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Ca^{2+} Translocation in Ehrlich Ascites Tumor Cells

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Summary. Ca^{2+} uptake into Ehrlich ascites tumor cells was studied at 0 °C in the presence of mitochondrial inhibitors, conditions that minimized complications caused by sequestration of Ca^{2+} into organelles or by excretion. Under these conditions Ruthenium Red inhibited Ca^{2+} uptake, but other previously implicated ions, such as P_i or Mg^{2+}, had no effect. Valinomycin either inhibited or slightly stimulated Ca^{2+} uptake depending on the presence of excess K^+ on the outside or inside of the cell, respectively. Nigericin inhibited Ca^{2+} transport. Based on these data we propose an electrogenic uptake of Ca^{2+}, possibly via a Ca^{2+}/H^+ antiport mechanism.

The observation that glucose inhibited Ca^{2+} uptake suggested that in Ehrlich ascites tumor cells an energy-driven Ca^{2+} expulsion mechanism is operative, similar to that in erythrocytes. Plasma membrane preparations of ascites tumor cells were found to contain a Ca^{2+}-dependent ATPase. These preparations, when incorporated into liposomes in an inside-out orientation, catalyzed an ATP-dependent uptake of Ca^{2+}.

Calcium plays a unique role in mammalian cells. At low concentrations it influences enzymatic reactions, the function of excitable membranes, cell permeability, cell communication, and cell growth (Carafoli *et al.*, 1975). Ca^{2+} is eminently suited to play such a critical role because the concentration of the free ion in the cytosol is very low compared to that of other physiological cations such as K^+, Na^+ or Mg^{2+}. The low Ca^{2+} concentration in the cytosol is maintained by several mechanisms, involving sequestration by intracellular organelles (mitochondria and microsomes) and expulsion from the cell in the other direction.

The complexity of the various movements of Ca^{2+} into and from organelles have complicated the analysis of the primary event, the entry of Ca^{2+} into the cell. It was shown by Cittadini, Scarpa and Chance (1973) that mitochondria are the major reservoir for Ca^{2+} in Ehrlich ascites tumor cells and that the entry into the cells is dependent on coupled respiration. The authors concluded that these cells "possess

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a plasma membrane permeable to Ca^{2+} and that Ca^{2+} uptake by mitochondria inside the cells accounts for the entire cellular Ca^{2+} accumulation." The question then arises how permeable is the plasma membrane and what is the mechanism of Ca^{2+} entry? The entry of Ca^{2+} into Ehrlich ascites tumor cells is in fact limited by the permeability of the plasma membrane. We have shown (McCoy, Resch & Racker, 1976) that in cells permeabilized by exposure to dextran sulfate the uptake of Ca^{2+} into the mitochondria in the presence of ATP[1] was much faster than in intact cells, even when compared with the rates at optimal conditions in the presence of succinate, rotenone, and P_i described by Landry and Lehninger (1976) and Resch Charlton and Wenner (1978).

In the present paper we report on investigations of the mechanism of Ca^{2+} entry into Ehrlich ascites tumor cells. To make these studies possible, we had to develop experimental conditions that minimized the contribution (stimulating or inhibiting) of other Ca^{2+} transport systems including movements into organelles and expulsion from the cell. In the course of these studies we obtained evidence for the presence of an energy-driven Ca^{2+} pump in the plasma membrane. Isolated plasma membrane reconstituted into liposomes catalyzed an ATP-dependent uptake of Ca^{2+}.

Materials and Methods

Cells and Media

Ehrlich ascites tumor cells were maintained in mice, harvested after 7 to 10 days of growth as described previously (Scholnick, Lang & Racker, 1973). They were washed 3 times with medium consisting of 120 mM NaCl, 5 mM KCl, 4 mM sodium P_i, 0.5 mM $MgCl_2$, 40 mM Hepes (pH 7.4) unless stated otherwise. After washing, the pellet was diluted to about 40 mg/ml in the same buffer. Protein was measured according to the method of Lowry *et al.* (1951) in the presence of 1% sodium deoxycholate with bovine serum albumin as a standard.

Ca^{2+} Uptake

Mitochondrial inhibitors antimycin A, 20 μM (Sigma Chemical Company, St. Louis, Mo.) and oligomycin, 20 μg/ml (Sigma), were added to cells at 0° for 5 min prior to assay. The uptake was started with the dilution of cells into various buffers (as indicated) containing 10 μM antimycin A and 10 μg/ml oligomycin, 200 μM $^{45}Ca^{2+}$ at a specific radioactivity of about 100 Ci/mole (ICN, Chemical Radioisotope Division, Irvine, Calif.). The

[1] *Abbreviations:* ATP – adenosine triphosphate; P_i – inorganic phosphate; HEPES – N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; EDTA – (ethylenedinitrilo)tetraacetic acid; ATPase – adenosine triphosphatase; EGTA – ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid.

incubations were done at 0° unless otherwise stated. The cell protein concentration in the assay was usually about 4 mg/ml. Samples were removed periodically and passed through Dowex 50W, 50X8-100 cation exchanger (Sigma Chemical Company, St. Louis, Mo.) which had been converted to the Tris form, and treated with bovine serum albumin (Sigma Stock Solution). After elution with 0.25 M sucrose as described (Gasko *et al.*, 1976), the samples were counted for radioactivity in the presence of ACS (Amersham, Arlington Heights, Ill.) in a Beckman liquid scintillation counter.

