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Ca^{2+} uptake by corpus-luteum plasma membranes

Evidence for the presence of both a Ca^{2+}-pumping ATPase and a Ca^{2+}-dependent nucleoside triphosphatase

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Plasma-membrane vesicles from rat corpus luteum showed an ATP-dependent uptake of Ca^{2+}. Ca^{2+} was accumulated with a $K_{\frac{1}{2}}$ (concn. giving half-maximal activity) of 0.2 μM and was released by the bivalent-cation ionophore A23187. A Ca^{2+}-dependent phosphorylated intermediate (M_r 100000) was detected which showed a low decomposition rate, consistent with it being the phosphorylated intermediate of the transport ATPase responsible for Ca^{2+} uptake. The Ca^{2+} uptake and the phosphorylated intermediate ($E \sim P$) displayed several properties that were different from those of the high-affinity Ca^{2+}-ATPase previously observed in these membranes. Both Ca^{2+} uptake and $E \sim P$ discriminated against ribonucleoside triphosphates other than ATP, whereas the ATPase split all the ribonucleoside triphosphates equally. Both Ca^{2+} uptake and $E \sim P$ were sensitive to three different Hg-containing inhibitors, whereas the ATPase was inhibited much less. Ca^{2+} uptake required added Mg^{2+} (K_m = 2.2 mM), whereas the ATPase required no added Mg^{2+}. The maximum rate of Ca^{2+} uptake was about 400-fold less than that of ATP splitting; under different conditions, the decomposition rate of $E \sim P$ was 1000 times too slow to account for the ATPase activity observed. All of these features suggested that Ca^{2+} uptake was due to an enzyme of low activity, whose ATPase activity was not detected in the presence of the higher-specific-activity Ca^{2+}-dependent ATPase.

INTRODUCTION

The relation between the high-Ca^{2+}-affinity ATPase and Ca^{2+} transport is simple in the erythrocyte; the Ca^{2+}-ATPase is primarily due to the Ca^{2+} pump, and the properties of one reflect closely those of the other (Sarkadi, 1980; Schatzmann, 1982). In some plasma membranes of more active tissues, the relationship also appeared to be simple: in smooth muscle a calmodulin-stimulated ATPase is present, and its properties were reported to reflect those of the plasma-membrane ATP-dependent Ca^{2+} pump (De Schutter *et al.*, 1984). Because of these examples, before 1984 researchers assumed that a high-Ca^{2+}-affinity ATPase represented an enzyme responsible for Ca^{2+} transport. Some subsequent studies indicate that this is not always the case: Ochs & Reed (1983, 1984) showed that neutrophil plasma-membrane vesicles, in the absence of Mg^{2+}, contained a high-Ca^{2+}-affinity ATPase with properties rather different from those of the Ca^{2+} transport seen in the presence of Mg^{2+}. Even in one type of smooth muscle, a Ca^{2+}-ATPase not involved in Ca^{2+} transport has been reported (Kwan *et al.*, 1986).

Liver plasma membranes represent another case in which the relationship between Ca^{2+} transport and Ca^{2+}-ATPase is complex and confusing. The liver plasma-membrane Ca^{2+} pump has a phosphorylated intermediate of M_r 100000 (Chan & Junger, 1983), much smaller than the M_r 140000 of the calmodulin-responsive Ca^{2+} pumps. A high-affinity Ca^{2+}-ATPase was first reported in liver plasma membranes by Lotersztajn *et al.* (1981). An ATPase, believed to be the same one, was subsequently purified to high specific activity by Lin & Fain (1984). Lin (1985*a*,*b*) has subsequently reported that this ATPase is not responsible for Ca^{2+} transport in rat liver plasma membranes. She showed that Ca^{2+} transport in reconstituted plasma membranes had a strict requirement for Mg^{2+} and ATP, whereas purified Ca^{2+}-ATPase did not require Mg^{2+} and could utilize a variety of nucleoside triphosphates (Lin, 1985*a*). The transport was also reported to be sensitive to vanadate, whereas most of the ATPase activity was not. On the other hand, the Pecker group has shown that their purified ATPase can support Ca^{2+} transport (C. Pavoine, S. Lotersztajn, A. Mallet & F. Pecker, personal communication). Clearly, additional experiments are necessary to determine the relationship between Ca^{2+} transport and Ca^{2+}-ATPase.

