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Calcium Transport in Protoplasts Isolated from *ml-o* Barley Isolines Resistant and Susceptible to Powdery Mildew[1]

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ABSTRACT

Free cytoplasmic calcium has been postulated to play a role in preventing powdery mildew in a series of homozygous *ml-o* mutants of barley, *Hordeum vulgare* L. Protoplasts isolated from 7-day-old plants of the *ml-o* resistant-susceptible (R-S) barley isolines, Riso 5678/3* × Carlsberg II R and S, were used to test for differences in fluxes of Ca^{2+} across the plasmalemma. Greater influx or lesser efflux might account for a higher free cytosolic Ca^{2+} postulated to exist in *ml-o* R mutants. Uniform patterns of uptake were maintained for 3 hours from solutions of 0.2 and 2 millimolar Ca^{2+}. Washout curves of $^{45}Ca^{2+}$ from R and S protoplasts revealed three compartments—presumed to represent release from the vacuole, organelles, and the cytoplasm (which included bound as well as free Ca^{2+}). Uptake and washout did not differ between isolines. On the basis of recent determinations of submicromolar levels of free cytoplasmic Ca^{2+} and our initial rates of ^{45}Ca-labeled Ca^{2+} uptake, we show that measurement of the unidirectional influx of Ca^{2+} across the plasmalemma is not feasible because the specific activity of the pool of free cytoplasmic calcium increases almost instantaneously to a level that would result in a significant, but unknown, efflux of label. Similarly, measurement of the efflux of Ca^{2+} across the plasmalemma is not possible since the activity of the pool of free cytoplasmic calcium is a factor of 350 smaller than the most rapid component of the washout experiment. This pool of cytoplasmic free Ca^{2+} will wash out too rapidly and be too small to detect under the conditions of these experiments.

In plants, calcium plays a role in the regulation of development and in many stress responses (12), and it has been implicated in disease resistance (3). Recent evidence (10, 26, 28) shows that the formation of papillae (parasite-elicited wall appositions on the inner surfaces of the cell walls of host plants) is enhanced in a series of agriculturally important homozygous *ml-o* barley mutants that universally exhibit near immunity to the incitant of powdery mildew disease, *Erysiphe graminis* f. sp. hordei (13, 14, 28). Papillae intercept encroaching pathogens. Disease resistance in *ml-o* resistant barley mutants is linked to the Ca^{2+}-mediated secretion phenomenon of early papilla formation, is specifically abolished by the chelation of Ca^{2+}, and is inhibited by low exogenous Ca^{2+} and treatments with auxin (1, 3, 10). Enhanced papilla formation in the resistant mutants was postulated to be due to an elevation of the level of cytoplasmic free Ca^{2+} as a result of an altered free cytosolic Ca^{2+} regulatory mechanism (1, 3). The level of cytosolic free Ca^{2+} in the cytoplasm will be influenced by transport across the membranes of organelles in addition to movement across the plasmalemma and tonoplast. We decided to begin with an attempt to look for differences in the fluxes of Ca^{2+} across the plasmalemma of resistant and susceptible isolines. This search led us to reexamine the experimental problems inherent in the measurement of Ca^{2+} fluxes in plant cells.

The problem that has received the most attention is the slow exchange of a significant fraction of the Ca^{2+} associated with the cell wall. Because of the difficulty inherent in distinguishing the slowly exchanging cell wall fractions from fluxes of Ca^{2+} across the plasmalemma, measurements of uptake and efflux are complicated (18, 20, 21, 27). By using isolated protoplasts in studies of Ca^{2+} fluxes, one can circumvent such interference from the slowly exchanging cell wall fractions. We have developed a method of isolating viable protoplasts from 7-d-old barley seedlings in a medium of relatively low (0.2 mM) calcium.

A second, more intractable problem arises from the relatively recent realization that the level of cytoplasmic free calcium in plants is in the submicromolar range (4, 5, 8, 9, 15, 30). This information strengthens the conclusion that the passive fluxes of calcium are driven by electrochemical gradients directed from both the apoplast and the vacuole toward the cytoplasm. However, the small size of the pool of cytoplasmic free calcium may lead to a violation of the assumption inherent in the measurement of the influx—namely that the cytoplasmic pool of the ion is sufficiently large to prevent its specific activity during the period of uptake, from reaching a value which is significant in comparison to the external specific activity.