Ca^{2+} Efflux

Cells (40 mg/ml) in 120 mM NaCl, 5 mM KCl, 4 mM sodium P_i, 0.5 mM $MgCl_2$, 40 mM HEPES (pH 7.4) were incubated with 200 μM $^{45}Ca^{2+}$ for 45 min at 0° in the presence of antimycin, 20 μM, and oligomycin, 20 μg/ml. After loading, the cells were diluted 100-fold with buffer of the same composition minus Ca^{2+}. $^{45}Ca^{2+}$ efflux from the cells at the indicated temperatures was measured after passing aliquots of cell suspensions through the Dowex columns as described above.

Preparation and Analysis of Mitochondria and Microsomes

Mitochondria were prepared (Wu & Sauer, 1967) by the Dounce homogenizer method. $^{45}Ca^{2+}$ uptake into mitochondria (0.2 mg/ml) was measured by the Dowex method after incubation at the indicated temperature in the presence of 120 mM KCl, 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 5 mM ATP and 100 μM $^{45}Ca^{2+}$.

Microsomes were prepared by centrifugation of the postmitochondrial supernatant for 20 min at $35,000 \times g$ then 30 min at $65,000 \times g$ and 60 min at $105,000 \times g$. The $35,000 \times g$ pellet was heavily contaminated with mitochondria. The $65,000 \times g$ pellet had only a small mitochondrial contamination and was analyzed for energy-dependent Ca^{2+} uptake. The $105,000 \times g$ pellet contained very little protein, no Ca^{2+} uptake activity and was discarded. Uptake of Ca^{2+} into the $65,000 \times g$ fraction was measured in the presence of 120 mM KCl, 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, and 20 μg/ml oligomycin. Ca^{2+} concentration in the assay was 100 μM, protein concentration 0.2 mg/ml.

Isolation of Plasma Membranes

Plasma membranes were isolated from 20 to 30 g wet wt of cells by the aqueous two-phase polymer procedure described by Brunette and Till (1971), except for a few minor modifications. Breakage of the cells was monitored in a phase-contrast microscope. Approximately 20 to 30 strokes with a Dounce homogenizer with a type-B pestle (Kontes, Vineland, N.Y.) were required. After homogenization, unbroken cells were removed by centrifugation at $200 \times g$ for 1 min. The isolated membranes were washed with 40 ml of 0.25 M sucrose, 1 mM EDTA (pH 7.4) three times by centrifugation at $12,000 \times g$ for 15 min and suspended in the same medium at a protein concentration between 7 and 10 mg/ml. Small aliquots were quickly frozen in dry ice-ethanol and stored at −70 °C. The preparation could be kept at least for one month with little loss of (Ca^{2+}) ATPase activity.

Assay and Reconstitution of the (Ca^{2+}) ATPase

Thawed plasma membranes (ca. 5 mg) were washed by centrifugation at $20,000 \times g$ for 15 min, two times with 40 ml of either 100 mM imidazole (pH 6.8), 100 mM NaCl,

5 mM $MgCl_2$ (for ATPase assay) or with 0.4 M potassium P_i, pH 7.4 (for reconstitution). They were suspended in the same buffer at about 5 mg/ml. (Ca^{2+}) ATPase activity was measured in the presence of 3 mM [^{32}P]ATP and 1 mM ouabain in 100 mM imidazole (pH 6.8), 100 mM NaCl and 5 mM $MgCl_2$. Fifty to 100 μg of protein were used per assay. After incubation at 37 °C for 10 min in the presence of Ca^{2+} at the indicated concentrations or in the presence of 2 mM EGTA, the reaction was terminated by the addition of trichloroacetic acid (5% final concentration). $^{32}P_i$ was extracted as described by Nelson, Nelson and Racker (1972). An aliquot of the extract was counted in the presence of ACS in a liquid scintillation counter. Reconstitution was usually done in a 0.2-ml volume by the freeze-thaw sonication technique (Kasahara & Hinkle, 1977). Acetone-washed soybean phospholipids (Kagawa & Racker, 1971) were sonicated in a bath-type sonicator (Laboratory Supplies Company, Hicksville, N.Y.) in 0.4 M potassium P_i (pH 7.4) at a concentration of 25 mg/ml. Plasma membranes in 0.4 M potassium P_i (pH 7.4) were added at a phospholipid to protein ratio of 20 to 1 or 40 to 1. The final phospholipid concentration was usually about 20 mg/ml. The mixture was quickly frozen in dry ice/ethanol for 1 min, thawed at room temperature for 20 min, and then sonicated for 20 sec at 0 °C. For Ca^{2+} uptake, vesicles were usually diluted 20-fold into 0.6 M sorbitol, 50 mM imidazole (pH 7.4), 5 mM $MgCl_2$, without or with 3 mM ATP. $^{45}Ca^{2+}$ was at 10 μM (about 100 Ci/mole) unless specified otherwise. After incubation at 37 °C, the reaction was terminated by passing the vesicles through serum albumin-treated cation exchanger. (Ca^{2+})ATPase in vesicles was measured in 0.6 M sorbitol, 50 mM imidazole (pH 7.4), 5 mM $MgCl_2$, 3 mM ATP in the presence of 10 μM Ca^{2+} or 2 mM EGTA (as a control).

