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DEMONSTRATION OF A HIGH AFFINITY Ca^{2+}-ATPase IN RAT LIVER PLASMA MEMBRANES*

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SUMMARY: Rat liver plasma membranes contained a high affinity Ca^{2+}-ATPase which had an apparent half saturation constant of 0.2 μM for calcium. The Ca^{2+}-ATPase was not stimulated by adding magnesium and/or calmodulin. Conversely, the addition of these substances diminished the calcium-stimulation of the ATPase. Orthovanadate (7 nM-2 mM), mitochondrial ATPase blockers (NaN_3,KCN,dicyclohexylcarbodiimide), Na^+,K^+ and ouabain had no effect on the ATPase activity. The ATPase was separated from nonspecific divalent cation stimulatable ATPase (Mg^{2+}-ATPase) by solubilization with Triton X-100 followed by a Sephadex G-200 column chromatography and showed an apparent molecular weight of 200,000.

The plasma membrane of a cell possesses two mechanisms to maintain cytoplasmic calcium concentration at submicromolar range. One of them is an ATP-requiring Ca^{2+}-pump which associates with ATPase activity. This Ca^{2+}-pumping ATPase has been extensively studied in the case of erythrocyte plasma membranes as the calcium-stimulated and magnesium-dependent ATPase ((Ca^{2+}+ Mg^{2+})-ATPase) (1,2). Recently, a variant type of (Ca^{2+}+ Mg^{2+})-ATPase has been reported in plasma membranes of adipocyte and corpus luteum (3,4). The (Ca^{2+}+ Mg^{2+})-ATPase of this type possesses a high affinity for Ca^{2+} with a half saturation constant of 0.2 to 0.4 μM, and does not respond to calmodulin or calmodulin-antagonists. The calcium-stimulation of this ATPase was abolished by the addition of magnesium. Nevertheless, this type of (Ca^{2+}+ Mg^{2+})-ATPase has been proposed as a Ca^{2+}-pumping ATPase of these plasma membranes.

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In liver, Cittadini and Van Rossum (5) have reported that Ca^{2+}-extrusion from liver slices requires oxidative phosphorylation and is dependent on magnesium. However, rat liver plasma membranes do not contain a classical Ca^{2+}-pumping ATPase such as (Ca^{2+}+ Mg^{2+})-ATPase of erythrocyte plasma membranes. In this communication, a high affinity calcium-dependent ATPase (Ca^{2+}-ATPase) distinct from the nonspecific divalent cation stimulatable ATPase (Mg^{2+}-ATPase) in rat liver plasma membranes will be described.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats, weighing 250 to 300 g, were employed. Plasma membranes of rat livers were prepared by the method of Coleman et al.(6). Calmodulin was purified to homogeniety from bovine brain by the method of Yazawa et al.(7). N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was kindly donated by Dr. H. Hidaka, Mie University School of Medicine. Na-ATP was a product of Sigma. Other chemicals were obtained from commercial sources.

ATPase was assayed in the reaction mixture (0.5 ml) which contained 10 μmol of Tris-piperazine-N,N'-bis(2-ethanesulfonic acid) buffer (Tris-PIPES buffer) (pH 7.5), 0.5 μmol of Na-ATP, different concentration of Ca^{2+} or Mg^{2+} and ATPase preparation. After the incubation was carried out at 37°C for 15 to 30 min, the reaction was stopped by adding 1 N H_2SO_4 and 1% SDS. Inorganic phosphate was determined colorimetrically (8). Protein was determined by the method of Bradford (9).

Free calcium and free magnesium concentrations were controlled by using ligands such as ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and trans-cyclohexane-1,2-diamine-N,N,N',N'-tetra acetic acid (CDTA). Logarithms of stability constants (log K) with EGTA for Ca^{2+} and with CDTA for Ca^{2+} and Mg^{2+} are 11.00, 12.50 and 10.32, respectively (10). The apparent stability constant (K') at any given pH was calculated as follows.

$$\log K' = \log K - \log \alpha H$$

$$\alpha H = 1 + \frac{[H^+]}{K_{a4}} + \frac{[H^+]^2}{K_{a4} \cdot K_{a3}} + \frac{[H^+]^3}{K_{a4} \cdot K_{a3} \cdot K_{a2}} + \frac{[H^+]^4}{K_{a4} \cdot K_{a3} \cdot K_{a2} \cdot K_{a1}}$$

K_{a1}, K_{a2}, K_{a3} and K_{a4} are dissociation constants with the forms of the ligand (L) with L^-, L^{2-}, L^{3-} and L^{4-}, respectively. We used following values: log K' with EGTA for Ca^{2+} and with CDTA for Ca^{2+} and Mg^{2+} of 7.66, 8.58 and 6.10 in pH 7.5 (assay condition), respectively. Although ATP can also be a ligand, its apparent stability constants for Ca^{2+} and Mg^{2+} were small and negligible.

