Hepatic microsomal Ca^{2+} -dependent ATPase

Calmodulin-dependence and partial purification

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The hepatic microsomal fraction contains tightly bound calmodulin as demonstrated by affinity chromatography. When this calmodulin was partially removed by EGTA treatment (0.5 mM-EGTA), the uptake of $^{45}\text{Ca}^{2+}$ by the microsomal vesicles was stimulated by added calmodulin and inhibited by trifluoperazine (TFP). The Ca^{2+} -dependent ATPase was partially purified on a calmodulin column. This partial purification resulted in a 500-fold increase in the specific activity of the enzyme when measured in the presence of added calmodulin. Antibodies prepared against calmodulin prevented this stimulatory effect. The fraction eluted from the calmodulin column contained several protein bands indicating that the specific activity of the Ca^{2+} -dependent ATPase is probably still underestimated. There are likely to be other calmodulin-sensitive processes present in the hepatic microsomal fraction.

The hepatic microsomal fraction, presumably derived from the endoplasmic reticulum, has been demonstrated to possess an ATP-dependent Ca^{2+} -sequestering system (Moore *et al.*, 1975; Bygrave, 1978; Dawson, 1982). This microsomal Ca^{2+} -ATPase is likely to be involved in the regulation of cellular calcium distribution. It has been shown that the microsomal fraction of the liver, together with mitochondria, can lower external free Ca^{2+} concentration to physiological levels (Becker *et al.*, 1980).

The hepatic microsomal Ca^{2+} -ATPase has been demonstrated to be hormone-sensitive. Glucagon stimulates the enzyme, whereas insulin is inhibitory (Andia-Waltenbaugh *et al.*, 1980; Taylor *et al.*, 1980). Glucocorticoids are inductive, although this is a delayed effect (Friedmann & Johnson, 1980). The hormone-sensitivity is an additional indication that the endoplasmic reticulum might participate in hormonally evoked calcium redistribution, which is often an integral part of the hormonal response.

The sarcoplasmic-reticulum Ca^{2+} -ATPase from cardiac tissue is analogous to the hepatic microsomal Ca^{2+} -ATPase, and has been shown to be regulated by both cyclic AMP- and calmodulin-dependent phosphorylation of specific proteins

(Kirchberger & Antonetz, 1982). A Ca^{2+} -ATPase present in the microsomal fraction of the rat parotid gland has also been recently described to be calmodulin-sensitive (Kanagasuntheram & Teo, 1982). In the liver, although addition of calmodulin to the microsomal fraction did not alter the activity of the Ca^{2+} -ATPase, the enzyme activity was sensitive to trifluoperazine as demonstrated by inhibition of $^{45}\text{Ca}^{2+}$ uptake. This suggested that calmodulin is involved in the regulation of this Ca^{2+} -ATPase (Famulski & Carafoli, 1982). The present paper reports that calmodulin is bound to the hepatic microsomal fraction. This characteristic was utilized for the partial purification of the Ca^{2+} -ATPase using a calmodulin affinity column.

Experimental

Chemicals

$^{45}\text{CaCl}_2$ was purchased from New-England Nuclear. Trifluoperazine was a gift from Smith, Kline and French Co. Adenosine 3'-phosphate (potassium salt) was from Sigma. All other reagents were of the purest quality available. Millipore filters (0.22 μm) were from Millipore Corporation. Asolectin was purchased from Associated Concentrates, Long Island, NY, U.S.A.

Abbreviation used: Ca^{2+} -ATPase, Ca^{2+} -dependent ATPase.

Preparation of microsomes

Livers from male fed Sprague–Dawley rats were excised and minced in ice-cold homogenization buffer containing 250 mM-sucrose, 10 mM-Hepes, 0.2 mM- CaCl_2 , pH 7.2, and homogenized in a loose-fitting motor-driven homogenizer with five strokes. Subsequently 100 mM-EGTA solution was added to give a final EGTA concentration of 0.05 mM. The microsomal fraction was then isolated by the protocol of Moore *et al.* (1975). The homogenate was first centrifuged at 3000 rev./min for 10 min in a Sorvall RC2-B centrifuge, followed by re-centrifugation for 20 min at 8000 rev./min. Finally the supernatant was centrifuged in a Beckmann L5-50 ultracentrifuge at 35000 rev./min for 60 min. The precipitate was resuspended in 120 mM-KCl/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 6.8, to give a final protein concentration of 20–30 mg/ml.

