The Plant Journal (1994) **5**(5), 683–694

Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from *Zea mays* L. roots

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Summary

Right-side-out plasma membrane vesicles isolated from *Zea mays* roots were used to study membrane potential ($\Delta\psi$)-dependent Ca^{2+} transport. Membrane potentials were imposed on the vesicles using either K^+ concentration gradients and valinomycin or SCN^- concentration gradients, and the size of the imposed $\Delta\psi$ was measured with [^{14}C]tetraphenylphosphonium. Uptake of $^{45}Ca^{2+}$ into the vesicles was stimulated by inside-negative $\Delta\psi$. The rate of transport increased to a maximum at a $\Delta\psi$ of about –80 mV and then declined at more negative $\Delta\psi$. When extravesicular Ca^{2+} concentration was varied, uptake was maximal in the range 100–200 μM Ca^{2+}. Neither dihydropyridine nor phenylalkylamine Ca^{2+} channel blockers had any effect on Ca^{2+} uptake but 30 μM ruthenium red was completely inhibitory with half maximal inhibition at 10–15 μM ruthenium red. Calcium transport was also inhibited by inorganic cations. Zn^{2+}, Gd^{3+} and Mg^{2+} inhibited by a maximum of 30% while La^{3+}, Nd^{3+} and Mn^{2+} inhibited by 70%. The inhibitory effects of La^{3+} and Gd^{3+} were additive. Lanthanum-insensitive Ca^{2+} tive Ca^{2+} transport was totally inhibited by 80 μM Gd^{3+} and showed maximum activity at a $\Delta\psi$ of –60 mV, with less uptake at both higher and lower $\Delta\psi$. Lanthanum and Gd^{3+} also inhibited Ca^{2+} uptake into protoplasts isolated from *Zea* roots and their individual and combined effects were similar in extent to those observed with plasma membrane vesicles. It is concluded that maize root plasma membrane contains two Ca^{2+}-permeable channels that can be distinguished by their susceptibility to inhibition by La^{3+} and Gd^{3+}. Both are inhibited by ruthenium red but not by other organic Ca^{2+} channel blockers.

Received 24 November 1993; revised 17 January 1994; accepted 20 January 1994.

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Introduction

Elevation of cytosolic free Ca^{2+} concentration has been implicated as an intermediary process in the physiological responses of plant cells to a variety of effectors (Bush, 1993; Hepler and Wayne, 1985; Johannes *et al.*, 1991; Kauss, 1987). This requires the maintenance of a low resting cytosolic Ca^{2+} concentration in normal conditions and its elevation in response to appropriate stimuli.

There are considerable transmembrane electrochemical gradients for the passive movement of Ca^{2+} into the cytosol from both the apoplast and intracellular stores such as the vacuole and endoplasmic reticulum. These gradients are maintained by efflux of Ca^{2+} across the plasma membrane into the apoplast via a Ca^{2+}-ATPase (Evans *et al.*, 1991), into the vacuole via a tonoplast Ca^{2+}/H^+ antiport (Blackford *et al.*, 1990) and into the lumen of the endoplasmic reticulum via a second Ca^{2+}-ATPase (Brauer *et al.*, 1990). Stabilization of cytosolic free Ca^{2+} at submicromolar concentrations is achieved by physiochemical buffering by calcium-binding proteins such as calmodulin (Hepler and Wayne, 1985). In principle, elevation of cytosolic free Ca^{2+} during signal transduction may occur either by controlled influx of Ca^{2+} across the plasma membrane, or by mobilization of Ca^{2+} from internal stores.

Entry of Ca^{2+} into the cytosol is probably a passive process mediated by ion channels (Tester, 1990). Two Ca^{2+} channels have been identified in the tonoplast using a combination of patch clamping and *in vitro* studies with membrane vesicles (Johannes *et al.*, 1992). However, membrane potential ($\Delta\psi$)-dependent passive transport of Ca^{2+} at the plasma membrane has received less attention but patch clamping has indicated that Ca^{2+}-permeable channels are present in this membrane (reviewed by Bush, 1993; Johannes *et al.*, 1991; Schroeder and Thuleau, 1991). The availability of methods for preparing highly purified right-side-out plasma membrane vesicles (Larsson, 1985) offers an opportunity to study Ca^{2+} transport at this membrane using *in vitro* techniques. In right-side-out plasma membrane vesicles, the uptake of radiolabelled $^{45}Ca^{2+}$ will be in the same direction as Ca^{2+}

influx into intact cells, making these vesicles a particularly useful system for studying inwardly directed transport. Thus far, however, the majority of studies with plasma membrane vesicles have been concerned with the processes of active Ca^{2+} extrusion, rather than the pathways of Ca^{2+} influx (e.g. Evans *et al.*, 1991; Giannini *et al.*, 1987; Graf and Weiler, 1989).

The aim of the work presented in this paper was to investigate the $\Delta\psi$-dependent Ca^{2+} influx pathways at the plasma membrane of *Zea mays* roots using plasma membrane vesicles and $^{45}Ca^{2+}$. Although $\Delta\psi$ have been imposed on plant plasma membrane vesicles in other studies (e.g. Bush, 1989; Williams *et al.*, 1990), there have been no attempts to quantify the size of the imposed $\Delta\psi$ and this limits the amount of useful information that can be gained. Therefore, in this study, particular attention was paid to quantitative estimation of $\Delta\psi$. In addition, Ca^{2+} channel blockers were used to determine whether multiple Ca^{2+} permeable pathways were mediating the measured Ca^{2+} influx. The results suggest that there are at least two pathways operative in Ca^{2+} influx across the plasma membrane of maize roots and these can be distinguished by their response to inhibition by inorganic cations.

Results

Plasma membrane purity and vesicle orientation

Two sequential separations in a two-phase aqueous partitioning system (Larsson, 1985) were used to separate a fraction enriched in right-side-out plasma membrane vesicles. Table 1 shows the activities and percentage recoveries of various marker enzymes in microsomes, the final plasma membrane-enriched fraction (U_2) and the contaminant-enriched lower phase from the first partition (L_1). The plasma membrane-specific markers, vanadate-sensitive ATPase (O'Neill and Spanswick, 1984) and glucan synthetase II (Ray, 1977) were enriched in U_2 relative to their activities in the microsomes.

Orientation of the plasma membrane vesicles in U_2 was determined by three different methods: (i) latency of NADH-ferricyanide reductase activity (Palmgren *et al.*, 1990); (ii) trypsin sensitivity of ATPase activity (Grouzis *et al.*, 1987); (iii) freeze-fracture electron microscopy. The latter technique depends on the distribution of intramembrane particles between the E- and P-faces of the plasma membrane *in vivo* (Marty, 1982) which allows vesicle orientation *in vitro* to be assessed from the shape of the vesicle fracture face (concave or convex) and the number of particles per unit membrane area. All methods indicated that the vesicles were 85–90% right-side-out (Table 2).