In the female reproductive system, the corpus luteum displays an interesting specialization of the plasma membrane, with the microvillus surface of the cells being enriched in human choriogonadotropin binding, 5′-nucleotidase, Mg^{2+}-dependent ATPase, Na^+/K^+-dependent Mg^{2+}-ATPase and Ca^{2+}-ATPase activities. In contrast, the basolateral surface membrane is enriched in the various forms of adenylate cyclase, but not in other plasma-membrane markers (Bramley & Ryan, 1980). In our original study of the Ca^{2+}-ATPase from these plasma membranes, we found it to have properties consistent with its being responsible for transport of Ca^{2+} (Verma & Penniston, 1981). In common with all other workers at that time, we supposed this ATPase to be a Ca^{2+}-extrusion pump.

We now report data on the relationship between Ca^{2+} transport and Ca^{2+}-ATPase; this indicates that the two activities have quite different properties, and are probably different enzymes.

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

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MATERIALS AND METHODS

Animals and materials

Immature female Holtzman rats, 22–25 days old and weighing 90–100 g, were obtained from Holtzman Co., Madison, WI, U.S.A. Pregnant-mare-serum gonadotropin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; human choriogonadotropin was from Ayerst, New York, NY, U.S.A.; carrier-free [γ-^{32}P]ATP was from New England Nuclear, Boston, MA, U.S.A.; and chemicals used in the enzmic assay were from Sigma.

Preparation of plasma membrane

The microvillus (light) membrane from corpus luteum was prepared from female rats which had been primed with pregnant-mare-serum gonadotropin, followed by human choriogonadotropin. The procedures for the priming and for the preparation of the membrane were as previously described (Verma & Penniston, 1981). All buffers used for the preparation of membrane contained 5 mM-benzamidine and 0.5 mM-phenylmethanesulphonyl fluoride. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Detection of phosphorylated intermediate

Plasma membranes (200 μg for electrophoresis or 20 μg for other experiments) were suspended in a reaction mixture containing 50 mM-Tes/triethanolamine, pH 7.4, 2 mM-EDTA/triethanolamine, pH 7.4, 0.1 mM-ouabain, 1.994 mM-Ca^{2+} (free [Ca^{2+}] was 3.1 μM). The suspension was preincubated at 37 °C for 20 min, and then cooled to 0 °C. The reaction (at 0 °C) was started by the addition of 2 μM-[γ-^{32}P]ATP (sp. radioactivity 100 μCi/nmol) to the reaction mixture (final volume 0.5 ml), and stopped after 15 s with 1 vol. of a cold solution containing 14% (w/v) trichloroacetic acid, 10 mM-H_3PO_4 and 4 mM-ATP (stopping solution).

In chase experiments (Table 1 and Figs. 3 and 4), after 15 s 60 mM non-radioactive nucleoside triphosphate was added to a final concentration of 6 mM, and precipitation by the stopping solution followed after the time indicated. After centrifugation for 10 min at 2400 *g* at 4 °C, the pellet was washed twice with the cold stopping solution and once with cold water, and then redissolved for gel electrophoresis or direct radioactivity counting. For the latter, the washed precipitate was dissolved in 150 μl of 5% (w/v) SDS/6 M-urea, and then 15 ml of scintillation liquid (Safety Solv) was added. The vials were counted for radioactivity in a scintillation counter.

