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EXCESS MAGNESIUM CONVERTS RED CELL (SODIUM+POTASSIUM) ATPase TO THE POTASSIUM PHOSPHATASE

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SUMMARY

1. The ATPase and phosphatase activities of red cell membranes were measured simultaneously as a function of the magnesium content of the medium.

2. It was found that when the magnesium concentration was greater than that of ATP, magnesium inhibited the ATPase and simultaneously stimulated the phosphatase. The concentrations of magnesium needed for half-maximal stimulation of the phosphatase and half-maximal inhibition of the ATPase were similar.

3. It is suggested that increasing the concentration of magnesium directly causes a change in the conformation of the enzyme from one which favours ATPase activity to one which favours phosphatase activity.

INTRODUCTION

Purified preparations of the sodium pump exhibit both (Na+K)ATPase and potassium phosphatase activities (Jørgensen, Skou & Solomonson, 1971; Jørgensen, 1977). However, these two reactions are affected differently by changes in the concentration of magnesium. Whereas the ATPase reaction shows a stimulation by low concentrations of magnesium followed by a pronounced inhibition at higher levels (Dunham & Glynn, 1961; Hexum, Samson & Himes, 1970; Robinson, 1974; Skou, 1974*a*), the phosphatase requires high levels of magnesium for activation (Robinson, 1969; Garrahan, Pouchan & Rega, 1969; Skou, 1974*b*). Since the phosphatase substrates have low magnesium affinities, it could be argued that high magnesium concentrations are needed for activation of the phosphatase because the magnesium-bound forms are the true substrates. This, however, is not true, since the same concentration of magnesium is needed for stimulation of the phosphatase when different substrates with different magnesium affinities are used (Rega & Garrahan, 1976; Robinson, 1969). Hence an alternative explanation needs to be found for the different magnesium requirements of the two reactions.

Stimulation of phosphatase and inhibition of ATPase have never been demonstrated simultaneously in the same preparation. In addition, the apparent affinities for stimulation and inhibition obtained separately have not been measured under the same conditions and thus are not comparable. In this paper we examine the effect of

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magnesium on the ATPase and phosphatase measured simultaneously and show that there is a direct correlation between the stimulation of phosphatase and inhibition of ATPase. We suggest that excess magnesium causes the enzyme to change from the native E_1 form, which favours the ATPase reaction, to the E_2 form which favours the phosphatase.

METHODS

The experiments were designed so that the ATPase and phosphatase activities could be measured in the same sample. ATPase was therefore measured by the release of $^{32}P_i$ from [γ-^{32}P]ATP and phosphatase by the release of *p*-nitrophenol from *p*-nitrophenolphosphate (*p*NPP). The ATPase and phosphatase activities specific to the sodium pump were determined either as the ouabain-sensitive activities in human red cell membranes or as the potassium-dependent activities in LK goat red cell membranes because of the lower ouabain sensitivity of the phosphatase in this case (Ellory & Lew, 1974).

Preparation of ghosts

Human red cell ghosts were prepared from freshly drawn blood which was lysed at 0 °C in twenty volumes of a medium containing 5 mM-Tris Cl (pH 7·5 at 0 °C), 2 mM-$MgCl_2$ and 0·1 mM-Tris EGTA. The ghosts were washed once in a similar medium containing 10 mM-Tris EGTA and then three times in a medium containing 2 mM-$MgCl_2$ and 5 mM-Tris Cl. After freezing and thawing twice the membranes were washed three times in a solution containing 5 mM-Tris Cl and 0·02 mM-Tris EGTA. They were resuspended in a volume of this medium equivalent to the original volume of packed cells ('100% haematocrit') and stored at −20 °C until used.

Ghosts were prepared from goat LK-type red cells by the method described by Ellory & Lew (1974). The ghosts were then treated with the specific antibody, anti-L. This stimulates the potassium-sensitive phosphatase activity without changing its affinity for potassium (see Ellory & Lew, 1974).

Simultaneous measurement of ATPase and phosphatase

The experiments on human red cell ghosts were carried out in an incubation medium which contained: 100 mM-NaCl, 10 mM-KCl, 0·1 mM-Tris EGTA, 10 mM-Tris Cl (pH 7·5 at 37 °C), 0·1–1·0 mM-ATP containing [γ-^{33}P]ATP (Amersham, specific activity 2000 Ci/m-mole), ±5 mM-*p*NPP, ±0·1 mM-ouabain and the indicated concentration of $MgCl_2$. Experiments with goat red cell ghosts were carried out in a medium containing: 100 mM-NaCl, 10 mM-Tris Cl (pH 7·5 at 37 °C), 0·1 mM-Tris EGTA, 5 mM-*p*NPP, between 0 and 8 mM-$MgCl_2$ and between 0 and 50 mM-KCl. The ionic strength was kept constant with choline chloride.

