Biochimica et Biophysica Acta, 339 (1974) 323–333

BBA 76572

CALCIUM BINDING TO PLACENTAL PLASMA MEMBRANES AS MEASURED BY RATE OF DIFFUSION IN A FLOW DIALYSIS SYSTEM

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(Received October 1st, 1973)

SUMMARY

The Ca^{2+}-binding properties of placental plasma membranes were studied using a flow dialysis system.

Ca^{2+}-binding was not detectable at pH 4.0, but increased at higher pH values to a maximum binding at pH 11.0.

Two types of Ca^{2+}-binding sites were identified: high-affinity sites with dissociation constant $K_s = 3.1 \cdot 10^{-5}$ M and a capacity of 26 nmoles per mg protein; low-affinity sites with $K_s = 1.1 \cdot 10^{-3}$ M and a capacity of 266 nmoles per mg protein.

The affinities of Mg^{2+} and Sr^{2+} for the high-affinity sites were 10-fold lower than that of Ca^{2+}, and for the low-affinity sites were 4- and 8-fold lower, respectively.

The placental plasma membranes contain sites for Ca^{2+} with capacity, specificity and affinity within the range reported for other membranes involved in an active transport of Ca^{2+} (mitochondria, sarcoplasmic reticulum, cardiac microsomes). The presence of high-affinity Ca^{2+} sites as well as Ca^{2+}-ATPase implicates these membranes in Ca^{2+} transport from the maternal to the fetal circulation.

INTRODUCTION

The regulation of intracellular Ca^{2+} concentration in a number of tissues has been shown to involve Ca^{2+}-ATPase (sarcoplasmic reticulum [1] and the red blood cell [2]). However, the mechanism by which Ca^{2+} is transported from one body compartment to another is still not clear. Such Ca^{2+} transport occurs across the placenta and is believed to be an active process [3, 4]. This concept is supported by the existence of a difference in ionized Ca^{2+} concentration between the maternal and fetal circulations [5]. The presence of a Ca^{2+}-ATPase in placental plasma membranes and its properties [5, 6] suggest a possible role for this enzyme in Ca^{2+} transport between body compartments.

The first step in Ca^{2+} transport is assumed to be a passive binding of Ca^{2+} to the membrane involved. In the present study we describe the Ca^{2+}-binding properties of placental plasma membranes. Instead of the more conventional techniques of ultrafiltration and equilibrium dialysis, we have used a more rapid method based on the measurement of the rate of diffusion in a flow dialysis system [7].

MATERIALS AND METHODS

Plasma membrane preparation

Guinea pig placental plasma membranes were isolated as described previously [5] and suspended in $5 \cdot 10^{-4}$ M imidazole-histidine, 5 mM Tris–HCl buffer (pH 8.0) and 100 mM NaCl.

Ca^{2+}-binding assay

The Ca^{2+}-binding properties were investigated in a flow dialysis system based on that described by Colowick and Womack [7]. The method is based on the principle that the rate of diffusion is proportional to the concentration of the free diffusible molecule; this rate will be constant when equilibrium is achieved with the macromolecule in the upper chamber of the dialysis cell. Equilibrium will be reached within seconds, and constant diffusion rate will occur within 1.5–2 min when the effluent volume pumped through the lower chamber is about five times its volume [7].

The dialysis cell was prepared as described by Colowick and Womack [7] using a standard cellophane dialysis membrane (Fisher Scientific Co.). The placental plasma membranes and $^{45}Ca^{2+}$ (New England Nuclear) were premixed, and the pH adjusted to 8.0 if necessary, before addition to the upper chamber. All experiments were conducted at room temperature (24 °C). Initial $^{45}Ca^{2+}$ concentration was $1.5 \cdot 10^{-5}$ M (approx. 0.04 Ci/l in final reaction mixture) unless indicated differently.

The dialysis buffer contained 20 mM Tris–HCl (pH 8.0) and 100 mM NaCl. The NaCl concentration was raised to 100 mM (as suggested by Reed [8]) in order to prevent an excessive non-specific binding to the dialysis membrane. The flow rate was maintained at 8 ml/min and the effluent was collected in 2-ml aliquots with a fraction collector. 1-ml samples were added to 10 ml scintillation fluid (Aquasol-New England Nuclear) and were counted in a Beckman model LS-233 liquid scintillation counter. Protein was determined by the procedure of Lowry et al. [9].

