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ASYMMETRIC CALCIUM INFLUX AND EFFLUX AT MATERNAL AND FETAL SIDES OF THE GUINEA-PIG PLACENTA: KINETICS AND SPECIFICITY

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SUMMARY

1. Unidirectional influx of calcium across maternal and fetal sides of the syncytiotrophoblast was investigated in the guinea-pig placenta by using a rapid (< 30 s) paired-tracer dilution technique. Experiments were performed in an *in situ* placenta artificially perfused through the umbilical vessels or in an isolated placenta in which both the maternal and fetal circulations were perfused.

2. At equimolar Ca^{2+} concentrations, unidirectional calcium influx was always significantly lower on the maternal side than on the fetal side. Saturation kinetics were observed: on the fetal side the estimated K_m was $1{\cdot}8 \pm 0{\cdot}7$ mM and V_{max} was $1{\cdot}66 \pm 0{\cdot}28$ μmol/min . g (mean ± S.E. of mean) and on the maternal side K_m ranged from 0·18 to 1·15 mM and V_{max} ranged from 0·12 to 0·59 μmol/min . g.

3. When the inhibition of calcium influx was investigated on the fetal side of the trophoblast by using competing cations, the following sequence was observed: $Ba^{2+} > Ca^{2+} \simeq Ni^{2+} > Sr^{2+} > Mg^{2+} \simeq Li^{+}$.

4. Efflux of $^{45}Ca^{2+}$ from the trophoblast into the ipsilateral circulation (backflux) was rapid (20–100 % in 6 min) and asymmetric since the fetal:maternal ratio was $1{\cdot}35 \pm 0{\cdot}11$ (mean ± S.E. of mean) in the presence of 0·1 mM-Ca^{2+}.

5. In the dually perfused placenta, transplacental transfer (6 min) of $^{45}Ca^{2+}$ varied over a wide range (0–80 %); however, it was similar to that of the extracellular reference tracer, $^{22}Na^{+}$, in either maternal-to-fetal or fetal-to-maternal directions. It is suggested that this is a consequence of the 'leakiness' of the dually perfused placenta since the transplacental transfer of $^{22}Na^{+}$ and D-[^{3}H]mannitol (or L-[^{14}C]glucose) measured simultaneously were also variable but similar. Transplacental transfer of $^{45}Ca^{2+}$ could not be used to characterize specific calcium-transport mechanisms, whereas highly sensitive trophoblast uptake measurements were provided by the single-circulation, paired-tracer technique.

6. Our findings suggest the presence of a specific carrier-mediated transport system for calcium on both maternal and fetal surfaces of the trophoblast. The asymmetries in unidirectional influx into the trophoblast and rapid backflux indicate a mechanism by which the net transfer of calcium from the maternal to the fetal circulation is maintained in favour of the fetus.

INTRODUCTION

There is evidence in mammals that during late gestation calcium is transported across the placenta from mother to fetus against a concentration gradient (Bawden, Wolkoff & Flowers, 1965; Delivoria-Papadopoulos, Battaglia, Bruns & Meschia, 1967; Woods, Thornburg & Faber, 1978). It appears that this transplacental calcium gradient in favour of the fetus is not the result of increased calcium-binding capacity of fetal serum proteins (Delivoria-Papadopoulos *et al.* 1967) nor is it dependent upon the presence of the fetus (Twardock & Austin, 1970). Furthermore, manoeuvres which produced either maternal hypocalcaemia (Payne & Sansom, 1963) or hypercalcaemia (Bawden & Wolkoff, 1967) did not affect the fetal blood calcium levels. These observations suggest that calcium is transferred to the fetus by means of active processes which may be regulated by the placenta itself. Little information has been obtained concerning the actual mechanisms involved, except for the identification of Ca-ATPase in guinea-pig (Shami & Radde, 1971) and human (Miller & Berndt, 1973; Whitsett & Tsang, 1980) placental plasma vesicles. However, the localization and orientation of this ATPase in the trophoblast and its function, whether the intracellular regulation of Ca^{2+} or the transplacental transport of calcium, remains unclear (Whitsett & Tsang, 1980).

The present study was aimed at characterizing the kinetics and specificity of the calcium-transport system at maternal and fetal sides of the trophoblast membrane of the intact guinea-pig placenta. By applying a single-circulation, paired-tracer dilution technique originally used for placental sugar and amino acid transport studies (Yudilevich, 1976*a*; Yudilevich, Eaton, Short & Leichtweiss, 1979; Yudilevich & Eaton, 1980), we were able for the first time to demonstrate that the unidirectional calcium influx across both surfaces of the trophoblast conformed to Michaelis–Menten saturation kinetics. Furthermore, the two sides of the placenta exhibited an asymmetry in both influx and efflux, which could result in a net transfer of calcium to the fetal circulation and the maintenance of the fetal-to-maternal calcium gradient. Preliminary communications of this work have been given (Sweiry & Yudilevich, 1982*a*, *b*; Sweiry, 1983*b*).

METHODS

Placental preparations

Isolated dually perfused placenta. Detailed surgical procedures have previously been described (Yudilevich *et al.* 1979; Eaton, Mann & Yudilevich, 1982) and involved a modification of the original preparation reported by Leichtweiss & Schröder (1971). Briefly, white Dunkin–Hartley guinea-pig dams in their last week of gestation were sedated with an intraperitoneal injection of 10 mg diazepam (Valium, Roche Laboratories) and then anaesthetized with sodium pentobarbitone 20–25 mg/kg (Nembutal, Abbott Laboratories) administered intravenously via a permanent limb vein cannula. The dam was then placed in a saline bath maintained at 37 °C, the uterus was exteriorized and a placenta isolated and then transferred to a small organ bath containing saline (0·9 % NaCl w/v) maintained at 37 °C.

In situ perfused placenta. This preparation in which the fetal side of the placenta is perfused with the maternal side remaining intact has been described by Money & Dancis (1960) and Reynolds & Young (1971). The initial procedure follows that described above, except that here a fetus was exteriorized via a small abdominal and uterine incision, a fetal artery and vein were cannulated and the fetal circulation of the placenta was artificially perfused with a non-recirculating system.

These preparations remained viable for the duration of experiments as judged by production of lactate and pyruvate, and the consistency of the Na^+/K^+ ratios in the venous effluents (Sweiry, 1983*a*).

