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Potassium-Activated Phosphatase from Human Red Blood Cells

The Effects of Adenosine Triphosphate

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Summary. The behavior of the red cell membrane K^+-activated phosphatase is significantly altered by ATP. When ATP is added, the apparent affinity of the enzyme for its substrate and for K^+ is lowered, whereas its sensitivity to ouabain is increased. Under these conditions, addition of Na^+ raises the apparent affinity of the enzyme for K^+ to values well above those found in the absence of Na^+ and ATP. The effect of Na^+ is blocked by hydroxylamine and oligomycin. Low concentrations of Ca^{++}, Sr^{++} or Ba^{++}, which have little effect in the absence of ATP, induce large increases in the K^+-dependent phosphatase activity in the presence of ATP. This effect is associated with the loss of ouabain sensitivity of the phosphatase. The velocity vs. divalent cation concentration curves of the K^+-dependent phosphatase and the $(Na^+ + K^+)$-independent ATPase activities are very similar. The effects of ATP seem to be specific for this nucleotide and are exerted at concentrations similar to those normally found in red cells. They may therefore be relevant to the proposed physiological role of the cell membrane phosphatase.

In the preceding papers of this series (Garrahan, Pouchan & Rega, 1969; Rega, Garrahan & Pouchan, 1970), we showed that the red cell membrane phosphatase is endowed with the asymmetrical requirements for substrate and cofactors necessary to participate in the cell membrane $(Na^+ + K^+)$-activated adenosine triphosphatase system ($Na^+ - K^+$-ATPase). This fact, together with the good correlation found between the activities of the $Na^+ - K^+$-ATPase and of the K^+-dependent phosphatase in red cells with different rates of pumping (Vigliocco, Rega & Garrahan, 1970), strongly support the idea that the phosphatase is indeed a partial reaction of the $Na^+ - K^+$-ATPase. Further evidence for the role of the K^+-activated phosphatase in the $Na^+ - K^+$-ATPase system comes from the observation

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that the behavior of the phosphatase is significantly altered by ATP (Rega, Garrahan & Pouchan, 1968; Askari & Koyal, 1968; Pouchan, Garrahan & Rega, 1969; Yoshida, Nagai, Ohashi & Nakagawa, 1969). In this paper, we wish to report the results of a detailed examination of the effects of ATP on the K^+-activated hydrolysis of p-nitrophenylphosphate by human red cell membranes and to discuss their physiological relevance. Preliminary accounts of some of these experiments have already been published (Rega *et al.*, 1968; Pouchan *et al.*, 1969).

Materials and Methods

Preparation of Fragmented Ghosts

"Hemoglobin-free" fragmented ghosts from human red cells were prepared following the procedure already described (Garrahan *et al.*, 1969). Blood from hematologically normal adults was used, either freshly drawn or as outdated blood-bank blood.

Estimation of the Phosphatase Activity

Phosphatase activity was estimated measuring the release of p-nitrophenol (p-NP) from p-nitrophenylphosphate (p-NPP) as previously described (Garrahan *et al.*, 1969). The composition of the incubation media differed in the different experiments but certain rules were observed; the total molarity was always 160 mM and the pH was kept at 7.8 (37 °C) with Tris–HCl. Unless otherwise indicated, the p-NPP concentration was 6.3 mM. The $MgCl_2$ concentration was 5 mM higher than the sum of the concentrations of ATP and ethyleneglycol-1-bis(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA). EGTA (0.5 mM) was always present to prevent the effects of trace amounts of Ca^{++}. All enzymatic assays were carried out at 37 °C for 30 min. The quantity of fragmented ghosts in the reaction mixture was that which gave a hematocrit of 15% calculated on the original volume of the cells. K^+-dependent phosphatase activity was calculated as the difference between the activities in the presence and absence of K^+.

Estimation of ATPase Activity

The ATPase activity of fragmented ghosts was assayed by measuring the amount of inorganic phosphate (Fiske & Subbarow, 1925) in the supernatant of the reaction mixture after deproteinization with trichloroacetic acid (final concentration 5 g/100 ml). Assays were carried out at 37 °C for 30 min.

Sources of Materials

ATP, adenosine diphosphate (ADP), inosine triphosphate (ITP), uridine triphosphate (UTP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), EGTA, hydroxylamine-HCl and oligomycin were obtained from Sigma. Nucleotides were freed of Na^+ by the procedure described in the preceding paper (Rega *et al.*, 1970), where the sources of other materials are also given.

Calculation of Free Ca^{++}

The amount of Ca^{++} in solutions containing known amounts of EGTA, ATP, $CaCl_2$ and $MgCl_2$ was calculated taking into account all possible equilibria between Mg^{++}, Ca^{++}, ATP and EGTA. The following apparent formation constants were used: (EGTA-Ca)/(EGTA)$(Ca^{++}) = 1.79 \times 10^8\ M^{-1}$; (EGTA-Mg)/(EGTA)$(Mg^{++}) = 4.82 \times 10^2\ M^{-1}$ (Portzehl, Caldwell & Ruegg, 1964); (ATP-Ca)/(ATP)$(Ca^{++}) = 5.88 \times 10^3\ M^{-1}$; (ATP-Mg)/(ATP)$(Mg^{++}) = 5.50 \times 10^4\ M^{-1}$ (Long, 1961). Equilibria between p-NPP and Ca^{++} or Mg^{++} were neglected. This seems justified because of the low values of the formation constants of Ca or Mg orthophosphate or mono-orthophosphate ester complexes.

