Does a calmodulin-dependent Ca^{2+} -regulated Mg^{2+} -dependent ATPase contribute to hepatic microsomal calcium uptake?

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Solubilization of microsomal proteins followed by calmodulin affinity chromatography resulted in the separation of two distinct Ca^{2+} - Mg^{2+} -ATPases (Ca^{2+} -regulated Mg^{2+} -dependent ATPases), one being insensitive to calmodulin (ATPase-1), the other being stimulated about 5-fold by calmodulin (ATPase-2). ATPase-2 accounts for only 8% of total microsomal Ca^{2+} - Mg^{2+} -ATPase-activity. ATPase-1 and -2 can also be distinguished by different pH optima, different sensitivity towards inhibition by vanadate and LaCl_3 , and different apparent M_r values of the phosphoenzyme intermediates (115000 and 150000 for ATPase-1 and ATPase-2 respectively). ATPase-1 from liver co-migrated with Ca^{2+} - Mg^{2+} -ATPase from rat skeletal-muscle sarcoplasmic reticulum, whereas ATPase-2 from liver co-migrated with calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase derived from rat skeletal-muscle sarcolemma. After separation of parenchymal and non-parenchymal liver cells, a calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase of M_r 150000 was found only in the non-parenchymal cells. The kinetic parameters of ATPase-2 and the similarity of the apparent M_r of its phosphoenzyme intermediate to that of skeletal-muscle sarcolemma Ca^{2+} - Mg^{2+} -ATPase makes it likely that the calmodulin-sensitive Ca^{2+} - Mg^{2+} -ATPase found in rat liver microsomal fractions reflects a contamination with plasma membranes (possibly from non-parenchymal cells) rather than a true location in the endoplasmic reticulum of parenchymal liver cells.

INTRODUCTION

The hepatic ATP-dependent Ca^{2+} -transporting systems of microsomes (microsomal fractions) and plasma membranes have been extensively studied. However, the question of involvement of calmodulin in Ca^{2+} sequestration and regulation of Ca^{2+} - Mg^{2+} -ATPases in liver is still a matter of debate. Both plasma membranes [1,2] and endoplasmic reticulum [3,4] from rat liver contain bound calmodulin. In contrast with the findings obtained with plasma membranes from other tissues [5–12], no direct effect of exogenous calmodulin on Ca^{2+} transport or Ca^{2+} -dependent ATPase activity has been detected in hepatic plasma membranes [13,14]. Conflicting reports exist concerning the role of calmodulin for Ca^{2+} sequestration by the endoplasmic reticulum in liver cells. Famulski & Carafoli [15] concluded that calmodulin had no effect on the Ca^{2+} - Mg^{2+} -ATPase and that the hepatic microsomal Ca^{2+} - Mg^{2+} -ATPase resembles that of skeletal muscle rather than that of heart muscle sarcoplasmic reticulum. Also Dawson & Fulton [4] found neither a stimulatory effect of exogenous calmodulin on the activity of the Ca^{2+} -ATPase in rat liver microsomes nor an inhibitory effect of TFP. On the other hand, Moore & Kraus-Friedmann [3] isolated a Ca^{2+} - Mg^{2+} -ATPase from rat liver microsomes by calmodulin affinity chromatography and postulated that the Ca^{2+} transport in the liver endoplasmic reticulum could be regulated by calmodulin in a way similar to the regulation of the Ca^{2+} - Mg^{2+} -ATPase in cardiac sarco-

plasmic reticulum [16]. This hypothesis was not supported by data and is highly unlikely, as liver tissue does not contain phospholamban. The results presented here identify the hepatic microsomal calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase as a contaminant plasma-membrane Ca^{2+} - Mg^{2+} -ATPase, most likely of non-parenchymal origin.

EXPERIMENTAL

Materials

MgATP and bovine brain extract (Folch Fraction 3, containing 80–85% phosphatidylserine) were purchased from Sigma (München, Germany), pepstatin, leupeptin and antipain were from the Peptide Institute (Osaka, Japan), and CNBr-activated Sepharose 4B and phenyl-Sepharose were from Pharmacia (Freiburg, Germany). Calmodulin was prepared from bull testis as described in ref. [17] and coupled to phenyl-Sepharose 4B as described in ref. [5]. Acrylamide and bisacrylamide were from Serva (Heidelberg, Germany). Other biochemicals were from Boehringer (Mannheim, Germany). All other chemicals (analytical grade) were purchased from E. Merck (Darmstadt, Germany). $^{45}\text{CaCl}_2$ (3 mCi/mmol) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) were obtained from Amersham-Buchler Co. (Braunschweig, Germany). Male Wistar rats were purchased from Mus Rattus G.m.b.H. (Brunnthal, Germany). They received a standard diet (Altromin-R; Altrogge (Lage, Lippe, Germany) and water *ad libitum*.

Abbreviations used: DTE, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Tos-Lys- CH_2Cl , *N*-*p*-tosyl-L-lysylchloromethane; Tos-Phe- CH_2Cl , L-1-tosylamido-2-phenylalanylchloromethane; Ca^{2+} - Mg^{2+} -ATPase, Mg^{2+} -dependent Ca^{2+} -regulated ATPase.

