BBA 75072

A 'LATENT' DINITROPHENOL-STIMULATED ATPase IN RED-CELL GHOSTS

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(Received March 15th, 1967)

SUMMARY

Some properties of the Mg^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) system of pig red-cell ghosts are described. When the membrane structure had not been much damaged during the preparative procedure, this activity was weak but could be stimulated up to 3-fold by 2,4-dinitrophenol and up to 5-fold by Sr^{2+}. Various kinds of injury to the membrane structure such as severe disruption or action of detergent led to a strong enhancement ('unmasking') of the normally latent ATPase activity. This was always accompanied by a loss of ability of the ATPase to be stimulated by 2,4-dinitrophenol and Sr^{2+}. The possible relationship of this ATPase activity to energy-linked functions of the red-cell membrane are discussed.

INTRODUCTION

In a previous paper[1], we have described in intact human red-cell ghosts a Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase (ATP phosphohydrolase, EC 3.6.1.3) system; this enzyme could be stimulated by Sr^{2+} as well as by Ca^{2+} and appeared to be distinct from the well-known (Na^+—K^+)-ATPase.

An interesting feature of the (Mg^{2+} + Ca^{2+})-dependent enzyme system was its sensitivity to 2,4-dinitrophenol; at concentrations up to 5 mM, this agent slightly stimulated the Mg^{2+}-dependent ATPase activity of the ghosts, whilst higher amounts of 2,4-dinitrophenol were inhibitory. On the other hand, the stimulating effect of Ca^{2+} and Sr^{2+} ions was impaired in the presence of 2,4-dinitrophenol.

It has been possible, since then, to modify the preparative procedure in such a way that much higher degrees of stimulation of the Mg^{2+}-dependent ATPase by 2,4-dinitrophenol could be obtained. In fact, it was found that the preparative technique greatly affected the activity pattern, particularly the sensitivity to 2,4-dinitrophenol. Data presented in this communication suggest a close analogy between this 2,4-dinitrophenol-stimulated enzyme system and mitochondrial ATPase.

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METHODS

Pig red cells have been used; the results were essentially the same as with human erythrocytes. Blood, obtained by exsanguination, was defibrinated. The red cells were centrifuged down, and, after two washings with isotonic NaCl, the packed cells were lysed by adding them to 5 volumes of 40 mM $MgCl_2$ (pH 7.1). It is known that in such conditions (moderately hypotonic haemolysis) the permeability properties of the membranes are preserved, indicating that the membrane structure is not much damaged[2]. Mg^{2+} was used in order to eliminate the bound Ca^{2+}, which can be displaced by excess Mg^{2+} (refs. 3, 4).

Ghosts were centrifuged down and washed twice with 8 volumes of suspending medium, the composition of which varied with the experiment. They were then submitted to various treatments, as indicated in the text, before ATPase assay.

ATPase activity was measured as described previously[1].

Protein was determined by the method of LOWRY *et al.*[5].

RESULTS AND DISCUSSION

The effects of 2,4-dinitrophenol on the Mg^{2+}-dependent ATPase of ghosts under different experimental conditions are shown in Fig. 1. Ghosts were washed with 25 mM Tris–HCl (pH 7.0).

When the ghost preparation had only been frozen and thawed once and was not aged, 2,4-dinitrophenol could induce up to 200% stimulation of the ATPase activity in the presence of Mg^{2+} alone (Curve a). The degree of stimulation was somewhat variable from experiment to experiment, especially when different samples of blood were used; it was lower when the blood had been stored for several days or had been contaminated with heavy metal ions.

It can be seen (Curve a) that concentrations of 2,4-dinitrophenol higher than 5 mM were inhibitory. This inhibition by excess 2,4-dinitrophenol was more marked when the medium used to wash the ghosts was more hypotonic.

In the presence of 0.5 mM $SrCl_2$ (Curve b), the activity was greatly enhanced but 2,4-dinitrophenol had little stimulating effect; concentrations higher than 1 mM were inhibitory, as already reported[1].