$^{45}\text{Ca}^{2+}$ uptake

$^{45}\text{Ca}^{2+}$ uptake was measured by the Millipore filtration technique. About 100–200 μg of protein/ml was incubated at 37°C in 1 ml of medium containing 100 mM-KCl, 20 mM-Hepes, 1 mM- NaN_3 , 1 mM- MgCl_2 , 3 mM-ammonium oxalate, 1 μM -Ruthenium Red and 100 μM - CaCl_2 containing $^{45}\text{Ca}^{2+}$, pH 6.8.

At a designated time a 100 μl sample was filtered through a 0.22 μm pre-wetted Millipore filter. The precipitate was washed with 120 mM-KCl/10 mM-Hepes, pH 6.8, transferred to a scintillation vial and counted for radioactivity.

Ca^{2+} -ATPase activity

The Ca^{2+} -ATPase activity was estimated in a reaction mixture containing 100 mM-KCl, 20 mM-Hepes, 1 mM- NaN_3 , 1 mM- MgCl_2 , 2 μM -Ruthenium Red and 2 mM-EGTA, pH 6.8, in the presence or in the absence of 3.6 mM- CaCl_2 . Protein was added (0.5–1 mg/assay). The volume of the incubation medium was 1 ml. The reaction was started by the addition of K^+ /ATP to give a final concentration of 1 mM. Incubation was carried out for 30 min in a Dubnoff shaking water bath at 37°C. At the end of the incubation the reaction was terminated by the addition of ice-cold 8% trichloroacetic acid. The difference in the amount of P_i released (Lanzetta *et al.*, 1979) in the presence and in the absence of Ca^{2+} was used for the calculation of enzyme activity. This assay system is a slight modification of the one previously used (Andia-Waltenbaugh *et al.*, 1980), which was shown to be linear with time and the protein concentration used.

Calmodulin preparation

Chicken gizzard calmodulin was prepared by the procedure described by Dedman *et al.* (1977) for rat testis calmodulin.

Calmodulin isolation from microsomes

Microsomes (4 ml) were extracted with 3 vol. of 75 mM-NaCl/40 mM-Tris/HCl, pH 7.3, also containing 2 mM-EDTA. The extract was centrifuged at 15000 rev./min in a Sorvall RC-5B centrifuge using the SS-34 rotor for 15 min. CaCl_2 was added to give a concentration of 4 mM and the extract was re-centrifuged at 15000 rev./min for 10 min. The clarified extract was applied to a fluphenazine–Sephacrose affinity column prepared by the procedure of Charbonneau & Cormier (1979). The column dimensions were 3.5 cm $\times$ 0.7 cm (5.4 ml of resin) and had the capacity to bind 6 mg of calmodulin per ml of resin. The column was washed with 500 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 2 mM- CaCl_2 . Bound protein was then eluted by washing the column with 75 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 2 mM-EGTA. Fractions were monitored by absorbance at 280 nm using a Gilford model 2600 spectrophotometer. The protein peak was pooled and dialysed against 5 mM-ammonium carbonate, pH 7.0, and freeze-dried for electrophoretic analysis.

Electrophoresis

The method described by Laemmli (1970) was followed with either a 9% or an 11% acrylamide lower running gel and a 3.5% acrylamide upper stacking gel. The gels were fixed in 7.5% acetic acid/10% propan-2-ol (final concentrations) for 2 h followed by staining with Coomassie Brilliant Blue R250 [7.5% acetic acid/0.025% stain/25% propan-2-ol (final concentrations)] overnight. Destaining was with the acetic acid/propan-2-ol mixture described.

Western blot analysis

Calmodulin presence in the microsomal extracts and in the EGTA-eluted material from the fluphenazine affinity matrix was determined by dot blot analysis using affinity-purified anti-calmodulin (Towbin *et al.*, 1979). Portions (40 μl) of the microsomal extract or the column effluent were spotted on 0.2 μm nitrocellulose paper. Standard chicken gizzard calmodulin was also spotted. Aldolase and microsomal extracts chromatographed on a calmodulin–Sephacrose affinity column were spotted as controls. The paper was washed with 5% bovine serum albumin solution in Dulbecco's phosphate-buffered saline for 2 h. The nitrocellulose paper was incubated with 100 μl of anti-calmodulin (0.3 A_{280} unit/ml) diluted to 40 ml with Dulbecco's phosphate-buffered saline containing 1 ml of bovine serum albumin for 4 h, followed by extensive washing with Dulbecco's phosphate-buffered saline. Colour was developed by incubation for 1 h with peroxidase-conjugated rabbit anti-sheep antibodies followed by reaction with 25 mg of *o*-dianisidine

hydrochloride in 100 ml of Dulbecco's phosphate-buffered saline containing 40 μl of 30% H_2O_2 . The paper was washed with water, dried and photographed.

