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CALCIUM ION-DEPENDENT DEPHOSPHORYLATION OF THE Ca^{2+}-ATPase OF HUMAN RED-CELLS BY ADP

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Summary

In the Ca^{2+}-ATPase of human red cells the rate of dephosphorylation of the phosphoenzyme is increased by ADP, provided Ca^{2+} is present. This effect suggests that phosphorylation of the Ca^{2+}-ATPase is a reversible process.

During the hydrolysis of ATP catalyzed by the Ca^{2+}-dependent ATPase of human red-cell membranes, the terminal phosphate of ATP is transferred in a Ca^{2+}-dependent reaction to the enzyme with the formation of an acyl-phosphate-like bond [1—3]. Mg^{2+} converts the phosphoenzyme into such a form that, when ATP is present in high concentrations (Garrahan, P.J. and Rega, A.F., unpublished), it undergoes rapid hydrolysis with liberation of inorganic phosphate [2]. If the phosphorylation reaction were reversible, in the presence of Ca^{2+} the phosphoenzyme would be able to react with ADP donating its phosphate moiety to form ATP. In this case it can be expected that adequate concentrations of ADP will increase the rate of dephosphorylation of the phosphoenzyme, this effect being strictly dependent on the presence of Ca^{2+} This prediction was tested in the experiments reported here.

Fragmented membranes from human red cells were prepared by the procedure already described [4]. Phosphorylation and dephosphorylation were performed by the procedure described previously [2] using [γ-^{32}P]ATP in media with the composition given in the legends to the figures. [γ-^{32}P]ATP was prepared according to Glynn and Chappell [5]. All rate constants for dephosphorylation were calculated assuming first order kinetics.

In the experiment shown in Fig. 1, membranes were phosphorylated in the absence of Mg^{2+} for 20 s. Phosphorylation was stopped by the addition of an excess of unlabelled ATP to half of the tubes and an excess of unlabelled

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ATP plus ethanedioxy-bis(ethylamine) tetraacetic acid (EGTA) to chelate Ca^{2+} to the rest of the tubes. Dephosphorylation was allowed to proceed for 2 s and then all the tubes received enough of a concentrated solution of ADP to attain a final concentration of 5 mM. 2 s later the reaction was stopped with trichloroacetic acid. Results show that addition of ADP to the tubes containing Ca^{2+} accelerates the rate of dephosphorylation of the phosphoenzyme. In fact, it can be calculated that the nucleotide increases the rate constant for dephosphorylation from 0.2 to 0.7 s^{-1}. In contrast with this result, when Ca^{2+} is chelated with EGTA dephosphorylation of the phosphoenzyme is unaffected by ADP, showing that the stimulatory effect of ADP depends on the presence of Ca^{2+}.

When phosphoenzyme is made in the presence of Mg^{2+} and chased with unlabelled ATP at the concentrations used in the experiment in Fig. 1, the rate

