Coupling of ATP Synthesis to Reversal of Rat Liver Microsomal Ca^{2+}-ATPase†

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ABSTRACT: The reversal of the rat liver microsomal Ca^{2+}-ATPase transport cycle was studied. Microsomes were loaded with $^{45}Ca^{2+}$ (~30 nmol/mg of protein) in an ATP-dependent process, and the time dependency of the microsomal $^{45}Ca^{2+}$ efflux was determined with various ADP and inorganic phosphate (P_i) concentrations. Pseudo-first-order rate constants (K'_e) for $^{45}Ca^{2+}$ efflux were determined. Although there was considerable $^{45}Ca^{2+}$ efflux in the absence of added ADP or P_i, the addition of ADP or P_i alone had minimal effects upon the K'_e; in contrast, a 2.5-fold increase in the K'_e was observed in the presence of both ADP and P_i. The apparent K_m values for ADP and P_i were 4 μM and 0.22 mM, respectively. Stimulation of $^{45}Ca^{2+}$ efflux by ADP and P_i was associated with ATP synthesis. The calcium ionophore A23187 prevented ATP synthesis, which indicates that the Ca^{2+} gradient facilitates the coupling of ATP synthesis to Ca^{2+} efflux.

The rat liver endoplasmic reticulum Ca^{2+}-ATPase plays an integral role in the maintenance of a low intracellular Ca^{2+} (Ca^{2+}_i) concentration (Becker et al., 1980; Murphy et al., 1980). The Ca^{2+}-ATPase has been implicated as a primary cellular target for several hepatotoxins, which are believed to induce toxic cell injury through alterations in intracellular Ca^{2+} homeostasis (Schanne et al., 1979; Farber, 1981; Jewell et al., 1982; Trump & Berezesky, 1984). Recent studies suggest that the endoplasmic reticulum or microsomal Ca^{2+} pool is the source of Ca^{2+}_i released upon α_1-agonist, vasopressin, angiotensin II, and glucagon stimulation and that *d*-myoinositol 1,4,5-triphosphate may serve as a messenger for these hormonal stimuli (Joseph et al., 1984; Berridge, 1984; Berridge & Irvine, 1984).

Despite the apparent physiological importance of the Ca^{2+}-ATPase in the Ca^{2+}_i homeostasis of rat hepatocytes, little information about the physical properties and mechanism of this ATPase is available. The proposed mechanism of the rat liver microsomal Ca^{2+}-ATPase is consistent with the ionmotive E_1E_2ATPase mechanism found in both the sarcoplasmic reticulum (deMeis & Tume, 1977) and the erythrocyte (Chiesi et al., 1984) Ca^{2+}-ATPases. The findings of a hydroxylamine-sensitive phosphoenzyme (Heilman et al., 1984), of inhibition of Ca^{2+}-ATPase activity by intravesicular Ca^{2+} accumulation (Brattin & Waller, 1983), and of enhanced phosphoenzyme dephosphorylation by Mg^{2+} (Heilmann et al., 1984) are consistent with the E_1E_2ATPase mechanism. The complete reversibility of the transport cycle with ATP synthesis coupled to the Ca^{2+} efflux, as observed with the sarcoplasmic reticulum Ca^{2+}-ATPase (Panet & Selinger, 1972; Hasselbach, 1978), is characteristic of this mechanism.

To further our understanding of the mechanism of the rat liver microsomal Ca^{2+}-ATPase and its role in intracellular Ca^{2+} homeostasis, the reversibility of the enzyme transport cycle was studied. We report that, although passive Ca^{2+} efflux is observed, Ca^{2+} efflux is associated with ATPase reversal and that ATP synthesis is coupled to this efflux.

Materials and Methods

Materials

[^{32}P]P_i[1] (1.0 Ci/mmol) was obtained as the potassium salt from New England Nuclear Co. (Boston, MA). Adenosine 5′-triphosphate (disodium salt) and adenosine 5′-diphosphate (sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). A23187 was a generous gift from Dr. W. R. Fields, Eli Lilly and Co., Indianapolis, IN. All other chemicals were of reagent grade and were obtained from commercial sources.