Solubilization of the Ca^{2+} -ATPase by Triton X-100

The microsomal fraction was prepared as described above and resuspended in about 5 ml of 120 mM-KCl/20 mM-Hepes, pH 7.2. This was added to 20 ml of solution containing 120 mM-KCl, 20 mM-Hepes, 0.5 mM- MgCl_2 , 50 μM - CaCl_2 , 2 mM-dithiothreitol, 2 mM-EDTA and 75 mg of Triton X-100, and incubated with continuous stirring in the cold room for 30 min. At the end of the incubation a solution containing 1 mg of asolectin was added. Asolectin, a phospholipid mixture, has been shown to preserve the activity of other Ca^{2+} -ATPases during purification (Caroni & Carafoli, 1981). The suspension was centrifuged at 35 000 rev./min for 30 min in a Beckman L5-50 ultracentrifuge. The supernatant containing the solubilized Ca^{2+} -ATPase was subsequently applied to a calmodulin affinity column.

Enzyme isolation by affinity chromatography

The microsomal Triton X-100 extract was adjusted to a total final calcium concentration of 2.1 mM and directly applied to a calmodulin-coupled Sepharose 4B-CL affinity column. Column dimensions were 5.4 cm $\times$ 1.0 cm and contained 5 mg of calmodulin bound per ml of resin. The column was washed with 120 mM-KCl/20 mM-Hepes, pH 6.85, containing 0.1 mM- CaCl_2 and 0.4% Triton X-100 until the absorbance at 280 nm returned to that of the buffer. The column was then washed with 120 mM-KCl/20 mM-Hepes buffer, pH 6.85, containing 2 mM-EGTA and 0.4% Triton X-100 to elute bound protein. The protein content in the eluted fractions was monitored by absorbance at 280 nm and by the Bradford (1976) protein assay. Enzyme activity was determined in selected fractions. Those fractions containing Ca^{2+} -ATPase activity were pooled, dialysed as described above, freeze-dried and analysed by gel electrophoresis.

Anti-calmodulin isolation

Antibody to rat testis calmodulin, prepared in sheep and affinity-purified by the method reported by Dedman *et al.* (1978), was a gift from Dr. J. R. Dedman, Departments of Medicine and Physiology and Cell Biology, University of Texas Medical School at Houston.

Results

Binding of calmodulin to microsomes

If calmodulin does play a role in the regulation of the microsomal Ca^{2+} -ATPase, then it seemed

probable that it would be found tightly associated with the microsomal fraction. Microsomal fraction was therefore prepared with 0.5 mM-EGTA treatment to remove superficially bound calmodulin. The material was re-extracted and applied to a fluphenazine-Sepharose affinity column, which has been shown to bind calmodulin (Charbonneau & Cormier, 1979); Fig. 1 shows the results. The microsomal fraction clearly contains a major

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of EDTA-extracted microsomes. Lane 1 shows EDTA-extracted microsomes chromatographed on the fluphenazine-Sepharose affinity column eluted by EGTA and electrophoresed on a 9% sodium dodecyl sulphate/polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R250 before photography; 10 μg of protein were loaded on the gel. Lane 2 shows 16 μg of purified standard chicken gizzard calmodulin electrophoresed similarly for comparison.

Coomassie Brilliant Blue R250 staining protein that migrates identically with authentic chicken gizzard calmodulin. To confirm further the presence of calmodulin in the microsomal extracts, a Western blot analysis was performed. As shown in Fig. 2, immunoreactive dots were observed for both the microsomal extract as well as the EGTA-eluted material from the fluphenazine–Sephacryl affinity column. No immunoreactive spot developed for the material bound and eluted from a calmodulin–Sephacryl affinity matrix or for aldolase.

