Calcium Transport and Phosphorylated Intermediate of (Ca$^{2+}$ + Mg$^{2+}$)-ATPase in Plasma Membranes of Rat Liver*

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We have identified and characterized calcium transport and the phosphorylated intermediate of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase in plasma membrane vesicles prepared from rat liver. The calcium transport did not absolutely require the presence of oxalate and was completely inhibited by 1 μM of ionophore A23187. Oxalate, which serves as a trapping agent in calcium uptake of skeletal muscle and liver microsomes, was not absolutely required to maintain the net accumulation of calcium. The $V_{max}$ and $K_{m}$ for calcium uptake were 35.2 ± 10.1 pmol of calcium/mg of protein/min, and 17.6 ± 2.5 nm of free calcium, respectively. Ten mM magnesium was required for the maximal accumulation of calcium. Substitution of 5 and 10 mM ADP, CTP, GTP, and UTP for ATP could not support calcium uptake. The calcium uptake was not affected by 0.5 mM ouabain, 20 mM azide, or 2 μg/ml of oligomycin but was inhibited in a dose-dependent fashion by vanadate, with a $K_{i}$ of ~20 μM for vanadate. The substrate affinities and specificities of this calcium-transport activity suggest that it is closely associated with the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase reported in the plasma membranes of liver (Lotersztajn, S., Hanoune, J., and Pecker, M. (1981) J. Biol. Chem. 256, 11509-11215). A calcium-stimulated and magnesium-dependent phosphoprotein was also demonstrated in the same membrane vesicles. The free calcium concentration at which its phosphorylation was half-maximal was 15.5 ± 5.6 mM. Sodium fluoride, oxalate, sodium azide, oligomycin, adriamycin, and N,N'-dicyclohexylcarbodiimide did not affect its formation while vanadate at 100 μM inhibited the calcium-dependent phosphorylation by ~60%. The properties of this phosphoprotein suggest that it may be the phosphorylated intermediate of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase in the plasma membranes of rat liver.

Regulation of the intracellular concentration of calcium is linked closely to the various metabolic responses of cells to hormones (1-4). The cytoplasmic free calcium ion concentration is approximately 0.1 μM, which is 100-fold lower than that in the extracellular fluid (5, 6). Such a low intracellular concentration of calcium can be maintained by several means. In addition to systems for extruding calcium from the cell by Na'/Ca$^{2+}$ exchange (7), a mechanism by which calcium transport is linked to a (Ca$^{2+}$ + Mg$^{2+}$)-ATPase has also been described in various tissues (8-12).

A calcium transport-related (Ca$^{2+}$ + Mg$^{2+}$)-ATPase (13) and its phosphorylated intermediate (14) have been studied in rat adipocytes. Both the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase activity and its calcium-dependent phosphorylation in isolated plasma membranes are inhibited when adipocytes are incubated with insulin. Recently, a high affinity (Ca$^{2+}$ + Mg$^{2+}$)-ATPase has been reported in plasma membranes of rat liver (15, 16). This (Ca$^{2+}$ + Mg$^{2+}$)-ATPase activity is inhibited by insulin (16). However, the association of this (Ca$^{2+}$ + Mg$^{2+}$)-ATPase with calcium transport has not been demonstrated.

We now report an ATP-dependent calcium-transport system in the plasma membranes of rat liver. The kinetics and the substrate specificity of the system suggest that it is closely related to the previously reported (Ca$^{2+}$ + Mg$^{2+}$)-ATPase in the plasma membranes of liver (15). In addition, we also describe a calcium-dependent phosphoprotein whose properties are consistent with its being the phosphorylated intermediate of this (Ca$^{2+}$ + Mg$^{2+}$)-ATPase. This is the first time that a high affinity calcium-transport system and the corresponding (Ca$^{2+}$ + Mg$^{2+}$)-ATPase phosphorylated intermediate has been described in plasma membranes of rat liver.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats, weighing 150-200 g, were obtained from Eldridge Laboratory Animals, Barnhart, MO. Materials—$^{44}$Ca (10-40 mCi/mg) and carrier-free ($^{32}$P)ATP (2000 Ci/mmol) were obtained from Amersham. A23187 was purchased from Calbiochem and a 20 mM stock solution was prepared in dimethyl sulfoxide. Tris, EGTA, ATP (Tris salt), ADP, CTP, GTP, UTP, ouabain, sodium azide, oligomycin, adriamycin, and N,N'-dicyclohexylcarbodiimide were purchased from Sigma. Ultrapure sucrose was obtained from Schwarz-Mann. All other reagents were purchased from Fisher Scientific Co. All aqueous solutions were prepared with water deionized by a double chambered mixed bed ion exchange resin system from Culligan, Inc., Northbrook, IL.