For electrophoresis, the sample was dissolved in 100 mM-sodium phosphate, pH 6.0, containing 1% SDS, 20 mM-dithiothreitol, 6 M-urea and 0.002% Bromophenol Blue. SDS/polyacrylamide-gel electrophoresis of the ^{32}P-labelled enzyme (150 μg of plasma membrane/gel tube) was done on 4%-acrylamide gels containing 96 mM-sodium phosphate buffer, pH 6.0, 0.1% SDS and 6 M-urea. The gels were run in 100 mM-sodium phosphate (pH 6.0)/0.1% SDS at 15 °C for 2 h at 2 mA/gel tube and for 3 h more at 3 mA/gel tube. The gels were then frozen on solid CO_2, and 1 mm slices were incubated with NCS solubilizer (Amersham Corp., Arlington Heights, IL, U.S.A.) at 50 °C for 2 h. After cooling, the digests were neutralized with acetic acid and then 10 ml of scintillation liquid (Safety Solv, Research Products International Corp., IL) was added. The vials were counted for radioactivity in a Beckman LS-100C scintillation counter.

Ca^{2+}-ATPase assay

The Ca^{2+}-ATPase activity was measured by monitoring the release of [^{32}P]P_i from [γ-^{32}P]ATP as previously described (Verma & Penniston, 1981). The reaction mixture, in a final volume of 0.5 ml, contained 50 mM-Tes/triethanolamine, pH 7.4, 2 mM-EDTA/triethanolamine, pH 7.4, 0.1 mM-ouabain, 1.994 mM-$CaCl_2$ (free Ca^{2+} 0.90 μM), 25–50 μg of plasma membrane, and 6 mM-[γ-^{32}P]ATP (Tris salt; sp. radioactivity 0.1 Ci/mol). The reaction was started by the addition of [γ-^{32}P]ATP. Ca^{2+}-stimulated activity was determined by subtracting the value in the absence of Ca^{2+} from that in its presence. Incubations were done at 37 °C for 30 min, and [^{32}P]P_i was determined by the extraction of phosphomolybdate complex into the organic phase and counting radioactivity in a scintillation counter.

Nucleotide specificity was assayed by utilizing 2 mM-ATP, -ITP, -CTP, -GTP and -UTP in the same medium as used in the Ca^{2+}-ATPase assay. After the released P_i was extracted into the organic phase, the non-radioactive P_i was measured colorimetrically by the Martin & Doty method as described by Lindberg & Ernster (1956).

Ca^{2+}-uptake assay

Plasma membranes (25 μg) were suspended in a reaction mixture containing 50 mM-Tes/triethanolamine, pH 7.4, 0.25 M-sucrose, 0.1 mM-ouabain, 5 mM-$MgCl_2$, 6 mM-ATP (Tris salt) in a total volume of 0.5 ml. In the experiment to determine the apparent affinity for Ca^{2+}, 0.2 mM-EGTA/triethanolamine, pH 7.4, was used to control the free Ca^{2+} concentration. The reaction was started by adding $^{45}CaCl_2$ (150000 c.p.m./nmol) to a concentration of 50 μM (free Ca^{2+} 4.86 μM). The reaction mixture was incubated at 37 °C for 30 min, except where otherwise noted. Ca^{2+} uptake into the vesicles was measured by separating the vesicles from their medium by centrifugation (Penniston, 1982). Then 1 ml of cold 50 mM-Tes/triethanolamine buffer, pH 7.4, containing 025 M-sucrose and 5 mM-EGTA was added to the assay medium. The suspension was centrifuged for 20 min at 100000 *g*, the pellet resuspended in 1.5 ml of cold 50 mM-Tes/triethanolamine buffer, pH 7.4, containing 0.25 M-sucrose (without EGTA) and centrifuged again in the same way. The resulting pellet was resuspended in 0.3 ml of water, and 10 ml of scintillation liquid (Safety Solv) was added. The vials were counted for radioactivity in a liquid-scintillation counter. ATP-dependent Ca^{2+} uptake was determined by subtracting the value in the absence of ATP from that in its presence.