Results

The experiment in Fig. 1 shows the phosphatase activity of fragmented ghosts plotted as a function of ATP concentration in the presence and absence of K^+. As ATP concentration is raised from 0 to 0.5 mM, it is clear that there is a progressive decline of the activity in the presence of K^+, whereas the activity in the absence of K^+ is only slightly affected. Increasing the concentration of ATP from 0.5 to 1 mM has only a slight effect, suggesting that excess ATP leads to a degree of maximal inhibition that is less than 100%. If the effect of 1.0 mM ATP were maximal, half-maximal inhibition of the K^+-dependent phosphatase would be obtained with approximately 0.1 mM ATP.

As the experiment in Fig. 1 was performed with nonsaturating concentrations of substrate and of K^+, the observed inhibition could have been due to changes induced by ATP in the reactivity of the enzyme toward the substrate or K^+. The effects of ATP on the phosphatase activity were therefore studied as a function of p-NPP or K^+ concentrations. Fig. 2

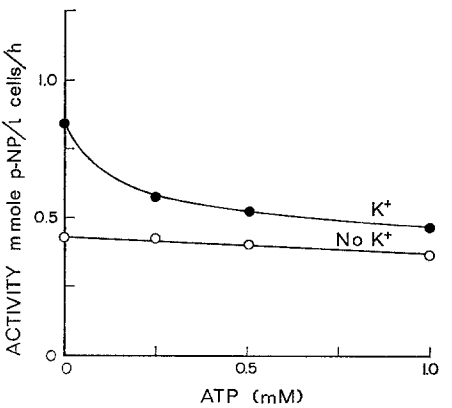

Fig. 1. Rate of p-NPP hydrolysis by fragmented ghosts in the presence and absence of 15 mM K^+ as a function of ATP concentration

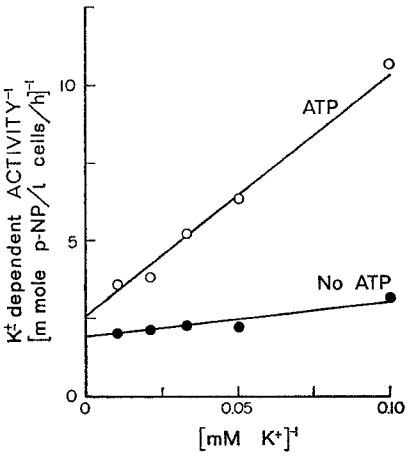

Fig. 2. A Lineweaver-Burk plot of K^+-dependent p-NPP hydrolysis by fragmented ghosts as a function of K^+ concentration, in the presence and absence of 0.25 mM ATP

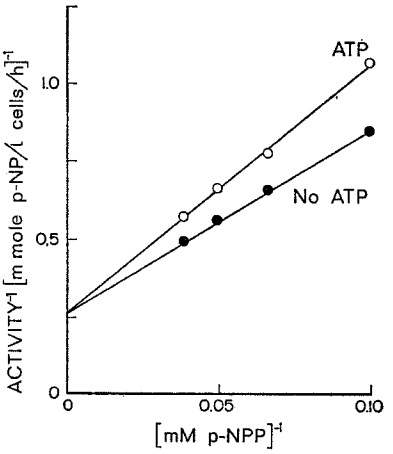

Fig. 3. A Lineweaver-Burk plot of the rate of p-NPP hydrolysis by fragmented ghosts as a function of p-NPP concentration, in the presence and absence of 0.25 mM ATP. The K^+ concentration was 100 mM

shows a Lineweaver-Burk plot of the K^+-dependent phosphatase activity as a function of K^+ concentration in the presence and absence of ATP. It is clear that: (1) the nucleotide induces a large decrease in the apparent affinity of the enzyme for K^+, the concentration of K^+ giving half-maximal activation raising from 6 to 27 mM; and (2) inhibition of the K^+-dependent phosphatase activity by ATP is still evident in the presence of non limiting amounts of K^+. The mechanism of inhibition under this condition was studied by measuring the effects of ATP on the phosphatase activity as a

function of substrate concentration. The Lineweaver-Burk plot of an experiment of this kind is shown in Fig. 3. Inspection of the curves shows that inhibition by ATP in the presence of nonlimiting concentrations of K^+ is due to a decrease in the apparent affinity of the enzyme for the substrate.

