Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3473–3477, April 1994
Plant Biology

Voltage-dependent Ca^{2+} influx into right-side-out plasma membrane vesicles isolated from wheat roots: Characterization of a putative Ca^{2+} channel

JIANWEI W. HUANG, DAVID L. GRUNES, AND LEON V. KOCHIAN*

U.S. Plant, Soil and Nutrition Laboratory, U.S. Department of Agriculture–Agricultural Research Service, Cornell University, Ithaca, NY 14853

Communicated by Emanuel Epstein, December 20, 1993 (received for review June 11, 1993)

ABSTRACT **We report on the identification of a voltage-dependent Ca^{2+} transport system that mediates Ca^{2+} influx across the plasma membrane (PM) of wheat (*Triticum aestivum*) root cells. The experimental approach involved the imposition of transmembrane electrical potentials (via K^+ diffusion potentials) in populations of purified, right-side-out PM vesicles isolated from wheat roots. Using $^{45}Ca^{2+}$ to quantify Ca^{2+} influx into the PM vesicles, the voltage-dependent characteristics of Ca^{2+} transport were found to be similar to those exhibited by L-type voltage-gated Ca^{2+} channels in animal cells. The putative PM Ca^{2+} channel opened upon depolarization of the membrane potential, and Ca^{2+} flux increased to a maximum upon further depolarization and then decreased back to zero upon further successive depolarizations. This channel was found to be selective for Ca^{2+} over Mg^{2+}, Sr^{2+}, K^+, and Na^+; was blocked by very low concentrations of La^{3+}; was unaffected by high concentrations of the K^+ channel blocker tetraethylammonium; and exhibited Michaelis–Menten-type transport kinetics. Based on these transport properties, we argue that this transport system is a PM Ca^{2+} channel. We suggest that the use of radiotracer flux analysis of voltage-clamped PM vesicles derived from plant roots is a straightforward approach for the characterization of certain voltage-gated ion channels functioning in cellular membranes of higher plant cells.**

The activity of cytosolic free calcium (Ca^{2+}) is essential for the regulation of a number of metabolic and developmental processes in plants. The transport systems involved in cytoplasmic Ca^{2+} homeostasis have been studied in greater detail in animal cells, where cytosolic Ca^{2+} concentrations rise as a result of entry via Ca^{2+} channels in the plasma membrane (PM) and release from internal stores through endomembrane-localized Ca^{2+} channels (1). The best-characterized Ca^{2+} channels in animal cells are the voltage-gated Ca^{2+} channels functioning across the PM. In general, voltage-gated Ca^{2+} channels are characterized by bell-shaped current–voltage relationships, with channel opening upon depolarization and subsequent increase in Ca^{2+} current to a maximum upon further successive depolarizations. The Ca^{2+} current then decreases upon further depolarization as the electrochemical driving force for Ca^{2+} influx diminishes (2, 3).

In higher plant cells, however, detailed information concerning the transport properties of PM Ca^{2+} channels is still lacking. Voltage clamp studies on tonoplast-free cells of *Nitellopsis obtusa* demonstrated the presence of a Ca^{2+} channel that activated upon depolarization, did not exhibit time-dependent inactivation, and was blocked by La^{3+} and nifedipine (4). Photoaffinity labeling of membrane proteins derived from carrot suspension cell protoplasts with a Ca^{2+} channel blocker from the phenylalkylamine family identified a protein possibly localized in the PM (5). This protein bound the phenylalkylamine azido derivative with a high affinity. Patch clamp studies, on liposomes containing this protein, showed the presence of an unstable Ca^{2+}-permeable channel that rapidly gave way to a nonselective ion channel permeable to Cl^- and Ca^{2+} ions (6). Finally, evidence has recently been presented for stretch-activated Ca^{2+} channels in the PM of *Vicia faba* guard cells and onion leaf epidermal cells (7, 8). However, presumably because Ca^{2+} channels functioning in the PM of higher plant cells exist in very low abundance and spend most of their time in a closed state in comparison with K^+ channels, they have proven to be difficult to identify and characterize using patch clamp techniques that have yielded considerable information concerning K^+ and anion channels. In this study, we report on the identification and characterization of a voltage-dependent Ca^{2+} transport system that mediates Ca^{2+} influx across the PM, using radiotracer flux analysis of voltage-clamped PM vesicles from wheat roots.

