Some properties of the Ca^{2+} -stimulated ATPase of a rat liver microsomal fraction

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1. The heavy microsomal fraction from rat liver apparently has very little Ca^{2+} -stimulated ATPase activity, although it has an active, ATP-driven Ca^{2+} accumulation system. 2. The addition of ionophore A23187 to the ATPase assay, to allow continuous Ca^{2+} recycling during the assay time, reveals the presence of a substantial Ca^{2+} -stimulated ATPase with V_{max} 160 nmol of P_i /10 min per mg of protein and K_m for Ca^{2+} 0.19 μM . 3. The Ca^{2+} -stimulated ATPase, but not the basal Mg^{2+} -stimulated ATPase, is potently inhibited by orthovanadate. Both the Ca^{2+} -stimulated ATPase and the vanadate inhibition are enhanced by the presence of Mg^{2+} . 4. Ca^{2+} -stimulated ATPase activity is not responsive to calmodulin or the calmodulin antagonist trifluoperazine.

The ATP-dependent Ca^{2+} accumulation system of rat liver microsomal fractions has been extensively studied in recent years because of its possible role in control of cytoplasmic Ca^{2+} concentration and involvement in hormonal control mechanisms (Moore *et al.*, 1975; Bygrave, 1978; Andia-Waltenbaugh *et al.*, 1980; Reinhart & Bygrave, 1981; Dawson, 1982). However, relatively little work has been done on the Ca^{2+} -stimulated ATPase activity of rat liver microsomes. Moore *et al.* (1975) and Andia-Waltenbaugh *et al.* (1980) reported the presence of a Ca^{2+} -stimulated ATPase activity in this fraction, but in both cases the extra ATPase activity found in the presence of Ca^{2+} was very small compared with the basal Mg^{2+} -stimulated ATPase, making detailed investigation difficult.

A possible explanation for the low levels of Ca^{2+} -stimulated ATPase activity found in liver microsomes stems from the work of Bygrave (1978) and Dawson (1982) on the time course of Ca^{2+} accumulation into microsomal vesicles. In the absence of a precipitating anion such as oxalate, there is an initial rapid Ca^{2+} accumulation ($V_0 = 16 \text{ nmol of Ca}^{2+}/\text{min per mg}$), followed by a transition into a steady-state level of Ca^{2+} accumulation where the microsomal Ca^{2+} content remains constant at about 20 nmol/mg. In this steady-state phase, the rate of ATP-driven Ca^{2+} accumulation is balanced by the rate of passive efflux of Ca^{2+} from the vesicles. However, the rate of passive efflux from microsomes under these conditions was found to be rather low (about 3 nmol of

$\text{Ca}^{2+}/\text{min per mg}$ at pH 6.8 and an internal Ca^{2+} load of 20 nmol/mg; Dawson, 1982). This suggests that the rate of ATP-driven Ca^{2+} accumulation decreases from an initial rate of 16 nmol/min per mg to a steady-state turnover rate of about 3 nmol/min per mg as the internal Ca^{2+} concentration in the vesicles increases with, presumably, a corresponding decrease in the rate of ATP hydrolysis. Niggli *et al.* (1981) found that the activity of the red blood cell $\text{Ca}^{2+}/\text{Mg}^{2+}$ -stimulated ATPase, after incorporation into liposomes, was greatly increased by the presence of ionophore A23187 to prevent the build-up of a Ca^{2+} concentration gradient. It seemed likely that the presence of the ionophore might also release the thermodynamic or kinetic restraint operating on the liver microsomal ATPase.

We report here that, in the presence of A23187, there is a readily detectable level of Ca^{2+} -stimulated ATPase activity present in the rat liver heavy microsomal fraction (as defined by Reinhart & Bygrave, 1981). The ATPase shows half-maximal activation by about 0.2 μM free Ca^{2+} and its activity is enhanced by the presence of Mg^{2+} . It is potently inhibited by vanadate but is apparently insensitive to calmodulin or the calmodulin antagonist trifluoperazine.

Experimental

Materials

$^{45}\text{CaCl}_2$ (650 Ci/mol) was obtained from Amersham International, Amersham, Bucks., U.K. Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Hopkin and Williams and

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Ruthenium Red was from BDH, Poole, Dorset, U.K. Ruthenium Red was purified by the method of Fletcher *et al.* (1961). A23187 was from Calbiochem and calmodulin was purified from human red cells by the method of Muallem & Karlish (1979). Trifluoperazine was from Sigma.

