A HIGH AFFINITY Ca^{2+}-ATPase IN C57 BLACK MOUSE LIVER PLASMA MEMBRANES

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1. Introduction

Plasma membranes of a cell contain 2 kinds of Ca^{2+}-extruding mechanisms to maintain $[Ca^{2+}]$ of cytoplasm at submicromolar levels, ATP-dependent and extracellular Na^+-dependent mechanisms. In plasma membranes of several tissues, the ATP-dependent Ca^{2+}-pumps associate with $(Ca^{2+} + Mg^{2+})$-ATPase activity [1–3]. In the case of erythrocyte plasma membrane, a reconstitution study has clearly demonstrated the identity of the $(Ca^{2+} + Mg^{2+})$-ATPase and the Ca^{2+}-extruding pump [4].

A variant type of $(Ca^{2+} + Mg^{2+})$-ATPase has been shown in adipocyte and corpus luteum plasma membranes [5,6]. This ATPase has a high affinity for Ca^{2+} with $K_{0.5}$ of 0.14–0.3 μM and some other characteristics consistent with those of erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase. However, this ATPase shows different sensitivity to Mg^{2+}, calmodulin and calmodulin-antagonists from that of erythrocyte enzyme.

In rat liver plasma membrane, no $(Ca^{2+} + Mg^{2+})$-ATPase had been detected although a Ca^{2+}-extruding activity in rat liver cells was shown to be ATP-dependent and extracellular Na^+-independent [7,8]. A $(Ca^{2+} + Mg^{2+})$-ATPase distinct from $(Ca^{2+} + Mg^{2+})$-ATPases of erythrocyte, adipocyte and corpus luteum has been reported in rat liver plasma membranes [9]. This enzyme has very high affinities for Ca^{2+} ($K_{0.5}$ = 13 nM) and Mg^{2+} ($K_{0.5}$ = <12 μM), and is activated by a protein distinct from calmodulin. The report suggests an occurrence of analogous Ca^{2+}-sensitive ATPase in mouse liver plasma membranes, which is different from the $(Ca^{2+} + Mg^{2+})$-ATPase possessing a low affinity for Ca^{2+} ($K_{0.5}$ = 0.3 mM) in [10].

Here, the occurrence and some characterization of a high affinity Ca^{2+}-ATPase in liver plasma membranes from C57 black mouse [C57BL/6(+/+)] and its mutant beige mouse [C57BL/6(bgj/bgj)] is described. The high affinity Ca^{2+}-ATPase had a high affinity for Ca^{2+} ($K_{0.5}$ = 50 nM) and app. $M_r \sim 200\,000$ estimated by gel filtration method. The ATPase did not require Mg^{2+} even in trace amounts (10^{-8} M), and was insensitive to calmodulin. Na^+, K^+, ouabain, NaN_3, KCN and dicyclohexylcarbodiimide showed no effect on the ATPase activity. The high affinity Ca^{2+}-ATPase from beige mouse showed the same properties as C57 black mouse.

2. Materials and methods

Adult mice, C57BL/6(+/+) and C57BL/6(bgj/bgj), were employed. Mouse liver plasma membranes were prepared as in [11]. The plasma membranes were purified ~14-fold starting from the first homogenate when 5′-nucleotidase was used as a plasma membrane marker enzyme to estimate the purity. Na-ATP was a product of Sigma. Other chemicals were obtained from commercial sources.

ATPase activity was assayed in the reaction mixture (0.5 ml) which contained 10 μmol Tris–Pipes buffer (pH 7.5), 0.5 μmol ATP, plasma membranes (75–300 μg protein) and desired concentration of divalent cation controlled with EGTA or CDTA. After incubation at 37°C for 15–30 min, the reaction was stopped by adding 1 N H_2SO_4 and 1% laurylsulfate to clarify the medium since the turbidity inter-

Abbreviations: Ca^{2+} and Mg^{2+}, free ionized calcium and magnesium; $K_{0.5}$, apparent half-saturation constant; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CDTA, *trans*-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid

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Table 1
True (K) and apparent (K') stability constants for the ligands EGTA, CDTA and ATP (M^{-1})

Cation	Ligand	log K			log K' (pH 7.5)		
		EGTA	CDTA	ATP	EGTA	CDTA	ATP
H^+	L^{4-}	9.46	11.70	6.50			
H^+	HL^{3-}	8.85	6.12	4.05			
Ca^{2+}	L^{4-}	11.00	12.50	3.60	7.69	8.28	3.56
Mg^{2+}	L^{4-}	5.21	10.32	4.00	1.90	6.10	3.96

feres with colorimetric assay of released P_i. The phosphate was determined colorimetrically as in [12]. ATPase activity was determined by subtracting values obtained with chelator alone from those with metal plus chelator, and represented as nmol ATP hydrolysis . min^{-1} . mg $protein^{-1}$ unless otherwise noted.