Methods

Microsome Preparation. Hepatic microsomes were prepared as previously described (Anders, 1968) from male Long Evans rats (Charles River Breeding Laboratories, Kingston, NY) weighing 250–350 g. Briefly, the rats were killed by decapitation, and the livers were perfused and then homogenized as a 20% solution (w/v) in ice-cold homogenization buffer (80 mM KCl, 50 mM PIPES, 5.0 mM NaN_3, and 1.0 mM $MgCl_2$, pH 6.8) with a Potter Elvehjem tissue homogenizer. The homogenate was centrifuged at 9000*g* for 20 min (4 °C), and the supernatant was filtered through gauze and centrifuged at 100000*g* (4 °C) for 60 min. The resultant microsomal pellet was resuspended with homogenization buffer to a concentration of 5–15 mg of protein/mL. Protein concentrations were determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Microsomal Ca^{2+} Influx and Efflux Determinations. Microsomes were loaded with $^{45}Ca^{2+}$ by incubating microsomes (~0.25 mg of protein/mL) in a buffer consisting of 50 mM PIPES, 5.0 mM NaN_3, 5.0 mM MgATP, and $^{45}CaCl_2$ (0.1–0.2 μCi), at pCa^{2+} 5.30, pH 6.8, and 37 °C. Portions (200 μL) were removed at several times and were quickly filtered through 0.45-μm Millipore HA filters (Millipore Corp., Bedford, MA), and the filters were immediately washed with 10 mL of ice-cold wash buffer (80 mM KCl, 50 mM PIPES, 5.0 mM NaN_3, 5.0 mM EGTA, 2.59 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 1.0 mM $HgCl_2$, pH 6.8). The amount of $^{45}Ca^{2+}$ retained on the filter was measured by liquid scintillation spectrophotometry.

For the efflux experiments, loaded microsomes (~30 nmol Ca^{2+}/mg of protein) were centrifuged at 10000*g* (4 °C) for 5 min in an Eppendorf centrifuge 3200. The pellet was resuspended in cold homogenization buffer at a protein concentration of ~2 mg of protein/mL. A sample of the suspension was subsequently added to incubation medium (50 mM PIPES, 5.0 mM NaN_3, 5.0 mM EGTA, and 1.0 mM

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_i, inorganic phosphate; PIPES, 1,4-piperazinediethanesulfonic acid; pCa^{2+}, $-\log [Ca^{2+}_i]$.

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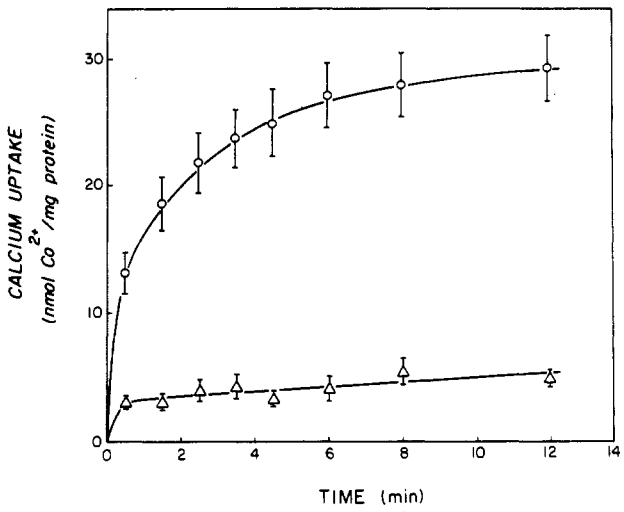


FIGURE 1: Time course of Ca^{2+} accumulation in rat liver microsomal vesicles. Incubation mixtures contained 5.0 μM $Ca^{2+}{}_i$ and 1.0 mM $Mg^{2+}{}_i$ in the presence (○) or absence (△) of 5.0 mM MgATP. Results are expressed as the mean ± SEM for six microsomal preparations.

Mg^{2+}, pCa^{2+} 7.0, pH 6.8) at 37 °C containing various concentrations of ADP and P_i. Samples (200 μL) were removed as a function of time and were washed as described above.