MATERIALS AND METHODS

PM Preparation. Wheat (*Triticum aestivum* Scout 66) seedlings were grown in nutrient solution as described (9). Roots (200 g) of 20 day-old plants were homogenized in a Waring blender containing 150 ml of buffer [250 mM sucrose/50 mM K_2SO_4/15 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol (DTT)/0.6% polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was then centrifuged at 50,000 × *g* for 40 min. The resulting microsomal fraction (MF) was suspended in 250 mM sucrose/5 mM K_2HPO_4, pH 7.8/3 mM KCl/1 mM DTT. The MF was partitioned using an aqueous two-phase system (10), with a final composition of 6.2% (wt/wt) dextran T500/6.2% (wt/wt) polyethylene glycol 3350/250 mM sucrose/5 mM K_2HPO_4, pH 7.8/3 mM KCl. The final upper phase (U_3) containing purified PM was mixed with dilution buffer (250 mM sucrose/50 mM K_2SO_4/5 mM Mes-Tris, pH 7.0/1 mM DTT), pelleted at 100,000 × *g* for 1 hr, and resuspended in the same dilution buffer.

Protein and Enzyme Analysis. Protein was assayed by the method of Bradford (11). Vesicle sidedness, based on latency of ATPase activity, was determined in isoosmotic medium with or without 0.02% (wt/vol) Brij 58 (12). Purity of PM preparations was determined by assaying for contaminating membrane marker enzyme activities (13).

Ca^{2+} Transport Assays. $^{45}Ca^{2+}$ influx was measured at 22°C in 1.0 ml of reaction medium consisting of 250 mM sucrose, 5 mM Mes-Tris (pH 7.0), 20 μg of membrane protein, 5 μM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: E_m, membrane potential; MF, microsomal fraction; PM, plasma membrane; TPP, tetraphenylphosphonium; U_3, upper phase from aqueous two-phase partitioning; TEA, tetraethylammonium.

*To whom reprint requests should be addressed.

valinomycin, 10–500 μM $CaCl_2$, and $^{45}Ca^{2+}$ (3.7×10^4 Bq·ml^{-1}). To examine the voltage dependence of Ca^{2+} influx, external K^+ (as K_2SO_4) was varied from 0.01 to 350 mM. For experiments with Ca^{2+} channel blockers or for cation (Sr^{2+}, Mg^{2+}, Ba^{2+}, K^+, Na^+) selectivity studies, the appropriate concentration of channel blocker or competing cation was added to the reaction mixture 3 min before the initiation of $^{45}Ca^{2+}$ flux. For studies of Ca^{2+} versus K^+ selectivity, the internal and external K^+ was varied in tandem to maintain the membrane potential (E_m) at −100 mV, while measuring Ca^{2+} influx from an uptake solution containing 100 μM Ca^{2+} and 0.1–10 mM K^+. The osmotic potentials of all experimental solutions were adjusted to 370 ± 10 mmol·kg^{-1} using sorbitol. Measurement of Ca^{2+} influx was initiated by adding $^{45}Ca^{2+}$-labeled Ca^{2+} solutions to the reaction medium, and uptake was terminated after 3 min by pipetting the reaction mixture into 3 ml of desorption solution (250 mM sucrose/50 mM K_2SO_4/5 mM Mes-Tris, pH 7.0/1 mM EGTA), in a vacuum filtration apparatus on top of a 0.45-μm cellulose nitrate filter (Whatman). After filtering, the PM vesicles were washed two more times with 3 ml of desorption solution. Filters were dissolved in 10 ml of scintillation solution (Ready Protein$^+$, Beckman), and radioactivity was determined by liquid scintillation counting.

E_m Measurement. K^+ diffusion potentials were imposed across PM vesicles via a K^+-valinomycin voltage clamp. These transmembrane E_m values were measured by the method of Rottenberg (14). The reaction mixture was identical in composition to the Ca^{2+} uptake solution except that it contained tetraphenylphosphonium {([^{3}H]TPP) 5 μM, 7.4×10^3 Bq·ml^{-1}}. [^{3}H]TPP accumulation into PM vesicles was terminated by vacuum filtration of vesicles on polysulfone membrane filters (0.45 μm; Supor, Gelman). For determination of PM vesicle internal volume, vesicles (200 μg of membrane protein) were incubated in 0.2 ml of the same reaction mixture described above containing ^3H_2O (9.3×10^4 Bq·ml^{-1}) and 5 μM [^{14}C]carboxydextran (M_r = 70,000; 1.7×10^4 Bq·ml^{-1}). The PM vesicles were incubated in the reaction mixture for 10 min at 22°C and then pelleted via centrifugation at 60,000 × g for 5 min in a tabletop ultracentrifuge (Optima TLX, Beckmaan).

RESULTS

Purity and Sidedness of PM Vesicle Preparation. The U_3 phase was highly enriched in PM, with a 4-fold enrichment over the MF. This phase was relatively free of contaminating endomembranes, with no detectable tonoplast contamination and trace amounts of mitochondrial, Golgi, and endoplasmic reticulum membranes. The total marker enzyme activity for contaminating endomembranes was <10% of K^+-stimulated ATPase activity associated with the PM in the U_3 phase. For reasons enumerated below, it is highly unlikely that the small amount of contaminating membranes could contribute significantly to the voltage-dependent Ca^{2+} influx activity characterized in this study.