Assays of the effect of Hg-containing inhibitors

The conditions were as described above, with the following variations: the reaction mixture, containing enzyme and inhibitor (but not the starting reagent) was preincubated at 37 °C for 20 min. EDTA was omitted from the reaction media, and the total $CaCl_2$ concentration was correspondingly lowered. The final $CaCl_2$ concentrations were 50 μM (1.2 μM free Ca^{2+}) for ATPase and 3.1 μM (3.0 μM free Ca^{2+}) for phosphorylation.

RESULTS

General properties of the Ca^{2+} uptake

Incubation of corpus-luteum light plasma-membrane

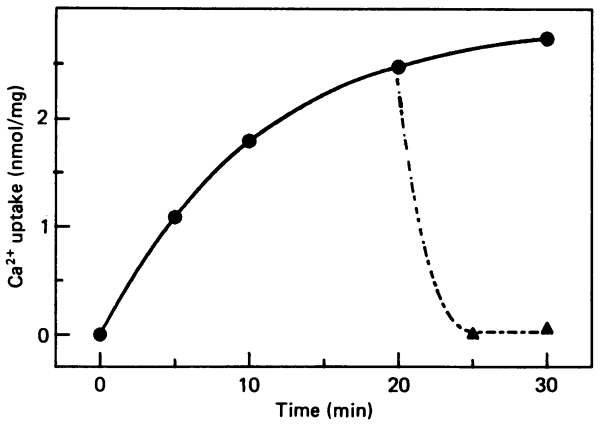

Fig. 1. Time course of ATP-dependent Ca^{2+} uptake by plasma-membrane vesicles

●, Ca^{2+} uptake in the presence of $MgCl_2$ and ATP. ▲, Effect of addition (after 20 min) of the bivalent cation ionophore A23187 (final concn. 10 μg/ml). Ca^{2+} uptake was measured in 50 mM-Tes/triethanolamine, pH 7.4, 0.25 M-sucrose, 0.1 mM-ouabain, 5 mM-$MgCl_2$, 6 mM-ATP (Tris salt) and 50 μM-$CaCl_2$. The reaction medium was preincubated without membrane for 5 min, and the reaction was started by addition of plasma membrane. Incubation was carried out at 37 °C. The A23187 was dissolved in dimethyl sulphoxide, which had no effect on measured uptake.

vesicles with Ca^{2+} and ATP showed an ATP-dependent and time-dependent uptake of Ca^{2+} (Fig. 1). In the absence of ATP, essentially no Ca^{2+} uptake occurred: the Ca^{2+} uptake was 0.28 nmol/mg, which did not vary significantly with time over the period 5–30 min. As shown in Fig. 1, addition of the bivalent-cation ionophore A23187 caused rapid and complete release of Ca^{2+}, indicating that the uptake was indeed active transport of Ca^{2+}.

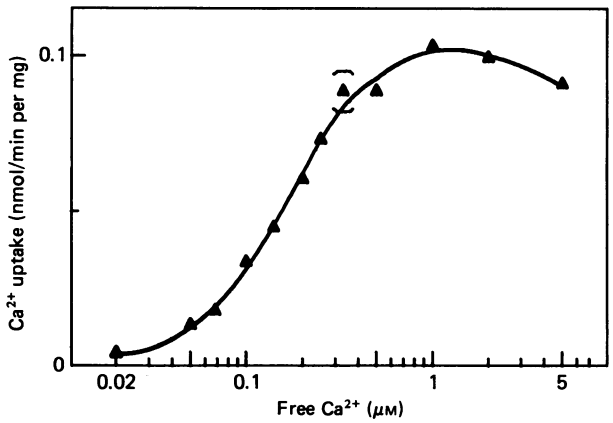

Fig. 2. Effect of free Ca^{2+} concentration on ATP-dependent Ca^{2+} uptake by plasma-membrane vesicles

Points are the average of two measurements; where the standard deviation of the two measurements exceeded 4% of full scale, it is shown by brackets. Ca^{2+} uptake was measured after 30 min of incubation at 37 °C in 50 mM-Tes/triethanolamine, pH 7.4, 0.25 M-sucrose, 0.1 mM-ouabain, 10 mM-$MgCl_2$, 6 mM-ATP (Tris salt), 0.2 mM-EGTA/triethanolamine, pH 7.4, and 0.02–5 μM free Ca^{2+}.

