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ATPase
calcium
human placenta
magnesium
mitochondrial inhibitors

Calcium Uptake and Binding by Membrane Fractions of Human Placenta: ATP-dependent Calcium Accumulation

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Summary

An ATP-dependent calcium (Ca^{2+}) sequestration activity was demonstrated in membrane vesicles prepared from the human term placenta. Microsomal and brush border membrane fractions accumulated Ca^{2+} within a vesicular space by a saturable process requiring Mg^{2+} and ATP. The "uptake" activity was enriched six-fold in a microsomal membrane fraction and was only 1.5-fold enriched in purified brush border membranes compared to the activity present in the filtered homogenate. Mitochondrial inhibitors such as azide and oligomycin did not inhibit Ca^{2+} uptake in these preparations. The process was temperature dependent and displayed Michaelis-Menten-like kinetics with respect to free Ca^{2+} concentrations. At 30°C, the V_{max} was 1.05 nmole/mg/min; K_m = 74 nM for free Ca^{2+} in the microsomal fraction. Oxalate and phosphate enhanced uptake in both fractions. Ca^{2+} uptake activity was not associated with Ca^{2+}-stimulated ATPase, alkaline phosphatase, or other brush border markers during cell fractionation. The characteristics of the Ca^{2+} uptake process contrasted sharply with those of Ca^{2+}-stimulated ATPase, and a Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase activity could not be identified in these membrane vesicle preparations.

Speculation

The ATP-dependent Ca^{2+} uptake activity and Ca^{2+} binding by placental vesicles are likely to be involved in the regulation of intracellular Ca^{2+} within the placenta and in the transcellular transport of Ca^{2+} to the fetus while serving to maintain low intracellular Ca^{2+} concentrations within the syncytium.

Calcium (Ca^{2+}) is transported transcellularly across the placenta from mother to fetus against a concentration gradient (5). The mechanisms involved in such asymmetrical transport have not been clarified. However, it is likely that both intracellular and transcellular Ca^{2+} are highly regulated by processes which sequester or bind intracellular free Ca^{2+}. Such Ca^{2+} "pump" activities might protect the syncytium from high intracellular Ca^{2+} concentrations and play a role in the transport of large quantities of Ca^{2+} to the fetus. Both Ca^{2+} binding and ATP-dependent Ca^{2+} sequestration activities have been described in numerous cell types (10, 12, 14, 15, 19, 24) and in various subcellular components including plasma membranes (19), mitochondria (2), microsomal membranes (14, 15), and sarcoplasmic reticulum (12). In several tissues, Ca^{2+} uptake activity is coupled to Ca^{2+}-stimulated ATPase activity which serves to transport Ca^{2+} at the expense of ATP hydrolysis (12, 19). These relationships, however, have not been demonstrated in the human placenta. Thus, although Ca^{2+}-ATPase activity has been demonstrated in human (13, 27) and guinea pig placenta (21), it remains unclear whether this enzyme is associated with placental Ca^{2+} transport or uptake in placental membranes.

"Brush border membranes" of the syncytium (which are exposed directly to maternal blood in the intervillous space) have been recently characterized. These membranes are 10- to 15-fold enriched in Ca^{2+}-ATPase and insulin receptors (27) and also contain a sodium gradient-dependent amino acid transport system (18) but do not contain adenylate cyclase activity (28). Because the first step in the movement of nutrients to the fetus occurs through the brush border membrane, it is likely that it might also have components which might interact with Ca^{2+}. Ca^{2+} and other cations have also been observed within vesicular structures (4, 16) within the cytosol of the human syncytium. These intracellular membranes or vesicles, as well as the plasma membranes associated with the trophoblastic basement membrane (facing the fetal circulation) might also play a role in the regulation of placental Ca^{2+}. Recently, a placental "microsomal" preparation which is distinct from brush border preparations has been described (26, 28). This preparation, which is presumed to be related to plasma membrane near the fetal circulation, is enriched in adenylate cyclase, Na^+, K^+-ATPase, and an ATP-dependent Ca^{2+} uptake activity. In the present study, vesicles were prepared both from the brush border and microsomal membrane fractions of the human placenta to demonstrate possible Ca^{2+} binding, Ca^{2+}-ATPase, and ATP-dependent Ca^{2+} uptake activity in the human placenta.

MATERIALS AND METHODS

PREPARATION OF BRUSH BORDER MEMBRANES

Brush border membranes were prepared by a modification of the method of Smith and Brush (23) as previously reported (27). Approximately 50 g of washed placental tissue was minced and placed in 300 ml iced buffer containing 140 mM NaCl-10 mM Tris-HCl (pH 7.2). The tissue was stirred with a magnetic stir bar at 4°C for 30 min. After filtering the tissue and medium through cheesecloth, the medium was centrifuged at 15,000 × *g* for 20 min. The supernatant was decanted and centrifuged at 100,000 × *g* for 60 min. The pellet obtained was then homogenized in iced isotonic sucrose-Tris buffer with a Potter homogenizer with a Teflon pestle and centrifuged at 15,000 × *g* for 15 min. This produced a pellet containing two distinct layers. The upper fraction consisted of a loosely packed white layer which contained the brush border membranes. This membrane was collected, resuspended by gentle homogenization, and diluted to final protein concentrations between 3 to 5 mg/ml. By electron microscopic examination,this preparation consisted primarily of spherical membrane vesicles as previously reported with no apparent mitochondrial contaminants (27).