The media were prepared in tubes at 0 °C and the enzyme was added just before the start of the incubation. The enzyme concentration was equivalent to a haematocrit of about 5% and was calculated to give no more than 20% hydrolysis of ATP by the end of the incubation. The reaction was started by transferring the tubes to a water bath at 37 °C. After 90 min the reaction was stopped by transferring the tubes back to the ice bath and adding a mixture of trichloroacetic acid and orthophosphoric acid to give final concentrations of 5% and 0·77 mM respectively. Phosphatase activity was determined by transferring 1 ml. of the TCA supernatant to a tube containing 2 ml. of a mixture of 1 M-NaOH and 20 mM-EDTA and measuring the absorbance at 410 nm. The ATPase activity was measured by adding 0·1 ml. of the TCA supernatant to a tube containing 1·4 ml. 1·43 N-sulphuric acid and 1·8% ammonium molybdate and 1·5 ml. isobutanol, all at 0–5 °C. The tube was vortexed for 15 sec and 1 ml. of the isobutanol extract was transferred to a vial containing 5 ml. Bray's solution and the ^{32}P determined by liquid scintillation counting.

RESULTS

Fig. 1 shows the ouabain-sensitive ATPase and phosphatase activities plotted as a function of the total concentration of magnesium in the medium. In the absence of any added magnesium the phosphatase showed no activity but the ATPase

operated at 15% of its maximal activity both in the presence and absence of *p*NPP. Increasing the magnesium concentration to 0·5 mM (equal to that of the ATP) resulted in maximal stimulation of the ATPase whereas the phosphatase activity rose to less than 12% of its maximum value. Further increase in the total magnesium concentration led to a progressive inhibition of the ATPase so that at 8 mM-magnesium the activity was only 28% of its maximum value. In an experiment using

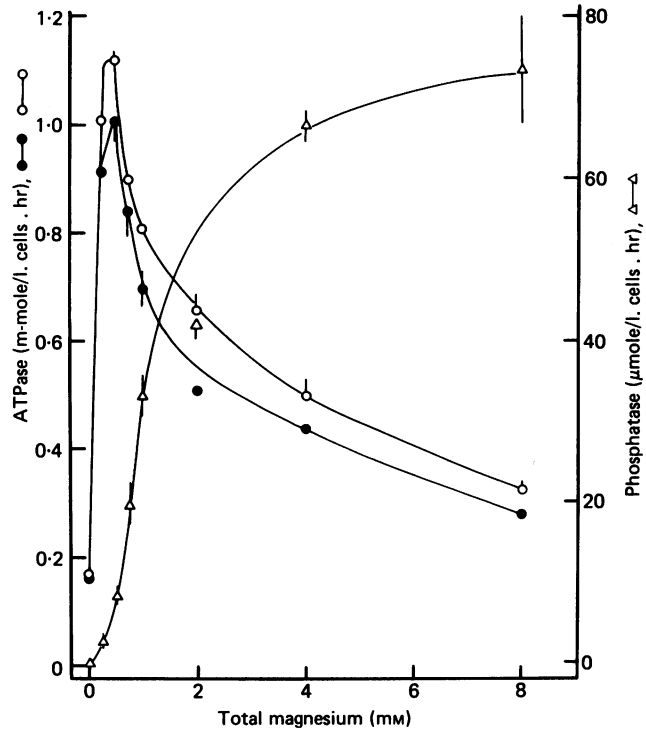

Fig. 1. The ouabain-sensitive phosphatase and ATPase activities of human red cell ghosts are plotted as a function of the total concentration of magnesium in the medium. The concentrations of ATP and *p*NPP in this experiment were 0·5 and 5·0 mM respectively. Each point represents the mean and S.E. of mean of triplicate measurements. ○—○, Ouabain-sensitive ATPase, in the absence of *p*NPP; ●—●, ouabain-sensitive ATPase, in the presence of 5 mM-*p*NPP; △—△, ouabain-sensitive phosphatase.

1 mM-ATP (and the usual 5 mM-*p*NPP), increasing the total magnesium concentration from 8 to 12 mM (equivalent to 5 and 8 mM ionized) led to very little further inhibition of ATPase activity (see Fig. 2), indicating saturation of the magnesium effect. During the inhibition of ATPase activity the phosphatase activity was stimulated. The curve relating phosphatase activity to total magnesium concentration was sigmoid. Also shown in Fig. 1 is a plot of the ATPase activity in the absence of *p*NPP. It can be seen that the curves in the presence and absence of *p*NPP are similar in shape, but that the activity in the presence of *p*NPP is lower at each total magnesium level.