Quantification of imposed $\Delta\psi$

To provide a driving force for Ca^{2+} uptake it was necessary to impose inside-negative $\Delta\psi$ on the plasma membrane vesicles and this was done using K^+ concentration gradients in the presence of valinomycin (Rottenberg, 1989). To impose a K^+-diffusion potential, plasma membrane vesicles were loaded with 4 mM K-gluconate and added to media with known K^+ concentrations. Independent estimates of $\Delta\psi$ were made using [^{14}C]tetraphenylphosphonium (TPP^+) which, when used at concentrations of 10 μM or less, equilibrates across the membrane in accordance with the Nernst equation (Rottenberg, 1989). To estimate the imposed $\Delta\psi$ it was necessary to calculate the intravesicular TPP^+ concentration which required an estimate of the intravesicular volume. The latter was obtained using ^3H_2O and [^{14}C]inulin (Rottenberg, 1989). The mean ± SE total, extravesicular and intravesicular volumes for four different plasma membrane preparations were 10.01 ± 0.93, 7.83 ± 0.60 and 2.18 ± 0.22 μl mg^{-1} protein, respec-

Table 1. Percentage recovery of marker enzymes in the upper phase (U_2) and the lower phase (L_1) from the two-phase aqueous partition system

Marker enzyme	Specific activity			% of microsomal activity recovered	
	Microsomes	U_2	L_1	U_2	L_1
Vanadate-sensitive ATPase pH 6.5	230 ± 30	800 ± 60	150 ± 10	80 ± 5	15 ± 3
Glucan synthetase II	500 ± 50	1800 ± 110	700 ± 80	80 ± 7	10 ± 3
Azide-sensitive ATPase pH 8.0	30 ± 5	0	40 ± 10	0	90 ± 9
Cytochrome c oxidase	3.6 ± 0.8	0.5 ± 0.1	4.7 ± 0.9	5 ± 2	95 ± 7
Nitrate-sensitive ATPase pH 8.0	60 ± 10	10 ± 3	90 ± 20	8 ± 1	100 ± 5
PPase	80 ± 10	0	100 ± 20	0	97 ± 7
Antimycin A-insensitive NADH cytochrome c reductase	70 ± 10	0	100 ± 10	0	104 ± 8
Latent IDPase	40 ± 10	0	60 ± 10	0	97 ± 5
Aspartate aminotransferase	170 ± 30	0	200 ± 20	0	110 ± 9

Specific activities are expressed as nmol mg^{-1} protein min^{-1} except for glucan synthetase II (c.p.m. 10^3 mg^{-1} protein min^{-1}) and cytochrome c oxidase (μmol mg^{-1} protein min^{-1}). Results are the mean ± SE from six experiments.

tively. Using the latter value, the TPP^+ concentration inside the vesicles was calculated and the $\Delta\psi$ was determined for a range of negative $\Delta\psi$ (Table 3). The imposed $\Delta\psi$ agreed well with the theoretically expected values down to −40 mV but at more negative values the K^+-diffusion potentials failed to clamp the $\Delta\psi$ at the calculated values and $\Delta\psi$ was less negative than anticipated from the Nernst equation. All negative $\Delta\psi$ quoted below are the actual values derived from measurements of TPP^+ accumulation (Table 3).

The effect of $\Delta\psi$ on Ca^{2+} uptake

The imposition of a $\Delta\psi$ of −74 mV stimulated the accumulation of $^{45}Ca^{2+}$ in the plasma membrane vesicles, compared with vesicles in which the $\Delta\psi$ was clamped at 0 mV (Figure 1). The amount of $^{45}Ca^{2+}$ accumulated increased with time and reached a steady state after 6 min. This relatively slow accumulation rules out the possibility that the uptake is due to binding at the outer surface of the vesicles as this would be a more rapid process. The stimulation of Ca^{2+} uptake by a $\Delta\psi$ of −74 mV indicates that the plasma membrane vesicles contain a voltage-dependent Ca^{2+} transport pathway. Following the attainment of a steady-state Ca^{2+} accumulation, the addition of the Ca^{2+} ionophore, A23187, caused an increase in Ca^{2+} uptake into the vesicles. This result can be explained if the plasma membrane *in vivo* has a low density of $\Delta\psi$-dependent Ca^{2+} transport pathways which results in only a small proportion of the isolated vesicles possessing these transporters, with the remainder being sealed to Ca^{2+}. Uptake of $^{45}Ca^{2+}$ in response to the $\Delta\psi$ will initially occur only in those vesicles possessing the Ca^{2+} transporter. However, the addition of A23187 makes all vesicles permeable to Ca^{2+} and the ion now accumulates in the whole vesicle population (Brosnan, 1990).

To determine the voltage-dependence of Ca^{2+} uptake into the vesicles a range of $\Delta\psi$ was imposed on the vesicles using either K^+/valinomycin or SCN^- gradients and uptake was measured from an external Ca^{2+} concentration of 10 μM. Using TPP^+ to determine the size of the $\Delta\psi$ generated by SCN^- gradients showed that they were up to −90 mV and so could be used to extend the range of the imposed $\Delta\psi$ beyond the −74 mV achievable with K^+/valinomycin. At a $\Delta\psi$ of +60 mV there was no uptake of Ca^{2+}, but as $\Delta\psi$ became more negative there was a steep increase in the initial rate of Ca^{2+} uptake with the maximum rate occurring at a $\Delta\psi$ of approximately −80 mV (Figure 2). As $\Delta\psi$ became more negative than −80 mV, the initial rate of Ca^{2+} uptake decreased implying that uptake of Ca^{2+} is not only voltage-driven but also voltage-gated.

The initial rate of uptake of Ca^{2+} into the vesicles increased with increasing extravesicular Ca^{2+} concentration and was maximal at 100–200 μM external Ca^{2+} (Figure 3). At higher Ca^{2+} concentrations the rate of accumulation decreased. A Hanes–Wolf plot of the rates between 0 and 100 μM Ca^{2+} yielded a K_m for Ca^{2+} of 48 μM.

Table 2. The orientation of plasma membrane vesicles isolated from maize roots, determined by three different methods

Method	% right-side-out
Latency of NADH-ferricyanide reductase	85 ± 6 (3)
Trypsinolysis of ATPase	90 ± 3 (4)
Freeze-fracture	85 ± 2 (4)

The number of different plasma membrane preparations measured for each method is given in parentheses.

Table 3. The relationship between the theoretical and actual $\Delta\psi$ imposed on maize root plasma membrane vesicles by K^+/valinomycin

Theoretical $\Delta\psi$ (mV)	TPP^+ uptake (nmol mg^{-1} protein)	$[TPP^+]_{in}$ (μM)	Actual $\Delta\psi$ (mV)
−120	0.20	91.7	−74
−100	0.17	78.0	−70
−80	0.15	68.8	−67
−60	0.08	36.7	−51
−40	0.06	27.5	−43
−20	0.02	9.2	−15

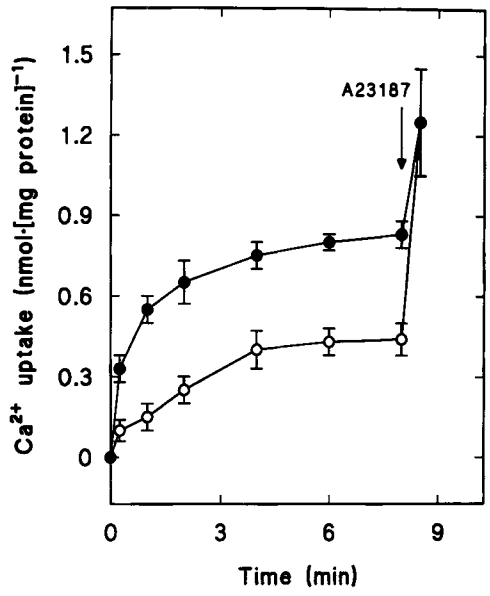

Figure 1. Time-dependent uptake of $^{45}Ca^{2+}$ into plasma membrane vesicles with imposed $\Delta\psi$ of 0 mV (○) or −74 mV (●).
The extravesicular Ca^{2+} concentration was 10 μM. Data are the mean ± SE of six experiments.

