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# Direct Measurement of Calcium Transport across Chloroplast Inner-Envelope Vesicles[1]

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**The initial rate of $Ca^{2+}$ movement across the inner-envelope membrane of pea (*Pisum sativum* L.) chloroplasts was directly measured by stopped-flow spectrofluorometry using membrane vesicles loaded with the $Ca^{2+}$-sensitive fluorophore fura-2. Calibration of fura-2 fluorescence was achieved by combining a ratiometric method with $Ca^{2+}$-selective minielectrodes to determine pCa values. The initial rate of $Ca^{2+}$ influx in predominantly right-side-out inner-envelope membrane vesicles was greater than that in largely inside-out vesicles. $Ca^{2+}$ movement was stimulated by an inwardly directed electrochemical proton gradient across the membrane vesicles, an effect that was diminished by the addition of valinomycin in the presence of $K^+$. In addition, $Ca^{2+}$ was shown to move across the membrane vesicles in the presence of a $K^+$ diffusion potential gradient. The potential-stimulated rate of $Ca^{2+}$ transport was slightly inhibited by diltiazem and greatly inhibited by ruthenium red. Other pharmacological agents such as $LaCl_3$, verapamil, and nifedipine had little or no effect. These results indicate that $Ca^{2+}$ transport across the chloroplast inner envelope can occur by a potential-stimulated uniport mechanism.**

$Ca^{2+}$ in plant cells has many key physiological functions; for example, as an intracellular second messenger it is especially important for the maintenance of cellular homeostasis and signal transduction pathways (Evans et al., 1991; Pineros and Tester, 1997). Therefore, the $[Ca^{2+}]_{cyt}$ must be strictly regulated. The sequestration of $Ca^{2+}$ into endomembrane compartments has been documented in detail for the ER and the vacuole (Evans et al., 1991). The chloroplast may also serve as a potential $Ca^{2+}$ sink (Brand and Becker, 1984; Evans et al., 1991).

In addition to the potential role of chloroplasts in maintaining low resting $[Ca^{2+}]_{cyt}$, it has been proposed that the free $[Ca^{2+}]$ in the stroma regulates several key enzymes involved in photosynthetic $CO_2$ assimilation, including Fru-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase (Kreimer et al., 1988) and $NAD^+$ kinase (Brand and Becker, 1984). $Ca^{2+}$ is also essential for $O_2$ evolution by PSII (Grove and Brudvig, 1998). Although the role of $Ca^{2+}$ in the stroma and thylakoids has been studied in detail (Brand and Becker, 1984), there have been relatively few investigations on $Ca^{2+}$ uptake by chloroplasts.

One study examining $Ca^{2+}$ movement into intact wheat chloroplasts (Muto et al., 1982) indicated that the $Ca^{2+}$ uptake occurs via an $H^+/Ca^{2+}$-antiport mechanism, and that the $K_m$ was only slightly higher than $[Ca^{2+}]_{cyt}$. Kreimer et al. (1985a, 1985b), who measured $Ca^{2+}$ fluxes across the envelope of intact chloroplasts isolated from spinach, reported that $Ca^{2+}$ transport into illuminated chloroplasts could occur via electrogenic uniport and that this was linked to photosynthetic electron transport.

The majority of the work done in this area has been carried out using intact chloroplasts; however, that system has some disadvantages. For example, it is difficult to monitor the nearly instantaneous influx or efflux of ions, and therefore it is difficult to resolve the initial rate kinetics of transport processes. In addition, the pH and ionic composition of the stroma are not easy to control. Another drawback to using intact chloroplasts is that it is difficult to examine the directionality of transport processes, because it is difficult to preload the chloroplasts with $Ca^{2+}$ to measure efflux.

An alternative approach, which we used in this study, involves the use of membrane vesicles prepared from inner-envelope membranes isolated from intact chloroplasts. Vesicles have been shown to be competent for studying ion and metabolite movement across membranes (Sze, 1985). There are several advantages to using membrane vesicles over intact organelles. For example, vesicles can be loaded with fluorescent probes, allowing for the continuous fluorometric measurement of substrate and ion transport. When used along with stopped-flow spectrofluorometry, these processes can be monitored almost instantaneously with measurement times of less than 2 ms, which allows for the determination of initial rate kinetics of transport. This method has been used to measure the symport movement of protons with glycolate and D-glycerate (Young and McCarty, 1993) and the rapid proton-linked diffusion of nitrite as nitrous acid (Shingles et al., 1996) across the chloroplast inner-envelope membrane. Another advantage of this method is that the pH and ionic content of the intravesicular and external media can be easily manipulated, a procedure commonly used to study transport mechanisms. Finally, because inner-envelope vesicles of either right-side-out or inside-out orientation can be prepared (Shingles and McCarty, 1995), the directionality of transport processes can be characterized in detail.

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Abbreviations: $\beta_{in}$, internal $Ca^{2+}$-buffering capacity; $\beta_{out}$, external $Ca^{2+}$-buffering capacity; $[Ca^{2+}]_{cyt}$, free $[Ca^{2+}]$ in the cytosol; $\Delta\Psi$, membrane potential difference; $F_s$, fluorescence at a Ca-sensitive wavelength; $F_{is}$, fluorescence at a Ca-insensitive wavelength.

Studies using liposomes loaded with the ratioable $Ca^{2+}$ fluorescent probe fura-2 have been performed to measure $Ca^{2+}$ transport catalyzed by reconstituted annexin ion channels (Berendes et al., 1993) and ionophores such as ionomycin and A23187 (Blau and Weissmann, 1988; Fasolato and Pozzan, 1989). In addition, fura-2 has been used to monitor voltage-dependent $Ca^{2+}$ movement across the erythrocyte plasma membrane (Soldati et al., 1997). Preliminary experiments using chloroplast inner-envelope vesicles loaded with $Ca^{2+}$-sensitive fluorophores indicated that these probes would be sensitive enough to detect small changes in intravesicular free [$Ca^{2+}$], and therefore could be used to monitor $Ca^{2+}$ movement across membranes (Cleveland and McCarty, 1995). In the present study this experimental procedure was used to directly calculate the initial rates of $Ca^{2+}$ movement and to characterize further the properties of $Ca^{2+}$ transport across the chloroplast inner envelope.

