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CALCIUM UPTAKE BY PLACENTAL PLASMA MEMBRANE VESICLES

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SUMMARY

The placental plasma membrane vesicles are capable of accumulating up to 190 mM Ca^{2+}. This is 24-fold higher than the external Ca^{2+} concentration.

This process is dependent on ATP hydrolysis by the placental Ca^{2+}-ATPase.

The P_i/Ca ratio is dependent on the external Ca^{2+} concentration, and reaches the value of 2 at 10 mM Ca^{2+}.

Phosphate (5 mM) can double Ca^{2+} uptake when measured in the presence of 5 mM Ca^{2+}.

Mg^{2+} increased Ca^{2+} uptake only at low Ca^{2+} concentrations, and had no significant effect at 5 mM Ca^{2+}.

INTRODUCTION

Calcium transport across the placenta is asymmetrical and is believed to be an energy-requiring process [1–3]. An essential step in such transport is translocation of the ion across a single plasma membrane.

In previous papers [4–6], it was demonstrated that placental plasma membrane vesicles contain Ca^{2+}-ATPase and high affinity sites for Ca^{2+}, with properties sufficiently similar to those of other membranes involved in active transport to suggest that these vesicles may also do so.

The present study investigates Ca^{2+} uptake by placental plasma membrane vesicles. This study was designed to answer very simple questions: (1) Can the placental plasma membrane vesicles accumulate calcium; if so, how efficiently? (2) Is the uptake dependent on ATP hydrolysis? (3) What is the effect of the external Ca^{2+} concentration on Ca^{2+} uptake? It is realized that many more questions must be answered before a firm conclusion can be made concerning the relationship of the Ca^{2+}-related properties of the placental plasma membranes to the active transport of Ca^{2+}. However, positive answers to these basic questions may suggest that the system is operating generally in the same manner as the sarcoplasmic reticulum [7] and cardiac microsomes [8].

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Before any measurement of Ca^{2+} uptake can be made, the term uptake must be defined and distinguished from binding. The most widely used working definitions of Ca^{2+} uptake and binding are given by Entman et al. [9] and Repke and Katz [8]. "Binding" is defined as Ca^{2+} accumulation in the presence of ATP, but in the absence of a calcium-precipitating anion (oxalate or phosphate). This process is rapid and reaches a maximum within 1 min. Uptake is Ca^{2+} accumulation in the presence of ATP and calcium-precipitating anion. This is a slower process. When calculating uptake the binding should be subtracted. This is done by extrapolating the uptake to time zero. According to this definition, the distinction between binding and uptake is possible only at time zero. Thus any event during the incubation period that alters the binding of calcium to the external surface of the vesicles from time zero value may distort the measurement of uptake. For example, the hydrolysis products of ATP (which are not present at time zero) can from a complex with Ca^{2+} (e.g. Ca^{2+}-ADP).

In addition, it has been found that in brain microsomes, ATP provides the phosphate for the formation of the phospholipid triphosphoinositide through the action of the enzyme diphosphoinositide kinase (EC 2.7.1.68) [10]. The stability constant of the complex between this phospholipid and Ca^{2+} is higher (10-fold) in the presence of ATP than in its absence [11]. The concentration of such complexes will be time dependent and so will their binding to the external surface of the vesicles. Thus, taking measurements later than time zero without being able to distinguish between binding and uptake, can overestimate Ca^{2+} uptake.

In this study binding and uptake are defined differently. Bound calcium is accessible for an immediate exchange and can be displaced by a competitor. Bound calcium is measured by labeling the membrane with $^{45}Ca^{2+}$ (in the absence of ATP) and displacing it with unlabeled calcium. Uptake is represented by the fraction of $^{45}Ca^{2+}$ which cannot be rapidly displaced by a large excess of $^{40}Ca^{2+}$. This definition enables one to distinguish between binding and uptake throughout the incubation period and overestimation of Ca^{2+} uptake is avoided. The advantage of using this definition is evident, especially in cases when the Ca^{2+}-accumulating system is not as efficient as the sarcoplasmic reticulum.