Preparation of microsomes

The heavy microsomal fraction was prepared from the livers of 200–250g male albino Wistar rats by the method of Reinhart & Bygrave (1981) and the protein concentration was determined as described previously (Dawson, 1982).

ATPase assay

The ATPase activity of the microsomal fraction was determined by measurement of P_i release from ATP in the presence of oligomycin to inhibit any contaminating mitochondrial ATPase. The temperature was 37°C. The incubation mixture contained, in a total volume of 1ml:100mM-KCl; 20mM-Hepes/KOH buffer, pH 6.8; 5mM-MgCl₂, 1µg of oligomycin; 1µg of A23187; CaCl₂ and EGTA, to give the required free Ca²⁺ concentration and about 0.7mg of microsomal protein. After pre-incubation at 37°C for 2 min, the reaction was started by the addition of 1mM-ATP. After 10min the assays were stopped by the addition of 0.5ml of 5% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and $[P_i]$ measured on a 1ml sample of the supernatant by the method of Fiske & SubbaRow (1925). Total Ca²⁺ content of the assay mixtures was measured, after precipitation with trichloroacetic acid, by atomic-absorption spectrophotometry. Free [Ca²⁺] was determined as described previously (Dawson, 1982).

Ca²⁺-uptake assays

These were done as described by Dawson (1982), except that the incubation medium was the same as that given for the assay of ATPase activity, with the addition of 1µCi of ⁴⁵Ca²⁺ and 1µM-Ruthenium Red and the omission of A23187.

Results

Detection of a Ca²⁺-stimulated ATPase activity

Fig. 1 shows the variation in microsomal ATPase activity with change in free Ca²⁺ concentration under several different experimental conditions. In agreement with the observations of Andia-Waltenbaugh *et al.* (1980), there is only a very small increment in ATPase activity on changing the free Ca²⁺ concentration from 0.01 to 1.0µM in the absence of oxalate and A23187. The addition of 5mM-oxalate to the incubation medium causes an apparent increase in the basal Mg²⁺-stimulated ATPase activity but also reveals the presence of a Ca²⁺-stimulated ATPase activity, amounting to

Fig. 1. Effects of oxalate and A23187 on ATPase activity

ATPase activity was determined as described in the Experimental section with the following modifications to the basic medium: ○, no A23187; ●, plus 1µg of A23187/ml; △, no A23187, plus 5mM-ammonium oxalate; ▲, plus 1µg of A23187/ml and 5mM-ammonium oxalate. The protein concentration was 0.9mg/ml. For the first datum point on each line, the total Ca²⁺ concentration was 18µM and EGTA was 1mM. For the remaining points, total Ca²⁺ was 81µM and EGTA was (in mM) 1, 0.62, 0.44, 0.31, 0.19, 0.1, 0.06, to give the free Ca²⁺ concentrations shown. The method for calculating free Ca²⁺ concentrations allows for formation of a Ca²⁺-oxalate complex.

about 50nmol of P_i /10min per mg, when the free [Ca²⁺] is increased from 0.01 to 1.0 µM. The presence of oxalate allows microsomal Ca²⁺ accumulation to continue throughout the assay time, although at a lower rate than observed during the first 90s of the progress curve (Bygrave, 1978; Dawson, 1982). The effect of oxalate on the basal Mg²⁺-stimulated ATPase is probably due to chelation of Mg²⁺ by oxalate. Increasing the Mg²⁺ concentration in the assay medium from 2 to 5mM in the absence of oxalate causes a small inhibition of the basal ATPase activity (results not shown). The addition of 5mM-oxalate to the medium decreases the free Mg²⁺ concentration from 4mM to 2mM and will therefore relieve the inhibition by high Mg²⁺.