Previous work on calcium fluxes in isolated protoplasts is scant. By radiolabeling solutions containing 1 mM Ca^{2+}, Mettler and Leonard (22) showed that the Ca^{2+} uptake into isolated protoplasts from tobacco suspension cells was passive and nonlinear over a 45 min uptake period with measurements taken at 5, 20, and 45 min, at which point the rate of uptake had tapered off. Åkerman *et al.* (2) reported linear uptake of Ca^{2+} by wheat leaf protoplasts for 40 min, after which uptake had leveled off. Like Mettler and Leonard, they used radiolabeled 1 mM Ca^{2+} solutions in buffered osmoticum. Das and Sopory (6) implicated phytochrome in regulation of Ca^{2+} uptake by maize protoplasts. They showed maximal uptake 240 s after introduction of radiolabel to protoplasts kept in the dark, whereas maximal uptake occurred earlier (after only 120 s) in protoplasts irradiated for 1 min with red light before the introduction of label into solutions containing no other source of calcium. Using the metallochromic indicator, arzenazo III, Heimann *et al.* (11) showed light-stimulated net Ca^{2+} influx into intact spinach protoplasts incubated in buffered solutions of 20 μM $CaCl_2$ and 20 mM KCl. By following changes in the free Ca^{2+} in the medium, they were able to show linear uptake of Ca^{2+} over a 10 min period. No saturation of uptake was observed within that time. However, saturation of

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$^{45}Ca^{2+}$ uptake into carrot protoplasts occurred in 10 to 20 min when Rincon and Boss (24) exposed protoplasts to labeled osmoticum containing no Ca^{2+} other than that introduced with the label. The saturation they observed could have been due partly to depletion of $^{45}Ca^{2+}$ in their uptake solutions.

With regard to measurements of calcium fluxes, the implications of the small pool of cytoplasmic free calcium activity do not appear to have been considered explicitly. We show that measurement of the unidirectional fluxes of Ca^{2+} across the plasmalemma is not feasible with current techniques.

MATERIALS AND METHODS

Plant Material. The *ml-o* R-S[2] barley (*Hordeum vulgare* L.) breeding lines, Riso 5678/3* × Carlsberg II R and S, used in this study were described previously (28). Seeds were planted in Bailey Hortorium Mix and grown 7 d in controlled environment chambers (model 63-10, Sherer Gillette Environmental Division, Marshall, MI 49068) which were set to cycle with 12 h days at 25°C and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, and 12 h nights at 20°C.

Plasticware. Because of the ion exchange properties of glass, we used plastic labware throughout all experiments to avoid contamination from Ca^{2+} that could leach out of glassware and to avoid problems with radiolabel adhering to a glass surface. All plasticware was soaked in Micro detergent (International Products Corporation, Trenton, NJ 08601), then rinsed with hot water followed by 5 rinses with distilled, deionized water.

Protoplast Isolation. The abaxial surfaces of leaves from both R and S seedlings were abraded with carborundum and floated on isolation buffer (0.5 M mannitol, 0.2 mM $CaCl_2$, 5 mM KCl, 0.5 mM DTT, 25 mM Mes, 0.1% [w/v] BSA at pH 5.5) for 5 min with vacuum applied in a vacuum desiccator. The resulting plasmolyzed leaves were blotted and floated on digestion buffer, the same buffer including only 4 mM KCl and supplemented with 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan) and 2% (w/v) CELF Cellulase (Cooper Biomedical, Inc., Malvern, PA 19355). Following a 50 min incubation in a shaker bath in the dark at 25°C and 50 rpm, the digested leaf slurries were diluted with isolation buffer and filtered through 88 μm mesh nylon. These protoplast suspensions were centrifuged 10 min at 50*g* in a swinging bucket centrifuge. The pellets were resuspended in isolation buffer and added to a 7 mL layer of the same buffer over a 17% (w/v) sucrose pad in a conical centrifuge tube. Again they were centrifuged 10 min at 50*g*. Protoplasts were removed with plastic Pasteur pipets from bands over the sucrose pads and slowly released into tubes of isolation buffer. These suspensions were centrifuged for 10 min at 50*g*, then the pellets were resuspended in isolation buffer and again centrifuged for 10 min at 50*g*. The supernatant was decanted, and the resulting protoplast pellets were suspended to a concentration of 1 to 2 × 10^6 protoplasts per mL of solution appropriate for a particular experiment. Fluorescein diacetate was used to check protoplast viability (17).