Results and Discussion

Contribution of Intracellular Organelles to the Rate of Ca^{2+} Uptake by Ehrlich Ascites Cells

It can be seen from Table 1 that mitochondria isolated from Ehrlich ascites tumor cells took up Ca^{2+} at 20 °C, but very little at 0 °C. Antimycin A and oligomycin inhibited Ca^{2+} uptake. Together they were more effective than either alone. Isolated microsomes also took up Ca^{2+} at 20 °C more rapidly than at 0 °C. Also recorded in Table 1 are data which show that the uptake of Ca^{2+} into vesicles reconstituted with plasma membrane fragments is very slow at 0 °C. As will be discussed later, we believe that these reconstituted vesicles are representative of the process that is responsible for the energy-dependent expulsion of Ca^{2+} from the cell. Measurements of Ca^{2+} entry into the cells were therefore performed at 0 °C in the presence of both antimycin and oligomycin. The uptake of Ca^{2+} into Ehrlich ascites tumor cells was almost linear for about 5 min and was inhibited about 50% by the mitochondrial inhibitors (Fig. 1). After 30 min about 3.3 nmol of Ca^{2+} per mg protein were taken up in the absence and 2.5 nmol in the presence of inhibitors.

Table 1. ATP-dependent uptake of Ca^{2+} into mitochondria, microsomes and reconstituted plasma membrane vesicles at 20 °C and at 0 °C

	Ca^{2+} uptake (nmol/mg · 3 min)	% inhibition
Expt. 1:		
Mitochondria at 20 °C	134.8	–
+ antimycin A 10 μM	33.9	75.0
+ oligomycin 10 μg/ml	0.8	99.4
Mitochondria at 0 °C	3.6	–
+ antimycin A 10 μM	1.5	58.3
+ oligomycin 10 μg/ml	0.9	75.0
Microsomes at 20 °C	1.0	
Microsomes at 0 °C	0.28	
Expt. 2:		
Mitochondria at 20 °C	377.0	–
+ oligomycin 20 μg/ml	4.7	98.8
+ oligomycin 20 μg/ml + antimycin 10 μM	1.1	99.7
Reconstituted[a] plasma membrane vesicles at 20 °C	4.5	
Reconstituted plasma membrane vesicles at 0 °C	0.11	

[a]Assayed in the presence of 20 μM Ca^{2+}.

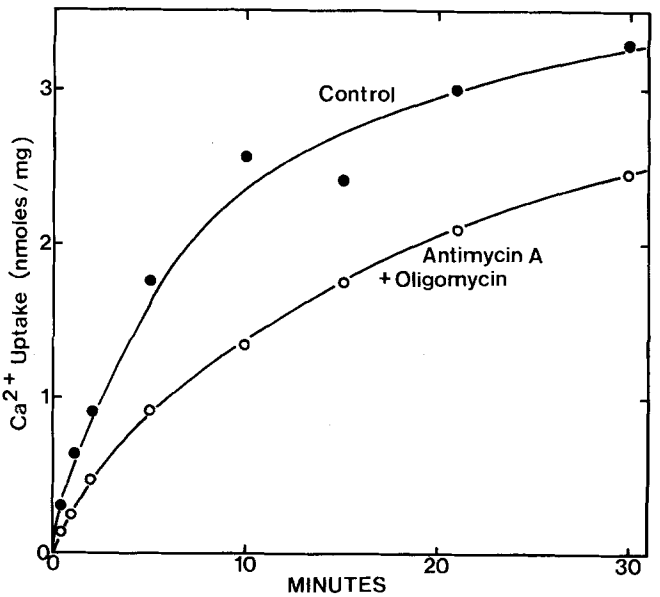

Fig. 1. Time course of Ca^{2+} uptake by Ehrlich ascites tumor cells in the presence and absence of antimycin A and oligomycin. Uptake of Ca^{2+} was measured at 0 °C in 120 mM NaCl, 5 mM KCl, 40 mM HEPES, 0.5 mM $MgCl_2$, 4 mM sodium P_i (pH 7.4) in the presence and absence of mitochondrial inhibitors as described under *Materials and Methods*. All values are expressed in terms of mg protein. One ml of packed cells contain 140 mg protein

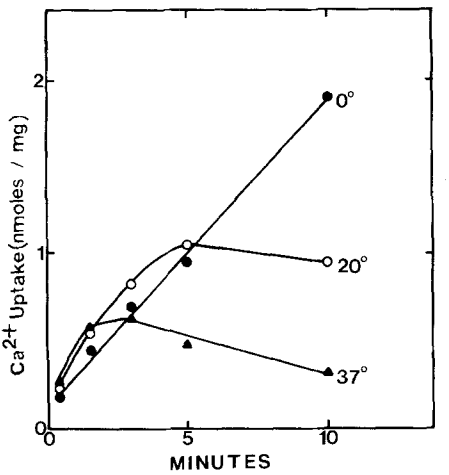

Fig. 2. Ca^{2+} uptake by Ehrlich ascites tumor cells at different temperatures. Ca^{2+} uptake was measured as described in the legend of Fig. 1. Assay temperatures were 0, 20, and 37 °C, respectively

Figure 2 shows the temperature dependency of Ca^{2+} uptake in the presence of mitochondrial inhibitors. The pattern of Ca^{2+} uptake at 0, 20, and 37 °C suggested the operation of an extrusion mechanism at the higher temperature. However, it was also possible that the cells have become leaky at the higher temperatures. Cell depleted of ATP by incubation with 2,4 dinitrophenol (Wu & Racker, 1959) were tested in the presence and absence of glucose. As shown in Fig. 3, Ca^{2+} uptake was faster in the absence of glucose. These experiments suggested the functioning of an energy-dependent expulsion mechanism which will be discussed later.