$[^{32}P]P_i$ Incorporation into $[\gamma\text{-}^{32}P]ATP$. $^{45}Ca^{2+}$-loaded microsomes were incubated with either 1.0 mM ADP or 1.0 mM $[^{32}P]P_i$ (1.0 Ci/mmol), or both, in the efflux medium described above. Samples (200 μL) were added to 20 μL of 50% (w/v) trichloroacetic acid and were centrifuged at 10000*g* for 5 min. The supernatant was withdrawn and stored at −70 °C. The supernatant (20 μL) was analyzed by reverse-phase high-pressure liquid chromatography (HPLC), and the amount of $[^{32}P]P_i$ incorporated into the ATP eluting fractions was determined by liquid scintillation spectrophotometry. The HPLC method of Ingebretson et al. (1982) was used; the eluent was 220 mM potassium phosphate (pH 5.0), 0.3 mM tetrabutylammonium hydrogen sulfate, and 1% methanol. The flow rate was 3.0 mL·min^{-1}, and absorbance was monitored at 254 nm. Free $Ca^{2+}{}_i$ and $Mg^{2+}{}_i$ concentrations were calculated with a program for solving multiple equilibria by the binding constants described by Fabiato & Fabiato (1979).

Results

The reversal of the Ca^{2+}-ATPase transport cycle was studied in Ca^{2+}-loaded rat liver microsomal vesicles. Rat liver microsomal vesicles accumulate Ca^{2+} in a Mg^{2+}- and ATP-dependent process to a value of ~30 nmol of Ca^{2+} (mg of microsomal protein)$^{-1}$ (Figure 1). The apparent Michaelis constant for $Ca^{2+}{}_i$ is 0.36 μM, and the maximum velocity is 10–20 nmol (mg of microsomal protein)$^{-1}$ min^{-1}; these values are in agreement with those of the Ca^{2+}-ATPases of other microsomal preparations (Dawson, 1982; Brattin et al., 1982). Present evidence, including a higher (1 order of magnitude) K_m for $Ca^{2+}{}_i$, a more acidic pH optimum, vanadate sensitivity, and oxalate stimulation (data not shown), indicates that the Ca^{2+}-ATPase of microsomal vesicles differs from the plasma membrane Ca^{2+}-ATPase (Kraus-Friedmann et al., 1982; Epping & Bygrave, 1984). The observed Ca^{2+} transport is ruthenium red insensitive, which precludes a mitochondrial source for this activity.

The rate of $^{45}Ca^{2+}$ efflux from preloaded microsomal vesicles was determined after dilution into an efflux medium (pCa^{2+} 7.00, pH 6.8). A typical experiment is shown in Figure 2. The apparent pseudo-first-order rate constants (K'_e) were determined from the slopes of semilogarithmic plots of the microsomal $^{45}Ca^{2+}$ content vs. time (Figure 2, inset). The basal K'_e was 0.19 ± 0.02 min^{-1} (mean ± SD, n = 5) and was not affected by various pCa^{2+} values (6.00–7.30) of the efflux medium (data not shown). This basal Ca^{2+} efflux from control microsomes probably represents passive Ca^{2+} efflux; whether or not this efflux occurs in vivo or is a result of the homogenization or incubation procedures remains unknown. The inclusion of P_i (10 mM), a potential precipitating anion, had no significant effect upon the basal K'_e (0.23 ± 0.04 min^{-1}, mean ± SD, n = 21). Addition of both ADP and P_i to the efflux medium increased the K'_e 2.5-fold. The K'_e at various ADP concentrations and in the presence of P_i (10 mM) was determined (Table I). Analysis of the data by double-reciprocal linear regression analysis yielded an apparent Michaelis constant for ADP of 4 μM (data not shown). Inclusion of ADP alone resulted in a slight increase in the K'_e (0.25 ± 0.02 min^{-1}), which is probably the result of a phosphoenzyme species or residual phosphate. The K'_e at various P_i concentrations in the presence of ADP (1.0 mM) was measured (Table II), and the apparent Michaelis constant was determined by double-reciprocal linear regression analysis to be 0.22 mM.