The orientation of the PM vesicles in the U_3 phase was 85–95% right-side-out, as determined from the proportion of K^+-stimulated ATPase activity in the presence and absence of 0.02% Brij 58 (12). By comparison, the percent sidedness of PM vesicles in the MF and first lower phase (L_1) was found to be 56% and 39%, respectively.

Measurement of PM Vesicle E_m Values. To study the voltage dependence of Ca^{2+} influx across the wheat root PM, K^+ diffusion potentials resulting in E_m values ranging from −236 to +32 mV (as determined by the Nernst equation) were imposed across the PM vesicles. The equilibrium distribution of TPP across the PM was measured to compare actual E_m values with those predicted by the Nernst equation. For E_m values between −50 and −150 mV, the measured voltages were in good agreement with the predicted ones (Table 1). When membrane potentials more negative than −150 mV were imposed, the measured values began to deviate from the predicted voltages. This discrepancy at more negative E_m values might result from a deviation, at equilibrium, of the internal K^+ concentration from the K^+ concentration initially imposed (100 mM). This technique did not allow us to measure E_m values more positive than −50 mV. Thus, for studies on the voltage dependency of Ca^{2+} influx, we have chosen to use the predicted E_m values, with the proviso that the most negative E_m values might represent a modest overestimation of actual E_m values. The E_m values were constant for the duration of the 3-min Ca^{2+} uptake period.

Table 1. Comparison of predicted and measured E_m values for PM vesicles

External K^+, mM	E_m, mV	
	Predicted*	Measured†
0.04	−200	−152 ± 11
0.28	−150	−130 ± 10
2.00	−100	−94 ± 8
14.2	−50	−59 ± 10

*Calculated using the Nernst equation.
†Values are mean ± SE (n = 4).

Voltage-Dependent Ca^{2+} Influx. Radiotracer Ca^{2+} influx into right-side-out PM vesicles was strongly voltage dependent, with little Ca^{2+} uptake at very negative potentials and increasing Ca^{2+} uptake upon depolarization of E_m to values less negative than −170 mV (Fig. 1). The voltage dependency of Ca^{2+} influx exhibited a maximum at −100 mV, and then Ca^{2+} influx decreased to near-zero as E_m was depolarized further. The membrane vesicle preparation used for these transport experiments was highly enriched in PM, with only trace amounts of contaminating membranes. To demonstrate that voltage-dependent Ca^{2+} transport activity was not due to a highly active endomembrane contamination, Ca^{2+} influx at an E_m of −100 mV was measured in the MF, the L_1 phase (enriched in endomembranes), and the U_3 phase (PM-enriched). Ca^{2+} influx (in 100 μM $CaCl_2$) into vesicles from the U_3 phase was 5.0 nmol of Ca^{2+} per (mg·min)$^{-1}$, while Ca^{2+}

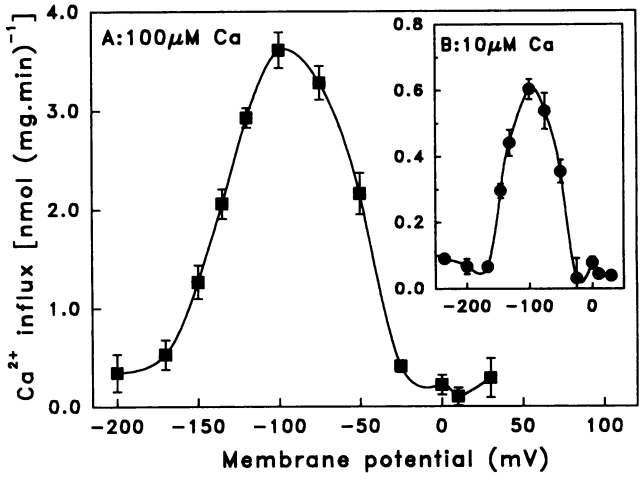

FIG. 1. Voltage dependence of Ca^{2+} influx into right-side-out PM vesicles of wheat roots. E_m values were imposed by K^+-valinomycin clamps with 100 mM K^+ (as K_2SO_4) inside the vesicles, and various external K^+ concentrations (0.01–350 mM K as K_2SO_4). Valinomycin (5 μM) was added to the incubation medium 5 min before the Ca^{2+} flux measurements. The uptake solution contained either 100 μM Ca^{2+} (*A*) or 10 μM Ca^{2+} (*B*). Each point is the mean of replicated measurements from two independent experiments. Error bars represent ±SE (n = 6).

influx into vesicles from the MF and L_1 phase was 1.30 and 0.75 nmol of Ca^{2+} per $(mg \cdot min)^{-1}$, respectively. These results strongly indicate that voltage-gated Ca^{2+} transport is occurring into membrane vesicles that are PM in origin.