Perfusates

The perfusate was a Krebs–Ringer solution of the following composition (mM): NaCl, 120; KCl, 5·3; $MgSO_4$, 1·2; KH_2PO_4, 0·5; $NaHCO_3$, 20–25; glucose, 5·5 and dextran (40000) 40 g/l (Sigma Chem. Co., Poole). In the *in situ* preparation, only the fetal circulation was perfused at 3 ml/min. In the fully isolated preparation, both maternal and fetal circulations were perfused at 3 ml/min. Perfusion pressures were generally below 50 mmHg and were similar on both sides in the isolated preparation. The perfusate was maintained at 37 °C and gassed with 95% O_2/5% CO_2 to a pH of $7{\cdot}40 \pm 0{\cdot}05$ ($n = 6$). The osmolarity ranged from 285 to 291 mosmol/l. Unlabelled calcium chloride was added to the perfusate as required for the kinetic studies. Before these experiments (Sweiry, 1983*a*), the ionized calcium (Ca^{2+}) concentration in the presence and absence of dextran in the perfusing solution was measured using a Ca^{2+} ion-selective electrode (Corning Membrane Electrode together with Corning M125 millivoltmeter). The results (not shown) indicated no significant loss of Ca^{2+} activity in the presence of dextran. All subsequent calculations of influx were therefore based on the total calcium concentration.

Measurement of unidirectional influx

The procedure for the measurement of cellular transport in the guinea-pig placenta by using a single-circulation, paired-tracer dilution technique has previously been described in detail for sugars (Yudilevich *et al.* 1979; Eaton, Mann & Yudilevich, 1980) and amino acids (Yudilevich & Eaton, 1980; Eaton & Yudilevich, 1981; Eaton *et al.* 1982). This method has also been applied to exocrine organs (Yudilevich & Mann, 1982) and more recently to the study of lactate transport in the perfused human cotyledon (Carstensen, Leichtweiss & Schröder, 1983). In the present study, a mixture of an extracellular marker, $^{22}Na^+$ (1 μCi), and $^{45}Ca^{2+}$ (2·5 μCi) in 100 μl of the appropriate perfusate was rapidly (1–2 s) injected into the arterial inflow of either the maternal or fetal circulation of the placenta. This was immediately followed by the collection of twenty-five to thirty successive three-drop (about 100 μl) samples of the venous effluent on the injection (donor) side. A final 4 min accumulated sample was then immediately collected for assessing tracer backflux, transplacental transfer (dually perfused placenta) and total tracer recovery. In the fully isolated placenta, the transplacental transfer of both tracers was monitored by either sequentially sampling the contralateral (acceptor) side or by collecting a cumulative 6 min sample.

Preparation of samples for liquid scintillation counting

The venous effluent samples, aliquots of the injection mixture, $^{22}Na^+$ and $^{45}Ca^{2+}$ channel standards, and background samples were prepared for liquid scintillation counting by the addition of 2 ml of either Aqualuma (Luma Systems AG) or Readysolve H.P. scintillant (Beckman). The data were analysed using a Basic program on a Commodore CBM microcomputer.

Analysis of paired-tracer dilution profiles

The activities of $^{22}Na^+$ and $^{45}Ca^{2+}$ in the successive venous effluent samples were expressed as a percentage of their respective injected doses. The cellular uptake (U, %) was determined from:

$$U = (1 - {}^{45}Ca^{2+}/{}^{22}Na^+) \times 100, \quad (1)$$

in each sample. From a plot of uptake *versus* sample number or time, a characteristic maximal uptake (U_{max}, see Fig. 1) was taken to be the unidirectional calcium uptake. The percentage backflux (b) of the label was estimated from:

$$b = (1 - U_T/U_{max}) \times 100, \quad (2)$$

where the over-all uptake, U_T (%), was calculated from the integrated tracer recoveries starting from the initial maximal uptake and including the final 4 min accumulated sample: $U_T = 1 -$ total $^{45}Ca^{2+}$/total $^{22}Na^+$.

The unidirectional influx (J, μmol/min . g) into the trophoblast was calculated from the fractional U_{max}, flow and the unlabelled calcium perfusate concentration, C (mM):

$$J = -F \ln (1 - U_{max})\, C \quad (3)$$

(Pardridge, Connor & Crawford, 1975; Bustamante, Mann & Yudilevich, 1981) where F is the perfusate flow per gram wet weight of placenta.

Validation of the use of $^{22}Na^+$ as an extracellular marker

The problems generally encountered in selecting an extracellular marker for the purpose of studying cellular uptake *in vitro* and *in vivo* have been extensively reviewed recently (Law, 1982). For the single-circulation, paired-tracer dilution technique, reference and test molecules should ideally be of equal charge, similar size and diffusibility, and should not be subject to intravascular separation (Taylor effect). In the placenta, an ideal pair of tracers were L-[^{3}H]glucose and D-[^{14}C]glucose, where the former is apparently totally excluded from the trophoblast (Yudilevich *et al.* 1979). The choice of an extracellular marker for the study of $^{45}Ca^{2+}$ transport is limited. However, $^{22}Na^+$ was thought to be appropriate based on physico-chemical characteristics (both sodium and calcium are positively charged and have similar free-diffusion coefficients in water) and the general concept that the tissue distribution of labelled sodium within a short time is in the extracellular compartment. The relatively small rate of transfer of $^{22}Na^+$ across the cell membrane would be insignificant in relation to the size of the sodium extracellular compartment.