To determine if an electrochemical Ca^{2+} gradient was associated with ATP synthesis through reversal of the Ca^{2+}-ATPase, the incorporation of $[^{32}P]P_i$ into ATP was studied. Unloaded or Ca^{2+}-loaded vesicles were incubated in efflux media containing either $[^{32}P]P_i$, ADP and $[^{32}P]P_i$, or ADP, $[^{32}P]P_i$, and A23187. Samples were removed as a function

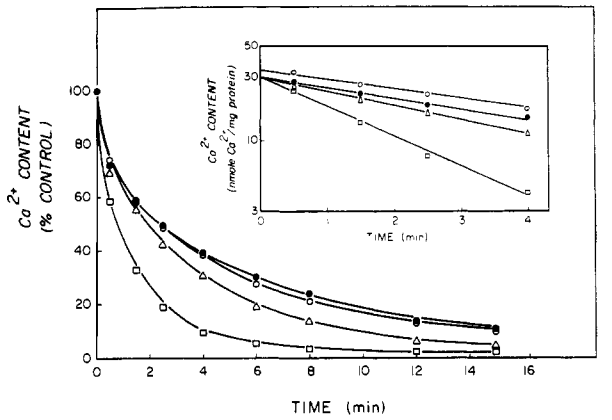


FIGURE 2: Representative plot of $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded microsomes. $^{45}Ca^{2+}$-loaded vesicles were incubated in efflux medium (pCa^{2+} 7.0, pH 6.8) containing buffer (●), 10 mM P_i (○), 2.5 mM ADP (△), or ADP and P_i (□). (Insert) Semilogarithmic plot of $^{45}Ca^{2+}$ content vs. time from which the pseudo-first-order rate constants for $^{45}Ca^{2+}$ efflux were determined.

Table I: Effect of Various ADP Concentrations on the Rate of $Ca^{2+}{}_i$ Efflux (K'_e) from $^{45}Ca^{2+}$-Loaded Microsomes

[ADP] (M)	K'_e (min^{-1})[a]	
	+ADP	+ADP and P_i[b]
1×10^{-6}	0.28 ± 0.03	0.28 ± 0.02
3×10^{-6}	0.28 ± 0.02	0.31 ± 0.01
1×10^{-5}	0.28 ± 0.02	0.34 ± 0.04
3×10^{-5}	0.33 ± 0.01	0.44 ± 0.02
1×10^{-4}	0.30 ± 0.02	0.48 ± 0.02
3×10^{-4}	0.31 ± 0.01	0.52 ± 0.02
2.5×10^{-3}	0.25 ± 0.01	0.49 ± 0.03

[a] Values were determined from the slopes of semilogarithmic plots as described under Materials and Methods. All values represent the mean ±SD for three microsomal preparations. The K'_e values for control microsomes incubated in the absence of P_i and ADP and for microsomes incubated in the presence of P_i alone were 0.19 ± 0.02 (n = 5) and 0.23 ± 0.04 min^{-1} (n = 21), respectively. [b] Inorganic phosphate (P_i) concentration was 10 mM.