The effect of Ca^{2+} channel blockers

A variety of Ca^{2+} channel antagonists were tested for their effects on Ca^{2+} transport into the plasma membrane vesicles in the presence of an imposed $\Delta\psi$ of −74 mV and an extravesicular Ca^{2+} concentration of 10 μM. Of the organic inhibitors tested, verapamil, nifedipine and diltiazem (all at 100 μM) had no effect (data not shown). However, 100 μM ruthenium red totally inhibited Ca^{2+} uptake with half-maximal inhibition at 10–15 μM and total inhibition at 30 μM ruthenium red (Figure 4). As ruthenium red is generally considered an endomembrane Ca^{2+} channel blocker (Reed and Bygrave, 1974; Ying *et al.*, 1991), it was necessary to ensure that it inhibited Ca^{2+} transport activity of the plasma membrane, rather than a minor contaminating endomembrane, even though marker enzyme analysis had indicated relatively low contamination by endomembranes (Table 1). This was done by comparing the ability of ruthenium red to inhibit Ca^{2+} transport activity in the U_2 (plasma membrane-enriched) and L_1 (contaminant-enriched) fractions from the two-phase aqueous partitioning system. All of the Ca^{2+} uptake in U_2 was inhibited by 100 μM ruthenium red, but only 25% of that in L_1 (Figure 5). Thus, the ruthenium red-sensitive Ca^{2+} uptake co-purified with the plasma membrane vesicles. It seems likely that the small ruthenium red-sensitive proportion of Ca^{2+} uptake in the lower phase is accounted for by plasma membrane vesicles in this phase (Table 1).

As Ca^{2+} transport into the plasma membrane vesicles was insensitive to organic Ca^{2+} channel blockers, a range of cations, all at 100 μM, were tested for their effects on Ca^{2+} influx. Cadmium and Cu^{2+} had no effect, Ba^{2+}, Cs^+,

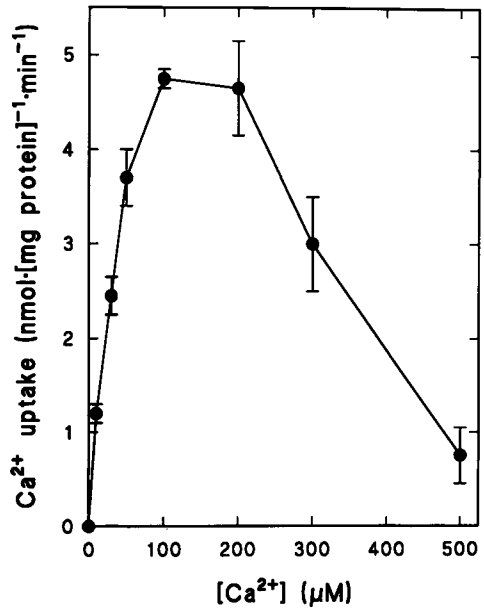

Figure 3. The effect of extravesicular Ca^{2+} concentration on the initial rate of Ca^{2+} influx into plasma membrane vesicles with an imposed $\Delta\psi$ of −74 mV.
Initial rate of Ca^{2+} uptake was determined 15 sec after the addition of $^{45}Ca^{2+}$. Data are the mean ± SE of four experiments.

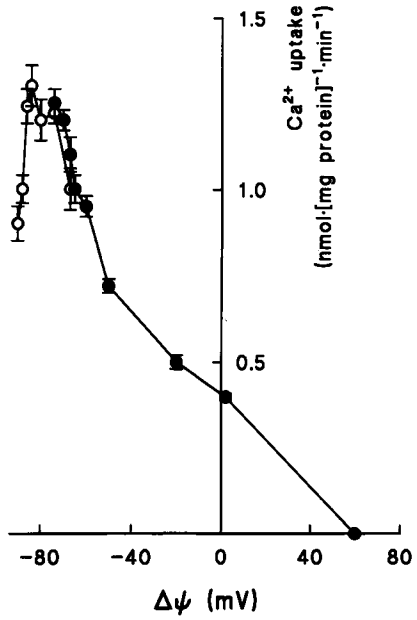

Figure 2. The relationship between initial rate of Ca^{2+} transport into plasma membrane vesicles and the $\Delta\psi$ imposed using either K^+/valinomycin (●) or SCN^- gradients (○).
The extravesicular Ca^{2+} concentration was 10 μM. The initial rate of Ca^{2+} uptake was measured 15 sec after the addition of $^{45}Ca^{2+}$. Data are the mean ± SE of five experiments.

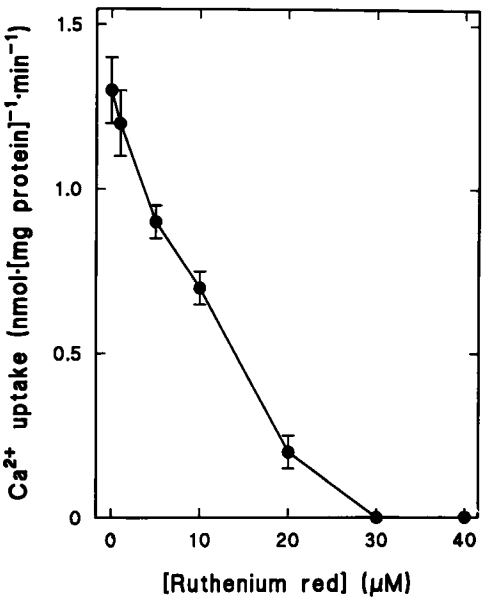

Figure 4. Ruthenium red inhibition of Ca^{2+} influx into right-side-out plasma membrane vesicles.
The extravesicular Ca^{2+} concentration was 10 μM. A $\Delta\psi$ of −74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with ruthenium red for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of five experiments.