RESULTS AND DISCUSSION

In an initial experiment, 3 mM $MgCl_2$ was added to the reaction mixture and no Ca^{2+}-ATPase activity was observed. A Ca^{2+}-ATPase activity was detected when no $MgCl_2$ was added. As shown in Fig. 1, Ca^{2+}-ATPase activity was composed of two kinds of ATPase when the concentration of

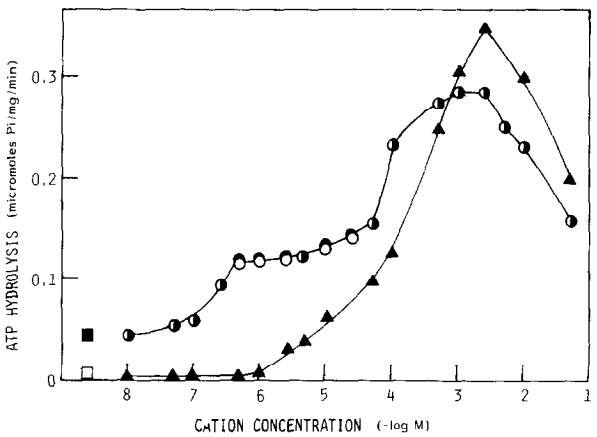

Fig. 1 Effects of free calcium and magnesium concentrations on rat liver plasma membrane ATPase activity. ATPase activity was assayed under standard conditions described under "EXPERIMENTAL PROCEDURES" in the presence of either Ca/EGTA (●), Ca/CDTA (o) or Mg/CDTA (▲) buffer system. ATPase activities were expressed by subtracting values obtained with ATP blanks whose reaction mixture contained no plasma membrane from that obtained with cation and ligand. Free calcium and magnesium concentrations were varied as specified under "EXPERIMENTAL PROCEDURES". (■) and (□) are respective basal activities in the presence of 2 mM EGTA and 2 mM CDTA. Each point is mean of triplicate determinations. Each standard error was less than 0.002.

free Ca^{2+} was controlled by EGTA. One had a high affinity for free Ca^{2+} with an apparent half saturation constant (K_a) of 0.2 μM, and its activity was little changed, if any, by varying the concentration of free Ca^{2+} from 0.5 to 5 μM. The other had a K_a of 50 μM for free Ca^{2+} and was maximally activated by 2 mM calcium. When the effect of free Mg^{2+} on the activity of membrane ATPase was examined by means of magnesium-CDTA system, only a low affnity ATPase was evident [K_a= 180 μM for free Mg^{2+}], which was maximally activated by 2 mM Mg^{2+}. Since Ca^{2+} and Mg^{2+} were equally effective in stimulating the low affinity ATPase, this ATPase was considered a nonspecific divalent cation stimulatable ATPase (Mg^{2+}-ATPase). Nearly identical results were obtained with Ca^{2+}-ATPases when CDTA was employed instead of EGTA to control free Ca^{2+} concentration. However, the basal activity obtained with CDTA was lower than that with EGTA (Fig. 1). This difference is probably due to the very low concentration of Mg^{2+} in the plasma membrane preparation since EGTA, in contrast to CDTA, has a low affinity for Mg^{2+} among divalent cations (10). Indeed, such reaction mixtures (containing 98 to 143 μg of membrane protein) con-