The use of extracellular markers other than $^{22}Na^+$, such as D-[^{3}H]mannitol, L-[^{3}H]glucose and ^{51}Cr-labelled EDTA was considered for the present calcium-transport study. These appeared less suitable since on the fetal side they might separate from calcium in crossing the capillary and interstitium. In previously reported experiments, the fetal capillary extraction for $^{22}Na^+$ (relative to ^{125}I-labelled albumin) in the *in situ* perfused guinea-pig placenta was higher than that for ^{51}Cr-labelled EDTA (0·84 compared to 0·67) but their rapid extravascular volumes of distribution (e.v.v.) were similar (Eaton, Yudilevich, Bradbury & Bailey, 1977). Since the parameter e.v.v. is a virtual space, molecules which penetrate the cell will have higher e.v.v. compared to a molecule that is confined to the interstitial space (Yudilevich, 1976*b*). As part of the present study, we measured e.v.v. for $^{22}Na^+$ and D-[^{3}H]mannitol simultaneously and they were found to be identical (mean ± S.E. of mean, n = four placentae): 0·15 ± 0·04 ml/g ($P > 0·10$) and hence no cellular uptake of $^{22}Na^+$ was apparent. In a further series of experiments, measurements were made for $^{22}Na^+$ and $^{45}Ca^{2+}$ simultaneously (Sweiry & Yudilevich, 1982*b*). When 0·5 mM-Ca^{2+} was present in the perfusate, the capillary extractions, relative to ^{125}I-labelled albumin, were high and similar (mean ± S.E. of mean, n = four placentae): 0·93 ± 0·03 and 0·96 ± 0·02 for $^{22}Na^+$ and $^{45}Ca^{2+}$ ($P > 0·12$), respectively but e.v.v. for $^{45}Ca^{2+}$ (0·28 ± 0·09 ml/g) was found to be about twice that for $^{22}Na^+$ (0·16 ± 0·05 ml/g; $P < 0·06$). When the perfusate calcium was raised to 5·0 mM, the extractions were not altered (0·92 ± 0·01 and 0·93 ± 0·01, respectively), whereas in contrast the e.v.v. for $^{45}Ca^{2+}$ was reduced by 50% to 0·14 ± 0·01 and approached that for $^{22}Na^+$ (0·11 ± 0·01; $P > 0·29$) measured simultaneously.

RESULTS

The results of an experiment with the paired-tracer method in a dually perfused placenta are illustrated in Fig. 1. The upper panels show the outflow dilution profiles obtained on the injection (donor) side following the intra-arterial injection of a bolus containing the two tracers ($^{22}Na^+$ and $^{45}Ca^{2+}$) into either the maternal (left panel) or fetal (right panel) circulation of the dually perfused placenta. It can be seen that initially the curve for $^{45}Ca^{2+}$ lies below that of the extracellular marker, $^{22}Na^+$ curve, indicating uptake of $^{45}Ca^{2+}$ into the trophoblast. During the downslope the test tracer tends to approach the reference tracer (and on the fetal side it rises above that of the reference tracer) indicating backflux from the trophoblast. The uptake (eqn. (1)) pattern of $^{45}Ca^{2+}$ (lower panels) shows that on both sides of the placenta there was an early unidirectional maximal uptake (U_{max}) followed by backflux of the label. However, the maximal uptake was higher and the backflux (estimated from eqn. (2)) more prominent on the fetal than on the maternal side. There were no difficulties in estimating maximal (unidirectional) $^{45}Ca^{2+}$ uptake since on both sides there was an

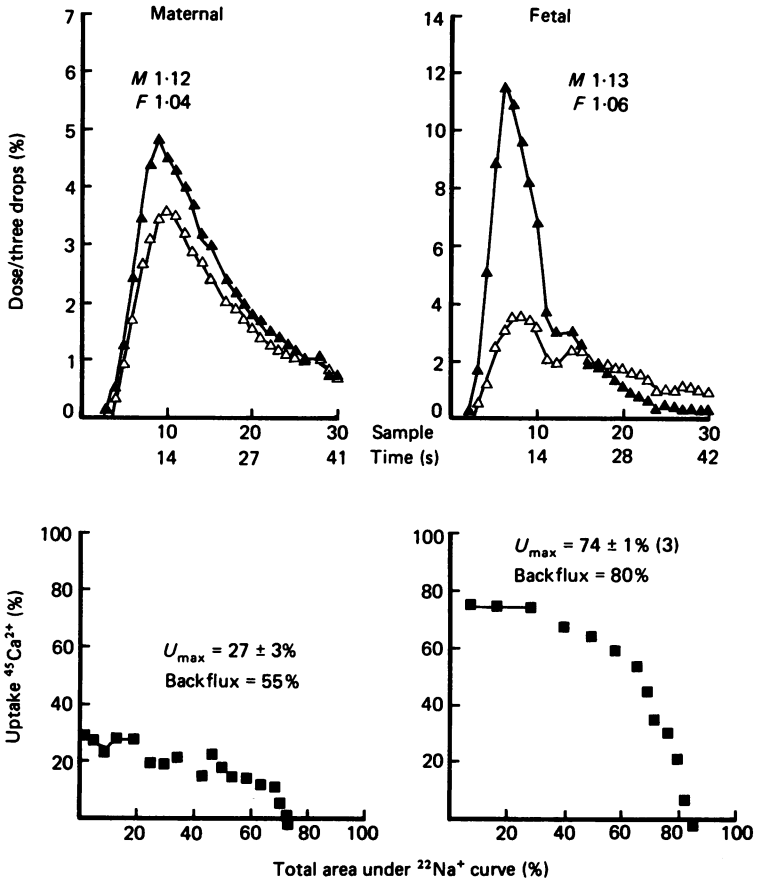

Fig. 1. Analysis of a paired-tracer dilution experiment. This Figure illustrates results obtained in the dually perfused placenta; however, the same fetal side patterns were observed in the *in situ* singly perfused preparation. An intra-arterial injection of a mixture of $^{22}Na^{+}$ (extracellular marker) and $^{45}Ca^{2+}$ was made into either the maternal (left) or fetal (right) side and both venous effluents were sequentially sampled. Upper panels: venous outflow profiles on the injection (donor) side: filled triangles, $^{22}Na^{+}$; open triangles, $^{45}Ca^{2+}$. The tracer activities in each sample were expressed as a percentage of the injected dose. Lower panels: $^{45}Ca^{2+}$ uptake pattern. The uptakes calculated from each sample were plotted against the area under the extracellular tracer curve. The maximal unidirectional uptake (U_{max}) is calculated from the mean of the initial samples before the uptake starts to decline (the points chosen are shown joined by a line). The $^{45}Ca^{2+}$ backflux quantifies the reduction in the apparent uptake which follows the initial maximal undirectional values. *M* and *F* refer to maternal and fetal venous outflows (ml/min . g), respectively.

initial maximal value which, even though it covered fewer points on the fetal side than on the maternal side, was always easily measurable. This is similar to previous findings with asymmetric uptake curves for amino acids (Yudilevich & Eaton, 1980; Eaton & Yudilevich, 1981). The difficulty reported by Štulc, Štulcova & Švihovec (1982), who attempted to use the paired-tracer technique to study [^{32}P]phosphate in the placenta, may have been due to the use of [^{14}C]sucrose as an extracellular

marker since the two molecules have very different physico-chemical characteristics and this may have influenced their distribution across the fetal capillary and interstitium.