When the ghosts had been sonicated (Curve c) or preincubated with 0.1% digitonin before ATPase assay (Curve d), or when they had been aged at $-10°$ for 15 days (Curve e), the ATPase activity was often quite high; however, 2,4-dinitrophenol had but little stimulating effect. At concentrations higher than 5 mM, 2,4-dinitrophenol was inhibitory, especially in digitonin-treated ghosts. It may be concluded that maltreatment of the membrane structure (*e.g.* severe disruption or the action of detergent) may lead to 'unmasking' of the Mg^{2+}-dependent ATPase activity. Unmasking is accompanied by a loss of ability of 2,4-dinitrophenol to stimulate the ATPase.

On the other hand, Sr^{2+} failed to stimulate the Mg^{2+}-dependent ATPase not only when 2,4-dinitrophenol was present but also when the ATPase activity was unmasked; this is shown in Table I. A good correlation can be observed between the 'latency' of the Mg^{2+}-dependent ATPase, its ability to be stimulated by 2,4-dinitro-

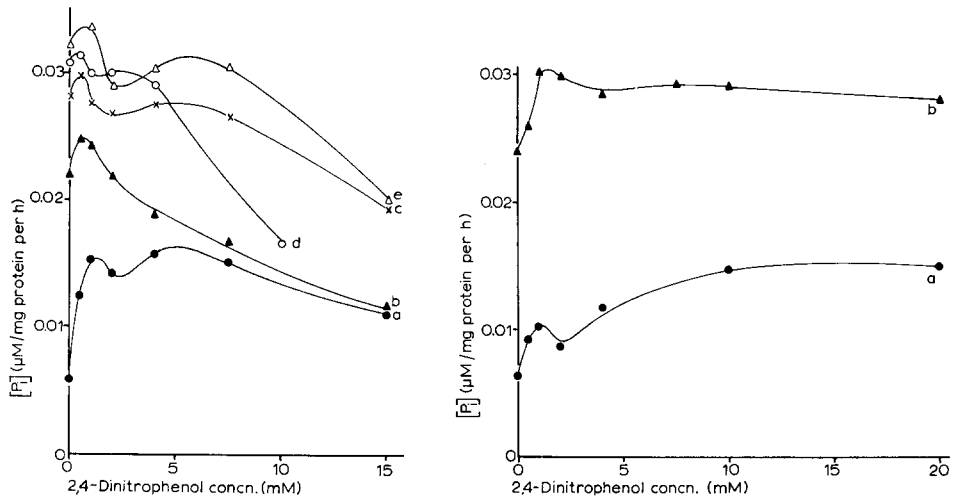

Fig. 1. Effect of 2,4-dinitrophenol on ATP splitting by ghosts under different conditions. The assay medium contained ATP, 3 mM; $MgCl_2$, 3 mM; Tris–HCl buffer, 70 mM (pH 7.0). Incubation 60 min at 37°. Curve a, a fresh preparation of ghosts was used, after freezing and thawing once. Curve b, same ghosts as in (a) but with 0.5 mM Sr^{2+} present in the incubating medium. Curve c, ghosts were sonicated during 40 sec (MSE 100-W ultrasonic disintegrator, $\nu = 20$ kc/sec). Curve d, ghosts were preincubated 1 h at 25° in the presence of 0.1% digitonin. Curve e, ghosts were aged at −10° for 15 days, with frequent freezing and thawing. ATPase activity is given in μmoles inorganic phosphate split per ml incubation mixture per mg protein and per h.

Fig. 2. Effect of 2,4-dinitrophenol on ATPase activity of ghosts washed in the presence of 4 mM EDTA. Conditions of experiment: see Fig. 1. Curve a, no Sr^{2+} added. Curve b, 0.5 mM Sr^{2+} present in the medium.

phenol and its ability to be stimulated by Sr^{2+}. This suggests that a common ATPase system is involved in the stimulation of the activity by 2,4-dinitrophenol and by Sr^{2+}. Moreover, the changes in sensitivity to 2,4-dinitrophenol and Sr^{2+} suggest that unmasking is linked to structural modifications affecting the ATPase system rather than to increased permeability of the membranes to ATP.

