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EFFECT OF CONCANAVALIN A ON THE ACTIVITY OF MEMBRANE-BOUND AND DETERGENT-SOLUBILIZED Mg^{2+}-ATPase

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Plasma membranes were isolated after binding liver and hepatoma cells to polylysine-coated polyacrylamide beads, and the effect of concanavalin A on the membrane-bound Mg^{2+}-ATPase and the Mg^{2+}-ATPase solubilized by octaethylene glycol monododecyl ether ($C_{12}E_8$) was studied. In the experiment of membrane-bound Mg^{2+}-ATPase, plasma membranes were pretreated with Concanavalin A and the activity was assayed. Concanavalin A stimulated the activity of both liver and hepatoma enzymes assayed above 20°C. Concanavalin A abolished the negative temperature dependency characteristic of liver plasma membrane Mg^{2+}-ATPase. On the other hand, Concanavalin A prevented the rapid inactivation due to storage at −20°C, which was characteristic of hepatoma plasma membrane Mg^{2+}-ATPase. With solubilized Mg^{2+}-ATPase from liver plasma membranes, the negative temperature dependency was not observed. Concanavalin A, which was added to the assay medium, stimulated the activity of the enzyme solubilized in $C_{12}E_8$ at a high ionic strength. However, Concanavalin A failed to show any effect on the enzyme solubilized in $C_{12}E_8$ at a low ionic strength. With solubilized Mg^{2+}-ATPase from hepatoma plasma membranes, Concanavalin A could not prevent the inactivation of the enzyme during incubation at −20°C.

Introduction

We have found previously that plasma membrane Mg^{2+}-ATPase activity was stimulated when monolayer cultured hepatoma cells came into contact with each other, whereas no such stimulation was observed in liver plasma membranes [1]. A biochemical study of Mg^{2+}-ATPase revealed several distinct differences between liver and hepatoma plasma membranes [2]. It has been found by some authors that Concanavalin A stimulated the plasma membrane Mg^{2+}-ATPase of rat liver [3,4], mammary gland [5], mammary-adenocarcinoma ascites cells [6], and transplantable human tumor cells [7]. In this report, we describe the change in Mg^{2+}-ATPase activity when cytoplasmic surfaces of liver and hepatoma plasma membranes were pretreated with Concanavalin A and sensitivity of solubilized enzyme to Concanavalin A which was added to the assay medium.

Materials and Methods

The cells under study were rat hepatocytes prepared from livers using a sodium citrate perfusion technique and Yoshida ascites hepatoma 66 cells maintained in the rats by serial transplantation. Plasma membranes were isolated after binding

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Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminoethane; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

cells to polylysine (Miles Lab)-coated polyacrylamide beads (Bio-Rad Lab) according to the method of Cohen et al. [8]. The procedures and the marker enzyme determination were described in detail previously [2].

Membranes attached to beads at approx. 0.6 mg of protein/ml were preincubated in the Mg^{2+}-ATPase assay medium (containing no ATP) in the absence or in the presence of Concanavalin A (Sigma) at a final concentration of 0.2 mg/ml at 37°C for 30 min. The membranes were washed before transfer to the enzyme assay mixture. After 30 min incubation at the temperature indicated, the amount of P_i (μmol) released from 3 mM Na_2ATP in the presence of 75 mM Tris-HCl, pH 8.0 and 5 mM $MgCl_2$, was quantitated by the method of Martin and Doty [9]. Proteins were solubilized by 3% solution of SDS and determined by the method of Lowry et al. [10].

Membranous Mg^{2+}-ATPase was solubilized in $C_{12}E_8$ (Nikko Chem. Co., Tokyo), $C_{12}E_8$-glycerol (20% v/v), and $C_{12}E_8$-glycerol-0.1 M KCl [11], respectively. $C_{12}E_8$ was dissolved in 10 mM Tes-NaOH, pH 7.5. The solubilized enzyme was concentrated to a small volume using an Amicon CF50A filter.

