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Transport Properties of the Tomato Fruit Tonoplast[1]

III. TEMPERATURE DEPENDENCE OF CALCIUM TRANSPORT

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ABSTRACT

Calcium transport into tomato (*Lycopersicon esculentum* Mill, cv Castlemart) fruit tonoplast vesicles was studied. Calcium uptake was stimulated approximately 10-fold by MgATP. Two ATP-dependent Ca^{2+} transport activities could be resolved on the basis of sensitivity to nitrate and affinity for Ca^{2+}. A low affinity Ca^{2+} uptake system (K_m > 200 micromolar) was inhibited by nitrate and ionophores and is thought to represent a tonoplast localized H^+/Ca^{2+} antiport. A high affinity Ca^{2+} uptake system (K_m = 6 micromolar) was not inhibited by nitrate, had reduced sensitivity to ionophores, and appeared to be associated with a population of low density endoplasmic reticulum vesicles that contaminated the tonoplast-enriched membrane fraction. Arrhenius plots of the temperature dependence of Ca^{2+} transport in tomato membrane vesicles showed a sharp increase in activation energy at temperatures below 10 to 12°C that was not observed in red beet membrane vesicles. This low temperature effect on tonoplast Ca^{2+}/H^+ antiport activity could only by partially ascribed to an effect of low temperature on H^+-ATPase activity, ATP-dependent H^+ transport, passive H^+ fluxes, or passive Ca^{2+} fluxes. These results suggest that low temperature directly affects Ca^{2+}/H^+ exchange across the tomato fruit tonoplast, resulting in an apparent change in activation energy for the transport reaction. This could result from a direct effect of temperature on the Ca^{2+}/H^+ exchange protein or by an indirect effect of temperature on lipid interactions with the Ca^{2+}/H^+ exchange protein.

Calcium is widely acknowledged as an important second messenger in eukaryotic organisms (11, 18). Calcium fluxes have been associated with major changes in metabolism in plants and animals (11, 13), and it has also been proposed that transient fluctuations in cytoplasmic Ca^{2+} activity may play a central role in chilling injury of chilling-sensitive plants (14, 21, 22). It is therefore of interest to obtain a complete understanding of mechanisms regulating subcellular Ca^{2+} distribution in plant cells. ATP-dependent transport of Ca^{2+} has been demonstrated in microsomal membrane preparations (9, 17, 24), in mitochondria (10), and in membrane fractions enriched in endoplasmic reticulum (5, 6, 8, 20) or tonoplast (8, 20).

In tomato fruit, Ca^{2+} regulation has been implicated in the regulation of fruit softening (7), calmodulin-mediated protein phosphorylation during fruit ripening (25), and in chilling injury (21, 22). Here we have investigated the mechanisms and temperature dependence of ATP-dependent Ca^{2+} transport in a tonoplast enriched membrane fraction isolated from mature-green tomato fruit.

MATERIALS AND METHODS

Plant Material. Mature green tomato (*Lycopersicon esculentum* Mill. cv Castlemart) were harvested from the field (summer) or greenhouse (winter) immediately prior to use. Fruits were sliced, the locular material removed, and the pericarp used for membrane isolation.

Membrane Preparation. A tonoplast-enriched membrane fraction from tomato was prepared as previously described (16), with minor modifications. Pericarp tissue (100 g) was homogenized in a chilled blender with 200 ml of 250 mM sucrose, 70 mM Tris/Mes (pH 8), 4 mM DTT, 3 mM EDTA, 0.1% (w/v) BSA, and 0.5% (w/v) soluble polyvinylpyrrolidine (PVP-40). The homogenate was filtered through cheesecloth and the filtrate centrifuged for 15 min at 13,000*g* in a Beckman SW 28 rotor at 4°C. The supernatant was retained and centrifuged at 80,000*g* for 45 min. The pellet was resuspended in 250 mM sucrose, 2 mM DTT, and 5 mM Tris/Mes (pH 7) and layered onto a discontinuous (16/26/34/40% w/v) or continuous (15–45% w/v) sucrose gradient containing 2 mM DTT and 5 mM Tris/Mes (pH 7). Sucrose gradients were centrifuged at 80,000*g* for 2 h. The tonoplast-enriched fraction was collected from the 16/26% (w/v) sucrose interface, diluted with an equal volume of 10 mM Tris/Mes (pH 7) and membranes pelleted at 80,000*g* for 30 min. The pellet was resuspended in 250 mM sucrose, 2 mM DTT, and 5 mM Tris/Mes (pH 7) to an average concentration of 1.6 mg protein/ml and frozen in liquid nitrogen. For the continuous sucrose gradient, 18 fractions (2 ml each) were collected and stored in liquid nitrogen until assayed. Beet tonoplast membranes were prepared as described (2).