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Preparation and subfractionation of rat liver microsomes

A crude microsomal fraction was prepared from the livers of overnight-starved male Wistar rats (body wt. 180–220 g) as described by Moore & Kraus-Friedmann [3] and Famulski & Carafoli [18], except that the homogenizing medium contained the following proteinase inhibitors (final concns.): PMSF, 0.1 mM; Tos-Lys-CH₂Cl, 0.5 mM; Tos-Phe-CH₂Cl, 0.1 mM; benzamidine, 1 mM; leupeptin, antipain and pepstatin, 4 µg/ml each; aprotinin, 500 kallikrein-inhibitory units/ml.

The 'crude microsomal fraction' was layered on top of a discontinuous sucrose gradient consisting of 5 ml of 54% (w/w), 13 ml of 38.5% (w/w) and 13 ml of 34% (w/w) sucrose in 120 mM-KCl/10 mM-Hepes/1 mM-DTE, pH 7.0, and centrifuged for 18 h at 105000 *g*_{max}. Fractions were collected from the top of the 34%-sucrose cushion (interphase 1), from the 34%/38.5% interphase (interphase 2) and from the 38.5%/54% interphase (interphase 3). The subfractions were diluted with 3 vol. of 120 mM-KCl/10 mM-Hepes/1 mM-DTE. EGTA was added to final concentrations of 0.1 mM (for the Ca²⁺-uptake studies) or 2 mM (for isolation of the Ca²⁺-Mg²⁺-ATPase). The membranes were subsequently sedimented at 100000 *g*_{max} for 60 min to remove most of the endogenous bound calmodulin from the membranes. The pellets were washed in 120 mM-KCl/10 mM-Hepes/1 mM-DTE, pH 7.0, to remove EGTA and finally suspended in the appropriate buffers for use in the Ca²⁺-uptake studies, the Ca²⁺-Mg²⁺-ATPase assays, or for ATPase solubilization.

Protein was determined by the dye-binding method of Bradford [19]. When Triton X-100 was present, this method was combined with that of Bensadoun & Weinstein [20] to remove the detergent. 5'-Nucleotidase [21], glucose-6-phosphatase [22] and RNA [23] were determined by standard methods. To check the morphology of the isolated vesicles and the purity of the fractions, samples were fixed in glutaraldehyde and OsO₄, embedded in Epon and subjected to electron microscopy.

Preparation of membranes from rat liver parenchymal and non-parenchymal cells

Hepatocytes from livers of male Wistar rats were prepared as in ref. [24] and fractionated into parenchymal and non-parenchymal cells by differential centrifugation as in ref. [25]. Purity of the separated cell fractions was checked microscopically. The non-parenchymal-cell fraction was essentially free from parenchymal cells; the parenchymal-cell fraction contained approx. 4% non-parenchymal cells. After disruption of the cells with an Ultra-Turrax, the homogenates were centrifuged for 5 min at 1000 *g*_{max}. The resulting supernatants were spun for 60 min at 100000 *g*_{max}. The omission of an intermediate 12500 *g*_{max} centrifugation step led to a considerable enrichment of plasma-membrane vesicles in the microsomal pellet, which ensured that phosphoenzyme intermediates of the plasma-membrane Ca²⁺-Mg²⁺-ATPases from both cell types could be detected. The sedimented membranes were used for the solubilization of Ca²⁺-Mg²⁺-ATPases as described for the liver microsomes.

Determination of ATP-dependent Ca²⁺ uptake into microsomal vesicles

Ca²⁺ uptake was measured by the Millipore-filtration technique [26] as modified [27]. For studying the effects of calmodulin or calmodulin antagonists on Ca²⁺ uptake, calmodulin (1–100 µg/ml) or trifluoperazine (50 µM final concn.) was added to the standard incubation assay.

Preparation of rat skeletal-muscle sarcoplasmic-reticulum and sarcolemma vesicles

Sarcoplasmic-reticulum and sarcolemmal vesicles from rat skeletal muscle were prepared by 'procedure 1' in ref. [28]. Sarcolemma vesicles were collected from the 0.6 M/0.25 M and 0.8 M/0.6 M interphases of the discontinuous sucrose gradient; the Ca²⁺-loaded sarcoplasmic-reticulum vesicles were collected from the 1.5 M/1.0 M interphase and from the pellet. For isolation of the Ca²⁺-Mg²⁺-ATPases, the sarcolemma vesicles were washed with EGTA as in ref. [8] to remove endogenous calmodulin.