Uptake of $^{45}Ca^{2+}$. Protoplasts were suspended in solutions containing different concentrations of $CaCl_2$ and KCl (0.2 mM Ca^{2+}, 5 mM K^+; 0.2 mM Ca^{2+}, 54 mM K^+; 1 mM Ca^{2+}, 9 mM K^+; 2 mM Ca^{2+}, 14 mM K^+; or 2 mM Ca^{2+}, 54 mM K^+) in 0.5 M mannitol, 0.5 mM DTT, and 25 mM Mes at pH 5.5, and preincubated 0.5 h at 25°C and 200 $\mu mol \cdot m^2 \cdot s^{-1}$ PAR in a reciprocating shaker bath set at 50 rpm. Labeling of these uptake solutions was with $^{45}Ca^{2+}$ as $CaCl_2$ in H_2O (ICN Biomedicals, Inc., Irvine, CA 92715). Gelman open filter holders (Gelman Sciences, Ann Arbor, MI 48106) were modified to serve as reservoirs to hold 4 mL of solution over glass fiber filters (Whatman 934AH) supported by 100 mesh stainless steel screens (Fig. 1A). Duplicate 0.2 mL samples of the radiolabeled protoplast suspensions (containing 1–4 × 10^5 protoplasts) were introduced into these wells filled with unlabeled uptake solution supplemented with 10 mM $CaCl_2$. Suction was applied and the protoplasts were rinsed with another 10 mL of unlabeled uptake solution containing 10 mM Ca^{2+}. Filters were mixed with 1 mL isopropanol, vortexed to break up the protoplasts, and then diluted to 10 mL with Liquiscint (National Diagnostics, Somerville, NJ 08835), vortexed again, and counted in a Beckman LS100 liquid scintillation counter.

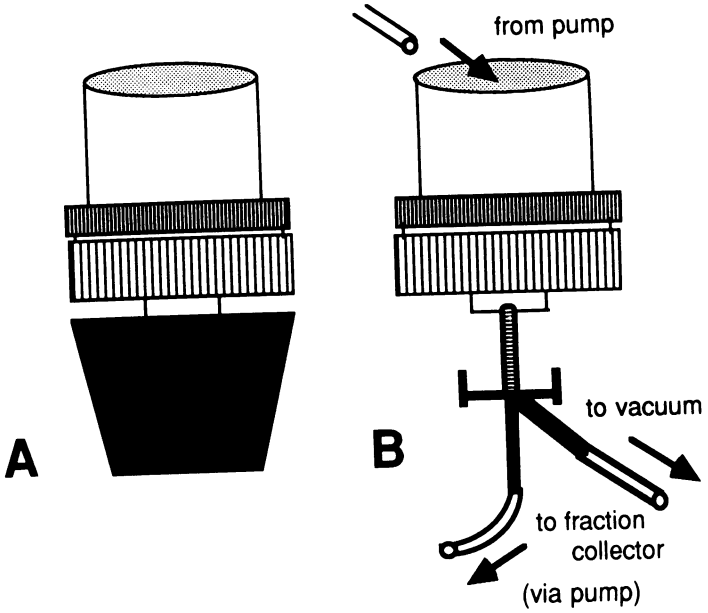

FIG. 1. Modified Gelman open filter holders containing Whatman 934AH glass fiber filters and fitted over vacuum line for rapid filtration of radiolabeled protoplasts from their uptake solutions (A) or connected via a 3-way valve to a vacuum line and fraction collector for gathering timed perfusate samples during the efflux experiments (B).

Washout of $^{45}Ca^{2+}$. Protoplasts were suspended and preincubated as in the uptake studies, labeled with $^{45}Ca^{2+}$, and incubated 1 h at 25°C, 50 rpm and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Labeled protoplast suspensions were introduced in 1 mL aliquots (containing 1–2 × 10^6 protoplasts) into wells (Fig. 1B) of unlabeled uptake solution (washout solution). The protoplasts were held in the mesh of the glass fiber filters, quickly rinsed via suction with 50 mL of washout solution, and then continuously perfused with washout solution via a peristaltic pump while eluate rinsing over the protoplasts was continuously collected, via the second channel of the same peristaltic pump, into scintillation vials in a fraction collector (FRAC 100, Pharmacia, Inc., Piscataway, NJ 08854). Perfusate was collected initially for 0.5, then 1, and finally 5 min intervals up to 110 min before the wells were emptied of washout solution. The filters containing the protoplasts were removed from the wells, mixed with isopropanol, and then Liquiscint, and counted along with the collected eluates which were also mixed with Liquiscint.