Table 1 summarizes paired (maternal and fetal) injection data similar to those illustrated in Fig. 1. It can be seen that the asymmetry of influx and backflux was highly significant.

TABLE 1. Summary of paired maternal and fetal $^{45}Ca^{2+}$ transport parameters in the dually perfused placenta. The results are from twenty-two preparations in which the perfusate contained 0·1 mM-calcium. In addition, this table shows the retention of $^{45}Ca^{2+}$ over a 6 min collection period (see text)

	Maternal (M)	Fetal (F)	P
U_{max} (%)	33±2	82±2	< 0·001
Unidirectional influx (μmol/min . g)	0·032±0·003	0·15±0·001	< 0·001
Backflux (%)	74±5	89±3	< 0·01
Backflux ratio, $F:M$	1·35±0·11		
Tissue retention (% dose)	12±2	9±1	N.s.

Values are mean±S.E. of mean, compared using paired t test for maternal and fetal injections. N.s. = not significant ($P > 0·05$).

Unidirectional calcium influx

In order to investigate the saturability of calcium uptake, placentae were perfused, in random order, successively with solutions containing 0·05, 0·1, 0·5, 1, 1·5, 1·75, 2·5 and 5·0 mM-Ca^{2+}. Unidirectional influx of calcium was estimated using eqn. (3). The results obtained on the maternal interface in three different placentae are shown in Fig. 2. Michaelis–Menten parameters were estimated by fitting (Cleland, 1967) each set of data points with an unweighted single rectangular hyperbola and a curve was drawn based on the calculated kinetic constants. Saturation was always observed; however, due to inter-animal variability the values were not pooled but kinetic constants were separately estimated for each placenta. Individual kinetic estimates at the maternal side from four additional placentae were: K_m (mM): 0·18, 0·45, 1·12 and 1·15; V_{max} (μmol/min . g): 0·12, 0·21, 0·54 and 0·39, respectively.

In contrast to the maternal side, the fetal side exhibited much less variability since the kinetic constants were of the same order of magnitude. Hence the results from four placentae were pooled (Fig. 3) and a single rectangular hyperbola weighted for the standard error at each mean was fitted to the data (Cleland, 1967). The kinetic constants for the fetal interface were: $K_m = 1·8 \pm 0·7$ mM and $V_{max} = 1·66 \pm 0·28$ μmol/min . g.

Inhibition of influx by strontium and other cations

The effects of strontium on calcium influx were investigated on the fetal side in four *in situ* placentae. Four dually perfused placentae were used to study the maternal interface. Inhibition was determined by comparing the U_{max} for $^{45}Ca^{2+}$ obtained in the presence of 0·1 mM-Ca^{2+} and when increasing concentrations of either unlabelled

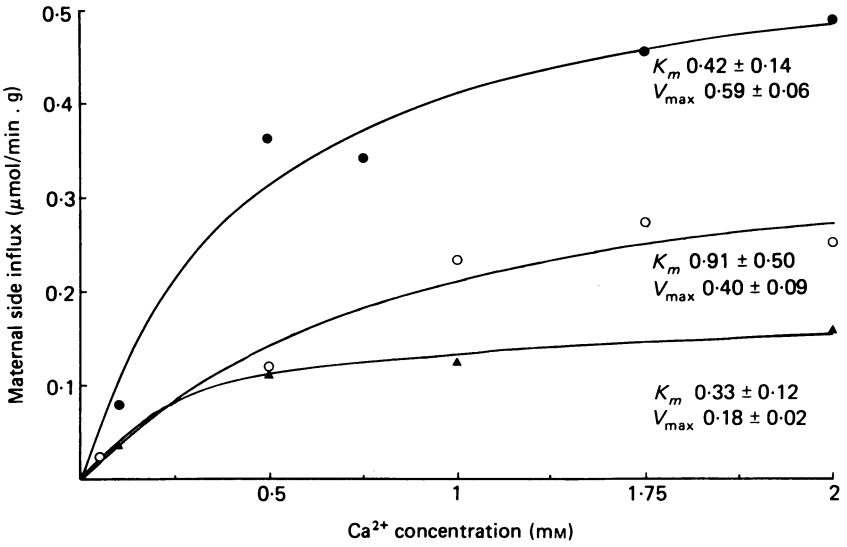

Fig. 2. Unidirectional influx kinetics of calcium across the maternal side of the trophoblast. A single hyperbola was fitted (see text) to data points from each of three placentae and shown individually. In each experiment influx was saturable, although inter-animal variation was observed. The units for K_m and V_{max} are mM and μmol/min . g, respectively. Results from four additional experiments are given in the text.