Ni^{2+}, Zn^{2+}, Mg^{2+} and Gd^{3+} inhibited by 20–30%, and La^{3+}, Nd^{3+} and Mn^{2+} all inhibited by approximately 70% (Table 4). Half-maximal inhibition was obtained with 5 μM Mn^{2+}, 20 μM Nd^{3+} and 40 μM La^{3+} or Gd^{3+} but even with concentrations as high as 500 μM the inhibition by La^{3+}, Nd^{3+} or Mn^{2+} never exceeded 70% and that by Gd^{3+} never exceeded 30 % (Figure 6). The La^{3+}-insensitive portion of Ca^{2+} uptake was completely inhibited by 100 μM Gd^{3+} (Figure 7) with half-maximal and total inhibition at 20 and 80 μM, respectively (Figure 8). Lanthanum-insensitive Ca^{2+} uptake appeared to be both voltage-driven and voltage-gated (Figure 9). At a $\Delta\psi$ of approximately +60 mV there was no uptake of Ca^{2+}, but as the $\Delta\psi$ became more negative there was an increase in Ca^{2+} influx with the maximum rate occurring at a $\Delta\psi$ of approximately –60 mV. As the $\Delta\psi$ became more negative than –60 mV, the Ca^{2+} influx decreased. The Gd^{3+}-sensitive, La^{3+}-insensitive Ca^{2+} uptake co-purified with plasma membrane vesicles isolated in U_2 (Figure 10) suggesting that the Gd^{3+} sensitive, La^{3+}-insensitive Ca^{2+} is associated with the plasma membrane and not with a minor contaminant in the plasma membrane fraction.

To determine whether La^{3+} and Gd^{3+} were also able to inhibit Ca^{2+} transport into intact cells, their effect on $^{45}Ca^{2+}$ uptake into maize root protoplasts was investigated (Figure 11). One millimolar La^{3+} inhibited Ca^{2+} uptake by 56.8 ± 13.7% (n = 5) and 1 mM Gd^{3+} inhibited by 26.5 ± 6.3% (n = 5). When both cations were added together they inhibited almost totally (94 ± 6%, n = 2). Attempts were also made to demonstrate a $\Delta\psi$-dependence of Ca^{2+} transport into the protoplasts using metabolic inhibitors but CN^-, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, and carbonyl cyanide 3-chlorophenylhydrazone had no effect (not shown).

Table 4. Effect of various inorganic cations on Ca^{2+} influx into right-side-out plasma membrane vesicles from maize roots

Cation	% inhibition of Ca^{2+} influx
Cd^{2+}	0
Cu^{2+}	0
Ba^{2+}	15 ± 5
Cs^{+}	19 ± 9
Ni^{2+}	22 ± 5
Zn^{2+}	28 ± 3
Gd^{3+}	30 ± 3
Mg^{2+}	34 ± 6
La^{3+}	70 ± 2
Nd^{3+}	70 ± 3
Mn^{2+}	71 ± 3

Each cation was used at a concentration of 100 μM and Ca^{2+} uptake was measured from an external Ca^{2+} concentration of 10 μM. A $\Delta\psi$ of –74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with the cations for 10 min before uptake was initiated by the addition of ^{45}Ca. Calcium influx in the absence of an inhibitor was 1.4 ± 0.2 nmol mg^{-1} protein min^{-1}. Data are the mean ± SE of five experiments.

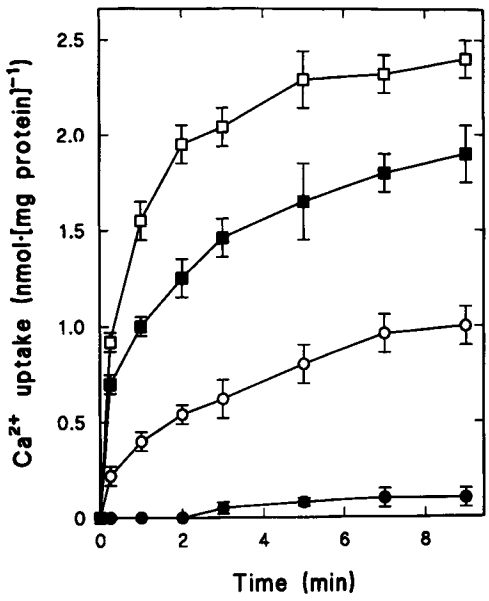

Figure 5. Ruthenium red inhibition of Ca^{2+} uptake into membrane vesicles from the U_2 (plasma membrane-enriched) and L_1 (contaminant-enriched) fractions.
Calcium uptake was measured from an external Ca^{2+} concentration of 10 μM in the presence (●, ■) or absence (○, □) of 100 μM ruthenium red using vesicles from the upper (U_2) phase (plasma membrane-enriched: ●, ○) or from the lower (L_1) phase (contaminant-enriched: ■, □) from the two-phase aqueous polymer partitioning system. A $\Delta\psi$ of –74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with ruthenium red for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of four experiments.

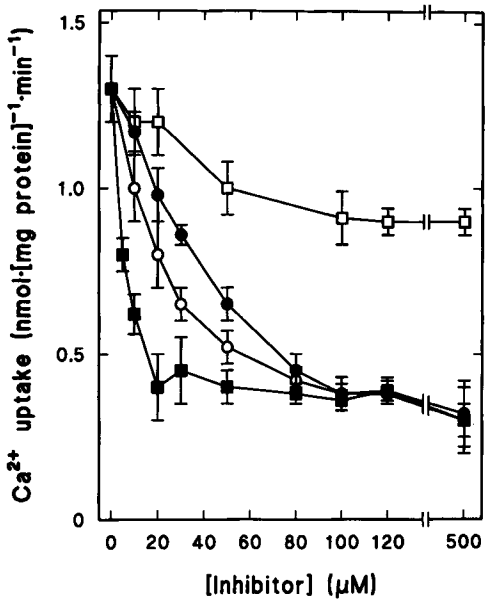

Figure 6. The inhibition of Ca^{2+} uptake into plasma membrane vesicles by 100 μM La^{3+} (●), Nd^{3+} (○), Mn^{2+} (■), or Gd^{3+} (□).
The extravesicular Ca^{2+} concentration was 10 μM. A $\Delta\psi$ of -74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with the blockers for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of four experiments.

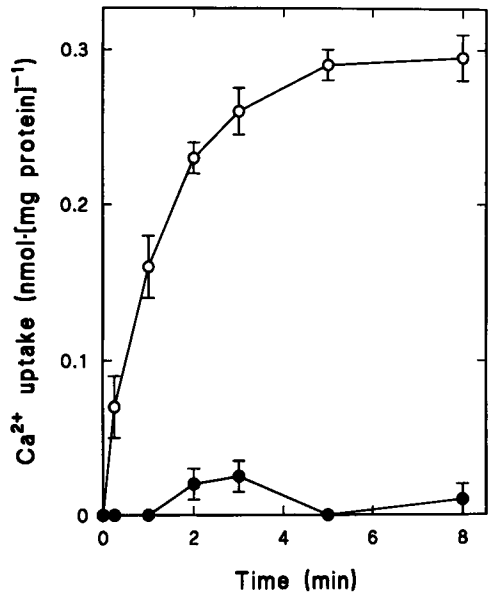

Figure 7. The effect of Gd^{3+} on La^{3+}-sensitive Ca^{2+} uptake into right-side-out plasma membrane vesicles.
The extravesicular Ca^{2+} concentration was 10 μM and all assays contained 100 μM La^{3+} and either 0 (○) or 100 (●) μM Gd^{3+}. A Δψ of −74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with the La^{3+} and Gd^{3+} for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of four experiments.