## MATERIALS AND METHODS

### Reagents

Fura-2 was purchased from Molecular Probes (Eugene, OR). All other reagents were of the highest grade commercially available. Stock solutions of buffer components were passed through a column containing Chelex-100 to reduce $Ca^{2+}$ content before buffer preparation.

### Plant Material

Pea (*Pisum sativum* L. cv Laxton's Progress No. 9) plants were grown from seed for 16 to 18 d in vermiculite in a controlled-environment growth cabinet (Revco, Asheville, NC) set for 16-h day (24°C)/8-h night (20°C) periods. Spinach was obtained at local markets.

### Membrane Isolation

Intact chloroplasts were isolated according to the method of Joy and Mills (1987). Inner envelopes were prepared as described by Keegstra and Yousif (1986). Frozen, intact chloroplasts, equivalent to between 80 and 120 mg of chlorophyll, were thawed at 4°C, refrozen at −20°C, and thawed again at 4°C. Chloroplast rupture was facilitated by gentle homogenization using a pestle tissue grinder. The homogenate was centrifuged at 3,150*g* for 15 min. The resulting supernatants were collected and centrifuged at 27,000*g* for 90 min. Pellets were resuspended in 0.2 M Suc and placed on top of a 0.45/0.80/1.0 M Suc step gradient and centrifuged at 105,000*g* for 18 h. Inner-envelope membrane vesicles were recovered from the 0.80/1.0 M Suc interface. All of the operations described above were performed at 4°C. Inner envelopes were stored under liquid nitrogen.

### Vesicle Preparations

Suspensions of purified inner envelopes or asolectin (20 mg) were diluted 4-fold in the appropriate resuspension buffer. The membranes were pelleted by centrifugation at 144,000*g* for 1 h at 4°C and then resuspended in the same buffer before vesicle-preparation protocols.

Membrane vesicles were also prepared using a hand-held, small-volume extrusion apparatus (Shingles and McCarty, 1995). Trace amounts of $Ca^{2+}$ in the filters and apparatus were removed by passing through the apparatus a total of 5 mL of resuspension buffer containing 10 mM K-Hepes, pH 8.0, 100 mM KCl, 100 mM Suc, and 100 $\mu$M EGTA. A total of 1.0 to 2.0 mL of a membrane suspension containing 1.2 mM fura-2 and about 1 mg of inner-envelope protein or 15 to 20 mg of asolectin was then passed 9 to 11 times through the extrusion apparatus with a polycarbonate filter (100-nm pore size) in place.

Inner-envelope vesicles, predominantly of the inside-out orientation, were prepared by the freeze/thaw method described by Young and McCarty (1993) in a resuspension buffer containing 10 mM K-Hepes, pH 8.0, 50 mM KCl, 50 mM choline chloride, 100 mM Suc, 100 $\mu$M EGTA, and 1.2 mM fura-2.

All vesicle preparations were passed through a 1.6- × 10-cm Sephadex G-50 column equilibrated with the appropriate resuspension buffer to remove most of the external fura-2. To eliminate trace amounts of external probe, the eluted vesicles were diluted 4-fold in resuspension buffer and centrifuged at 144,000*g* for 20 min at 4°C. The pellet comprising fura-2-loaded membrane vesicles was resuspended in the same buffer and allowed to equilibrate for 1 to 2 h at 4°C.

### $Ca^{2+}$ Minielectrodes

The $Ca^{2+}$ electrode was constructed as described by Baudet et al. (1994). A 3-cm length of 1.67-mm (o.d.) polyethylene tubing was dipped into a mixture of polyvinyl chloride and potassium tetrakis chlorophenyl borate dissolved in tetrahydrofuran and the $Ca^{2+}$ ionophore *N*,*N*,*N*′,*N*′-tetracyclohexyl-3-oxapentanediamide dissolved in 2-nitrophenyl octyl ether and allowed to dry overnight. Dried electrodes were filled with 28.5 mM nitrilotriacetic acid, pH 8.0, and 1.13 mM $CaCl_2$ to give approximately 1 $\mu$M free $Ca^{2+}$ and an ionic strength of about 0.10 M. The electrodes were allowed to equilibrate in this buffer for at least 3 d before use.

### Fluorescence Measurements

Fura-2 fluorescence emission was monitored at 512 nm with excitation at 340 nm ($F_s$) or 359 nm ($F_{is}$) using a modified spectrofluorometer (model SLM-SPF-500C, Olis, Bogart, GA) and a stopped-flow apparatus (Olis). All slits were set at 10 nm with a cutoff filter (LP47, Oriel, Stamford, CT) placed over the entrance to the emission monochromator. Chamber A contained 2.0 mL of vesicle suspension, and chamber B contained 2.0 mL of buffer of predetermined pH and composition so that the intravesicular osmotic and ionic strengths were closely balanced with those of the external medium. Chamber B also contained $CaCl_2$ when used. Mixing was achieved by a nitrogen-driven piston at 80 p.s.i. All measurements were taken at 25°C.

### Internal Buffering Capacity Measurements

To determine the $\beta_{in}$ of inner-envelope vesicles known amounts of $CaCl_2$ were added to the vesicles in the stopped-flow apparatus and the resulting changes in external pCa were monitored over time. The $\beta_{in}$ relates the total number of moles of $Ca^{2+}$ that cross the vesicle membrane in a given number of vesicles and the intravesicular pCa change that results under experimental conditions; accordingly, it is expressed in nanomoles of $Ca^{2+}$ per pCa unit per milligram of inner-envelope protein.