Solubilization of the Ca²⁺-Mg²⁺-ATPases and separation by calmodulin affinity chromatography

Vesicles derived from rat liver microsomes, liver parenchymal and non-parenchymal membranes or rat muscle sarcoplasmic reticulum and sarcolemma (10 mg of protein/ml) were partially solubilized by a 20 min incubation at 4 °C in a medium containing 0.5% (v/v) Triton X-100 (corresponding to 0.5 mg of detergent/mg of protein), 20% (v/v) glycerol, 120 mM-KCl, 20 mM-Hepes, pH 7.2, 100 µM-CaCl₂, 2 mM-DTE and the proteinase-inhibitor 'cocktail' mentioned above. After solubilization, sonicated brain lipid extract was added (final concn. 0.5 µg/ml), and the insoluble material was sedimented at 105000 *g*_{max} for 60 min. The supernatants were applied to a Sepharose 4B-calmodulin column (1.5 cm × 4 cm; 1 mg of calmodulin coupled to 1 ml of resin), which had been equilibrated with buffer A (120 mM-KCl, 20 mM-Hepes, 2 mM-DTE, 100 µM-CaCl₂, 0.5 mg of brain extract/ml, 20% glycerol and 0.5% Triton X-100; final pH 7.2). The column was then washed with buffer A and subsequently with buffer B (same as buffer A, but with 0.05% Triton X-100) until the A₂₈₀ had returned to that of the buffer. Then the buffer was changed to buffer C (same as buffer B, but with 2 mM-EGTA instead of 0.1 mM-CaCl₂). To each fraction (1.5 ml) eluted with EGTA, CaCl₂ was added immediately to a final concentration of 2 mM. The fractions were analysed for ATPase activity and phosphoenzyme formation immediately after column chromatography.

Assay of Ca²⁺-Mg²⁺-ATPase activity

Ca²⁺-Mg²⁺-ATPase activity was determined as described in ref. [29] in a standard medium consisting of (final concns.) 120 mM-KCl, 20 mM-Hepes, pH 7.0, 1 mM-Na₃ and 1 mM-[γ-³²P]ATP (sp. radioactivity 40 µCi/mmol). The concentrations of MgCl₂, CaCl₂ and other reagents were varied as described in the Figure legends. The desired free Ca²⁺ concentrations were established with the aid of an EGTA/Ca²⁺ buffer system by using the absolute stability constants given by Bartfai [30] and correcting for pH. The total assay volume was 0.5 ml. All additions to the reaction mixture were adjusted to the desired pH. Ca²⁺-Mg²⁺-ATPase activity

was defined as the rate of P_i formation at the indicated Ca^{2+} concentrations minus the rate of P_i formation in the presence of 2–10 mM EGTA (free $[\text{Ca}^{2+}] < 1 \text{ nM}$).

Analysis of the phosphoenzyme intermediates

To obtain the phosphoenzyme intermediates of the Ca^{2+} - Mg^{2+} -ATPases, the enzyme-containing fractions (10–50 μg of protein) were incubated at 0°C in 0.2–0.5 ml of a basal medium consisting of (final concns.) 120 mM-KCl and 20 mM-Hepes (pH 7.0). Other additions are indicated in the Figure legends. The reactions were started by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (final concn. 10 nM, sp. radioactivity 3000 Ci/mmol). The reactions were terminated after 4 s by the addition of 1 vol. of ice-cold 20% (w/v) trichloroacetic acid containing 1 mM- NaH_2PO_4 . To this mixture, 1.5 vol. of cold acetone (-20°C) was added to remove Triton X-100. After standing overnight, the phosphoproteins were pelleted by centrifugation at 20000 g_{max} for 10 min and washed in cold 1 mM- NaH_2PO_4 (pH 5). The samples were divided into two. One half was suspended in 0.8 M-hydroxylamine/150 mM-sodium acetate, pH 5.0, and incubated for 20 min at room temperature. The other half was treated in parallel with 150 mM-sodium acetate/150 mM-Tris/acetate, pH 5.0. Both samples were subsequently washed once in 1 mM- Na_2HPO_4 (pH 5.0) and resuspended in a solution consisting of 10 mM-sodium phosphate (pH 7.0), 3% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 15% (v/v) glycerol and 0.05% (w/v) Bromophenol Blue. After incubation for 12 h at room temperature, the samples were subjected to SDS/polyacrylamide-gel electrophoresis. Heat denaturation of the proteins was omitted, to avoid aggregation of ATPases [31].

Electrophoresis and autoradiography

Because of the sensitivity of the phosphoenzyme intermediates towards alkaline pH [32], the phosphoprotein patterns of the fractions containing the ATPase were analysed by SDS/polyacrylamide-gel electrophoresis as described by Weber & Osborn [33] on 7.5%-polyacrylamide slab gels containing 0.1 M-sodium phosphate buffer, pH 7.2, and 0.1% SDS. Gels were run for 24 h at 40 mA constant current per slab at room temperature. M_r standards used were myosin heavy chain (M_r 205000), β -galactosidase (M_r 116000), phosphorylase *b* (M_r 96000), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000) and carbonic anhydrase (M_r 29000). The gels were dried under

vacuum and autoradiographed by using Fuji RX-films at -80°C .