Calculation of Ca^{2+} binding

The difference between the control diffusion rate without plasma membranes and the diffusion rate observed in the presence of plasma membranes represents the fraction of bound Ca^{2+} in the medium. However, the control diffusion rate is not constant because dilution occurs with each addition to the upper chamber, and because of cpm loss during the experiment. For these reasons the initial control diffusion rate must be corrected for dilution and cpm loss for each step. The dilution effect can be duplicated in the control simply by adding the same volumes to the upper chamber without plasma membranes present. The cpm loss cannot be duplicated because differences in the diffusion rates in the presence and absence of plasma membranes produce different rates of loss from the upper chamber.

For each experiment, the loss of cpm for each step was calculated from the cpm found in the effluent collected in each step. These figures were then subtracted from the initial cpm in the upper chamber to give actual cpm concentration for each step. The total loss of cpm during experiments of 25–30 min, with a flow rate of 8 ml/min was up to 10%. The control diffusion rate for each step was calculated by multiplying the experimental initial control diffusion rate by the dilution factor and by the cpm loss factor.

RESULTS

Evaluation of the method

The suitability of the flow dialysis method for Ca^{2+}-binding studies is demonstrated in Fig. 1. The addition of $^{45}Ca^{2+}$ to the upper chamber in the absence of plasma membranes (upper curve) caused a linear increase in diffusion rate, suggesting that only a small fraction of $^{45}Ca^{2+}$ was bound. The lower curve in Fig. 1 represents the results obtained under the same conditions but in the presence of plasma membranes in the upper chamber. The difference in the diffusion rates in the absence and the presence of plasma membranes is due to $^{45}Ca^{2+}$ binding by the placental plasma membranes.

The addition of a large excess of $^{40}Ca^{2+}$ ($1 \cdot 10^{-2}$ M) in the absence of plasma membranes produced a sharp spike in the diffusion rate as a result of $^{45}Ca^{2+}$ displacement from the dialysis membrane by $^{40}Ca^{2+}$. However, the amount bound

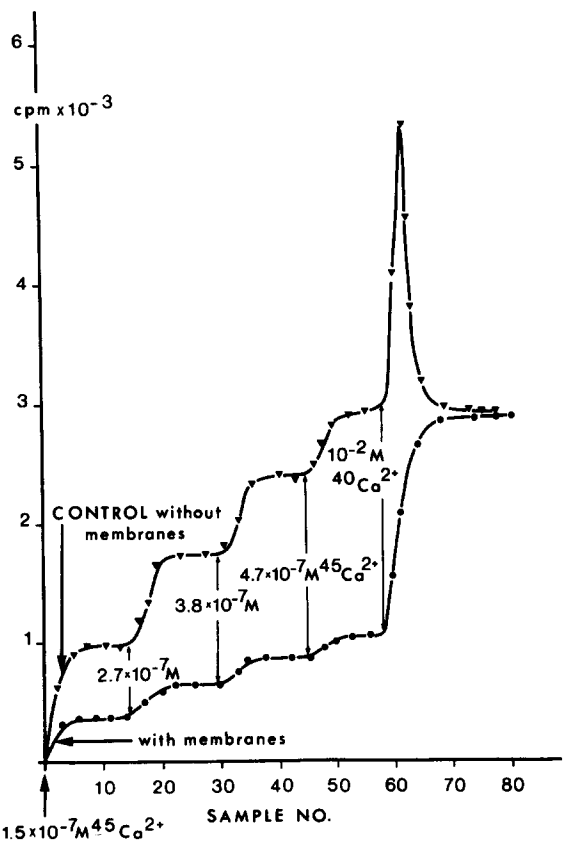

Fig. 1. Measurement of $^{45}Ca^{2+}$ diffusion rate at various $^{45}Ca^{2+}$ concentrations (▼–▼) without plasma membranes, (●–●) with plasma membranes. The medium and dialysis buffer contained 20 mM Tris–HCl (pH 8.0), 100 mM NaCl. Protein concentration was 2.52 mg/ml, the effluent was collected in 2-ml fractions of which 1 ml was counted for $^{45}Ca^{2+}$. Each step was allowed 15 samples before increasing $^{45}Ca^{2+}$ concentration. The results are expressed in cpm per ml effluent.

