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Quantitative Competition of Calcium with Sodium or Magnesium for Sorption Sites on Plasma Membrane Vesicles of Melon (*Cucumis melo* L.) Root Cells

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Abstract. The presence of Ca^{2+} ions in solution is vital for root growth. The plasma membrane is one of the first sites where competition between Ca^{2+} and other ions occurs. We studied the competition between Ca^{2+} and Na^+ or Mg^{2+} for sorption sites on the plasma membrane of melon root cells.

Sorption of $^{45}Ca^{2+}$ to right-side-out PM vesicles of melon (*Cucumis melo* L.) roots (prepared by aqueous two-phase partitioning) was studied at various Ca^{2+} concentrations, in the presence of increasing concentrations of Na^+ or Mg^{2+} chlorides. Experimentally determined amounts of Ca^{2+} sorbed to the plasma membrane vesicles agreed fairly well with those calculated from a competitive sorption model. The best fit of the model to the experimental data was obtained for an average surface area of 370 $Å^2$ per charge, and binding coefficients for Na^+, Mg^{2+} and Ca^{2+} of 0.8, 9 and 50 M^{-1}, respectively.

Our results suggest that nonphospholipid components in the plasma membrane contribute significantly to Ca^{2+} binding. The high affinity of Ca^{2+} binding to the plasma membrane found in this study might explain the specific role of Ca^{2+} in relieving salt stress in plant roots.

Key words: Ca^{2+} binding — Plasma membrane — Root cells — Surface charge density

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Introduction

The presence of Ca^{2+} in the external solution is of prime importance for proper root elongation under saline conditions (LaHaye & Epstein, 1969). Calcium plays a structural role in cell membranes to prevent damage and leakage (Clarkson & Hanson, 1980). Leakage of K^+ from cells in high-salt solutions is a well-established phenomenon (LaHaye & Epstein, 1969; Cramer, Lauchli & Polito, 1985). The presence of Ca^{2+} has been shown to prevent K^+ leakage from cultured citrus cells under saline conditions (Ben Hayyim, Kafkafi & Ganmore-Newman, 1987). In that study, the initial change in membrane permeability was attributed to displacement of membrane-associated Ca^{2+} by Na^+. Direct evidence for Ca^{2+} displacement by Na^+ has been reported by Cramer et al. (1985) in intact cotton-root hairs, and by Lynch and Lauchli (1987) in maize root protoplasts. However, both studies used a microfluorometric technique which enabled only qualitative determinations. Lynch and Lauchli (1988) suggested that Na^+ displaces Ca^{2+} associated with intracellular membranes via activation of the phosphoinositide system and depletion of intracellular Ca^{2+} pools. Zidan et al. (1991) measured sodium influx into maize root segments, and concluded that Na^+ does not compete with Ca^{2+} in saturating PM sites.

Most biomembranes are negatively charged, mainly due to the presence of acidic phospholipids (Moller, Lundborg & Berczi, 1984; Gennis, 1989). Negative charges are also contributed by other membrane components, such as gangliosides and proteins (Gennis, 1989). Studies with phospholipid membranes have provided mutually consistent results for cation interactions with negatively charged surfaces using several different

experimental procedures, such as the measurement of radioisotopes (e.g., $^{45}Ca^{2+}$) and atomic absorption of sorbed cations (Newton et al., 1978; Nir, Newton & Papahadjopoulos, 1978), infrared absorption (Dluhy et al., 1983), nuclear magnetic resonance (Kurland et al., 1979; McLaughlin, 1982; Seelig, MacDonald & Scherer, 1987; Roux & Bloom, 1990), electrokinetic measurements (Eisenberg et al., 1979; McLaughlin et al., 1981; Bentz et al., 1988) and surface potential measurements (Ohki & Kurland, 1981). Numerous studies have established that a combination of specific binding and the Gouy-Chapman equations can explain cation sorption to phospholipid membranes consisting of a single component (Nir et al., 1978; Eisenberg et al., 1979; Cohen & Cohen, 1981), as well as mixed ones (Duzgunes et al., 1981; Nir, 1984). Critical evaluation of attempts at consideration of discrete charges led to the conclusion that models based on smeared charge are adequate (Winiski et al., 1986). However, the analysis of specific binding of cations to more complex biological membranes has not been reported so far.

The present study was undertaken to quantify the effect of Na^{+} and Mg^{2+} on the sorption of Ca^{2+} to the PM of melon root cells. We constructed PM vesicles and performed experiments involving $^{45}Ca^{2+}$ association with these vesicles under conditions where most of the association was due to Ca^{2+} sorption to the vesicle membranes. The results were analyzed using a competitive sorption model that was previously used in studies with phospholipid vesicles (Nir, 1984) and adapted here for studies on the more complex biological membranes.