RESULTS

Ca^{2+} uptake into rat liver microsomal subfractions and effect of calmodulin

The Ca^{2+} uptake into the crude microsomal preparation was $4.2 \pm 1.2 \text{ nmol/min per mg}$ of protein (mean value $\pm$ S.E.M.; $n = 6$). We subfractionated the crude microsomes further by sucrose-gradient centrifugation (see the Experimental section), resulting in the separation of an interphase-1 fraction, highly enriched in plasma membranes, and two fractions containing mainly endoplasmic reticulum (see Table 1). One of these (interphase 2) also contained some plasma-membrane markers; the other (interphase 3) was almost free from contamination by plasma membranes. The high amount of RNA in the interphase-3 fraction characterizes this fraction as rough endoplasmic reticulum. This is in accordance with the electron micrographs, which showed mostly vesicles with ribosomes attached to their outer surface (results not shown). In contrast, the vesicles in the interphase-2 fraction were almost free of ribosomes.

The interphase-2 fraction exhibited the highest specific ATP-dependent Ca^{2+} -uptake rates (Table 1). The interphase-3 fraction showed a much lower Ca^{2+} -uptake rate, and the Ca^{2+} -uptake rate of interphase-1 fraction was intermediate. The latter finding can be explained by the high contamination of the interphase-1 fraction with plasma membranes (Table 1), which possesses little Ca^{2+} -transporting activity at pH 6.8 [34]. Addition of calmodulin (1–100 $\mu\text{g/ml}$) did not stimulate Ca^{2+} uptake into vesicles, and the anti-calmodulin agent trifluoperazine (up to 10 μM) had essentially no effect (results not shown).

To exclude the possibility that the system was already saturated with endogenous membrane-bound calmodulin, the microsomal vesicles were washed with 0.5 mM-EGTA to remove most of the endogenous calmodulin. After treatment with EGTA, the Ca^{2+} -uptake rate was indeed decreased by more than 70%. Electron-microscopic analysis revealed, however, that most of the vesicles had been disrupted by the treatment with EGTA. A milder treatment, with only 0.05 mM-EGTA, left most of the vesicles morphologically intact and diminished the Ca^{2+} -uptake rate only by about 20%

Table 1. Characterization of rat microsomal subfractions

Activities of marker enzymes, RNA, Ca^{2+} uptake, Ca^{2+} - Mg^{2+} -ATPase and the effect of calmodulin on the activity of the Ca^{2+} - Mg^{2+} -ATPase were measured in microsomal subfractions derived from rat liver microsomes after centrifugation on a discontinuous sucrose gradient (see the Experimental section for details). Results are means $\pm$ S.E.M. from four experiments. The numbers in parentheses refer to the relative increase (as percentage over controls) of microsomal Ca^{2+} - Mg^{2+} -ATPase activity caused by addition of 100 μg of calmodulin (CAM)/ml.

Fraction analysed	Protein (mg)	5'-Nucleotidase (μg of P_i /30 min per mg)	RNA ($\mu\text{g}/\text{mg}$)	Ca^{2+} uptake (nmol/min per mg)	Ca^{2+} - Mg^{2+} -ATPase (nmol of P_i /min per mg)	
					No CAM	+ CAM
Interphase 1	7.2 ± 2.4	237 ± 11.4	81 ± 3.5	3.4 ± 0.5	6.64 ± 1.8	6.88 ± 2.0 (+3.7%)
Interphase 2	13.2 ± 3.2	25.2 ± 1.2	158 ± 7.2	4.5 ± 0.5	9.37 ± 1.8	18.59 ± 3.0 (+98%)
Interphase 3	14.0 ± 4.0	5.5 ± 0.4	184 ± 8.4	1.9 ± 0.4	9.13 ± 2.0	11.0 ± 2.8 (+20%)

Fig. 1. Activation of the Ca^{2+} - Mg^{2+} -ATPase in microsomal subfractions by exogenous calmodulin

ATPase activity was determined as described in the Experimental section. The concentration of free Ca^{2+} was $20 \mu\text{M}$, and that of MgCl_2 4 mM . Each point represents the mean $\pm$ S.E.M. from four experiments with different preparations. Samples were derived from the interphases of the sucrose gradient: ○, interphase 1; △, interphase 2; □, interphase 3.

in all three subfractions. Exogenous calmodulin (up to $100 \mu\text{g/ml}$) had no effect on the Ca^{2+} uptake by EGTA-treated vesicles (0.5 mM - or 0.05 mM -EGTA), suggesting that the loss of Ca^{2+} -uptake activity of the EGTA-washed vesicles had not resulted from the removal of endogenous calmodulin.

Activity of Ca^{2+} - Mg^{2+} -ATPases in microsomal subfractions; effect of calmodulin on the membrane-bound enzyme

In a preparation of crude liver microsomes (washed with 0.5 mM -EGTA), the activity of the Ca^{2+} - Mg^{2+} -ATPase (V_{max}) in the presence of $20 \mu\text{M}$ - Ca^{2+} and without exogenous Mg^{2+} was about $11 \pm 2.5 \text{ nmol}$ of

Fig. 2. Affinity chromatography of Triton-X-100-solubilized microsomes on a calmodulin-Sepharose 4B column

Details are given in the Experimental section. Ca^{2+} - Mg^{2+} -ATPase activity was assayed at $20 \mu\text{M}$ free Ca^{2+} and 4 mM - Mg^{2+} in the absence (----) and in the presence (-----) of added calmodulin ($20 \mu\text{g/ml}$ final concn.); —, A_{280} . Arrow A indicates the equilibrium buffer A, containing $100 \mu\text{M}$ - Ca^{2+} and 0.5% Triton X-100. Arrow S marks the application of the solubilized material; arrow B marks the application of buffer B, containing $100 \mu\text{M}$ - Ca^{2+} and 0.05% Triton X-100, and arrow C indicates the change to buffer C, containing 2 mM -EGTA instead of Ca^{2+} and 0.05% Triton X-100.