The flow dialysis system which was used previously for measuring Ca^{2+} binding [6] was adapted for measuring Ca^{2+} uptake in this paper.

MATERIALS AND METHODS

Measurement of Ca^{2+} uptake

Placental plasma membrane vesicles were incubated at 24 °C in 20 mM Tris – HCl buffer, pH 8.0, 100 mM NaCl, 10^{-5} M $^{45}Ca^{2+}$ and $^{40}Ca^{2+}$ as indicated, with or without 5 mM ATP (final volume 1.5 ml). At the end of the incubation period 3×0.1-ml aliquots were taken for the determination of P_i release. 1 ml of the incubation medium was introduced to the upper chamber of the flow dialysis cells [6], 0.5 ml of 1 M $^{40}Ca^{2+}$ was added, and the diffusion rate was measured. The diffusion rate of the control without ATP was also measured. The difference in the diffusion rates represents the fraction of calcium which is not available for displacement; thus Ca^{2+} uptake can be calculated. After each measurement the upper chamber was emptied and washed three times with the effluent buffer (20 mM Tris – HCl, pH 8.0, 100 mM NaCl) before the next sample was introduced. ^{45}Ca counting was carried out

as described previously [6], protein was determined by the method of Lowry et al. [12] and P_i released by the Gomori method [13].

RESULTS

Evaluation of the flow dialysis method for measuring Ca^{2+} uptake

The usefulness of the method for measuring calcium uptake was tested by incubating vesicles for 2 h in the presence of ATP. Displacement of $^{45}Ca^{2+}$ by $3 \cdot 10^{-1}$ M $^{40}Ca^{2+}$ was measured in the flow dialysis cell. The results are represented in Fig. 1. It is evident that in the presence of 5 mM ATP, 5 mM Ca^{2+} and the placental plasma membrane vesicles (Fig. 1, column B), part of the $^{45}Ca^{2+}$ (25 %) is not available for displacement after 2 h of incubation at 24 °C. From the results of the various controls, it is concluded that this fraction of non-displaceable Ca^{2+} represents uptake by the vesicles and is not the result of non-specific unexchangeable binding to any of the constituents of the incubation fluid. Thus the suitability of the modified flow dialysis method for Ca^{2+} uptake studies is demonstrated.

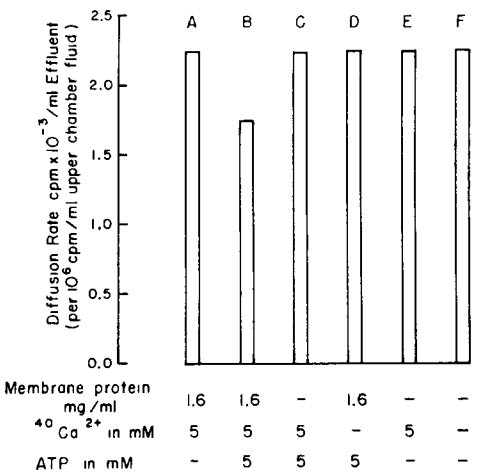

Fig. 1. The effect of the major constituents of the incubation medium on $^{45}Ca^{2+}$ displacement by $3 \cdot 10^{-1}$ M Ca^{2+}. The samples were incubated for 2 h in 20 mM Tris—HCl, pH 8.0, 100 mM NaCl, 10^{-5} M $^{45}Ca^{2+}$, and different combinations of ATP, $^{40}Ca^{2+}$, and protein as indicated. $^{40}Ca^{2+}$ ($3 \cdot 10^{-1}$ M) was added after 2 h of incubation at 24 °C, and the diffusion rate obtained was divided by the cpm concentration in the upper chamber to give cpm/ml effluent per 10^6 cpm/ml upper chamber fluid.