Time-Dependent Kinetics of Voltage-Gated Ca^{2+} Influx. For studies characterizing Ca^{2+} influx via the putative PM Ca^{2+} channel, all experiments were run at an E_m value of -100 mV. Ca^{2+} uptake was linear for the first 2–3 min and then began to saturate (Fig. 2). These results indicate either that the PM vesicles do not leak accumulated radiotracer and Ca^{2+} channels close as the internal $[Ca^{2+}]$ rises or that the vesicles do leak $^{45}Ca^{2+}$ and as Ca^{2+} is accumulated a significant Ca^{2+} efflux occurs, resulting in Ca^{2+} flux equilibrium.

This was investigated further by allowing PM vesicles to accumulate radiotracer for 4 min, and then vesicles (with uptake solution) were transferred to a new isoosmotic solution where external $[K^+]$ was altered to yield an E_m of 0 mV. As shown in Fig. 2, the PM vesicles did not lose accumulated $^{45}Ca^{2+}$ over the next 10 min, indicating that they were not leaky for Ca^{2+}. When the Ca^{2+} chelator EGTA (1 mM) was included in the uptake termination solution, an initial loss of $\approx$25% of the accumulated $^{45}Ca^{2+}$ was observed, and then no further loss of radiolabel occurred.

To verify that the $^{45}Ca^{2+}$ was being transported into the vesicle lumen and not merely binding to the outer surface of the membrane, PM vesicles were allowed to accumulate $^{45}Ca^{2+}$ for 4 min, uptake was terminated, and then the Ca^{2+} ionophore A23187 was added. As depicted in Fig. 2, all of the radiolabel accumulated after 4 min of uptake was released upon addition of A23187, indicating that we were indeed studying the transport of Ca^{2+} across the wheat root-cell PM.

Cation Specificity and Studies with Ca^{2+} Channel Blockers. The effects of La^{3+}, nifedipine, and verapamil on Ca^{2+} influx via this transport system were investigated. As shown in Fig. 3, 5 μM La^{3+} completely blocked voltage-dependent Ca^{2+} influx in 100 μM Ca^{2+}. A 50% inhibition of Ca^{2+} was elicited by 2 μM La^{3+}. On the other hand, nifedipine and verapamil had little inhibitory effect on this putative plant PM Ca^{2+} channel (Fig. 3 *Inset*). At lower concentrations (<100 μM), both verapamil and nifedipine caused a modest stimulation of Ca^{2+} influx, while for verapamil, only very high concentrations (500 or 1000 μM) inhibited Ca^{2+} influx (30–50% inhibition).

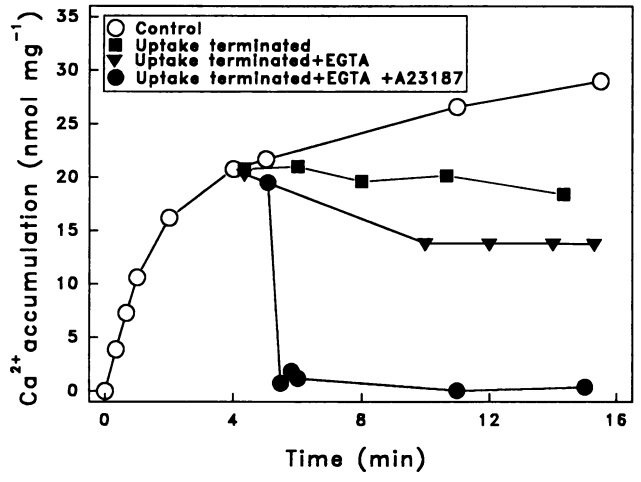

FIG. 2. Representative time course of Ca^{2+} accumulation into right-side-out PM vesicles from wheat roots. The uptake solution contained 100 μM Ca^{2+} and the E_m was -100 mV. The open circles represent the control data for voltage-dependent Ca^{2+} accumulation. The closed symbols represent data from an experiment in which $^{45}Ca^{2+}$ accumulation was terminated after 4 min by transferring aliquots of the reaction mixture to the appropriate solutions such that the E_m was shifted from -100 to 0 mV, and the $^{45}Ca^{2+}$ was diluted (1:5 dilution). When used, the final concentrations of EGTA and A23187 were 1 mM and 5 μM, respectively.