Calcium Transport Assays. Calcium transport was assayed by measuring the uptake of radiolabeled Ca^{2+} ($^{45}CaCl_2$ was obtained from New England Nuclear [164 × 10^7 Bq/mg Ca^{2+}] or Amersham [90 × 10^7 Bq/mg Ca^{2+}]). The specific activity of $^{45}Ca^{2+}$ ranged from 30 × 10^5 to 164 × 10^7 Bq/mg Ca^{2+} in the assay, depending on the Ca^{2+} concentration in the assay. Assays were conducted in 101 μl volume containing 50 mM KCl, 6 mM $MgSO_4$, 25 mM K_2SO_4, 3 mM Tris/ATP, 69 mM sucrose, 0.6 mM DTT, 1.4 mM Tris/Mes (pH 7) and approximately 20 μg membrane protein. The water used in all reaction stocks was double deionized and passed through a Dowex 50 column to eliminate Ca^{2+} contamination. Inhibitors soluble in methanol (*i.e.* A23187, valinomycin, nigericin, gramicidin, oligomycin, and DCCD[4])

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[4] Abbreviation: DCCD: *N,N'*-dicyclohexylcarbodiimide.

were added after membrane addition to a final methanol concentration of 0.1% (v/v). Assays were carried out in disposable polystyrene test tubes maintained at 28°C in a water bath. Except for the time course study, Ca^{2+} uptake was measured after a 15 min incubation. The reaction was stopped by dilution with 4 ml of chilled 250 mM sucrose, 50 mM KCl, and 25 mM Tris/Mes (pH 7). The diluted vesicles were immediately filtered onto 0.45 μm Millipore HAWP filters, and the test tube and filter rinsed with an additional 4 ml of buffer. Filters were transferred to scintillation vials and radioactivity determined by liquid scintillation counting. For experiments determining temperature dependence of Ca^{2+} uptake the temperature range was achieved using an aluminum temperature gradient block. The dimensions of the block were 13 cm wide, 8 cm high, and 60 cm long with 10 sets of 5 evenly spaced holes drilled along the top of the block and equipped with channels to allow temeprature-regulated water to be circulated at either end of the block. After temperature equilibration the block maintained a linear temperature gradient whose endpoints were determined by the temperature of the water circulated at each end.

Calcium Efflux. Calcium retained in vesicles following passive efflux at different temperatures and time intervals was monitored. Vesicles were preloaded with $^{45}Ca^{2+}$ by incubation at 28°C for 40 to 60 min in a medium containing 50 mM KCl, 25 mM K_2SO_4, 6 mM $MgSO_4$, 3 mM ATP, 50 mM Hepes/Tris (pH 7), and 1 μM $^{45}Ca^{2+}$. Calcium loading was terminated by the addition of 95 μM EGTA and tubes then transferred to the appropriate temperature. At the indicated times the efflux assay was terminated and the Ca^{2+} retained in the membrane vesicles was determined in the manner described for Ca^{2+} uptake assays.

H^+ Transport. Development of interior acid pH gradients was monitored as the quenching of a fluorescent permeant amine dye (acridine orange) with a Perkin Elmer fluorescence spectrophotometer (16). Temperatures of the assays were controlled between 2 and 21°C with a circulating refrigerated water bath connected to the cuvette water jacket. Temperatures in the cuvette were monitored with a Cu/Cd thermocouple and microvoltmeter, using an ice bath reference. Approximately 47 and 150 μg membrane protein were used for individual tomato and red beet H^+ transport assays, respectively. The reaction medium was 50 mM KCl, 5 mM $MgSO_4$, 222 mM sucrose, 4.5 mM Tris/Mes (pH 7), 1.8 mM DTT, and 3 mM Tris/ATP. Assays were started by addition of ATP, and quench of acridine orange fluorescence was monitored at excitation and emission wave lengths of 472 and 525 nm, respectively. Total quench (% Q) was measured as the proportion of full quench attained when the quench curve reached its minimum level. The initial rate of quench (% Q min^{-1}) was estimated from the initial slope of the quench trace upon addition of ATP.

Enzyme, Protein, and Chlorophyll Assay. Nitrate and vanadate sensitive ATPase activities were measured as tonoplast and plasma membrane markers, respectively (2). ATPase activity was assayed at 28°C as previously described (16). The reaction contained 30 mM Tris/Mes (pH 6.5), 6 mM $MgSO_4$, 50 mM KCl, 3 mM Tris/ATP, either 25 mM K_2SO_4 or 50 mM KNO_3, and 50 μM vanadate where indicated. The temperature range was achieved using the aluminum temperature gradient block described above.

Antimycin A-insensitive NAD(P)H-Cyt *c* reductase activity was assayed as an endoplasmic reticulum marker enzyme (12). The assay contained 47 mM phosphate buffer (pH 7.5), 0.8 mM NaCN, 7 μM antimycin A, 20 μM Cyt *c*, and 0.2 mM NADPH in 1.5 ml. The reaction was started by the addition of a 100 μl aliquot of membrane fraction and reduction of Cyt *c* measured at 550 nm.

UDPase activity was assayed as a marker for Golgi membranes (15). The reaction contained 0.1% (v/v) Triton X-100, 1.5 mM $MnSO_4$, 30 mM bis-tris-propane/Mes (pH 7), and 1.5 mM UDP in 0.5 ml. The reaction was run for 30 min at 28°C and liberation of inorganic phosphate determined by the method of Ames (1), except that 4% SDS (w/v) was included in the reagent to avoid interference by Triton X-100.

