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$(Ca^{2+}+Mg^{2+})$-ACTIVATED ATPases IN THE PLASMA MEMBRANE OF MOUSE LIVER CELLS

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SUMMARY

1. Purified plasma membranes from dissociated adult mouse liver cells possess a $(Ca^{2+}+Mg^{2+})$-stimulated ATPase (EC 3.6.1.3) activity.

2. Enzyme activity is at a maximum with the addition of 0.3 mM Ca^{2+} and 3 mM Mg^{2+}.

3. Using medium devoid of alkali metal ions $(Ca^{2+}+Mg^{2+})$-ATPase enzyme activity was observed with $K_{m_1} = 0.35 \cdot 10^{-3}$ M at a substrate concentration of 1 mM or less and an apparent $K_{m_2} = 0.88 \cdot 10^{-3}$ M at higher substrate concentrations.

4. In the presence of Na^+ and 4 mM ATP, an increase in activity was seen, suggesting the presence of a $(Ca^{2+}+Mg^{2+}+Na^+)$-activated ATPase.

5. In the presence of both Na^+ and K^+ the $(Ca^{2+}+Mg^{2+})$-dependent enzyme activity was further increased, indicating that a $(Ca^{2+}+Mg^{2+}+K^+)$-stimulated ATPase may also be present.

INTRODUCTION

Ca^{2+} is important in controlling the structure and adhesiveness of cells [1] and in regulating cellular activity and metabolism [2]. Normal body fluid concentration is 10^{-3} M while cellular Ca^{2+} concentration is 10^{-5} M or less [3]. That is not to say that cellular Ca^{2+} levels remain static, for fluctuations have been seen through the cell cycle of, for instance, sea urchin eggs [4]. Ca^{2+}-ATPases have been considered to play a role in the regulation of cellular Ca^{2+} concentration.

Such ATPases have been demonstrated in the plasma membrane of embryonic chick fibroblasts [5], of cells of rat kidney cortex [6] and guinea pig placenta [7], and of egg cells of freshwater fish [8] and sea urchins [9]. The $(Ca^{2+}+Mg^{2+})$-ATPases of the above cells were all thought to be involved in the active uptake of Ca^{2+}. Several investigations have also indicated the existence of $(Ca^{2+}+Mg^{2+})$-ATPases

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in the plasma membrane of the red blood cell [10–16], including that of Schatzmann and Rossi [17], who describe a complex consisting of several Ca^{2+}-activated enzymes. The $(Ca^{2+}+Mg^{2+})$-ATPase of red blood cell membranes is considered to be involved in active extrusion of Ca^{2+}.

Similarly, van Rossum [18] has reported that rat liver slices extrude Ca^{2+}, using high energy compounds, by a mechanism independent of Na^+ or K^+ transport. These results imply the possible existence of a Ca^{2+}-stimulated ATPase in the rat liver plasma membrane.

The presence of a $(Ca^{2+}+Mg^{2+})$-ATPase in mouse liver plasma membranes has also been implied. Neifakh and Vasilets [19] isolated an actomyosin-like protein from mouse liver plasma membranes and tissue cells, including mouse liver cells, have been shown to possess an intramembranous smooth-muscle-like myosin [20–22]. This myosin would be expected to exhibit $(Ca^{2+}+Mg^{2+})$-ATPase activity [23]. The aim of the present study has therefore been to investigate the Ca^{2+}-activated ATPases of the plasma membrane from cells isolated from adult mouse liver.