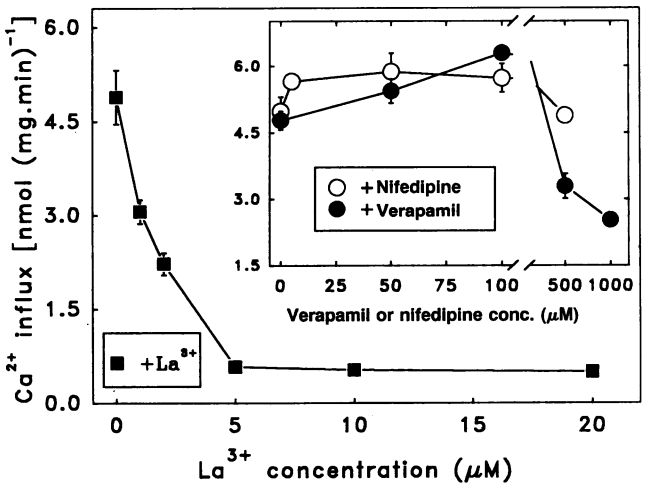

FIG. 3. Influence of varying concentrations of the inorganic Ca^{2+} channel blocker La^{3+} and the organic channel blockers verapamil and nifedipine (*Inset*) on Ca^{2+} influx into right-side-out PM vesicles from wheat roots. The uptake solution contained 100 μM Ca^{2+} and the E_m was -100 mV. Each point is the mean of replicate measurements from two independent experiments. Error bars represent $\pm$SE (n = 4).

Studies of Ca^{2+} uptake in solutions containing up to 10 mM K^+ and Na^+ indicated that the Ca^{2+} transport system was selective for Ca^{2+} over these monovalent cations (Fig. 4). The observation that up to 10 mM TEA had little effect on Ca^{2+} influx also strongly suggests that the major pathway for Ca^{2+} transport across the PM is not via a K^+ channel. Ca^{2+} uptake in the presence of increasing concentrations of Mg^{2+} or Sr^{2+} showed a modest inhibition of transport (Fig. 4 *Inset*).

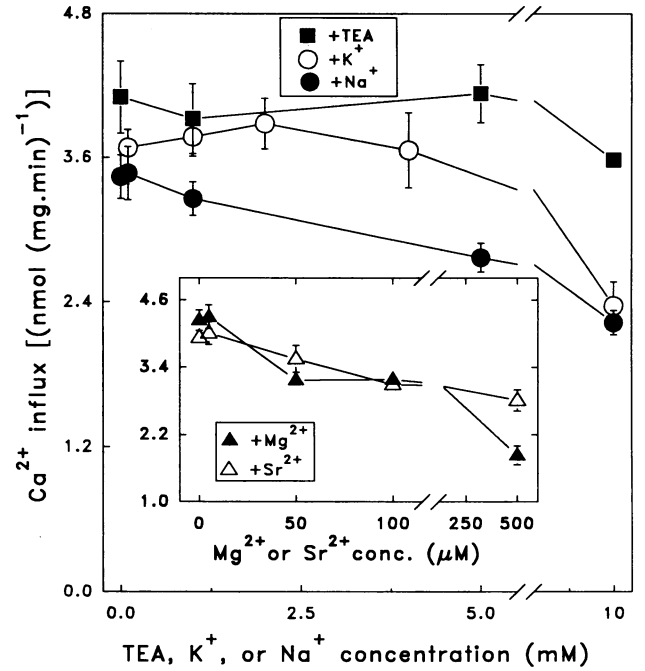

FIG. 4. Influence of varying concentrations of K^+, Na^+, and tetraethylammonium (TEA) on Ca^{2+} influx into right-side-out PM vesicles from wheat roots. Additionally, the *Inset* depicts the influence of varying concentrations of Mg^{2+} or Sr^{2+} on Ca^{2+} influx under the same conditions. All uptake solutions contained 100 μM Ca^{2+}. Each point is the mean of replicate measurements from two independent experiments. Error bars represent $\pm$SE (n = 6).

Exposure to 100 μM Mg^{2+} or Sr^{2+} elicited a 20% inhibition of Ca^{2+} influx, while 500 μM Mg^{2+} or Sr^{2+} inhibited Ca^{2+} uptake by 30–55%. Also, exposure to Ba^{2+} was studied (data not shown); 100 μM Ba^{2+} caused a modest stimulation of Ca^{2+} uptake, while 500 μM Ba^{2+} had little effect on Ca^{2+} transport.