Results and Discussion

Fig. 1a shows that pretreatment of plasma membranes with Concanavalin A stimulated the activity of both liver and hepatoma Mg^{2+}-ATPase in the range, 20–37°C, however, as shown in Fig. 1b, Concanavalin A had no effect below 18°C. Stimulation of the enzyme by Concanavalin A was prevented by 0.3 M α-methyl-D-glucoside (data not shown). Fig. 1a also shows that the activity of liver plasma membranes declined markedly with increasing temperature above 30°C, whereas the activity of hepatoma plasma membranes continued to increase. The most remarkable effect of Concanavalin A was observed in the activity of liver plasma membranes assayed at 37°C; the decline in the activity was prevented and the negative temperature dependency was abolished by Concanavalin A. As shown in Fig. 1b, the Arrhenius plot of Mg^{2+}-ATPase activity of plasma membranes presented two straight lines between 8 and 30°C, with a break occurring at 18°C in liver

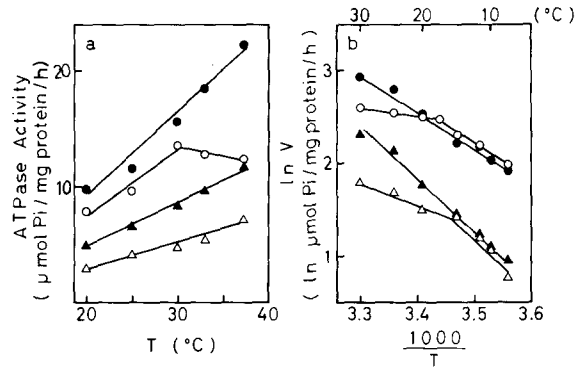

Fig. 1. (a) Influence of temperature on the membrane-bound Mg^{2+}-ATPase activity with or without pretreatment with Concanavalin A (0.2 mg/ml). (b) Arrhenius kinetics of membrane-bound Mg^{2+}-ATPase activity with or without pretreatment with Concanavalin A (0.2 mg/ml). ○——○, Activity in liver plasma membranes; ●——●, activity in Concanavalin A-treated liver plasma membranes; △——△, activity in hepatoma plasma membranes; ▲——▲, activity in Concanavalin A-treated hepatoma plasma membranes.

enzyme, and at 16°C in hepatoma enzyme, respectively. Below the break point, the enzyme no longer showed any increase of the activity due to Concanavalin A. The cause for this remains obscure, but it may result from a conformational change of the enzyme. Although a break in the enyzymic activity was explained as representing a conformational change effected by the membrane lipids in the vicinity of the enzyme, recent evidence suggests that temperature-sensitive change in protein conformation independent of the membrane lipids is also important [12,13]. It is, therefore, quite possible that below the break point the conformation of the Mg^{2+}-ATPase becomes inaccessible to Concanavalin A. Plasma membranes used in this experiment were the membranes attached to beads, therefore, extracellular surfaces of the membranes became inaccessible to Concanavalin A, whereas cytoplasmic surfaces were exposed and accessible to it.

The activity of Mg^{2+}-ATPase decreased with increasing storage after isolation of plasma membranes. Fig. 2 summarized the results of experiments measuring the stability of the activity during storage at −20°C for up to 3 days, with or without pretreatment with Concanavalin A. Storage of plasma membranes caused rapid and re-