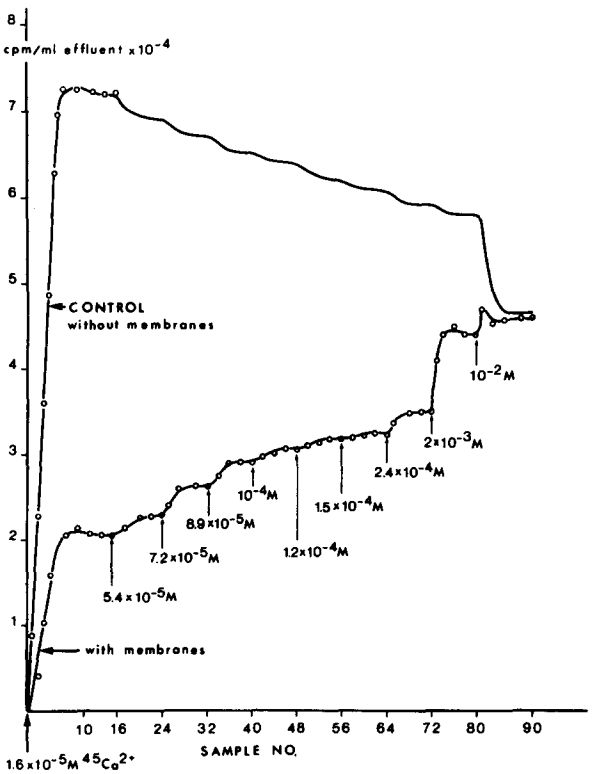

Fig. 2. Flow dialysis profiles of Ca^{2+} binding at various Ca^{2+} concentrations: The medium and dialysis buffer contained 20 mM Tris–HCl (pH 8.0), 100 mM NaCl. At time zero $1.6 \cdot 10^{-5}$ M $^{45}Ca^{2+}$ was added to the medium in the presence or absence of plasma membranes. The lower curve represents the diffusion rate profile of $^{45}Ca^{2+}$ in the presence of plasma membranes (protein concentration 2.4 mg/ml) at various $^{40}Ca^{2+}$ concentrations, as indicated under the arrows. The upper curve is a control curve in the absence of plasma membranes. Up to sample No. 16 the control curve represents experimental values. The remainder is a corrected control curve compensated for dilution and cpm loss for each step. The flow rate was 8 ml/min, each step was allowed 8 samples and results are expressed as cpm per ml effluent.

was negligible (0.1% of the total $^{45}Ca^{2+}$ present) and after a few samples the diffusion rate returned to the same level as before the addition of $^{40}Ca^{2+}$. In the presence of plasma membranes, excess $^{40}Ca^{2+}$ ($1 \cdot 10^{-2}$ M) increased the diffusion rate to the level of the control, indicating complete displacement of $^{45}Ca^{2+}$ from the plasma membranes. The absence of a spike in the presence of plasma membranes is due to a reduced binding of $^{45}Ca^{2+}$ to the dialysis membrane because of the higher affinity of the plasma membranes for the available $^{45}Ca^{2+}$.

A typical $^{45}Ca^{2+}$ diffusion rate profile is presented in Fig. 2. The control diffusion rate after the first step (samples Nos 1–15) was corrected for dilution and loss of $^{45}Ca^{2+}$, as described in Materials and Methods. For all experiments, the data were first plotted in this manner, to allow calculation of the extent of Ca^{2+} binding for each step. The Ca^{2+} binding was then replotted against the different parameters used in subsequent experiments.