$[Ca^{2+}]$ and $[Mg^{2+}]$ were adjusted by means of metal–metal chelator buffer system using EGTA and CDTA as chelators. The common logarithms of true and apparent stability constants with EGTA, CDTA and ATP for Ca^{2+}, Mg^{2+} and H^+ are given in table 1. Composition of each metal–metal chelator buffer system was determined by a calculation using these stability constants as in [5]. Details of each buffer system are shown in table 2. The reaction mixture (containing 75–300 μg membrane protein) contained <1 μM total calcium as measured by atomic absorption spectroscopy. Since the buffering capacity of metal–metal chelator buffer systems increases in enlarging the amount ratio of chelator/metal, the very low concentrations of Ca^{2+} adjusted with EGTA or CDTA were little affected by such a contaminated calcium as follows: 10^{-7}, 10^{-8} and 10^{-9} M adjusted with EGTA and 10^{-7}, 10^{-8} and 10^{-9} M with CDTA, where 1.15–21.40 excess amounts of chelators over those of the metal were present, were changed to 1.061×10^{-7}, 1.015×10^{-8}, 1.000×10^{-9}, 1.236×10^{-7}, 1.030×10^{-8} and 1.015×10^{-9} M Ca^{2+} by the presence of contaminated calcium (1 μM), respectively.

M_I-Value of the high affinity Ca^{2+}-ATPase was

Table 2
Composition of calcium-EGTA, calcium-CDTA and magnesium-CDTA buffers used in the experiments of fig.1,2

Calcium-EGTA			Calcium-CDTA			Magnesium-CDTA		
Ca^{2+} (M)	$CaCl_2$ (μM)	EGTA (μM)	Ca^{2+} (M)	$CaCl_2$ (μM)	CDTA (μM)	Mg^{2+} (M)	$MgCl_2$ (μM)	CDTA (μM)
3.5×10^{-9}	100	692	2.5×10^{-9}	100	309	2.2×10^{-8}	1.7	62
6.9×10^{-9}	100	396	5.0×10^{-9}	100	205	4.3×10^{-8}	3.4	63
1.0×10^{-8}	100	297	2.6×10^{-8}	100	120	1.1×10^{-7}	8.5	68
1.4×10^{-8}	100	248	5.8×10^{-8}	100	109	2.2×10^{-7}	16.9	77
3.5×10^{-8}	100	159	1.8×10^{-7}	100	103	5.1×10^{-7}	33.8	94
6.8×10^{-8}	100	130	6.8×10^{-7}	100	100	1.1×10^{-6}	84.5	144
1.3×10^{-7}	100	115	3.2×10^{-6}	100	97	2.2×10^{-6}	169	228
7.4×10^{-7}	100	102				4.3×10^{-6}	338	396
1.4×10^{-6}	100	100				8.3×10^{-6}	676	732
5.4×10^{-6}	100	95						
1.0×10^{-5}	100	90						
8.9×10^{-5}	100	57						
1.0×10^{-4}	200	100						
5.0×10^{-4}	1000	500						
1.0×10^{-3}	2000	1000						
5.0×10^{-3}	6000	1000						

Apparent stability constants for Ca^{2+} and Mg^{2+} with each ligand are listed in table 1

determined as in [13] using a Sephadex G-200 column after the solubilization of the enzyme with Triton X-100. Marker proteins employed were bovine catalase, human γ-globulin and bovine serum albumin with M_r-values of 240 000, 160 000 and 68 000, respectively.

Total calcium and magnesium concentrations were determined by atomic absorption spectroscopy (Perkin-Elmer, model 403).

Protein was assayed as in [14] with bovine serum albumin as a reference protein.