Pi/min per mg of protein. Addition of 4 mM - MgCl_2 to the assay raised the enzyme activity to approx. $30 \pm 6.4 \text{ nmol}$ of Pi/min per mg of protein. Addition of calmodulin ($100 \mu\text{g/ml}$ final concn.) to the vesicles increased the Ca^{2+} - Mg^{2+} -ATPase activity to 16 ± 3.0 and $36 \pm 7.2 \text{ nmol/min}$ per mg of protein ($n = 6$) in the absence and presence of exogenous Mg^{2+} respectively, corresponding to a 45% and 20% stimulation (results not shown).

Table 2. Balance sheet for the distribution of protein and Ca^{2+} - Mg^{2+} -ATPase activity and effects of calmodulin (CAM) on the enzymes in the fractions derived from solubilized rat liver microsomes

A typical distribution is shown (mean values $\pm$ S.E.M. from six experiments) starting from 100 mg of microsomal protein.

Fraction	Total protein (mg)	Specific ATPase activity (nmol/min per mg)		Total ATPase activity				Total calmodulin-stimulated ATPase activity	
		No CAM	+CAM	(nmol/min)		(%)		(nmol/min) (%)	
				No CAM	+CAM	No CAM	+CAM	(nmol/min)	(%)
Crude microsomes	100	8.7 ± 1.2	9.6 ± 2.0	869 ± 112	960 ± 192	100	100	91	100
Triton X-100 extract	33	18.6 ± 3.0	20.3 ± 3.4	615 ± 99	672 ± 112	71	70	57	63
Column peak 1	32	18.0 ± 2.8	18.0 ± 2.8	578 ± 87	578 ± 87	67	60	0	0
Column peak 2 eluted with EGTA	0.065	253 ± 25	1206 ± 126	16.6 ± 1.7	78.3 ± 7.8	1.9	8.2	78	86*

* The higher value for total calmodulin-stimulated ATPase activity in the peak 2 fraction compared with the values given for the Triton X-100 extract results most likely from the inhibitory effect of Triton X-100, which was present at a higher concentration (0.5%) in the microsomal extract than in the peak-2 fraction (0.05%).

After subfractionation of the microsomal vesicles on a discontinuous sucrose gradient (see the Experimental section), the highest Ca^{2+} - Mg^{2+} -ATPase activity ($V_{\text{max}} = 9.4$ nmol/min per mg) and the highest degree of stimulation by calmodulin were found in the interphase-2 fraction (see Table 1 and Fig. 1). The activity of the enzyme was almost doubled in this fraction by the addition of calmodulin ($V_{\text{max}} = 18.6$ nmol/min per mg). This contrasts with the inefficiency of calmodulin to stimulate Ca^{2+} uptake in crude microsomes and in the subfractions as mentioned above. The high activity of Ca^{2+} - Mg^{2+} -ATPase in the interphase-2 fraction paralleled the high Ca^{2+} -uptake rate in this fraction (see Table 1).

Solubilization of Ca^{2+} - Mg^{2+} -ATPases from microsomes and partial purification of a calmodulin-binding Ca^{2+} - Mg^{2+} -ATPase by affinity chromatography

When several detergents commonly used for the solubilization of membrane-bound ATPases (sodium deoxycholate, β -octyl glycoside and Triton X-100) were compared, 0.5% (v/v) Triton X-100 (0.5 mg of detergent/mg of protein) gave the best results with regard to yield and stability of the Ca^{2+} - Mg^{2+} -ATPase from rat liver microsomes. Treatment with Triton X-100 solubilized about 32% (range 26–38%; $n = 22$) of the total microsomal protein and about 70% (range 62–78%; $n = 14$) of the total Ca^{2+} - Mg^{2+} -ATPase activity of calmodulin-depleted microsomes (Table 2). The solubilized Ca^{2+} - Mg^{2+} -ATPase activity could be separated into two fractions by calmodulin affinity chromatography (Fig. 2). The distribution of ATPase activities among the fractions derived from 100 mg of microsomal protein is given in Table 2. Most of the protein (97%) applied on to the column in the presence of Ca^{2+} -containing buffer did not bind to the calmodulin affinity column. This

Fig. 3. Activity of the two Ca^{2+} - Mg^{2+} -ATPases as a function of pH

The activities of ATPase-1 (○) and ATPase-2 (△) were assayed in the standard medium (120 mM-KCl/20 mM-Hepes) at 20 μM free Ca^{2+} and 4 mM- Mg^{2+} , and (for ATPase-2 only) in the presence of calmodulin (20 $\mu\text{g}/\text{ml}$). The desired pH was adjusted at 37 °C with KOH and the reactions were started by the addition of ATP, which had been adjusted also to the desired pH. ATPase activity is plotted as percentage of the maximal activity, which corresponds to 20 and 650 nmol/min per mg for ATPase-1 and ATPase-2 respectively. Results are means from three separate experiments.