Measurements of Ca^{2+} uptake at various concentrations, revealed two phases. The rapid phase had an apparent K_m of about 50 μM (in the presence or absence of mitochondrial inhibitors). The V_{max} was considerably greater in the absence of inhibitors (Fig. 4). The second and slower phase was observed at concentrations higher than 200 μM, resulting in Ca^{2+} accumulation nearly proportional to the external concentration over the range studied (up to 1 mM, Fig. 4). Ca^{2+} uptake experiments were therefore usually performed at 200 μM Ca^{2+}.

Effect of Inhibitors on Ca^{2+} Uptake

Ruthenium Red, an inhibitor of Ca^{2+} transport in mitochondria (Moore, 1971) was shown to be without effect on Ca^{2+} uptake in microso-

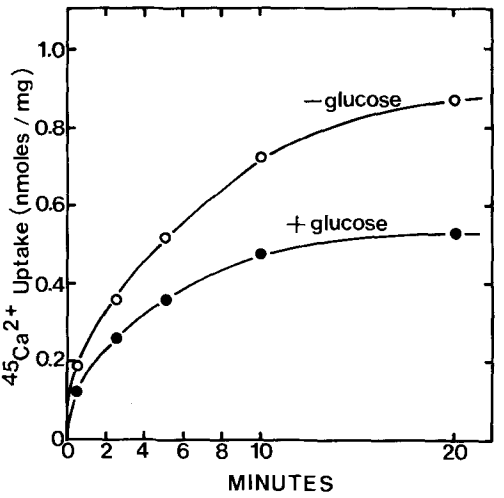

Fig. 3. Effect of glucose on Ca^{2+} uptake in ATP-depleted cells. Cells suspended in 120 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 4 mM sodium P_i, 40 mM HEPES (pH 7.4) were incubated for 10 min at 37 °C in the presence of 200 μM 2,4-dinitrophenol and mitochondrial inhibitors. Glucose (10 mM) was then added to one aliquot and both aliquots were further incubated for 10 min at 37 °C. Ca^{2+} uptake was measured at 0 °C in the presence of 1 mM Ca^{2+} as described under *Materials and Methods*

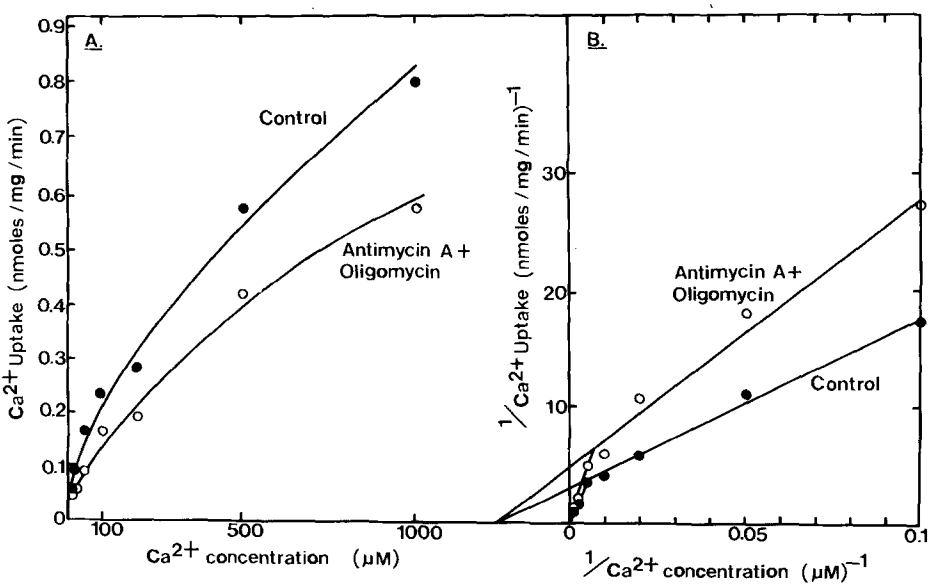

Fig. 4. Effect of Ca^{2+} concentration on Ca^{2+} uptake by Ehrlich ascites tumor cells. (*A*): Cells suspended in 120 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 4 mM sodium P_i, 40 mM HEPES (pH 7.4) were assayed at the Ca^{2+} concentration indicated, in the presence or absence of mitochondrial inhibitors, at 0 °C. (*B*): Double reciprocal plot of *A*