We determined the intrinsic and the actual surface area per charge on the membrane, and binding coefficients of Ca^{2+}, Na^{+} and Mg^{2+} which are independent of cation concentrations. We demonstrate that it is possible to predict the sorbed amounts of these cations for any combination of their concentrations.

Materials and Methods

Plant Material

Melon (*Cucumis melo* L.) seeds (cv. Eshkolit Ha'Amaqim muskmelon, kindly provided by Dr. H. Nerson, Neve Ya'ar Experimental Station, Israel) were soaked in 5% NaOCl for 5 min. After thoroughly rinsing in tap water, the seeds were placed on a glass plate between two germination papers saturated with tap water. The glass plates were placed in a slanted position in an incubator set at 27 ± 1°C, 100% relative humidity. The germination papers were kept wet throughout the experiment. After six days, the primary roots were 10 to 15 cm long. Roots were cut for PM isolation.

Isolation of PM Vesicles

Right-side-out PM vesicles were isolated according to Larsson, Widell and Sommarin (1988) with slight modifications: The PIPES suspension buffer used was titrated by 2 M Tris to pH 7.2. The PM vesicles were stored at 4°C until use in the sorption studies. ATPase activity was measured as described by Ben Hayyim and Ran (1990). Addition of Triton X-100 to the membrane preparation increased ATPase activity seven- to eightfold, indicating the presence of a high proportion of right-side-out vesicles in our membrane preparations (Larsson et al., 1988). The amount of Ca^{2+} sorbed to PM vesicles immediately after vesicle isolation was almost the same as that found after 15 hr (Table 1). All other sorption experiments were performed at 15 ± 1 hr after vesicle isolation.

Sorption Studies

Unless otherwise specified, 50 µl of $^{45}Ca^{2+}$ solution (containing 0.15 µCi) were added to a 50 µl solution containing 0.5 M sucrose, Ca^{2+} and Na^{+} or Mg^{2+} chlorides at the indicated concentrations. The sorption reaction was started with the addition of 50 µl PM vesicles containing 25 ± 2 µg protein. The final volume of the reaction solution was 150 µl, containing 0.25 M sucrose. The pH of the solution was 7.2 buffered by 5 mM PIPES titrated by 2 M Tris. All sorption studies were performed at 24 ± 2°C. Measurements were made in triplicate, and every experiment was repeated at least three times.

Sorption experiments at incubations of up to 2 hr indicated the sorption reactions to be nearly complete after 10 min (Table 1). All sorption reactions were therefore run for 30 min, after which the suspensions were transferred to Microfilterfuge™ tubes (0.2 µm; Rainin), and centrifuged for 15 min at 8,000 × *g* in a Hettich centrifuge. Radioactivity was determined in the upper part of the tube, containing the filter and PM vesicles, and in the lower part, containing the equilibrium solution. A determination of filtrate protein content showed that 97% of the PM vesicles had remained on the filter after centrifugation. Radioactivity was determined by liquid scintillation spectroscopy in 4 ml of scintillation fluid (Scintillator 299™, Packard).

To account for the equilibrium solution remaining with the vesicles on the filter, we separated and weighed the two parts of the microfilterfuge tube before and after centrifugation, each in their own scintillation bottle, to an accuracy of 0.1 mg. This information was used to correct for the radioactivity remaining in the filter as free solution by subtracting it from the total counts. The $^{45}Ca^{2+}$ as free solution remaining with the vesicles was in the range of 2–10% of the total counts, depending on the treatment. The amount of Ca^{2+} sorbed onto the microfilterfuge tube which was determined without the vesicles, did not exceed 2% of total $^{45}Ca^{2+}$ and was also taken into account.

Desorption Studies

The sorbed $^{45}Ca^{2+}$ was released from the PM vesicles after the sorption reaction by exchange with $LaCl_3$. A solution containing 10 mM $LaCl_3$ and 0.25 M sucrose at pH 7.2 was added at a volume of 0.1 ml to the upper part of the tube (containing the vesicles), which was attached to a new, empty, lower part. The tube was centrifuged for 15 min. This procedure was repeated three times for each sample. The $^{45}Ca^{2+}$ released from the PM was counted for every leaching cycle, as well as that remaining in the upper part after the final leaching cycle.

Protein Assay

Protein content was determined by the method of Bradford (1979) using BSA as a protein standard.