The effect of incubation time on Ca^{2+} uptake and ATP hydrolysis

Calcium uptake and ATP hydrolysis by the placental plasma membrane vesicles showed similar kinetic behaviour (Fig. 2A). No Ca^{2+} uptake was detected in the absence of ATP. The ratio between ATP hydrolysis and Ca^{2+} uptake was 7.7 (Fig. 2B). Thus for every 7.7 μmol ATP hydrolyzed, 1 μmol Ca^{2+} was taken up. This ratio was constant throughout the incubation time. These results demonstrate Ca^{2+} uptake in the absence of calcium-precipitating ion (except for the P_i released). However, the process was slow (even after 2 h of incubation, steady-state conditions

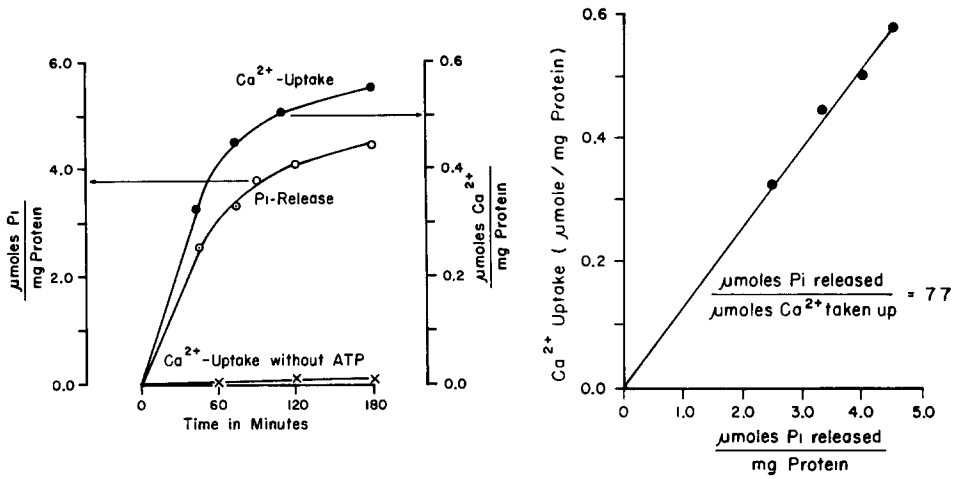

Fig. 2. (A) The effect of incubation time on Ca^{2+} uptake and ATP hydrolysis. The samples were incubated under standard conditions. Protein concentration was 1.78 mg/ml. ●–●, Ca^{2+} uptake in the presence of 5 mM ATP and 5 mM Ca^{2+}; ○–○, P_i release under the same conditions; ×–×, Ca^{2+} uptake in the absence of ATP. (B) The relationship between P_i release and Ca^{2+} uptake. The data to construct this figure were obtained from A.

were not reached) and required high protein concentration (1.5–2.0 mg protein/ml).

Sonication (sonic dismembrator, Artek, N.Y.) for up to 30 min at 40 W, 20 kHz, did not affect the efficiency of Ca^{2+} uptake. However, it was observed that Ca^{2+} uptake by freshly prepared vesicles was low. "Aging" the vesicles by storing for

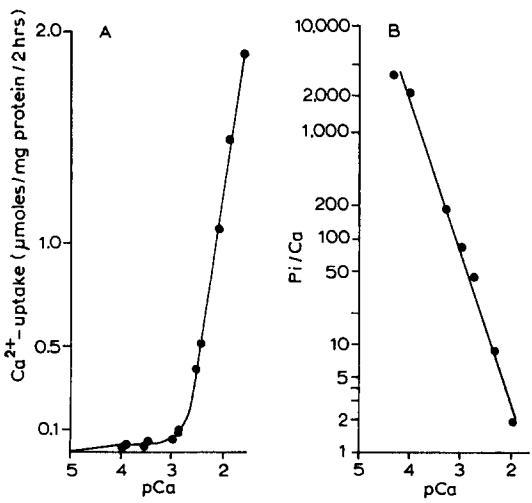

Fig. 3. (A) The effect of Ca^{2+} concentration on Ca^{2+} uptake. The samples were incubated for 2 h under the standard conditions with different concentrations of Ca^{2+}. The results are expressed in µmol Ca^{2+} accumulated per mg protein. Protein concentration was 1.72 mg/ml. (B) The effect of Ca^{2+} concentration on P_i/Ca ratio. P_i release is expressed in µmol/mg protein (was measured in the same samples which were used for constructing Fig. 3A) and Ca^{2+} uptake in µmol/mg protein.