In this assay fura-2 was present as an indicator of the external pCa. Spinach chloroplast inner-envelope vesicles were prepared via the freeze/thaw procedure described previously with the following exceptions. The resuspension buffer consisted of 10 mM K-Hepes, pH 8.0, 100 mM KCl, 100 mM Suc, and 100 $\mu$M EGTA. The intravesicular fura-2 was replaced by 1.2 mM 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid to avoid interference with the fluorescence of the external fura-2. Finally, the EGTA concentration in the elution buffer used to equilibrate the Sephadex G-50 column and to dilute the eluted vesicles before centrifugation was reduced to 20 $\mu$M. However, the concentrations of KCl, Suc, and K-Hepes, pH 8.0, in the elution buffer were identical to those in the resuspension buffer.

Vesicle suspensions were diluted with an equal volume of elution buffer supplemented with approximately 10 $\mu$M fura-2. The resulting mixture was loaded into chamber A of the stopped-flow apparatus. Chamber B contained buffer containing 100 mM choline chloride plus 32 $\mu$M $CaCl_2$. The fluorescence of fura-2 was monitored for 2 min. The decrease in pCa immediately after mixing was used to calculate the $\beta_{out}$. This decrease was followed by a gradual increase in external pCa for the next 2 min as the external and internal $[Ca^{2+}]$ reached equilibrium. The final overall extent of change in pCa allowed for the calculation of the combination of $\beta_{in}$ and $\beta_{out}$. From these data, $\beta_{in}$ was calculated.

### Assays

The modified TCA-Lowry procedure of Bensadoun and Weinstein (1976) was used to determine the amount of protein in the inner-envelope membrane vesicle preparations.

## RESULTS

### Calibration of Vesicles Loaded with Fura-2

The minielectrode used in this study was calibrated in a buffer containing 12 mM EGTA, 15 mM nitrilotriacetic acid, and 10 mM $CaCl_2$. At the start of the calibration process, the pH of the buffer was 4.0. Under these conditions, almost all of the $Ca^{2+}$ was unchelated. The concentration of free $Ca^{2+}$ in the buffer was changed by varying the pH of the buffer from 4.0 to 9.0. This procedure allowed for the calibration of the minielectrode at millimolar and submicromolar $[Ca^{2+}]$. The electrode gave a Nernstian response at both of these $[Ca^{2+}]$ ranges (Fig. 1A). At each pH the pCa was calculated using the MaxChelator program (Bers et al., 1994).

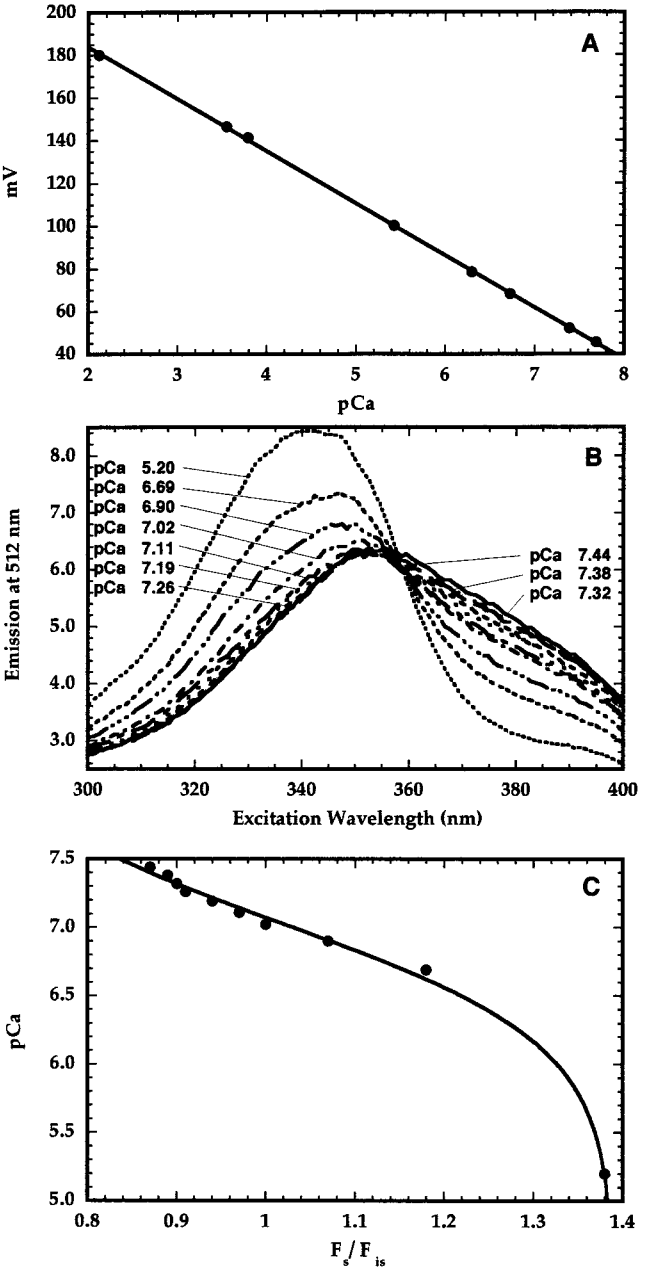


**Figure 1.** $Ca^{2+}$ minielectrode calibration. The pH of the calibration buffer containing 15 mM nitrilotriacetic acid, 12 mM EGTA, and 10 mM $CaCl_2$ was varied from 4.0 to 9.0, thereby decreasing the free $[Ca^{2+}]$ (expressed as pCa) that was calculated using computer software. At each pH the reading from the electrode was recorded (A). Afterward, the excitation spectra of a suspension of inner-envelope vesicles loaded with 1.2 mM fura-2 were recorded in the presence of 1 $\mu$M ionomycin between 300 and 400 nm, with emission monitored at 512 nm after adding small amounts of $CaCl_2$ (B). The pCa of the vesicle suspension was recorded using the minielectrode after each addition. The $F_s$ (340 nm) and $F_{is}$ (359 nm) data from each of the spectra were used to produce the pCa versus $F_s/F_{is}$ calibration curve (C).