Preparation of Subcellular Fractions from Rat Liver—Liver plasma membranes were prepared according to Pillets et al. (17). All procedures were carried out at 4 °C. The purity of the plasma membranes was evaluated by assaying the plasma membrane specific marker enzyme, 5'-nucleotidase (18) and the microsomal enzyme, glucose-6-phosphatase (19). The plasma membrane fraction showed a 2-fold enhancement in the 5'-nucleotidase activity and its microsomal enzyme in the crude homogenate. The plasma membranes were resuspended in either 50 mM Tris-Cl, pH 8.0, 0.25 M sucrose, or 1 mM NaHCO3, 1 mM EDTA, frozen in a dry ice/methanol bath and stored at ~70 °C until use. The microsomes were prepared according to Moore et al. (20). Protein was determined by the method of Lowry et al. (21).

Calcium Uptake—Calcium uptake by liver plasma membrane vesicles was assayed as previously described (7).

The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TDAB, tetradeyltrimethylammonium bromide; PMSF, phenylmethylsulfonyl fluoride; CDTA, trans-1,3-diaminocyclohexane-N,N',N'-tetraacetic acid.

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icles was assayed according to the procedure of Brune et al. (22) in a 500-ml medium containing 50 mm Tris-Cl, pH 8.0, at 37°C, 0.25 mM sucrose, 0.2 mm EGTA, 0-0.2 mm CaCl₂, 2 μCi/ml of ⁴⁰Ca, 10 mm MgCl₂, 20 mm sodium azide, 10 mm Tris-ATP, and 4 mm Tris-oxalate. The mixture was preincubated for 5 min at 37°C. The reaction was started by the addition of 50-100 μg of plasma membrane proteins (resuspended in 50 mm Tris-Cl, pH 8.0, 0.25 mM sucrose), and the mixture was incubated at 37°C in a shaking water bath. At appropriate time intervals, 450-pl samples were removed and vacuum filtered on 0.45-μm pore Millipore cellulose acetate filters that had been soaked in 0.25 M KCl for at least 30 min. The filters were washed three times with 5 ml of 0.25 M sucrose containing 40 mM NaCl. The membranes were trapped on the filter were dried and placed in 3 ml of 3 μCi/ml of ⁴⁰Ca. Calcium uptake was expressed as picomoles of ⁴⁰Ca/mg of protein/min. The ATP-dependent uptake was determined from the difference in radioactivity bound to the filter in the presence and absence of ATP. All uptake values were corrected for nonspecific radioactivity bound to the filter when an identical reaction mixture without plasma membranes was filtered. This usually accounted for less than 1% of the total measured radioactivity.

**Phosphorylation Assay:** Phosphorylation was performed (in 1.5-ml polypropylene microcentrifuge tubes) in a 300-μl reaction mixture containing 50 mm Tris-Cl, pH 8.0, at 4°C, 5 μM [γ-³²P]ATP (20 Ci/mmol), 4 mM EGTA, and varying concentrations of calcium chloride. Following the addition of 50 μg of plasma membranes, the mixture was incubated at 4°C for 30 s (unless otherwise indicated) and the reaction was terminated by the addition of 600 μl of ice-cold 20% trichloroacetic acid, 1.5 mM ATP, 10 mM HEPES. The precipitated protein was collected by centrifugation, washed with 10% trichloroacetic acid, 1.0 mM ATP, 10 mM HEPES, and solubilized in 100 μl of 0.1 M HEPES/NaOH, 1 M NaOH buffer, pH 4.0, containing 0.25 mM sucrose, 20 mM Tris-oxalate, 150 mM sodium azide, and 10 μl of 60% trichloroacetic acid. For hydroxylation treatment, the trichloroacetic acid-washed precipitate was resuspended in 300 μl of freshly prepared 150 mM hydroxyamine, 150 mM sodium acetate, pH 6.0, the controls were treated with 150 mM NaCl, 150 mM sodium acetate, pH 6.0. After a 10-min incubation at room temperature, the reaction was terminated by the addition of 1 ml of 20% trichloroacetic acid, 1 mM ATP, and 10 mM HEPES. The precipitated proteins were processed for polyacrylamide gel electrophoresis.