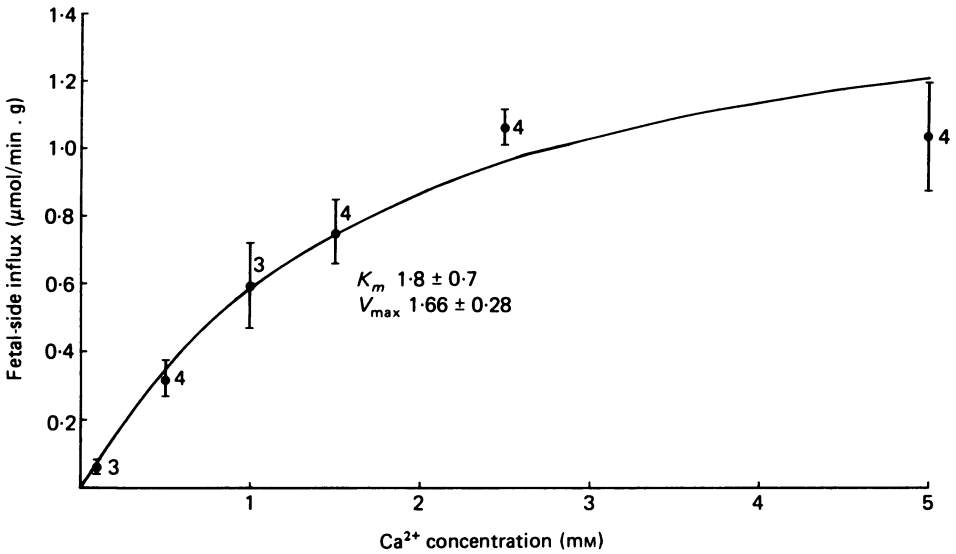

Fig. 3. Unidirectional influx kinetics of calcium across the fetal side of the trophoblast in the *in situ* perfused placenta. Results from a total of four placentae were pooled and a single rectangular hyperbola weighted for 1/S.E. of mean was fitted to the data to estimate the kinetic constants. Values are mean ± S.E. of mean; number of placental preparations is shown. The units for K_m and V_{max} are mM and μmol/min . g, respectively.

strontium or calcium were added to the perfusate. The inhibition kinetics illustrated in Fig. 4 demonstrate that on both sides of the trophoblast strontium inhibited the uptake of $^{45}Ca^{2+}$ and that the effect increased with concentration. Compared to calcium, strontium appeared to be less effective on the maternal side than on the fetal side.

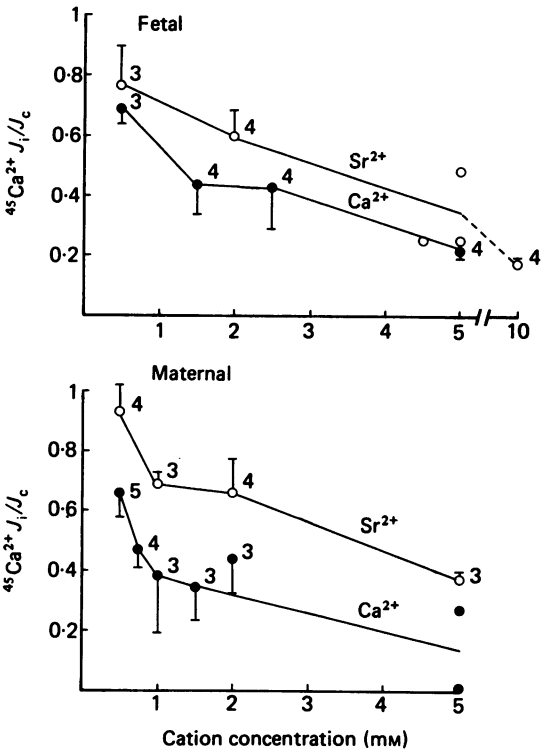

Fig. 4. Inhibition of $^{45}Ca^{2+}$ influx by either unlabelled Ca^{2+} or Sr^{2+}. The inhibition is expressed as the ratio between a control influx in the presence of 0·1 mM-Ca^{2+} (J_c) and the influx (J_i) due to 0·1 mM-Ca^{2+} measured at various additional perfusate concentrations of the unlabelled cation. Upper panel: fetal-side data from singly perfused placentae. Lower panel: maternal-side data from isolated dually perfused placentae. Values are mean ± S.E. of mean, the number of placental preparations is shown.

To characterize further the specificity of the calcium-transport system, similar inhibition experiments were performed to compare the effects of various monovalent and divalent cations at one perfusate concentration (5 mM) in the *in situ* preparation. In these experiments, 0·1 mM-adenosine was added to all perfusates to prevent the vasoconstriction initially observed with some of the cations. Control Ca^{2+} influx at the fetal side of the trophoblast was measured at an unlabelled calcium perfusate concentration of 0·1 mM. The concentrations of the other cations normally present in the perfusate (see Methods) were not altered. The relative effectiveness of the inhibitory cations (Fig. 5*A*) was: $Ba^{2+} > Ca^{2+} \simeq Ni^{2+} > Sr^{2+} > Mg^{2+} \simeq Li^{+}$. The control backflux was 92 ± 2% and compares favourably with the value (89 ± 3%)

measured on the fetal side in the dually perfused placenta (Table 1). In the presence of the different cations, backfluxes were respectively: 97 ± 2, 82 ± 9, 88 ± 12, 96 ± 3, 87 ± 9, 93 ± 1 %. Interestingly, the low inhibition of calcium influx caused by Mg^{2+} on the fetal side (15 ± 4 %, Fig. 5*A*) was also observed on the maternal side (16 ± 6 %, $n = 4$ dually perfused placentae).

When the cations were arranged in the order of inhibition expected (Ca^{2+} greatest) due to non-specific charge binding in model membranes (see Bianchi, 1968), it became apparent that this phenomenon could not explain our observed inhibition sequence. Plotting the data as a function of the effective hydrated-ion radius (Deon, 1979)

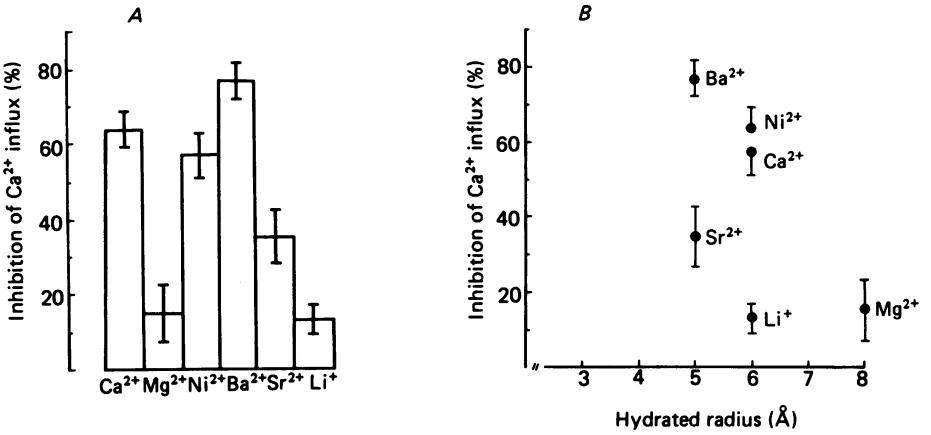

Fig. 5. *A*, the effect of various cations (5·0 mM) on unidirectional calcium influx on the fetal side. Values are mean ± S.E. of mean, n = three placentae. *B*, the inhibition data are plotted against the effective hydrated size of the cations, illustrating that charge as well as size are important in determining the interaction of the competitor cations at the calcium-uptake site.

indicated that the nearer, in both size and valency (e.g. Ba^{2+} and Ni^{2+} but not Li^{+}) the cation was to Ca^{2+}, the more effective was the inhibition (Fig. 5*B*). Sr^{2+} appeared to be an exception and was less effective than Ba^{2+} and Ni^{2+} in reducing calcium influx.