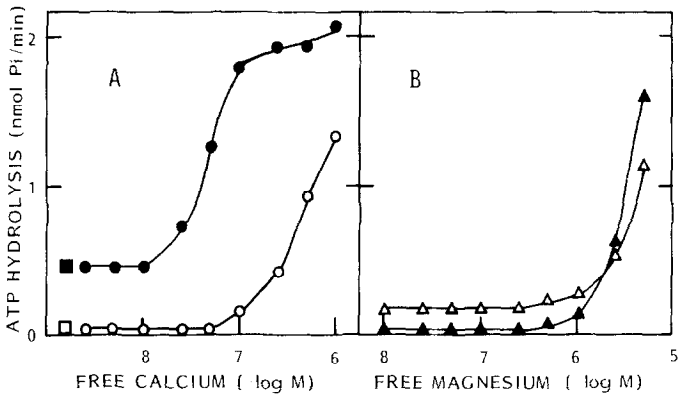

Fig. 2 Effects of calcium and magnesium concentrations controlled by EGTA or CDTA employing the apparent stability constants with each ligand proposed by Pershadsingh and McDonald (3) on rat liver plasma membrane ATPase. Free calcium and magnesium concentrations were varied by means of EGTA and CDTA buffer system employing respective logarithms of apparent stability constant with EGTA for calcium and with CDTA for calcium and magnesium of 7.83, 6.98 and 6.45. ATPase activity in the presence of either Ca/EGTA (●), Ca/CDTA (o), Ca/Mg/CDTA (△) or Mg/CDTA (▲) buffer system was assayed under standard conditions and similarly expressed as Fig. 1. (■) and (□) are respective basal activities of ATPase in the presence of 2 mM EGTA and 2 mM CDTA. In Ca/Mg/CDTA buffer system, free calcium and ligand concentrations were fixed at 0.5 μM and 60 μM, respectively. Plasma membranes (45 μg of protein) were employed. Each point is mean of triplicate determinations. Each standard error was less than 0.02.

tained 3 to 3.6 μM magnesium as measured by atomic absorption spectroscopy. The difference in basal ATPase activities between CDTA and EGTA buffer systems corresponded to the activity given by 2.5 μM free Mg^{2+} in the magnesium-CDTA system (Fig. 1).

For further examination of Mg^{2+}-dependence of the high affinity Ca^{2+}-ATPase, the apparent stability constants for Ca^{2+} and Mg^{2+} with EGTA and CDTA proposed by Pershadsingh and McDonald (3) were employed to control free Ca^{2+} concentrations. Employment of this calcium-EGTA buffer system shifted the dose response curve to the left, and demonstrated the high affinity of Ca^{2+}-ATPase for free calcium (K_a = 0.05 μM). Apparently as in the case of adipocyte plasma membrane (Ca^{2+} + Mg^{2+})-ATPase (3), activity of the high affinity Ca^{2+}-ATPase was abolished by the presence of CDTA when the free Ca^{2+} concentration was less than 0.1 μM (Fig. 2A). To determine whether Mg^{2+} was the endogenous divalent cation required by the high affinity Ca^{2+}-ATPase, we compared the ATPase activity in the calcium-magnesium-CDTA buffer system with that in the magnesium-CDTA system (3,4).

Table I
Effects of calmodulin and W-7 on
the high and low affinity Ca^{2+}-ATPases

Additions	ATPase activity (μmol Pi/mg/min)	
	High affinity	Low affinity
None	0.44 + 0.02	1.76 + 0.01
1.2 μM calmodulin	0.44 + 0.01	1.76 + 0.01
12 μM calmodulin	0.35 + 0.01	1.79 + 0.01
120 μM calmodulin	0.17 + 0.01	1.84 + 0.01
100 μM W-7	0.30 + 0.01	1.16 + 0.01
100 μM W-7 plus 1.2 μM calmodulin	0.29 + 0.01	1.14 + 0.01
100 μM W-7 plus 12 μM calmodulin	0.23 + 0.01	1.36 + 0.01
100 μM W-7 plus 120 μM calmodulin	0.09 + 0.01	1.65 + 0.02

ATPase activity was assayed under standard conditions in the presence of Ca/EGTA buffer of 0.5 μM free calcium for the high affinity Ca^{2+}-ATPase or 2 mM calcium for the low affinity one. ATPase activities were determined by subtracting values of controls whose reaction mixture contained 2 mM EGTA and no divalent cation from that of each Ca/EGTA buffer system. Results are mean + standard error of triplicate determinations.

However, as shown in Fig 2B, we failed to show any Mg^{2+}-dependence of this ATPase. Thus, the high affinity Ca^{2+}-ATPase of rat liver plasma membrane may not be magnesium-dependent.

The high affinity Ca^{2+}-ATPase was clearly distinguished from the low affinity Ca^{2+}-ATPase by its different optimum pH (7.2 -7.5 vs 8.0). Furthermore, a high concentration of calmodulin inhibited the high affinity Ca^{2+}-ATPase, but slightly stimulated the low affinity one (Table I). Calmodulin-antagonist W-7 (11) inhibited both ATPases in a dose dependent manner. While calmodulin diminished inhibition of the low affinity Ca^{2+}-ATPase by W-7, it increased inhibition of high affinity Ca^{2+}-ATPase by this agent (Table I).