MICROSOMAL MEMBRANE PREPARATION

The microsomal membrane fraction was prepared as previously described (27) except that EDTA was omitted from buffer solutions. Placentas were obtained with maternal consent immediately

following term, normal vaginal deliveries and placed on ice. Decidual tissue was removed, and placental fragments were scraped from the fetal vascular tree with a dull scalpel and washed extensively in iced buffer containing 250 mM sucrose-10 mM Tris-HCl (pH 7.2). Approximately 20 g of washed tissue was homogenized with a Tekmar tissuemizer in 200 ml of sucrose buffer at 4°C by four ten-sec bursts. The homogenate was poured through four layers of cheesecloth and centrifuged at 15,000 × *g* for 15 min. The supernatant was centrifuged at 40,000 × *g* for 40 min. The pellet obtained was resuspended by gentle homogenization in 100 ml iced buffer and centrifuged again at 40,000 × *g* for 40 min. This washed pellet was resuspended in 250 mM sucrose-10 mM Tris-HCl (pH 7.2) to a final membrane protein content of 3 to 5 mg/ml by the method of Lowry *et al.* (11). Samples were assayed for Ca^{2+} uptake on the day of preparation or within three days after freezing in dry ice-acetone bath and storage at −30°C. Previously thawed samples were discarded. This fraction consisted primarily of spherical membrane vesicles by electron microscopic examination. However, mitochondrial and ribosomal contaminants were also evident.

Marker enzyme analysis was performed as previously described (27). Alkaline phosphatase (EC 3.1.3.1) was determined by hydrolysis of paranitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in 2 mM $MgCl_2$ and in 20 mM monoethanolamine (pH 10.5). Ca^{2+} and Mg^{2+}-ATPase (EC 3.6.1.3) were determined in the Ca^{2+} uptake media (pH 7.0) under Ca^{2+} uptake conditions or at pH 8.1 under optimal conditions (27) by the liberation of inorganic phosphate determined by the method of Fiske and Subbarow (6). Glutamate dehydrogenase (EC 1.4.1.3) activity was assessed at 24°C as previously described (27).

Ca^{2+} UPTAKE

Standard Ca^{2+} uptake was assessed at 30°C in a 6 ml final assay volume containing 0.2 to 0.6 mg/ml membrane protein, 100 mM KCl, 20 mM imidazole buffer (pH 7.0), 20 mM sodium azide, 5 mM oxalic acid, 5 mM Mg-ATP (Sigma Chemical Co.), 0.05 mM $CaCl_2$, and $^{45}CaCl_2$ (0.1 to 4.0 μCi/ml) (New England Nuclear, Boston, MA). The specific activity of $^{45}Ca^{2+}$ was kept constant during experiments in which increasing concentrations of $CaCl_2$ were required. After preincubation at 30°C for 10 min, the reaction was initiated by the addition of membrane protein (300 μl). Aliquots were removed immediately and every two min for 10 to 20 min and filtered under negative pressure through Millipore filters. The filters were washed four times with 1-ml aliquots of iced isotonic sucrose buffer, dried at room temperature, and counted in Aquasol (New England Nuclear). An aliquot of the incubation media was also spotted on a Millipore filter, dried, and counted under identical conditions. Uptake rates were estimated from at least six points for each uptake experiment and analyzed in relation to final membrane protein concentration (0.2 to 0.6 mg/ml). Binding in the absence of ATP was complete by 2 min and was subtracted to give ATP-dependent accumulation values. Contaminant calcium was similar in microsomal and brush border fractions and was approximately 0.005 mM elemental calcium as determined by flame spectroscopy in the final membrane preparation. All ligands and reactants were buffered prior to addition to the assay and varied as described in individual experiments.

Maximal uptake was always observed in freshly prepared vesicles within several hr after preparation. Vesicles stored at 2°C lost activity rapidly and were devoid of ATP-dependent Ca^{2+} uptake activity after two days. Samples which were rapidly frozen and stored at −30°C retained 50 to 70% of the starting activity for at least one wk. Experiments were performed on the day following preparation, unless otherwise noted. Boiling the sample assay at 60°C, and multiple freeze-thaw cycles each abolished uptake activity.

VESICULAR SPACE

Fresh brush border and microsomal vesicles were incubated with 1 to 10 mM, 3-*O*-methyl-D-glucose (Sigma Chemical Co.) and [^{3}H]3-*O*-methyl-D-glucose (2 μCi/ml) (New England Nuclear). The vesicular space was determined as described by Kletzien *et al.* (9) after Millipore filtration and wash in iced isotonic buffer containing 1 mM phloridzin (Sigma Chemical Co.).

Ca^{2+}-EGTA BUFFER SYSTEM

Free Ca^{2+} concentrations during these uptake experiments were estimated from the reaction conditions in relation to Ca^{2+}, Mg^{2+}, K^+, ATP, and 0.5 or 1.0 mM EGTA at pH 7.0. The affinity constants and equations used for these calculations have been previously reported by Potter and Gergely (17).