The ATP-Dependent Activation by Na^+

Na^+ is a competitive inhibitor of the activating effects of K^+ on the red cell membrane phosphatase (Garrahan *et al.*, 1969). Results in Fig. 4 show that the effects of Na^+ on the membrane phosphatase are markedly altered by ATP; i.e., as Na^+ concentration is raised, the K^+-dependent

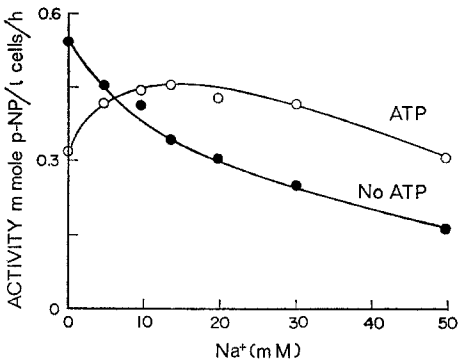

Fig. 4. Effects of Na^+ on the K^+-dependent hydrolysis of p-NPP by fragmented ghosts in the presence and absence of 0.5 mM ATP. The K^+ concentration was 15 mM

activity is enhanced, reaching a broad maximum between 10 and 20 mM Na^+. The Na^+ concentration giving half-maximal activation lies around 3 mM. Also, if the concentration of Na^+ is raised above 30 mM, the activity drops along a curve paralel to that of the control experiment (Fig. 4), suggesting that inhibition by Na^+ has not disappeared but that at low concentrations it is masked by the more prominent ATP-dependent activating effect. Na^+ has no effect in the absence of K^+. In other experiments (not shown), it was found that the ATP concentration for half-maximal effect of 20 mM Na^+ in the presence of 15 mM K^+ lies around 0.2 mM.

In the experiment shown in Fig. 5, phosphatase activity was measured as a function of K^+ concentration in the presence of ATP and of ATP + Na^+. The curves show that addition of Na^+ in the presence of ATP more than overcomes the effect that ATP alone has on the apparent affinity for K^+, the K^+ concentration giving half-maximal activation dropping from 27 to less than 2.5 mM, a value which is at least two times less than

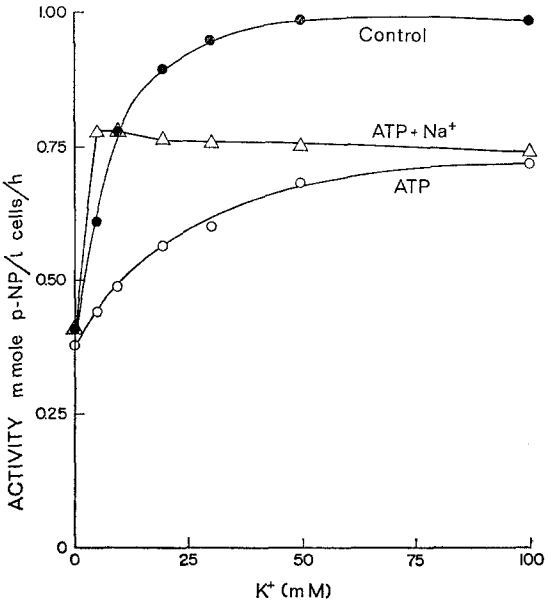

Fig. 5. p-NPP hydrolysis by fragmented ghosts as a function of K^+ concentration in the presence and absence of 0.25 mM ATP $\pm$ 20 mM Na^+

that found in the absence of Na^+ and ATP. On the other hand, Na^+ does not change the K^+-dependent phosphatase activity when nonlimiting amounts of K^+ are present. The ATP-dependent activation by Na^+ is therefore indirect and mediated through an increase in the apparent affinity of the enzyme for K^+.

Phosphatase activity in the presence of ATP and ATP + Na^+ was assayed in the presence of hydroxylamine, a compound which irreversibly displaces phosphate transferred from ATP to membrane protein (Nagano, Kanazawa, Mizuno, Tashima, Nakao & Nakao, 1965; Hokin, Sastry, Galsworthy & Yoda, 1965; Bader, Sen & Post, 1966). Results in Table 1 show that hydroxylamine only partially abolishes the ATP-dependent activation by Na^+, but if Ca^{++} is added, although the activity in 0 mM Na^+ is also inhibited, the effect of Na^+ is almost completely abolished by 60 mM hydroxylamine. These results seem to agree with the observation by Bader and Broom (1967) that hydroxylamine has no effect on the $Na^+ - K^+$-ATPase activity unless small amounts of Ca^{++} are present.

The activating effect of Na^+ is also abolished by oligomycin (Table 2) in concentrations similar to those which inhibit the $Na^+ - K^+$-ATPase activity without affecting its phosphorylation step (Whittam, Wheeler & Blake, 1964; Hokin *et al.*, 1965). This result confirms earlier observations by Askari and Koyal (1968) on a phosphatase preparation from rat brain.