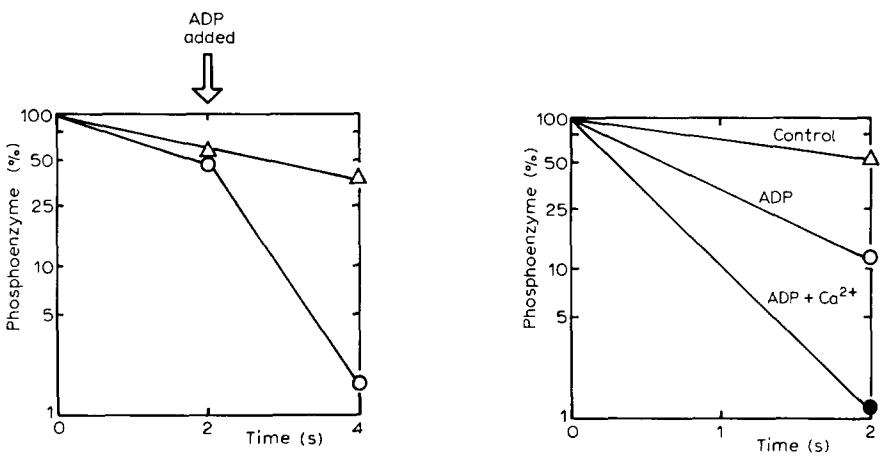

Fig. 1. The effects of ADP in the presence (○) and absence (△) of Ca^{2+} on Ca^{2+}-dependent phosphoenzyme made in the absence of Mg^{2+}. Phosphoenzyme was prepared by incubating fragmented membranes (3.2 mg protein) in 0.4 ml of a reaction mixture containing (mM): [γ-^{32}P]ATP, 0.03; $CaCl_2$, 0.5; EGTA, 0.05; Tris · HCl (pH 7.4 at 0°C), 150. After 20 s of incubation (zero time in the figure) 0.1 ml of a chase solution containing salts and unlabelled ATP was added to bring the final composition of the reaction mixture to (mM): ATP, 0.5; $CaCl_2$, 1; EGTA, 0.05; Tris · HCl (pH 7.4 at 0°C), 150, in the Ca^{2+} containing samples and to (mM): ATP, 0.5; EGTA, 30; $CaCl_2$, 0.04; Tris · HCl (pH 7.4 at 0°C), 150, in the Ca^{2+}-free samples. After 2 s all tubes received 0.2 ml of a solution having the same composition of the reaction mixture but with enough ADP added to bring the final concentration of ADP to 5 mM. The reaction was terminated by the addition of 3 ml of 300 mM trichloroacetic acid, 1 mM ATP and 50 mM H_3PO_4. Throughout the procedure the temperature was kept between 0 and 3°C. Ca^{2+}-dependent phosphoenzyme is the difference between the amount of ^{32}P bound to the membranes in the above mentioned media and in media with identical composition except that $CaCl_2$ was omitted. All determinations were performed by quintuplicate. Phosphoenzyme values were expressed taking as 100% the amount of phosphoenzyme measured after 20 s phosphorylation, which in this experiment was 0.8 pmol ^{32}P/mg membrane protein.

Fig. 2. The effects of ADP and ADP plus Ca^{2+} on dephosphorylation of Ca^{2+}-dependent phosphoenzyme made in the presence of Mg^{2+}. Phosphoenzyme was prepared by incubating fragmented membranes (3.2 mg protein) in 0.4 ml of a reaction mixture containing (mM): [γ-^{32}P]ATP, 0.001; EGTA, 0.1; $CaCl_2$, 0.15; $MgCl_2$, 0.5; Tris · HCl (pH 7.4 at 0°C), 150. After 20 s of incubation (zero time in the figure) 0.2 ml of a chase solution was added. The composition of the chase solution was adjusted as to bring the final composition of the reaction mixture to (mM): ATP, 0.03; $CaCl_2$, 0.1; EGTA, 0.067; $MgCl_2$, 0.34; Tris · HCl (pH 7.4 at 0°C), 150, in the control medium. The ADP plus Ca^{2+} and the ADP media had the same composition as the control medium except that 1 mM ADP and 1 mM ADP plus 20 mM EGTA, respectively, were also present. For other details see legend to Fig. 1. In this experiment the initial amount of phosphoenzyme was 0.4 pmol ^{32}P/mg membrane protein.

of hydrolysis is so fast that it falls below the limits of detection of our procedure (Garrahan, P.J. and Rega, A.F., unpublished). Therefore, to study the effect of ADP on phosphoenzyme made in the presence of Mg^{2+}, phosphorylation was carried out with 1 μM ATP. This allowed us to stop phosphorylation with only 30 μM unlabelled ATP, a concentration at which the stimulatory effect of the nucleotide on phosphoenzyme hydrolysis is still small (Garrahan, P.J. and Rega, A.F., unpublished). The results of an experiment using these conditions are shown in Fig. 2. It can be seen that ADP induces an increase in the rate of dephosphorylation that is considerably larger than the stimulatory effect of ADP on dephosphorylation of phosphoenzyme made in the absence of Mg^{2+}. This difference may perhaps be related to the fact that in phosphoenzyme made in the presence of Mg^{2+} the effect of ADP can be separated into two components: one persists in the presence of enough EGTA to chelate all the Ca^{2+} present and might perhaps show the ability of ADP to mimick the stimulatory effect ATP has on the hydrolysis of the phosphoenzyme. The other depends on the presence of Ca^{2+}. This feature relates this component to the reversal of the phosphorylation reaction. From the difference between the rate constants for dephosphorylation in the presence of ADP and of ADP plus EGTA, the rate constant of the Ca^{2+}-dependent dephosphorylation in the presence of ADP can be calculated to be 0.4 s^{-1}. This value is not very different to that observed in the phosphoenzyme made in the absence of Mg^{2+}.

Our results, which agree with previous findings in the Ca^{2+}-ATPase of sarcoplasmic reticulum [6], suggest that both in the presence and absence of Mg^{2+}, the reaction of transference of the terminal phosphate of ATP to the Ca^{2+} pump of human red-cell membranes is reversible.

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