The addition of A23187, in either the presence or the absence of oxalate, reveals the presence of a substantial Ca²⁺-stimulated ATPase activity. In the experiment shown in Fig. 1, this amounts to about 140nmol of P_i /10min per mg of protein. The increment in ATPase activity on increasing the free Ca²⁺ concentration from 0.01µM to 1.0µM is the same, irrespective of the presence or absence of oxalate, although, as discussed above, the basal Mg²⁺-stimulated ATPase activity is different in the two cases. For eight different microsome preparations, the ATPase activities were as follows (means ± S.E.M.): Mg²⁺-stimulated ATPase (at

Fig. 2. Effects of pH on ATPase activity

The composition of the assay medium was as described in the Experimental section, except that the pH was adjusted to the required value with KOH. The protein concentration was 0.8 mg/ml. In all cases the total Ca^{2+} concentration was 11 μM . (a) ATPase activity, in the presence (○) and absence (●) of 1 mM-EGTA. Because of the pH-dependence of the association constant for Ca^{2+} and EGTA, the free Ca^{2+} concentration in (○) varied from 90 nM at pH 6.2 to 0.13 nM at pH 7.9. For (●) the corresponding value is 10.1 μM at both pH values. (b) Ca^{2+} -stimulated ATPase activity. The difference between the two curves in (a) is plotted.

0.01 μM - Ca^{2+}), $0.45 \pm 0.02 \mu\text{mol}$ of P_i /10 min per mg; Mg^{2+} / Ca^{2+} -stimulated ATPase (at 1.0 μM - Ca^{2+}) $0.61 \pm 0.03 \mu\text{mol}$ of P_i /10 min per mg; the paired difference for the eight preparations, giving the Ca^{2+} -stimulated ATPase activity, was $0.16 \pm 0.01 \mu\text{mol}$ of P_i /10 min per mg. The free Ca^{2+} concentration giving half-maximal stimulation of the ATPase was $0.19 \pm 0.04 \mu\text{M}$ ($n = 4$). The comparative data for Ca^{2+} accumulation reported previously (Dawson, 1982) are V_{max} , 16 nmol/min per mg and K_m 0.2 μM for free Ca^{2+} .

Fig. 2 shows the pH profile of the Ca^{2+} -stimulated ATPase activity. In agreement with the data for pH-dependence of Ca^{2+} -accumulation (Dawson, 1982), the optimum pH is about 7.0, with a steep decline in activity below pH 6.8 but a more gradual decline on the alkaline side. In contrast, the Mg^{2+} -stimulated ATPase at low free $[\text{Ca}^{2+}]$ shows a very weak pH-dependence over this range.

Effects of vanadate and Mg^{2+} on ATPase activity

Orthovanadate has been found to be a potent inhibitor of various ATPase systems, including the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase (Josephson & Cantley, 1977) and the Ca^{2+} -translocating ATPases of sarcoplasmic reticulum (Wang *et al.*, 1979), red blood cell membranes (Bond & Hudgins, 1980; Niggli *et al.*, 1981), heart sarcolemma (Caroni & Carafoli, 1981) and intestinal smooth-muscle microsomes (Wibo *et al.*, 1981). Fig. 3 shows the effect of orthovanadate, in the presence of 5 mM- Mg^{2+} , on liver microsomal ATPase activity in the presence and absence of Ca^{2+} . In the absence of Ca^{2+} ($< 0.01 \mu\text{M}$ free) there is very little effect of vanadate on ATPase activity. However, the Ca^{2+} -stimulated activity is very potently inhibited by vanadate, addition of 0.5 μM -vanadate causing 50% inhibition. Fig. 3 also shows that under similar conditions, but in the absence of A23187, the initial rate of Ca^{2+} accumulation is inhibited by vanadate to the same extent as ATPase activity. Ca^{2+} accumulation measured over a longer time period is less affected by vanadate, suggesting that a high intravesicular Ca^{2+} concentration reverses the inhibition.

As in the case of the Ca^{2+} / Mg^{2+} -stimulated ATPase of red blood cell membranes (Bond & Hudgins, 1980; Niggli *et al.*, 1981) and sarcoplasmic reticulum (Pick, 1982), inhibition by vanadate is dependent on the presence of Mg^{2+} (Fig. 4), maximal inhibition being obtained at about 3 mM- Mg^{2+} . Fig. 4 also shows that added Mg^{2+} enhances the activity not only of basal ATPase, observed at low free Ca^{2+} concentrations, but also of the Ca^{2+} -stimulated ATPase activity. The dependence of Ca^{2+} -stimulated ATPase activity on Mg^{2+} concentration appears to be very similar to that for the basal ATPase.