MATERIALS AND METHODS

A suspension of liver cells, free of red blood cells, was prepared from adult laboratory mice according to the perfusion method of ap Gwynn et al. [24]. Membranes were then isolated from these cells using essentially the method of Warren et al. [25]. In this technique, isolated cells were washed with 50 mM Tris · HCl buffer, pH 7.4, and allowed to swell in the cold for 10 min, while suspended in the same buffer containing 5 mM $MgCl_2$. The membranes were then stripped using a Dounce homogeniser and the homogenate centrifuged at $1000 \times g$ for 1 min to remove whole cells and debris, including nuclei. The supernatant was then centrifuged at $4000 \times g$ for 10 min to remove mitochondria and other fine cell debris and the membranes obtained by sedimentation at $6000 \times g$ for 15 min. These were suspended in 30 % (w v) sucrose and layered onto a discontinuous gradient consisting of 65 %, 55 %, 45 % and 40 % (w/v) sucrose. After centrifugation at $90\,000 \times g$ for 5 h, the plasma membranes were harvested from the interface between the 55 % (w/v) and 45 % (w/v) sucrose solutions. They were then dialysed against 50 mM Tris · HCl buffer, pH 7.4, to remove sucrose and pelleted at $6000 \times g$ for 15 min.

The purity of the plasma membranes produced was determined by looking for nuclei under the phase contrast microscope and by assaying for enzymes characteristic of other possible contaminating cell fractions. Glucose-6-phosphatase (EC 3.1.3.9) is characteristic of rough endoplasmic reticulum and the activity of this enzyme was determined according to the method of Harper [26]. Mitochondria contain the enzyme succinate dehydrogenase (EC 1.2.99.1) and the activity of this enzyme was determined by following, spectrophotometrically at 600 nm, the reduction of 2,6-dichlorophenolindophenol [27]. The presence of other intracellular membranes was evaluated by measuring the activity of NADH dehydrogenase (EC 1.6.2.1) [28]. Crude cell homogenates were used as controls in all cases to ensure the efficacy of the enzymic reactions. A positive check, in combination with the above tests for the presence of plasma membranes was performed by assaying for $(Na^+ + K^+)$-ATPase activity in a medium containing Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $MgCl_2$ (5 mM) and ATP (4 mM), according to the method of Emmelot et al. [29].

The membranes at a protein concentration of 0.1–0.5 mg/ml were incubated in 2 ml of the appropriate reaction mixture for 1 h at 37 °C. These reaction mixtures were based on Emmelot's medium [2]–medium A. Choline chloride was employed to replace the NaCl and KCl where necessary [17]–medium B. Tris/ATP was used in determinations of ATPase activity, which was estimated by the release of inorganic phosphate [30] and routinely expressed as μmol P_i/mg protein per h. The Folin phenol test, as modified by Lowry et al. [31], was used for the estimation of protein, after alkaline hydrolysis of the membranes to release insoluble protein. Control flasks were always used to show the natural breakdown of ATP during the incubation time.

The cardiac glycoside, ouabain, which is a specific inhibitor of the $(Na^+ + K^+)$-ATPase [29] was obtained from Sigma and all reagents used in this study were of analytical grade.

RESULTS

The tests for succinate dehydrogenase, glucose-6-phosphatase and NADH dehydrogenase were in working order because crude cell homogenates gave a positive result. Since assays for these enzymes on the membrane preparations were negative, it was concluded that the isolation and purification procedures had removed cytoplasmic organelles. Microscopy indicated the absence of nuclei.

As will be seen from Table I, the total $(Na^+ + K^+_?)$-ATPase activity of the liver cell plasma membranes in an incubation medium containing Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $MgCl_2$ (5 mM) and ATP (4 mM) was 10.9 ± 0.9 μmol P_i/mg protein per h. The presence of this enzyme showed the preparation did indeed contain plasma membranes. When ouabain (0.05 mM) was added to the incubation medium the $(Na^+ + K^+)$-ATPase activity was inhibited by 97 %. On the addition of Ca^{2+} (0.3 mM) to medium A, an enhanced enzyme activity was observed, resulting in the formation of 21.1 ± 2.1 μmol P_i/mg protein per h (Table I) and this was reduced to 11.1 ± 0.38 μmol P_i/mg protein per h in the presence of 0.05 mM ouabain. Thus a Ca^{2+}-activated ATPase was present in mouse liver plasma membranes.