Fig. 4. Inhibition of Ca^{2+} - Mg^{2+} -ATPases 1 and 2 by vanadate

Ca^{2+} - Mg^{2+} -ATPases were assayed in the standard medium at pH 7.0 (ATPase-1, ○) and pH 7.4 (ATPase-2, △). The final concentrations of vanadate are indicated. Vanadate was added 2 min before the reaction was started by addition of ATP. Results are means $\pm$ S.E.M. from three separate experiments.

fraction (peak 1) contained Ca^{2+} - Mg^{2+} -ATPase with a specific activity of 18 nmol/min per mg, which could not be further activated by calmodulin; this activity will be termed 'ATPase-1'. ATPase-1 accounted for 94% of the total Ca^{2+} - Mg^{2+} -ATPase activity applied to the column or 66.5% of the total activity in the crude microsomes (578 nmol/min). The Ca^{2+} - Mg^{2+} -ATPase fraction that was retained on the column and eluted with EGTA (peak 2) accounted for 0.2% of the protein applied to the column or 0.065% of the total microsomal protein. The specific activity of Ca^{2+} - Mg^{2+} -ATPase in peak 2 was 253 nmol per min/mg when measured in the absence of calmodulin; this activity will be termed 'ATPase-2'. In contrast with ATPase-1, ATPase-2 was stimulated about 5-fold by calmodulin, to a maximal activity of 1200 nmol/min per mg of protein (Table 2), indicating a 125-fold purification by the calmodulin affinity chromatography, which compares well with purification factors of 80–100 given for muscle sarcolemma Ca^{2+} - Mg^{2+} -ATPase [7,8]. The activity of ATPase-2 might be underestimated, as the ATPase was subjected to a Ca^{2+} -free medium during the elution from the column with EGTA, a circumstance in which Ca^{2+} - Mg^{2+} -ATPases have been shown to undergo rapid inactivation [35]. We have tried to minimize inactivation in our preparations by using low concentrations of Triton X-100 and introducing 20% glycerol for stabilization of the enzyme during the elution process. As depicted in Table 2, the total activity of ATPase-2 accounted for only 2.7% (i.e. 16.6 nmol/min) of the total Ca^{2+} - Mg^{2+} -ATPase activity in the Triton X-100 extract applied to the column, or 1.9% of the total activity in the microsomes when measured without calmodulin, reaching maximal values of 11.7% (i.e. 78.3 nmol/min) of the Triton X-100 extract and 8.2% of the total activity in the microsomes after stimulation with calmodulin.

Fig. 5. Comparison of the phosphoenzyme intermediates of the Ca^{2+} - Mg^{2+} -ATPases from rat liver microsomes and rat skeletal-muscle sarcoplasmic reticulum and sarcolemma

Preparation of liver microsomal and skeletal-muscle sarcoplasmic-reticulum and sarcolemma vesicles, as well as separation of the solubilized proteins and phosphorylation, were performed as described in the Experimental section. The autoradiograph shows the phosphoprotein patterns of protein fractions solubilized from liver microsomes (a) and from skeletal-muscle membranes (b). In (a) lanes 1 and 2 represent ATPase-1 and lanes 3–5 ATPase-2 from the calmodulin affinity chromatography of solubilized liver microsomes. In (b) lanes 6 and 7 represent the solubilized proteins from skeletal-muscle sarcoplasmic reticulum, and lanes 8–10 the calmodulin-binding proteins from skeletal-muscle sarcolemma. The samples in lanes 4 and 9 were assayed in the presence of EGTA ($[\text{Ca}^{2+}] < 1 \text{ nM}$), demonstrating the Ca^{2+} -dependency of phosphoprotein formation. In lanes 1, 5, 7 and 10 the phosphoproteins were treated with hydroxylamine before electrophoresis. The positions of M_r standards are indicated on the right, and those of the ATPase phosphoenzyme intermediates on the left.

These data clearly indicate that most of the Ca^{2+} - Mg^{2+} -ATPase activity in rat liver microsomes represents ATPase-1 and not the calmodulin-sensitive ATPase-2.

Effect of pH on the activities of ATPase-1 and ATPase-2

The pH/activity profiles are clearly different. Whereas the maximum enzyme activity for ATPase-1 was at pH 7.0, that for ATPase-2 was found at pH 7.4 (Fig. 3). The different pH optima of the two Ca^{2+} - Mg^{2+} -ATPases point to different origins of the enzymes. The ATPase-1 shows the typical pH/activity profile of an endoplasmic-reticulum Ca^{2+} - Mg^{2+} -ATPase [3,4,36], whereas the pH/activity profile of ATPase-2 is more of the plasma-membrane type [13,14].