We could not find any evidence that the Ca^{2+} uptake was responsive to calmodulin. In six different preparations of light plasma membrane (prepared in homogenization buffer containing 1 mM-EDTA), the Ca^{2+} uptake in the presence and absence of calmodulin (40 μg/ml) was compared. For these preparations, the average activity in the presence of calmodulin was 105% ±26% (S.D.) of that in its absence. Trifluoperazine (20 μM) also did not affect the ATPase activity of light plasma membrane prepared in the absence of EDTA.

The Ca^{2+}-dependence of Ca^{2+} uptake is shown in Fig. 2. The free Ca^{2+} was controlled by an EGTA buffer system, in which added Ca^{2+} was varied. Half-maximal activation of Ca^{2+} transport occurred at about 0.2 μM free Ca^{2+}; this value is consistent with that found for other physiologically important plasma-membrane Ca^{2+} pumps.

The Ca^{2+} uptake showed an absolute requirement for Mg^{2+}. The dependence of Ca^{2+} uptake on Mg^{2+} obeyed the Michaelis–Menten equation; the K_m for Mg^{2+} was 2.2 mM.

Detection of an acyl phosphate intermediate (E ~ P)

Low-temperature incubation with highly labelled [γ-^{32}P]ATP allowed the detection of a labile phosphorylated intermediate of the type frequently associated with transport ATPases (Fig. 3). Also shown is the 'chase' of this phosphorylated intermediate by excess of unlabelled ATP. The assay was carried out in the absence of Mg^{2+}, a condition which encourages the detection of such phosphorylated intermediates in transport ATPases, even though these enzymes require Mg^{2+} for rapid turnover (Garrahan *et al.*, 1976). Measurements on several different preparations showed that this phosphorylated intermediate migrated with M_r 100000, a value lower than that of the Ca^{2+}-ATPase of erythrocyte plasma membranes. The Ca^{2+} requirement of E ~ P is

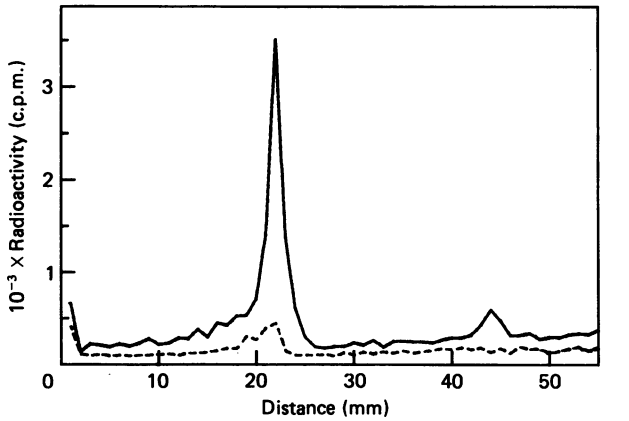

Fig. 3. Detection of acyl phosphate intermediate after electrophoresis

The phosphorylated intermediate was detected under conditions optimal for its labelling (low ATP, no Mg^{2+}). The details of the conditions are described in the Materials and methods section. ——, distribution of ^{32}P radioactivity after phosphorylation for 15 s at 0 °C; ----, that after chase with 6 mM unlabelled ATP for 60 s at 0 °C. The M_r values of standards were compared with that of this phosphorylated intermediate in a control electrophoresis experiment conducted at the same time; the phosphorylated intermediate had M_r 100000.