**Polyacrylamide Gel Electrophoresis and Autoradiography:** The cationic detergent polyacrylamide gel electrophoresis at acid pH was performed at 4°C at a constant current of 40 mA according to the method described by Amory et al. (23). Following electrophoresis, the gels were soaked in 1% (v/v) glycerol for 5 min, dried, and exposed to Kodak XR-5 x-ray film. The calcium-dependent phosphoprotein band was excised from the gel and the radioactivity was quantitated according to the method of Landt and McDonald (24).

**Determination of Total and Free Calcium or Magnesium Concentrations of Reaction Mixtures:** The total calcium and magnesium concentrations of the reaction mixture were determined by atomic absorption spectrophotometry. The contaminating calcium in a reaction mixture with no added calcium was -13 nm. The amount of magnesium present in the reaction mixture with no added magnesium was -9 μM. The free concentrations of calcium and magnesium were determined (in the presence of oxalate for about 10 min, after which it leveled off resulting in a lower total calcium uptake at the end of the 30-min incubation (Fig. 1B). The absence of oxalate had no effect on the basal activity.

**Calcium Uptake by Membrane Vesicles**

**Time Course, Cofactor Requirements, and Membrane Specificities**—The time course of calcium uptake by liver plasma membrane vesicles is shown in Fig. 1. The amount of calcium bound to the plasma membranes at zero time was between 0.15-0.2 nmol/mg of protein both in the absence and presence of ATP. In the absence of 1.0 μM of A23187, the ATP-dependent calcium uptake proceeded in a linear fashion for about 10-15 min before it began to level off (Fig. 1A). The addition of 1.0 μM of A23187 at the start of the experiment completely eliminated the basal and ATP-dependent calcium accumulation by the vesicles. When A23187 was added after 15 min of incubation, both the basal and ATP-dependent calcium uptake was inhibited with a rapid release of the accumulated calcium into the medium. When oxalate (4 mM) was omitted from the incubation medium, the initial rate of ATP-dependent calcium uptake paralleled that in the presence of oxalate for about 10 min, after which it leveled off resulting in a lower total calcium uptake at the end of the 30-min incubation (Fig. 1B). The absence of oxalate had no effect on the basal activity.

**Cation Requirements and Nucleotide Specificity**—The ATP-dependent calcium uptake by liver plasma membrane vesicles was measured as a function of the free calcium concentration in the reaction medium (Fig. 2). The calcium uptake exhibited saturable kinetics reaching a maximum at a free calcium concentration of approximately 30 nm. The Kₘ and Vₚ for ATP were calculated from the double reciprocal plot of the data from 3 separate membrane preparations were 17.6 ± 2.5 nm and 35.2 ± 13.1 pmol of calcium/mg of protein/min, respectively.

The ATP-dependent calcium uptake by liver plasma membrane vesicles was also measured as a function of magnesium concentration at a constant Tris-ATP concentration of 10 mM and free calcium concentration of ~15 nm (Fig. 3). No ATP-dependent calcium uptake occurred when magnesium was omitted from the assay. Starting at a magnesium concentration of 2.5 mM, calcium uptake proceeded precipitously, reaching a maximum at a magnesium concentration of 10 mM when it started to decline. The free calcium concentration varied...
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only slightly from 12 to 16 nm as total magnesium was varied from 2.5 to 10 mM. The reason for the apparent decline in \(Ca^{2+}\) transport at magnesium concentrations above 20 mM is unknown.