From the time at which the solutions were radiolabeled, duplicate 0.1 mL samples of labeled suspension were taken at 30 min intervals to measure uptake. The resulting values served to check that the label had reached saturation and that the protoplasts used in the washout studies remained viable for the duration of the experiment.

Elemental Analyses. Protoplasts were resuspended in washout solution containing 2 mM Ca^{2+} and 5 mM K^+, incubated 2 h in a reciprocating shaker bath set at 25°C, 50 rpm, and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, and then centrifuged 10 min at 50*g*. Pellets were resuspended to 5 mL in washout solution lacking calcium.

[2] Abbreviations: R, resistant; S, susceptible.

Protoplasts were counted with a hemocytometer, their diameters were measured, and then they were centrifuged for 10 min at 50 *g*. The supernatants were poured off and 0.1 mL of concentrated HNO_3 was added to each pellet. The tubes were vortexed until the protoplasts were digested. Digests were diluted to 5 mL with distilled deionized water, vortexed, and centrifuged. The decanted supernatants were analyzed with an inductively coupled atomic emission spectrophotometer (ARL 3400, Applied Research Laboratories, Division of Bausch and Lomb, Sunland, CA 91040).

RESULTS

Uptake. Initially, within a single experiment considerable variability was seen in uptake values at long time periods (data not shown). However, by maintaining a K^+ to Ca^{2+} ratio of more than 5:1 in the external solutions, uniform patterns of $^{45}Ca^{2+}$-labeled Ca^{2+} uptake by protoplasts isolated from R and S barley seedlings were exhibited over a 3 h period. From solutions of low calcium concentration (0.2 mM Ca^{2+}, 5 mM K^+) the pattern of uptake was linear for 3 h (Fig. 2A). No difference was observed in uptake between protoplasts isolated from R and S. Raising the external potassium concentration (0.2 mM Ca^{2+}, 54 mM K^+) reduced uptake by both R and S protoplasts, and resulted in saturation and eventual decline of a formerly linear pattern (Fig. 2B). Maximal uptake was less than half that at 5 mM K^+ (Fig. 2A). Again, uptake by R protoplasts was not different from that by S protoplasts.

From solutions of 1 mM Ca^{2+} and 9 mM K^+, uptake of ^{45}Ca-labeled Ca^{2+} by both R and S protoplasts showed saturation with time (Fig. 2C). This pattern was maintained from solutions of even higher calcium concentration (2 mM Ca^{2+}, 14 mM K^+) as shown in Figure 2D. In the presence of 54 mM potassium, uptake of ^{45}Ca-labeled Ca^{2+} by protoplasts showed saturation by 1 h, followed by a gradual decline (Fig. 2E). No differences in uptake were discerned between R and S protoplasts.

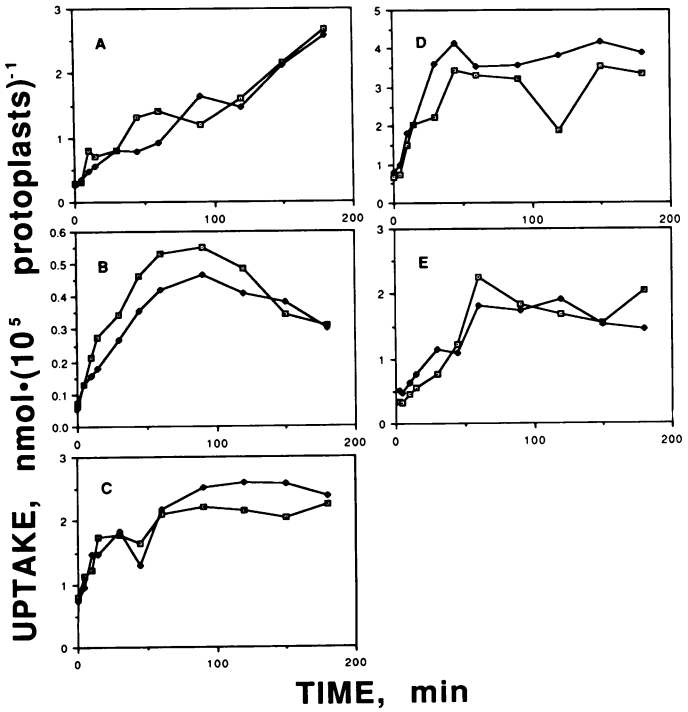

FIG. 2. Uptake of ^{45}Ca-labeled Ca^{2+} by protoplasts isolated from seedlings of the Riso 5678 resistant (□) and susceptible (♦) isolines of barley as a function of time from solutions of A, 0.2 mM Ca^{2+} and 5 mM K^+, B, 0.2 mM Ca^{2+} and 54 mM K^+, C, 1 mM Ca^{2+} and 9 mM K^+, D, 2 mM Ca^{2+} and 14 mM K^+, or E, 2 mM Ca^{2+} and 54 mM K^+ in 0.5 M mannitol, 0.5 mM DDT, and buffered with 25 mM Mes to pH 5.5.