When small amounts of $CaCl_2$ were added to membrane vesicles loaded with fura-2 in the presence of 1 $\mu$M iono-

mycin, thereby sequentially decreasing the intravesicular pCa (i.e. increasing the internal $Ca^{2+}$ concentration), significant changes in the excitation spectrum were observed (Fig. 1B). Although ionomycin catalyzes $Ca^{2+}/H^+$ exchange, the changes in the spectrum were not attributable to pH effects, because fura-2 is insensitive to pH. The fluorescence change at 512 nm brought on by $CaCl_2$ addition was greatest at an excitation wavelength of 340 nm, so this wavelength was used as the $F_s$. However, with excitation at 359 nm, the fluorescence was relatively insensitive to the change in $[Ca^{2+}]$ inside the vesicles, so this isoexcitation point was taken as the $F_{is}$. After each addition of $CaCl_2$, the fluorescence values at $F_s$ and $F_{is}$ were determined from excitation spectra and the final pCa values were measured with the $Ca^{2+}$ minielectrode. The plot of $F_s/F_{is}$ versus pCa produced the curve shown in Figure 1C.

According to Grynkiewicz et al. (1985), the fluorescence ratio of fura-2 ($R$ or $F_s/F_{is}$) can be related to $[Ca^{2+}]$ by the following equation:

$$[Ca^{2+}] = K_d \times \frac{(R - R_{min})}{(R_{max} - R)}. \quad (1)$$

The plot shown in Figure 1C yielded a curve that fit the rearranged form of Equation 1:

$$pCa = pK_d - \log\frac{[(F_s/F_{is}) - (F_s/F_{is})_{min}]/(F_s/F_{is})_{max}}{1 - [(F_s/F_{is})/(F_s/F_{is})_{max}]} \quad (2)$$

where $(F_s/F_{is})_{max}$ is the fluorescence ratio at a pCa value at which essentially all of the fura-2 is bound to $Ca^{2+}$, and $(F_s/F_{is})_{min}$ is the ratio observed when all of the fura-2 is in the unchelated form. From the plot of this particular curve, the $pK_d$ was determined to be 7.0 and the $(F_s/F_{is})_{min}$ and $(F_s/F_{is})_{max}$ were equal to 0.66 and 1.4, respectively. These values were observed to vary only slightly among vesicle preparations; however, this calibration process was performed for each set of experiments. In each preparation the relationship between $Ca^{2+}$ chelation by fura-2 and pCa was approximately linear between pCa 6.5 and 7.5.

### Determination of the Internal Buffering Capacity of Inner-Envelope Vesicles

The internal buffering capacity of the inner-envelope vesicles must be known to directly calculate the actual rate of $Ca^{2+}$ influx. The stopped-flow method used by Young and McCarty (1993) was used to determine buffering capacity by mixing a known amount of $CaCl_2$ with a vesicle suspension. Fura-2 was present in the weakly buffered external medium as an indicator of the external pCa. The observed decrease in pCa immediately after mixing was used to calculate $\beta_{out}$. This decrease was followed by a slow increase in pCa (the rebound phase) over the next 2 min as the extravesicular and intravesicular pCa reached equilibrium. The final change in pCa allows for the calculation of the internal and external buffering capacities.

In our experiments an average $\beta_{in}$ value of 7.0 ± 1.0 $\mu$M pCa unit$^{-1}$ was obtained. Because the concentration of inner-envelope protein in the stopped-flow apparatus was 0.07 mg mL$^{-1}$, the internal buffering capacity was calculated to be 100 ± 14 nmol $Ca^{2+}$ pCa unit$^{-1}$ mg$^{-1}$ protein. The calculation of $\beta_{in}$ allows for the direct calculation of the initial rate of $Ca^{2+}$ movement as the product of the initial rate in pCa units per second and the internal buffering capacity.

### $Ca^{2+}$ Movement across Vesicle Membranes

To investigate the diffusive component of $Ca^{2+}$ influx, asolectin vesicles loaded with fura-2 were mixed with $CaCl_2$ and the fluorescence emission after excitation at 340 nm ($F_s$) and 359 nm ($F_{is}$) was monitored over time. Asolectin is a protein-free lipid mixture, so $Ca^{2+}$ would traverse these membranes via passive diffusion. When these vesicles were mixed with approximately 0.35 $\mu$M free $Ca^{2+}$, no decrease in internal pCa was observed for the first 30 s (Fig. 2A). Even when the free $[Ca^{2+}]$ was increased to 10 to 25 $\mu$M, no change in fura-2 fluorescence was observed (data not shown), suggesting that the diffusive movement of $Ca^{2+}$ across nonproteinaceous membranes was quite low. However, when 0.2 $\mu$M ionomycin was added to the asolectin vesicles and subsequently mixed with a buffer containing free $Ca^{2+}$ at a concentration of 0.35 $\mu$M, a rapid $Ca^{2+}$ influx at a rate of 1.0 pCa unit s$^{-1}$ was observed (Fig. 2A), confirming that fura-2 was indeed present inside these vesicles and was still sensitive to changes in intravesicular pCa.

Membranes that were extruded were shown to be predominantly right-side-out, whereas membranes subjected to a freeze/thaw treatment were largely inside-out in orientation (Shingles and McCarty, 1995). The addition of 1.0 $\mu$M free $Ca^{2+}$ to inner-envelope membrane vesicles prepared by extrusion resulted in a decrease in intravesicular pCa during the first 30 s (Fig. 2B). The initial rate of $Ca^{2+}$ transport under these conditions was equal to 0.06 pCa unit s$^{-1}$. However, when inner-envelope membrane vesicles prepared by the freeze/thaw method were used (Fig. 2C), the observed initial rate of $Ca^{2+}$ influx was approximately 0.03 pCa unit s$^{-1}$. When vesicles of either orientation were mixed with $Ca^{2+}$ in the presence of 0.2 $\mu$M ionomycin, the added $Ca^{2+}$ equilibrated within the 1st s (data not shown).