Concentration-Dependent Kinetics for Voltage-Gated Ca^{2+} Transport. Ca^{2+} uptake via this voltage-gated transporter yielded Michaelis–Menten concentration-dependent kinetics (in 10–500 μM Ca^{2+}) (Fig. 5). The Ca^{2+} influx kinetic data were analyzed via Hanes–Woolf linear transformation (15) and yielded a K_m of 167 μM and V_{max} of 12 nmol of Ca^{2+} per $(mg \cdot min)^{-1}$ for Ca^{2+} transport. The concentration-dependent kinetics for Ca^{2+} influx in the presence of La^{3+} (1–5 μM) and Mg^{2+} (100 or 500 μM) indicated that both ions competitively inhibited Ca^{2+} transport (Fig. 5). For example, Ca^{2+} influx in the presence of 0, 1, and 2 μM La^{3+} increased the K_m for Ca^{2+} uptake from 167 to 305 and 380 μM, respectively, while having no significant effect on the V_{max}. Exposure to 100 and 500 μM Mg^{2+} caused the K_m to increase to 240 and then 332 μM, while again having little effect on the V_{max}. These results are consistent with competitive inhibition of Ca^{2+} uptake arising from La^{3+} or Mg^{2+} blockade of the channel, or possibly by competitive transport of Mg^{2+} through the channel.

DISCUSSION

In this study, we have developed a radiotracer ($^{45}Ca^{2+}$) flux technique using purified, right-side-out PM vesicles from wheat roots that has enabled us to identify and characterize a voltage-dependent Ca^{2+} influx across the PM. We feel that a strong case can be made that this transport system is a voltage-gated Ca^{2+} channel based on the arguments made below.

(*i*) *Voltage dependency.* The dramatic voltage dependence of this Ca^{2+} transport system, depicted in Fig. 1, is certainly characteristic of voltage-gated ion channels. We do not know of any non-channel ion transport systems that exhibit such a voltage dependency. Furthermore, the voltage dependency, which is characterized by an activation of Ca^{2+} influx upon depolarization in a steeply voltage-dependent manner, with Ca^{2+} influx rising to a maximum and then declining to zero flux upon successive depolarizations of E_m, is most similar to the responses of voltage-gated Ca^{2+} channels that have been characterized in some detail in animal cells (1, 2).

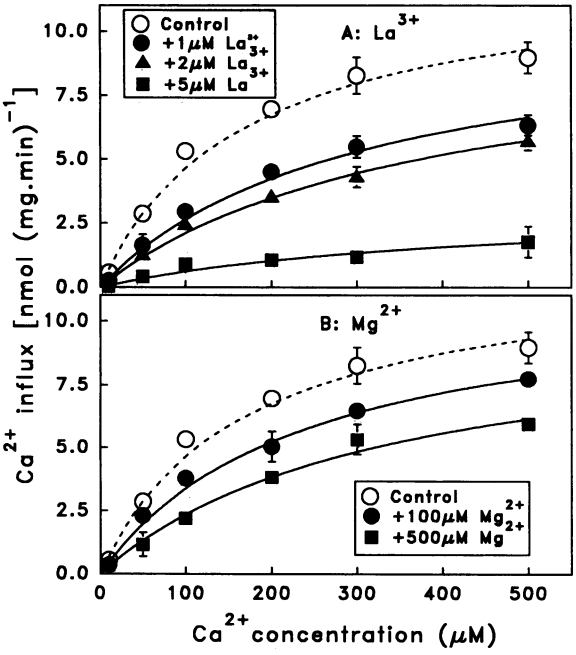

FIG. 5. Influence of La^{3+} (*A*) or Mg^{2+} (*B*) on concentration-dependent kinetics for Ca^{2+} influx into right-side-out PM vesicles from wheat roots. The E_m was −100 mV. Each point is the mean of replicate measurements from three independent experiments. Error bars represent ±SE (n = 6).

We feel we have unequivocally demonstrated a true Ca^{2+} influx into right-side-out PM vesicles, based on the purity and latency of the membrane preparations, and the ability to release nearly all of the accumulated $^{45}Ca^{2+}$ from the vesicles with the Ca^{2+} ionophore A23187 (Fig. 2). In any living cell, the two best candidates for a voltage-gated ion channel that would mediate Ca^{2+} influx would be a Ca^{2+} channel or, possibly, an inward-rectifying K^+ channel. Fairley-Grenot and Assmann (16) have demonstrated that inwardly rectifying K^+ channels in the guard cell PM can mediate Ca^{2+} influx. However, the voltage characteristics presented here are not consistent with the functioning of inwardly rectifying K^+ channels, which open upon hyperpolarization of E_m. In the aforementioned work of Fairley-Grenot and Assmann, they did describe some U-shaped current–voltage characteristics for what appears to be an inward K^+ current. However, this was only seen in high (10 mM) external Ca^{2+}. In the work presented here, the bell-shaped voltage dependency of Ca^{2+} influx was observed even in very low external Ca^{2+} (10 μM).