After staining nonradiolabeled protoplasts with fluorescein diacetate and filtering them as in an uptake experiment, fluorescence microscopy showed brightly fluorescent protoplasts enmeshed in the glass fiber filters—a good indication of viability. We also used a microscope to check the filtrate for broken protoplasts and organelles and found none.

Elemental Analyses. Total protoplast calcium ± SD was 4.6 ± 1.4 nmol·(10^5 protoplasts)$^{-1}$ for both R and S. Total potassium content ± SD was 430 ± 79.3 nmol·(10^5 protoplasts)$^{-1}$. These values were obtained by using hemocytometer counts of protoplasts per mL, and the Ca^{2+} content in ppm obtained from the analysis with the inductively coupled atomic emission spectrophotometer.

The close correspondence between the Ca^{2+} content of protoplasts incubated in a solution containing 2 mM Ca^{2+} (*i.e.* 4.6 nmol·[10^5 protoplasts]$^{-1}$) and the value estimated from the saturated uptake of $^{45}Ca^{2+}$ (Fig. 2D) means that isotopic equilibrium is reached in less than 1 h. Therefore, compartmental analysis can be justifiably applied to estimate the pool sizes derived from washout curves of protoplasts loaded with $^{45}Ca^{2+}$.

Washout and Compartmental Analysis. Washout of ^{45}Ca-labeled Ca^{2+} from both R and S protoplasts clearly showed three phases—a small, rapid component, an intermediate component, and a slower third component interpreted as release from the vacuole (Fig. 3A). For each original logarithmic plot the linear portion was fitted between 50 and 110 min with a regression equation (Fig. 3B), and the resulting zero time intercept was used to estimate the size of the vacuolar pool (Table I). The half-time for exchange of the pool was also calculated (Table I). The content of the vacuolar pool, represented by the regression equation, was subtracted from the total content (data points, Fig. 3A) to give the content of the remaining pools (Fig. 3B). Since a rapidly exchanging pool was evident in Figure 3B, we repeated the process and fitted regression equations to the points between 5 and 30 min (Fig. 3B) to show the efflux from the most rapid compartment (Fig. 3C). The sizes and half-times of the intermediately and rapidly exchanging pools are given in Table I. The apparent initial increase in counts seen in the fast compartment (Fig. 3C) is due to the lag in collecting radiolabeled perfusate introduced by the void volume of the peristaltic pump/efflux well system.

When viewed with fluorescence microscopy, nonlabeled protoplasts perfused with fluorescein diacetate, after being exposed to the conditions of an efflux experiment, fluoresced brightly—an indication that they were intact, viable protoplasts.

DISCUSSION

Entry of Ca^{2+} into plant cells is passive and downhill along an electrochemical potential gradient (19). Even though the problem of Ca^{2+} binding to cell walls can be overcome by using protoplasts, the significance for flux measurements of the recent demonstration of small cytoplasmic pools of free Ca^{2+} does not seem to have been fully appreciated. We show that measurement of the unidirectional fluxes of Ca^{2+} across the plasmalemma is not feasible because the specific activity of the pool of free cytoplasmic calcium changes almost instantaneously.

If we assume that the observed initial uptake rate represents the unidirectional influx across the plasmalemma, then we can calculate a minimal estimate of how quickly the Ca^{2+} in the cytoplasm would turn over. We have measured Ca^{2+} uptake by 10^5 protoplasts to be about 2 nmol in 10 min (Fig. 2C), and the average radius of both R and S protoplasts to be 20 μm. If we assume, for the sake of argument, that the volume of the cytoplasm is 5% of protoplast volume and that all Ca^{2+} taken up remains free in the cytoplasm, then free cytosolic Ca^{2+} would