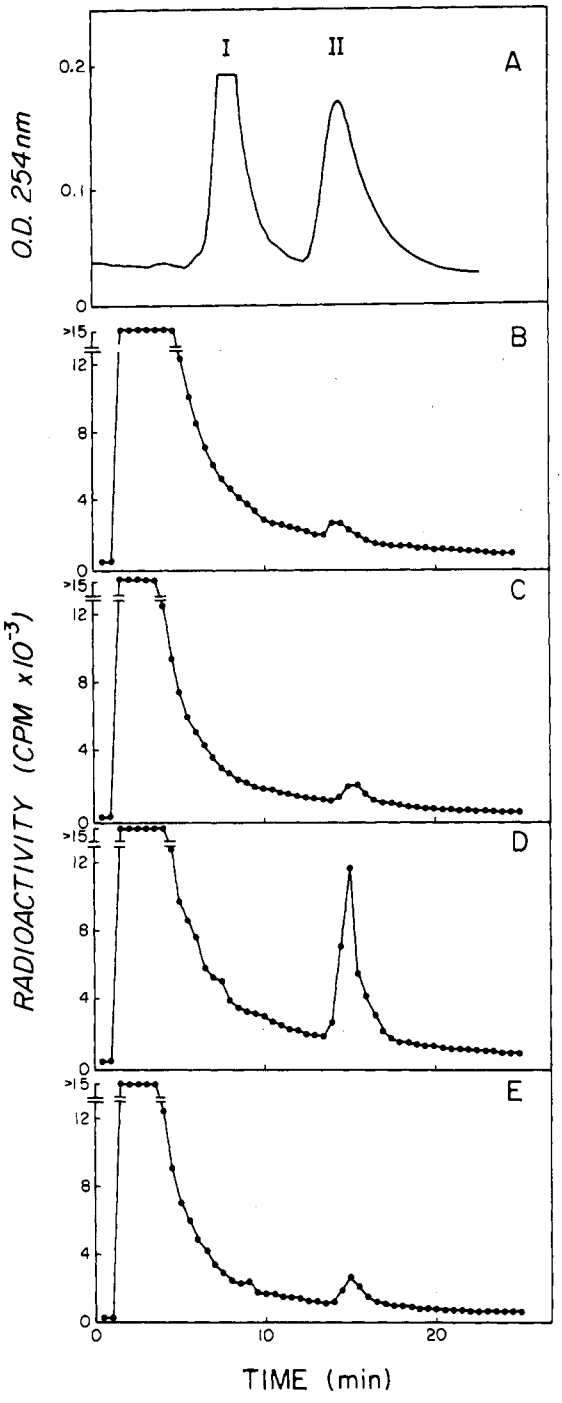


FIGURE 3: HPLC chromatograms for the determination of $[^{32}P]P_i$ incorporation into $[\gamma\text{-}^{32}P]ATP$: (A) chromatogram of ADP (I) and ATP (II); (B) chromatogram of incubation mixture of unloaded microsomes with ADP (1.0 mM) and $[^{32}P]P_i$ (1.0 mM); (C) chromatogram of incubation mixture of loaded microsomes with $[^{32}P]P_i$ (1.0 mM); (D) chromatogram of incubation mixture of loaded microsomes with ADP (1.0 mM) and $[^{32}P]P_i$ (1.0 mM); (E) chromatogram of incubation mixture of loaded microsomes with ADP (1.0 mM) and $[^{32}P]P_i$ (1.0 mM) containing A23187 (2 μg/mL).

of time, and the amount of $[^{32}P]P_i$ incorporated into ATP was determined by HPLC and liquid scintillation spectrophotometry (Figure 3). Unloaded microsomal vesicles incubated in the presence of ADP and $[^{32}P]P_i$ incorporated a small amount of $[^{32}P]P_i$ into ATP (Figure 3B). A similar amount of $[^{32}P]P_i$ incorporation was observed when Ca^{2+}-loaded vesicles were incubated in the absence of ADP (Figure 3C), which suggests that some ADP remained associated with the enzyme. When loaded vesicles were incubated in the presence of ADP and

Table II: Effect of Various P_i Concentrations on the Rate of ADP-Induced Ca^{2+}_i Efflux from $^{45}Ca^{2+}$-Loaded Microsomes

$[P_i]$ (M)	K'_e $(min^{-1})^a$	$[P_i]$ (M)	K'_e $(min^{-1})^a$
3×10^{-5}	0.24 ± 0.03	1×10^{-3}	0.46 ± 0.01
1×10^{-4}	0.29 ± 0.01	3×10^{-3}	0.47 ± 0.02
3×10^{-4}	0.36 ± 0.02	1×10^{-2}	0.48 ± 0.02
6×10^{-4}	0.42 ± 0.02		

[a] Values were determined from the slopes of semilogarithmic plots as described under Materials and Methods. All values represent the mean ±SD for three microsomal preparations. The ADP concentration was 1×10^{-3} M. The K'_e values for control microsomes incubated in the presence of ADP alone and for control microsomes incubated in the presence of P_i alone were 0.25 ± 0.02 and 0.20 ± 0.01 min^{-1}, respectively.