RESULTS

Calcium uptake by microsomal and brush border vesicles was dependent on ATP and magnesium and was enhanced by the addition of a trapping ion such as phosphate or oxalate (Fig. 1). In the absence of ATP, Ca^{2+} was rapidly "bound" by the membrane in a process which was complete within 2 min at 30°C and was proportional to membrane protein. However, in the presence of ATP and Mg^{2+}, calcium was rapidly accumulated by the vesicles in a process linearly dependent upon membrane protein, between 0.2 and 0.6 mg/ml. Equilibrium was established within 15 min (Fig. 2) and was maintained up to 60 min. The initial uptake rates (0 to 10 min) were nearly linear in the presence of phosphate or oxalate, and the uptake rates were therefore esti-

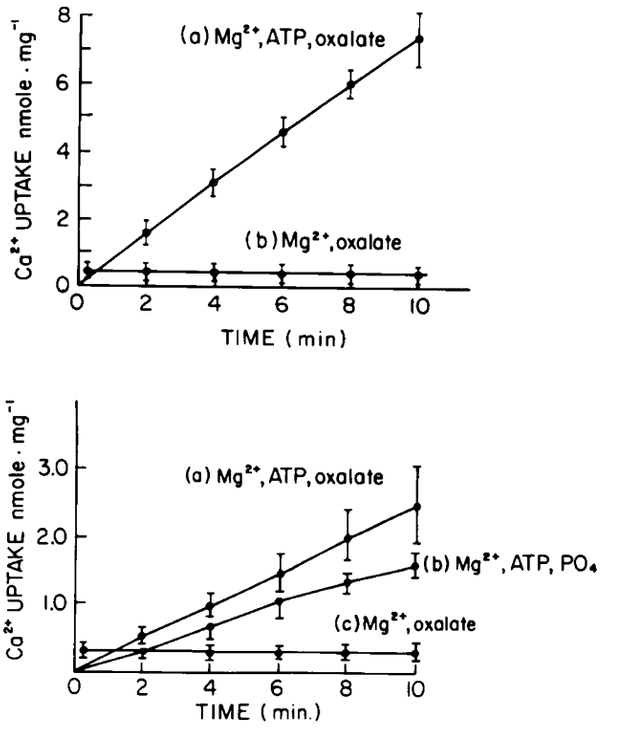

Fig. 1. Time course of ATP-dependent Ca^{2+} uptake by *A*, microsomal and *B*, microvillus placental fractions. The Ca^{2+} uptake by frozen placental fractions was assessed in a standard assay containing 50 μM $CaCl_2$, $^{45}CaCl_2$ (0.5 μCi/ml), 5 mM $MgCl_2$, 5 mM ATP, 10 mM sodium azide, 5 mM oxalate or phosphate, and 20 mM imidazole (pH 7.0). Ca^{2+} binding in the absence of ATP was subtracted from uptake in the presence of ATP to give uptake values. The reaction was initiated by additions of membrane fraction and was terminated by rapid filtration through Millipore filters. Phosphate was as effective as oxalate in enhancing Ca^{2+} uptake in the microsomal fraction. Values represent the mean ± S.D. of ten placental preparations for each fraction. Ca^{2+} uptake was entirely ATP dependent in both membrane fractions and increased nearly linearly between 2 and 10 min. The specific activity was always higher in the microsomal fraction as compared to the brush border fraction.

mated from the slope of the 0 to 10 min uptake curve for each determination of rate. The rate of Ca^{2+} uptake by fresh microsomal and brush border vesicles was approximately double that of once-frozen vesicles. Kinetic characteristics and requirements of Ca^{2+} uptake were similar in both membrane fractions, and no distinct property was found which distinguished microsomal from brush border Ca^{2+} uptake except for the lower activity in the latter.

The vesicular nature of the uptake process was demonstrated by performing the incubation in increasing concentrations of sucrose which theoretically reduced the vesicular volume. Ca^{2+} uptake was inversely related to increasing sucrose concentration (Fig. 3). The vesicular space of these preparations was estimated from the equilibration of 3-*O*-methyl-D-glucose with fresh brush border and microsomal vesicles. Brush border vesicular space was not different from that of the microsomal fraction, and spaces were, respectively, 3.95 ± 0.32 and 4.08 ± 0.11 μl/mg protein (mean ± S.D.; $n = 4$). Thus, in the present study, at equilibrium, Ca^{2+} was accumulated within the vesicles up to 100-fold greater than the concentration present in the medium. Further support for the vesicular nature of the uptake was the finding that Ca^{2+} uptake was enhanced by trapping ligands such as oxalate or phosphate and that uptake activity was destroyed by conditions which might disrupt vesicle formation such as heating, freeze-thaw cycles, and storage at 2°C.

SUBCELLULAR DISTRIBUTION

Calcium uptake activity was enriched approximately six-fold in the microsomal fraction above that in the crude homogenate, whereas it was only slightly enriched in the brush border fraction as represented by Table 1. Because the vesicular volumes were similar in microsomal and brush border fractions, the enrichment values for Ca^{2+} uptake are likely to represent differences in the intrinsic Ca^{2+} pump activities of these membrane preparations; however, differences related to the orientation or "sidedness" of the Ca^{2+} pump were not clarified in these experiments.

Ca^{2+} uptake was not coenriched with Ca^{2+}-stimulated ATPase or alkaline phosphatase activity during subcellular fractionation. These latter activities were best enriched in the brush border membranes. Mitochondrial contamination was assessed with the marker enzyme glutamate dehydrogenase, which was present in microsomal but absent from brush border fractions. However, azide and oligomycin, which are known potent inhibitors of mitochondrial Ca^{2+} uptake, had no effect on Ca^{2+} uptake in either placental fraction. Nevertheless, 10 mM azide was added to the uptake media during all experiments to control for a possible mitochondrial contribution to Ca^{2+} uptake.