### Effect of a Proton Gradient on $Ca^{2+}$ Influx into Inner-Envelope Vesicles

To investigate the possibility that a proton gradient may stimulate $Ca^{2+}$ uptake, $Ca^{2+}$ influx into inner-envelope vesicles prepared by extrusion was monitored in the stopped-flow apparatus under several conditions. In these assays the free external $[Ca^{2+}]$ was equal to 0.4 $\mu$M. When the external and internal pH were equivalent, the initial rate of $Ca^{2+}$ transport was determined to be 1.1 pCa units mg$^{-1}$ protein s$^{-1}$ (Fig. 3). However, when the vesicles in pH 8.0 buffer (containing 100 mM KCl) were mixed with the same buffer, giving a final external pH of 7.0 in the presence of $Ca^{2+}$, the initial rate increased to approximately 2.3 pCa units mg$^{-1}$ protein s$^{-1}$ (Fig. 3). This observation may be consistent with a $Ca^{2+}/H^+$-symport mechanism for $Ca^{2+}$ movement across inner-envelope vesicles, or it may reflect the dependence of $Ca^{2+}$ uptake on pH. Because the proton gradient has a $\Delta\Psi$ component, the

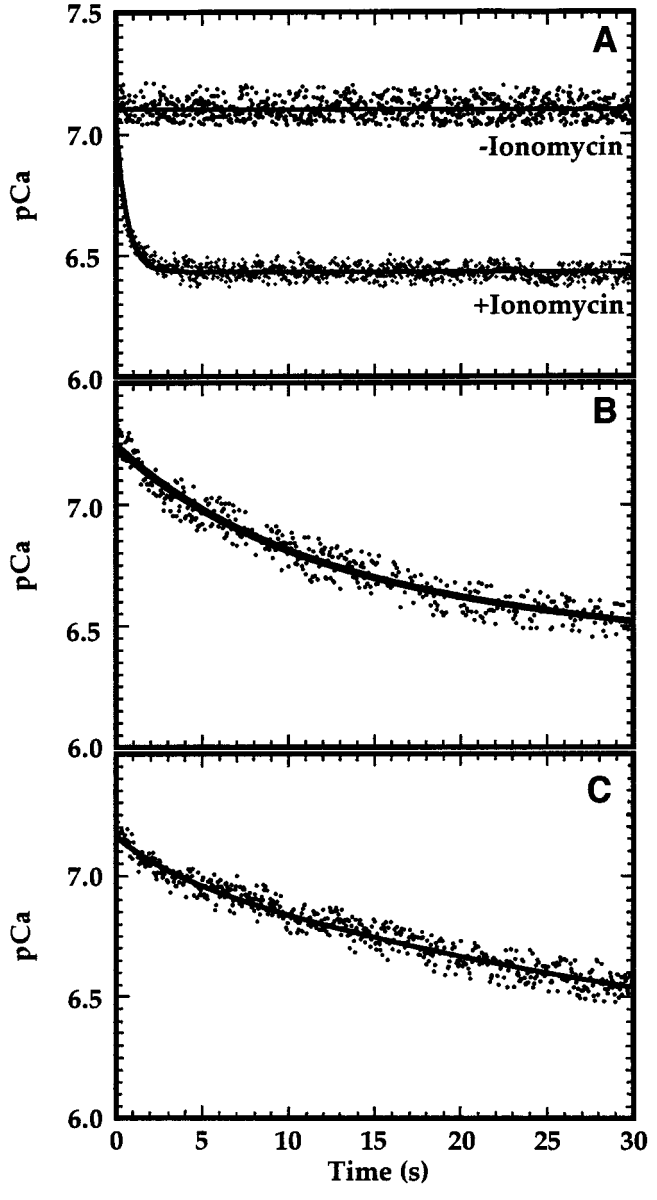


**Figure 2.** $Ca^{2+}$ influx in asolectin and chloroplast inner-envelope vesicles. A, Asolectin vesicles at pH 8.0 were mixed with pH 8.0 buffer containing $CaCl_2$ such that the free external [$Ca^{2+}$] after mixing was 0.35 $\mu$M. When used, the asolectin vesicles were preincubated with 0.2 $\mu$M ionomycin for 15 min. B, Inner-envelope vesicles (50 $\mu$g of protein) prepared by extrusion were mixed under the same conditions as in A with 1.0 $\mu$M free $Ca^{2+}$. C, Inner-envelope vesicles (50 $\mu$g of protein) prepared by the freeze/thaw method were mixed with 1.0 $\mu$M free $Ca^{2+}$. Data were fit to the single exponential equation using the graphing program Kaleidagraph (Synergy Software, Reading, PA).

results are also consistent with the potential-stimulated uniport mechanism described by Kreimer et al. (1985b). A pH difference of 1.0 unit corresponds to a $\Delta\Psi$ of $-59$ mV (lumen negative) at 25°C. In the presence of 2 nM valinomycin, which dissipates the potential gradient while essentially leaving the magnitude of the pH gradient unaffected, the stimulation of $Ca^{2+}$ movement by the pH increase disappeared (Fig. 3). Finally, when the vesicles were mixed with pH 8.0 buffer containing 100 mM choline chloride in the presence of valinomycin, resulting in the formation of a $\Delta\Psi$ of about $-16$ mV, the initial rate of $Ca^{2+}$ uptake was equivalent to that produced in the pH-increase experiment (Fig. 3). Therefore, it appears that membrane potential, rather than external pH or the pH gradient, is the cause of the stimulation of $Ca^{2+}$ uptake by a pH increase.

### The Effect of the Membrane Potential on $Ca^{2+}$ Influx

It is interesting to note that a $\Delta\Psi$ of $-59$ mV resulted in the same $Ca^{2+}$ uptake rate as a $\Delta\Psi$ of $-16$ mV (Fig. 3). To further investigate the effect of $\Delta\Psi$ on $Ca^{2+}$ uptake, $Ca^{2+}$ influx was measured as the magnitude of $\Delta\Psi$ was varied and the external free [$Ca^{2+}$] was kept constant at 0.65 $\mu$M. As illustrated in Figure 4, a significant increase in the initial rate was observed as the magnitude of the membrane potential was increased from 0 to $-15.8$ mV (lumen negative). Furthermore, the stimulation of $Ca^{2+}$ uptake by $\Delta\Psi$ appears to be nearly saturated at the highest $\Delta\Psi$ used.