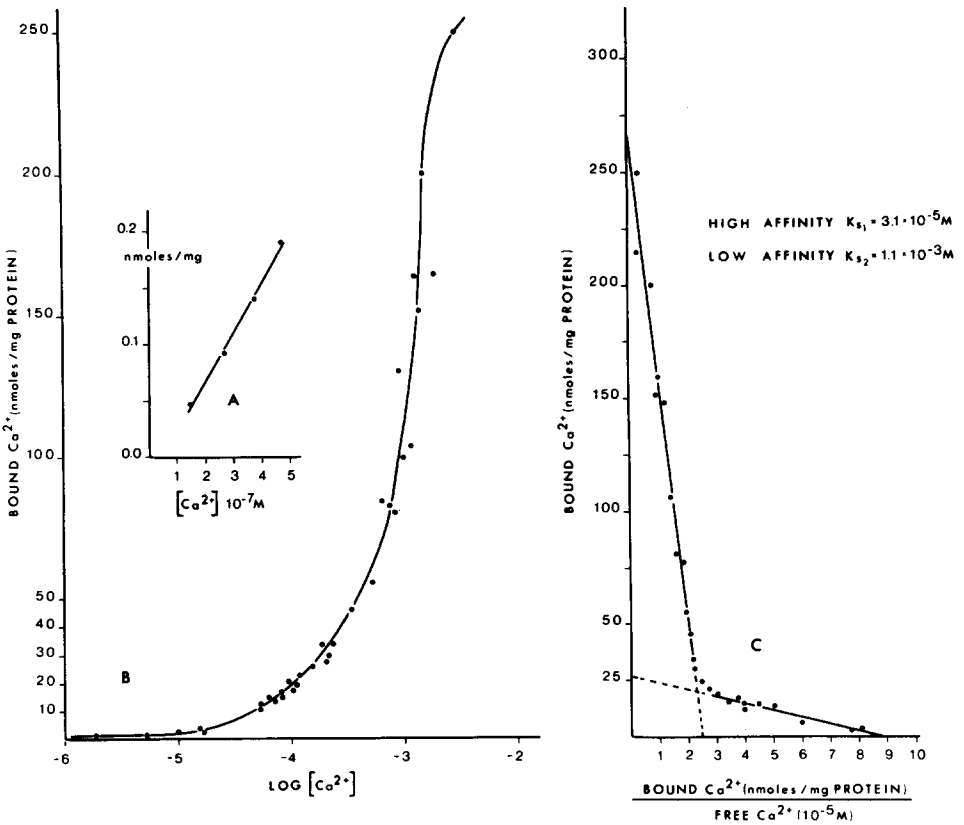

Fig. 3. Ca^{2+} concentration effect on Ca^{2+}-binding levels by placental plasma membranes. (A) Ca^{2+} binding at low Ca^{2+} concentration ($1 \cdot 10^{-7}$–$5 \cdot 10^{-7}$ M). This curve was derived from Fig. 1. The results are expressed in nmoles Ca^{2+} bound per mg protein. (B) Ca^{2+} binding at higher Ca^{2+} concentrations ($1 \cdot 10^{-6}$–$1 \cdot 10^{-2}$ M). This curve was derived from four different experiments carried out under the same conditions as described in Fig. 2. The Ca^{2+} concentration was varied slightly from one experiment to another to cover the wide range. The bound calcium is expressed in nmoles Ca^{2+} per mg protein. (C) Scatchard plot of Ca^{2+} binding derived from the steady-state values which were used to construct (B).

Ca^{2+} binding by placental plasma membranes

At low Ca^{2+} concentrations ($1 \cdot 10^{-7}$–$5 \cdot 10^{-7}$ M) Ca^{2+} binding per mg protein was linear (Fig. 3A: data derived from Fig. 1), indicating a constant percentage bound in this concentration range. In a double reciprocal plot (not shown) the line passed through the origin, suggesting non-saturable kinetics.

At higher Ca^{2+} concentration ($1 \cdot 10^{-6}$–$1 \cdot 10^{-2}$ M) the amount of Ca^{2+} bound per mg protein increased sigmoidally (Fig. 3B). A Scatchard plot of Ca^{2+} binding revealed two types of sites: high-affinity sites accommodating 26 ± 2 (mean $\pm$ S.E.) nmoles per mg protein and dissociation constant $3.1 \pm 0.4 \cdot 10^{-5}$ M; and low-affinity sites accommodating 266 ± 27 nmoles per mg protein and dissociation constant $1.1 \pm 0.1 \cdot 10^{-3}$ M (Fig. 3c).

Time, temperature, protein concentration and pH effects

The maximal diffusion rate was achieved after 1.5 min, and when no further additions or treatments were performed, the diffusion rate was constant (after compensating for cpm loss) for at least 30 min. Thus Ca^{2+} binding by placental plasma membranes reached a maximum within 1.5 min, and thereafter was independent of incubation time.

Reducing the temperature from 24 to 4 °C did not change significantly the extent of Ca^{2+} binding.