The initial velocity of calcium uptake by the liver plasma membrane vesicles was also studied as a function of ATP concentration (Fig. 4). In these studies, no more than 10% of the initial ATP was allowed to be hydrolyzed. Under these conditions, and in the absence of ATP, a small amount of \(Ca^{2+}\) transport at magnesium concentrations above 20 mM is described for the red cell (28), adipocyte endoplasmic reticulum vesicles specifically required ATP. The presence of the Tris-ATP in supporting the calcium transport (data not shown).

Calcium-dependent Phosphoprotein in Membrane Vesicles: Identification and Time Course of Phosphorylation

Plasma membranes were phosphorylated with \([γ^3P]ATP\) in a 2 mM EGTA containing buffer and in the presence or absence of 2 mM calcium. When the membranes were soluble translocation experiments by double reciprocal plots yielded a broken line with 2 components. The \(K_a\) values for the high and low affinity components were extrapolated as 81 ± 16 μM and ~10 mM, respectively.

The calcium uptake by the liver plasma membrane vesicles in the presence of ATP was also compared to that measured in the presence of other nucleotides. Calcium uptake by these vesicles specifically required ATP. The presence of 5 or 10 mM of the Tris salts of ADP, CTP, GTP, or UTP could not replace the Tris-ATP in supporting the calcium transport (data not shown).

Calcium-dependent Phosphoprotein in Membrane Vesicles: Identification and Time Course of Phosphorylation

Plasma membranes were phosphorylated with \([γ^3P]ATP\) in a 2 mM EGTA containing buffer and in the presence or absence of 2 mM calcium. The free calcium concentrations were calculated as described in the text. The ordinate represents ATP-dependent calcium uptake, i.e. the difference in calcium uptake in the presence and absence of Tris-ATP. Each point represents the mean ± S.E. of triplicate sample determinations.

Calcium uptake by liver plasma membrane vesicles: dependence on calcium. Calcium uptake was assayed as described under “Experimental Procedures” after 30 min of incubation at 37 °C in a standard medium of 50 mM Tris-Cl, pH 8.0, 0.25 M sucrose, 0.2 mM NaN₃, 10 mM MgCl₂, 0 or 10 mM Tris ATP, and 4 mM Tris-oxalate as described in the legend to Fig. 1 and in the text. The free calcium concentrations were varied as shown. Each point represents the mean ± S.E. of triplicate sample determinations.

Calcium uptake by liver plasma membrane vesicles: dependence on magnesium. Calcium uptake was assayed as described under “Experimental Procedures” after 30 min of incubation at 37 °C in a standard medium of 50 mM Tris-Cl, pH 8.0, 0.25 M sucrose, 0.2 mM EGTA, 0.18 mM CaCl₂, 1-2 μCi/ml of \(^{45}\)CaCl₂, 20 mM NaN₃, 4 mM Tris-oxalate, 0-15 mM MgCl₂, and in the presence or absence of 10 mM Tris-ATP. The ordinate represents ATP-dependent calcium uptake, i.e. the difference in calcium uptake in the presence and absence of Tris-ATP. Each point represents the mean ± S.E. of triplicate sample determinations.

Calcium uptake by liver plasma membrane vesicles: dependence on ATP concentration. Calcium uptake was assayed as described in the legend to Fig. 1, except that the total ATP concentration was varied as shown. Each point represents the mean ± S.E. of triplicate sample determinations.

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The calcium uptake by liver plasma membrane vesicles specifically required ATP. The presence of 10 mM Tris-ATP in a 2 mM Tris-Cl, pH 8.0, 0.25 M sucrose, 0.2 mM EGTA, 0.18 mM CaCl₂, 1-2 μCi/ml of \(^{45}\)CaCl₂, 20 mM NaN₃, 4 mM Tris-oxalate, 0-15 mM MgCl₂, and in the presence or absence of 10 mM Tris-ATP. The ordinate represents ATP-dependent calcium uptake, i.e. the difference in calcium uptake in the presence and absence of Tris-ATP. Each point represents the mean ± S.E. of triplicate sample determinations.

Calcium uptake by liver plasma membrane vesicles: dependence on ATP concentration. Calcium uptake was assayed as described in the legend to Fig. 1, except that the total ATP concentration was varied as shown. Each point represents the mean ± S.E. of triplicate sample determinations.