### $Ca^{2+}$ Concentration Dependence of Initial Rates of $Ca^{2+}$ Movement

To determine the relationship between the initial rate of $Ca^{2+}$ influx and external free [$Ca^{2+}$], inner-envelope vesicles were mixed with buffers so that the $\Delta\Psi$ was main-

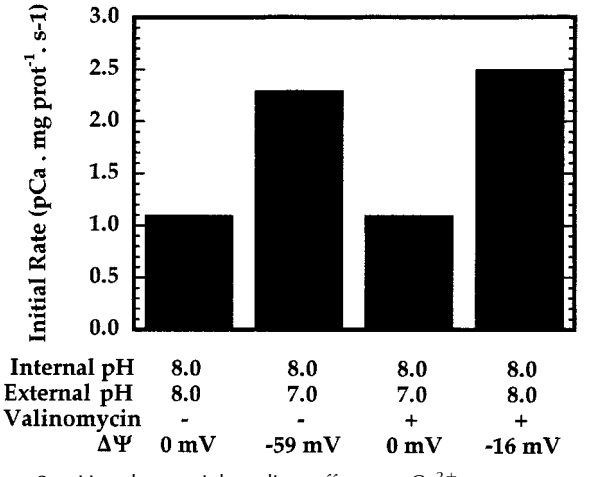


**Figure 3.** pH and potential gradient effects on $Ca^{2+}$ transport across inner-envelope vesicles. Extruded inner-envelope vesicles (15 $\mu$g of protein) at pH 8.0 were mixed with various buffers containing $Ca^{2+}$ such that after mixing the free external [$Ca^{2+}$] was approximately 0.4 $\mu$M. When the external pH was 7.0 in the absence of valinomycin, the $\Delta\Psi$ was calculated to be $-59$ mV using the Nernst equation. When used, 2 nM valinomycin was preincubated with vesicles for 30 min on ice. A membrane potential of about $-16$ mV was imposed across the vesicle membranes by mixing vesicles in 100 mM KCl, 10 mM K-Hepes, 100 mM Suc, and 100 $\mu$M EGTA with an equal volume of the same buffer in which the 100 mM KCl was replaced with 100 mM choline chloride. The instantaneous equilibration of $K^+$ in the presence of valinomycin resulted in a negatively charged vesicle interior.

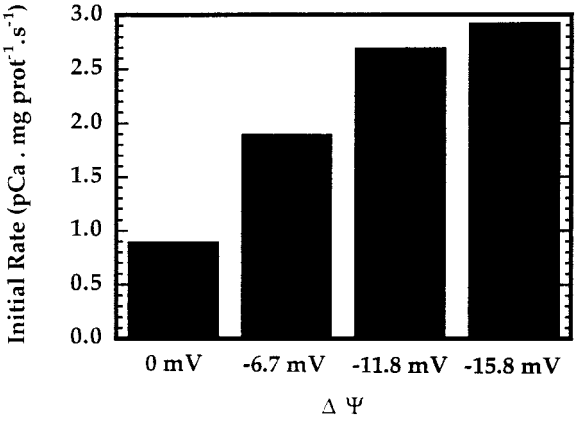


**Figure 4.** Potential gradient effect on $Ca^{2+}$ transport across inner-envelope vesicles. $Ca^{2+}$ influx into inner-envelope vesicles prepared by extrusion was monitored under varying magnitudes of $\Delta\Psi$ generated by adjusting the KCl and choline chloride solutions used in Figure 3. The intravesicular $[K^+]$ was maintained at 110 mM. The $\Delta\Psi$ was varied by changing the external $[K^+]$. Inner-envelope vesicles (15 $\mu$g of protein) were incubated with 2 nM valinomycin for 30 min before each experiment.

tained at approximately −16 mV while free $[Ca^{2+}]$ was varied in the submicromolar range. As seen in Figure 5, the observed increase in initial rate with respect to increasing free $[Ca^{2+}]$ was linear over the range studied. Also, because the initial internal pCa in the majority of the vesicle preparations ranged from 7.2 to 7.3 units, it follows that the initial rate of $Ca^{2+}$ influx would be minimal when the external $[Ca^{2+}]$ was equal to 0.06 $\mu$M (Fig. 5), which corresponds to an external pCa of 7.22 units. Both Muto et al. (1982) and Kreimer et al. (1985b) used $Ca^{2+}$ concentrations in their studies that exceeded the estimated physiological concentration of 0.05 to 0.4 $\mu$M in the plant cell cytosol (Evans et al., 1991). In fact, the $K_m$ for $Ca^{2+}$ uptake by isolated chloroplasts was determined to be 188 $\mu$M (Kreimer et al., 1985b), which indicates that $Ca^{2+}$ transport would not be saturable at low physiological levels, as seen in Figure 5. A limitation of these experiments is that $Ca^{2+}$ concentrations higher than about 1 $\mu$M cannot be measured reliably, because fura-2 fluorescence becomes saturated when the intravesicular $[Ca^{2+}]$ exceeds this value.