The binding of Ca^{2+} increased linearly with protein concentration over the range 0.25–2.7 mg protein/ml when [Ca^{2+}] was $1.5 \cdot 10^{-5}$ M (Fig. 4).

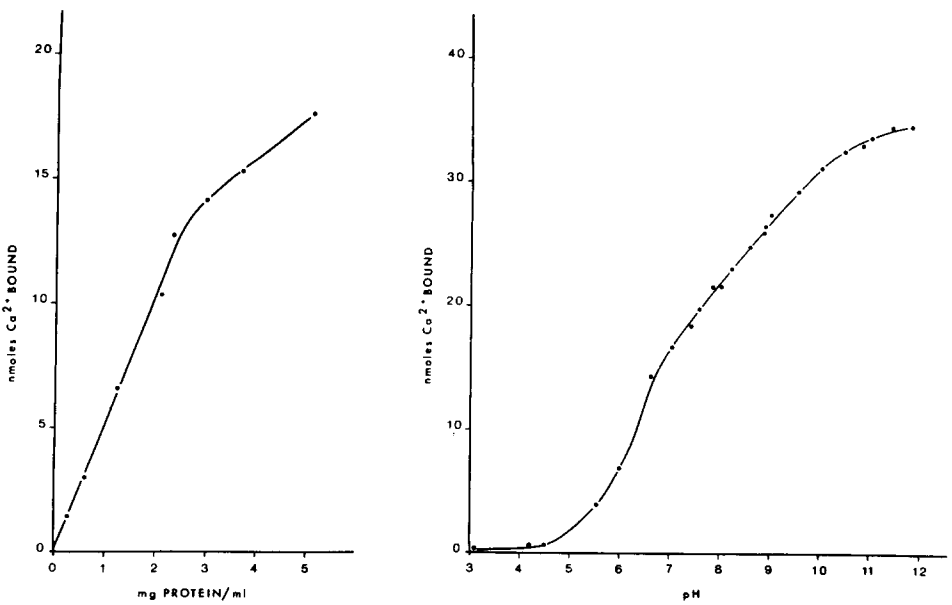

Fig. 4. Ca^{2+} binding as a function of placental plasma membrane protein concentration. The medium contained 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, and $1.5 \cdot 10^{-5}$ M $^{45}Ca^{2+}$. The plasma membranes were added to the upper chamber to give the indicated concentration. The results are expressed as total nmoles Ca^{2+} bound.

Fig. 5. pH effect on Ca^{2+} binding. The pH in the upper chamber was measured with a pH electrode, and controlled by addition of HCl or NaOH (0.1 M). The plasma membranes were exposed to each pH for not more than 2.5 min. The medium contained 20 mM Tris–HCl, 100 mM NaCl, $2.2 \cdot 10^{-5}$ M $^{45}Ca^{2+}$ and protein concentration was 2.5 mg/ml. The results are expressed as total nmoles Ca^{2+} bound.

Ca^{2+} binding was dependent on pH, practically no binding could be detected at pH 4.0. The binding increased with increasing pH up to pH 11.0, and leveled off (Fig. 5). Since the membranes were subjected to each pH for no more than 2.5 min, denaturation effects of extreme pH values were minimized; the pH effect was reversible over the entire pH range (3.0–11.8).

Ca^{2+} displacement from its sites by Mg^{2+} and Sr^{2+}

Fig. 6A shows the $^{45}Ca^{2+}$ displacement from its sites by $^{40}Ca^{2+}$, Mg^{2+}, and