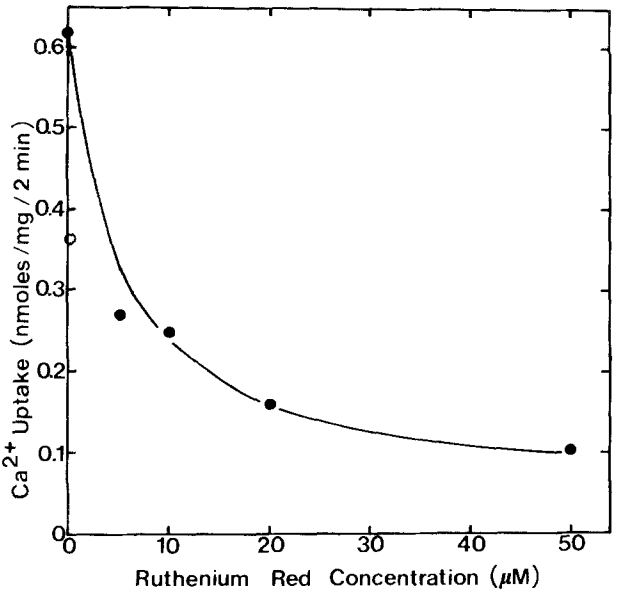

Fig. 5. Effect of Ruthenium Red on Ca^{2+} uptake by Ehrlich ascites tumor cells. Assays were performed at 0 °C in the presence of mitochondrial inhibitors as described in the legend of Fig. 1. Ruthenium Red at the indicated concentrations was added to cells 3 min prior to addition of $^{45}Ca^{2+}$

mes from rat liver (Bygrave, 1978) or on Ca^{2+} binding to plasma membranes from adipose tissue (McDonald, Bruns & Jarett, 1976). On the other hand, Ruthenium Red was previously shown to inhibit Ca^{2+} uptake in Ehrlich ascites tumor cells (Cittadini *et al.*, 1973). The authors left open whether the dye acts at the level of the plasma membrane or the mitochondria or both. We observed that Ruthenium Red was effective at 0 °C in the presence of inhibitors which virtually eliminated mitochondrial activity (Fig. 5), thus pointing to the plasma membrane as a target for its action. Ca^{2+} accumulation was inhibited about 50% by both mersalyl and $HgCl_2$ (Table 2). La^{3+} and Tb^{3+} at 100 μM slightly stimulated Ca^{2+} uptake which perhaps can be explained by the observation that La^{3+} inhibited efflux of Ca^{2+} from cells (data not shown). Na^+, K^+, choline or Cl^- in the medium had little effect on Ca^{2+} uptake at 200 μM Ca^{2+}. Mg^{2+}, up to 10 mM, had little effect under these conditions.

Effect of P_i

Stimulation of Ca^{2+} uptake by P_i in the presence of succinate has been observed (Landry & Lehninger, 1976; Resch-Charlton & Wenner,

Table 2. Effect of various ions and sulfhydryl reagents on Ca^{2+} uptake

	Ca^{2+} uptake (nmol/mg·3 min)	% inhibition or stimulation
Expt. 1:		
Control	0.51	0
Mersalyl (1 mM)	0.24	−53
$HgCl_2$ (1 mM)	0.27	−46
La^{3+} (100 μM)	0.66	+30
Tb^{3+} (100 μM)	0.64	+27
Expt. 2:		
Control, no Mg^{2+}	0.46	
10 mM Mg^{2+}	0.45	−2

Uptake was assayed in the presence of mitochondrial inhibitors, 200 μM Ca^{2+} at 0 °C. Inhibitors were added together with $^{45}Ca^{2+}$.

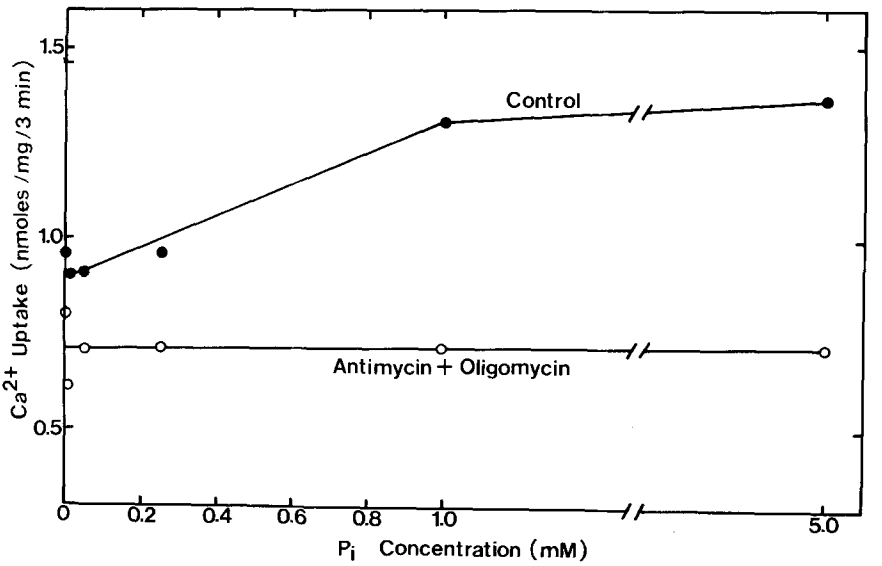

Fig. 6. Dependence of Ca^{2+} uptake on phosphate concentration. Ca^{2+} uptake was measured at 0 °C with or without antimycin A and oligomycin in the presence of 120 mM NaCl, 5 mM KCl, 40 mM HEPES (pH 7.4), 0.5 mM $MgCl_2$, and different sodium P_i concentrations as indicated