Protein was determined by the method of Schaffner and Weissman (19).

RESULTS

Preliminary experiments were carried out with 0.7 μM $^{45}CaCl_2$ to determine the time course of ATP-dependent Ca^{2+} uptake in the tonoplast-enriched membrane fraction isolated from tomato fruit (Fig. 1). We have previously characterized a NO_3^--sensitive H^+-ATPase associated with this membrane fraction (16) and others have characterized Ca^{2+}/H^+ antiport activity in plant tonoplast preparations (4, 8, 20). Our results indicated that ATP stimulated Ca^{2+} uptake approximately 10-fold and this uptake was reversible by the calcium ionophore, A23187. However, under the conditions employed, Ca^{2+} uptake was stimulated rather than inhibited by NO_3^-, suggesting that the uptake mechanism was not coupled to the activity of the NO_3^--sensitive tonoplast H^+-ATPase.

When a wider range of Ca^{2+} concentration was examined both NO_3^--sensitive and NO_3^--insensitive Ca^{2+} uptake could be resolved (Fig. 2). At concentrations below 1 μM Ca^{2+}, NO_3^- either had no effect or stimulated Ca^{2+} uptake (Figs. 1 and 2A). However, at concentrations above 10 μM Ca^{2+}, the NO_3^--insensitive component of Ca^{2+} uptake saturated and NO_3^- inhibition of Ca^{2+} uptake became evident (Fig. 2A). When a Hanes-Woolf plot of NO_3^--insensitive Ca^{2+} uptake was plotted, a K_m of approximately 6 μM Ca^{2+} was estimated for this component of Ca^{2+} uptake (Fig. 2B). To examine the NO_3^--sensitive component of Ca^{2+} uptake, a much wider concentration range was employed (Fig. 2, C and D). Over this high concentration range, NO_3^- inhibition of Ca^{2+} uptake was evident and the Hanes-Woolf transformation of NO_3^--sensitive Ca^{2+} uptake indicated a K_m of approximately 250 μM (Fig. 2D).

Interestingly, we found that attempts to assay the high affinity (NO_3^--insensitive) component of Ca^{2+} uptake in glass test tubes was confounded by high levels of Ca^{2+} released from the glass surface. When assayed in the presence of 0.7 μM exogenous Ca^{2+}, uptake was consistently lower when assayed in glass rather than

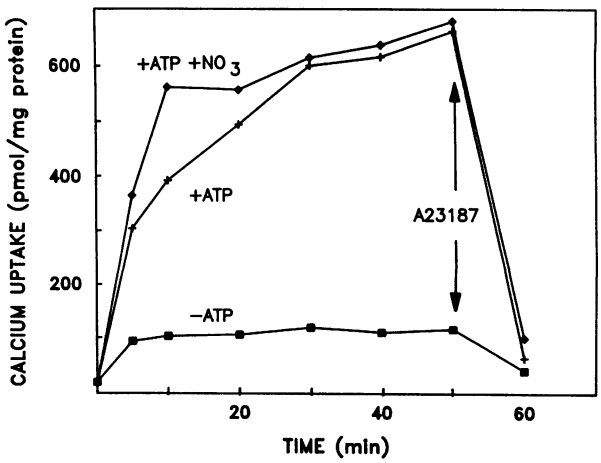

FIG. 1. Time course of Ca^{2+} uptake by tonoplast-enriched membrane vesicles from tomato fruit. Uptake was measured in polystyrene test tubes at a Ca^{2+} concentration of 0.7 μM in the absence (■) or presence (+) of 3 mM ATP or in the presence of 3 mM ATP and 50 mM KNO_3 (◇). When added, 50 mM KNO_3 replaced 25 mM K_2SO_4 in the reaction medium. At 55 min, 5 μM A23187 was added to the reaction medium. Data are means of duplicate assays.