Effects of vanadate and La^{3+} on the activities of ATPase-1 and ATPase-2

The concentration of vanadate required for half-maximal inhibition (K_i) is very different for different ATPases. Fig. 4 shows that the two Ca^{2+} - Mg^{2+} -ATPases isolated from liver microsomes could be clearly distinguished on the basis of their sensitivity towards vanadate: whereas ATPase-1 was strongly inhibited at low concentrations of vanadate ($K_i = 2 \mu\text{M}$), ATPase-2

required higher concentrations for inhibition ($K_i = 75 \mu\text{M}$). The sensitivity of ATPase-1 to inhibition by vanadate resembles that of the microsomal Ca^{2+} -ATPase described by Dawson & Fulton [4]. The low vanadate sensitivity of ATPase-2, on the other hand, resembles the behaviour of the Ca^{2+} -ATPase from rat liver plasma membrane [14,37–39]. In contrast with vanadate, La^{3+} at concentrations up to $150 \mu\text{M}$ had no inhibitory effect on ATPase-1 activity, whereas ATPase-2 was inhibited by La^{3+} in a dose-dependent manner [half-maximal and complete inhibition at 30 ± 10 and $100 \pm 16 \mu\text{M}$ - La^{3+} ($n = 3$) respectively; results not shown]. The sensitivity of ATPase-2 towards inhibition by LaCl_3 resembles that of the Ca^{2+} - Mg^{2+} -ATPase from bile-canalicular plasma membranes [14] and from skeletal-muscle sarcolemma [9].

Comparison of the phosphoenzyme intermediates of ATPase-1 and ATPase-2 with those of the Ca^{2+} - Mg^{2+} -ATPases from rat skeletal-muscle sarcoplasmic reticulum and sarcolemma

Fig. 5 shows the autoradiographs of the phosphorylated proteins from rat liver microsomes (a) and from rat skeletal-muscle sarcoplasmic reticulum and sarcolemma (b). The hydroxylamine-sensitive phosphoenzyme intermediates of ATPase-1 and ATPase-2 from rat liver

Fig. 6. Phosphoenzyme intermediates and enzyme activities of plasma-membrane $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPases}$ from parenchymal (a) and non-parenchymal (b) cells after calmodulin affinity chromatography

Plasma-membrane-enriched microsomal fractions were prepared from isolated parenchymal and non-parenchymal cells as given in the Experimental section. After solubilization with 0.5% Triton X-100, the extracts were separated by calmodulin affinity chromatography as in Fig. 2. The flow-through fractions (I) and the fractions eluted with EGTA (II) were analysed for $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activity in the absence and presence of added calmodulin (CAM; 20 $\mu\text{g}/\text{ml}$); values (nmol of P_i /min) are given below the autoradiographs. Moreover, fractions (I) and fractions (II) were analysed for the phosphoenzyme intermediates of plasma-membrane-type $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ (M_r 150000), before and after treatment with hydroxylamine, by Weber & Osborn [33] SDS/polyacrylamide-gel electrophoresis (see the autoradiographs). (a) Material from parenchymal cells: lane 1, flow-through fraction; lane 2, flow-through fraction after treatment with hydroxylamine; lane 3, EGTA-eluted fraction; lane 4, EGTA-eluted fraction after treatment with hydroxylamine. (b) Material from non-parenchymal cells: lane 5, flow-through fraction; lane 6, flow-through fraction after treatment with hydroxylamine; lane 7, EGTA-eluted fraction; lane 8, EGTA-eluted fraction after treatment with hydroxylamine.

microsomes differ in their apparent M_r (115000 for ATPase-1; 150000 for ATPase-2; Fig. 5a). As indicated in Fig. 5(b), ATPase-1 from liver co-migrated with the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ from muscle sarcoplasmic reticulum, whereas ATPase-2 from liver co-migrated with the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ from muscle sarcolemma. In addition to the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPases}$, several other phosphoproteins were found in the two fractions from rat liver microsomes and in the muscle sarcolemma fraction, whereas in muscle sarcoplasmic reticulum ^{32}P was almost exclusively incorporated into the hydroxylamine-sensitive ATPase molecule. Since these phosphoproteins were hydroxylamine-resistant, they represent, most probably, phosphoester bonds resulting from the action of endogenous protein kinases.

$\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activities in rat liver parenchymal and non-parenchymal cells

In three experiments, plasma-membrane-enriched microsomal fractions were prepared from parenchymal and non-parenchymal liver cells. The solubilized membranes were processed by calmodulin affinity chromatography and analysed for plasma-membrane-type

$\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPases}$. Less than 0.5% of the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activity from parenchymal-cell membranes was retained on the affinity column, in contrast with 7.2% of the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPases}$ from non-parenchymal-cell membranes (both values were obtained in the presence of calmodulin). Although the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ from the parenchymal-cell membranes contained significant amounts of plasma-membrane-type $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$, as indicated by the M_r of 150000 (Fig. 6a), this $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activity was not affected by the addition of 50 μg of calmodulin/ml. In contrast, for solubilized membranes from non-parenchymal cells, all the $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ of M_r 150000 was retained by the affinity column (Fig. 6b) and could be eluted with EGTA. The eluted $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ could be stimulated 3.6-fold by calmodulin. The $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activity of this fraction was 1530 nmol/min per mg when measured at pH 7.5, but only 431 nmol/min per mg when measured at pH 6.8. The higher activity at alkaline pH is in line with its plasma-membrane origin. Moreover, its apparent M_r and calmodulin-sensitivity corresponds well to the properties of ATPase-2 obtained from total liver microsomes.