The inhibitor constant (K_i) may be determined by means of a 'Velocity Ratio' (see Neame & Richards, 1972) in which the uninhibited rate of influx may be related to the inhibited rate, provided the inhibition is competitive:

$$K_i = iv_iK_m/(v-v_i)(S+K_m),$$

where i is the competitor concentration, v_i the influx due to 0·1 mM-calcium measured in the presence of the competitor, v is the control influx due to 0·1 mM-calcium, S is the substrate concentration (0·1 mM-calcium) and K_m is the affinity constant for the substrate. The K_m of 1·8 mM obtained on the fetal side (see Fig. 3) was substituted into the above equation and the results are shown in Table 2. In one out of the three experiments no inhibition was observed with Mg^{2+} and Li^{+}, and hence K_i tends to infinity.

TABLE 2. Kinetic inhibitory constants (K_i) for various cations in the *in situ* perfused placenta. They are arranged in the order of inhibition of control calcium influx observed (Ba^{2+} greatest). The calculation of K_i was based on the K_m of 1·8 mM measured for Ca^{2+} (see Fig. 3). (In one out of the three experiments no inhibition was observed with Mg^{2+} and Li^+ and hence the K_i tends to infinity.) The ratio K_m/K_i is also tabulated for direct comparison of the affinity of each cation with that of Ca^{2+}

Cation	K_i (mM)	K_m/K_i
Ba^{2+}	0·9±0·11	0·5±0·1
Ca^{2+}	2·0±0·4	1·1±0·1
Ni^{2+}	2·8±0·5	1·5±0·3
Sr^{2+}	4·8±1·4	2·7±0·8
Mg^{2+}	8·3, 12·4	4·6, 6·9
Li^+	19·1, 10·1	10·6, 5·6

All cations were present in the perfusate at 5 mM. Values are mean±S.E. of mean for three placentae.

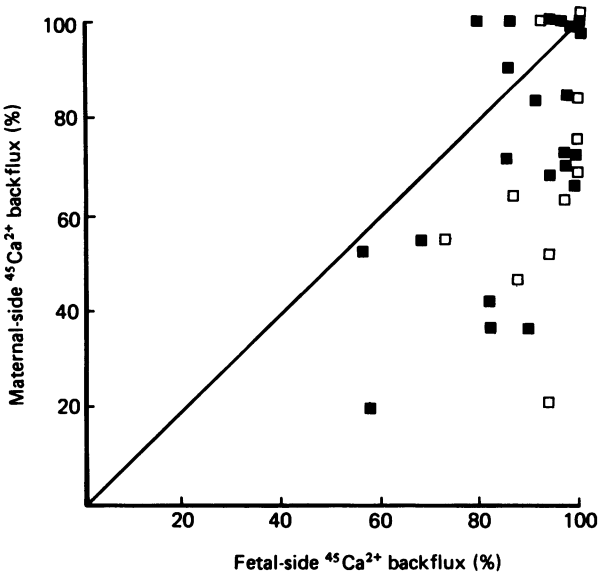

Fig. 6. Efflux of $^{45}Ca^{2+}$ from the trophoblast towards the injection side (backflux). Each point represents successive (paired) tracer injections, one in the maternal circulation (ordinate) and the other in the fetal circulation (abscissa). The data were obtained at two different perfusate calcium concentrations, 0·1 mM (filled squares) and 0·5 mM (open squares). The line of equality is shown.

Calcium backflux

Data for calcium efflux from the trophoblast into the injection side circulation (backflux) in the dually perfused placenta are shown in Fig. 6. It can be seen that on the fetal side, $^{45}Ca^{2+}$ backflux was above 60% whereas the maternal backflux varied between 20 and 100%. Fetal:maternal $^{45}Ca^{2+}$ backflux ratios as high as 5·0 were observed, although the average ratio was 1·35 (Table 1).

Transplacental transfer of $^{45}Ca^{2+}$ and simultaneously measured $^{22}Na^{+}$

Fig. 7*A* shows that in the dually perfused placenta, transplacental movement of the two tracers from the maternal to fetal circulation, and also in the reverse direction, was highly variable and ranged between 0 and 80% of the injected dose. The transplacental recoveries of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ were correlated even though in most cases there was some excess recovery of $^{22}Na^{+}$, which averaged $5{\cdot}0 \pm 0{\cdot}1$% dose (n = ninety-seven paired-tracer runs in forty-seven placentae). In order to understand better these results we conducted experiments in which we compared the transplacental passage of $^{22}Na^{+}$ with that of D-[^{3}H]mannitol or L-[^{14}C]glucose which had