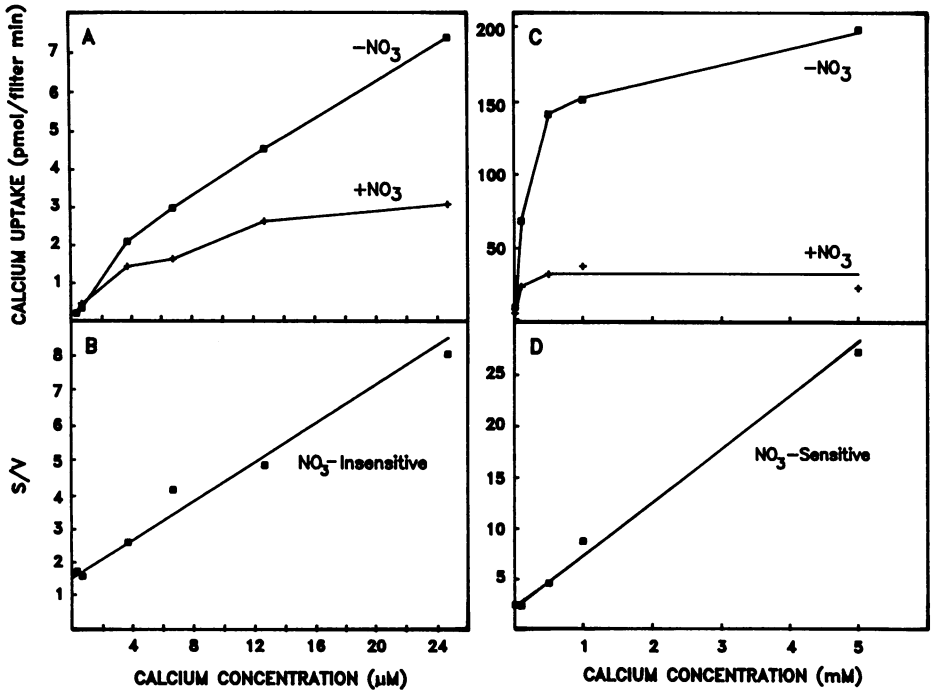

FIG. 2. Calcium concentration dependence of ATP-dependent Ca^{2+} uptake by tonoplast-enriched membrane vesicles from tomato fruit. Assays were carried out over the concentration range between 0.35 and 24.7 μM (panel A) or between 50 and 5000 μM (panel C). ATP-dependent Ca^{2+} uptake was determined as the difference between uptake in the absence and presence of 3 mM ATP; these are the values plotted. Over both concentration ranges Ca^{2+} uptake was measured in the absence and presence of 50 mM KNO_3. Data for NO_3^--insensitive Ca^{2+} uptake over the low concentration range were determined as the uptake measured in the presence of 50 mM KNO_3 and this data replotted using a Hanes-Woolf transformation (panel B). NO_3^--sensitive Ca^{2+} uptake over the high concentration range was determined as the difference in uptake measured in the absence and presence of 50 mM KNO_3 and this data replotted using a Hanes-Woolf transformation (panel D).

plastic test tubes. Furthermore, NO_3^- inhibited Ca^{2+} uptake when assayed in glass test tubes but stimulated Ca^{2+} uptake when assayed in plastic test tubes (data not shown). Based on the results shown in Figures 1 and 2 this difference between glass and plastic test tubes can be readily explained by the release of Ca^{2+} from the glass surface. This release of Ca^{2+} decreased the specific activity of $^{45}Ca^{2+}$ in the reaction, leading to an apparent decrease in Ca^{2+} uptake rate and increased the Ca^{2+} concentration to a range where NO_3^- inhibition became apparent. We estimate that sufficient Ca^{2+} was released from glass test tubes to raise the Ca^{2+} concentration in the assay to approximately 20 μM.

To further characterize the two components of ATP-dependent Ca^{2+} uptake, inhibitor sensitivity of Ca^{2+} uptake was determined at low (0.7 μM) or high (210 μM) Ca^{2+} concentration (Table I). At high Ca^{2+} concentration uptake was strongly inhibited by NO_3^-, DCCD, and ionophores but was insensitive to inhibition by vanadate or mitochondrial ATPase inhibitors, N_3, and oligomycin. In contrast, at low Ca^{2+} concentration Ca^{2+} uptake was stimulated by NO_3^-, inhibited by vanadate and DCCD, showed reduced sensitivity to ionophores but was similarly uneffected by the mitochondrial ATPase inhibitors, N_3, and oligomycin. These results indicate that the two Ca^{2+} uptake mechanisms can be distinguished on the basis of Ca^{2+} concentration dependence and inhibitor sensitivity. The NO_3^- and ionophore sensitivity of the low affinity Ca^{2+} uptake system suggests that this uptake represents the activity of a tonoplast localized Ca^{2+}/H^+ antiport coupled to the activity of the NO_3^--sensitive H^+-ATPase. This type of system has been characterized in several other tissues including red beet (4, 8, 20). For comparison, we also assayed Ca^{2+} uptake at high concentration in red beet tonoplast vesicles and found inhibitor characteristics almost identical to those of the low affinity Ca^{2+} uptake system in tomato fruit membrane vesicles (Table I). This supports our view that this system is indeed comparable to the Ca^{2+}/H^+ antiport characterized in other systems. The high affinity system of Ca^{2+} uptake (NO_3^--insensitive) had inhibitor characteristics similar to Ca^{2+}-ATPase-dependent Ca^{2+} uptake that has been localized to the endoplasmic reticulum in other tissues (8, 20).

Table I. *Effect of Inhibitors on Ca^{2+} Uptake in Tomato and Red Beet Tonoplast-Enriched Membrane Fractions*

Calcium uptake was measured at 0.7 and 210 μM Ca^{2+} in the presence of the indicated inhibitors. All assays contained 0.1% methanol. Data are the means and standard deviations for duplicate assays from two independent experiments.