Effects of calmodulin and trifluoperazine

Calmodulin has been shown to have a direct effect on the activity of Ca^{2+} -transporting ATPase systems from a variety of sources, such as red blood cells (Gopinath & Vicenzi, 1977; Jarrett & Penniston, 1977), heart sarcolemma (Caroni & Carafoli, 1981) and parotid endoplasmic reticulum (Kanasuntheram & Teo, 1982). In view of the possibility of liver endoplasmic reticulum playing a part in controlling cytoplasmic Ca^{2+} concentration (Berthon *et al.*, 1981) it was important to investigate the effects of calmodulin on the liver microsomal Ca^{2+} -stimulated

Fig. 3. Effects of vanadate on ATPase activity and Ca²⁺ accumulation

The ATPase assay and Ca²⁺ accumulation measurements were as described in the Experimental section, except that the microsomes were pre-incubated at a concentration of 0.9 mg/ml in the assay medium with the required vanadate concentration for 12 min before addition of ATP. (a) ATPase activity: ○, plus 1 mM-EGTA and 28 μM-Ca²⁺ (free [Ca²⁺] = 16 nM); ●, plus 0.1 mM-EGTA and 77 μM-Ca²⁺ (free [Ca²⁺] = 1.76 μM). (b) Comparative effects on ATPase and Ca²⁺ accumulation. ○, Percentage activity of Ca²⁺-stimulated ATPase; ●, percentage activity of Ca²⁺ accumulation measured 20 s after initiation of uptake with ATP; Δ, percentage activity of Ca²⁺ accumulation measured 45 s after initiation of uptake with ATP. For the uptake measurements, conditions were as for (a), with a free Ca²⁺ concentration of 1.76 μM, with the omission of A23187 but with the addition of 1 μCi of ⁴⁵Ca²⁺/ml and 1 μM-Ruthenium Red.

Fig. 4. Dependence of ATPase activity and vanadate inhibition on Mg²⁺ concentration

(a) Total ATPase activity. Microsomes (0.7 mg/ml) were pre-incubated for 10 min in the presence (Δ and ▲) or absence (○ and ●) of 2 μM-orthovanadate before ATP addition. The ATPase assay medium was the same as that given in the Experimental section, with various Mg²⁺ concentrations and Ca²⁺ concentrations of: ○ and Δ, 0.008 μM (1 mM-EGTA, 14.5 μM-Ca²⁺ total); ● and ▲, 0.84 μM (0.1 mM-EGTA, 65 μM-Ca²⁺ total) at no added Mg²⁺, changing to 0.96 μM-Ca²⁺ at 3 mM-Mg²⁺. (b) Ca²⁺-stimulated ATPase activity: ○, no vanadate present; ●, plus 2 μM-vanadate. The points (○) are derived from the data points (● minus ○) in (a), and the points (●) are derived from the data points (▲ minus Δ) in (a).

ATPase. Pre-incubation of microsomes with 5 μg of human red cell calmodulin/ml has no effect on the Ca²⁺-stimulated ATPase activity over the Ca²⁺ concentration range used in Fig. 1. However, it has

been reported that rat liver microsomes contain endogenous calmodulin (Carafoli *et al.*, 1980). We have found that after boiling the microsomes used in this study for 10 min at 5 mg of protein/ml in 5 mM-

Hepes/KOH, pH 6.8, the supernatant contains a calmodulin-like activity that is equivalent in its effect on the red-cell Ca^{2+} -stimulated ATPase to about $1\mu\text{g}$ of calmodulin/ml of supernatant. To remove endogenous calmodulin a procedure based on that described by Caroni & Carafoli (1981) was used. The microsomes were extracted with 1 mM-EGTA/5 mM-Hepes/KOH (pH 6.8), followed by centrifugation and resuspension in 1 M-NaCl/1 mM-EGTA/5 mM-Hepes/KOH (pH 6.8). After centrifugation, the microsomes were put through the same two extraction media again and were finally washed and resuspended in 250 mM-sucrose/10 mM-KCl 1 mM-dithiothreitol 5 mM-Hepes/KOH (pH 6.8). This treatment decreased the endogenous calmodulin-like activity by >70% compared with the original microsomes. The specific activity of the basal ATPase ($\text{Ca}^{2+} = 0.01\mu\text{M}$) is increased approx. 2-fold by the washing procedure to $0.85\mu\text{mol}$ of P_i /10 min per mg, presumably by removal of extrinsic membrane proteins. However, the Ca^{2+} -stimulated component of the ATPase is very similar in size to that in untreated microsomes ($0.15\mu\text{mol}$ of P_i /10 min per mg) indicating that, relative to the basal ATPase, approximately half of the Ca^{2+} -stimulated activity has been lost. Even though the extraction apparently causes an inactivation of the Ca^{2+} -stimulated ATPase, addition of $5\mu\text{g}$ of calmodulin/ml has essentially no effect on its activity, suggesting that the loss of Ca^{2+} -stimulation is not connected with the removal of endogenous calmodulin. Furthermore, the calmodulin antagonist trifluoperazine, at concentrations up to $100\mu\text{M}$, has no effect on the Ca^{2+} -stimulated ATPase activity of non-extracted microsomes over the Ca^{2+} concentration range used in Fig. 1. Trifluoperazine causes a small decrease in the ATPase activity in the absence of Ca^{2+} , but at the concentrations of inhibitor used this is likely to be a non-specific effect.