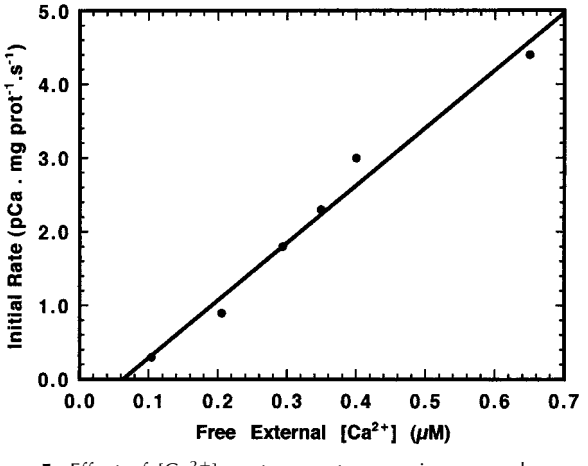


**Figure 5.** Effect of $[Ca^{2+}]$ on transport across inner-envelope vesicles. Extruded inner-envelope vesicles (15 $\mu$g of protein) were mixed with buffer containing various concentrations of free $Ca^{2+}$, and $Ca^{2+}$ influx was monitored. In each experiment a membrane potential of approximately −16 mV was imposed across the vesicle membranes, as described in Figure 3.

**Table I.** *Effect of inhibitors on $Ca^{2+}$ influx into pea chloroplast inner-envelope membranes prepared by extrusion*

$Ca^{2+}$ movement was monitored for 30 s in the absence and presence of known $Ca^{2+}$-channel blockers at a concentration of 10 $\mu$M. In each experiment a membrane potential of −16 mV (when used) was imposed across the vesicle membranes and the $[Ca^{2+}]$ in the external medium was equal to 0.4 $\mu$M. The amount of inner-envelope protein in each experiment was approximately 15 $\mu$g.

| Inhibitor | pCa | |
|---|---|---|
| | Initial rate | Inhibition |
| | *units $mg^{-1}$ protein $s^{-1}$* | *%* |
| No membrane potential | | |
| Control | 1.05 | 0 |
| + Diltiazem | 0.16 | 85 |
| + Ruthenium red | 0.10 | 90 |
| Membrane potential of −16 mV | | |
| Control | 2.80 | 0 |
| + $LaCl_3$ | 2.93 | 0 |
| + Diltiazem | 2.13 | 25 |
| + Ruthenium red | 0.13 | 96 |

### Effect of Inhibitors on $Ca^{2+}$ Influx

In a previous study (Kreimer et al., 1985a, 1985b) $Ca^{2+}$ influx into intact chloroplasts was shown to be inhibited by micromolar amounts of ruthenium red. Similar results were observed in this study. When 0.4 $\mu$M $Ca^{2+}$ was present in the external medium and $\Delta\Psi$ was −16 mV (lumen negative), the initial rate of $Ca^{2+}$ influx was equal to 2.8 pCa units $mg^{-1}$ protein $s^{-1}$. In the presence of 10 $\mu$M ruthenium red, the observed $Ca^{2+}$-transport activity was 0.1 pCa unit $mg^{-1}$ protein $s^{-1}$, an inhibition of 96% (Table I).

Other pharmacological agents known to block $Ca^{2+}$-channel activity were also tested for their effects on $Ca^{2+}$ movement. Diltiazem inhibited the non-potential-dependent $Ca^{2+}$ influx by approximately 85%, but only inhibited potential-stimulated $Ca^{2+}$ influx by 25% (Table I). $LaCl_3$ and two other known $Ca^{2+}$-channel blockers, nifedipine and verapamil, had little inhibitory effect on $Ca^{2+}$ transport. Other divalent cations that might compete for $Ca^{2+}$ uptake were not tested because of their known effects on fura-2 fluorescence. However, an independent study on divalent cation uptake could be performed using fura-2-loaded membrane vesicles.

## DISCUSSION

In this study we have demonstrated that a sensitive $Ca^{2+}$ probe, fura-2, can be loaded into chloroplast inner-envelope vesicles and used to monitor $Ca^{2+}$ transport across this membrane. This system has several advantages, including control of the buffer components on both sides of

the membrane, the analysis of $Ca^{2+}$ movement under essentially zero *trans* conditions, and sensitivity to $Ca^{2+}$ at submicromolar levels. Combined with stopped-flow spectrofluorometry, the movement of $Ca^{2+}$ can be followed with a resolution time of 2 ms (Fig. 2). In addition, previous methods used to produce membrane vesicles of right-side-out or inside-out orientation (Shingles and McCarty, 1995) allow for the evaluation of the sidedness of $Ca^{2+}$ movement.

Previous studies using intact chloroplasts have demonstrated light-stimulated $Ca^{2+}$ uptake (Muto et al., 1982; Kreimer et al., 1985b). The mechanism by which $Ca^{2+}$ influx occurs in chloroplasts is in dispute. Muto et al. (1982) claimed the presence of a $Ca^{2+}/H^{+}$ antiporter, whereas Kreimer et al. (1985b) concluded that this process was mediated by an electrogenic uniport-carrier system. In our inner-envelope vesicle preparations we were not able to measure any $Ca^{2+}$-linked, proton transport using membrane vesicles loaded with the pH-sensitive fluorophore pyranine (data not shown). This would seem to indicate that $Ca^{2+}$ does not move across these membrane vesicles by a $Ca^{2+}/H^{+}$-antiport mechanism. Huang et al. (1993) reported that an antibody raised against a portion of a putative $Ca^{2+}$-ATPase recognizes a 95-kD polypeptide in chloroplast inner-envelope preparations, suggesting that $Ca^{2+}$ pumping might also be a mechanism for moving $Ca^{2+}$ across this membrane. However, they were not able to show any $Ca^{2+}$-dependent ATPase activity or ATP-dependent $Ca^{2+}$ uptake in their inner-envelope preparations from pea chloroplasts. Similarly, we were not able to measure either of these activities using fura-2-loaded vesicles or a $^{45}Ca$ assay in the presence or absence of added calmodulin (data not shown).