In Fig. 3, the same data as in Fig. 1 have been plotted as a function of the ionized

magnesium in the medium, and the activities have been normalized to their respective maxima. The plot of phosphatase activity against ionized magnesium is now a rectangular hyperbola where the concentration needed to give half-maximal stimulation is about 0·55 mM. At this level of ionized magnesium the ATPase, measured in the presence of *p*NPP, had been inhibited and was about 57% of its maximal level.

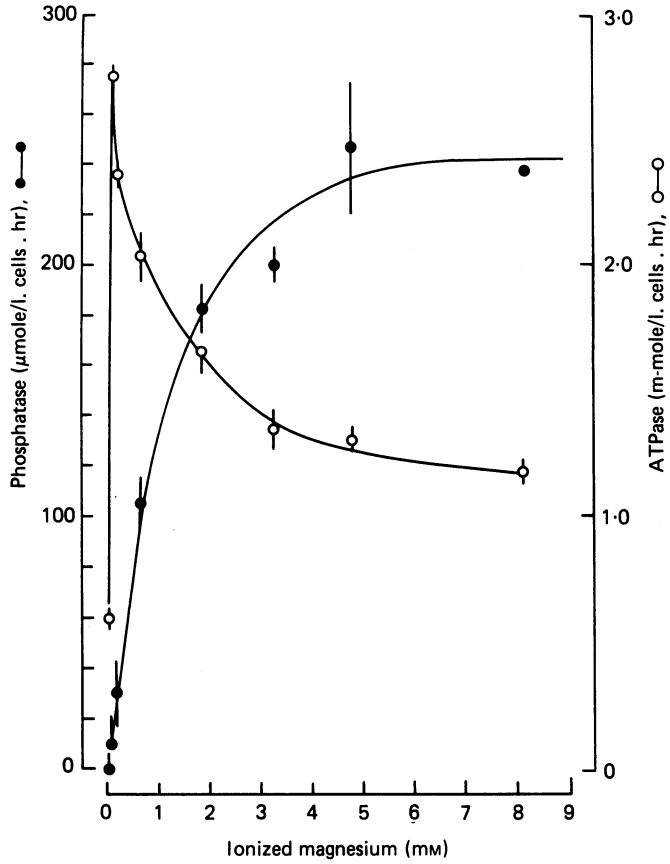

Fig. 2. The ouabain-sensitive phosphatase and ATPase activities of human red cell ghosts have been plotted as a function of the concentration of ionized magnesium in the medium. In this experiment the concentrations of ATP and *p*NPP were 1·0 and 5·0 mM respectively. The concentration of ionized magnesium was calculated from the total concentrations of ATP, *p*NPP, and magnesium, assuming that the dissociation constants for MgATP and Mg*p*NPP were 0·083 (Berger, Jänig, Gerber, Ruckpaul & Rapoport, 1973) and 5·9 mM (Robinson, 1969) respectively. ○—○, Ouabain-sensitive ATPase in the presence of *p*NPP; ●—●, ouabain-sensitive phosphatase.

This represents 50% inhibition of ATPase activity when allowance is made for the saturation of the magnesium effect. 50% inhibition of the total ouabain-sensitive ATPase activity occurred at about 0·9 mM ionized magnesium. The same Figure also shows that the inhibition of ATPase activity by *p*NPP is a genuine effect of *p*NPP rather than due to the chelation of magnesium. Half-maximal inhibition of ATPase by magnesium in the absence of *p*NPP occurs at about 2·5 mM ionized magnesium.

It is apparent that the direct inhibition of ATPase activity by *p*NPP is much smaller during the activation than during the inhibition by magnesium. Similar patterns of mirror-image stimulation of potassium phosphatase and inhibition of ATPase by magnesium were also observed at ATP concentrations of 1·0 and 0·1 mM (see Figs. 2 and 4). With 1·0 mM-ATP, half-maximal stimulation of phosphatase and inhibition of ATPase occurred at 0·85 mM ionized magnesium (2·3 mM total) and with 0·1 mM-