Discussion

The ATPase activity of rat liver microsomes appears to contain at least two components. There is an Mg^{2+} -stimulated ATPase with a specific activity of $0.45\mu\text{mol}$ of P_i /10 min per mg of protein and an additional, smaller, Ca^{2+} -stimulated activity with a specific activity of $0.16\mu\text{mol}$ of P_i /10 min per mg of protein, observable in the presence of A23187 to prevent the development of a Ca^{2+} concentration gradient. Although both activities have similar dependence on Mg^{2+} concentration, they appear to reflect the presence of two different enzymes, since the pH profiles of the two are different and the Ca^{2+} -stimulated activity is potently inhibited by vanadate, whereas the Ca^{2+} -independent activity is not. Although the function of the Ca^{2+} -independent ATPase is obscure, the Ca^{2+} -stimulated ATPase

seems to be involved in Ca^{2+} pumping across the microsomal membrane. The pH-dependence, inhibition by vanadate and K_m for Ca^{2+} of the ATPase are all very similar to the equivalent parameters measured for Ca^{2+} -uptake by microsomes (Dawson, 1982). The V_{max} of 160nmol of P_i /10 min per mg, taken with the V_{max} for Ca^{2+} -uptake (16nmol of Ca^{2+} /min per mg), suggests a stoichiometry of 1Ca^{2+} /ATP, similar to that reported by Niggli *et al.* (1981) for the erythrocyte membrane Ca^{2+} / Mg^{2+} -stimulated ATPase.

Apart from its insensitivity to calmodulin, other properties of the enzyme, such as the potency of vanadate inhibition, pH profile and K_m for Ca^{2+} are very similar to the equivalent properties, tabulated by Caroni & Carafoli (1981), for the heart sarcolemma and erythrocyte plasma-membrane Ca^{2+} / Mg^{2+} -stimulated ATPases. In contrast, Ca^{2+} / Mg^{2+} -stimulated ATPases derived from sarcoplasmic reticulum or analogous structures show a higher K_i for vanadate and a somewhat higher K_m for Ca^{2+} (Caroni & Carafoli, 1981; Wibo *et al.*, 1981). To this extent it would thus appear possible that the liver microsomal Ca^{2+} -transport system was derived from plasma-membrane fragments. A Ca^{2+} -stimulated ATPase has been detected in liver plasma membranes (Lotersztajn *et al.*, 1981; Iwasa *et al.*, 1982) but in native membranes this is only observable in the absence of added Mg^{2+} . The purified Ca^{2+} -stimulated ATPase from plasma membranes was found to be Mg^{2+} -dependent but had a very high affinity for Ca^{2+} ($K_{0.5} = 13\text{ nM-Ca}^{2+}$; Lotersztajn *et al.*, 1981) compared with the value reported here for microsomes. The plasma-membrane Ca^{2+} -stimulated ATPase studied by Iwasa *et al.* (1982) also showed a rather higher affinity for Ca^{2+} , was apparently Mg^{2+} -independent and was not inhibited by vanadate (albeit in the absence of added Mg^{2+}). The Ca^{2+} -stimulated ATPase of rat liver microsomes appears therefore to differ in some important respects from the rat liver plasma-membrane enzyme and hence is likely to originate from a different, presumably intracellular, membrane type.

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