Because the rate at which $Ca^{2+}$ diffuses across nonproteinaceous membranes is very low (Fig. 2A), the movement of $Ca^{2+}$ across the chloroplast inner-envelope membrane observed in this study indicates the presence of a $Ca^{2+}$ uniporter in our vesicle preparations. The influx of $Ca^{2+}$ into predominantly right-side-out inner-envelope vesicles was stimulated by a negative $\Delta\Psi$ and was very sensitive to micromolar amounts of ruthenium red (Table I). These results are in agreement with those of previous studies using intact chloroplasts (Kreimer et al., 1985a, 1985b). Furthermore, a high magnitude of $\Delta\Psi$ may not be necessary to fully stimulate $Ca^{2+}$-transport activity (Fig. 4). The observation that $Ca^{2+}$ influx does not increase at voltages more negative than $-11.8$ mV indicates that uptake is not dependent on an electrophoretic driving force, but, rather, may occur through a voltage-dependent channel. In addition, the fact that $Ca^{2+}$ influx does increase with $Ca^{2+}$ concentration at a constant $\Delta\Psi$ of $-16$ mV (Fig. 5) indicates that this channel would be $Ca^{2+}$ specific.

The initial rate of $Ca^{2+}$ influx at a free $Ca^{2+}$ concentration of 0.2 to 0.4 $\mu$M was determined to range between 1.0 and 3.0 pCa units $mg^{-1}$ protein $s^{-1}$ (Fig. 5). Under these conditions the $\beta_{in}$ of the vesicles was equal to approximately 100 nmol $Ca^{2+}$ pCa $unit^{-1}$ $mg^{-1}$ inner-envelope protein. Assuming that there is 0.1 mg of inner-envelope protein per mg of chlorophyll (Young and McCarty, 1993), the initial rate was calculated to range from 0.54 to 1.62 $\mu$mol $Ca^{2+}$ $h^{-1}$ $mg^{-1}$ chlorophyll at 25°C. These rates are 10-fold lower than the rates calculated by Kreimer et al. (1985b); however, we used a $[Ca^{2+}]$ of up to 100 times lower than that used in the previous study.

Antagonists to $Ca^{2+}$ channels have been frequently used to characterize transport processes. Various drugs such as verapamil, nifedipine, and diltiazem have been frequently used as inhibitors of voltage-dependent $Ca^{2+}$ channels (Triggle, 1990; Takahashi et al., 1997). In this study $Ca^{2+}$ influx under nonpotential conditions was greatly inhibited by diltiazem and ruthenium red (Table I). The weak inhibition by diltiazem under potential gradient conditions suggests that there may be more than one pathway for $Ca^{2+}$ uptake across chloroplast inner envelopes. Furthermore, $LaCl_3$ can be used to inhibit a ruthenium-red-insensitive $Ca^{2+}/H^{+}$ exchanger in liver and kidney mitochondria (Saris and Allshire, 1989). In chloroplast inner-envelope membranes $LaCl_3$ had no effect, indicating that $Ca^{2+}$ does not move by a $Ca^{2+}/H^{+}$ antiporter. In contrast, ruthenium red, which inhibits the plant mitochondrial $Ca^{2+}$ uniporter (Wilson and Graesser, 1976), almost completely inhibited $Ca^{2+}$ movement across chloroplast inner-envelope vesicles (Table I).

Chloroplasts have been reported to contain from 4 to 23 mM total $Ca^{2+}$ (Portis and Heldt, 1976). The concentration of free $Ca^{2+}$ in the stroma of chloroplasts kept in the dark was determined to be 2.4 to 6.3 $\mu$M (Kreimer et al., 1988), which indicates that most of the $Ca^{2+}$ is sequestered at binding sites within the chloroplast. Johnson et al. (1995), using a transgenic tobacco line in which expressed apoaequorin was targeted to the chloroplast stroma, estimated that under light conditions, chloroplasts maintain basal free $[Ca^{2+}]$ in the low nanomolar range (150 nM), indicating that even more $Ca^{2+}$-binding sites, such as those on PSII (Grove and Brudvig, 1998), become available during illumination. However, when plants are transferred from light to dark conditions, a transient increase in free stromal $[Ca^{2+}]$, which peaks at 5 to 10 $\mu$M, takes place (Johnson et al., 1995). The transient nature of the stromal $[Ca^{2+}]$ increase as plants are switched from light to dark suggests that there may be a mechanism for $Ca^{2+}$ efflux across the inner envelope, and also indicates that $Ca^{2+}$ efflux may be slower than influx. Under certain conditions, Kreimer et al. (1985a, 1985b) observed an efflux rather than a net influx of $Ca^{2+}$ into intact chloroplasts; however, they were unable to conclude whether efflux occurred via a reversal of uniport.

Inner-envelope membranes prepared by the freeze/thaw method produce vesicles of predominantly inside-out orientation (Shingles and McCarty, 1995). When vesicles in pH 8.0 buffer prepared by the freeze/thaw method were mixed with 1 $\mu$M free $Ca^{2+}$, a significant $Ca^{2+}$ influx was observed (Fig. 2C). This movement was also inhibited by ruthenium red but not as strongly as in predominantly right-side-out vesicles (data not shown). Therefore, it is possible that the $Ca^{2+}$ uniporter may be reversed and may catalyze the net efflux of $Ca^{2+}$ after chloroplasts are transferred from light to dark conditions. However, because the initial rates measured with predominantly inside-out vesicles were one-third of those measured with predominantly right-side-out vesicles, there is a directional preference to

$Ca^{2+}$ transport across the chloroplast inner envelope favoring $Ca^{2+}$ uptake.

In this study the initial rate of $Ca^{2+}$ uniport activity across the pea chloroplast inner envelope under physiological free [$Ca^{2+}$] was measured to be 0.5 to 1.6 $\mu$mol $h^{-1}$ $mg^{-1}$ chlorophyll and was demonstrated to be stimulated by a negative membrane potential. It has been demonstrated that an inwardly directed pH gradient of 0.25 to 0.5 unit can be maintained across the chloroplast envelope by the $H^+$-ATPase present in the inner-envelope membrane (Shingles and McCarty, 1994). This $H^+$ gradient also represents a $\Delta\Psi$ of 15 to 30 mV, enough to couple this ATPase to the potential-stimulated $Ca^{2+}$ uniporter. This mechanism could result in the efficient uptake of $Ca^{2+}$ by the chloroplasts as well as the activation of photosynthetic $CO_2$ assimilation.

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