Isolation of the high affinity Ca^{2+}-ATPase would provide direct evidence for its separate nature. As shown in Fig. 3, the high affinity Ca^{2+}-ATPase was separated from Mg^{2+}-ATPase (low affinity Ca^{2+}-ATPase) by solubilization with Triton X-100 followed by chromatography over Sephadex

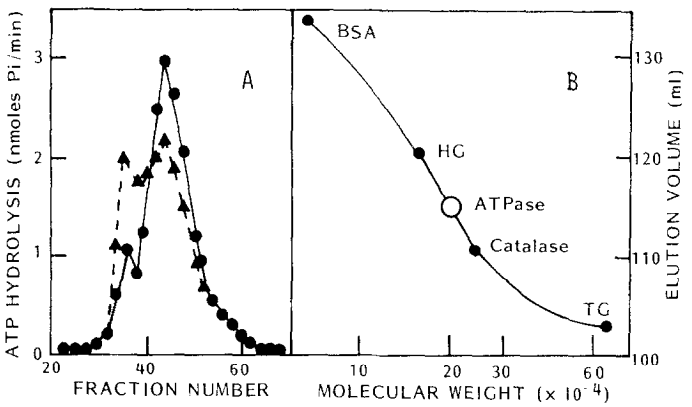

Fig. 3 Resolution of the high affinity Ca^{2+}-ATPase on a Sephadex G-200 column chromatography. A: Rat liver plasma membranes (23 mg of protein) in 20 ml of 20 mM Tris-HCl buffer (pH 7.5) were solubilized with 0.4% Triton X-100 at 4°C for 20 min, and were then centrifuged at 105,000 x g for 60 min. The supernatant was concentrated in a Amicon ultrafiltration cell equipped with a PM-10 filter to 4.7 ml. 4.3 ml of the concentrated sample was charged on a Sephadex G-200 column (2.2 x 80 cm) equilibrated with 20 mM Tris-HCl and 0.4% Triton X-100 at pH 7.5. Elution was performed with same buffer. Fractions of 3 ml each were collected. Each fraction was assayed for ATPase in the presence of either Ca/EGTA (0.5 μM free calcium) (●—●) or Mg/CDTA (2 mM free magnesium) (▲—▲) buffer system under standard conditions. B: Molecular weight of the high affinity Ca^{2+}-ATPase was estimated by gel filtration analysis on the same column of experiment A employing four marker proteins (bovine serum albumin (BSA), human γ-globulin(HG), bovine catalase (Catalase) and bovine thyrogrobulin (TG) with molecular weights of 67,000, 160,000, 240,000 and 670,000, respectively)

G-200 and DEAE-cellulose columns. The Mg^{2+}-ATPase was eluted near the void volume and the high affinity Ca^{2+}-ATPase at a position corresponding to a molecular weight of about 200,000. Neither column caused a major loss of activity; however, the ATPase became very unstable, losing 80% of its activity on storage at 4°C for 24 h after the anion-exchange chromatography. By these purification procedures, specific activity of the high affinity Ca^{2+}-ATPase was increased from 18.5 nmol/mg/min to 70.5 nmol/mg/min. The partially purified high affinity Ca^{2+}-ATPase was also shown to be Mg^{2+}-independent by an experiment similar that of Fig. 2. Orthovanadate (up to 2 mM), a potent inhibitor for many ATPases (12), had no effect on either the crude or partially purified high affinity Ca^{2+}-ATPase. Although insensitivity to orthovanadate is also observed for mitochondrial ATPase, mitochondrial ATPase blockers such as NaN_3, KCN and dicyclohexylcarbodiimide also had no effect on the high affinity Ca^{2+}-ATPase. Ouabain Na^+ and K^+ also showed no effect.

The high affinity Ca^{2+}-ATPase of rat liver plasma membrane described in this report resembls the erythrocyte plasma membrane Ca^{2+}-pumping ATPase with respect to optimum pH, K_a for Ca^{2+} and molecular weight; however, unlike Ca^{2+}-pumps of plasma membrane from various sources (2, 13-19) it is not sensitive to magnesium and orthovanadate, or inhibited by calmodulin. Further examinations should be needed to identify this Ca^{2+}-ATPase as a Ca^{2+}-pumping ATPase of liver plasma membrane.

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