2 weeks at 4 °C improved substantially the efficiency of Ca^{2+} uptake and the P_i/Ca ratio dropped to 4 (in the presence of 5 mM ATP and 5 mM Ca^{2+}).

The effect of external Ca^{2+} concentration on Ca^{2+} uptake and P_i/Ca ratio

At Ca^{2+} concentrations below 10^{-3} M, Ca^{2+} uptake was very low (Fig. 3A) (less than 0.05 μmol/mg protein per 2 h) and P_i/Ca ratio values were very high, indicating extremely low efficiency (Fig. 3B). Ca^{2+} uptake showed a sharp increase at Ca^{2+} concentrations between 10^{-3} and 10^{-2} M (Fig. 3A), with a similar increase in Ca^{2+} uptake efficiency. The P_i/Ca ratio dropped to 2 at 10^{-2} M Ca^{2+} (Fig. 3B).

Estimation of calcium concentration in the vesicles

The vesicular volume of a sarcoplasmic reticulum preparation, using ultracentrifugation, was estimated by Weber et al. [14] to be 10 μl per mg membrane protein. Lower vesicular volume was reported by Inesi [7] 7 μl/mg protein; Duggan and Martonosi [15] found it to be 4–5 μl/mg protein. In order to avoid overestimation of intravesicular calcium concentration, we used the most conservative estimate of 10 μl/mg protein. Using this approximate figure, the intravesicular Ca^{2+} concentration for placental vesicles was calculated to be 190 mM (1.9 μmol/10 μl, after 2 h of incubation. The initial Ca^{2+} concentration was 10 mM and after 2 h, 20 % was taken vesicles, so that the final external Ca^{2+} concentration dropped to by the 8 mM. Thus the intravesicular calcium was concentrated approx. 24-fold (190/8 = 23.8). The data was obtained from Fig. 3A. When the vesicles were incubated in the presence of 5 mM Ca^{2+} and 5 mM P_i, the vesicles concentrated calcium 39-fold (calculated from Table I).

Phosphate effect on Ca^{2+} uptake

To exclude the possibility that Ca^{2+} accumulation in the presence of ATP is due to the release of P_i which acts as a calcium-precipitating ion, samples were incubated with 0.3 and 5 mM Na_2HPO_4 in the absence of ATP under the standard conditions (5 mM Ca^{2+} for 2 h). No Ca^{2+} uptake was found. When the samples were incubated with P_i (5 mM) and ATP (5 mM), a marked increase in Ca^{2+} uptake was observed and the P_i released/Ca^{2+} uptake ratio dropped from 4.0 to 1.7. The results so obtained are presented in Table I.

TABLE I

THE EFFECT OF PHOSPHATE ON Ca^{2+} UPTAKE

The samples were incubated for 2 h in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM Ca^{2+}, ATP and P_i as indicated. Protein concentration was 2.1 mg/ml.

Additions	Ca^{2+} uptake (μmol/mg protein)	P_i release (μmol/mg protein)	P_i/Ca (μmol P_i/ μmol Ca)
5 mM ATP	0.52	2.2	4.0
5 mM ATP+0.3 mM P_i	0.52	2.2	4.0
5 mM ATP+5 mM P_i	1.2	2.2	1.70

TABLE II

THE EFFECT OF 5.4 mM Mg^{2+} ON Ca^{2+} UPTAKE

The samples were incubated for 2 h at 24 °C in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10^{-5} M $^{45}Ca^{2+}$, and 2.2 mg membrane protein, in final volume of 1.85 ml. The concentrations of Ca^{2+}, Mg^{2+}, and ATP were varied as indicated.