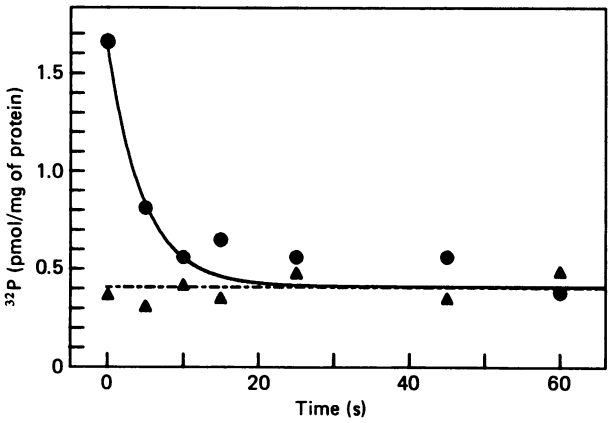

Fig. 4. Decay of Ca^{2+}-dependent phosphorylated intermediate in the presence of excess ATP

●, Phosphorylation in the presence of Ca^{2+}; ▲, that in the absence of Ca^{2+}. The details of the experimental conditions are described in the Materials and methods section. After 15 s incubation with [γ-^{32}P]ATP in media with and without Ca^{2+}, 60 mM non-radioactive ATP (final concn. 6 mM) was added, and the reaction continued. The reaction was terminated at the indicated times by adding stopping solution. Incubation was at 0 °C.

shown in Fig. 4, which also deals with the turnover rate of the enzyme, and is discussed further below.

Comparison of the properties of Ca^{2+} uptake and of E ~ P with those of the Ca^{2+}-ATPase

As one approach to this comparison, it was useful to look at the nucleotide specificity and the effects of Hg-containing inhibitors. It was not possible to measure these three activities under identical conditions, because of the special requirements for the assay of each activity. For example, the low-affinity Mg^{2+}-ATPase had a high activity under the conditions necessary for Ca^{2+} transport, thus preventing measurement of the high-affinity Ca^{2+}-ATPase. Despite this difficulty, it seems unlikely that the nucleotide specificity of the activities, or their sensitivity to Hg-containing inhibitors, would be drastically changed by the small changes in the assay conditions. As Table 1 shows, Ca^{2+} uptake and formation of E ~ P were rather specific for ATP, whereas the Ca^{2+}-ATPase split all the substrates equally well.

Table 1. Nucleoside triphosphate specificity of the three activities

For ATPase and uptake, a final concentration of 2 mM of each nucleotide was used. For E ~ P, the effectiveness of each nucleotide in a 10 s chase experiment was compared. The percentage of maximum activity was calculated as $(x_0-x)/x_0-x_{ATP})$, where x indicated the radioactivity (c.p.m.) remaining 10 s after initiation of the chase, x_0 the radioactivity in a control with no added nucleotide, and x_{ATP} the radioactivity when ATP was the chasing nucleotide.

	Activity (% of maximum)		
Nucleotide	Ca^{2+} uptake	E ~ P	Ca^{2+}-ATPase
ATP	100	100	98
ITP	45	46	80
CTP	28	33	90
GTP	4	27	84
UTP	4	10	100

The effects of three different Hg-containing compounds are shown in Table 2; the high-affinity Ca^{2+}-ATPase was relatively insensitive to all three, whereas Ca^{2+} uptake was completely inhibited even by 10 μM of any of these reagents. Formation of E ~ P was also sensitive to these compounds, but somewhat less sensitive than Ca^{2+} uptake.

In order to determine whether E ~ P could account for the Ca^{2+}-stimulated ATPase activity, the turnover of the E ~ P was compared with the ATPase activity under the same incubation conditions. Fig. 4 shows the amount of phosphorylated intermediate, with and without Ca^{2+}, as a function of time after excess unlabelled ATP was added. Zero time in Fig. 4 corresponds to the addition of excess unlabelled ATP. From the decay of the phosphorylated intermediate, a rate constant of 0.21 ± 0.01 (S.D.) s^{-1} was calculated. On the basis of the simple scheme:

$$E + ATP \underset{ADP}{\rightleftharpoons} E \sim P \xrightarrow{k} E + P_i$$

one can calculate the velocity of the ATPase activity, which would correspond to this decomposition rate of the phosphorylated intermediate, by using the equation:

$$v_{calc.} = k \times [EP]$$

where k is the rate constant and [EP] the steady-state concentration of phosphorylated intermediate. The concentration of phosphorylated intermediate is the difference between the phosphorylated intermediate observed in the presence of Ca^{2+} and the background observed in its absence; this was 1.25 pmol/mg. From the rate constant of 0.21 s^{-1}, the calculated rate of ATP splitting was 16 pmol/min per mg.

Under identical conditions, with 6 mM labelled ATP and incubation at 0 °C, the ATPase activity was measured at incubation times of 5, 10, 20, 40 and 60 min. ATP splitting was linear with time, and the Ca^{2+}-ATPase activity was 16.7 nmol/min per mg, over 1000 times faster than could be accounted for by turnover of the phosphorylated intermediate.

It is not possible to compare directly the turnover of the phosphorylated intermediate with the rate of transport, since detection of transport requires higher ATP concentrations, which make it impractical to detect the phosphorylated intermediate. However, at 37 °C and 6 mM-ATP, Ca^{2+} transport is 85 pmol/min per mg. This can be corrected to 0 °C by using the activation energy determined for the erythrocyte plasma-membrane Ca^{2+} pump (54 kJ/mol) (Penniston *et al.*, 1980). Making this correction yielded a calculated rate of 5 pmol/min per mg, comparable with the turnover of the phosphorylated intermediate.

The effects of vanadate on transport and ATPase were also compared, but the vanadate effect proved to be more dependent on the concentration of ATP than on the nature of the activity tested. At 6 mM-ATP, neither ATPase nor Ca^{2+} transport was inhibited significantly by

Table 2. Effect of Hg-containing inhibitors on Ca^{2+}-ATPase, Ca^{2+} uptake and formation of enzyme ~ phosphate intermediate

Details of the conditions used are given in the Materials and methods section.

Inhibitor	Concn.	ATPase (nmol/min per mg)	Ca^{2+} uptake (pmol/min per mg)	Phosphorylation (pmol/mg)
None		59	66	2.2
p-Chloromercuribenzoate	10 μM	49	1	1.6
	100 μM	41	−2	0.0
p-Chloromercuribenzenesulphonate	10 μM	51	−1	1.7
	100 μM	39	−3	−0.1
$HgCl_2$	10 μM	39	−3	1.1
	100 μM	20	1	0.0

concentrations of vanadate of up to 200 μM, whereas at 20 μM-ATP both ATPase and formation of the phosphorylated intermediate were inhibited by 100 μM-vanadate. It was not possible to assay the phosphorylated intermediate at high [ATP], because of the huge amounts of radioactive ATP that would be required. At low [ATP], Ca^{2+} transport was not detectable. Because of the dependence of inhibition on ATP concentration and these limitations on the measurements that could be made, vanadate was not a useful agent for determining the relationship between ATPase, transport and the phosphorylated intermediate.

DISCUSSION

As is the case in liver plasma membrane, so also in corpus luteum the presence of different types of Ca^{2+}-ATPase activities makes it difficult to assess unequivocally the role of each. The present data, combined with those previously published (Verma & Penniston, 1981) requires, at a minimum, three enzyme activities: (1) a high-Ca^{2+}-affinity Ca^{2+} transporter of low specific activity; (2) a high-Ca^{2+}-affinity nucleoside triphosphatase of somewhat higher specific activity; and (3) a low-affinity (Ca^{2+} or Mg^{2+}) nucleoside triphosphatase of relatively high specific activity. Since some of these cannot be assayed under the conditions required to detect the others, their roles are difficult to disentangle. Despite this difficulty, the data presented here show striking differences in the properties of activities (1) and (2). For the same enzyme to be responsible for both activities it would, as Mg^{2+} was decreased, have to become uncoupled from transport, change its nucleotide specificity and its sensitivity to Hg-containing inhibitors. This is unlikely, and it seems more reasonable to attribute the high-Ca^{2+}-affinity ATPase activity to a separate enzyme from that responsible for the Ca^{2+} transport.