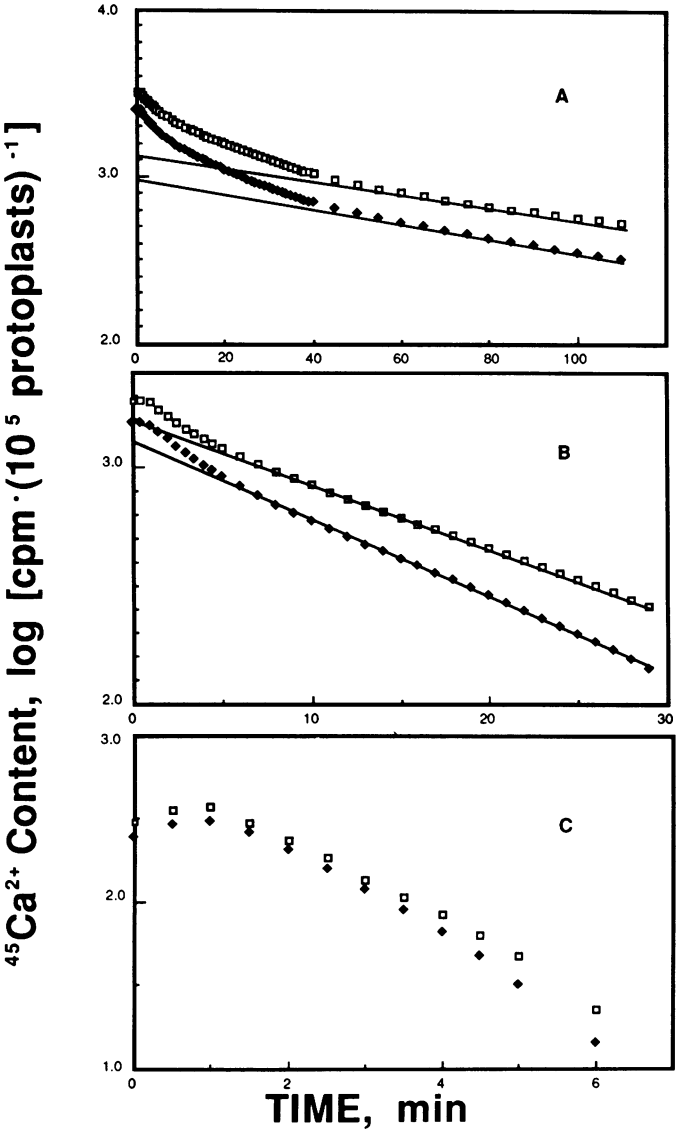

FIG. 3. Washout curve of $^{45}Ca^{2+}$ from protoplasts isolated from the Riso 5678 resistant (□) and susceptible (♦) isolines of barley in solutions of 1 mM Ca^{2+}, 9 mM K^+ in 0.5 M mannitol, 0.5 mM DTT, and buffered with 25 mM Mes to pH 5.5. A, Logarithmic plot of CPM remaining, indicating three compartments; y intercepts from the regression equations ($y = 3.127 - 0.0037x$ for R and $y = 3.0009 - 0.0046x$ for S) were used to estimate the sizes of the vacuolar compartments. B, The data in A were replotted, following subtraction of the slowly exchanging compartment, and the y intercepts were used to estimate the size of the intermediately exchanging pool. The regression equations were $y = 3.1999 - 0.0271x$ for R, and $y = 3.1088 - 0.0328x$ for S. C, The data were replotted following subtraction of the slowly and intermediately exchanging compartments. Again, the y intercepts were used to estimate the sizes of the rapidly exchanging pools.

reach a concentration of 12 mM! Raven (23) lists measurements of protoplast and vacuolar volumes. A great deal of variability is exhibited, as values for the cytoplasmic volume of higher plants range from 0.5 to 61%. None of these measurements is on a graminaceous species. Thus, by taking these uncertainties into account in our calculations, if we assume that the volume of the cytoplasm is 50% of protoplast volume, and again, assume that all Ca^{2+} taken up remains free in the cytoplasm, then free cytosolic Ca^{2+} would reach a concentration of 1.2 mM in 10 min, still significantly higher than recently reported free cytosolic calcium concentrations (4, 5, 8, 9, 15, 30).

Alternatively, if we assume that the Ca^{2+} activity in the cytoplasm is 1 μM and that the cytoplasm is 5% of the protoplast volume, then the free cytoplasmic Ca^{2+} content of 10^5 protoplasts will be 1.7×10^{-13} mol. Since the initial rate of uptake is 33×10^{-13} mol·s^{-1}· (10^5 protoplasts)$^{-1}$, the cytosolic free Ca^{2+} will reach a pseudo steady state in a fraction of a second. Thus, almost instantaneously, the efflux of label will become a significant but unknown fraction of the influx.