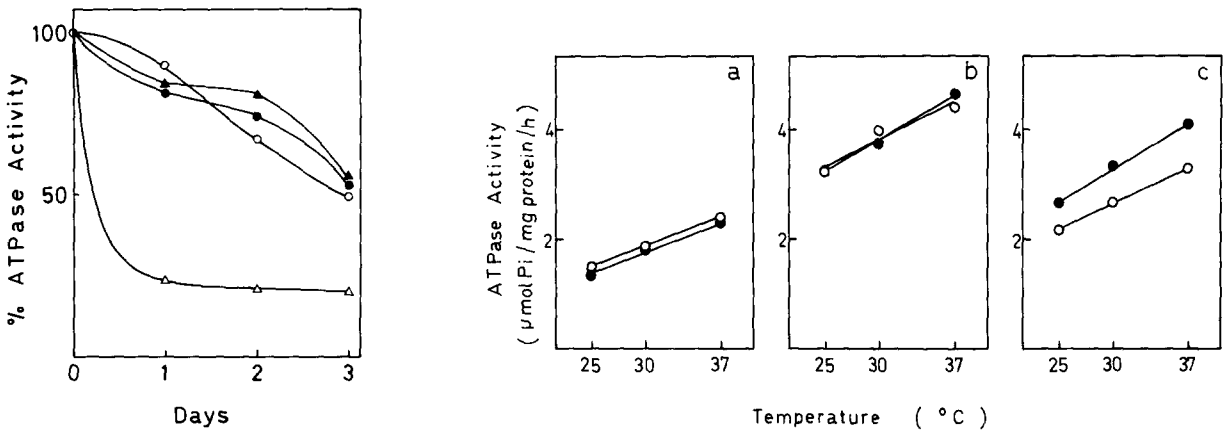

Fig. 2. Effect of Concanavalin A-pretreatment on the stability of membrane-bound Mg^{2+}-ATPase to storage. Plasma membranes were incubated at 37°C for 30 min in the absence or in the presence of Concanavalin A (0.2 mg/ml). After washing, the membranes were kept at −20°C, and the activity was assayed at 37°C on the day indicated. Initial enzyme specific activities were 9.48, 16.76, 5.70, and 7.94 μmol Pi/mg protein per h for liver plasma membranes O——O, Concanavalin A-treated liver plasma membranes ●——●, hepatoma plasma membranes △——△, and Concanavalin A-treated hepatoma plasma membranes ▲——▲, respectively.

Fig. 3. Influence of temperature and effect of Concanavalin A on solubilized Mg^{2+}-ATPase from liver plasma membranes. (a) Solubilized by $C_{12}E_8$. (b) Solubilized by $C_{12}E_8$-glycerol. (c) Solubilized by $C_{12}E_8$-glycerol-0.1 M KCl. $C_{12}E_8$ was dissolved in 10 mM Tes-NaOH, pH 7.5. The concentrations of protein and $C_{12}E_8$ were 0.6 and 0.5 mg/ml, respectively. The detergent was removed as much as possible by ultrafiltration before the enzyme assay. Concanavalin A was added to the assay medium at a final concentration of 0.2 mg/ml. O——O, Activity of untreated enzyme; ●——●, activity of Concanavalin A-treated enzyme.

markable decrease in the activity of hepatoma enzyme, whereas the activity of liver enzyme steadily but modestly declined. The inactivation observed with hepatoma enzyme could be effectively blocked by pretreatment of plasma membranes with Concanavalin A; the level of activity remained essentially similar to that of liver enzyme.

To further investigate the effect of Concanavalin A, the following series of experiment were performed; the Mg^{2+}-ATPase was solubilized from liver plasma membranes by $C_{12}E_8$ dissolved in an electrolyte medium of ionic strength ($I = 0.005$), $C_{12}E_8$-glycerol, and $C_{12}E_8$-glycerol-0.1 M KCl ($I = 0.105$), respectively. A high ionic strength in the detergent was more effective in solubilizing the enzyme; 80% of the membrane-bound enzyme were solubilized in $C_{12}E_8$-glycerol-0.1 M KCl, 61% in $C_{12}E_8$-glycerol, and 48% in $C_{12}E_8$, respectively. As can be seen in Fig. 3, there was no significant difference in the activity between three preparations, but it is noted that these preparations have low specific activity. The reason for this is due to the fact that the enzymic activity is partly inhibited by the detergent. In contrast to the membrane-bound enzyme (Fig. 1a), the activity of the solubilized enzyme increased with increasing temperature above 30°C. It seems reasonable, therefore, to conclude that the negative temperature dependency is characteristic of the plasma membrane-bound Mg^{2+}-ATPase of liver cells. It is proposed that the negative temperature dependency is due to a conformational change of the enzyme, possibly as a result of a modification of the physical state of the liver plasma membrane above 30°C. Also, it is proposed that Concanavalin A is able to stabilize the enzyme conformation via its effect on the membrane. In fact, high doses of Concanavalin A have been shown to reduce membrane fluidity, or increase ordering of membrane [4]. Fig. 3c shows that the activity of the enzyme solubilized in $C_{12}E_8$ at a high ionic strength was stimulated by adding Concanavalin A to the assay medium, the degree of stimulation being similar to that of the membrane-bound enzyme