Table 1. *Effects of hydroxylamine ($HONH_2$) and of hydroxylamine + Ca^{++} on the ATP-dependent activation by Na^+ of p-NPP hydrolysis by fragmented ghosts*

Additions	p-NPP Hydrolysis[a] (mmole p-NP/liter cells/hr)		Inhibition of Na^+ activation (%)
	0 mM Na^+ media	20 mM Na^+ media	
None	0.77	0.91	–
$HONH_2$ (30 mM)	0.75	0.88	8
$HONH_2$ (60 mM)	0.74	0.83	38
$CaCl_2$ (0.7 mM)	1.72	1.90	–
$HONH_2$ (30 mM), $CaCl_2$ (0.7 mM)	1.48	1.53	67
$HONH_2$ (60 mM), $CaCl_2$ (0.7 mM)	1.32	1.35	83

[a] All media contained 0.25 mM ATP and 15 mM KCl.

Table 2. *Effect of different oligomycin concentrations on the ATP-dependent activation by Na^+ of p-NPP hydrolysis by fragmented ghosts*

Oligomycin concentration[a] (μg/ml)	p-NPP Hydrolysis[b] (mmole p-NP/liter cells/hr)	
	0 mM Na^+ media	20 mM Na^+ media
0.0	1.02	1.22
2.5	1.00	1.13
5.0	1.03	1.07

[a] Oligomycin was added as a concentrated solution in ethanol. The final concentration of ethanol in both control and oligomycin media was 1 %.

[b] All media contained 0.25 mM ATP and 15 mM KCl.

Changes in the Reactivity to Ouabain Induced by ATP

Fig. 6 shows a comparison of the effects of different ouabain concentrations on the K^+-dependent phosphatase activity in the presence and absence of ATP and of ATP + Na^+. The results make clear that inhibition by ATP of the K^+-dependent phosphatase activity is associated with a large increase in its ouabain sensitivity, the concentration of ouabain giving 50% inhibition falling from 5×10^{-5} to 8×10^{-7} M.

The decrease in the apparent affinity for K^+ does not seem to be the main mechanism responsible for the increase in ouabain sensitivity induced

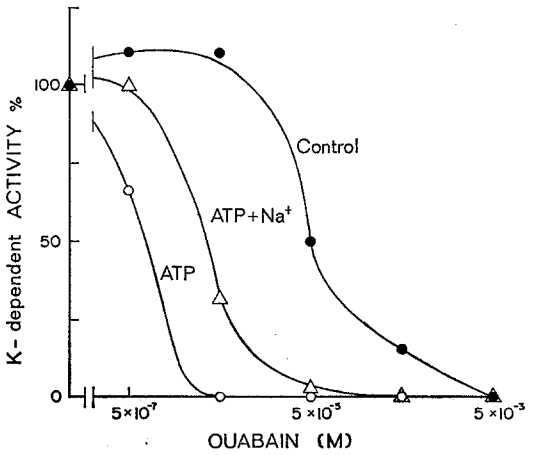

Fig. 6. The effects of ouabain on the K^+-dependent p-NPP hydrolysis by fragmented ghosts in the presence and absence of 0.25 mM ATP and of 0.25 mM ATP + 20 mM Na^+. The K^+ concentration was 15 mM

by ATP. This assertion is based on the fact that in the presence of amounts of Na^+ adequate to increase the apparent affinity for K^+ well above the values found in the absence of ATP, the ouabain concentration giving 50% inhibition is still much lower than in the control experiment.

Specificity of the Effects of ATP

In the experiment shown in Table 3 the effects of near-optimal concentrations of ATP were compared, under identical conditions, with those

Table 3. *Comparison of the effect of different nucleotides on p-NPP hydrolysis by fragmented ghosts in the presence and absence of Na^+*

Nucleotide (0.25 mM)	p-NPP Hydrolysis[a] (mmole p-NP/liter cells/hr)		ΔNa^+[b] (mmole p-NP/liter cells/hr)
	0 mM Na^+ media	20 mM Na^+ media	
None	1.05	—	—
ATP	0.78	0.93	0.15
ADP[c]	1.16	1.05	−0.11
CTP	1.56	1.56	0.00
ITP	1.10	0.91	−0.19
UTP	0.97	0.84	−0.13
GTP	0.94	0.81	−0.13

[a] The K^+ concentration was 15 mM. [b] ΔNa^+ is the difference between the activities with and without Na^+. [c] When the effects of ADP were tested, the ghosts were freed of adenylate kinase activity following the procedure described by Sen and Post (1964).

of other nucleotide triphosphates and ADP. Although the results do not eliminate the possibility that at concentrations higher than 0.25 mM other nucleotides might be effective (*cf.* Askari & Koyal, 1968; Yoshida *et al.*, 1969), it is clear that at this concentration only ATP appreciably inhibits the phosphatase activity and changes its reactivity toward Na^+. Results in Table 3 also show that CTP activates the membrane phosphatase, but no further studies have been done on this phenomenon.