If we assume that the observed initial rate of uptake is a minimal estimate of the Ca^{2+} influx, then the influx would be 0.6 pmol·cm^{-2}·s^{-1}. If we further assume that all of the uptake goes into the vacuole and that the vacuolar Ca^{2+} concentration is about 1 mM, then our measured uptake of 2×10^{-9} mol·(10^5 protoplasts)$^{-1}$ in 10 min means that, when compared to the calcium content of 4.6×10^{-9} mol·(10^5 protoplasts)$^{-1}$, uptake of radioactivity should begin to level off after a few tens of minutes, exactly as we have observed (Fig. 2, C and D). From 0.5 mM $CaSO_4$ solutions, Siddiqi and Glass (25) showed greatest rates of ^{45}Ca-labeled Ca^{2+} uptake into intact barley seedlings over 10 min, lower rates over 30 min, and lowest rates over 60 min of uptake. The fact that they obtained decreasing rates of uptake as the time was increased to 60 min also indicates that the specific activity of the tissue Ca^{2+} approached that of the Ca^{2+} in the labeling solution. Their work demonstrated that saturation of uptake also occurs in intact tissue.

Confirmation that the specific activity of the protoplast Ca^{2+} approached that of the external solution can be obtained by comparing the ^{45}Ca-labeled Ca^{2+} uptake with the Ca^{2+} content calculated from chemical analyses. The calcium content, determined spectrophotometrically from protoplasts incubated in a solution containing 2 mM Ca^{2+} and 5 mM K^+, was 4.6 nmol·(10^5 protoplasts)$^{-1}$, similar to the value in Figure 2D, of about 4.0 nmol·(10^5 protoplasts)$^{-1}$, determined with radiolabel. Thus, the results from the radiotracer studies are comparable to those obtained analytically.

The 80 and 50% repression in overall Ca^{2+} uptake seen in the presence of 54 mM K^+ at 0.2 and 2 mM Ca^{2+} (Fig. 2, B and E), respectively, was probably caused by a depolarization of the plasma membrane which would interfere with the driving force for Ca^{2+} uptake. The uniform patterns of uptake seen at K^+/Ca^{2+} ratios greater than 5:1 are indicative of physiologically stable systems. At 10 min, uptake is 0.5 nmol·(10^5 protoplasts)$^{-1}$ from solutions of 0.2 mM Ca^{2+}, and 2.0 nmol·(10^5 protoplasts)$^{-1}$ from solutions of 2 mM Ca^{2+} (Fig. 2, A and D, respectively). The fact that the initial rate of ^{45}Ca-labeled Ca^{2+} uptake did not increase proportionately with the external concentration does not mean that the influx behaved in the same way—again, because the specific activity of the pool of cytoplasmic free Ca^{2+} quickly reaches a pseudo steady state. The value of the specific activity depends on the relative values of the calcium influx and the sum of the effluxes from the cytoplasm out of the cell and into internal compartments. If, as the external Ca^{2+} concentration is raised, the calcium fluxes across the plasmalemma increase more than the other fluxes, the specific activity of free cytoplasmic calcium will rise to a value closer to that of the external solution. The result is that the ratio of the efflux to influx of label at the higher external calcium concentration will be greater than that at the low concentration. Consequently, the net initial rate of uptake of label will not be proportional to the increase in the influx.

Macklon (19) bathed excised onion roots in ^{45}Ca-labeled Ca^{2+} and then did washout experiments. He identified five components of efflux, three of them extracellular. He suggested that the components represent slow release from the vacuole, next slowest release from the cytoplasm, then release from the Donnan free space, efflux from the water free space in the cell walls (anion free space), and lastly the most rapid release from a surface film. He then calculated the amount of ion in each compartment and

Table I. *Estimates of Cellular Pool Sizes and Half Times Based on Compartmental Analysis Calculations*

Values are means of 3 experiments ± SD.

ml-o Isoline	Slow Compartment		Intermediate Compartment		Fast Compartment	
	Half-time	Pool size	Half-time	Pool size	Half-time	Pool size
	min	*nmol·(10⁵ protoplasts)⁻¹*	*min*	*nmol·(10⁵ protoplasts)⁻¹*	*min*	*nmol·(10⁵ protoplasts)⁻¹*
R	60.7 ± 19.5	0.16 ± 0.10	10.1 ± 1.2	0.18 ± 0.11	2.5 ± 0.0	0.055 ± 0.008
S	56.5 ± 9.9	0.15 ± 0.10	8.3 ± 0.7	0.18 ± 0.07	2.5 ± 0.5	0.061 ± 0.008

determined the cytoplasm to be 7 mM Ca^{2+}, a value higher by a factor of 10^3 to 10^4 than current estimates of cytoplasmic free Ca^{2+}. He interpreted this discrepancy as being due to pools of calcium in the cytoplasm being in rapid exchange with the free calcium in the cytoplasm.