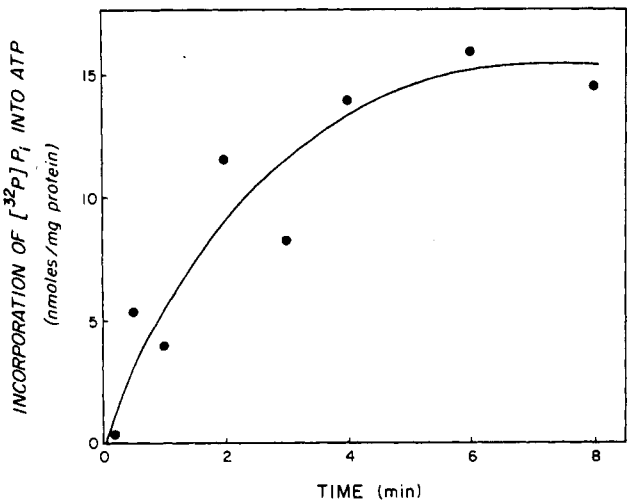


FIGURE 4: Time course of $[^{32}P]P_i$ incorporation into $[\gamma\text{-}^{32}P]ATP$. The amount of $[^{32}P]P_i$ incorporated into ATP, which was present in fractions eluting from 13 to 19 min, was determined by liquid scintillation spectrophotometry. Values represent the net amount of $[^{32}P]P_i$ incorporated into ATP by Ca^{2+}-loaded microsomes incubated with ADP and $[^{32}P]P_i$ (Figure 3D) minus the amount incorporated by unloaded microsomes under the same conditions (Figure 3B).

$[^{32}P]P_i$, a greater than 10-fold increase in the amount of $[^{32}P]P_i$ incorporated into ATP was observed (Figure 3D). This increase was abolished by the inclusion of A23187 in the efflux medium (Figure 3E), which indicates that an electrochemical Ca^{2+} gradient facilitates the coupling of Ca^{2+} efflux to ATP synthesis.

The amount of $[^{32}P]P_i$ incorporated into ATP was a function of time (Figure 4). The $[^{32}P]P_i$ incorporation reached a plateau within 4–6 min, which agrees with the rate of $^{45}Ca^{2+}$ efflux (Figure 2). The synthesis of approximately 15 nmol of $[\gamma\text{-}^{32}P]ATP$ (mg of protein)$^{-1}$ was associated with the efflux of Ca^{2+}, which indicates a stoichiometry of Ca^{2+} efflux to ATP synthesis of 2:1. This stoichiometry of 2:1 has also been observed for the sarcoplasmic reticulum Ca^{2+}-ATPase (Hasselbach, 1978). Because the passive Ca^{2+} efflux represents nearly 50% of the total Ca^{2+} efflux, the stoichiometry between active Ca^{2+} efflux and ATP synthesis would be 1:1. More accurate determinations of the stoichiometry are precluded, because the mechanism for the basal Ca^{2+} efflux is unknown and because hydrolysis of newly synthesized ATP may occur by the Ca^{2+}-ATPase, by the basal Mg^{2+}-ATPase, or by the recently described proton-translocating ATPase (Rees-Jones & Al-Awqati, 1984) activities.

The effect of the ATP/ADP ratio on the rate of active Ca^{2+} efflux ($\Delta K'_e$) was studied (Figure 5). As the ATP/ADP ratio is decreased from 30 to 1, there is a large increase in the $\Delta K'_e$. Conditions leading to decreases in the normal cytosolic ATP/ADP ratio of ~10 (Schwenke et al., 1981) would be