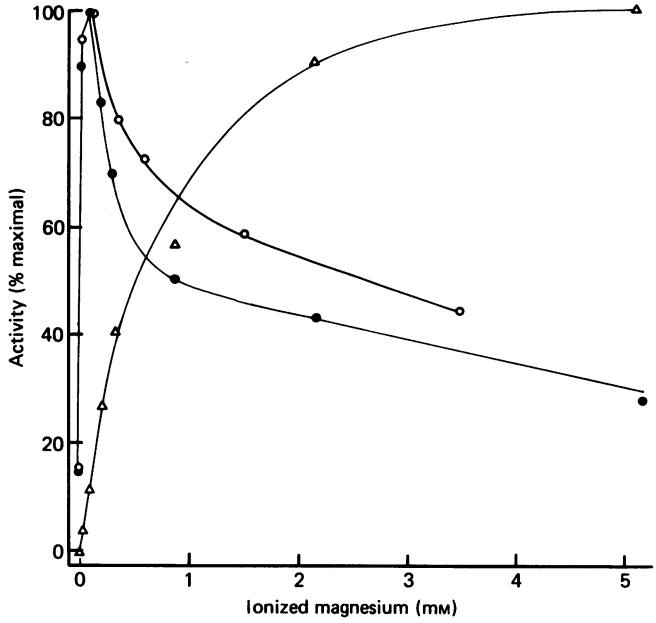

Fig. 3. The ouabain-sensitive phosphatase and ATPase activities shown in Fig. 1 have been replotted as a function of the concentration of ionized magnesium in the medium and the activities have been expressed as a percentage of their respective maxima. The concentrations of ATP and *p*NPP were 0·5 and 5·0 mM respectively. The concentration of ionized magnesium was calculated as described in the legend to Fig. 2. Each point is the mean and S.E. of mean of three measurements. △—△, Ouabain-sensitive phosphatase; ●—●, ouabain-sensitive ATPase in the presence of *p*NPP; ○—○, ouabain-sensitive ATPase in the absence of *p*NPP.

ATP about 0·65 mM ionized magnesium was required (1·2 mM total). In addition, this same pattern was observed for the potassium-dependent phosphatase (Ellory & Lew, 1974) and ATPase measured simultaneously in LK goat red cell membranes. In this latter case, mirror-image stimulation and inhibition by magnesium in the presence of 0·5 mM-ATP were observed at four K^+ concentrations (2, 10, 25 and 50 mM) including those at which K^+ is inhibitory to the phosphatase (25 and 50 mM).

DISCUSSION

Post, Hegyvary & Kume (1972) suggested that the potassium-occluded form of the enzyme, E_2K, was responsible for the phosphatase action. This E_2K form is normally produced (see Fig. 5) from the native (E_1) enzyme by phosphorylation in the presence of Mg^{2+}, ATP and sodium, followed by a K^+-stimulated dephosphory-

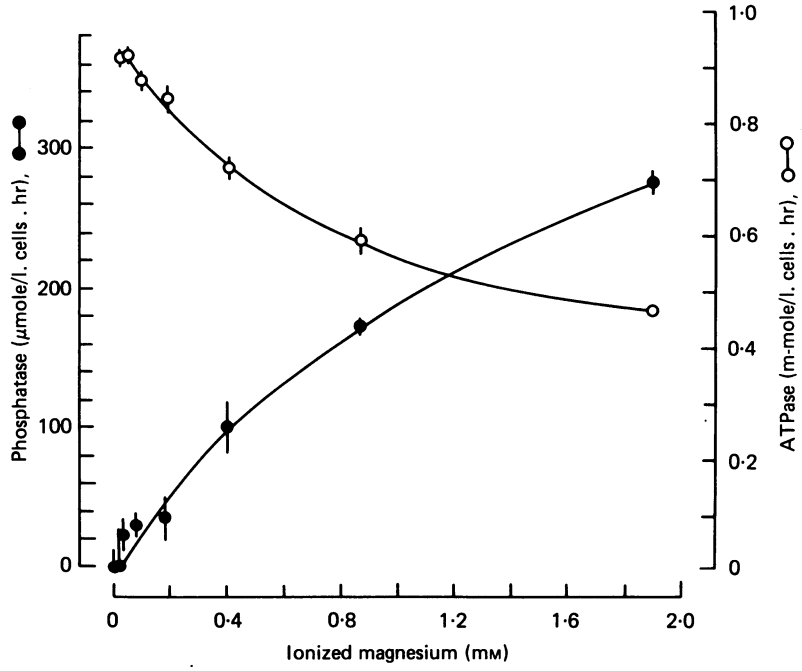

Fig. 4. The ouabain-sensitive phosphatase and ATPase activities of human red cell ghosts are plotted as a function of the concentration of ionized magnesium in the medium. The concentrations of ATP and *p*NPP in this experiment were 0·1 and 5·0 mM respectively. For details of calculation see the legend to Fig. 2. Each point represents the mean and S.E. of mean of triplicate measurements. ○—○, Ouabain-sensitive ATPase in the presence of *p*NPP; ●—●, ouabain-sensitive phosphatase.