The ATP-Dependent Effects of Alkali Earth Ions

Fig. 7 shows the effects of increasing concentrations of $CaCl_2$ on the phosphatase activity of fragmented ghosts incubated in solutions containing either 0 mM ATP (Fig. 7a) or 0.25 mM ATP (Fig. 7b). In the ab-

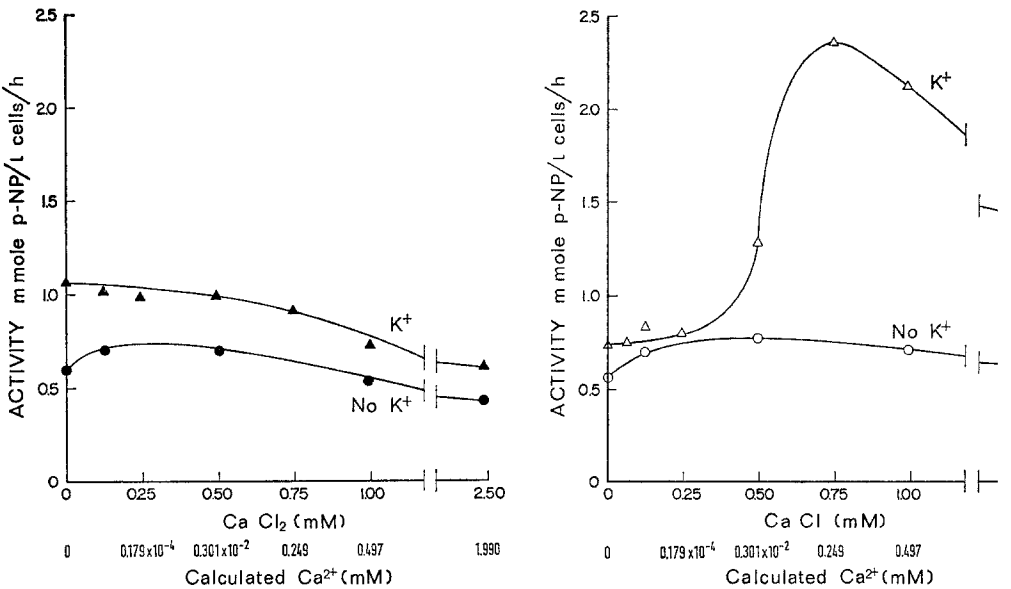

Fig. 7a and b. p-NPP hydrolysis by fragmented ghosts in the presence and absence of 100 mM K^+ at different $CaCl_2$ concentrations in media without (a) and with (b) 0.25 mM ATP. The calculated concentration of ATP-Ca (*see* Methods) was 1.4×10^{-3} mM in the media with 0.75 mM $CaCl_2$ and 1.1×10^{-2} mM in the media with 2.5 mM $CaCl_2$

sence of ATP, Ca^{++} has little effect on the phosphatase activity, but when ATP is present, increasing Ca^{++} concentrations from 0 to about 0.7 mM leads to an activation of the phosphatase that is large enough not only to overcome the inhibition by ATP but also to increase the activity well

Table 4. *Comparison of the effect of different nucleotides on p-NPP hydrolysis by fragmented ghosts in the presence and absence of $CaCl_2$*

Nucleotide (0.25 mM)	p-NPP Hydrolysis[a] (mmole p-NP/liter cells/hr)	
	0 mM $CaCl_2$ media	0.7 mM $CaCl_2$ media
ATP	0.78	2.40
ITP	1.10	0.76
UTP	0.98	0.72
GTP	0.94	0.73

[a] The K^+ concentration was 15 mM.

above control values. The plot of phosphatase activity against the calculated Ca^{++} concentration (Fig. 7b) suggests that this ion is the activating species. The ATP-dependent activation by Ca^{++} needs the presence of K^+ as shown by the small effect Ca^{++} has in the absence of this ion. In other experiments (not shown), it was found that half-maximal activation by 0.7 mM $CaCl_2$ was reached with about 0.1 mM ATP.

Specificity for ATP of the ATP-dependent activation by Ca^{++} was studied in the experiment shown in Table 4. It is clear that in the presence of ITP, UTP or GTP in concentrations similar to those optimal for ATP, addition of Ca^{++} depresses rather than activates the phosphatase activity.

The effect of other alkali earth cations was also tested (*see* Fig. 10). In the presence of ATP, Sr^{++} or Ba^{++} activates the phosphatase in the same range of concentrations in which Ca^{++} is effective. Control experiments demonstrated that activation by Sr^{++} or Ba^{++} also requires the presence of K^+.

The sensitivity to ouabain of the membrane phosphatase in the presence of Ca^{++}, ATP, or ATP+Ca^{++} is illustrated in Fig. 8. It is clear that, in contrast with the inhibitory effect of ATP which is accompained by an increase in ouabain sensitivity, the ATP-dependent activation by Ca^{++} is associated with loss of ouabain sensitivity of the K^+-dependent phosphatase activity. Ca^{++} alone does not significantly change the concentration of ouabain giving half-maximal inhibition (*cf.* Figs. 8 & 6). Essentially similar results were obtained replacing Ca^{++} by Ba^{++} or Sr^{++}.

Further insight concerning the ATP-dependent effects of alkali earth cations was obtained by measuring phosphatase activity as a function of substrate or K^+ concentrations. The ATP-dependent effects of Ca^{++} on the substrate vs. velocity curves of the phosphatase are given in Fig. 9.