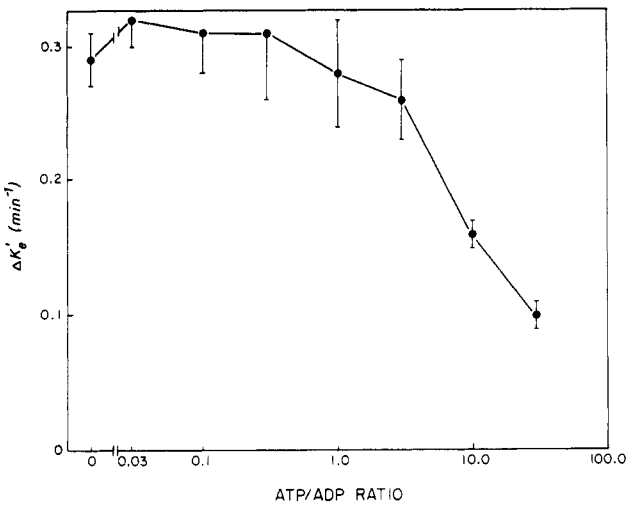
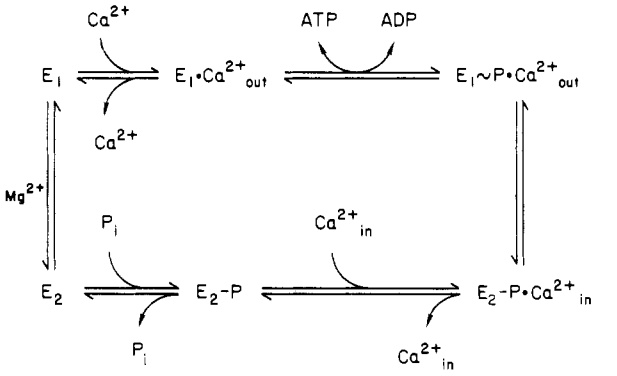


FIGURE 5: Effect of the ATP/ADP ratio on the rate of active Ca^{2+} efflux. Active Ca^{2+} efflux ($\Delta K'_e$) was determined by subtracting the rate of $^{45}Ca^{2+}$ efflux in the presence of various ATP/ADP ratios and P_i (10 mM) from the rate of $^{45}Ca^{2+}$ efflux in the presence of P_i alone. Total nucleotide concentration was 1.0 mM. Values represent the mean ± SEM for three to four different microsomal preparations.

expected to promote Ca^{2+} release by Ca^{2+}-ATPase reversal. Such conditions leading to a decrease in the liver cytosolic ATP/ADP ratio or in the adenylate energy charge include hypoxia (Kinnula & Hassinen, 1978; Aw & Jones, 1982), fasting (Soboll et al., 1984), trialkyltin (Weibkin et al., 1982) and ethionine (Farber, 1967) toxicity, and septicemia (Tanaka et al., 1982).

Discussion

The mechanism of the Ca^{2+}-ATPase of sarcoplasmic reticulum (deMeis & Vianna, 1979) and of erythrocyte plasma membrane (Chiesi et al., 1984) is well characterized. This general scheme for the ion-motive E_1E_2ATPase enzymes has been proposed:



where E~P and E–P denote the reactive and low-energy acyl phosphate conformations of the phosphoenzyme, respectively. Characteristics of this E_1E_2ATPase mechanism include inhibition by high intravesicular Ca^{2+} concentrations, Mg^{2+}-enhanced enzyme dephosphorylation (E_2–P), the formation of a phosphoenzyme intermediate, complete reversibility of the transport cycle, and the requirement for Ca^{2+} in the synthesis of ATP during the Ca^{2+}-ATPase reversal.

The Ca^{2+}-ATPase of rat liver endoplasmic reticulum vesicles shares many features of this ion-motive E_1E_2ATPase mechanism. Intravesicular accumulation of Ca^{2+} inhibits Ca^{2+}-ATPase activity (Brattin & Waller, 1983). The formation of a hydroxylamine-sensitive phosphoenzyme intermediate with a M_r of 118 000 and the Mg^{2+}-enhanced dephosphorylation of this phosphoenzyme have also been demonstrated (Heilmann et al., 1984, 1985). In addition to the similarities associated with the E_1E_2ATPase mechanism, the tryptic and chymotryptic digests of the sarcoplasmic reticulum and endoplasmic reticulum Ca^{2+}-ATPase indicate extensive homology (Heilmann et al., 1984). The results presented herein provide evidence for the reversibility of the transport cycle associated with the Ca^{2+}-ATPase and for the coupling of ATP synthesis to Ca^{2+} efflux. Disruption of the Ca^{2+} gradient with A23187 and the associated loss of ATP synthesis indicate a requirement for a Ca^{2+} gradient for ATP synthesis.