RELATIONSHIP OF UPTAKE TO FREE Ca^{2+}

The rate of ATP-dependent Ca^{2+} uptake was nonlinearly related to the free Ca^{2+} concentration in both microsomal and brush border fractions (Fig. 4). Free Ca^{2+} concentration was varied with a Ca^{2+}-EGTA buffer system between 0.11 and 62.9 × 10^{-8} free Ca^{2+}. Under these conditions, the uptake process was saturable with respect to free Ca^{2+}. Data from such experiments were fit directly to the equation $V = \frac{V_{max}S}{S + K_m}$ using nonlinear regression

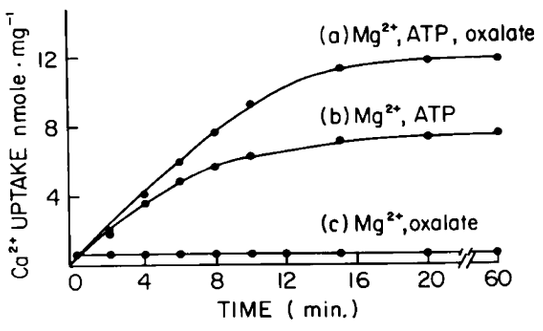

Fig. 2. Time course of Ca^{2+} uptake in microsomal placental membranes with or without ATP or oxalate. Ca^{2+} uptake was assessed in the standard assay as described in "Materials and Methods" at 30°C. *a*, presence of Mg^{2+}, 5 mM oxalate, and 5 mM ATP; *b*, 5 mM ATP and Mg^{2+}; and *c*, oxalate and Mg^{2+} in the absence of ATP. Ca^{2+} uptake reached equilibrium after 20 min and remained stable for up to 60 min. ATP-dependent uptake was enhanced by oxalate. Further addition of ATP (10 mM final concentration) at 20 min did not further enhance uptake. *Curves*, experiments from four separate placental preparations.

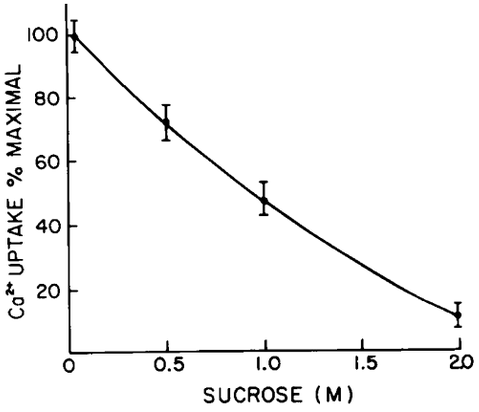

Fig. 3. ATP-dependent Ca^{2+} uptake in relation to osmolality (sucrose). Increasing concentrations of sucrose were added to standard Ca^{2+} uptake assay with microsomal membrane to final concentrations (0.1 to 2.0 mM) sucrose. The reaction was terminated by rapid filtration at 20 min. *Points*, mean ± S.D. of triplicate determinations at each concentration. Ca^{2+} uptake varied inversely with the osmolality and therefore positively with increasing vesicular space.

Table 1. *Distribution of Ca^{2+} uptake, Ca^{2+}-ATPase, and marker enzymes in placental fractions*[1]

Fraction	Ca^{2+} uptake oxalate (nmole/mg/min)	Ca^{2+} uptake phosphate (nmole/mg/min)	Ca^{2+}-ATPase (pH 8.1) (μmole/mg/min)	Alkaline phosphatase (μmole/mg/min)	Glutamate dehydrogenase (μmole/mg/min)	Total ATPase (μmole/mg/min)
Whole	1.44 ± 0.02[2]	ND[3]	0.13 ± 0.03	0.25 ± 0.04	0.015 ± 0.005	ND
Microsomal	7.4 ± 0.82	7.01 ± 1.2	0.59 ± 0.15	1.33 ± 0.39	0.016 ± 0.004	0.156 ± 0.01
Brush border	2.37 ± 0.71	1.6 ± 0.18	1.46 ± 0.16	3.20 ± 0.30	<0.001	0.164 ± 0.02

[1] Distribution of Ca^{2+} uptake, Ca^{2+}-ATPase, alkaline phosphatase, and glutamate dehydrogenase in placental fractions. Ca^{2+} uptake activity was assessed in each fraction at 30°C in ten placental preparations under standard assay conditions (pH 7.0) as described in "Materials and Methods"; 5 mM phosphate was substituted for 5 mM oxalate as indicated. Ca^{2+}-ATPase and alkaline phosphatase were determined under optimal conditions (pH 8.1 and 10.5, respectively) at 37°C. Total ATPase was assessed under standard uptake conditions at pH 7.0 in the presence of 50 μM $CaCl_2$, 10 mM azide, 5 mM oxalate, and 5 mM Mg-ATP. The addition of 100 μM EGTA in the presence of 100 μM $CaCl_2$ did not inhibit ATPase activity nor did the addition of $CaCl_2$ (100 μM) stimulate Mg^{2+}-ATPase in at least 10 experiments with six separate placental preparations.

[2] Mean ± S.D.

[3] ND, not done.