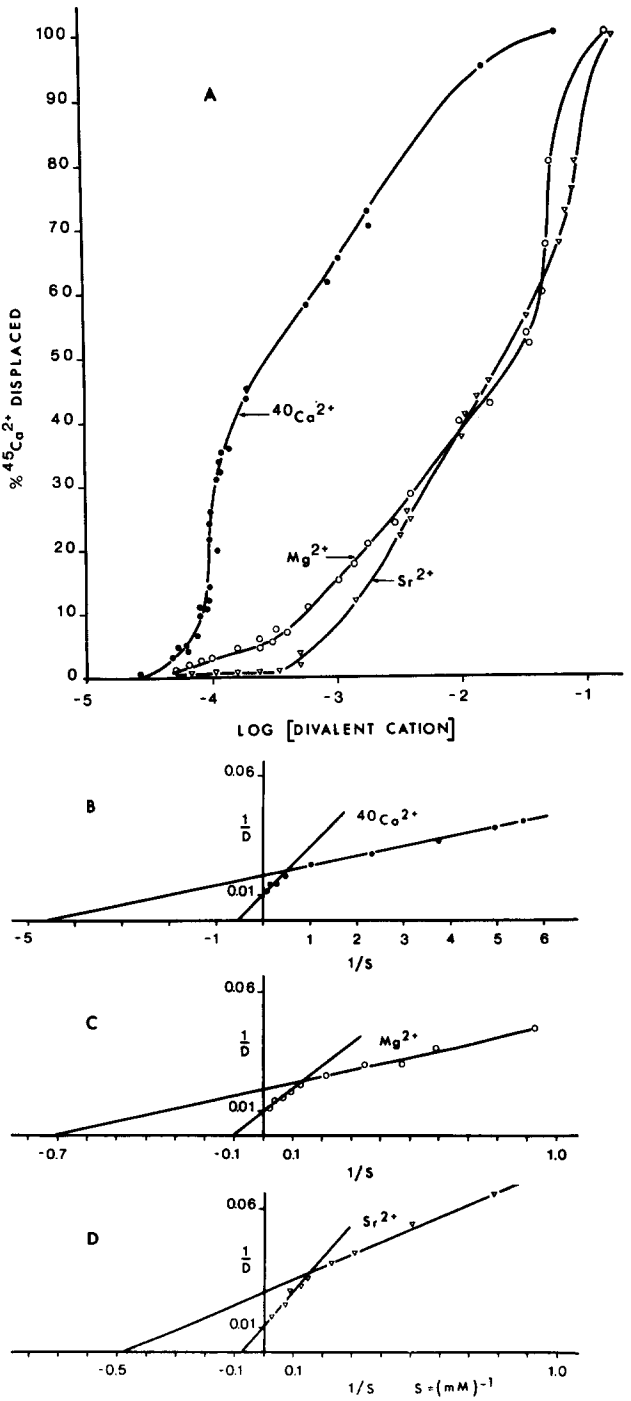

Fig. 6. $^{45}Ca^{2+}$ displacement by divalent cations. (A) The experiments were carried out as described for Fig. 2. $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+} were added in increasing concentrations to the upper chamber as indicated. The results are expressed as a percentage of $^{45}Ca^{2+}$ displaced from the plasma membranes. Zero displacement is defined as the amount of $^{45}Ca^{2+}$ bound at the end of the first step. (B), (C) and (D) Double reciprocal plots for $^{45}Ca^{2+}$ displacement by $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+}, respectively. D is expressed as percent $^{45}Ca^{2+}$ displaced and [S] as millimolar divalent cation. These plots were derived from data used in (A).

Sr^{2+}. The percent $^{45}Ca^{2+}$ bound at the end of the first step was regarded as zero percent displaced. The ability of Mg^{2+} and Sr^{2} to displace $^{45}Ca^{2+}$ from its sites was much lower than that of $^{40}Ca^{2+}$ (Fig. 6A).

The double reciprocal plots for displacement of $^{45}Ca^{2+}$ from the plasma membranes by $^{40}Ca^{2+}$, Mg^{2+}, and Sr^{2+} (Figs 6B, 6C and 6D) revealed two apparent K_m values for each ion. These values appear in Table I. The affinity of Mg^{2+} and Sr^{2+} for the high-affinity Ca^{2+} sites was approx. 10-fold lower than that of $^{40}Ca^{2+}$. The affinity of Mg^{2+} for the high-affinity Ca^{2+} sites was higher than that of Sr^{2+}. The wide gap of affinities was narrower for the low affinity sites (4-fold lower for Mg^{2+} and 8-fold lower for Sr^{2+}). These results indicate high specificity of these sites for Ca^{2+}, with greater specificity in the high-affinity sites.

TABLE I

APPARENT K_m VALUES FOR $^{45}Ca^{2+}$ DISPLACEMENT BY $^{40}Ca^{2+}$, Mg^{2+}, Sr^{2+}

The K_m values were calculated from the double reciprocal plots presented in Figs 6 B–6 D.