The comparison of the E ~ P kinetics with those of the ATPase demonstrates that the phosphorylated intermediate cannot be responsible for the ATPase activity. It is not possible to identify E ~ P conclusively as an intermediate of the Ca^{2+} transport without purification and reconstitution. However, the concurrence of several pieces of evidence points insistently toward the association of E ~ P with Ca^{2+} transport: both activities are sensitive to mercurials, and both have similar nucleotide specificity. E ~ P has a similar M_r and similar turnover properties to those of the phosphorylated intermediate of other ion transporters. Finally, the rate of reaction calculated from the decomposition rate of E ~ P is comparable with the rate of Ca^{2+} transport.

This last comparison is only approximate, because of the necessary differences in assay conditions for E ~ P and Ca^{2+} uptake, and because factors such as sidedness and leakiness of the vesicles may decrease Ca^{2+} transport somewhat. However, none of these factors would be expected to account for the 1000-fold difference between the transport rate and the rate of the Ca^{2+}-ATPase.

The different sensitivities to Hg-containing inhibitors of E ~ P and of Ca^{2+} transport might seem to separate them. However, this difference can be resolved when it is taken into account that formation of the phosphorylated intermediate is a partial reaction of the entire Ca^{2+}-transport cycle. The existence of two sites for Hg inhibition of the Ca^{2+}-transport ATPase would cause a differential sensitivity, such as is observed. One site would inhibit a step leading to formation of the phosphorylated intermediate, and the other site a step following formation of the phosphorylated intermediate. Action of Hg-containing inhibitors at either site would disrupt the catalytic cycle and prevent Ca^{2+} uptake, thus accounting for the high sensitivity of Ca^{2+} transport. Action at the second site would not inhibit formation of the phosphorylated intermediate, but action at the first site would, accounting for the lesser sensitivity of the phosphorylated intermediate.

A wide variety of tissues appear to have plasma-membrane Ca^{2+} pumps which resemble that of the erythrocyte. Both a calmodulin-responsive Ca^{2+}-ATPase (or Ca^{2+} transport) and a phosphorylated intermediate (or purified enzyme) of M_r 140000 have been demonstrated in brain (Papazian *et al.*, 1984; Hakim *et al.*, 1982), skeletal muscle (Michalak *et al.*, 1984), heart muscle (Caroni & Carafoli, 1981), smooth muscle (De Schutter *et al.*, 1984), intestinal epithelium (De Jonge *et al.*, 1981; Nellans & Popovitch, 1981), kidney (De Smedt *et al.*, 1981, 1984), pancreas (Ansah *et al.*, 1984) and Ehrlich ascites cells (Wetzker *et al.*, 1986). In addition, calmodulin-responsiveness of Ca^{2+}-ATPase or Ca^{2+} transport has been demonstrated in osteosarcoma cells (Shen *et al.*, 1983), thyroid (Kasai & Field, 1982), macrophages (Lew & Stossel, 1980), lymphocytes

(Lichtman *et al.*, 1981) and chloroplast envelope (Nguyen & Siegenthaler, 1985). In the light of all these reports, the failure to demonstrate calmodulin-stimulated ATPase or transport in liver and in corpus luteum is rather striking, as is the different M_r of the phosphorylated intermediate. In yet another case (platelets), where the primary phosphorylated intermediate showed an M_r of about 100000, study of patterns of proteolysis showed that two types of Ca^{2+} pumps were present. A non-proteolysed pump of the endoplasmic-reticulum type was mixed with a partially proteolysed (but still active) pump of the erythrocyte type (Enyedi *et al.*, 1986). Additional work will be necessary to determine the source of the Ca^{2+}-transporter studied in the present paper.

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