TABLE I

ATPase ACTIVITY OF MOUSE LIVER PLASMA MEMBRANES

The membranes (protein concentration 0.1–0.5 mg/ml) were incubated in 2 ml of the appropriate reaction mixture for 1 h, at 37 °C. The reaction was initiated by the addition of ATP and stopped by rapidly lowering the temperature to 4 °C. The mean values (in μmol P_i/mg protein per h) $\pm$S.E. are the results of four experiments.

Incubation medium	Enzyme activity
Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $MgCl_2$ (5 mM), ATP (4 mM)	10.9 ± 0.9
Above medium + ouabain (0.05 mM)	0.3 ± 0.05
Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $MgCl_2$ (5 mM), $CaCl_2$ (0.3 mM), ATP (4 mM)	22.2 ± 2.1
Above ouabain + ouabain (0.05 mM)	11.1 ± 0.4

Effect of Mg^{2+} concentration and Ca^{2+} concentration on total Ca^{2+}-activated ATPase activity

Using medium A containing 0.05 mM ouabain, the added Ca^{2+} concentration was maintained at 0.3 mM and the Mg^{2+} concentration was varied from 0 to 6.25 mM. Within this range a curve was obtained peaking at 3 mM (Fig. 1). When the Mg^{2+} concentration was maintained at this level and Ca^{2+} added at concentrations increasing to 1 mM, maximum activity was obtained at 0.3 mM (Fig. 1). These optimal concentrations were used in the following experiments.

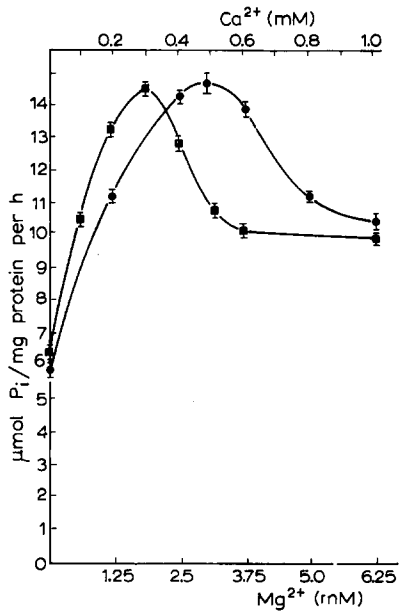

Fig. 1. Total Ca^{2+}-ATPase activity as a function of Mg^{2+} and Ca^{2+} concentration. Membranes (protein concentration 0.1–0.5 mg/ml) were incubated at 37 °C for 1 h, in 2 ml of medium, pH 7.4, containing Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $CaCl_2$ (0.3 mM), ouabain (0.05 mM), ATP (4 mM) and $MgCl_2$ varying in concentration from 0 to 6.25 mM (●—●) or in a similar medium where the $MgCl_2$ was maintained at a concentration of 3 mM and increasing levels of $CaCl_2$ added (■—■). Zero Ca^{2+} was achieved by omitting $CaCl_2$ from the medium. The mean values ±S.E. are the result of three experiments.

Effect of pH

When the activity of the plasma membrane (Ca^{2+}+Mg^{2+})-activated ATPase was measured over the pH range of 6.5 to 7.8, it was observed that P_i production was adversely affected only at the extremes of the range. pH values between 7.2 and 7.6 gave maximal enzyme activity and consequently a pH of 7.4 was employed in later experiments.

Effect of substrate concentration on the total Ca^{2+}-stimulated enzyme activity

Mouse liver plasma membranes were placed in medium A, modified to give optimal ionic and pH conditions and containing 0.05 mM ouabain. The ATP concentration was varied from 0.1 to 8 mM and a substrate-dependent curve was ob-