Inhibitor	Ca^{2+} Uptake		
	Tomato		Red beet
	0.7 μM Ca^{2+}	210 μM Ca^{2+}	210 μM Ca^{2+}
	% of control		
Control	100	100	100
+NO_3^- (50 mM)	126 ± 4	23 ± 5	29 ± 4
+Vanadate (50 μM)	53 ± 6	99 ± 15	113 ± 14
+DCCD (100 μM)	51 ± 8	10 ± 4	14 ± 2
+A23187 (5 μM)	05 ± 5	10 ± 3	15 ± 5
+Nigericin (1 μM)	70 ± 4	23 ± 9	21 ± 6
+Gramicidin (1 μM)	77 ± 5	46 ± 15	52 ± 33
+Valinomycin (0.1 μM)	104 ± 3	110 ± 4	182 ± 15
+N_3 (200 μM)	103 ± 4	103 ± 6	103 ± 6
+Oligomycin (1 μg/mL)	101 ± 6	99 ± 11	114 ± 18

To assess the subcellular localization of high affinity ATP-dependent Ca^{2+} uptake, a continuous sucrose gradient was analyzed (Fig. 3). Nitrate- and vanadate-sensitive ATPase activities were resolved on the gradient (panel B); however, the distribution of high affinity Ca^{2+} uptake (panel C) did not coincide with either ATPase activity, showed NO_3^--stimulation rather than NO_3^--inhibition in all fractions and showed a prominent low-density peak that would be expected to be collected with our tonoplast-enriched membrane fraction taken from a 16/26% sucrose interface. This low-density peak of high affinity Ca^{2+} uptake coincided with a low-density peak of NAD(P)H Cyt *c* reductase activity (panel D), suggesting that the high affinity Ca^{2+} uptake was localized in the endoplasmic reticulum. A second, high-density peak of NAD(P)H Cyt *c* reductase was not associated with a peak of Ca^{2+} uptake (panel D), suggesting the existence of density gradient-resolvable endoplasmic reticulum domains, only some of which possess high affinity Ca^{2+} uptake.

Because increases in cytoplasmic Ca^{2+} have been implicated

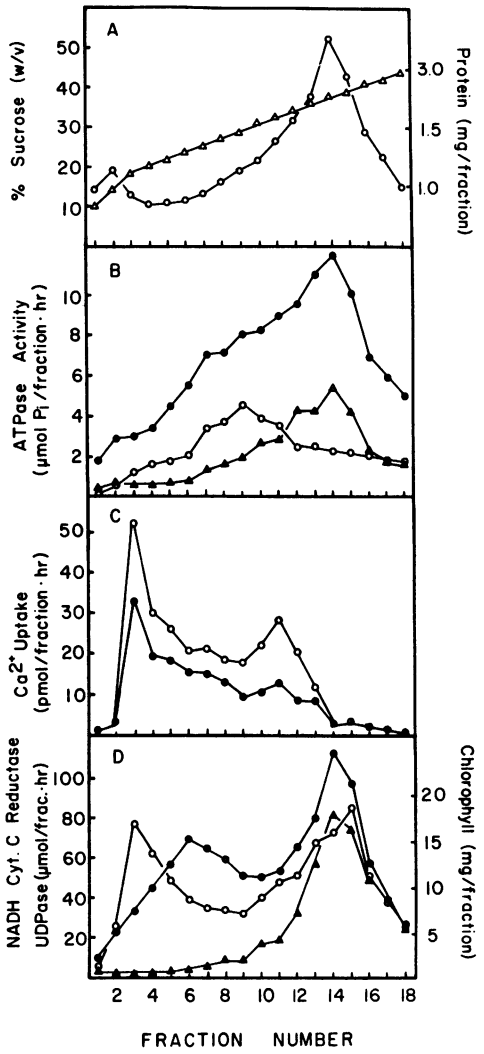

FIG. 3. Distribution of high affinity Ca^{2+} uptake activity and marker enzymes associated with tomato fruit microsomal membranes on a continuous sucrose gradient. Panel A, % sucrose (w/v) (△), and protein (○); panel B, total ATPase activity (●), NO_3^--sensitive ATPase activity (○), and vanadate-sensitive ATPase activity (▲); panel C, ATP-dependent Ca^{2+} uptake in the absence (●) or presence (○) of 50 mM KNO_3. Calcium uptake was measured at 0.7 μM Ca^{2+}. Panel D, Chl (▲), UDPase activity (●), and NAD(P)H Cyt *c* reductase activity (○).