1978). It was suggested by Landry and Lehninger (1976) that P_i is a major counter-anion for Ca^{2+} entry. We confirmed the stimulation of Ca^{2+} entry by P_i at 0 °C (Fig. 6) as well as at 25 °C (data not shown), but the effect was not observed in the presence of mitochondrial inhibitors

Table 3. Effect of valinomycin and tetraphenylphosphonium (TPP) on $^{45}Ca^{2+}$ uptake

	Ca^{2+} uptake (nmol/mg·15 min)	% inhibition
Assay conditions		
120 mM KCl buffer[a]	2.01	–
120 mM KCl + valinomycin 5 μg/ml	0.95	53
120 mM NaCl buffer[b]	1.89	–
120 mM NaCl + TPP 1 mM	1.02	46
120 mM NaCl + TPP 5 mM	0.69	63

[a] 120 mM KCl, 0.5 mM $MgCl_2$, 4 mM potassium P_i, 40 mM HEPES, pH 7.4.
[b] 120 mM NaCl, 0.5 mM $MgCl_2$, 4 mM sodium P_i, 40 mM HEPES, pH 7.7.

Assays were performed at 0 °C in the presence of mitochondrial inhibitors as described under *Materials and Methods*.

(Fig. 6). We therefore conclude that the effect of P_i on Ca^{2+} accumulation is on the mitochondria rather than on the plasma membrane as was suggested also by Resch-Charlton and Wenner (1978).

Effect of Ionophores on Ca^{2+} Uptake

Conditions which have been reported to alter the membrane potential in Ehrlich ascites tumor cells (Laris, Pershadsingh & Johnstone, 1976) affected Ca^{2+} uptake. In a K^+ medium, valinomycin markedly inhibited Ca^{2+} uptake (Table 3). Tetraphenyl phosphonium, a positively charged lipophilic cation (kindly provided by Dr. H.R. Kaback), inhibited Ca^{2+} transport at 5 mM. As shown in Fig. 7, valinomycin inhibited when K^+ was the major cation in the medium, whereas in a predominantly Na^+ medium valinomycin stimulated Ca^{2+} uptake. Nigericin, which catalyzes a H^+/K^+ or H^+/Na^+ exchange, consistently inhibited Ca^{2+} transport (Fig. 8). Most pronounced inhibitions were observed in the presence of both valinomycin and nigericin in either Na^+ or K^+ medium. 2,4 Dinitrophenol also markedly inhibited Ca^{2+} uptake between pH 6.0 and 8.5. These findings are consistent (but do not prove) that the electrogenic movement of Ca^{2+} is via a Ca^{2+}/H^+ exchange. Consistent with this formulation is the effect of pH on Ca^{2+} transport. As shown in Fig. 9 the rate of Ca^{2+} uptake increases with rising pH between 6 and 8. At a more alkaline pH the rate decreases, however, suggesting a more complicated role of the external pH. Attempts are now being made

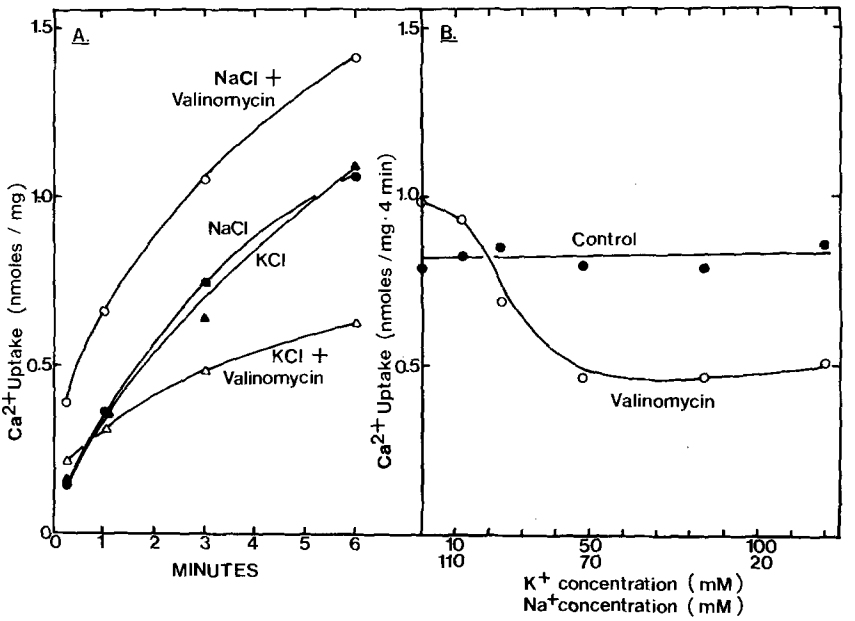

Fig. 7. Effect of a superimposed membrane potential on Ca^{2+} uptake by Ehrlich ascites tumor cells. (*A*): Ca^{2+} uptake was assayed at 0 °C either in the presence of 120 mM NaCl or 120 mM KCl, 0.5 mM $MgCl_2$, 40 mM HEPES, 4 mM sodium or potassium P_i (pH 7.4) in the presence of mitochondrial inhibitors with and without valinomycin (5 µg/ml). (*B*): Assay was done at 0 °C with mixtures of NaCl or KCl medium in the presence of mitochondrial inhibitors, 5 µg/ml valinomycin