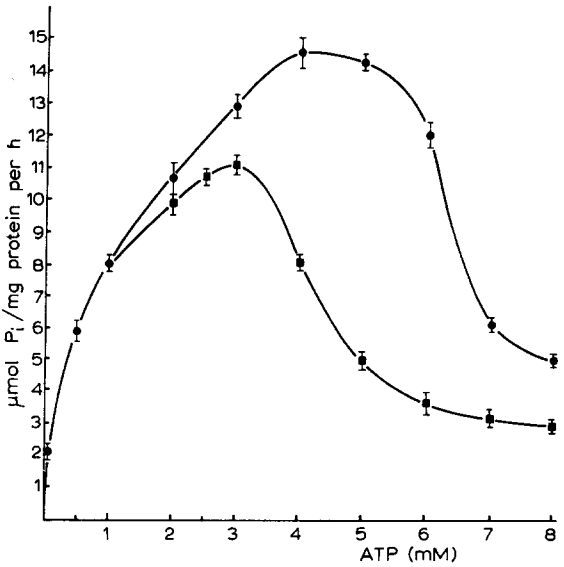

Fig. 2. Total Ca^{2+}-ATPase activity as a function of ATP concentration. Membranes (protein concentration 0.1–0.5 mg/ml) were incubated at 37 °C for 1 h, in 2 ml of medium, pH 7.4, containing either Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.3 mM), ouabain (0.05 mM) (Curve A, ●—●) or Tris · HCl (25 mM), choline chloride (99 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.3 mM) (Curve B, ■—■) and levels of ATP increasing from 0.1 mM to 8 mM. The mean values ±S.E. are the results of five experiments.

tained (Fig. 2A). On examination, these results showed that the maximum ATPase activity was obtained at an ATP concentration of 4 mM. However, the shape of this curve was not a simple hyperbola and thus the results could represent the activities of several $(Ca^{2+}+Mg^{2+})$-activated ATPase systems. Replacing the NaCl and KCl of the incubation medium with choline chloride, Curve B (Fig. 2) was obtained, having a maximal activity at 3 mM ATP. This curve represents $(Ca^{2+}+Mg^{2+})$-

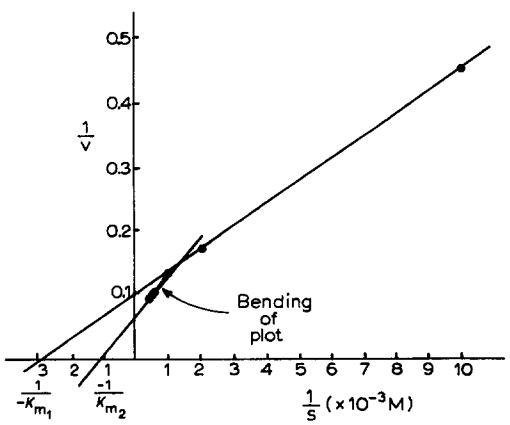

Fig. 3. Lineweaver-Burk plot of $(Ca^{2+}+Mg^{2+})$-ATPase activity represented by Curve B of Fig. 2.

ATPase activity. Plotting the results, represented by Curve B, as a Lineweaver-Burk plot (Fig. 3) the curve appeared to bend. A K_m of $0.35 \cdot 10^{-3}$ M was obtained when the ATP concentration was less than 2 mM (K_{m_1}), while for higher substrate concentrations the K_m appeared to be $0.88 \cdot 10^{-3}$ M (K_{m_2}).

Effect of alkali metal ions on the Ca^{2+}-stimulated ATPase

When the mouse liver plasma membranes were incubated in media varying in alkali metal ions and containing 4 mM ATP, different ($Ca^{2+}+Mg^{2+}$)-stimulated ATPase activities were observed. In medium B, which was devoid of Na^+ and K^+, an ATPase activity of 8.2 ± 0.34 μmol P_i/mg protein per h was obtained. This ATPase activity was increased to 9.0 ± 0.4 μmol P_i/mg protein per h (Table II) in a medium containing Na^+ and, in the presence of both K^+ and Na^+, the activity was further increased to 14.6 ± 0.5 μmol P_i/mg protein per h.