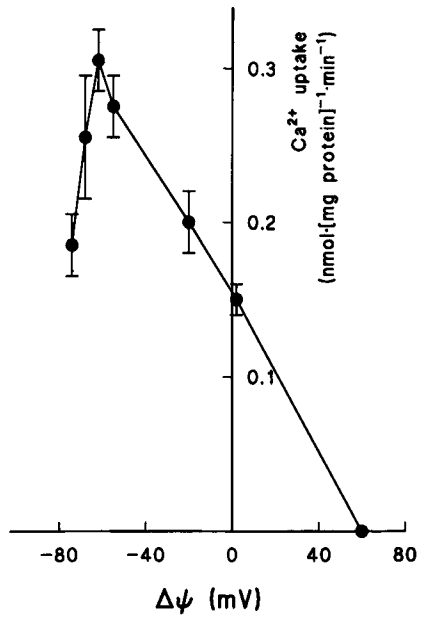

Figure 9. The Δψ-dependence of La^{3+}-insensitive Ca^{2+} influx into plasma membrane vesicles.
The extravesicular Ca^{2+} concentration was 10 μM and La^{3+} was present at a concentration of 100 μM. Membrane potentials were imposed with K^+/valinomycin and vesicles were pre-incubated with the La^{3+} for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of four experiments.

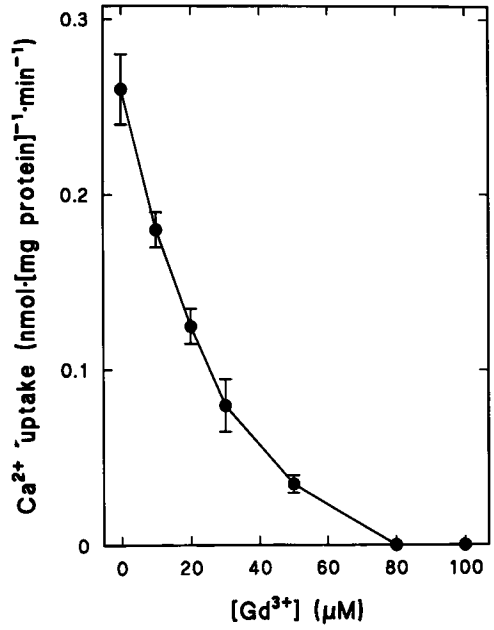

Figure 8. Concentration-dependence of Gd^{3+} inhibition of La^{3+}-insensitive Ca^{2+} uptake into plasma membrane vesicles.
The extravesicular Ca^{2+} concentration was 10 μM and all assays contained 100 μM La^{3+}. A Δψ of −74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with La^{3+} and the indicated concentration of Gd^{3+} for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of four experiments.

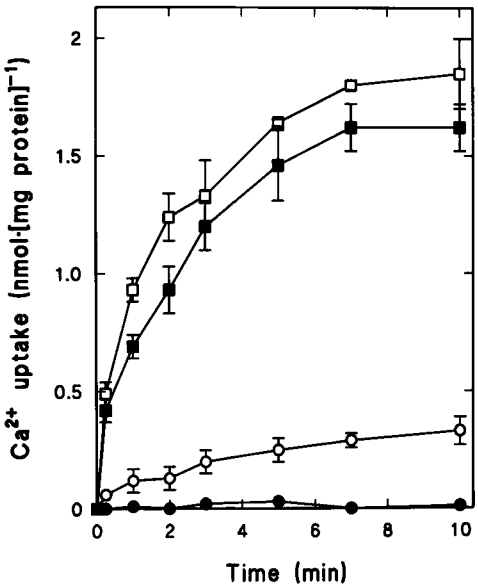

Figure 10. Gadolinium inhibition of the La^{3+}-insensitive Ca^{2+} uptake into membrane vesicles from the U_2 and L_1 fractions from the two-phase aqueous polymer partitioning system.
Calcium uptake was measured from an extravesicular Ca^{2+} concentration of 10 μM with 100 μM La^{3+} and in the presence (●, ■) or absence (○, □) of 100 μM Gd^{3+}. Measurements were made on vesicles from the U_2 (plasma membrane-enriched; ●, ○) or L_1 (contaminant-enriched; ■, □) fractions. A Δψ of −74 mV was imposed with K^+/valinomycin and vesicles were pre-incubated with the La^{3+} and Gd^{3+} for 10 min before uptake was initiated by the addition of ^{45}Ca. Data are the mean ± SE of four experiments.

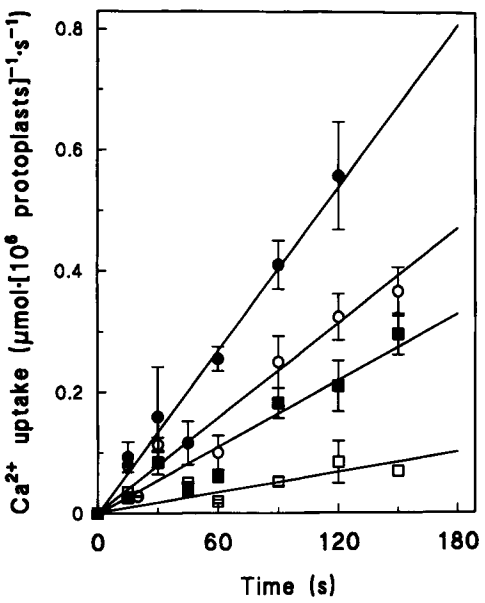

Figure 11. Inhibition of Ca^{2+} transport into *Zea* root protoplasts by lanthanides.
Protoplasts were pre-incubated with 100 μM $^{45}Ca^{2+}$ for 1 min at 25°C in the presence of no cations (●), 1 mM Gd^{3+} (○), 1 mM La^{3+} (■), or 1 mM La^{3+} plus 1 mM Gd^{3+} (□). Data are the mean ± SE of two to six experiments.

Discussion

The experiments reported in this paper show the usefulness of right-side-out plasma membrane vesicles for studying $\Delta\psi$-dependent Ca^{2+} transport *in vitro*. Although such preparations have previously been used to investigate the $\Delta\psi$-dependence of solute transport (Bush, 1989; Williams *et al.*, 1990), the comparison of actual and theoretical values of imposed $\Delta\psi$ (Table 3) demonstrates that care needs to be employed when using K^+/valinomycin gradients to impose $\Delta\psi$ and quantitative relationships require the measurement of the actual $\Delta\psi$. The lack of agreement between theoretical and actual $\Delta\psi$ is likely to be due to carry over of K^+ from the loading medium and/or its leakage from the vesicles resulting in a K^+ gradient less steep than anticipated. The lipophilic cation, TPP^+, gives reasonable estimates of $\Delta\psi$ and there was no evidence of TPP^+ binding to the plasma membrane vesicles. Thus, although the accuracy of TPP^+ uptake as a measure of $\Delta\psi$ in intact cells has been questioned (e.g. Ballarin-Denti *et al.*, 1989; Barts *et al.*, 1980; Boxman *et al.*, 1982; Komor *et al.*, 1979), the observations reported here agree with the findings of Ballarin-Denti *et al.* (1989) who showed that in liposomes a steady-state distribution of TPP^+ occurred after 5 min and correlated with $\Delta\psi$.