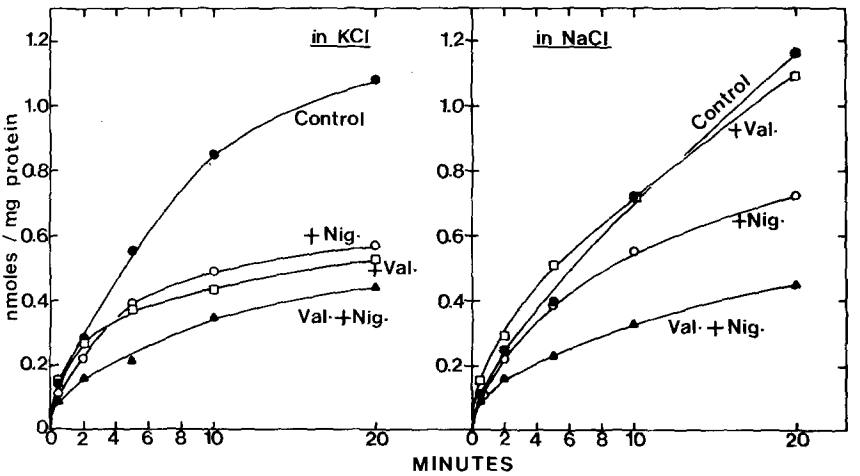

Fig. 8. Effect of nigericin and valinomycin on Ca^{2+} uptake. Ca^{2+} uptake was assayed at 0 °C in either NaCl buffer or KCl buffer as described in the legend of Fig. 7, in the presence of mitochondrial inhibitors, with and without valinomycin (2 µg/ml), nigericin (5 µg/ml) and with both. Val = valinomycin; Nig = nigericin

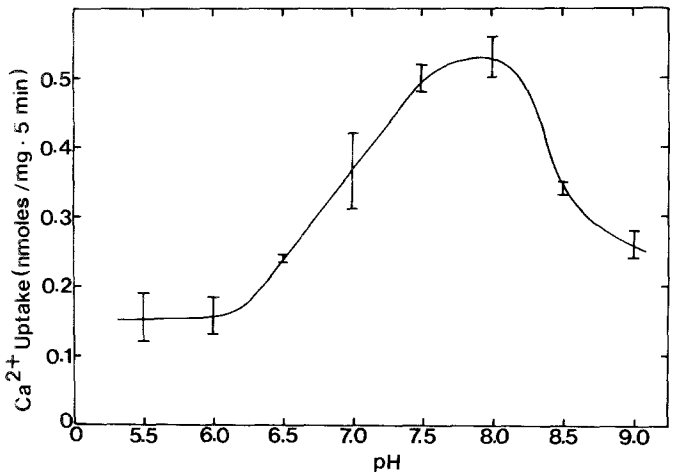

Fig. 9. Effect of pH on Ca^{2+} transport. Cells suspended in pH 7.4 buffer as described in the legend of Fig. 1 were assayed at 0 °C at various external pH values in the presence of mitochondrial inhibitors and 200 μM Ca^{2+}

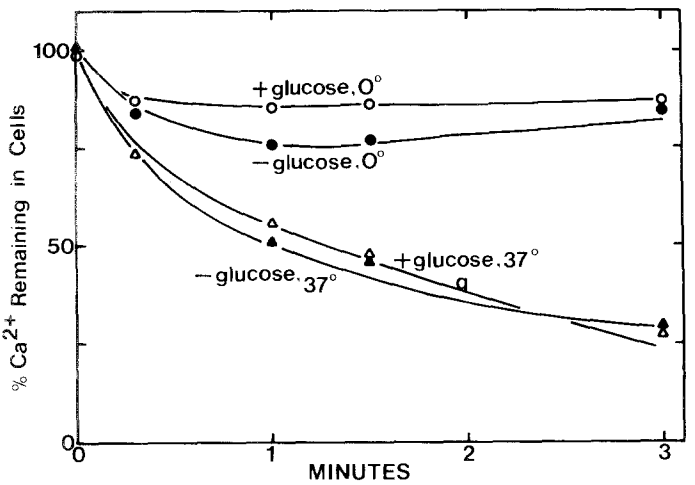

Fig. 10. Efflux of Ca^{2+} from loaded cells at 0 °C and at 37 °C, in the presence and absence of glucose. Ca^{2+} efflux was measured as described under *Materials and Methods*. Assays were performed in the presence of mitochondrial inhibitors with and without glucose (10 mM). It should noted that passages through the columns were performed at room temperature

to isolate from plasma membranes of Ehrlich ascites tumor cells a transporter which catalyzes an electrogenic transport of Ca^{2+}. Although the data shown in Table 1 and Fig. 6 speak against residual mitochondrial activity having an effect on Ca^{2+} uptake, it is difficult to completely exclude this possibility.