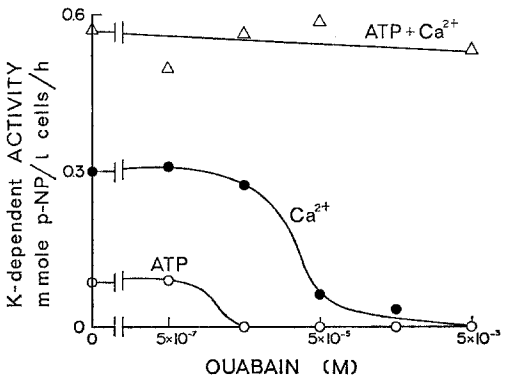

Fig. 8. The effect of ouabain on the K^+-dependent p-NPP hydrolysis by fragmented ghosts in the presence of 0.7 mM $CaCl_2$, 0.25 mM ATP or 0.7 mM $CaCl_2$ + 0.25 mM ATP

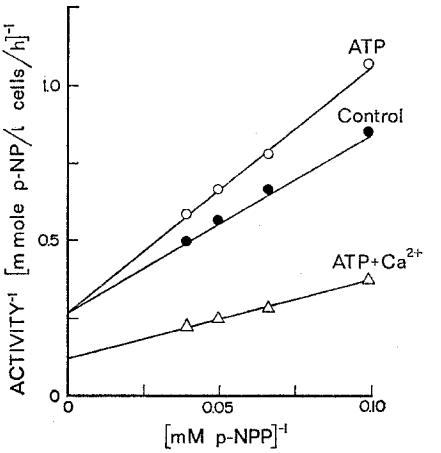

Fig. 9. A Lineweaver-Burk plot of p-NPP hydrolysis by fragmented ghosts as a function of p-NPP concentration in the presence and absence of 0.25 mM ATP and of 0.25 mM ATP + 0.7 mM $CaCl_2$

Addition of Ca^{++} increases both the turnover rate and the apparent affinity of the enzyme for p-NPP. As the apparent affinity for p-NPP only reaches the control value, ATP-dependent activation by Ca^{++} must be ascribed to the increase in turnover rate. The effects of optimally activating concentrations of Ca^{++} on the apparent affinity of the phosphatase for K^+ are given in Table 5. The results show that: (1) although lowered, the decrease in apparent affinity for K^+ induced by ATP is still evident in the presence of Ca^{++}; and (2) Ca^{++} significantly lessens, but does not abolish, the ATP-dependent increase induced by Na^+ on the apparent affinity of the phosphatase for K^+.

Table 5. *Effect of Ca^{++} on the apparent affinity for K^+ of the cell membrane phosphatase*[a]

Additions[b]	K^+ Concentration giving half-maximal activation (mM)
None	6.0
ATP	25.0
ATP + $CaCl_2$ (0.7 mM)	18.2
ATP + Na^+ (20 mM)	2.5
ATP + $CaCl_2$ (0.7 mM) + Na^+ (20 mM)	10.8

[a] With the exception of that in the presence of ATP + Na^+ (*see* Fig. 5), all the values were obtained from reciprocal plots of K^+-dependent p-NPP hydrolysis as a function of K^+ concentrations. [b] The ATP concentration was 0.25 mM.