TABLE I

EFFECT OF VARIOUS TREATMENTS OF GHOSTS ON THE SENSITIVITY OF ATPase TO 2,4-DINITROPHENOL AND TO Sr^{2+}

Conditions of experiment: see Fig. 1.

Treatment of ghosts	*Activity of ATPase in the presence of*			*Per cent activation (+) or inhibition (−) due to 2,4-dinitrophenol*	*Per cent activation (+) or inhibition (−) due to Sr^{2+}*
	Mg^{2+}, 3 mM	*Mg^{2+}, 3 mM + 2,4-dinitrophenol, 5 mM*	*Mg^{2+}, 3 mM + Sr^{2+}, 0.5 mM*		
Freezing and thawing once	0.0065	0.0179	0.0245	+177	+275
Washing in the presence of EDTA + freezing and thawing once	0.0090	0.0171	0.0348	+ 90	+287
Sonication	0.0282	0.0281	0.0288	0	+ 2
Digitonin treatment	0.0306	0.0255	0.0285	− 17	− 7
Ageing at −10°	0.0326	0.0324	0.0357	0	+ 9

In other respects, the $(Na^+ + K^+)$-dependent activity was found to be generally unaffected under conditions of unmasking of the Mg^{2+}-dependent component. So far, the two systems do not appear to have any kind of relationship.

In the experiment reported in Fig. 2, ghosts were washed, once with 100 mM Tris–HCl (pH 7.0) and once with 100 mM Tris–HCl + 4 mM EDTA (pH 7.0); they were frozen and thawed once. It can be seen that in these conditions higher amounts of 2,4-dinitrophenol were needed to obtain the optimal ATPase activity (Curve a); moreover, excess 2,4-dinitrophenol, up to 20 mM, had no inhibitory effect. In the presence of 0.5 mM Sr^{2+} (Curve b), the ATPase activity was considerably enhanced, as could be expected; but surprisingly, 2,4-dinitrophenol induced a further stimulation and failed to induce a marked inhibition when it was in excess.

It might be concluded that pretreatment of ghosts with EDTA prevents 2,4-dinitrophenol from reaching the sites where it normally exerts its inhibitory action; this may be linked to the fact that EDTA causes the membrane to be in a 'contracted' state: indeed, EDTA induces a marked shrinkage of ghosts[4] even when they have been rendered leaky by freezing and thawing.

The possible rôle of the 2,4-dinitrophenol-stimulated enzyme can be appreciated by considering its striking similarities with mitochondrial ATPase. Both are Mg^{2+}-dependent and 2,4-dinitrophenol-stimulated[6]; moreover, some kinds of injury such as ageing or disruption, to the mitochondrial membrane structure also result in the unmasking of ATPase activity. This corresponds to a loss of sensitivity to 2,4-dinitrophenol, as well as to a loss of ability of the mitochondria to undergo 'active' shrinkage[7]. Such an energy-linked shrinkage has been observed in red-cell ghosts too[4]; it was suggested that the phenomenon involved the activity of actomyosin-like proteins, the presence of which in ghosts has been reported[8].

We have observed that in ghosts as in mitochondria, active shrinkage could not be obtained under conditions of unmasking of the ATPase activity. We might thus speculate that unmasking corresponds to an uncoupling between contraction and ATP splitting by the myosin-like protein. However, we dare not state that the ATPase described here may be ascribed to the myosin-like protein, particularly in view of the fact that the ATPase activity of this protein can be observed in the presence of Ca^{2+} alone[8] whilst the ATPase activity of whole ghosts absolutely requires Mg^{2+}, even after unmasking.

An alternative way of appreciating the rôle of the Mg^{2+}-dependent ATPase system would be to consider a possible coupling of this activity with ion or electron transfer reactions. This point is now being investigated.

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