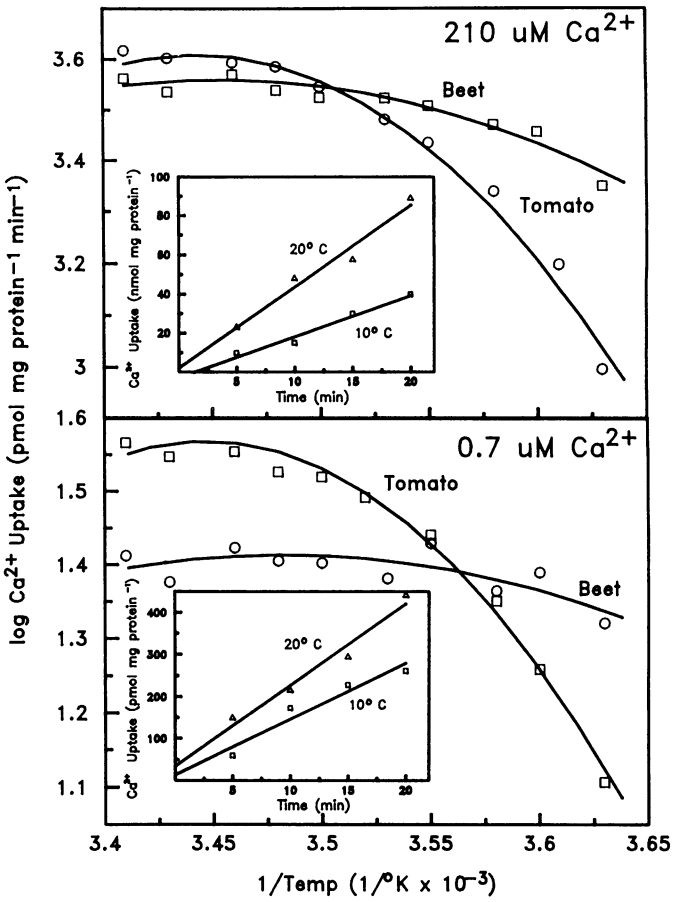

FIG. 4. Arrhenius plots of the temperature-dependence of ATP-dependent Ca^{2+} uptake at 0.7 and 210 μM Ca^{2+} by red beet and tomato tonoplast-enriched membrane vesicles. Curves were fit by polynomial regression. Equations for the polynomial fit were: 0.7 μM Ca^{2+}, beet ($-40.297 + 23.955x - 3.44x^2$); 0.7 μM Ca^{2+}, tomato ($-152.955 + 89.669x - 13.009x^2$); 210 μM$Ca^{2+}$, beet ($-66.175 + 40.378x - 5.845x^2$); 210 μM Ca^{2+}, tomato ($-190.38 + 112.674x - 16.361x^2$). Insets are Ca^{2+} uptake in tomato membrane vesicles over the time course of the experiments at both low and high temperatures. Each data point is the mean of 3 replicates.

as an early event in chilling injury (14, 21, 22) we assessed the temperature dependence of Ca^{2+} uptake in tonoplast-enriched vesicles from both tomato and red beet. Calcium uptake in tomato membrane vesicles was linear over a time course of 20 min at both high and low temperature (Fig. 4, insets). Calcium uptake in beet membrane vesicles was also linear (data not shown).

The data were plotted as an Arrhenius plot to reveal anomolous effects of temperature on Ca^{2+} uptake (Fig. 4). Calcium uptake in beet membranes showed a low temperature dependence with a relatively low activation energy. In contrast, Ca^{2+} uptake in tomato membrane vesicles had an activation energy similar to beet at high temperatures but this apparent activation energy increased significantly at temperatures below 10 to 12°C (Fig. 4). There is considerable controversy about the way to fit Arrhenius plots (14). We chose the simple approach by fitting with polynomial regression. The apparent energy of activation can be calculated by taking the derivative of the quadratic equation fit to the data. Clearly this model is not perfect, particularly at the high temperature range of the fit. It is, however, suitable for our purposes in contrasting the two genotypic responses.

Because the low affinity Ca^{2+} uptake system represents a complex reaction consisting of H^+ transport driven by the H^+-ATPase and subsequent Ca^{2+}/H^+ exchange, the effect of low temperature could be exerted at any of a number of sites in the reaction sequence. We examined the temperature dependence of individual steps in the reaction sequence by first assaying ATPase activity in the tomato and red beet membrane fractions (Fig. 5). The assays were carried out at saturating and half-saturating ATP concentrations as determined for each enzyme previously (3, 16). Both ATPases had similar activation energies that showed no apparent changes over the temperature range examined. Thus, the low temperature effects on Ca^{2+} uptake in tomato membranes cannot be attributed to an effect of temperature on the H^+-ATPase, at least as assessed by ATP hydrolytic activity.

The temperature dependence of H^+ transport catalyzed by the tomato and red beet H^+-ATPase was also examined using the fluorescent, ΔpH probe, acridine orange (Fig. 6). In both membranes the initial rate of H^+ transport was greatly reduced when temperature was decreased from 21 to 6°C. However, the total extent of ΔpH formation was similar at both temperatures. The