Calmodulin sensitivity of $^{45}\text{Ca}^{2+}$ uptake by microsomal vesicles

Because partial removal of calmodulin seemed likely to reveal the calmodulin sensitivity of the Ca^{2+} -ATPase, 0.5 mM-EGTA was included in the medium used for the preparation of the microsomal vesicles. Indeed these vesicles (Fig. 3) showed a more than 2-fold stimulation of $^{45}\text{Ca}^{2+}$ uptake upon the addition of calmodulin at physiological concentration. In some of the preparations $^{45}\text{Ca}^{2+}$ -uptake was entirely calmodulin-dependent, because no uptake was observed in the absence of added calmodulin. Thus, in some preparations the EGTA treatment depleted the vesicles of more calmodulin than in others.

In agreement with a previous report (Famulski & Carafoli, 1982) the anti-calmodulin drug trifluoperazine decreased the uptake of Ca^{2+} . This suggests that calmodulin still present in the vesicles was responsible for the low rate of uptake observed. This

Fig. 2. Western blot analysis of microsomal EDTA extract before chromatography and elution from the fluphenazine affinity matrix

(1), 40 μl of fluphenazine-column-eluted microsomal proteins; (2), 40 μl of microsomal proteins eluted from a calmodulin–Sephacryl column; (3), 40 μl of microsomal extract before chromatography; (4), (5) and (6), 40, 80 and 120 μl respectively of standard chicken gizzard calmodulin (0.09 mg/ml); (7), 40 μl of aldolase (1 mg/ml).

observation is in agreement with the data in Figs. 1 and 2 showing that the microsomal fraction does contain tightly bound calmodulin.

The inhibitory effect of trifluoperazine could best be demonstrated in vesicles into which calmodulin was reincorporated (Fig. 4). In such a system dose-dependent inhibition of $^{45}\text{Ca}^{2+}$ uptake by trifluoperazine was seen.

Partial purification of the Ca^{2+} -ATPase

The calmodulin sensitivity of the microsomal Ca^{2+} -ATPase suggested the feasibility of isolating the enzyme on a calmodulin affinity column as has been achieved with other calmodulin-sensitive Ca^{2+} -ATPase enzymes, e.g. from the red blood cell (Niggli

Fig. 3. Effects of calmodulin (CaM) and trifluoperazine (TFP) on microsomal $^{45}\text{Ca}^{2+}$ uptake

$^{45}\text{Ca}^{2+}$ uptake was measured as described in the Experimental section. Time zero represents the amount of Ca^{2+} bound to the vesicles when incubated for 1 min before the addition of ATP. At zero time ATP was added to give a final concentration of 1 mM. $\circ$, Basal uptake, with no addition. Calmodulin was included in the incubation medium at a concentration of 1 $\mu\text{g}/\text{ml}$. Trifluoperazine was included in the medium at a final concentration of 1 μM . Bars represent S.E.M.; four experiments were performed.

et al., 1979) and from cardiac sarcolemma (Caroni & Carofoli, 1981). In Fig. 5 the result of one such isolation is presented. Most of the protein that entered the column was eluted with Ca²⁺, indicating a lack of binding to the calmodulin-Sephadex matrix. This fraction contained minimal Ca²⁺-ATPase activity. The major peak of Ca²⁺-ATPase, however, was eluted with EGTA. Indeed, the calmodulin-sensitivity of the enzyme was most apparent in this fraction. Calmodulin increased the activity of the enzyme almost 10-fold, and antibodies prepared against calmodulin prevented this stimulation (Table 1).

The partial purification process greatly increased the specific activity of the enzyme. The activity of the native enzyme was 1.1 μmol/mg of protein per h and increased by the addition of calmodulin to 1.7 μmol of P_i formed/mg of protein per h. The activity of the fraction eluted with EGTA from the

Fig. 4. Dose-dependent inhibitory effect of trifluoperazine (TFP) on calmodulin-stimulated ⁴⁵Ca²⁺ uptake in microsomes

⁴⁵Ca²⁺ uptake was measured in the presence of calmodulin (CaM; 1 μg/ml) and two concentrations of trifluoperazine. Bars represent S.E.M.; four experiments were performed.

calmodulin affinity column was 50 μmol of P_i/mg of protein per h. Addition of calmodulin increased the specific activity of the enzyme to 565 μmol of P_i/mg of protein per h.