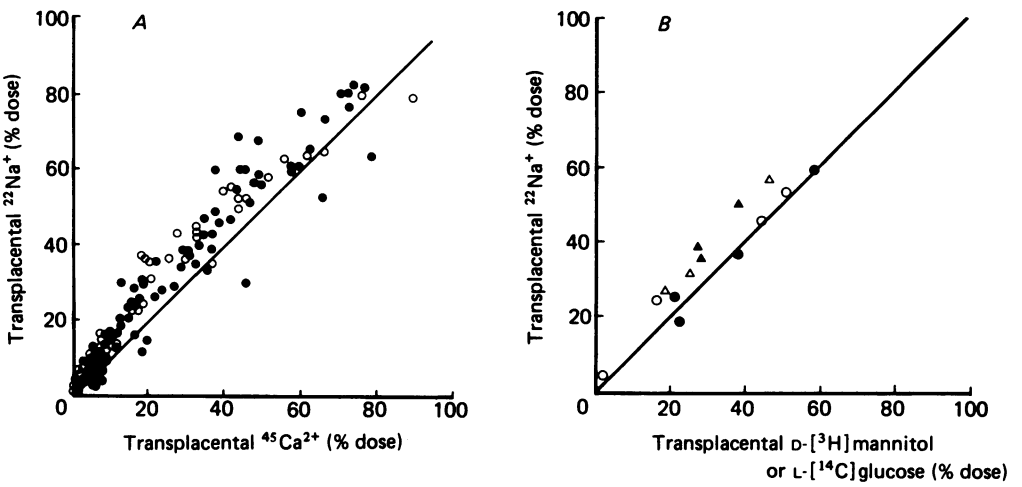

Fig. 7. Accumulated (6 min) transplacental transfer. *A*, $^{22}Na^{+}$ *versus* simultaneously measured $^{45}Ca^{2+}$ in the maternal-to-fetal (closed circles) and fetal-to-maternal (open circles) directions across the dually perfused placenta. Data from ninety-seven paired-tracer runs in forty-seven placentae. *B*, similar transplacental transfer of $^{22}Na^{+}$ *versus* either D-[^{3}H]mannitol (open symbols) or L-[^{14}C]glucose (closed symbols) in the maternal-to-fetal (circles) or in the reverse (triangles) direction. Data from fourteen paired-tracer runs in seven placentae. The line represents equal recoveries of the tracers.

previously been used to demonstrate the 'leakiness' of the dually perfused placenta (Yudilevich *et al.* 1979). The data (Fig. 7*B*) show that transplacental $^{22}Na^{+}$ transfer (6 min) was similar to the transfer of the inert molecule. The small difference in transfer rate for $^{22}Na^{+}$ which again appeared in some cases, could be explained by the difference in charge and in free-diffusion coefficients of the two molecules.

Placental $^{45}Ca^{2+}$ retention

This was measured from the total recovery (6 min) of $^{45}Ca^{2+}$ and $^{22}Na^{+}$ in the venous effluents from the ipsilateral and contralateral sides from: $^{45}Ca^{2+}$ retention = total $^{22}Na^{+}$ − total $^{45}Ca^{2+}$ (percentage dose). These results are given in Table 1, where it can be seen that 9–12% of $^{45}Ca^{2+}$ dose was still retained in the placenta 6 min after the tracer injection. In the same period most of the $^{22}Na^{+}$ had been recovered in the venous outflows (data not shown). There was no significant difference in the $^{45}Ca^{2+}$ retention following the administration of tracer on the maternal or fetal circulation.

DISCUSSION

Unidirectional calcium influx and efflux

In our intact placental preparation (*in situ* singly perfused or isolated dually perfused), unidirectional influx of calcium was saturable at both interfaces of the trophoblast and apparently mediated by a carrier system over the concentration range (0·05–5 mM) investigated. On the maternal side the kinetic analysis indicated affinity constants (0·18–1·12 mM) lower than those estimated on the fetal side (1·8 mM) and the transport capacity at the fetal interface (1·66 μmol/min . g) far exceeded that measured on the maternal side (0·12—0·59 μmol/min . g). The only other available kinetic data stem from studies in placental membrane vesicles. In their investigation of calcium binding to plasma membranes isolated from guinea-pig placentae, Shami, Messer & Copp (1974) identified two types of Ca^{2+}-binding sites: a high-affinity site with a dissociation constant (K_S) of 0·03 mM and a low-affinity site with a K_S of 1·1 mM – which we think could be related to our K_ms found on maternal and fetal sides, respectively. However, these authors discussed their two affinity constants as representing influx (blood to trophoblast) and efflux, since only some of the vesicles were inside-out. Whitsett & Tsang (1980) employed two types of membrane vesicles derived from human placentae: a brush-border fraction and a microsomal fraction which, on the basis of histochemical observations, appear to be related to the fetal side of the trophoblast (Whitsett & Lessard, 1978). These two membranes exhibited ATP-dependent, saturable calcium-uptake kinetics with similar K_m values (0·07 and $0{\cdot}05 \times 10^{-3}$ mM) and very different V_{max} values (1·05 and 0·2 nmol/min . g) for 'fetal' and 'brush-border', respectively. Whitsett & Tsang suggested that the low K_m could be related to the low Ca^{2+} concentration within the syncytium, hence implying that the orientation of these vesicles was inside-out and therefore their uptake measurements probably reflected the activity of a transport system moving calcium out of the trophoblast. Interestingly, our methods in the intact placenta provide measurements for the initial rapid uptake which must represent unidirectional influx across a specific pole of the syncytiotrophoblast. Even though we could not measure the kinetics of efflux, the tracer backflux qualitatively reflects the exit of transported calcium, which appeared to be faster at the fetal side (Fig. 6). This resembled the asymmetric efflux observed previously for a number of neutral and basic amino acids (Eaton & Yudilevich, 1981).

Further characterization of the calcium transport system was performed by studying the effects of different unlabelled cations on unidirectional calcium influx on the fetal side of the placenta. The following inhibition sequence was observed (see Fig. 5*A*): $Ba^{2+} > Ca^{2+} \simeq Ni^{2+} > Sr^{2+} > Mg^{2+} \simeq Li^{+}$. The literature lacks data to compare the calculated K_i (Table 2) for the various cations, even for the most common competitor, strontium. The poor inhibition of calcium influx by Mg^{2+} is similar to that observed in cardiac sarcolemmal vesicles (Bers & Langer, 1979; Trosper & Philipson, 1983). However, the latter study also showed that the effect of Sr^{2+} was greater than that of Ba^{2+}, which is opposite to our observation. In the intestinal brush-border membrane vesicles of the chick, the inhibition sequence was $Ca^{2+} > Sr^{2+} > Mg^{2+}$ (Wilson & Lawson, 1980), while in the erythrocyte extracellular Sr^{2+}, but not Mg^{2+}, competes with Ca^{2+} transport (Schatzmann & Vincenzi, 1969; Sarkadi, Macintyre & Gardos, 1978).

In view of its use as a model cation for calcium-transport systems, we further investigated the kinetics of Sr^{2+} competition with Ca^{2+} uptake at maternal and fetal interfaces of the trophoblast. Although Sr^{2+} was a good inhibitor on both sides of the trophoblast, the fetal side appeared to be less discriminatory (Fig. 4).