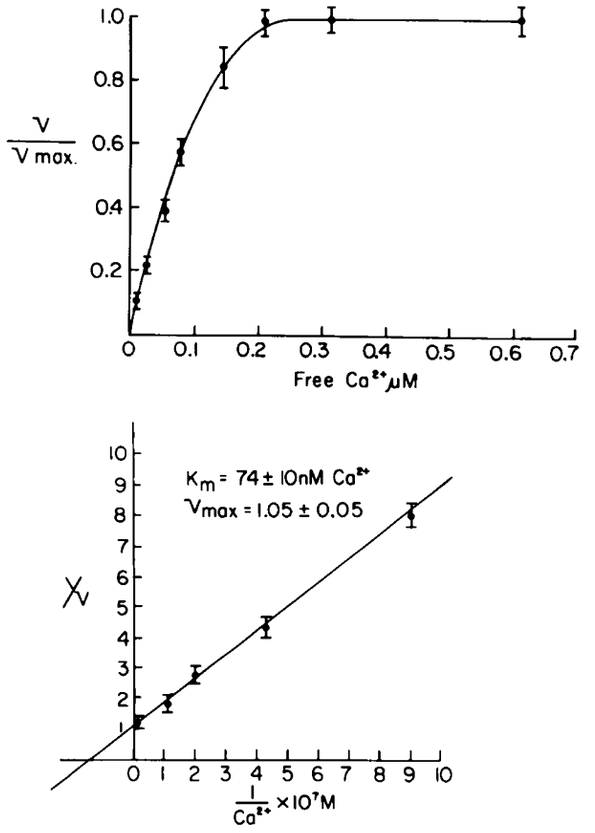

Fig. 4. ATP-dependent Ca^{2+} uptake in relation to estimated free Ca^{2+}. Free Ca^{2+} was regulated by addition of 0.5 or 1.0 mM EGTA to the standard uptake assay in the presence of 5 mM phosphate. Increasing $CaCl_2$ (0.050 to 1.0 mM) was added to vary free Ca^{2+} from 0.11 to 62.8 $\times 10^{-8}$ M. The specific activity of $CaCl_2$ was maintained constant, and the uptake rate at each Ca^{2+} concentration was determined from at least six data points from four separate microsomal membrane preparations. The free Ca^{2+} kinetic values were calculated as described in "Materials and Methods." The data from *A* were analyzed as described in "Materials and Methods"; *B*, Lineweaver-Burke plot from these data. ATP-dependent Ca^{2+} uptake was a saturable process dependent on free Ca^{2+} concentrations.

curve fitting procedures as suggested by Wilkinson (29). The K_m for free Ca^{2+} was 74 ± 10 nM in the microsomal fraction and 50 ± 6 nM in the brush border fraction; V_{max} was 1.05 ± 0.046 nmole/mg and 0.2 ± 0.01 nmole/mg/min, respectively (mean ± S.D.; $n = 4$). High concentrations of Ca^{2+} (greater than 1 mM) always resulted in an inhibition of uptake activity.

MAGNESIUM

Ca^{2+} uptake in both fractions was highly dependent on Mg^{2+} (Fig. 5). ATP-dependent Ca^{2+} uptake was not supported in the absence of Mg^{2+} in either fraction, nor did the addition of increasing concentrations of $CaCl_2$ (0.50 to 5.0 mM) support ATP-dependent Ca^{2+} uptake. Maximal Ca^{2+} uptake was observed at 5 mM $MgCl_2$ in the presence of 5 mM ATP. Higher Mg^{2+} concentrations (10 mM) did not further enhance Ca^{2+} uptake. Half-maximal activation was noted at approximately 1.25 mM Mg^{2+} under standard assay conditions.

NUCLEOTIDE REQUIREMENTS

Ca^{2+} uptake was best supported in both fractions by ATP as compared to other nucleotides. However, ADP, AMP, and GTP (5 mM) also supported Ca^{2+} uptake in the absence of ATP under standard assay conditions. The fraction of uptake for each nucleotide in relation to the rate observed in 5 mM ATP was ADP = 0.35 ± 0.14, AMP = 0.25 ± 0.08, and GTP = 0.25 ± 0.07 (mean ± S.D.) in five Ca^{2+} uptake experiments with the microsomal fraction.

pH DEPENDENCY

The pH dependency of Ca^{2+} uptake and Ca^{2+}-ATPase activity is represented by Figure 6. Maximal Ca^{2+} uptake in brush border and microsomal fractions was observed between 6.5 and 7.0. In contrast, the pH optimum of Ca^{2+}-ATPase activity was pH 8.1. At this pH, Ca^{2+} uptake was markedly inhibited.

TEMPERATURE

The time course of the ATP-dependent Ca^{2+} uptake process was assessed at 2, 10, 20, 30, 37, and 60°C. Uptake was abolished at 2 and 60°C. The reaction was highly temperature dependent; maximal uptake rates were noted at 37°C and approximately doubled for each 10°C increment above 2°C.

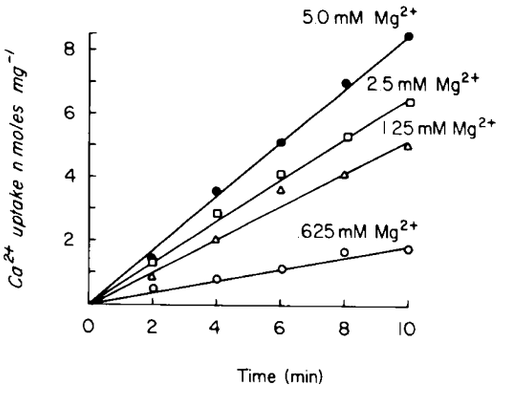

Fig. 5. Magnesium dependency of ATP-dependent Ca^{2+} uptake. Ca^{2+} uptake was assessed in the standard assay with microsomal membrane in the presence of 5 mM oxalate. Magnesium was varied from (0 to 5 mM) by the addition of $MgCl_2$. Maximal Ca^{2+} uptake was demonstrated at 5 mM $MgCl_2$, and higher final concentrations did not further enhance uptake.