Siddiqi and Glass (25) analyzed washout curves of $^{45}Ca^{2+}$ from intact barley roots and delineated three compartments—wall, cytoplasm, and vacuole with half-lives for exchange of 3, 25, and 167 min, respectively. These results are particularly interesting when compared to Macklon's (18) results for excised onion roots. He calculated cytoplasmic and vacuolar half-times for exchange of 55 and as high as 1780 min, respectively. Siddiqi and Glass' results (25) show much more rapid exchange rates, more in line with our estimates (Table I), which are for the same species, though a different tissue and preparation.

Interpretation of washout curves is usually tentative at best (31). Our compartmental analysis estimates of slowly, intermediately, and rapidly exchanging calcium pool sizes of 0.16 nmol·(10^5 protoplasts)$^{-1}$, 0.18 nmol·(10^5 protoplasts)$^{-1}$, and 0.06 nmol·(10^5 protoplasts)$^{-1}$, could be interpreted as representing release from the vacuole, some of the organelles, and cytoplasm, respectively (Table I). The rapid component would normally be interpreted as diffusion from the free space. However, protoplasts lack free space associated with cell walls, usually the largest component of free space. The initial rapid wash designed to remove extracellular calcium should have been sufficient to remove any free space component associated with an unstirred layer around the individual protoplasts. Because of a more complex structure in intact tissue, such washing to remove extracellular calcium would be more difficult, presumably take longer, and might also account for Macklon's delineation of three extracellular components in his $^{45}Ca^{2+}$ washout curves from onion roots (18).

Assuming the cytoplasm occupies 10% of the protoplast, when the values in Table I are converted to concentrations, they become 0.05 and 0.15 mM for vacuolar and total cytoplasmic Ca^{2+} levels, respectively. The value for total cytoplasmic calcium, 0.15 mM, is still 10^2 to 10^3 times greater than accepted values for the free calcium pool. Rather than representing merely cytosolic free Ca^{2+}, this pool probably includes bound Ca^{2+} as well as some of the most rapidly exchanging organellar pools of Ca^{2+} associated mainly with the endoplasmic reticulum and perhaps the mitochondria (7) and chloroplasts (16). The inherent limitations of the compartmental analysis technique would then conceivably allow these bound and organellar pools (*i.e.* the most rapidly exchanging ones) to be measured in the total cytoplasmic calcium pool (29, 31).

With the techniques we used we could not detect the small pool made up only of free calcium in the cytoplasm. As mentioned earlier, our calculations concerning the rapidity with which the specific activity of this pool would change should apply also to the efflux situation, in which case the specific activity will shift rapidly downward due to the entry of unlabeled calcium from the external solution. To measure the efflux across the plasmalemma, the transient contribution of the free cytoplasmic Ca^{2+} pool to the washout curve must be determined. Since the size of this cytoplasmic free Ca^{2+} pool, 1.7×10^{-13} mol·(10^5 protoplasts)$^{-1}$ (estimated earlier on the basis of 1 μM free Ca^{2+} concentration), is a factor of 350 smaller than the most rapid component of the washout experiment (Table I), this pool will wash out too rapidly and be too small to detect under the conditions of these experiments.

A surprising result is that the intermediate, presumed more slowly exchanging organellar, pools are slightly larger than those of the vacuolar pools. When the small volume of the organelles is considered (a crude estimate is 50% of the cytoplasmic volume of the protoplasts), the concentrations of Ca^{2+} in these organellar pools could be as much as an order of magnitude larger than the concentrations of the vacuolar and cytoplasmic pools. Such high concentrations of Ca^{2+} in these presumed organellar pools would seem consistent with the potentially significant role of organelles in buffering cytosolic free Ca^{2+} and in enabling the cell to maintain a constant submicromolar level of the free ion.

The inherent difficulty in measuring unidirectional calcium flux across the plasmalemma, a result of the small pool of cytoplasmic free calcium, has been recognized only recently in the animal literature (7). We have demonstrated that similar problems apply to plant cells. Because these techniques did not give us reliable information about influx and efflux across the plasmalemma, we cannot make any statements about our original hypothesis. Alternative methodologies will be needed to investigate Ca^{2+} regulation in these *ml-o* isolines.

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