with Concanavalin A pretreatment. However, Concanavalin A failed to show any stimulatory effect on the enzyme solubilized in $C_{12}E_8$ at a low ionic strength (Fig. 3a, b). Similar results were obtained in the solubilized enzyme from hepatoma plasma membranes (data not shown). What is particularly significant in our results is that Concanavalin A acts on the enzyme directly. However, why $C_{12}E_8$-glycerol-0.1 M KCl extracted enzyme alone is stimulted by Concanavalin A is unknown. Presumably, the polar molecules containing the carbohydrate moiety of the glycoprotein enzyme could be easily solubilized by salting-in. The Mg^{2+}-ATPase was solubilized in $C_{12}E_8$-glycerol or $C_{12}E_8$-glycerol-0.1 M KCl from the liver plasma membranes pretreated with Concanavalin A, and the activity was assayed at 37°C without Concanavalin A addition. The activity was similar to that of the enzyme solubilized from untreated membranes (data not shown).

The Mg^{2+}-ATPase was solubilized from hepatoma plasma membranes and Concanavalin A was added to the enzyme solution, then the stability of the activity during storage at −20°C was studied. As shown in Fig. 4a, b, Concanavalin A could not prevent the inactivation of the enzyme due to storage. With the solubilized enzyme from liver plasma membranes, the time-course profiles of the inactivation, in the presence or in the absence of Concanavalin A, were identical each other, and similar to those of the membrane-bound enzyme (data not shown). Based on the data presented in Fig. 2. and 4, it seems likely that Concanavalin A can serve to stabilize the hepatoma enzyme molecule when added to the plasma membranes, possibly as a result of a modification of the physical state of the membrane, thereby counteracting a tendency to storage-dependent, rapid inactivation, which is characteristic of the hepatoma enzyme.

The ability of Concanavalin A to bind Mg^{2+}-ATPase was tested by the affinity binding assay which utilizes Concanavalin A-agarose beads. Solubilized enzyme was incubated with Concanavalin A-agarose beads, and after sedimenting the beads the activity was assayed for the beads and for the supernatant, respectively. Fig. 5 shows that the Mg^{2+}-ATPase activity in the supernatant after treatment with Concanavalin A-agarose beads decreased with increasing the beads and, in contrast to this, the activity on the beads increased with increasing the beads, indicating the ability of