Calcium-dependent Phosphoprotein in Membrane Vesicles: Identification and Time Course of Phosphorylation

Plasma membranes were phosphorylated with \([γ^3P]ATP\) in a 2 mM EGTA containing buffer and in the presence or absence of 2 mM calcium. When the membranes were solubi-
lized and analyzed by TDBA polyacrylamide gel electrophoresis at acid pH followed by autoradiography, a single major $^{32}$P-labeled polypeptide with a molecular weight of 105,000 ± 5,800 was observed (Fig 5). In the absence of calcium, the phosphoprotein band was only barely visible. With added calcium, phosphorylation increased to 4-5-fold that observed in the presence of EGTA alone. The migration of the 105,000-Da phosphoprotein band was not altered when plasma membranes were prepared in PMSF or when 1 mM PMSF was added directly to the reaction mixture (data not shown). This suggests that this 105,000-Da phosphoprotein is not a degradative product of a larger polypeptide as a result of protease activity. Both the calcium-dependent and -independent phosphorylation of the 105,000-Da protein showed rapid incorporation pattern with labeling reaching a plateau between 20-30 s at 4 °C (data not shown). Further incubation up to 70 s did not significantly increase the phosphorylation. The maximal calcium-dependent phosphorylation was approximately 1.0 pmol of P/mg of protein.

**Calcium-dependent Phosphorylation**

**Caten and Nucleotide Requirements**—The phosphorylation was performed at different MgATP concentrations for 30 s at 4 °C. Total calcium was maintained at 2 mM with a free calcium of ~1 μM. Even with 0.25 μM of MgATP, less than 10% of the initial total ATP was hydrolyzed during the 30-s incubation. The ATP concentration required for maximal calcium-dependent phosphorylation was approximately 5 μM (data not shown).

The dependence of phosphorylation of the 105,000-Da plasma membrane protein on free calcium concentration in the medium was studied by performing the experiments in the presence of 2 mM EGTA and 0-1.90 mM CaCl₂ (0-32 mM free calcium). Maximal calcium-dependent phosphorylation of the 105,000-Da protein occurred at a free calcium concentration of 20-32 nM (Fig. 6). Analysis of data by double reciprocal plots showed that the free calcium concentration at which calcium-dependent phosphorylation was half-maximal was 15.5 ± 5.6 nM ($n = 4$).

The calcium-dependent phosphorylation occurred in the absence of exogenously added magnesium. However, at ~9 μM of endogenous magnesium was present in the reaction mixture, the chelator, CDTA, which has a high affinity for both calcium and magnesium (31), was employed to assess the magnesium requirement. In medium buffered with 2 mM CDTA, the basal phosphorylation activity was eliminated and

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**Fig. 6.** Calcium-dependent phosphorylation of the 105,000-Da plasma membrane protein: dependence on free calcium concentration. Plasma membranes were phosphorylated at 4 °C for 30 s in the EGTA buffer system in the absence or presence of varying amounts of CaCl₂, subjected to TDAB-polyacrylamide gel electrophoresis, and autoradiographed as described in the legend to Fig. 5. The 105,000-Da phosphoprotein band was excised and the radioactivity was quantitated. The ordinate represents the calcium-dependent phosphorylation, i.e. the difference in phosphorylation in the presence and absence of calcium, as expressed in picomoles of P/mg of protein. Each point represents the mean ± S.E. of triplicate sample determinations.

**Fig. 7.** Calcium-dependent phosphorylation of the 105,000-Da plasma membrane protein: requirement of free magnesium. Plasma membranes were phosphorylated at 4 °C for 30 s either in a EGTA (4 mM) buffer system containing 0 or 3.8 mM CaCl₂ (free calcium 32 mM) or in a CDTA (4 mM) buffer system and in the absence or presence of calcium, magnesium, or both. The 105,000-Da phosphorylated membrane protein was separated on TDAB polyacrylamide gel and quantitated for the $^{32}$P radioactivity as described previously in the legend to Fig. 5. Each point represents the mean ± S.E. of triplicate determinations. The free calcium and/or magnesium concentrations in the two buffer systems were calculated as described in the text.