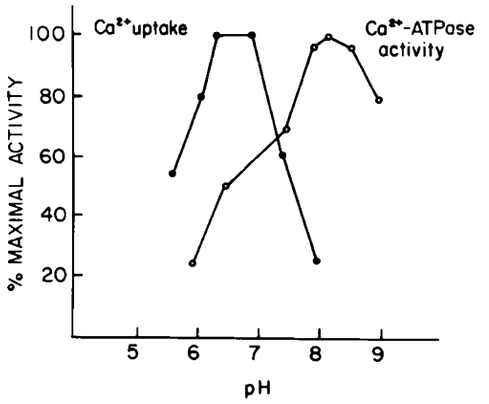

Fig. 6. The pH dependency of ATP-dependent Ca^{2+} uptake and Ca^{2+} ATPase. The pH of the assay medium was adjusted between 6.0 and 9.5. Ca^{2+} uptake was determined under standard assay conditions at 30°C in both fractions in four separate experiments at each pH. Ca^{2+}-ATPase was assessed at 37°C by determination of inorganic phosphate as described in "Materials and Methods." The pH dependency of Ca^{2+} uptake was identical in brush border and microsomal fractions. The pH maximum of Ca^{2+} uptake was 6 to 7 and contrasted with the pH maximum of Ca^{2+}-stimulated ATPase (pH 8.1).

INHIBITORS AND THE EFFECTS OF OTHER LIGANDS

The effects of various agents on ATP-dependent Ca^{2+} uptake were assessed in at least four experiments with each agent in the microsomal fraction. Ethacrynic acid, which nonspecifically binds membrane-bound sulfhydryl groups, was a potent inhibitor of ATP-dependent Ca^{2+} uptake in both fractions; 50% inhibition was noted at 1 mM ethacrynic acid. In contrast, placental Ca^{+2}-ATPase was only minimally inhibited by 1 mM ethacrynic acid (10%). Azide (10 mM) and oligomycin (1 μg/ml), which are effective inhibitors of mitochondrial Ca^{2+} uptake, had no effect on microsomal and brush border membrane Ca^{2+} uptake. DL-Phenylalanine (5 mM) markedly inhibited placental alkaline phosphatase (approximately 50%) but did not alter Ca^{2+} uptake. Ouabain (10^{-3} M), ruthinium red, (10^{-5} M) strontium (0.1 to 1.0 mM), and manganese (0.02 to 0.1 mM) also had no effect on Ca^{2+} uptake. Likewise, the substitution of 100 mM NaCl in the assay medium did not alter Ca^{2+} uptake in either fraction. Prostaglandins E_1, E_2, and $F_{2\alpha}$ (10^{-5} M) had no effect on Ca^{2+} uptake when added directly to the incubation media. Dibutyryl cyclic adenosine monosphosphate (1 mM) also had no effect on Ca^{2+} uptake.

Ca^{2+}-ATPase

Ca^{2+}-stimulated ATPase was determined in each fraction under optimal conditions (pH 8.1) and during Ca^{2+} uptake experiments (pH 7.0). Ca^{2+}-stimulated ATPase activity was 10-fold enriched in the brush border fraction and four-fold enriched in the microsomal fraction. Such Ca^{2+}—ATPase was not Mg^{2+} dependent in the presence of 5 mM $CaCl_2$ and 5 mM ATP. The apparent K_m for $CaCl_2$ was 0.37 ± 0.02 mM (mean ± S.D.) in the presence of 5 mM ATP from four placental preparations under these conditions. The total ATPase activity also determined during the Ca^{2+} uptake experiments and was approximately 0.17 ± 0.02 μmole/mg/min in the microsomal fraction. Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase could not be demonstrated in the microsomal fraction under a variety of assay conditions designed to elicit such activity.

Ca^{2+} BINDING TO PLACENTAL MEMBRANES

Non-ATP-dependent Ca^{2+} binding increased with the calcium concentration in the assay to increasing microsomal and brush border protein concentration (Fig. 7). Binding was rapid at 30°C and was completed within 2 min. The specific activity of non-ATP-dependent Ca^{2+} binding was always higher in the microsomal as compared to brush border fractions. The binding of Ca^{2+} to the microsomal fraction increased as a "complex function" of free Ca^{2+} and was not saturable at free Ca^{2+} concentrations up to 3 mM. Ca^{2+} binding to brush border membranes also increased with increasing Ca^{2+}; these binding sites were also not saturated up to 3 mM Ca^{2+}.