Table 1. Effect of various treatments on the amount of Ca^{2+} sorbed to PM vesicles (mean ± SD)

Treatment[a]	Ca^{2+} sorbed (μmol/g protein)
Control	1,097 ± 95
Reaction time 10 min	967 ± 80
Reaction time 2 hr	1,056 ± 99
Immediately after vesicle isolation	1,157 ± 105
Addition of A23187 before sorption started	993 ± 112
Addition of A23187 after sorption started	1,051 ± 106
Freeze/thaw	995 ± 93
At 4°C	1,045 ± 94
Addition of MgATP 3.75 mM at 0.01 mM Ca^{2+}	4 ± 2
Addition of MgATP 3.75 mM at 1.0 mM Ca^{2+}	554 ± 46
Addition of $LaCl_3$ 0.67 mM at 0.1 mM Ca^{2+}	20 ± 3

[a] Experiments were carried out at a total Ca^{2+} concentration of 1.0 mM, at a temperature of 24°C, 15 hr after vesicle isolation, reaction time 30 min, unless otherwise indicated (control). The final concentration of the ionophore A23187 was 5 μM. Six freeze-thaw cycles were used by freezing the vesicles in liquid nitrogen and thawing at 20°C. All other details are described in Materials and Methods.

Sorption Model

The model used is essentially similar to that of Gouy-Chapman-Stern, but it accounts explicitly for the fact that when cations, e.g., Ca^{2+}, sorb, their concentrations in solution are reduced. In extreme cases, the resulting solution concentrations may be orders of magnitude below their total concentration in the system. The full set of equations of the model is given in Nir (1984). The Gouy-Chapman equation provides a solution for the electrical potential, ψ and the surface charge density, σ, in terms of the quantities S_i, the solution concentrations of ions far away from the surface (McLaughlin, 1977). Due to cation binding to the negatively charged surface, e.g., the PM, partial charge neutralization occurs. Consequently, σ is smaller in absolute magnitude than σ_0, which denotes the charge per area of unneutralized sites on the surface. The amount of sorbed cations consists of two parts, cations residing in enhanced concentrations in the double layer region and cations which are chemically bound to the surface. The tightness of the chemical binding is expressed by the magnitude of the intrinsic binding constants, K_i. The binding of cations to singly charged negative sites P^- on the surface, is given by

$$P^- + X_i^+ = PX_i \tag{1}$$

i.e.,

$$K_i = \frac{[PX_i]}{[P^-][X_i^+]} \tag{2}$$

In Eq. (2), the concentrations are given in mol/liter, or M. The unit of K_i is M^{-1}. At the surface, the concentration of the cation X_i is $X_i(0)$, and the electrical potential is ψ_0.

$$X_i(0) = S_i \exp - e\psi_0 Z_i/KT = S_i y_0^{Zi} \tag{3}$$

in which e is the absolute magnitude of an electron charge, Z_i is the valence of an ion, and $y_0 = \exp -e\psi_0/KT$. Equations (1) to (3) give

$$[PX_i] = K_i[P^-]S_i y_0^{Zi} \tag{4}$$

Divalent cations can form a 1–1 complex which will be denoted $[PX_j^+]$ since it is positively charged and a 2–1 complex which is neutral and will be denoted $[PX_j]$. The corresponding binding constants will be denoted K_{j1} and K_{j2}, respectively. The ratio between the actual surface charge density, σ, and the intrinsic value, σ_0, is given in Eq. (5). In this equation which is based on Eq. (4) both 1–1 and 2–1 complexes are considered and $K_j = K_{j1} + K_{j2}$.

$$\frac{\sigma}{\sigma_0} = \frac{1 - y_0^2 \sum K_{j1} S_j^{2+}}{1 + y_0 \sum K_i S_i^+ + y_0^2 \sum K_j S_j^{2+}} \tag{5}$$

If the quantities S_i, S_j, K_i and K_j are known then the combination of the Gouy-Chapman equation with Eq. (5) can yield the unknowns σ and y_0. However, in most cases the independent quantities are the total concentrations C_i, C_j, whereas the quantities S_i, S_j are modified due to sorption.

Computational Procedure

An iterative procedure is used for the determination of y_0, the amount of surface sites bound by each cation, and the solution concentrations S_i, S_j. It is assumed that the quantities C_i, C_j are known. It should be noted that if divalent cations, such as Ca^{2+} or Mg^{2+} form 1–1 complexes $[PX_j^+]$, then according to Eq. (5) charge reversal can occur. However, results with mesophyll protoplasts (Obi et al., 1989) demonstrate that charge reversal does not occur with Ca^{2+}. Hence, we considered only 2–1 complexes. The total concentration of a cation, C_i consists of three terms, (i) solution concentration, S_i, (ii) concentration of bound cations, PX_i, and (iii) cations residing in the double layer region, in excess of solution concentration.