DISCUSSION

The results presented here are not compatible with the view that ATP-driven Ca^{2+} uptake by rat liver microsomes is regulated by calmodulin. In accordance with Moore *et al.* [26], we determined the highest Ca^{2+} -uptake rates in a microsomal subfraction which contained mainly smooth endoplasmic reticulum. In this fraction we also observed the highest Ca^{2+} - Mg^{2+} -ATPase activity and a Ca^{2+} /ATP ratio of approx. 1:2 for ATP-driven Ca^{2+} uptake. Although calmodulin did not stimulate ATP-driven Ca^{2+} uptake, the moderate stimulation of total microsomal Ca^{2+} - Mg^{2+} -ATPase by calmodulin indicated the presence of a calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase. After solubilization from the membranes, a calmodulin-sensitive Ca^{2+} - Mg^{2+} -ATPase (ATPase-2) could be purified by calmodulin affinity chromatography. However, even after maximal stimulation with calmodulin, the calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase activity did not exceed 8% of the total Ca^{2+} - Mg^{2+} -ATPase activity of hepatic microsomes. Thus the activity of the calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase is much too low to account for the Ca^{2+} uptake measured for hepatic microsomal vesicles. The low amount of ATPase-2 isolated here does not result from a more rapid inactivation of this enzyme in comparison with overall Ca^{2+} - Mg^{2+} -ATPase activity. This is indicated by the high recovery of this enzyme activity (approx. 86%). The relative amount of the ATPase-2 protein in rat liver microsomes represented less than 0.065% of the total membrane protein, compared with 0.3% in skeletal-muscle sarcolemma [7] and 0.1% in erythrocyte membranes [5].

Thus we cannot confirm the findings by Moore & Kraus-Friedmann [3], who reported that the major portion of Ca^{2+} - Mg^{2+} -ATPase activity solubilized from rat liver microsomal membranes did bind to calmodulin affinity columns. This discrepancy cannot result from an insufficient binding capacity of our calmodulin column, because: (1) the capacity of the column was extremely high (approx. 1 mg of calmodulin coupled to 1 ml of resin); (2) the recovery of calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase activity was almost 90% when related to the corresponding activity of crude microsomes; (3) no calmodulin-activated Ca^{2+} - Mg^{2+} -ATPase activity appeared in the flow-through of the calmodulin affinity column, although it contained 94% of total microsomal Ca^{2+} - Mg^{2+} -ATPase activity. The two hepatic microsomal Ca^{2+} - Mg^{2+} -ATPases clearly represent two different enzymes. The calmodulin-independent Ca^{2+} - Mg^{2+} -ATPase (ATPase-1) resembles the Ca^{2+} - Mg^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum, whereas the calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase (ATPase-2) resembles the Ca^{2+} - Mg^{2+} -ATPase from muscle sarcolemma and other plasma membranes. The following parameters have been compared: (1) the different pH-dependency of the two enzymes; (2) the different sensitivity of the two enzymes towards inhibitors; (3) the different apparent M_r values [ATPase-1 migrated in the same position as rat skeletal-muscle sarcoplasmic reticulum Ca^{2+} - Mg^{2+} -ATPase (M_r 115000), whereas ATPase-2 co-migrated with rat skeletal-muscle sarcolemma Ca^{2+} - Mg^{2+} -ATPase (M_r 150000)]. Our observation is consistent with those of others, who also obtained lower apparent M_r values for Ca^{2+} - Mg^{2+} -ATPases from endoplasmic reticulum [32] or

sarcoplasmic reticulum [40–43] than for Ca^{2+} - Mg^{2+} -ATPases from sarcolemma [7,10–12] or plasma membranes of erythrocytes [6,44,45] or from liver [14,15,46].

We believe therefore that the calmodulin-independent hepatic microsomal Ca^{2+} - Mg^{2+} -ATPase (ATPase-1) represents the true Ca^{2+} - Mg^{2+} -ATPase of the endoplasmic reticulum. The calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase-2, on the other hand, shares so many properties with Ca^{2+} - Mg^{2+} -ATPases from plasma membranes that we believe that it reflects a contamination with membranes from a non-microsomal (most likely a plasma-membrane) compartment. A contamination with plasma membranes from hepatic parenchymal cells seems less likely, since calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPases could not be detected in any of the different regions of plasma membranes from liver parenchymal cells [13,14,34]. It seems more likely that the calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase found in the liver represents a contamination with plasma membranes from non-parenchymal cells, which make up to 20% of total liver cells. Although additional experiments are needed, our experiments performed with Ca^{2+} - Mg^{2+} -ATPases from separated parenchymal and non-parenchymal liver cells indicate that only the non-parenchymal liver cells contain significant calmodulin-dependent Ca^{2+} - Mg^{2+} -ATPase activity and that this activity originates from the plasma membrane, as can be concluded from the M_r of its phosphoenzyme intermediate and its pH-sensitivity.

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