**Table I**

Calcium-dependent phosphorylation of the 105,000-Da plasma membrane protein: properties of the phosphate linkage

Liver plasma membranes were prepared and phosphorylated in the presence or absence of ~1 μM of free calcium as described in the text. For the ATP chase, 30 μl of 100 mM unlabeled ATP in either the EGTA or calcium-EGTA buffer were added after the 30-s standard incubation and the reaction was stopped by the addition of trichloroacetic acid. Hydroxylamine treatment, the phosphorylation was terminated after the standard 30-s incubation at 4 °C. The phosphorylated plasma membranes were collected by centrifugation, washed once with trichloroacetic acid, and resuspended for 10 min at room temperature in 300 μl of either 150 mM Tris-Cl and 150 mM sodium acetate, pH 6.0 (control), or 150 mM hydroxylamine hydrochloride and 150 mM sodium acetate, pH 6.0. At the end of the incubation, the plasma membrane proteins were precipitated with 10% trichloroacetic acid. The $^{32}$P radioactivity associated with the 105,000-D protein from either the ATP-chased or hydroxylamine-treated samples was quantitated as described previously. The mean ± S.E. of measurements on 3 membrane preparations are presented.

| Treatment          | Calcium-dependent phosphorylation of 105,000-D protein (pmol P/mg protein) |
|--------------------|---------------------------------------------------------------------------|
| Addition of unlabeled ATP |                                                                 |
| Control            | 0.709 ± 0.033                                                             |
| 10 s               | 0.586 ± 0.037                                                             |
| 10 mM ATP          | 0.137 ± 0.014                                                             |
| 10 s               | 0.047 ± 0.007                                                             |
| Hydroxylamine      | 0.398 ± 0.054                                                             |
| Control            | 0.064 ± 0.007                                                             |
| 150 mM hydroxylamine| 0.162 ± 0.05                                                             |

**Note:** The phosphorylation was half-maximal was 15.5 ± 5.6 nM ($n = 4$).

The calcium-dependent phosphorylation occurred in the absence of exogenously added magnesium. However, at ~9 μM of endogenous magnesium was present in the reaction mixture, the chelator, CDTA, which has a high affinity for both calcium and magnesium (31), was employed to assess the magnesium requirement. In medium buffered with 2 mM CDTA, the basal phosphorylation activity was eliminated and...
Table II

Effect of inhibitors on calcium transport and calcium-dependent phosphorylation

Calcium transport and calcium-dependent phosphorylation of the 105,000-Da protein were measured in liver plasma membranes as described under “Experimental Procedures.” Results are expressed as per cent of the activity present in the standard incubation medium without inhibitors. Each value represents the mean (± S.E.) of 2-5 membrane preparations.

| Inhibitor            | Calcium transport (%) | Calcium-dependent phosphorylation (%) |
|----------------------|-----------------------|---------------------------------------|
| None                 | 100                   | 100                                   |
| Ouabain (0.5 mM)     | 105.4 ± 2.0           | 107 ± 11                              |
| Oligomycin (2 µg/ml) | 96.0 ± 4.0            | 112.4 ± 5.7                           |
| Vanadate (0.1 mM)    | 15.8 ± 5.1            | 44.0 ± 13.3                           |
| Sodium azide (20 mM) | 102 ± 4.5             | 91.0 ± 3.0                            |
| Adriamycin (200 µM)  | 121.0 ± 12.3          | 85.0 ± 8.0                            |

Fig. 8. Calcium uptake by liver plasma membrane vesicles: inhibition by vanadate. ATP-dependent calcium uptake was measured as described in the legend to Fig. 1 and in the text and in the absence or presence of varying concentrations of orthovanadate. Each point represents the mean ± S.D. of results from 2 membrane preparations.

Effect of Inhibitors on Calcium Transport and Calcium-dependent Phosphorylation

The effectiveness of potential inhibitors on calcium transport and/or calcium-dependent phosphorylation of the 105,000-Da protein in liver plasma membranes is shown in Table II. Ouabain and oligomycin did not have any effect on the calcium transport or calcium-dependent phosphorylation whereas both processes were significantly inhibited by 100 µM of orthovanadate. The inhibition by orthovanadate on the calcium uptake was dose-dependent with a 50% inhibition at an orthovandate concentration of ~20 µM (Fig. 8).