(*ii*) *Time-dependent kinetics of Ca^{2+} influx.* Because ion channels transport ions very rapidly, moving millions of ions per second, and can open and close very rapidly, sophisticated microelectrode techniques such as patch clamp methods are usually required to monitor ion transport processes occurring in milliseconds to seconds. At first glance, the relatively slow time course of $^{45}Ca^{2+}$ accumulation depicted in Fig. 2, where apparent isotopic equilibration occurs in minutes and not seconds, seems to argue against the operation of an ion channel. However, as noted by Hille (3), the open probability of most Ca^{2+} channels is <0.1. For example, in the recent study of a tonoplast Ca^{2+} channel in red beet (17), the open probability was 0.06 and the mean open time was <2% of the mean closed time. Therefore, in one of our measurements of Ca^{2+} influx, we are monitoring $^{45}Ca^{2+}$ influx into a population of $\approx 5 \times 10^9$ vesicles, which would consist of some vesicles containing zero Ca^{2+} channels and some vesicles with one or more channels. Over the 3-min time period for which Ca^{2+} flux is measured, we are monitoring a Ca^{2+} flux averaged over a very large number of putative Ca^{2+} channels that are closed for the majority of the uptake measurement. Using the mean closed time for the tonoplast Ca^{2+} channel (17), over the 3 min of $^{45}Ca^{2+}$ uptake, the PM Ca^{2+} channels would be open <3 sec.

There are two examples in the literature where ion channel activity was studied with radioisotopes in membrane vesicles. Garty *et al.* (18) developed a counter flux technique to study $^{22}Na^+$ fluxes in gramicidin channels incorporated into liposomes, and Na^+ channels in toad bladder and rat brain membrane vesicles, which yielded similar time-dependent kinetics to those presented here. Subsequently, Wang *et al.* (19) used the same technique to identify a K^+ channel in the chloroplast inner envelope. In that study, the same relatively slow time course was observed for $^{86}Rb^+$ influx.

(*iii*) *Ca^{2+} selectivity of transport system.* The methods employed here to quantify voltage-dependent Ca^{2+} fluxes in PM vesicles do not allow us to quantify directly the permeability ratios for this putative channel for Ca^{2+} over other cations. However, it is possible to estimate indirectly and qualitatively the cation selectivity of this transporter by measuring Ca^{2+} influx in the presence of increasing concentrations of competing ions. The results of these experiments indicate that the transporter is highly selective for Ca^{2+}; it appears to prefer Ca^{2+} over Mg^{2+}, Sr^{2+}, and Ba^{2+} (Figs. 4 and

5). These results are surprising, since the animal PM Ca^{2+} channels often exhibit a higher permeability to Sr^{2+} and especially, Ba^{2+}, over Ca^{2+} (3). In only one other study of putative plant PM Ca^{2+} channels has the divalent cation selectivity been investigated in detail. Shiina and Tazawa (4) found that a voltage-dependent inward Ca^{2+} current across the *Nitellopsis* PM was maintained when external Ca^{2+} was replaced with Sr^{2+}, but not when Mg^{2+}, Ba^{2+}, or Mn^{2+} was substituted. Although it is difficult to reconcile our results with those previously presented for animal PM Ca^{2+} channels, the results presented here and in the work of Shiina and Tazawa suggest that plant PM Ca^{2+} channels are different from their animal counterparts.

The work of Fairley-Grenot and Assmann (16) indicated that K^+ channels can be a transport pathway for Ca^{2+} influx. Therefore, we investigated the effect of the presence of up to 10 mM K^+ and Na^+ in the uptake solution on Ca^{2+} influx. As shown in Fig. 4, even the presence of 10 mM K^+ or Na^+ had only a moderate effect on Ca^{2+} influx from 0.1 mM Ca^{2+}. We also investigated Ca^{2+} influx in the presence of the K^+ channel blocker TEA. As shown in Fig. 4, 10 mM TEA had little effect on Ca^{2+} uptake. Taken together, these results along with the observed voltage characteristics that are distinctly different from those exhibited by voltage-gated K^+ channels, strongly suggest that the Ca^{2+} influx observed in this study is mediated by a Ca^{2+} channel, and not K^+ channels.

(*iv*) *Pharmacology of Ca^{2+} transport system.* Sensitivity to the 1,4-dihydropyridines has been one of the defining criteria for L-type Ca^{2+} channels in animal cells (1, 3). In animal cells, these compounds are specific for Ca^{2+} channels when applied in submicromolar concentrations. They are known to bind to other channels and receptors and to have nonspecific effects when applied at concentrations of 1 μM or greater (20). To date, in plant systems, these compounds appear to have effects only when applied at concentrations higher than 1 μM (see, for example, ref. 17). It is clear that in the plant literature, the response of Ca^{2+} channels to organic Ca^{2+} channel blockers can be quite variable. These responses range from inhibition of Ca^{2+} influx or Ca^{2+} channel activity to a stimulation of Ca^{2+} influx (4, 21–23). This situation is further complicated by the recent observation that the phenylalkylamine and dihydropyridine Ca^{2+} channel blockers also block outward rectifying K^+ channels in the PM of *Amaranthus* protoplasts (24). Based on these observations, those authors proposed that verapamil, bepridil, and the 1,4-dihydropyridines are not specific antagonists of Ca^{2+} channels in the plant cell PM.