TABLE II

ATPase ACTIVITY OF MOUSE LIVER PLASMA MEMBRANES

The membranes (protein concentration 0.1–0.5 mg/ml) were incubated in 2 ml of the appropriate reaction mixture for 1 h, at 37 °C. The reaction was initiated by the addition of ATP and stopped by rapidly lowering the temperature to 4 °C. The mean values (in μmol P_i/mg protein per h) ±S.E. are the results of five experiments.

Incubation medium	Enzyme activity
Tris · HCl (25 mM), NaCl (66 mM), KCl (33 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.3 mM), ATP (4 mM), ouabain (0.05 mM)	14.6 ± 0.5
Tris · HCl (75 mM), NaCl (66 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.3 mM), ATP (4 mM)	9.0 ± 0.4
Tris · HCl (25 mM), choline chloride (99 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.3 mM), ATP (4 mM)	8.2 ± 0.3

DISCUSSION

Phase contrast microscopy showed that the membrane preparations were free of contaminating nuclei, while the lack of enzyme activities characteristic of internal membranous structures indicated that the method of isolation produced a relatively pure plasma membrane preparation. That these membranes possessed an enzymic activity characteristic of plasma membranes was shown by the results obtained for the activity of the ($Na^+ + K^+$)-ATPase. The value of 10.9 ± 0.9 μmol P_i/mg protein per h for this enzyme compared favourably with the range of 9.7–13.5 μmoles P_i obtained by Emmelot et al. [29].

The bend in the Lineweaver-Burk plot of ATPase activity in a Na^+ and K^+ free incubation medium might possibly represent two enzymes or a single enzyme where the configuration of the active site is altered above 1 mM ATP.

Incubation of plasma membrane in a Na^+-rich but K^+-free medium containing 4 mM ATP gave an ATPase activity of 9 ± 0.4 μmol P_i/mg protein per h, which

exceeded the activity obtained in alkali metal-free medium by 10 % (t-test: $P < 0.05$). This enhanced response probably represented the sum of activities of a $(Ca^{2+}+Mg^{2+}+Na^{+})$-stimulated ATPase and a $(Ca^{2+}+Mg^{2+})$-ATPase. However, the total Ca^{2+}-stimulated activity observed in the medium containing both Na^{+} and K^{+}, in the presence of 4 mM ATP, exceeded the activity observed in either of the above media, indicating that a $(Ca^{2+}+Mg^{2+}+K^{+})$-ATPase may also be present. Regarding the possibility that the $(Ca^{2+}+Mg^{2+})$-activated ATPase may represent a complex system, Wins and Schoffeniels [11] reported that an extra divalent cation-stimulated ATPase activity was observed in red cell membranes incubated in medium containing K^{+} and this was later supported by Schatzmann and coworkers [12, 17], who claimed the existence of $(Ca^{2+}+Mg^{2+}+K^{+})$- and $(Ca^{2+}+Mg^{2+}+Na^{+})$-activated ATPases in the plasma membrane of red blood cells.

Liver cells have been shown to possess a low intracellular Ca^{2+} concentration [32] and it has been suggested [18] that this is maintained by active extrusion of Ca^{2+} by a process independent of Na^{+} and K^{+} transport. The $(Ca^{2+}+Mg^{2+})$-ATPase in the red cell membrane is thought to control the Ca^{2+} efflux [33–36] and in the light of the present experiments it is possible that a similar situation exists in liver cells.

However several $(Ca^{2+}+Mg^{2+})$-activated enzymes have been demonstrated in the mouse liver plasma membranes and as myosin has been shown to exist in these membranes one of these enzymes may be associated with the muscle-like protein. This is supported by the finding that actomyosin extracted from mouse liver plasma membranes exhibited a $(Ca^{2+}+Mg^{2+})$-ATPase activity [19].

The present results have therefore revealed a complex enzyme system, possibly consisting of two $(Ca^{2+}+Mg^{2+})$-ATPases, a $(Ca^{2+}+Mg^{2+}+Na^{+})$- and a $(Ca^{2+}+Mg^{2+}+K^{+})$-activated ATPase, the functions of which have yet to be ascertained in detail.

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