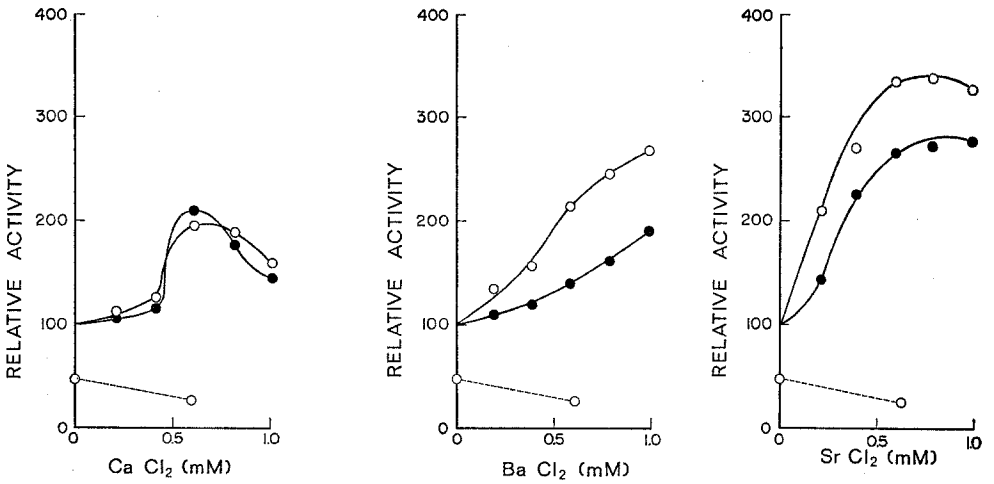

Fig. 10a–c. Comparison of the effects of (a) $CaCl_2$, (b) $BaCl_2$, and (c) $SrCl_2$ on the total p-NPPase activity (•——•) and on the total (o——o) and ($Na^+ + K^+$)-dependent (o---o) ATPase activities of fragmented ghosts. Phosphatase activity was assayed in the presence of 100 mM K^+ and of 0.25 mM ATP. Total ATPase activity was assayed in a medium containing: 0.25 mM ATP, 5.75 mM $MgCl_2$, 30 mM KCl, 80 mM NaCl, 45 mM Tris–HCl (pH 7.8), and 0.5 mM EGTA. ($Na^+ + K^+$)-dependent ATPase activity is the difference between the activity in the above medium and the activity in a medium in which all the KCl and NaCl were replaced by an equivalent amount of Tris-HCl. Phosphatase and ATPase activities are expressed as percent of the respective total activities in the absence of alkali earth cations. All activities were assayed simultaneously and on the same preparation of fragmented ghosts

Fig. 10 shows a comparison of the effects of alkali earth cations on the phosphatase and the ATPase activities of fragmented ghosts. Activities were measured simultaneously on the same preparation of ghosts under

conditions chosen to make the assays of both activities as comparable as possible; i.e., ATP and Mg^{++} concentration as well as total molarity and pH were the same, and in both assays nonlimiting concentrations of activating ions were present. Inspection of the curves shows three things. (1) Confirming earlier findings (Dunham & Glynn, 1961; Wins & Schoffeniels, 1966), alkali earth cations increase the cell membrane ATPase activity. (2) For each cation, there is a striking similarity between the velocity vs. concentration curves of the phosphatase and ATPase activities. (3) Activation of the ATPase is associated with a decrease in its $(Na^+ + K^+)$-dependent fraction which is not abolished by divalent cations in concentrations close to those giving maximal activation (*cf.* Dunham & Glynn, 1961). Control experiments done under conditions in which the K^+-dependent phosphatase activity is no longer sensitive to ouabain (*see* Fig. 8) confirmed earlier findings (Dunham & Glynn, 1961) in the sense that the residual $Na^+ - K^+$-ATPase activity is still completely blocked by 10^{-4} M ouabain.

Discussion

The experiments reported in this paper show that the behavior of the red cell membrane K^+-activated phosphatase is significantly altered by ATP. When ATP is present, the rate of p-NPP hydrolysis drops. Kinetic analysis shows that this drop is due to an increase of the K_m of the phosphatase without change in the maximum velocity. At face value, this result suggests competition between ATP and the phosphatase substrate and has been interpreted as such by other authors (Bader & Sen, 1966; Israel & Titus, 1967; Nagai, Izumi & Yoshida, 1966). Our results showing that inhibition by ATP is associated with large changes in the reactivity if the phosphatase toward K^+, Na^+ and ouabain are very difficult to explain without assuming the existence of an enzymatically active phosphatase-ATP complex and hence of different sites for p-NPP and ATP. This idea is also consistent with the observation that increasing ATP concentrations do not seem to lead to complete inhibition of p-NPP hydrolysis. The possibility that ATP, apart from combining at its site, competes with the phosphatase substrate becomes unlikely since nucleotide triphosphates other than ATP seem to be ineffective as inhibitors of the membrane phosphatase. If the active site of the phosphatase were unable to discriminate between ATP and p-NPP, as competition implies, it seems rather improbable that it would be able to distinguish ATP from other nucleotide triphosphates.

In view of the role the membrane phosphatase activity seems to play in the overall $Na^+ - K^+$-ATPase reaction, it is tempting to identify the site for ATP of the phosphatase with the active site of the $Na^+ - K^+$-ATPase system. Although no direct evidence in favor of this hypothesis is yet available, the following facts seem suggestive. (1) For all the observed effects of ATP on the phosphatase, the concentration of the nucleotide giving half-maximal effect is of the same order of magnitude as the reported value of the Michaelis constant (Dunham & Glynn, 1961) of the $Na^+ - K^+$-ATPase system. (2) The effects of ATP on the phosphatase seem to be specific for this nucleotide. (3) At least for the ATP-dependent effects of Na^+, the site for ATP of the phosphatase seems to be located, like the active site of the ATPase, on the internal surface of the membrane (Rega *et al.*, 1970).

The fact that ATP affects the interaction of the enzyme with Na^+ and K^+ is interesting because it suggests that the nucleotide, apart from providing the necessary energy for active transport, also plays a role in promoting the cyclic changes in selectivity for these ions that most active transport schemes require.