Uptake of Ca^{2+} into the plasma membrane vesicles is voltage-driven and the characteristics of uptake are suggestive of the involvement of Ca^{2+} channels. The stimulation of uptake by A23187 suggests that relatively few vesicles possess the $\Delta\psi$-dependent transport pathways thus they are present at relatively low abundance in the plasma membrane, as is the case for most ion channels because of their high rates of turnover (Hille, 1992). Further, the initial rate of total Ca^{2+} uptake into plasma membrane vesicles increased as $\Delta\psi$ became more negative, with maximum influx at a $\Delta\psi$ of −80 mV and then decreased at more negative $\Delta\psi$. This is suggestive of the operation of voltage-gated Ca^{2+}-permeable channels. The sensitivity of Ca^{2+} influx to lanthanides indicates that two different Ca^{2+}-permeable pathways contribute to total uptake. One is sensitive to La^{3+} but insensitive to Gd^{3+} while the other has the reverse sensitivity to these ions. As the La^{3+}-sensitive influx contributes 70% of total uptake (Figure 6), its voltage sensitivity will dominate the flux–voltage relationship of total uptake. This is confirmed by the voltage sensitivity of the smaller Gd^{3+}-sensitive flux which showed maximal activity at −60 mV rather than the −80 mV of total uptake (compare Figures 2 and 9). Overall, the results are consistent with the notion that at a plasma membrane resting potential of approximately −150 mV (Johannes *et al.*, 1991), the Ca^{2+}-permeable channels reside predominantly in a closed state. However, a depolarization of the membrane to about −80 mV would cause the La^{3+}-sensitive channel to open and allow Ca^{2+} to enter the cytosol. Further depolarization of the membrane would activate the Gd^{3+} sensitive channel. In this context it is noteworthy that the Charophyte plasma membrane Ca^{2+} channel opens upon depolarization of the membrane to approximately −100 mV (Berestovskii *et al.*, 1987; Lunevsky *et al.*, 1983; MacRobbie and Banfield, 1988; Shiina and Tazawa, 1987; Tsutsui *et al.*, 1987).

Attempts to demonstrate a $\Delta\psi$-dependency of Ca^{2+} uptake into maize root protoplasts by using metabolic inhibitors to decrease $\Delta\psi$ generated by the plasma membrane electrogenic H^+-pump were unsuccessful. This may be because the H^+-pump, which is required to generate large negative $\Delta\psi$ in plant cells, may be relatively inactive in protoplasts so their $\Delta\psi$ is dominated by a K^+-diffusion potential. In this situation, inhibitors would have no effect on $\Delta\psi$ (except those that discharged the K^+ gradient) and therefore Ca^{2+} uptake would not be stimulated in the way expected from the voltage sensitivity of Ca^{2+} transport in the plasma membrane vesicles. In patch-clamped wheat root protoplasts, two populations of $\Delta\psi$ can be defined, one more negative than the other suggesting that some protoplasts have a $\Delta\psi$ dominated by the H^+-pump while in others it is determined by passive ion distributions (Tyerman, Findlay, Garrill and Skerrett, personal communication).

Currently there is no known Ca^{2+} channel that has been shown to have the inhibitor sensitivities reported in this paper. Most studies of Ca^{2+} uptake in plants have shown a sensitivity to one or other of the dihydropyridine or phenyl-

alkylamine Ca^{2+} channel blockers (Andrejauskas *et al.*, 1985; Graziana *et al.*, 1988; Harvey *et al.*, 1989; MacRobbie and Banfield, 1988; Shiina and Tazawa, 1987; Thuleau *et al.*, 1990; Tsutsui *et al.*, 1987). However, Rincon and Hanson (1986) reported that Ca^{2+} uptake in maize root segments, was strongly inhibited by La^{3+} and only weakly sensitive to verapamil. Also, Siebers *et al.* (1990) reported that Ca^{2+} influx into plasma membrane vesicles from *Commelina communis* L. could be blocked by La^{3+}. Lanthanum is a very strong inhibitor of Ca^{2+} channel activity in animal cells (Kusaka and Matsushita, 1987; Reed and Bygrave, 1974) and has been shown to block plasma membrane Ca^{2+} fluxes in plants (Rincon and Hanson, 1986; Siebers *et al.*, 1990). Gadolinium is an inhibitor of stretch-activated Ca^{2+} channels in animals (Yang and Sachs, 1989) and inhibits cytokinin-evoked Ca^{2+} currents at the plasma membrane of *Funaria* (Saunders, 1986). Recently it has been shown to inhibit a voltage-gated Ca^{2+} channel at the tonoplast of higher plants (Johannes *et al.*, 1991). In animal cells, the closest corresponding Ca^{2+} channel with properties similar to those reported here is that of the mitochondrion which has been shown to be inhibited by both ruthenium red and La^{3+} (Reed and Bygrave, 1974).

The sensitivity of Ca^{2+} influx at the plasma membrane to ruthenium red and lanthanides suggests that these compounds cannot be used to distinguish unequivocally between the contributions of external and internal Ca^{2+} stores to increases in cytosolic Ca^{2+} concentration. Recently, Knight *et al.* (1992) found that a wind-induced rise in cytosolic Ca^{2+} concentration in *Nicotiana plumbaginifolia* was inhibited by ruthenium red but not by Gd^{3+} or La^{3+} whereas a cold-induced increase in cytosolic Ca^{2+} was sensitive to Gd^{3+} or La^{3+} but not to ruthenium red. Based on the sensitivity of animal Ca^{2+} channels to these compounds, they argued that this indicated that wind caused release of Ca^{2+} from internal stores whereas cold caused Ca^{2+} influx across the plasma membrane. Our results with plasma membrane vesicles indicate that this conclusion may not be valid and that all of the compounds used by Knight *et al.* (1992) may affect Ca^{2+} influx at the plasma membrane thus both wind- and cold-induced changes in cytosolic Ca^{2+} concentration may involve changes in Ca^{2+} at the plasma membrane.

The results presented here raise several questions about the physiological relevance of the two Ca^{2+}-permeable pathways described in maize root plasma membrane. For instance, what are the functions of the two types of Ca^{2+}-permeable channels in the plasma membrane of root cells and how are their activities integrated with those of Ca^{2+} transport systems in other cellular membranes such as the tonoplast? One possibility is that the two pathways reside in different cells (e.g. endodermis and cortex) and so are on different populations of plasma membrane vesicles that are not separated by the two-phase aqueous partitioning technique. On the other hand, the presence of more than one class of Ca^{2+} channel in the plasma membrane of single mammalian cells is a common feature. In heart cells, both L- and T-type Ca^{2+} channels co-exist in the plasma membrane while L-, T-, and N-type Ca^{2+} channels are found in the plasma membrane of neuronal cells (Tsien and Tsien, 1990). It is thought that, since each channel type is modulated by a different hormone and/or effector, they are involved in the transduction of contrasting physiological responses and provide the cell with a greater range of sensitivity to effectors as well as the ability to respond with a more flexible series of outputs such as rises of cytosolic Ca^{2+} concentrations of differing durations. By analogy, the numerous growth regulators suggested to elicit Ca^{2+} influx into plant cells from the apoplast might exert their effects via different Ca^{2+}-permeable channels.