The fractions containing the calmodulin-dependent high-specific-activity Ca²⁺-ATPase were pooled, protein-precipitated by cold acetone to remove Triton X-100, dialysed and applied to a sodium dodecyl sulphate/polyacrylamide gel in order to visually detect its components. Upon staining (Fig. 6) it became evident that the pooled fractions contained several proteins, including the Ca²⁺-ATPase, but it has yet to be clarified which band corresponds to the enzyme.

Fig. 5. Affinity chromatography of Triton X-100-solubilized microsomes on a calmodulin-coupled Sephadex-4B-CL affinity column (as described in the Experimental section)

----, Protein concentration as determined by the Bradford assay. —, Ca²⁺-ATPase activity assayed in the presence of added calmodulin (1 μg/ml)., ⁴⁵Ca²⁺-ATPase activity measured in the absence of added calmodulin.

Table 1. Effects of calmodulin and calmodulin antibody on Ca²⁺-ATPase activity purified by affinity chromatography

Results are means ± S.E.M. Ca²⁺-ATPase activity purified by affinity chromatography was assayed in four tubes containing EGTA-buffer-eluted fractions and the results were pooled. The concentrations of calmodulin was 1 μg/ml and that of calmodulin antibody 95 μg/ml.

Activity (μmol of P _i /mg of protein per h)		
Basal activity	+ Calmodulin	+ Calmodulin antibody
32 ± 6	180 ± 21	77 ± 8

Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of calmodulin-Sepharose-binding proteins extracted from isolated microsomes

Microsomes were extracted with Triton X-100 as described in the Experimental section and the extract applied to a calmodulin-Sepharose affinity column. Protein eluted from the matrix with 2 mM-EGTA was precipitated with 5 vol. of acetone (at -20°C), redissolved in Laemmli buffer (Laemmli, 1970) and electrophoresed on an 11% sodium dodecyl sulphate/polyacrylamide gel. Molecular-weight markers are: 220 000, myosin heavy chain; 95 000, 1-actinin; 68 000, bovine serum albumin; 43 000, actin; 17 000, calmodulin. Values shown in the Figure are $10^{-3} \times \text{mol.wt.}$

Discussion

The results presented here demonstrate that the hepatic microsomal Ca^{2+} -ATPase is regulated by

calmodulin. This conclusion is supported by the following observations. (1) The microsomal fraction contains bound calmodulin. (2) Addition of calmodulin to partially calmodulin-depleted vesicles increases $^{45}\text{Ca}^{2+}$ uptake and Ca^{2+} -ATPase activity by the vesicles. (3) Trifluoperazine and calmodulin antibody inhibit the effects of calmodulin. (4) It was possible to partially purify the enzyme on a calmodulin column.

The calmodulin dependence of the hepatic microsomal Ca^{2+} -ATPase is similar to reports for the Ca^{2+} -ATPases isolated from the parotid gland (Kanagasuntheram & Teo, 1982) and cardiac sarcoplasmic reticulum (Kirchberger & Antonetz, 1982). Our data also agree with the previous observation that trifluoperazine inhibits the hepatic microsomal Ca^{2+} -ATPase (Famulski & Carafoli, 1982).

The regulatory effect of calmodulin on the hepatic microsomal Ca^{2+} -ATPase is another characteristic that distinguishes it from the hepatic plasma-membrane Ca^{2+} -ATPase, which is not influenced by calmodulin but possesses another activator (Lotersztajn *et al.*, 1981). Thus, the plasma-membrane and the microsomal Ca^{2+} -ATPases might be simultaneously but separately regulated.

According to our data (Fig. 6) the effluent eluted from the calmodulin affinity column, which contained the Ca^{2+} -ATPase activity, apparently had only three major proteins present. Thus considerable purification of the Ca^{2+} -ATPase was achieved by calmodulin affinity chromatography. It is unclear at present if the Ca^{2+} -ATPase is represented by the band at mol.wt. 95 000–100 000 only or if the enzyme is composed of several subunits. Other Ca^{2+} -ATPases (MacLennan & Holland, 1975; Van Winkle *et al.*, 1978) have been shown to have a molecular weight on sodium dodecyl sulphate/polyacrylamide gels of nearly 100 000. Thus, the band at this molecular weight may in fact be the Ca^{2+} -ATPase. Other calmodulin acceptor proteins present in the extract from the microsomal fraction may represent additional calmodulin-sensitive enzymes or play a role in regulation of the Ca^{2+} -ATPase *in situ*.

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