Ion	$K_{m,1}$ (mean±S.E.)	$K_{m,2}$ (mean±S.E.)
$^{40}Ca^{2+}$	$2.2\pm0.3 \cdot 10^{-4}$ M	$2\pm0.2 \cdot 10^{-3}$ M
Mg^{2+}	$1.4\pm0.15 \cdot 10^{-3}$ M	$9\pm1 \cdot 10^{-3}$ M
Sr^{2+}	$2.2\pm0.25 \cdot 10^{-3}$ M	$1.5\pm0.1 \cdot 10^{-2}$ M

DISCUSSION

The flow dialysis method was found in the present study to be a very useful tool for studying Ca^{2+} binding by placental plasma membranes. The process of binding has met the requirements for the use of this method as defined by Colowick and Womack [7]: (1) chemical equilibrium was achieved within a few seconds; (2) a constant diffusion rate was established after 1.5 min as predicted; (3) the dissociation constants of this binding reaction were within the effective range of the method ($1 \cdot 10^{-3}$–$1 \cdot 10^{-6}$ M). The use of $^{45}Ca^{2+}$ as the diffusible molecule, which has a relatively high diffusion rate through the dialysis membrane, made this system sensitive to changes in free $^{45}Ca^{2+}$ in the upper chamber.

The flow dialysis system offers several advantages for Ca^{2+}-binding measurements over equilibrium dialysis and ultrafiltration. It is faster, since each step can be completed within 2 min. Washing the membrane, which is necessary in the ultrafiltration method, is avoided here; this eliminates a possible displacement of $^{45}Ca^{2+}$, particularly from the low-affinity sites. The continuous monitoring of the $^{45}Ca^{2+}$ diffusion rate gives a clearer picture of the kinetic behaviour of the investigated reaction. In studying pH effects, the membranes are exposed to each pH for not more than 2.5 min. Thus denaturation effects by extreme pH values can be minimized. Using the other methods, the exposure to each pH is much longer and denaturation effects cannot be excluded. However, in calculating Ca^{2+} binding using the flow dialysis method two effects must be taken into account: the dilution effect and cpm loss during the experiments. These two factors should be calculated for each step to give the actual control diffusion rate for each step. An excessive non-specific Ca^{2+}

binding to the dialysis membrane was successfully prevented by raising NaCl concentration up to 100 mM, as suggested by Reed [8]. In contrast to the ultrafiltration method the flow dialysis method is not sensitive enough to measure Ca^{2+} uptake. Because uptake is a relatively slow process, the effect on the diffusion rate will be small, and difficult to detect. Another limitation of this method is that it requires high protein concentrations (2–3 mg/ml).

Two types of Ca^{2+}-binding sites can be distinguished on the placental plasma membranes: high-affinity sites with a capacity of 26 nmoles per mg protein and $K_s = 3.1 \cdot 10^{-5}$ M, and low affinity sites with a capacity of 266 nmoles per mg protein and $K_s = 1.1 \cdot 10^{-3}$ M. Membranes from several sources show more than one class of Ca^{2+}-binding sites. The sarcoplasmic reticulum has been most intensively studied with respect to its Ca^{2+}-binding properties, but even with this one type of membrane, different methods have yielded conflicting results. Cohen and Selinger [10] reported two classes of sites with the same dissociation constant ($K_s = 4 \cdot 10^{-5}$ M), while a more recent study by Chevallier and Butow [11] revealed three classes of sites with the following dissociation constants: $1.3 \cdot 10^{-6}$, $3.2 \cdot 10^{-5}$, $3.2 \cdot 10^{-4}$ M.

Despite the difficulties one faces trying to compare Ca^{2+}-binding properties of preparations obtained by the various methods the existence of high-affinity sites is typical of membranes that are involved in the active regulation of intracellular Ca^{2+}. In addition to the sarcoplasmic reticulum, high-affinity sites for Ca^{2+} are found in the mitochondria [12] and in cardiac microsomes [13]. Lower affinity sites are found in rat liver plasma membranes [14] ($K_s = 2.5 \cdot 10^{-4}$ and $3.1 \cdot 10^{-3}$ M), and in red blood cell membranes [15] ($K_s = 2.8 \cdot 10^{-4}$ M). The placental plasma membranes, in respect to Ca^{2+}-binding affinity ($K_s = 3.1 \cdot 10^{-5}$ M), rank among the sarcoplasmic reticulum, mitochondria and cardiac microsomes. It is interesting that liver plasma membranes [14] have sites with 10-fold higher dissociation constant than the placental plasma membranes. It is tempting to attribute this difference in affinities for Ca^{2+} to the fact that the liver plasma membranes, unlike the placental plasma membranes, are not involved in active Ca^{2+} transport between body compartments.