Further evidence for the physiological role of the putative Ca^{2+} channels identified in this study might be obtained by examining the effect of La^{3+} and Gd^{3+} on effector-mediated rises in cytosolic Ca^{2+} concentrations in maize roots. If Ca^{2+} influx is an important contributor to the rise in cytosolic Ca^{2+} it should be possible to inhibit responses with both La^{3+} and Gd^{3+} and mixtures of these should be as inhibitory as ruthenium red. In addition, depolarization of the membrane to -80 mV should induce Ca^{2+} influx and this should also be inhibited by the La^{3+}, Gd^{3+} and ruthenium red. Transport studies with isolated vesicles cannot prove that fluxes are mediated by ion channels and confirmation that La^{3+} and Gd^{3+} are inhibiting two different Ca^{2+} channels will require the application of the patch clamp technique (Hedrich and Schroeder, 1989). None the less, the results presented here provide a strong basis for proceeding with such studies and indicate areas for fruitful investigation by patch clamping. Such studies would enable the ionic selectivity of the Ca^{2+}-conducting pathways to be defined thereby discriminating between transport through Ca^{2+}-specific pathways from that through more general cation channels.

Experimental procedures

Plant material and chemicals

Maize seeds (*Zea mays* L. cv. Bastille or Fronica) were surface-sterilized with 10% (v/v) sodium hypochlorite, imbibed in running tap water for 4–6 h then germinated in the dark at 25°C. Seeds were layered on muslin suspended 5 cm above a vigorously aerated solution of 0.5 mM $CaSO_4$ and were harvested 5 days later. Chemicals were from BDH Chemicals or Sigma (both of Poole, Dorset, UK), unless otherwise stated. Radiochemicals were from Amersham International (Little Chalfont, Bucks, UK). ATP and PPi were converted to their 1,3-bis(tris(hydroxy-methyl)methyl-amino)-propane (BTP) salts using Dowex cation exchange resin (H^+-form) and titration with BTP.

Plasma membrane isolation

Plasma membrane vesicles were isolated using the technique of aqueous polymer two-phase partitioning (Larsson, 1985). The isolation procedure was carried out at 4°C. Maize roots were homogenized with mortar and pestle in a medium containing 250 mM sucrose, 10% (w/v) glycerol, 0.5% (w/v) bovine serum albumen (BSA), 2 mM dithiothreitol (DTT), 2 mM ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM phenyl-methylsulphonyl fluoride, 0.5% (w/v) polyvinylpolypyrollidone and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes)–BTP, pH 7.4. The final ratio of medium to roots was 4 ml g^{-1} fresh weight. The homogenate was filtered through four layers of muslin then centrifuged at 10 000 ***g*** (R_{max}) for 15 min (Type 35 rotor; Beckman, High Wycombe, Bucks, UK). The pellet was discarded and the supernatant was centrifuged at 80 000 ***g*** (R_{max}) for 30 min (Beckman Type 35 rotor) to sediment the microsomal membranes which were then resuspended in buffer (1 mg protein ml^{-1}) containing 250 mM sucrose, 10% (w/v) glycerol, 1 mM EGTA, 1 mM DTT and 10 mM N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine (Tricine)–BTP, pH 7.4. The resuspended microsomes were added to a two-phase system which contained 6.0% (w/w) Dextran T500 (Pharmacia, Milton Keynes, Bucks, UK), 6.0% (w/w) polyethylene glycol 3350 (PEG), 250 mM sucrose, 30 mM KCl, and 5 mM K phosphate buffer, pH 7.8. After phase separation the PEG-enriched upper phase (U_1) was removed and repartitioned on to a fresh lower phase. The resulting upper phase (U_2) and the initial lower phase (L_1) were retained and separately diluted fivefold with dilution buffer containing 250 mM sucrose, 1 mM DTT, and 10 mM Tricine-BTP, pH 7.4, and centrifuged at 380 000 ***g*** (R_{max}) for 15 min (Beckman 70.1 Ti rotor). Membranes were resuspended in 1 ml of dilution buffer and were used immediately or stored under liquid N_2 until required. Typical yield was 1–2 mg of protein in the plasma membrane-enriched U_2 phase.

Biochemical assays

ATPase activity was determined by measuring the release of Pi from ATP using the Pi assay of Ames (1966). The reaction medium (final volume 0.5 ml) contained 250 mM sucrose, 50 mM KCl, 1.5 mM ATP–BTP, 1.5 mM $MgSO_4$, 2.5 μg ml^{-1} gramicidin-D, 100 μg ml^{-1} lysophosphatidylcholine (lysoPC), 300 μM ammonium molybdate and 20 mM BTP-2-(N-morpholino)ethanesulphonic-acid (Mes), pH 6.5 or 8.0. Vanadate (100 μM), sodium azide (1 mM) or KNO_3 (50 mM) were also added to some assays. Reaction media were pre-incubated at 25°C and the reaction was started by the addition of membranes (5 μg protein). Control treatments contained boiled membranes. Reaction was stopped after 60 min at 25°C by the addition of Pi assay reagent (Ames, 1966) and the absorbance at 820 nm was measured after 30 min. Trypsin-sensitive ATPase was determined by incubating plasma membrane vesicles (100 μg protein) at 25°C in a medium (final volume 2 ml) containing 250 mM sucrose, 25 μg ml^{-1} trypsin, 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)–HCl, pH 7.0, and 0 or 100 μg ml^{-1} lysoPC. Reaction was started by the addition of the membranes and stopped by the removal of 200 μl aliquots which were added to 200 μl of 250 mM sucrose, 75 μg ml^{-1} soybean trypsin inhibitor (Sigma). Aliquots of this were assayed for ATPase activity as described above. Pyrophosphatase (PPase) activity was determined by measuring the Pi released from PPi as described by Pope and Leigh (1987). Latent IDPase was determined by the method of Green (1983) after the membranes had been stored at 4°C for 72 h. NADH-ferricyanide reductase was assayed by measuring the reduction of ferricyanide (Buckhout and Hrubec, 1986). Latency of this activity was determined by adding 100 μg ml^{-1} lysoPC or 200 μg ml^{-1} Triton X-100. Cytochrome c oxidase and antimycin A-insensitive NADH-cytochrome c reductase were measured using the methods of Hodges and Leonard (1974). Aspartate aminotransferase was assayed with a commercial kit (Sigma). Glucan synthetase II was measured by the method of Kauss and Jeblick (1985). Protein was assayed by the method of Bradford (1976) using BSA as a standard.

Freeze-fracture electron microscopy

Modifications of the methods of Marty (1982) and Robards and Clarkson (1984) were used for membrane vesicles and whole roots, respectively. The plasma membrane pellet was resuspended in 200 μl of 250 mM sucrose, 20 mM Tricine–BTP, pH 7.4. Specimens were placed in Cu specimen holders, immersed in a liquid N_2 slurry and were kept below −100°C while being transferred into specimen cups and then into a Leybold-Heraeus Biotech 2005 automatic freeze-etching unit. Freeze-fracture and replica formation were performed with a vacuum of 10^{-5} Pa or better and with a specimen temperature of −110 to −115°C at the time of fracturing. Coating with Pt (at 45° to the sample) and then with C took place immediately after fracturing. The specimen holders were removed from the freeze-fracture machine and the replicas carefully washed three times in distilled water and then placed in 50% (w/v) chromic acid overnight. The replicas were washed three further times in distilled water, mounted on C-coated Cu grids and were viewed in a Hitachi HU12A electron microscope. All images were recorded at standard magnifications to allow direct comparisons of micrographs.