We define

$$S^{(2)} = \sum_{\text{divalent}} S_j \tag{6}$$

and a similar expression exists for monovalent cations. Hence,

$$C_j = S_j + [PX_j]/2 + Q_2 S_j/S^{(2)} \tag{7}$$

Where Q_2 is calculated analytically (Nir et al., 1978) and represents the excess concentration of divalent cations in the double layer region. Hence,

$$S_j^{2+} = \frac{C_j^{2+}}{1 + [P^-]y_0^2(K_{j1} + K_{j2}/2) + Q_2/S^{(2)}} \tag{8}$$

A similar relationship holds for the monovalent cations. S_i, S_j, y_0, and σ can be solved by an iterative procedure (Nir, 1984) based on the knowledge of the quantities C_i, C_j, the total concentration of surface sites, PT, and σ_0. In this procedure, the user assumes values for the binding constants K_i, K_j, which are determined from the best fit between the experimental and calculated amounts of sorbed cations.

Determination of Binding Coefficients

The procedure of determination of binding coefficients has been well established in previous studies with phospholipid membranes (Nir et al., 1978; Kurland et al., 1979; Ohki & Kurland, 1981). In those cases, the concentrations of cations in solution were experimentally known. Applications to systems where the concentrations of cations

in solutions are not a priori known, are given in Nir (1984), Nir et al. (1986) Hirsch, Nir and Banin (1989) and Baruch et al. (1991). The situation existing in sorption studies on biological membranes is more complex. Unlike studies on phospholipid membranes or clays, the concentration of surface sites and the area per charged site are unknown for most biological membranes. While the program can handle calculations for surfaces consisting of several sites with different affinities for the cations under consideration, and different areas per charged species, we preferred to approximate the surface charge using an effective concentration of charged sites and an effective area per site, thereby avoiding the use of additional parameters. The effective concentration of charged surface sites (assuming a single charge per site) was found from the case in which no Na^+ had been added and Ca^{2+} concentration was 1 mM. In this case, regardless of the parameter values, the surface is assumed to be saturated and neutralized by Ca^{2+} (Nir et al., 1978; Nir, 1984). No charge reversal is assumed to occur under these conditions (Obi et al., 1989). This means that the experimental ratio of Ca^{2+} (sorbed) per site should equal 1/2.

We first studied the sorption of Ca^{2+} in a binary system Ca^{2+} + Na^+. In estimating the surface area per site, we considered a range of values from 50 to 600 Å^2. For each chosen value of the surface area, we determined the parameters K_{Ca} and K_{Na} that gave the best agreement between the calculated and experimental values of sorbed Ca^{2+}, as described in previous studies (Nir, 1984; Nir et al., 1986; Baruch et al., 1991). We then chose the value of surface area per site that gave the best agreement between the experimental and calculated values of the amounts of Ca^{2+} sorbed. We utilized 20 experimental values that included 20- and 100-fold variation in the total concentrations of Ca^{2+} and Na^+, respectively. In studies with the Ca^{2+} + Mg^{2+} system, we already utilized the previously determined values of K_{Ca} and σ_0. Thus, we had to determine K_{Mg} from seven data points.

Results

Sorption of Ca^{2+} onto PM Vesicles

The effects of various treatments on the amount of Ca^{2+} sorbed to right-side-out PM vesicles are presented in Table 1. It is clear that the amount of $^{45}Ca^{2+}$ associated with the vesicles was similar at 4 and 24°C and that conditions favoring Ca^{2+} uptake (Giannini, Ruiz-Cristin & Briskin, 1987), e.g., the addition of 3.75 mM MgATP at 1.0 mM Ca^{2+}, reduced the association by 50%. The ionophore A23187 added before or after the sorption reaction at a final concentration of 5 μM did not change the amount of Ca^{2+} sorbed to the vesicles (Table 1). The total amount of Ca^{2+} released in three successive leaching cycles with 10 mM $LaCl_3$ was only 64 ± 7% of the total sorbed Ca^{2+} (Table 2). Over 70% of the total exchangeable Ca^{2+} was released in the first leaching cycle and consisted of about half of the total sorbed. In the presence of 0.67 mM La^{3+} and 0.1 mM Ca^{2+}, Ca^{2+} sorption was inhibited by 96% (*see* Fig. 2).

The amounts of Ca^{2+} sorbed to PM vesicles at various Na^+ and initial Ca^{2+} concentrations are presented in Fig. 1. The amount of sorbed Ca^{2+} decreased with increasing Na^+ concentration at a given initial Ca^{2+} concentration, and increased with increasing initial Ca^{2+} concentration at a fixed concentration of Na^+. Generally, Mg^{2+} had a similar effect on Ca^{2+} sorption (Fig. 2), but it competes more strongly with Ca^{2+} for the sorption sites on the PM than Na^+. Only 5 mM Mg^{2+}, *vs.* 100 mM Na^+, were required to reduce the amount of sorbed Ca^{2+} from 1.1 to 0.4 mmol/g protein in the presence of 1 mM Ca^{2+} (Figs. 1 and 2).