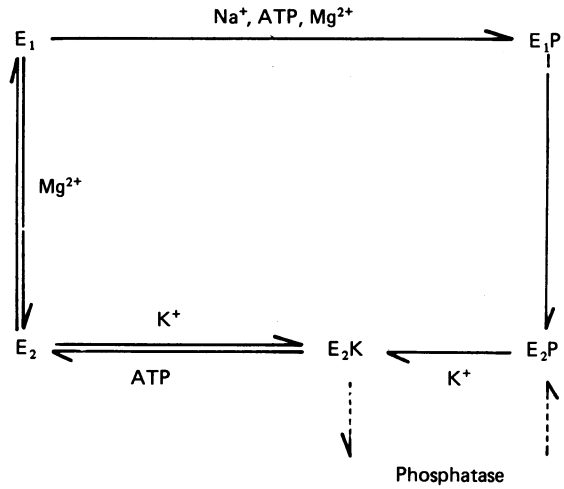

Fig. 5. A hypothetical scheme for the sodium-pump enzyme showing the proposed configurations for ATPase and phosphatase activities. The scheme presented here is based on the recent literature (Sen, Tobin & Post, 1969; Post *et al.* 1972, 1975; Karlish *et al.* 1978) and the findings of the present work. The continuous clockwise arrows represent the ATPase reaction and the dotted arrows represent the phosphatase reaction where the enzyme accepts the phosphate group from *p*NPP in the E_2 form. For details of scheme see text.

lation. E_2K then slowly and spontaneously breaks down to form E_1 again, the rate of reaction being increased by ATP acting at a low affinity site (Karlish, Yates & Glynn, 1978). Alternatively, E_2K can be formed directly from E_1 by reversal of the cycle shown in Fig. 5 (see Beaugé & Glynn, 1980). Agents which stabilize the E_2K form either by increasing its formation or inhibiting its breakdown can stimulate the phosphatase and inhibit the ATPase.

In this paper we have shown that magnesium stimulates phosphatase and inhibits ATPase and that the half-maximal concentrations of magnesium needed for these two effects are very similar. We therefore suggest that high magnesium concentrations stabilize the E_2K form of the enzyme. This stabilization could be brought about in two ways. First, it is possible that only free ATP can act as the substrate at the low affinity site which regulates the breakdown of the E_2K form of the enzyme. Hence increasing the concentration of magnesium will stabilize the E_2K form by reducing the level of free ATP. This hypothesis is difficult to test because of the complex relationship between Mg^{2+}, MgATP and free ATP (see Skou, 1974*a*). Secondly, magnesium may directly induce the conversion of E_1 to E_2 by reversal of the cycle outlined in Fig. 5. There are several pieces of evidence to support this scheme. Sen, Tobin & Post (1969) showed that magnesium increases the binding of ouabain to the enzyme in the absence of ATP. Since an E_2 form of the enzyme is believed to bind ouabain, magnesium would appear to promote the E_1 to E_2 conversion. More recently Post, Toda, Kume & Taniguchi (1975) have been able to reverse the entire ATPase cycle and have produced the phosphorylated enzyme directly from E_1 and phosphate. The yield of phosphoenzyme was markedly improved by washing the enzyme in $MgCl_2$, again probably due to stabilization of the E_2 form of the enzyme.

Recently it has been suggested that the sodium-pump enzyme consists of functional subunits (Hansen, Jensen & Ottolenghi, 1979; Ottolenghi, 1979). The enzyme has ATPase activity when the subunits are interacting and phosphatase activity when there is no subunit interaction. Sodium and ATP favour subunit interaction and hence ATPase activity, whereas potassium, partial delipidation of the enzyme or treatment of the enzyme with thimerosal prevents subunit interaction and hence favour phosphatase activity. Moreover, it was found that thimerosal activates phosphatase activity whilst inhibiting ATPase activity. In this scheme, magnesium would prevent subunit interaction (rather like thimerosal) and hence stimulate the phosphatase activity whilst inhibiting the ATPase activity. Thus, magnesium might control the activity of the sodium pump *in vivo* by altering its quaternary structure. Although Hansen *et al.* (1979) did not compare this scheme with those of a more conventional type, such as that shown in Fig. 5, it seems likely that abolition of subunit interaction is equivalent to the E_1 to E_2 conversion.

Finally, the inhibition of the ATPase activity by *p*NPP itself may be due to a stabilizing effect of *p*NPP on the E_2 form of the enzyme.

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