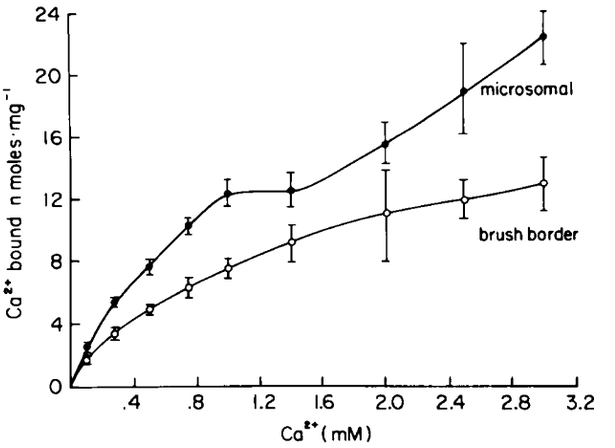

Fig. 7. Non-ATP-dependent Ca^{2+} binding to brush border and microsomal placental membrane. Ca^{2+} binding was determined under standard assay conditions (phosphate) with the omission of both ATP and oxalate. The incubation was terminated by rapid filtration at 20 min at 30°C. The Ca^{2+} concentration was varied by the addition of $CaCl_2$ to final concentrations (0.1 to 3.0 mM) $CaCl_2$. *Points*, mean ± S.D. of triplicate determinations from five separate brush border and microsomal preparations. Non-ATP-dependent Ca^{2+} binding was higher in microsomal than brush border membrane. Binding varied as a complex function of Ca^{2+} and was not saturable up to 3 mM added $CaCl_2$.

DISCUSSION

In the present study, it has been demonstrated that (1) vesicles prepared from membrane fractions of human placenta both bind Ca^{2+} and contain an ATP-dependent Ca^{2+} sequestration activity; (2) ATP-dependent Ca^{2+} uptake is dependent upon temperature and Mg^{2+} and is enhanced by trapping ligands such as oxalate and phosphate; (3) the process is saturable at low Ca^{2+} concentrations. Because of the low K_m (10^{-7} to 10^{-8} M Ca^{2+}), it is suggested that it might be activated by low Ca^{2+} concentrations, such as would be expected within the cytosol of the syncytium; (4) ATP-dependent Ca^{2+} uptake is maximally enriched in the microsomal placental fraction in comparison to the activity in purified brush border membranes; and finally (5) uptake is not associated with Ca^{2+}-stimulated ATPase which is most highly enriched in brush border membranes.

The asymmetrical transport of Ca^{2+} from the mother to fetus is likely to occur transcellularly across the syncytium. Large quantities of Ca^{2+} move from the mother to fetus at extracellular free Ca^{2+} concentrations which are in excess of 10^{-3} M. However, intracellular Ca^{2+} is generally maintained at concentrations which are much lower than extacellular Ca^{2+}. The molecular mechanisms which might be involved in intracellular Ca^{2+} regulation and in Ca^{2+} transport in the placenta are unclear at present but may include passive transcellular diffusion, binding to subcellular fractions, or energy-dependent uptake by subcellular elements as have been suggested in other epithelial Ca^{2+} transport systems (25). A rational hypothesis regarding the molecular mechanism of transplacental Ca^{2+} transport should include a mechanism by which intracellular placental Ca^{2+} is regulated at low concentrations (10^{-7} to 10^{-8} M). Thus, Ca^{2+} may enter the syncytium by passive diffusion down a chemical gradient or by a mechanism which involves Ca^{2+} binding to cell membrane which is subsequently internalized. Once within the cell, its concentration may be (1) regulated by its binding to intracellular sites such as to placental calcium binding protein which has been recently described in rat placental cytosol (1); (2) sequestered within membrane-bound structures such as vesicles, mitochondria, or other intracellular organelles; (3) regulated by its exit from the cell either by exocytosis or by being actively pumped to the fetus at the trophoblastic basement membrane (against a concentration gradient) by a plasma membrane-bound Ca^{2+} pump. The ATP-dependent Ca^{2+} pump activity presently described might be hypothesized to play a role in such energy-dependent intracellular Ca^{2+} sequestration or "pumping" at the fetal-placental interface.

ATP-dependent Ca^{2+} pump activity can be demonstrated in numerous tissues, and the characteristics of the Ca^{2+} uptake in placental vesicles described in the present studies are similar in many respects to those described in other tissues (10, 13, 14, 19, 24). The K_m for free Ca^{2+} demonstrated in the present study is extremely low, although there is some disagreement in the estimation of free Ca^{2+} in the presence of EGTA and ATP (the K_m is approximately five-fold higher when estimated with the calculations as described by Katz *et al.* (8). Nevertheless, the K_m is low by either calculation, and it is therefore likely that the ATP-dependent Ca^{2+} uptake activity is activated by low calcium concentrations, as would be expected within the cytosol of the syncytium.

The characteristics of placental Ca^{2+} uptake are similar to those of Ca^{2+} uptake activities in liver (14), kidney (15), and intestine (7), but contrast sharply to Ca^{2+} uptake described in a report on

guinea pig placental membranes as assessed by stop flow dialysis (20). Human placental Ca^{2+} uptake was saturable with respect to free Ca^{2+} and displayed Michaelis-Menten like kinetics with respect to free Ca^{2+}. Furthermore, uptake required Mg^{2+} and ATP and was highly pH dependent (maximum 7.0). In contrast, the Ca^{2+} uptake of guinea pig placental membranes did not require Mg^{2+}, was stimulated by Mg^{2+} at low Ca^{2+} concentration, and did not appear to be a saturable process (20).