Calculation of the Effective Concentration of Charged Surface Sites

As explained in Materials and Methods, the effective concentration of charged surface sites was found from the case in which no Na^+ had been added and Ca^{2+} concentration was 1 mM. In this case, the amount of sorbed Ca^{2+} was 1.09 mmol/g protein (Fig. 1), or 27.25 nmol Ca^{2+} sorbed to vesicles containing 25 μg protein per sample of 150 μl. Since Ca^{2+} is a divalent cation, the total amount of negatively charged sites on the membrane is 54.5 nmol for 25 μg protein, and the concentration of the charged sites is 0.363 mM (54.5 nmol in 150 μl).

Binding Constants of Ca^{2+}, Na^+, Mg^{2+} and Surface Charge Density of Melon Root PM

The experimentally determined sorption of Ca^{2+} to the biomembrane is fairly well explained by the sorption model (Tables 3 and 4). The Ca^{2+} ion/charged site (Ca/e) value represents the ratio between the number of sorbed Ca^{2+} ions and the total number of charged sites on the vesicles. Experimental Ca/e values (Tables 3 and 4) were obtained by dividing the amount of sorbed Ca^{2+} by the total number of charged sites. The total number of charge sites, the determination of surface area per charge and binding coefficients are explained in Materials and Methods. Good agreement between the calculated and the experimental values of sorbed Ca^{2+} was obtained for a surface area of 370 ± 30 Å^2 per charge, and binding coefficients of 0.8 ± 0.2, 9 ± 3, and 50 ± 10 M^{-1} for K_{Na}, K_{Mg} and K_{Ca}, respectively (Tables 3 and 4).

Discussion

Calcium Sorption to Vesicles *vs.* Active Uptake

Several studies have shown accumulation of Ca^{2+} inside PM vesicles. Such studies were performed using inside-out vesicle preparations, and Ca^{2+} and MgATP at concentrations of 0.01 and 3.75 mM, respectively (Gianni-

Table 2. Relative amounts of Ca^{2+} (mean ± SD) released from PM vesicles by La^{3+} at two initial Ca^{2+} concentrations. The La^{3+} was added following the sorption reaction in a 0.1 ml solution containing 10 mM $LaCl_3$ and 0.25 M sucrose at pH 7.2

Ca^{2+} concentration (mM)	Relative Ca^{2+} released			
	Leaching no. 1	Leaching no. 2	Leaching no. 3	Total released
	(% of total Ca^{2+} sorbed)			
0.5	48.3 ± 3.0	12.8 ± 2.1	4.1 ± 1.1	65.2 ± 6.2
1.0	45.7 ± 3.2	12.3 ± 2.5	5.1 ± 0.9	63.1 ± 6.8

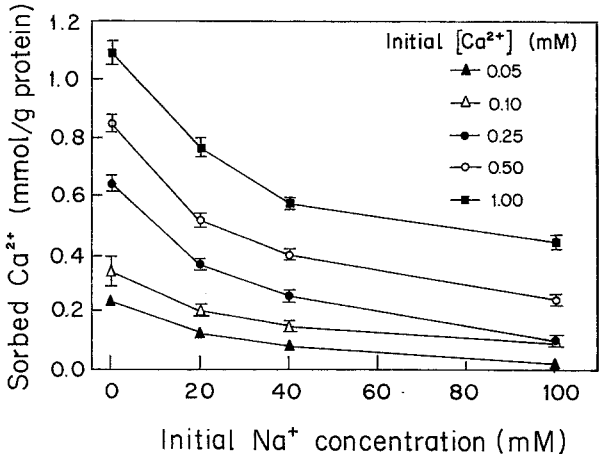

Fig. 1. Amount of Ca^{2+} sorbed to plasma membrane vesicles from solutions containing initially various Ca^{2+} and Na^{+} concentrations.

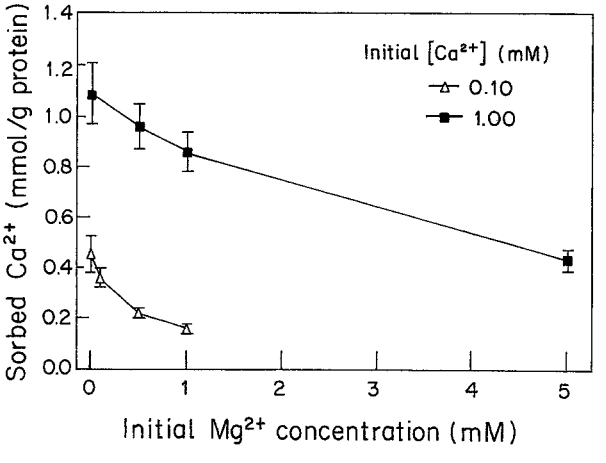

Fig. 2. Amount of Ca^{2+} sorbed to plasma membrane vesicles from solutions containing initially various Ca^{2+} and Mg^{2+} concentrations.