Therefore, it is not surprising that we observed a lack of inhibition of the Ca^{2+} transport system by nifedipine and verapamil (Fig. 3). It appears that La^{3+} is probably the most effective Ca^{2+} channel blocker that is currently available, when used at low concentrations ($\leq$5 μM). In the above mentioned study of Terry *et al.* (24), they found that La^{3+} also could cause a moderate inhibition of outward-rectifying K^+ channels, but this was observed at La^{3+} concentrations between 10 and 100 μM. In this study, low concentrations (1–5 μM) of La^{3+} very effectively inhibited voltage-dependent Ca^{2+} influx in wheat root PM vesicles (Figs. 3 and 5). On the other hand, TEA and Ba^{2+}, which are widely used to block K^+ channels, had no effect on voltage-dependent Ca^{2+} transport. These results further support the proposition that the voltage-gated Ca^{2+} transport characterized here involves a Ca^{2+} channel.

In summary, we report here on an alternative method to the patch clamp technique for the investigation of voltage-dependent ion transport systems in plant membranes. Although this method cannot provide information concerning many of the biophysical properties of ion channels, it is a very straightforward way of studying voltage-gated transporters that are not readily amenable to study by the patch clamp technique. Using this approach, we report on the characterization of a voltage-gated Ca^{2+} influx across the PM of wheat root cells that we argue is mediated by a voltage-gated Ca^{2+} channel.

We thank Dr. Clyde Wilson [U.S. Salinity Lab, U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS), Riverside, CA], Dr. Daniel Bush (USDA–ARS Photosynthesis Research Unit, University of Illinois, Urbana, IL), and Dr. Michael Grusak (USDA–ARS Children's Nutrition Research Center, Houston) for valuable discussions concerning the isolation of root PM vesicles. This research was supported by U.S. Department of Agriculture/National Research Initiative Competitive Grant 93-37100-8874 to L.V.K.

1. Tsien, R. W. & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* **6**, 715–760.
2. Pelzer, D., Pelzer, S. & McDonald, T. F. (1990) *Rev. Physiol. Biochem. Pharmacol.* **114**, 107–207.
3. Hille, B. (1992) *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA).
4. Shiina, T. & Tazawa, M. (1987) *J. Membr. Biol.* **96**, 263–276.
5. Thuleau, P., Graziana, A., Canut, H. & Ranjeva, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 10000–10004.
6. Thuleau, P., Graziana, A., Ranjeva, R. & Schroeder, J. I. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 756–769.
7. Cosgrove, D. J. & Hedrich, R. (1991) *Planta* **186**, 143–153.
8. Ding, J. P. & Pickard, B. G. (1993) *Plant J.* **3**, 83–110.
9. Huang, J. W., Grunes, D. L. & Kochian, L. V. (1993) *Agron. J.* **85**, 867–873.
10. Buckhout, T. J. & Terry, C. H. (1986) *Protoplasma* **135**, 144–154.
11. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
12. Palmgren, M. G., Sommarin, M., Ulvskov, P. & Larsson, C. (1990) *Biochim. Biophys. Acta* **1021**, 133–140.
13. Briskin, D. P., Leonard, R. T. & Hodges, T. K. (1987) *Methods Enzymol.* **148**, 542–558.
14. Rottenberg, H. (1979) *Methods Enzymol.* **55**, 547–569.
15. Segel, I. H. (1976) *Biochemical Calculations* (Wiley, New York), p. 236.
16. Fairley-Grenot, K. A. & Assmann, S. M. (1992) *J. Membr. Biol.* **128**, 103–113.
17. Gelli, A. & Blumwald, E. (1993) *Plant Physiol.* **102**, 1139–1146.
18. Garty, H., Rudy, B. & Karlish, S. J. D. (1983) *J. Biol. Chem.* **258**, 13094–13099.
19. Wang, X.-C., Berkowitz, G. A. & Peters, J. S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4981–4985.
20. Zernig, G. (1990) *Trends Pharmacol. Sci.* **11**, 38–44.
21. Tester, M. (1990) *New Phytol.* **114**, 305–340.
22. Reid, R. J. & Tester, M. (1992) *Philos. Trans. R. Soc. London B* **338**, 73–82.
23. Reid, R. J. & Smith, F. A. (1992) *Plant Physiol.* **100**, 637–643.
24. Terry, B. R., Findlay, G. P. & Tyerman, S. D. (1992) *J. Exp. Bot.* **43**, 1457–1473.