3. Results

3.1. *Demonstration of a high affinity Ca^{2+}-ATPase*

When Ca^{2+}-stimulated ATPase activity in C57 black mouse liver plasma membranes was examined in the presence of 3 mM $MgCl_2$, unlike in [10], we could not detect any Ca^{2+}-stimulated ATPase activity. Then, the effect of $[Ca^{2+}]$ on ATPase activity in the membrane was examined in the absence of added Mg^{2+}. The Ca^{2+}-stimulated ATPase activity was evident in fig.1 and the Ca^{2+} concentration-dependent ATPase activity could be divided into 2 components. A high affinity component was evident at <0.1 μM Ca^{2+}. Over 0.1–10 μM Ca^{2+}, there was little change in ATPase activity. This high affinity component showed a $K_{0.5}$ of 50 nM for Ca^{2+} (high affinity Ca^{2+}-ATPase). At >10 μM Ca^{2+}, ATPase activity again rose sharply. This latter low affinity component showed a $K_{0.5}$ of 100 μM (low affinity Ca^{2+}-ATPase). The low affinity Ca^{2+}-ATPase was considered a non-specific divalent cation-stimulated ATPase similar to that reported for rat liver plasma membrane [8] since Mg^{2+} (at >10 μM) could substitute effectively for Ca^{2+} in the stimulation of this enzyme.

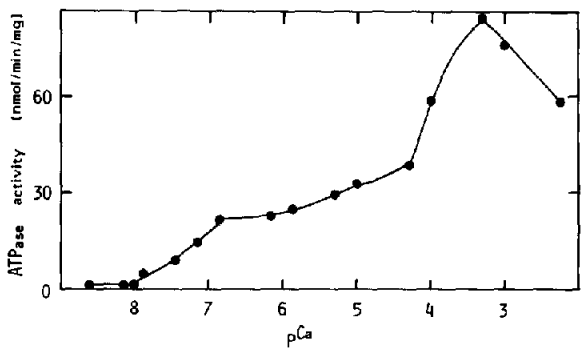

Fig.1. Effect of $[Ca^{2+}]$ on ATPase activity. ATPase activity was determined under standard conditions in the presence of various $[Ca^{2+}]$ indicated. The $[Ca^{2+}]$ were controlled with EGTA as specified in section 2. $[Ca^{2+}]$ at >10 μM represent the sum of Ca^{2+} and calcium-ATP moieties since the effect of ATP on $[Ca^{2+}]$ cannot be neglected over a range above this $[Ca^{2+}]$.

3.2. *Is the high affinity Ca^{2+}-ATPase Mg^{2+}-dependent?*

Although Mg^{2+} was not exogenously added to the reaction medium, it is likely that Mg^{2+} contaminating the experimental solutions and the plasma membranes was sufficient to satisfy a Mg^{2+} requirement of the high affinity Ca^{2+}-ATPase. The assay medium including membranes contained 2–9 μM total magnesium as determined by atomic absorption spectroscopy. To examine Mg^{2+}-dependence of the high affinity Ca^{2+}-ATPase, CDTA was used. This chelator possesses extremely high affinities for both Ca^{2+} and Mg^{2+}, unlike EGTA which has a selectively high affinity for Ca^{2+} only (section 2). Therefore, in the presence of EGTA, Mg^{2+} remains uncomplexed by the chelator, whereas in the presence of CDTA, contaminated Mg^{2+} was almost complexed by CDTA leaving nearly 10^{-8} M Mg^{2+} in the medium. Fig.2 shows the dependence of the enzyme on $[Ca^{2+}]$ and $[Mg^{2+}]$ in different systems; calcium-EGTA, calcium-CDTA and magnesium-CDTA systems. $[Ca^{2+}]$ controlled with EGTA and CDTA showed the same effect on the high affinity Ca^{2+}-ATPase, suggesting a Mg^{2+}-independence of the enzyme. The basal activity obtained with EGTA

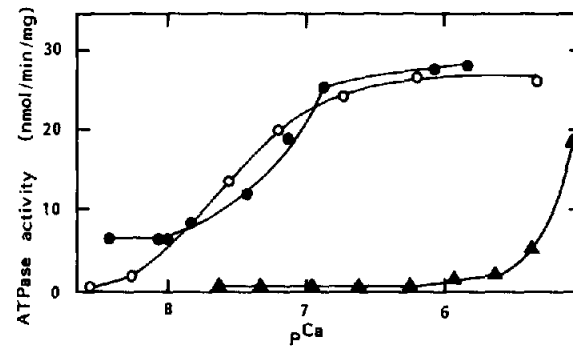

Fig.2. Effect of calcium-EGTA, calcium-CDTA and magnesium-CDTA systems on ATPase activity. ATPase activities in the presence of various concentrations of Ca^{2+} controlled with EGTA (•), Ca^{2+} with CDTA (○) or Mg^{2+} with CDTA (▲) were assayed under standard conditions. ATPase activities were represented by subtracting values obtained with no plasma membrane from those with the membranes plus metal and chelator.

was 6.2 nmol . min^{-1} . mg^{-1} higher than that with CDTA. The value corresponded to an ATPase activity obtained with 4 μM Mg^{2+} controlled with CDTA. Furthermore, externally added Mg^{2+} diminished the Ca^{2+}-stimulated ATPase activity as mentioned in section 3.1. It is, therefore, likely that the high basal activity of the high affinity Ca^{2+}-ATPase obtained with EGTA is due to the trace activity of the non-specific divalent cation-stimulated ATPase evoked by a contaminated Mg^{2+} in the reaction mixture. Low concentrations of Mg^{2+} could not substitute for Ca^{2+} to stimulate the high affinity Ca^{2+}-ATPase unlikely to the case of the low affinity Ca^{2+}-ATPase (fig.2).