Measurement of intravesicular volume

Intravesicular volume of isolated plasma membrane preparations was estimated using a modification of the method of Rottenberg (1989). The plasma membrane vesicles (400 μg membrane protein) were suspended in a medium (total volume 200 μl) containing 250 mM sucrose, 3.7 kBq ml^{-1} ^3H_2O, 37 kBq ml^{-1} [^{14}C]inulin and 10 mM Tricine–BTP, pH 7.4. A 100 μl aliquot of the suspension was layered over a mixture (ratio 3:1) of silicone fluid DC 550 and dinonyl phthalate in 1.5 ml conical-bottomed polyallomer centrifuge tubes (Beckman). These were then centrifuged for 10 min at 380 000 ***g*** (R_{max}; TLS-55 swinging bucket rotor, Beckman TL-100 ultracentrifuge). Twenty microlitres of supernatant were removed and then the pelleted membranes were recovered by cutting off the end of the tube as near to the pellet as possible. The excised end of the tube was placed in a scintillation vial and 5 μl of 1% (w/v) SDS were added to dissolve the pellet. The ^{3}H and ^{14}C in the supernatant and the pellet were determined by liquid scintillation counting. Calculations of total, extravesicular and intravesicular volumes were as described by Rottenberg (1989).

Imposition of $\Delta\psi$ and measurement of Ca^{2+} transport

Membrane potentials were usually imposed using a K^+/valinomycin clamp. The plasma membrane vesicles were loaded with 4 mM K^+-gluconate by resuspending them in 250 mM sucrose, 1 mM DTT, 4 mM K-gluconate and 20 mM Tricine–BTP, pH 7.4, and then incubating at 37°C for 30 min, then on ice for 10 min. The extravesicular K^+ was removed by washing the membranes twice in K^+-free medium (380 000 ***g*** for 15 min, R_{max}; 70.1 Ti

rotor). The $\Delta\psi$ was imposed by diluting the K-gluconate-loaded vesicles into buffer containing 0.04–400 mM K-gluconate, 100 nM valinomycin, 20 mM Tricine–BTP, pH 7.4, and sufficient sucrose to ensure that all solutions were iso-osmotic. This resulted in the imposition of $\Delta\psi$ with theoretical values of between –120 and +60 mV, assuming $\Delta\psi$ is clamped at the equilibrium potential for K^+. For the experiment in Figure 2, more negative membrane potentials were imposed using SCN^- gradients by diluting plasma membrane vesicles into medium containing 250 mM sucrose, up to 10 mM KSCN and 20 mM Tricine–BTP, pH 7.4.

The formation of an inside-negative $\Delta\psi$ within the vesicles was measured with [^{14}C]TPP^+ (Höfer and Künemund 1984; Perlin *et al.*, 1988). The time course of [^{14}C]TPP^+ uptake was measured at 25°C in a reaction medium (final volume 1 ml) containing 250 mM sucrose, 5 µM [^{14}C]TPP^+ (18.5 kBq ml^{-1}), 0.04–400 mM K-gluconate, 100 nM valinomycin, 10 µM $CaSO_4$ and 20 mM Tricine–BTP, pH 7.4. The reaction was started by the addition of the vesicles (100–200 µg protein). Duplicate samples were removed at specific time intervals and filtered through Whatman (Maidstone, Kent, UK) WCN cellulose nitrate membrane filters (0.45 µm pore diameter). The procedure consisted of wetting the filters with 1 ml ice-cold wash medium (250 mM sucrose, 30 µM unlabelled TPP^+, and 20 mM Tricine–BTP, pH 7.4), rapidly filtering the sample under vacuum and rinsing with a further 5 ml of wash medium. The filters were dried and the radioactivity was determined by liquid scintillation counting.

Accumulation of $^{45}Ca^{2+}$ was measured at 25°C in a reaction medium (final volume 1 ml) containing 250 mM sucrose, 10 µM $^{45}CaCl_2$, 2.5 µg ml^{-1} gramicidin-D, variable concentrations of K^+-gluconate and 20 mM Tricine-BTP, pH 7.4 and plasma membrane vesicles (100–200 µg protein). The reaction was started by the addition of the ^{45}Ca (approximately 75 kBq ml^{-1} in uptake medium; activity at time of delivery and all batches were used within one half-life). Duplicate 100 µl samples were removed at specific time intervals and filtered as described above using a wash medium containing 250 mM sucrose and 20 mM Tricine–BTP, pH 7.4. Initial rates of Ca^{2+} uptake were measured by filtering the vesicles 15 sec after the addition of the ^{45}Ca. In experiments where the effect of channel blockers was investigated, plasma membrane vesicles were pre-incubated for 10 min at 25°C with the inhibitor, Ca^{2+} uptake was initiated by the addition of 10 µM $^{45}Ca^{2+}$ and was stopped after 15 sec by filtration.

Isolation of protoplasts and measurement of Ca^{2+} transport

Protoplasts were isolated from 5-day-old maize roots by a modification of the method of Kasai and Bayer (1991). Roots were excised from the plant and chopped with a razor blade and then were placed in the digestion medium (0.6 M mannitol, 1 mM $CaCl_2$, 0.05% (w/v) BSA, 0.5 mM DTT, 0.1% (w/v) macerase-pectinase (Calbiochem), and 0.05% (w/v) cellulysin (Calbiochem), pH 5.6) at a tissue: medium ratio of 1 g ml^{-1}. The roots were incubated for 4 h on a rotary shaker (50 r.p.m.) at room temperature. The mixture was then filtered through three layers of muslin and centrifuged at 180 ***g*** for 6 min. The supernatant was discarded and the pellet was resuspended in 12 % (w/v) Ficoll, 0.63 M mannitol, 0.1 mM $CaCl_2$, 1.25 mM Tris–HCl, pH 6.5 and the suspension was placed in a centrifuge tube and overlaid with similar solutions containing 8, 5, and 0% (w/v) Ficoll. This step gradient was centrifuged at 360 ***g*** for 12 min and the protoplasts were recovered from the interfaces between the 0 and 5% and the 5 and 8% Ficoll layers. No difference could be discerned between the protoplasts recovered from these two interfaces either by light microscopy or flow cytometry and therefore they were pooled, diluted with 0.63 M mannitol, 0.1 mM $CaCl_2$, 1.25 mM Tris–HCl, pH 6.5 and centrifuged at 180 ***g*** for 6 min. The supernatant was discarded and the pellet was resuspended in uptake medium consisting of 0.63 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM KCl, and 10 mM Tris–HCl, pH 7.5. Once resuspended, protoplasts were kept in the dark at 4°C for 1–3 h before the experiment. The mean protoplast yield was $3.6 \times 10^5 \pm 1.22 \times 10^5$ cells g^{-1} fresh weight ($n = 13$).

Acknowledgements

We thank Dr David Cooke (Long Ashton Research Station) for advice on the aqueous two-phase partitioning technique, and Peter Crosby and Meg Stark (University of York) for assistance with freeze fracturing. J.M. was the recipient of an AFRC postgraduate research studentship held at both Rothamsted and York. A.C. was supported by a fellowship from the Spanish Ministry of Education.

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