ni et al., 1987). Under their conditions, 15 µmol $^{45}Ca^{2+}$/g protein was accumulated in the vesicles within 30 min, and the addition of the Ca^{2+} ionophore A23187 decreased accumulation of Ca^{2+}. We duplicated these conditions in our PM vesicles to determine

Table 3. Number of sorbed Ca^{2+} ions per charged site (Ca/e) on PM vesicles of melon roots as a function of total added concentrations of Ca^{2+} and Na^{+}.

Treatment		Experimental	Calculated[a]	
Ca^{2+}	Na^{+}	Sorbed[b]	Sorbed[b]	Bound[c]
(mM)		(Ion/charge)		
0.05	1	0.107 ± 0.007[d]	0.137	0.129
0.05	20	0.057 ± 0.007	0.081	0.076
0.05	40	0.035 ± 0.005	0.047	0.045
0.05	100	0.009 ± 0.003	0.017	0.016
0.1	1	0.155 ± 0.025	0.268	0.242
0.1	20	0.092 ± 0.011	0.132	0.124
0.1	40	0.067 ± 0.010	0.080	0.076
0.1	100	0.041 ± 0.009	0.031	0.030
0.25	1	0.293 ± 0.014	0.427	0.333
0.25	20	0.166 ± 0.009	0.210	0.192
0.25	40	0.115 ± 0.011	0.142	0.133
0.25	100	0.046 ± 0.010	0.066	0.062
0.5	1	0.388 ± 0.014	0.454	0.343
0.5	20	0.235 ± 0.012	0.264	0.236
0.5	40	0.181 ± 0.011	0.195	0.180
0.5	100	0.111 ± 0.011	0.105	0.100
1.0	1	0.500 ± 0.022	0.470	0.350
1.0	20	0.350 ± 0.017	0.312	0.272
1.0	40	0.261 ± 0.008	0.247	0.224
1.0	100	0.202 ± 0.011	0.155	0.146

[a] Calculations used a surface area of 370 Å^2 per charge. The binding coefficients K_{Na} and K_{Ca} were 0.8 and 50 mM^{-1}, respectively (*see text for further details*).

[b] Sorbed Ca^{2+} includes the bound plus the amount of the cations in the diffuse double-layer region.

[c] Bound represents only cations chemically bound to the surface.

[d] SD.

possible active transport of Ca^{2+} and found that under the above conditions the amount of Ca^{2+} associated with the vesicles was negligible (Table 1). Even at 1.0 mM Ca^{2+}, the addition of 3.75 mM MgATP decreased the Ca^{2+} associated by 50% (Table 1). It is evident that no increase observed under conditions favoring Ca^{2+} uptake, and that the decrease in sorption of Ca^{2+} is due to the presence of Mg^{2+}. Addition of ionopohore

Table 4. Number of sorbed Ca^{2+} ions per charged site (Ca/e) on PM vesicles of melon roots as a function of total added concentrations of Ca^{2+} and Mg^{2+}.

Treatment		Experimental	Calculated[a]	
Ca^{2+}	Mg^+	Sorbed[b]	Sorbed[b]	Bound[c]
(mM)		(Ion/charge)		
0.01	2.0	0.004 ± 0.001[d]	0.007	0.006
0.1	0	0.209 ± 0.031	0.268	0.242
0.1	0.1	0.165 ± 0.017	0.231	0.197
0.1	0.5	0.101 ± 0.010	0.137	0.117
0.1	1.0	0.075 ± 0.006	0.094	0.081
1.0	0	0.500 ± 0.052	0.470	0.350
1.0	0.5	0.440 ± 0.042	0.390	0.300
1.0	1.0	0.393 ± 0.036	0.340	0.270
1.0	5.0	0.199 ± 0.019	0.180	0.155

[a] The calculations assume a total Na^+ concentration of 1 mM and a surface area of 370 Å^2 per charge. The binding coefficients K_{Na}, K_{Mg} and K_{Ca} were 0.8, 9 and 50 M^{-1}, respectively (*see text for further details*).

[b] Sorbed Ca^{2+} includes the bound plus the amount of the cations in the diffuse double-layer region.

[c] Bound represents only cations chemically bound to the surface.

[d] SD.