The mechanism of the transformation of the electrochemical Ca^{2+} gradient into the chemical energy required for ATP synthesis is not understood. In a number of preparations, including the Ca^{2+}-ATPase of sarcoplasmic reticulum (Knowles & Racker, 1975) and of erythrocyte plasma membranes (Chiesi et al., 1984), the synthesis of ATP by Ca^{2+}-ATPase is accomplished in the absence of a Ca^{2+} gradient. Ca^{2+} appears to function in the transfer of the P_i from the phosphoenzyme to ADP, rather than in the formation of the E_2–P species. In both the sarcoplasmic reticulum (de Meis & Inesi, 1982) and the erythrocyte (Chiesi et al., 1984) Ca^{2+}-ATPases, dimethyl sulfoxide enhances the phosphorylation of the ATPase by P_i to form E_2–P. However, an increase in the water activity and high concentrations of Ca^{2+} are required to observe the phosphorylation of ADP, presumably by the reactive E_1~P conformation. Indeed, an increase in the water activity has been shown to enhance the formation of the E_1~P conformation from the E_2–P conformation (de Meis & Inesi, 1982). Hence, it is postulated that high concentrations of Ca^{2+} facilitate the transition of the low-energy E_2–P conformation to the reactive E_1~P conformation, which is capable of transferring P_i to ADP. Either a high concentration of Ca^{2+} in unloaded, permeable Ca^{2+}-ATPase vesicles or a Ca^{2+} gradient in intact Ca^{2+}-ATPase vesicles, where millimolar concentrations of Ca^{2+} are achieved, will likely drive the reversal of the transport cycle through the interaction of Ca^{2+} with the low-affinity site of the E_2–P conformation.

The synthesis of ATP by the rat liver microsomal Ca^{2+}-ATPase has recently been described (Heilmann et al., 1985). When the E~P·Ca^{2+}_{out} phosphoenzyme species, formed from [γ-^{32}P]ATP, was incubated in the presence of ADP, phosphate transfer to ADP was observed. Low Ca^{2+} concentrations were required for ATP synthesis, because preincubation with EGTA inhibited phosphoenzyme breakdown. The Ca^{2+} gradient, which is required for ATP synthesis in our preparation, may serve to convert the low-energy acyl phosphate (E_2–P) phosphoenzyme conformation to the reactive conformation (E_1~P). The possibility that the Ca^{2+}-ATPase is phosphorylated by P_i and that the phosphoenzyme species formed is able to transfer phosphate to ADP, both in the absence of a Ca^{2+} gradient, is currently under investigation.

The physiological significance of Ca^{2+}-ATPase reversal is uncertain, although during toxic insult alterations in the ATP concentration and ATP/ADP ratio have been observed (Bridges et al., 1983). Ca^{2+}-ATPase reversal, with the possibility of cytosolic Ca^{2+} accumulation, may result from alterations in the ATP/ADP ratio. It remains to be determined if Ca^{2+}-ATPase reversal is associated with alterations in cytosolic calcium homeostasis, which may represent one of many possible mechanisms involved in cell death.

Elucidation of the mechanism and characteristics of the endoplasmic reticulum Ca^{2+}-ATPase will enhance our understanding of its role in intracellular Ca^{2+} homeostasis. It is interesting to speculate that many of the hormones that exert their glycogenolytic effect through alterations in the Ca^{2+}_i

homeostasis and the subsequent activation of phosphorylase A may also affect the endoplasmic reticulum Ca^{2+}-ATPase. Inhibition or stimulation of the Ca^{2+}-ATPase could either prolong or shorten the glycogenolytic activity. Such effects have been observed with the rat liver plasma membrane (Ca^{2+}+Mg^{2+})-ATPase (Prpic et al., 1983; Lotersztajn et al., 1984).

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