3.3. *Effects of various agents on the high affinity Ca^{2+}-ATPase*

Many Ca^{2+}-pumping ATPases of plasma membrane require calmodulin as well as Mg^{2+} for their full activity [1–3]. Effect of calmodulin on the high affinity Ca^{2+}-ATPase was then examined. The enzyme was not stimulated by externally added calmodulin over 0.1–200 μg/ml. The membrane may have contained a sufficient amount of calmodulin for satisfying its requirement for the enzyme. Trifluoperazine, a calmodulin-antagonist, inhibits the Ca^{2+}-pumping ATPase in erythrocyte membrane [16]. This effect is thought to be because of binding of the drug to calmodulin. Then, effect of trifluoperazine on the high affinity Ca^{2+}-ATPase was studied. Trifluoperazine up to 100 μM, a concentration inhibiting 100% of the (Ca^{2+} + Mg^{2+})-ATPase of erythrocyte [16], caused no significant inhibition of the enzyme activity (fig.3). Higher concentrations of the drug inhibited the

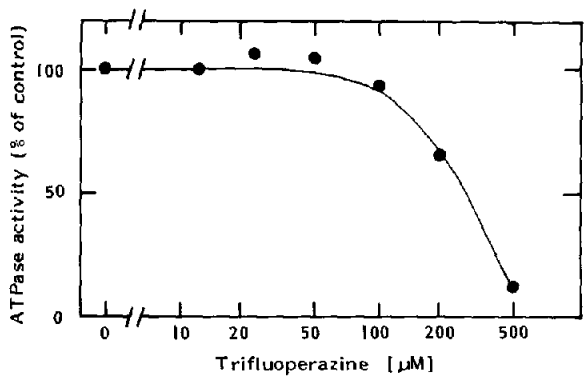

Fig.3. Effect of trifluoperazine on the high affinity Ca^{2+}-ATPase. ATPase activity was determined in the presence of various concentrations of trifluoperazine as indicated and 0.13 μM Ca^{2+} controlled with EGTA.

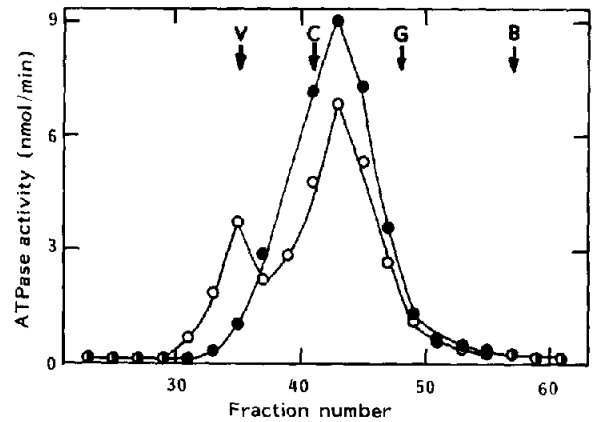

Fig.4. Resolution of the high affinity Ca^{2+}-ATPase by gel filtration upon a Sephadex G-200 column. The plasma membranes (final conc. 15 mg protein/ml) were partially solubilized in a solution containing 20 mM Tris–HCl, 1 mM NaN_3 and 0.4% Triton X-100, at pH 7.5, on ice with continuous stirring for 30 min. The solubilizate was centrifuged for 60 min at 100 000 × *g*. The supernatant was concentrated by an Amicon ultrafiltration cell equipped with a PM-10 filter, and then subjected to a Sephadex G-200 column (2.2 × 80 cm) equilibrated with 20 mM Tris–HCl, 1 mM NaN_3 and 4% Triton X-100, at pH 7.5. The elution was performed with the same buffer. Fractions of 2.9 ml each were collected. A 0.4 ml aliquot of each fraction was assayed for the high affinity Ca^{2+}-ATPase (•) and the non-specific divalent cation-stimulated ATPase (○) in the presence of 0.13 μM Ca^{2+} and 1 mM $MgCl_2$ plus 1 mM EGTA, respectively. The elution positions indicated with V, C, G and B are the void volume, bovine catalase, human γ-globulin and bovine serum albumin, respectively.

enzyme activity. However, this effect was considered a non-specific interaction of the drug with this ATPase [15]. Other agents such as 30 mM Na^+ and K^+, 1 mM ouabain, 20 mM NaN_3, 10 mM KCN and 0.1 mM dicyclohexylcarbodiimide had no effect on the high affinity Ca^{2+}-ATPase.