Other inhibitors that have been reported to inhibit mitochondrial ATPases (sodium azide, Adriamycin) (34, 35) or other ATPases (N,N'-dicyclohexylcarbodiimide) (36, 37) had little or no effect on the calcium-dependent phosphorylation.

Discussion

We have described an ATP-dependent calcium-transport system and the phosphorylated intermediate of the (Ca2+ + Mg2+)-ATPase associated with this transport system in the plasma membranes of rat liver. The experimental evidence presented in this study strongly suggest that the calcium uptake is plasma membrane-specific and cannot be attributable to microsomal or mitochondrial contaminant in the plasma membrane fraction. This contention was demonstrated by several means. First, the plasma membrane specific marker enzyme, 5'-nucleotidase was enhanced by 4-fold in the plasma membrane fraction whereas the microsomal marker enzyme, glucose-6-phosphatase, decreased by 50% over those in the crude homogenate. Second, inhibitors of mitochondrial ATPase and calcium uptake (azide and oligomycin) did not affect the calcium-transport activity. Third, oxalate, which serves as the trapping agent for calcium accumulation, was not absolutely required for net calcium uptake. The oxalate requirement is consistent with that observed with sarcolemma (38) but in contrast with that reported with liver microsomes (20). Fourth, the magnesium concentration for optimal calcium uptake by plasma membrane vesicles was different from that required by the liver microsomes that were enriched in endoplasmic reticulum (Table III). In addition, the Km values for free calcium and ATP for the plasma membrane system (17.6 nM free calcium, 81 µM ATP) were vastly different from those for liver microsomal transport system (4.6 µM free calcium, 1.8 mM ATP) (20) but closely resembled those reported for the (Ca2+ + Mg2+)-ATPase (13 nM free calcium, 21 µM ATP) in plasma membranes of liver (15).

Finally, the most convincing evidence comes from our study which measured simultaneously the 5'-nucleotidase, (Ca2+ + Mg2+)-ATPase, Ca2+-dependent phosphorylation, and Ca2+-transport activities in the plasma membrane and microsomal fractions of rat liver. Results indicate that their activities in liver microsomal fraction all represented only 30-40% of their respective activities in the plasma membranes (Table IV). These data clearly demonstrate that these activities identified in the microsomal fraction could only be accounted for by plasma membrane contamination.

Also in this study, we demonstrated the presence of a single major calcium-sensitive phosphoprotein in the plasma membranes of liver. The molecular weight of the phosphoprotein was demonstrated to be 105,000 ± 5,000. Although this differs from that of erythrocyte Ca2+-ATPase (120,000-150,000 Da) (39-44), addition of PMSF to the assay medium or the use of plasma membranes prepared in the presence of 1 mM PMSF did not result in altered migration of this protein on polyacrylamide gels. Moreover, a similar molecular weight was reported for the phosphorylated intermediate of (Ca2+ + Mg2+)-ATPase in plasma membranes of adipocytes (14).

The similarities between the properties of this phosphoprotein and the previously reported (Ca2+ + Mg2+)-ATPase in plasma membranes of rat liver suggests that it could represent the phosphorylated intermediate of this enzyme (Table III).
TABLE III
Comparison of high affinity (Ca$^{2+}$ + Mg$^{2+}$)-ATPase, calcium-dependent 105,000-Da phosphoprotein and calcium transport of plasma membranes and microsomes from rat liver

| Enzyme properties | Plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase (Ref. 18) | Calcium-dependent phosphoprotein | Calcium transport (Ref. 37) |
|-------------------|--------------------------------------------------------|---------------------------------|-----------------------------|
| $K_m$ for free calcium | 13 ± 5 μM                                             | 15.5 ± 6.9 μM                   | 17.6 ± 2.5 μM               |
| $K_i$ for ATP      | 21 ± 9 μM                                             | ~1 μM                           | 81 ± 16 μM                  |
| Magnesium requirement | Micromolar                                           | Micromolar                      | 10 μM                       |
| Oxalate requirement | Not determined                                        | Not determined                  | Absolutely required         |

Values and characteristics listed for the calcium-dependent phosphoprotein and calcium transport were determined in the studies described in this paper. The data for (Ca$^{2+}$ + Mg$^{2+}$)-ATPase of plasma membranes and calcium transport by microsomes were from Ref. 15 and 20, respectively.