Additions	A	B	C	D	E
mM Ca^{2+}	5.4	5.4	5.4	0.02	0.02
mM Mg^{2+}	5.4	—	5.4	—	5.4
mM ATP	—	5.0	5.0	5.0	5.0
Ca^{2+} uptake $\left(\frac{\text{nmol } Ca^{2+}}{\text{mg protein}}\right)$	—	934	986	—	1.4

Mg^{2+} effect on Ca^{2+} uptake

The effect of Mg^{2+} (5.4 mM) on Ca^{2+} uptake was studied when the vesicles were incubated in the presence of high (5.4 mM) Ca^{2+}, and low ($2 \cdot 10^{-5}$ M) Ca^{2+}. Mg^{2+} (5.4 mM) had no effect on Ca^{2+} uptake when the external medium contained 5.4 mM Ca^{2+}. However, when the external medium contained only $2 \cdot 10^{-5}$ M Ca^{2+} and Mg^{2+} (5.4 mM), the vesicles accumulated about 1.4 nmol Ca^{2+} per mg protein. No Ca^{2+} uptake could be detected when the incubation medium contained $2 \cdot 10^{-5}$ M Ca^{2+} in the absence of Mg^{2+}.

DISCUSSION

The flow dialysis method, which was used successfully for studying Ca^{2+} binding [6], was also found suitable for measuring Ca^{2+} uptake.

The first question which this study was designed to answer related to the ability of the vesicles of the placental plasma membrane to accumulate calcium. Clearly these vesicles are capable of accumulating calcium, even without a calcium-precipitating ion (except for the P_i released due to ATP hydrolysis). After 2 h of incubation, the concentration of calcium in the vesicles was calculated to be 190 mM and in the external medium 8 mM. Thus, calcium was concentrated approx. 24-fold. A 40-fold increase in Ca^{2+} concentration was observed when the vesicles were incubated in the presence of 5 mM Ca^{2+} and 5 mM P_i. In this study the use of oxalate as calcium-precipitating ion was avoided, since it would result in a major deviation from the physiological conditions.

Calcium accumulation by the sarcoplasmic reticulum was calculated to reach a concentration of 50 mM (500-fold over the external Ca^{2+} concentration) in the absence of oxalate [16]. Meisner [17] calculated the total calcium concentration in the vesicles of sarcoplasmic reticulum to be 30–35 mM, and the free calcium concentration 13–18 mM [7]. In less specialized calcium-accumulating systems such as platelet membranes, the intravesicular Ca^{2+} can reach only 1 mM, a 10-fold increase over the external Ca^{2+} concentration, in the absence of oxalate and in the presence of ATP [18]. Evidently, the calcium concentration within the placental plasma membrane vesicles (as measured in the absence of oxalate) exceeds that of the sarcoplasmic

reticulum and platelet membrane vesicles. However, the concentrating capability falls far behind that of the sarcoplasmic reticulum, although it is higher than that of the platelet membrane vesicles.

The stoichiometry of Ca^{2+} uptake was somewhat different from one preparation to another, and fluctuated between 4 and 8 ATP molecules hydrolyzed for each Ca^{2+} molecule taken up (measured in the presence of 5 mM Ca^{2+}). A constant ratio of 2 molecules of Ca^{2+} taken up for each ATP molecule hydrolyzed, has been repeatedly reported for sarcoplasmic reticulum [14, 19, 20]. However, when the vesicles are leaky, higher P_i/Ca ratios (4–8) were reported for the sarcoplasmic reticulum [21, 22]. For platelet membranes, the P_i/Ca ratio is between 10 and 20 [18]. The lowest P_i/Ca ratio observed in this study was 1.8 (when the incubation medium contained 10 mM Ca^{2+}). The high P_i/Ca ratio can be explained as follows: (1) The percentage of open vesicles as seen by electron microscopy is approx. 50 %. However, it is impossible to tell if the other 50 % are leaky or not since all the particles will hydrolyze ATP. But only variable fractions are tightly sealed and accumulated Ca^{2+}, thus the P_i/Ca ratio varies. (2) The calcium which is complexed with ATP is not taken up by the vesicles. Only free Ca^{2+} is available for uptake; when the Ca^{2+} concentration is 10^{-3} M, very little calcium is available. On the other hand, Ca^{2+}-ATP is the substrate for the Ca^{2+}-ATPase, with $K_m = 0.25$ mM [4]. Thus, ATP hydrolysis will approach a maximum when significant Ca^{2+} uptake begins to occur. We agree that there is a discrepancy between the K_m of Ca^{2+} for the Ca^{2+}-ATPase and Ca^{2+} transport; however, one should keep in mind that these are only the apparent K_m values using total Ca^{2+} concentration, rather than the concentration of the real substrate. We know that the substrate for the enzyme is Ca^{2+}-ATP complex and although the substrate for the transport system is not exactly defined, it is assumed to be free calcium. Further, we might postulate that the enzyme is not identical with the transporting system, that is to say it is possible that we have two systems in which the ATPase provides only the energy and it has to be coupled to the transport system before uptake will occur. The efficiency of uptake (P_i/Ca ratio) will be dependent in such a situation on the degree of coupling between the two systems. This might explain the logarithmic decrease in P_i/Ca ratio as the external Ca^{2+} concentration rises. All of the above factors will tend to increase the apparent P_i/Ca ratio so that the actual P_i/Ca may be much lower and might even be as low as was reported for the sarcoplasmic reticulum (0.5) [14].