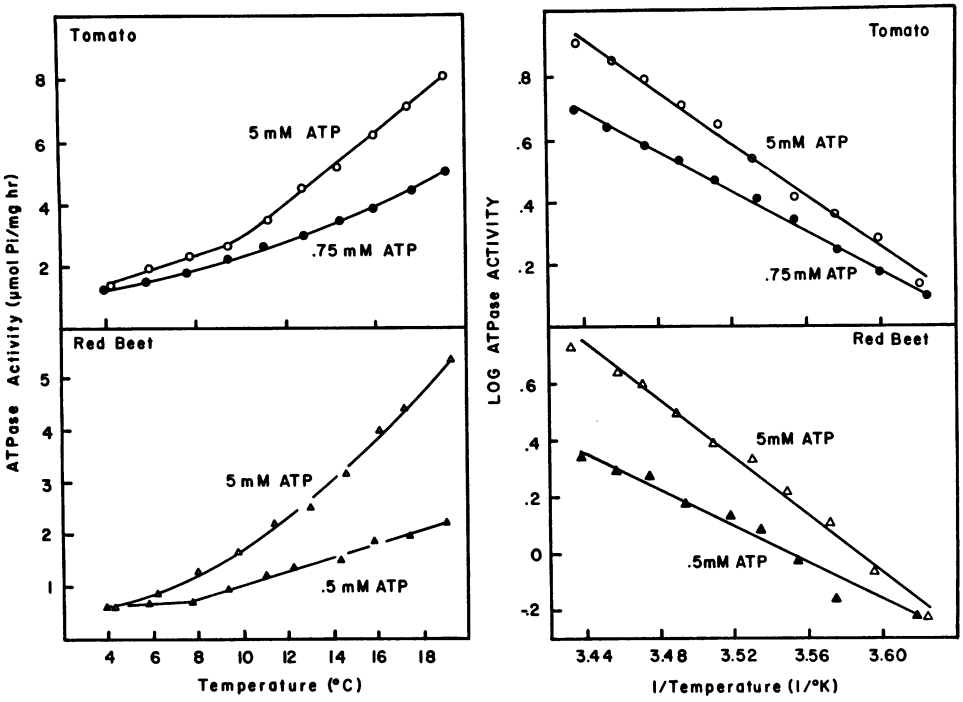

FIG. 5. Temperature-dependence of ATPase activity associated with tonoplast-enriched membrane fractions from red beet and tomato fruit. ATPase activity was assayed at the indicated temperature in the presence of saturating (5 mM) or half-saturating (0.5 mM-beet, 0.75 mM-tomato) ATP concentration. The data were transformed and Arrhenius plots of the data constructed.

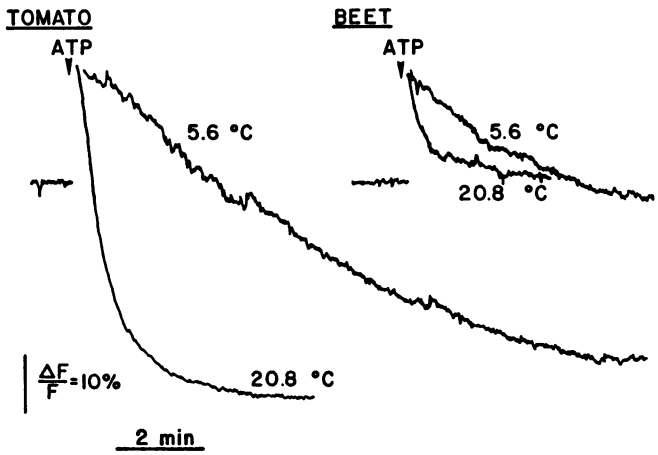

FIG. 6. ATP-dependent H^+ transport activity associated with tonoplast-enriched membranes isolated from tomato or red beet. Proton transport was assayed using the fluorescent ΔpH probe acridine orange and either 47 μg (tomato) or 150 μg (beet) membrane protein as described in "Materials and Methods." Temperature was regulated using a refrigerated water bath and a water-jacketed cuvette.

temperature dependence of the initial rate of H^+ transport over the temperature range of 3 to 21°C is shown in Figure 7. An Arrhenius plot of the data (Fig. 7B) does not indicate sufficiently large enough changes in the activation energy for H^+ transport to account for the low temperature effect on Ca^{2+} uptake. Because low temperature may induce ion leaks we also examined the extent of ΔpH formation (*i.e.* total fluorescence quench) over the same temperature range (Fig. 8). The extent of ΔpH formation was only slightly temperature dependent. We interpret the extent of ΔpH formation to reflect the steady state balance between the rate of active H^+ influx and the rate of passive H^+ efflux. Because the steady state ΔpH changed only slightly over a temperature range where the rate of active H^+ influx changes dramatically, we conclude that passive H^+ efflux decreased approximately in parallel with the decrease in active H^+ influx activity. This indicates that both tomato and beet membrane vesicles become less permeable to H^+ as the temperature decreases. Overall, decreases in ΔpH cannot completely account for the low temperature effect on Ca^{2+} uptake in tomato membranes, because there is only a 21% reduction in steady state ΔpH formation, whereas Ca^{2+} transport in the tonoplast was reduced by 62%.

The effect of low temperature on passive Ca^{2+} efflux was also examined by preloading vesicles with $^{45}Ca^{2+}$ and measuring Ca^{2+} retention at time intervals after the addition of EGTA (Figs. 9 and 10). Initial experiments examined Ca^{2+} efflux after 20 min incubation at a range of temperatures (Fig. 9). As with passive H^+ efflux, passive Ca^{2+} efflux progressively decreased with decreasing temperature. This was also observed when passive Ca^{2+} efflux was measured over a 3 h time period (Fig. 10). For both tomato and beet membranes, passive Ca^{2+} efflux was reduced at 5°C relative to 20°C. These results indicate that low temperature decreases rather than increases passive Ca^{2+} efflux and so a temperature effect on this process contributing to net Ca^{2+} uptake cannot account for the low temperature reduction of Ca^{2+} uptake in tomato membrane vesicles.