The pH-dependent Ca^{2+}-binding profile by the placental plasma membranes shows a pH optimum of 11.0, and at pH 4.0 there is practically no binding. Up to pH 7.0 the pH profile agrees with that already described for liver plasma membranes [14], sarcoplasmic reticulum [10] and muscle microsomes [16]. Above pH 7–8 a decrease was reported [14, 10]. In the present study no such decrease was observed even above pH 11.0. These differences can be at least partly accounted for by the different methods used. The time of exposure to each pH varied from 40 min (10 min incubation and 30 min centrifugation) [10] up to 42 h [14] using the equilibrium dialysis method. The use of flow dialysis reduced the exposure time to 2.5 min, minimizing long term pH effects. In addition no attempt to reverse the pH effect was mentioned in these studies [10, 14] so that long term effects cannot be excluded.

The affinity of Mg^{2+} and Sr^{2+} for the Ca^{2+} high-affinity sites was found to be 10-fold lower than that of Ca^{2+}. The binding of Mg^{2+} and Sr^{2+} was not studied, so that the possibility of specific sites for these ions cannot be excluded. The effect of Na^+ on Ca^{2+} binding could not be studied in detail since it was necessary to maintain a high Na^+ concentration to prevent non-specific Ca^{2+} binding to the dialysis membrane. However, in several short experiments when NaCl (100 mM) was added

after the first step (before measurable amounts of $^{45}Ca^{2+}$ were bound to the dialysis membrane, which is time dependent [8]) no significant changes in $^{45}Ca^{2+}$ diffusion rate could be detected. The more efficient displacement of $^{45}Ca^{2+}$ by Mg^{2+} and Sr^{2+} from the low-affinity sites indicates a reduced specificity of Ca^{2+}.

The specificity of Ca^{2+}-binding sites of different membranes shows wide variations. These sites on the liver mitochondria [12] are insensitive to Mg^{2+}, but Sr^{2+} displaces Ca^{2+} quite effectively. The sarcoplasmic reticulum [10] sites are unaffected by Mg^{2+} up to 10^{-2} M, while Sr^{2+} displaces at a level close to that found in this study. The Ca^{2+}-binding sites of another plasma membrane (derived from liver [14]) are insensitive to K^+ and Na^+ while Mg^{2+} reduced Ca^{2+} binding to the low-affinity sites only. The degree of specificity observed in the present study enables us to conclude that at physiological concentrations of Mg^{2+}, Ca^{2+} and normal levels of Sr^{2+}, practically no binding other than Ca^{2+} to these sites will be detected.

The concept behind this study is that a passive binding of Ca^{2+} to the membrane is an essential first step in the process of an active Ca^{2+} transport. The placental plasma membranes were found to contain sites for Ca^{2+} with capacity, specificity and affinity within the range reported for other membranes involved in an active transport of Ca^{2+}. Unlike the sarcoplasmic reticulum and mitochondria, which are exposed to only two different Ca^{2+} concentrations, the placental plasma membranes are exposed to three different Ca^{2+} concentrations. The outer-plasma membranes on the maternal side are exposed to a Ca^{2+} concentration of 2.2 mequiv/l, and on the fetal side to 3.1 mequiv/l [5]. The inner surface of the membranes is exposed to a low Ca^{2+} concentration, estimated to be around 10^{-5}–10^{-6} M. It is premature to assign the distribution of the two classes of sites to the different surfaces of the placental plasma membranes. However, it should be noticed that the dissociation constants obtained are close to the physiological concentrations of Ca^{2+} to which the membranes are exposed. Although both high-affinity Ca^{2+} sites and Ca^{2+}-ATPase are present in the placental plasma membranes, a direct involvement of these membranes in the active transport of Ca^{2+} from the maternal to the fetal circulation remains speculative in this stage. The role of ATP and Ca^{2+}-ATPase in Ca^{2+} binding and uptake is the subject of work in progress.

ACKNOWLEDGEMENTS

The authors thank Dr V. Palaty, Department of Anatomy, University of British Columbia, for his helpful comments on the manuscript. This study was supported by a grant from the Medical Research Council of Canada.

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