Although Ca-ATPase activity has been demonstrated in human (Miller & Berndt, 1973; Whitsett & Tsang, 1980) and guinea-pig (Shami & Radde, 1971) placental membrane vesicles, it is not clear whether this enzyme is associated with placental calcium transport. It has been shown that the sulphydryl reagent ethacrynic acid, an inhibitor of the Ca–Mg-ATPase (see Schuurmans-Stekhoven & Bonting, 1981) inhibits this enzyme (Shami & Radde, 1971; Miller & Berndt, 1973) as well as ATP-dependent Ca^{2+} uptake in placental vesicles (Whitsett & Tsang, 1980), whereas ouabain was ineffective on either the enzyme or calcium-uptake activity. In a recent study (McKercher, Derewlany & Radde, 1983), the effects of ethacrynic acid (2 mM) on the *in vivo* transplacental transfer of calcium in the guinea-pig were found to be negative. In this context, it is interesting that in preliminary experiments (Sweiry, 1983*a*, *b*) perfusion of the placenta with the sulphydryl reagent *N*-ethylmaleimide, an inhibitor of the Ca–Mg-ATPase (see Schuurmans-Stekhoven & Bonting, 1981), and potassium cyanide indicated that there was no inhibition of calcium influx (on the contrary, there was an apparent increase) whereas there was an inhibition of the efflux which appeared to be more prominent on the maternal interface. Contrary to the effects of the sulphydryl reagents, Ruthenium Red, a potent inhibitor of mitochondrial calcium transport (Moore, 1971), appeared to slightly inhibit the influx without significantly affecting the efflux of calcium from the trophoblast (J. H. Sweiry & D. L. Yudilevich, unpublished observations).

Calcium–sodium counter-transport has not been observed in placental membrane vesicles in conditions where ouabain (10^{-3} M) was present, or in the absence of sodium (Whitsett & Tsang, 1980). In a recent study, the net uptake (arteriovenous difference) of calcium by dually perfused guinea-pig placenta was investigated during steady-state perfusion of both maternal and fetal circulations with $^{45}Ca^{2+}$ (van Kreel & van Dijk. 1983). They showed that although replacement of sodium in the perfusate by choline or potassium caused a transient increase in the uptake of $^{45}Ca^{2+}$, this effect was not due to a calcium/sodium antiporter. The absence of this antiporter has been reported in other non-excitable tissues such as the liver (van Rossum, 1970), the kidney (see review by Suki, 1979) and the red blood cell (see review by Ferreira & Lew, 1977).

In van Kreel & van Dijk's study the $^{45}Ca^{2+}$ outflow concentrations rapidly approached the inflowing concentration and therefore hardly any tracer uptake was observed in control conditions. When cyanide was added to the perfusate there was an apparent increase in the $^{45}Ca^{2+}$ uptake, which was interpreted in terms of the inhibition of calcium pumps moving calcium out of the trophoblast. However, since they did not utilize an extracellular marker to correct for the presence of 'leak' pathways, their method could not separate influx from efflux or distinguish effects on active transport from passive diffusion.

Tissue retention of calcium

Most of the $^{45}Ca^{2+}$ which entered the placenta was rapidly returned to the circulation (Fig. 6) so that the accumulated 6 min tissue retention amounted to an average of only 9–12 % of the injected dose (Table 1). The presence of calcium or other

cations within the trophoblast (encased in membranes or vesicular structures) has been reported (Croley, 1973; Ockleford & Whyte, 1977). Analysis of $^{45}Ca^{2+}$ in subcellular particles has been performed in the guinea-pig placenta by van Kreel & van Dijk (1983), who observed that the radioactivity distribution was mainly in the nuclei (48%) and bound to cytoplasmic proteins (28%) with about 10% in mitochondria.

Transplacental calcium transfer and placental 'leakiness'

This additional information, which could be obtained with the paired-tracer experiments in the dually perfused placenta, showed that unlike sugars (Yudilevich *et al.* 1979) and amino acids (Eaton & Yudilevich, 1981), transplacental transfer of $^{45}Ca^{2+}$ in excess of the extracellular marker ('leakage') could not be observed in the 6 min collection period. In fact less $^{45}Ca^{2+}$ than $^{22}Na^{+}$ was recovered in the contralateral circulation and this was observed in either direction across the placenta. Since the transplacental transfer of $^{22}Na^{+}$ and the inert molecules D-[^{3}H]mannitol (or L-[^{14}C]glucose) measured simultaneously was also similar, it is concluded that the transplacental tracer data could not indicate any specific transport mechanism.

Generally, transplacental 'leakage' of inert molecules as well as of molecules whose transport into the trophoblast is carrier-mediated, is of interest. *In situ* perfusion of the guinea-pig placenta can cause an increase in its porosity or leakiness compared to the intact organ (Bradbury & Hedley, 1979) and it has been suggested (Faber & Hart, 1967; Hedley & Bradbury, 1980) that positively charged ions, such as Na^{+} (or K^{+}), may interact with negative charges on the leak pathway. In the perfused human placenta, Schneider, Mohlen, Challier & Dancis (1979) observed that transplacental transfer of D-glutamic acid was higher than the transfer of the natural isomer, and they concluded that D-glutamic acid defined the non-specific transplacental transfer and that L-glutamic was trapped (and metabolized) within the trophoblast. When, in a study similar to the present one, the transport of choline into and across the trophoblast was investigated (Sweiry & Yudilevich, 1984) we observed that transplacental transfer of this monovalent cation was equal to the 'leak' marker (unpublished observations).

In conclusion, the present investigation has demonstrated a specific carrier-mediated calcium influx on both sides of the trophoblast, although an asymmetry exists between the maternal and fetal sides. A further asymmetry was observed in the backflux of transported calcium, which was generally greater on the fetal than on the maternal side. These membrane mechanisms together with placental cytoplasmic factors may interact in the homoeostasis of fetal calcium. Our results emphasize the value of the paired-tracer technique and the possibility of expanding our studies to the regulatory mechanisms which may operate at the two poles of the trophoblast. The use of inhibitors and hormones such as vitamin D and its metabolites should permit further characterization of calcium transport. The present studies in the guinea-pig placenta could be applied to the perfused human placenta which is now being increasingly employed for transport studies.

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