate to a similar extent as Ca^{2+}-dependent ATPase, strongly favour the first alternative since, had *p*-nitrophenyl phosphate been able to drive Ca^{2+} extrusion, Ca^{2+} efflux would have been inhibited by *p*-nitrophenyl phosphate to a lesser extent than Ca^{2+}-dependent ATP hydrolysis.

A striking property of the membrane phosphatase is its strict dependence on ATP for activation by Ca^{2+}. As we have pointed out elsewhere (Garrahan *et al.*, 1970) the effects of ATP on the phosphatase require the existence of an enzymically active phosphatase–ATP complex and hence of different sites for ATP and *p*-nitrophenyl phosphate. As suggested above, the site at which ATP has to combine to promote Ca^{2+}-dependent phosphatase activity may perhaps be identified with the active centre of the ATPase. The existence of distinct and separate sites for ATP and *p*-nitrophenyl phosphate in the same system, together with the fact that *p*-nitrophenyl phosphate inhibits Ca^{2+}-dependent ATP hydrolysis, is consistent with the idea that the reaction pathway for *p*-nitrophenyl phosphate hydrolysis represents only a part of the reaction pathway for ATP hydrolysis. If this hypothesis is assumed to be true, the (ATP+Ca^{2+})-dependent phosphatase activity associated to the Ca^{2+}-dependent ATPase would

indicate that hydrolysis of ATP by this enzyme is a multi-stage process, involving intermediate compounds, some of which may be replaced by *p*-nitrophenyl phosphate.

One of the best characterized Ca^{2+}-transport systems is that of the sarcoplasmic reticulum of skeletal muscle. Like erythrocyte membranes, sarcoplasmic membranes catalyse a Ca^{2+}-dependent hydrolysis of *p*-nitrophenyl phosphate (Inesi, 1971). It should be pointed out, however, that, in contrast with our observations in erythrocyte membranes, Ca^{2+}-dependent *p*-nitrophenyl phosphate hydrolysis by sarcoplasmic membranes does not require the presence of ATP and is able to drive by itself active Ca^{2+} movements (Inesi, 1971), suggesting that different mechanisms may be involved in Ca^{2+} transport across both membranes.

Apart from the (ATP+Ca^{2+})-dependent phosphatase, erythrocyte membranes also possess a K^+-activated phosphatase which belongs to the system responsible for active Na^+ and K^+ transport (see Glynn *et al.*, 1971). Although there are some significant differences between the properties of both phosphatase activities, whether they represent the behaviour of a single or of distinct systems remains an open question which cannot be answered by the results presented in this paper.

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