Ca^{2+} Efflux

Cells preloaded with $^{45}Ca^{2+}$ showed almost no efflux at 0 °C but lost accumulated Ca^{2+} readily at 37 °C (Fig. 10). This temperature dependency is in line with the observations recorded in Fig. 2, showing less Ca^{2+} uptake at higher than at lower temperature. We did not observe an effect of glucose on the rate of efflux. Cells without glucose either have enough energy source present to extrude Ca^{2+}, even in the presence of mitochondrial inhibitors, or our experimental conditions induce a nonspecific leakage of Ca^{2+}. In favor of the first possibility are observations showing that very low concentrations of ATP are required for the extrusion of Ca^{2+} by the ATP-driven pump of squid axons (Beaugé & DiPolo, 1978). Although we could not show an energy-dependent efflux directly, the data recorded in Figs. 2 and 3 nonetheless suggest the existence of an energy-dependent extrusion mechanism.

Energy-Dependent Ca^{2+} Uptake in Reconstituted Plasma Membrane Vesicles

In view of the data described above, suggesting an active excretion of Ca^{2+}, we examined plasma membranes isolated from Ehrlich ascites tumor cells for (Ca^{2+})ATPase activity. They catalyzed a Ca^{2+}-dependent hydrolysis of ATP with a specific activity of 50 nmol/mg/min. This activity is similar to that of (Ca^{2+})ATPase of erythrocyte membranes (Farrance & Vincenzi, 1977*a*; Schatzmann, 1975).

We have recently shown (Haaker & Racker, 1979) that the (Ca^{2+}) ATPase from erythrocytes plasma membranes can be reconstituted into liposomes in the inside-out configuration, thus pumping Ca^{2+} into the vesicles in the presence of ATP. We have reviewed in this paper previous evidence in favor of an activator protein (calmodulin) as a component of the (Ca^{2+})ATPase.

Plasma membranes from Ehrlich ascites tumor cells have been reconstituted into liposomes by the same methods used for the erythrocyte membrane. These vesicles catalyzed an ATP-dependent uptake of Ca^{2+}, which was linear for 5 min (Fig. 11). As shown in Table 4, treatment of the membrane preparations with EGTA, which removes the activator protein from the erythrocyte membranes (Farrance & Vincenzi, 1977*b*), had no effect on ascites tumor membranes; nor did addition of calmodulin have any effect on the (Ca^{2+})ATPase or pump activity. Ca^{2+}

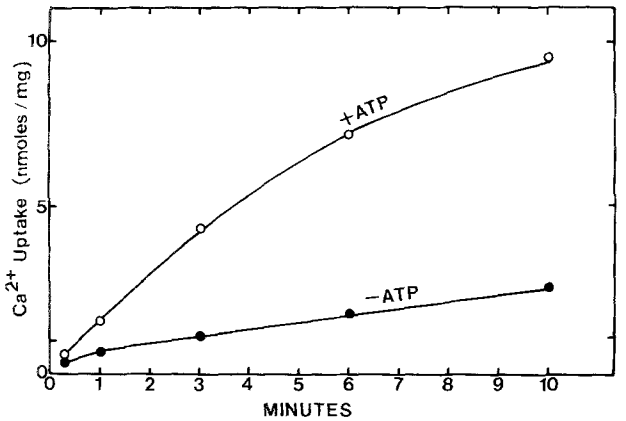

Fig. 11. ATP-dependent Ca^{2+} uptake into reconstituted plasma membrane vesicles. Reconstitutions and assays were done as described under *Materials and Methods*. Ca^{2+} concentration was 10 μM, assay temperature 37 °C

Table 4. Effect of calmodulin on Ca^{2+} uptake in untreated and EGTA-treated reconstituted plasma membrane vesicles

Treatment of plasma membranes	Additions	Ca^{2+} uptake (μmol/mg protein/3 min)	Ca^{2+} uptake (μmol/mg lipid/3 min)
None	−ATP	2.4	0.057
None	+ATP	11.0	0.262
None + calmodulin[a]	−ATP	2.2	0.052
None + calmodulin	+ATP	12.9	0.307
EGTA[b]	−ATP	2.1	0.050
EGTA	+ATP	10.7	0.255
EGTA + calmodulin	−ATP	2.7	0.064
EGTA + calmodulin	+ATP	11.5	0.274
Control without protein[c]	−ATP	–	0.066
Control without protein	+ATP	–	0.062

[a] Reconstituted plasma membrane vesicles (12 μg protein) were incubated in the presence of calmodulin (3.2 μg) and 200 μM Ca^{2+}. The incubation was for 45 min at 37 °C.

[b] Plasma membranes (3 mg) were washed once with 100 ml of 5 mM EGTA, 0.25 M sucrose (pH 7.4), and 2 times with 40 ml of 0.4 M potassium P_i, prior to reconstitution. Incubation with calmodulin was as under *a*.

[c] Control liposomes without plasma membrane protein were assayed for ^{45}Ca uptake.

Ca^{2+} uptake was measured in the presence of 20 μM $^{45}Ca^{2+}$ with and without 3 mM ATP at 37 °C as described under *Materials and Methods*.

uptake into the reconstituted vesicles was inhibited 50% by 3 mM N-ethylmaleimide, but oligomycin at 10 μg/ml and Ruthenium Red at 100 μM were without effect. It thus appears that the Ca^{2+} pump of Ehrlich ascites tumor cells is distinctly different from the pump of erythrocytes and resembles more the Ca^{2+} pump of sarcoplasmic reticulum.

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