CELLULAR LOCALIZATION

The precise cellular localization of the Ca^{2+} uptake system is complicated by the numerous cell types present in human placenta. For example, smooth muscle Ca^{2+} uptake has been previously demonstrated. However, the characteristics of Ca^{2+} uptake in vesicles from smooth muscle of the umbilical artery are distinct from Ca^{2+} uptake in placental vesicles (3). The former is highly ATP specific and inactive at pH less than 7. In contrast, placental Ca^{2+} uptake was supported by nucleotides other than ATP and was active at pH less than 7.0. Thus, it appears unlikely that smooth muscle contamination accounted for the Ca^{2+} uptake of placental vesicles.

SUBCELLULAR LOCALIZATION

ATP-dependent Ca^{2+} uptake activity was most highly enriched in microsomal membrane fractions of the placenta where it was enriched six- to seven-fold above the activity present in a crude placental homogenate. Microsomal membrane is likely to contain membranes from both apical and basilar plasma membranes of the placental as well as membrane from intracellular organelles (22, 26–28). However, the ATP-dependent Ca^{2+} uptake is not likely to originate primarily from brush border membranes which in the present study were not highly enriched in Ca^{2+} uptake activity. This difference in Ca^{2+} uptake activity between brush border and microsomal fractions could not be explained by differences in vesicular space. The vesicular space in each fraction was similar and in agreement with previously reported values for brush border membranes (18). Theoretically, the decreased Ca^{2+} uptake activity in the brush border fractions might also relate to a difference in the orientation of the Ca^{2+} pump, which would depend on the "sidedness" of the vesicles in each preparation. The orientation of these placental vesicles has not been clarified at present. Furthermore, the rate of efflux from the brush border preparations might be more rapid than in the microsomal fraction, although oxalate was present during these studies to minimize this possibility. Mitochondrial concentration to the Ca^{2+} uptake is unlikely because neither azide nor oligomycin inhibited Ca^{2+} uptake in either membrane fraction. Finally, it is possible that the observed differences in uptake rates were related to intrinsic properties of the components of the membrane or the intrinsic pump activity which is higher in the microsomal fraction. We therefore speculate that Ca^{2+} uptake activity results from transport vesicles or membranous organelles present within the placental cytosol or from plasma membrane Ca^{2+} pumps at the trophoblastic basement membrane where Ca^{2+} would be pumped to the fetal circulation.

Membranous and vesicular structures containing Ca^{2+} or other cations have been demonstrated within the cytosol of the human placenta by histochemical techniques (4, 16), and a role of such structures in Ca^{2+} transport has been previously suggested. Numerous vesicles and marked endocytotic activity are noted in the apical region (brush border side) of the syncytiotrophoblast (syncytium). Apical plasma membrane, possibly containing bound Ca^{2+}, is internalized in the intermicrovillous region of the brush border possibly related to an interaction of the plasma membrane with "coated vesicles." The plasma membrane associated with vesicles can be identified even as the vesicle moves through the syncytium towards the fetus and cations have been identified within these vesicles by histochemical techniques (16). Whether the vesicles sequester Ca^{2+} by an ATP-dependent process and/or are related to the Ca^{2+} uptake process noted in the present study remains to be clarified.

LACK OF RELATIONSHIP TO Ca^{2+} ATPase

ATP-dependent Ca^{2+} uptake has been related to Ca^{2+}-stimulated, MG^{2+}-dependent ATPase in several tissues (12, 19). A relationship of Ca^{2+} uptake to Ca^{2+}-ATPase activity could not be demonstrated in placental fractions. In sarcoplasmic reticulum and the erythrocyte, Ca^{2+} transport is closely coupled to Ca^{2+}-stimulated ATPase activity (12, 19). No Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase could be demonstrated in the present study of placental vesicles, but it is possible that the high activity of nonspecific Mg^{2+}-ATPase, Ca^{2+}-ATPase, and alkaline phosphatase obscures a possible Ca^{2+} transport-related ATPase. In fact, if Ca^{2+} uptake were tightly coupled to ATPase activity, the low activity of Ca^{2+}-stimulated ATPase activity which relates to Ca^{2+} transport would not be easily demonstrated by the usual chemical techniques because of the high nonspecific Mg^{2+}-ATPase activity present in placental fractions. The characteristics of Ca^{2+}-stimulated ATPase (present mainly in brush border membrane) were distinct from the Ca^{2+} uptake process which was present mainly in microsomal membrane. Furthermore, if Ca^{2+} uptake resulted from this high K_m, non-Mg^{2+}-dependent ATPase, the coupling ratio of ATP hydrolysis to Ca^{2+} transport activity in these placental membrane fractions would be extremely high. Finally, because the K_m (Ca^{2+}), pH maximum, and the effects of inhibitors are discrepant for Ca^{2+} uptake and Ca^{2+}-ATPase activity, we suggest that Ca^{2+}-ATPase is not directly related to Ca^{2+} transport in placental vesicles.

CONCLUSION

The ATP-dependent Ca^{2+} sequestration activity presently described in microsomal and brush border human placental membrane fractions is likely to be a mechanism for regulation of intracellular Ca^{2+}. Regulation of intracellelar Ca^{2+} can be achieved either by pumping Ca^{2+} into vesicular or membrane-bound structures within the placental cytosol or by actively pumping Ca^{2+} from the cytosol to the fetus at the expense of ATP hydrolysis at the trophoblastic basement membrane. Ca^{2+} binding to placental membranes and uptake into placental membrane vesicles may also represent a step in the "active" Ca^{2+} transport process which occurs across the human placenta.

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