Combination of ATP with the phosphatase leads to a large decrease in its apparent affinity for K^+. However, in this condition Na^+ becomes able to react with the system in a way that increases its apparent affinity for K^+ beyond the values found in the absence of Na^+ and ATP. This change may be the cause of the ATP-dependent activation by Na^+ reported in phosphatase preparations from other tissues (Nagai & Yoshida, 1966; Askari & Koyal, 1968). Since our initial observation (Rega *et al.*, 1968), this interpretation has been confirmed in a guinea pig kidney preparation by Yoshida *et al.* (1969). In the presence of Na^+ and Mg^{++}, ATP is able to phosphorylate membrane protein (*see* Glynn, 1968). The ATP-dependent increase in the apparent affinity for K^+ induced by Na^+ could conceivably be a result of such a phosphorylation. Indirect support for this view comes from the fact that the effect of Na^+ is blocked by hydroxylamine. However, if the phosphorylated protein were the natural substrate of the phosphatase, it becomes difficult to explain why its presence does not lead to further inhibition of the hydrolysis of artificial substrates like p-NPP. If the role of the K^+-activated phosphatase in the overall $Na^+ - K^+$-ATPase reaction is taken for granted, lack of inhibition of p-NPP hydrolysis in the presence of the natural substrate must mean that under our experimental conditions, interaction of the natural substrate with the phosphatase is fully impeded by p-NPP. Under this condition, the ATPase reaction should therefore be blocked. This prediction has not been tested,

although there is evidence in other preparations that phosphatase substrates are inhibitors of the $Na^+ - K^+$-ATPase (Bader & Sen, 1966; Fujita, Nakao, Tashima, Mizuno, Nagano & Nakao, 1966; Israel & Titus, 1967). The ATP-dependent effect of Na^+ is selectively abolished by oligomycin in concentrations similar to those that block active transport in human red cells (Garrahan & Glynn, 1967*a*). Since oligomycin seems to have no effect on the formation of phosphorylated protein (Whittam *et al.*, 1964; Hokin *et al.*, 1965), if phosphorylation is involved in the increase in apparent affinity for K^+, an oligomycin-sensitive reaction subsequent to phosphorylation has to be postulated. Such a reaction was proposed by Garrahan and Glynn (1967*b*) to explain their observation that, in the absence of K^+, the Na^+ pump in red cells exchanges internal for external Na^+ through an oligomycin-sensitive mechanism that needs, but does not consume, ATP. It has been suggested that this mechanism involves phosphorylation followed by an oligomycin-sensitive step, tentatively identified with a reaction replacing selective affinity for Na^+ by selective affinity for K^+ (Garrahan & Glynn, 1967*b*).

In sharp contrast with the effects ATP has alone, when it is added together with Ca^{++}, Sr^{++} or Ba^{++}, K^+-dependent phosphatase activity is enhanced and its ouabain sensitivity is abolished. Although a possible explanation for the changed response of the enzyme is that under these conditions ATP combines with the phosphatase at a different site, our results do not seem to demand such a difference. This assertion is based on the following: (1) although lowered, the ATP-dependent effects on the apparent affinity for K^+ of the phosphatase persist in the presence of divalent cations; and (2) the effects of divalent cations need ATP at similar concentrations and seem to have the same nucleotide specificity as other ATP-dependent effects. Therefore, until further evidence becomes available, it seems simpler to regard the ATP-dependent effects of divalent cations as the expression of one of the properties of the proposed phosphatase-ATP complex. The effects of Ca^{++}, Sr^{++} and Ba^{++} may be interpreted as suggestive that when alkali earth cations inhibit the $Na^+ - K^+$-ATPase reaction, the phosphatase is "uncoupled" from the ATPase system. Such an interpretation may explain why, under these conditions, the phosphatase more efficiently hydrolyzes artificial substrates and loses its ouabain sensitivity. A possible mechanism for the "uncoupling" of the phosphatase is suggested by the similarity between the effects that Ca^{++}, Ba^{++} and Sr^{++} have on the K^+-dependent phosphatase and on the $(Na^+ + K^+)$-*in*dependent ATPase activities. A tentative way to account for this similarity might be to assume that the $(Na^+ + K^+)$-dependent and the $(Na^+ + K^+)$-

*in*dependent ATPase activities are different modes of behavior of the same enzyme system. If this were the case, it may be that "uncoupling" of the phosphatase takes place because divalent cations are able to drive the ATPase reaction through a pathway which, being insensitive to Na^+ and K^+, does not involve the K^+-activated phosphatase.

In the presence of almost optimally activating concentrations of alkali earth cations, about 50% of the original Na^+-K^+-ATPase activity remains and is still sensitive to ouabain. This result implies that in this condition about 50% of the phosphatase should remain "coupled" and thus be sensitive to ouabain. Although this seems to be in contradiction with the loss of ouabain sensitivity of the phosphatase in the presence of divalent cations, it must be remembered that if the rate of the "coupled" phosphatase were similar to that found in the absence of Ca^{++}, it would be about 10 times less than that of the "uncoupled" phosphatase (*see* Fig. 7b). In this case, the ouabain-sensitive phosphatase would contribute a small and not easily detectable fraction to the observed K^+-dependent activity. This fraction might have been responsible for the small drop in K^+-dependent activity observed when ouabain was added in the presence of Ca^{++}.

If the above reasoning were true, the effects of ATP on the apparent affinity of the phosphatase for K^+ in the presence of Ca^{++} would not be accounted for by the residual "coupled" phosphatase and thus, to fit with our observations, the "uncoupled" phosphatase should retain, albeit to a lesser degree, the effects of ATP.

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