The exact role of ATP was not revealed in this study. However, the profile of ATP hydrolysis as a function of incubation time was the same as the profile of Ca^{2+} uptake, and no uptake of Ca^{2+} was detected in the absence of ATP. The most obvious role of ATP is to serve as the source of energy for the uptake process. Nakamura and Konishi [10] observed that for brain microsomes, though no Ca^{2+} uptake occurred without ATP, Ca^{2+} uptake did not follow the profile of P_i release. Their conclusion was that the brain microsomes exhibit ATP-dependent Ca^{2+} uptake without the participation of (Ca^{2+}, Mg^{2+})-dependent ATPase. They suggested that the role of ATP is to provide the phosphate for the formation of the phospholipid triphosphoinositide. This phospholipid has 10 times higher affinity for Ca^{2+} in the presence of ATP than in its absence [11], and this might explain the role of ATP in Ca^{2+} uptake by brain microsomes. The profiles of P_i release and Ca^{2+} uptake by placental plasma membrane vesicles show the same kinetic behavious, unlike the case of brain

microsomes. Thus, it is unlikely that the role of ATP in the uptake process is other than acting as a source of energy.

The increase in Ca^{2+} uptake in the presence of 5 mM P_i and 5 mM ATP, is most likely due to precipitation of calcium phosphate in the vesicles, thereby decreasing the leakage of Ca^{2+}.

A positive effect of Mg^{2+} on Ca^{2+} uptake was noted only at low Ca^{2+} concentration, and is interpreted as follows. Mg^{2+} frees Ca^{2+} from the complex with ATP. Since Ca^{2+} is the substrate of the uptake system, there is more Ca^{2+} available. The enzymes can still be activated by Mg^{2+}-ATP so that the overall result will be an increase in Ca^{2+} uptake. However, this is true only when the Ca^{2+} concentration is very low compared with that of Mg^{2+}. At 5 mM Ca^{2+} and 5 mM Mg^{2+}, the increase in Ca^{2+} uptake was not significant.

Ca^{2+} uptake by the sarcoplasmic reticulum is dependent on Mg^{2+}, as in the ATPase activity [14, 19, 20]. No such dependence was noted in this study, provided that calcium concentration was above 10^{-3} M as in the ATPase [4].

The physiological role of this system in Ca^{2+} transport across the placenta cannot be defined clearly. The objective of this study and the previous ones [4–6] was to study the relation between Ca^{2+} and the membrane involved in Ca^{2+} transport. However, one can speculate that the membrane of the vesicles which are loaded with Ca^{2+}, may be an in vivo part of the continuous tubular network which has been seen with electronmicroscope in the trophoblast [23]. (Croley [24] localized Ca^{2+} histochemically in this network within the human trophoblast. Since the human placenta and guinea pig placenta have the same basic structure (haemochorial), one can expect similar localization of Ca^{2+} in the guinea pig trophoblast.) Thus, fusion of segments of this network with the basal plasma membrane will provide the link for Ca^{2+} transport between the maternal and fetal circulation.

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