3.4. *Solubilization of the high affinity Ca^{2+}-ATPase*

The high affinity Ca^{2+}-ATPase lost 75% of its activity by solubilization with 0.4% Triton X-100 as in [17]. The solubilizate was subjected to gel filtration upon a Sephadex G-200 column to estimate M_r-value of the high affinity Ca^{2+}-ATPase. The high affinity Ca^{2+}-ATPase was eluted at the position corresponding to M_r 200 000 as a single symmetrical peak (fig.4). The non-specific divalent cation-stimulated ATPase showed 2 peaks, one at the void volume and the other at the same position with the high affinity Ca^{2+}-ATPase.

3.5. *The high affinity Ca^{2+}-ATPase from beige mouse*

Beige mouse, a mutant strain of C57 black mouse, shows a membrane abnormality as well as giant lysosomes [18]. Properties of the high affinity ATPase from beige mouse were compared to those of the enzyme from control C57 black mouse. No difference could be detected in our studies.

4. Discussion

The results obtained indicate the occurrence of a high affinity Ca^{2+}-ATPase in liver plasma membranes from C57 black and beige mice, showing no difference in properties of this enzyme between 2 strains. The localization of the high affinity Ca^{2+}-ATPase in plasma membrane is confirmed by the evidence; copurification with a plasma membrane marker enzyme 5′-nucleotidase and unsusceptibility to mitochondrial ATPase blockers.

Since ATP-dependent Ca^{2+}-extruding mechanism has not yet been explored in rat liver plasma membranes, it is difficult at this time to conclude that the high affinity Ca^{2+}-ATPase is a Ca^{2+}-pumping ATPase. Nevertheless, by analogy to erythrocyte Ca^{2+}-pumping ATPase, properties of the high affinity Ca^{2+}-ATPase such as high affinity for Ca^{2+}, non-susceptibility to mitochondrial ATPase blockers and M_r-value (M_r 200 000 corresponds to the dimer of $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocyte) suggest the possibility of the high affinity Ca^{2+}-ATPase as a Ca^{2+}-pumping ATPase.

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References

[1] Roufogalis, B. D. (1979) Can. J. Physiol. Pharmacol. 57, 1331–1349.
[2] Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263–3270.
[3] Lichtman, A. H., Segel, G. B. and Lichtman, M. A. (1981) J. Biol. Chem. 256, 6148–6154.
[4] Niggli, V., Adunyah, E. S., Penniston, J. T. and Carafoli, E. (1981) J. Biol. Chem. 256, 395–401.
[5] Pershadsingh, H. A. and McDonald, J. M. (1980) J. Biol. Chem. 255, 4087–4093.
[6] Verma, A. K. and Penniston, J. T. (1981) J. Biol. Chem. 256, 1269–1275.
[7] Cittadini, A. and Van Rossum, G. D. V. (1978) J. Physiol. 281, 29–43.
[8] Chambaut, A., Leray-Pecker, F., Feldmann, G. and Hanoune, J. (1974) J. Gen. Physiol. 64, 104–126.
[9] Lotersztajn, S., Hanoune, J. and Pecker, F. (1981) J. Biol. Chem. 256, 11209–11215.
[10] Garnett, H. M. and Kemp, R. B. (1975) Biochim. Biophys. Acta 382, 526–533.
[11] Coleman, R., Michell, R. H., Finean, J. B. and Hawthorne, J. N. (1967) Biochim. Biophys. Acta 135, 573–579.
[12] Ames, B. N. (1966) Methods Enzymol. 8, 115–118.
[13] Andrews, P. (1965) Biochem. J. 96, 595–606.
[14] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[15] Schuurmans Stekhoven, F. and Bonting, S. L. (1981) Physiol. Rev. 61, 1–76.
[16] Hinds, T. R., Raess, B. U. and Vincenzi, F. F. (1981) J. Membr. Biol. 58, 57–65.
[17] Gennaro, R., Mottola, C., Schneider, C. and Romeo, D. (1979) Biochim. Biophys. Acta 567, 238–246.
[18] Haak, R. A., Ingraham, L. M., Baehner, R. L. and Boxer, L. A. (1979) J. Clin. Invest. 64, 138–144.