TABLE IV
Distribution of the S'-nucleotidase, (Ca$^{2+}$ + Mg$^{2+}$)-ATPase, Ca$^{2+}$-dependent phosphorylation, and Ca$^{2+}$-transport activities between the plasma membrane and microsomal fractions of rat liver

Plasma membrane and microsomal fractions were isolated from rat liver according to Pilkis et al. (17) and Moore et al. (20), respectively. (Ca$^{2+}$-Mg$^{2+}$)-ATPase was assayed according to Lottersztajn et al. (15). Ca$^{2+}$-dependent phosphorylation was measured at 4°C for 30 s in a Ca$^{2+}$/EGTA buffer containing 50 mM Tris-Cl, pH 8.0, 4 mM EGTA, 5 μM [γ-32P]ATP and in the presence or absence of 4 mM CaCl$_2$. The phosphorylated plasma membrane proteins were separated on TDA-polycrylamide gel electrophoresis. The incorporation of 32P into the 105,000-Da calcium-dependent protein was quantitated by counting the excised band. Ca$^{2+}$-transport activity was assayed at 37°C in a medium containing 50 mM Tris-Cl, pH 8.0, 0.25 M sucrose, 0.2 mM EGTA, 0.18 mM CaCl$_2$, 1-2 μCi/ml of $^{45}$CaCl$_2$, 10 mM MgCl$_2$, 4 mM Tris-oxalate, 10 mM Tris-ATP, and 20 mM Na$_2$SO$_4$. The Ca$^{2+}$-oxalate trapped inside the vesicles was collected by filtration on 0.45-μm Millipore filters and counted. Results represent the mean ± S.E. of 6 determinations from 2 preparations. The number in parentheses expresses the activity as a percentage of that for plasma membranes.

In addition to having divalent ion and ATP requirements similar to the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase, the phosphoprotein also possesses properties that readily distinguishes it from the products formed by protein kinase reactions. These include the rapid breakdown of the phosphoryl bond upon addition of unlabeled ATP to the assay medium (14, 23, 32, 33, 40). The properties of these proteins are consistent with those found in other ATPases and suggest strongly that the phosphoprotein represents the phosphorylated intermediate of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase.

Like other ATPase-associated transported systems, both calcium uptake and formation of the phosphoprotein were inhibited by orthovanadate (45). Although the $K_i$ for orthovanadate (~20 μM) for calcium transport is significantly higher than the 2 μM for the calcium pump in other plasma membranes (38) and since the inhibitory effect of vanadate is greatly enhanced by the presence of potassium (46), our finding may simply reflect either such a difference in the two incubation conditions or a major difference between two vastly functionally and metabolically different tissues.

Thus far, we have characterized the phosphorylated intermediate and the calcium-transport system associated with the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase of plasma membranes of rat liver. The role of plasma membrane calcium regulation in terms of cellular metabolic function is poorly understood. Plasma membranes, mitochondria, and endoplasmic reticulum all have been suggested to play an important role in regulating the intracellular calcium concentration. Evidence suggests that the calcium uptake of calcium by liver mitochondria is governed by the total calcium content above a critical total concentration exceeding the physiological value (47). Thus, plasma membranes or membranes of the endoplasmic reticulum which communicate with extracellular fluid could be the primary regulators involved in fine adjustment regulation of calcium levels that may play a crucial role in control of permeability or of certain enzymes in the intermediary metabolic pathways (48-51). Moreover, the influence of hormone on high affinity (Ca$^{2+}$ + Mg$^{2+}$)-ATPase has been documented in plasma membranes of adipocytes (13, 14) and liver (16). Perhaps in hormone-sensitive tissues the Ca$^{2+}$-ATPase-associated calcium-transport system may also be involved in hormone action. The identification and characterization of the phosphorylated intermediate of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase as well as the calcium-transport system in plasma membranes of liver can therefore provide a valuable means by which their role in hormone action can be further pursued.

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