A23187 did not change the amount of Ca^{2+} associated with the vesicles. Furthermore, the membranes used in our study were mostly in the right-side-out orientation and the amount of Ca^{2+} associated with the vesicles was 1.1 mmol/g protein when the Ca^{2+} concentration in the medium was 1 mM. Assuming globular vesicles with a maximum radius of 100 nm (Vom Dorp, Volkmann & Scherer, 1986) and a Ca^{2+} concentration of 1 mM inside the vesicles, the calculated amount of Ca^{2+} in the free solution trapped inside the vesicles is only a few percent of the measured sorbed Ca^{2+} fraction. Hence, it can be concluded that most of the Ca^{2+} sorbed to PM vesicles rather than accumulated in free solution inside the vesicles.

In sorption studies with phospholipid vesicles, Ca^{2+} was found to bind only to the outer surface unless ionophores were provided or the integrity of the membrane was disrupted (Newton et al., 1978; Nir et al., 1978; Nir, 1984). That the addition of the ionophore did not increase the amount of Ca^{2+} sorbed to our vesicles (Table 1) suggests that Ca^{2+} ions crossed the biomembrane and were sorbed to both sides of it. This conclusion was further supported by the results obtained in the following two experiments:

First, we performed freeze-thaw cycles, which have been shown to convert right-side-out vesicles to inside-out ones (Palmgren et al., 1990). This treatment did not change the amount of Ca^{2+} sorbed to the vesicles (Table 1).

Second, lanthanum, which is a trivalent cation whose affinity to the charged sites is higher than that of Ca^{2+} (Obi et al., 1989), as was also found in our sorption competition experiment between Ca^{2+} and La^{3+} (Table 1), released only two-thirds of the total sorbed Ca^{2+} (Table 2). The inability of La^{3+} to release all the sorbed Ca^{2+} from the membrane could be explained in two ways: (i) Lanthanum does not cross the membrane. In this case, Ca^{2+} ions sorbed to the inner vesicle surfaces are not exposed to La^{3+}. (ii) Lanthanum does cross the membrane and exchanges with the Ca^{2+} sorbed to the inner side of the vesicles, but the released Ca^{2+} is not able to get out of the vesicles because of the known effect of La^{3+} as a Ca^{2+} channel blocker (Reuter, 1983).

Binding Constants of Ca^{2+}, Na^+ and Mg^{2+}

The binding constants used in this study are in the range of previously reported values for phospholipid vesicles obtained by different methods. In the case of phosphatidylserine vesicles, Nir et al. (1978) determined binding constants of $K_{Na} = 0.8$ M^{-1}, $K_{Ca} = 35$ M^{-1} and $K_{Mg} = 20$ M^{-1} from an analysis of the amounts of Ca^{2+} and Mg^{2+} sorbed in dialysis experiments in solutions containing various concentrations of Na^+, Ca^{2+} and Mg^{2+}. Kurland et al. (1979) studied similar systems by measuring relaxation rates of $^{23}Na^+$ in nuclear magnetic resonance studies, and found K_{Na} values from 0.6 to 1.2 M^{-1}, and K_{Ca} 35 M^{-1}. Eisenberg et al. (1979) studied the binding of monovalent cations using microelectrophoresis, and found a K_{Na} value of 0.6 M^{-1}. From measurements of the surface potential of phosphatidylserine monolayers, Ohki and Kurland (1981) deduced a binding constant of 0.6 M^{-1} for Na^+. Binding constants of $K_{Na} = 0.8$ M^{-1}, $K_{Mg} = 10$ M^{-1} and $K_{Ca} = 30$ M^{-1} were obtained by Nir (1984), based on an analysis of results from potentiometric titration, which measures the concentration of Ca^{2+} in solution.

The amount of sorbed cations is made up of two components: cations residing at enhanced concentrations in the double-layer region, and cations that are chemically bound to the surface. The degree of tightness of the chemical binding is reflected by the magnitude of the binding constants. Most of the Ca^{2+} ions sorbed to the PM vesicles were chemically bound to the membranes (Tables 3 and 4). In most cases, the amount of chemically bound Ca^{2+} ions was more then 90% of the total sorbed Ca^{2+}.

Existence of Nonphospholipid Charged Sites

The similarity between binding coefficient values from model vesicles and biomembranes for the sorption of divalent and monovalent metal ions may indicate that the affinities of biomembranes for Na^+, Ca^{2+} and Mg^{2+} are

essentially the same as those of phospholipid bilayers. However, the results reported here indicate that a great deal of Ca^{2+} sorption is due to nonphospholipid components of the PM. We pointed out that per 25 μg protein there are 54.5 nmol of singly charged sites. If these sites are assumed to be phospholipids with molecular weight (M_r) of 800, then 44 μg phospholipid per 25 μg protein must be found, i.e., a phospholipid to protein weight ratio of 1.8, which is several times higher than the phospholipid to protein ratio (0.2) found for melon root microsomal membranes (Borochov-Neori & Borochov, 1991). Furthermore, it should be noted that only a fraction of the phospholipids are negatively charged.