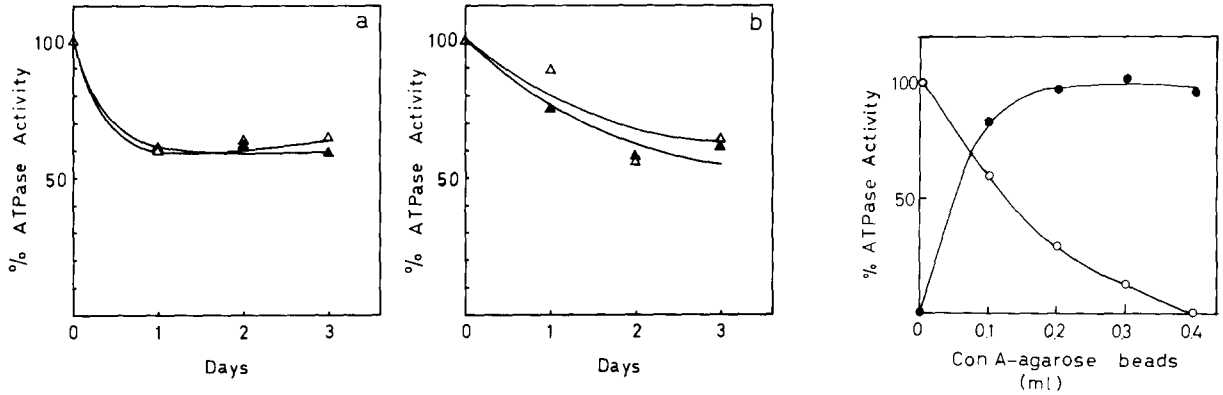

Fig. 4. Effect of Concanavalin A on the stability of solubilized Mg^{2+}-ATPase from hepatoma plasma membranes to storage. (a) Solubilized by $C_{12}E_8$. (b) Solubilized by $C_{12}E_8$-glycerol-0.1 M KCl. Concanavalin A was added to the enzyme solution. After incubation at −20°C the activity was assayed at 37°C. △——△, Activity of untreated enzyme; ▲——▲, activity of Concanavalin A-treated enzyme.

Fig. 5. Transfer of Mg^{2+}-ATPase activity from solubilized medium to Concanavalin A-agarose beads. Mg^{2+}-ATPase was solubilized in $C_{12}E_8$-glycerol-0.1 M KCl from liver plasma membranes, and incubated with various amounts of Concanavalin A-agarose beads (21 mg Concanavalin A per ml beads) for 30 min at 37°C. After sedimenting the Concanavalin A-agarose beads, the activity was assayed for the beads and for the supernatant in the presence of 3 mM ATP, 5 mM $MgCl_2$ and 75 mM Tris-HCl, pH 8.0. ●——●, Activity in Concanavalin A-agarose beads; ○——○, activity in supernatant.

TABLE I

SPECIFIC ACTIVITY OF Mg^{2+}-DEPENDENT HYDROLYSIS OF NUCLEOTIDES

In the presence of 5 mM $MgCl_2$ and 75 mM Tris-HCl, pH 8.0, ATP, GTP, UTP, CTP, and ITP, 3 mM were incubated with the enzyme preparation solubilized in $C_{12}E_8$-glycerol-0.1 M KCl from liver plasma membranes. Con A, Concanavalin A.

Additive	Activity (μmol P_i)/mg protein per h) (mean ± S.D.)				
	ATP	GTP	UTP	CTP	ITP
None	1.70 ± 0.16	5.96 ± 1.25	4.00 ± 0.26	2.88 ± 0.12	5.78 ± 0.85
Con A	2.10 ± 0.10	8.34 ± 0.93	4.36 ± 0.15	3.26 ± 0.09	5.98 ± 0.79

Concanavalin A to bind the enzyme.

Examination of the enzymic activity of solubilized liver plasma membranes was undertaken when several nucleotides were used as the substrate. Table I shows that GTP, UTP, CTP and ITP were better substrates than ATP. With Concanavalin A stimulation, GTP was more effective as a substrate than ATP; the activity was stimulated by Concanavalin A by 40% with GTP and by 24% with ATP, respectively. Stimulation by Concanavalin A occurred also with UTP, CTP and ITP, but to a lesser extent.

The preceding paper [2] has shown that the addition of ouabain (0.1–1 mM) to an assay medium had no influence on the activity, suggesting that the assay method described here excludes the possibility of $(Na^+ + K^+)$-ATPase activity. Although the physiological role of the plasma membrane Mg^{2+}-ATPase has not been defined, following experiments have recently performed. Solubilized enzyme was loaded on gradient polyacrylamide slab gels, electrophoresed, and the Mg^{2+}-ATPase was examined by means of histochemical activity-staining applied on gels. Three bands of 500, 480 and 370 kDa were present in liver plasma membrane Mg^{2+}-ATPase, however, only one major band of 500 kDa was visible in hepatoma plasma membrane enzyme. Furthermore, it has been found that 500 kDa Mg^{2+}-ATPase shows a myosin-like property in the presence of F-actin (unpublished experiments). It is, therefore, concluded that the inactivation of hepatoma enzyme during storage (Fig. 2, Fig. 4) is due to the instability of 500 kDa Mg^{2+}-ATPase.

In conclusion, Concanavalin A stimulation of the activity was observed in membrane-bound and detergent-solubilized Mg^{2+}-ATPase, however, the effect of Concanavalin A on negative temperature dependency and storage-dependent decline of the activity was observed in membrane-bound Mg^{2+}-ATPase alone.

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