Surface Charge Density

Information regarding surface charge density of PM isolated from plant cells is limited. Korner et al. (1985) measured the negative surface charge density at pH 7.0, using 9-aminoacridine fluorescence on right-side-out PM vesicles isolated from barley roots, and obtained a value of 852 $Å^2$ per charge. Using the same method, Moller et al. (1984) obtained 551 and 762–842 $Å^2$ per charge for oat and wheat root PM, respectively, and Oka et al. (1988) reported 340–516 $Å^2$ per charge for protoplasts isolated from elongating regions of *Vigna mungo* root. Obi et al. (1989) and Sack, Priestley and Leopold (1983) who determined surface charge density by particle electrophoresis obtained 2,247 $Å^2$ per charge for barley mesophyll protoplasts in a medium containing 6.5 mM NaCl (Obi et al., 1989) and 3,440 $Å^2$ for maize coleoptile amyloplasts in a medium containing 10 mM KCl (Sack et al., 1983). In the present study, the surface charge density value obtained for PM vesicles corresponds to 370 $Å^2$ per charge in the limit of no charge neutralization. Our program also yields the actual surface charge density, which depends on the composition of the solution and total concentration of surface sites (Nir, 1984). Thus, in the presence of 1 mM Na^{+} and 0.05 mM Ca^{2+}, the area per charge is 580 $Å^2$, whereas increasing the Ca^{2+} concentration to 0.5 mM gives an area of 1,225 $Å^2$ per charge. This example illustrates the difference (Eq. 5) between intrinsic and actual surface charge densities σ_0, and σ, respectively (Nir, 1984). Particle electrophoresis consistently gives lower values for the surface charge density, probably due to the presence of the shearing layer (Barber, 1982) and charge neutralization by chemically bound cations to the negatively charged surface. As shown in Table 3, in most cases more than 90% of the sorbed Ca^{2+} cation was tightly bound to the surface and neutralized its negative charge. The surface charge density values using 9-aminoacridine fluorescence were obtained after removing the divalent cations from the membrane surface by chelators. Therefore, the reduction of the surface charge density by bound divalent cations was not taken into account by this technique to estimate the overall intrinsic charge density (Chow & Barber, 1980; Moller et al., 1984; Korner et al., 1985; Oka et al., 1988). However, it is not clear whether the values obtained with 9-aminoacridine are absolute, because of overestimation of the charge density by monitoring local charge densities instead of an overall average (Chow & Barber, 1980; Moller et al., 1984). Since in this study we determined the overall intrinsic surface charge density, the results are independent of the type of cations used and their concentrations.

Concluding Remarks

The results presented in Tables 3 and 4 show that the sorption model used can simulate and predict cation sorption to the PM of melon root cells. In the Introduction, we presented an extensive, albeit not exhaustive, list of this model's (or variations of it) applications to phospholipid membranes. Part of the success of sorption models that ignore the discrete nature of the surface charges is due to the lateral mobility of membrane components which yields a smeared surface charge density, over a short period of time. The applicability of this model has also been demonstrated for cation sorption to a clay (Nir et al., 1986; Hirsch et al., 1989) and bile salt micelles (Baruch et al., 1991). However, studies on other biological membranes are needed to establish whether replacement of the wide variety of charged components by an effective single component is adequate.

The binding constant of Ca^{2+} to melon PM is 62 and 5.6 times greater than those of Na^{+} and Mg^{2+}, respectively. The same order of cation sorption affinity ($Ca^{2+} > Mg^{2+} > Na^{+}$) was found by Young and Kauss (1983), who examined displacement of $^{45}Ca^{2+}$ from suspension-cultured *Glycine max* cells, and by Obi et al. (1989), who measured the electrophoretic mobility of barley mesophyll protoplasts. A recent study on a plant system also concluded that Ca^{2+} was more effective in binding than other divalent cations (Kinraide et al., 1992). These results could explain the generally observed specificity of Ca^{2+} in protecting plants under salt stress, as described by LaHaye and Epstein (1969). While bean plants exposed to 50 mM Na^{+} and less than 1 mM Ca^{2+} were markedly damaged, these plants grew well in media with higher Ca^{2+} concentrations. Attempts to replace Ca^{2+} with Mg^{2+} failed, with Mg^{2+} causing further damage to that already observed with Na^{+} (LaHaye & Epstein, 1969). Our results suggest that Ca^{2+} acts as a specific electrostatic binder of the negative charges on PM surfaces. The displacement of Ca^{2+} by another cation may change the properties of the

membrane in relation to its permeability to ions (i.e., leakage). Such changes in the PM of root cells may subsequently determine the ability of the plant to grow under saline conditions.

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