DISCUSSION

Our results indicate that low-density membrane vesicles isolated from tomato fruit possess two mechanisms for ATP-dependent Ca^{2+} uptake. Calcium transport across the tonoplast appears to involve a Ca^{2+}/H^+ antiport coupled to activity of the H^+-ATPase. This conclusion is based on NO_3^--sensitivity and ionophore-sensitivity of the low affinity, high capacity uptake system. Similar tonoplast-localized Ca^{2+}/H^+ antiport systems have been described in oat (20), carrot (8), and red beet (4). Our results differ somewhat in that we find a K_m for Ca^{2+} uptake by this system to exceed 200 μM whereas two other papers report a K_m near 10 μM (8, 20). This discrepancy may be due to difficulty in controlling Ca^{2+} concentration as we observed when glass test tubes were used.

The high affinity, low capacity uptake system appears to be associated with a subpopulation of endoplasmic reticulum membranes. A similar system has also been reported to be associated with endoplasmic reticulum of other plant cells (8, 20). Vanadate sensitivity of this uptake system suggests that a Ca^{2+}-ATPase may be the mechanism responsible for Ca^{2+} uptake by this

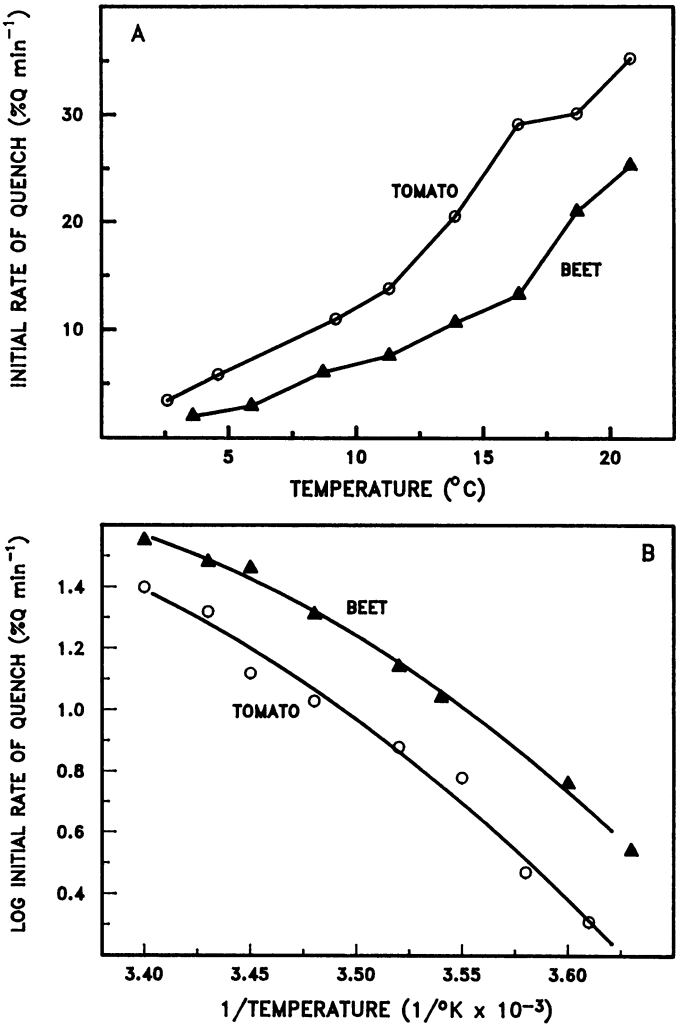

FIG. 7. Temperature-dependence of the initial rate of ATP-dependent H^+ transport associated with tonoplast enriched membranes isolated from tomato or red beet. The initial rate of fluorescence quenching of acridine orange was determined from data similar to that shown in Figure 7, at the indicated temperature and plotted (panel A). The data in panel A were transformed and an Arrhenius plot constructed with curves fitted by polynomial regression (panel B).

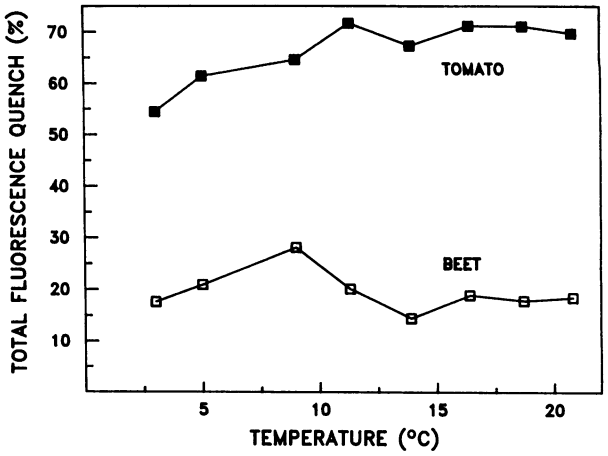

FIG. 8. Temperature-dependence of the extent of ΔpH formation in tonoplast-enriched membranes isolated from tomato or red beet. The total fluorescence quench of acridine orange was determined from data similar to that shown in Figure 7, at the indicated temperatures.

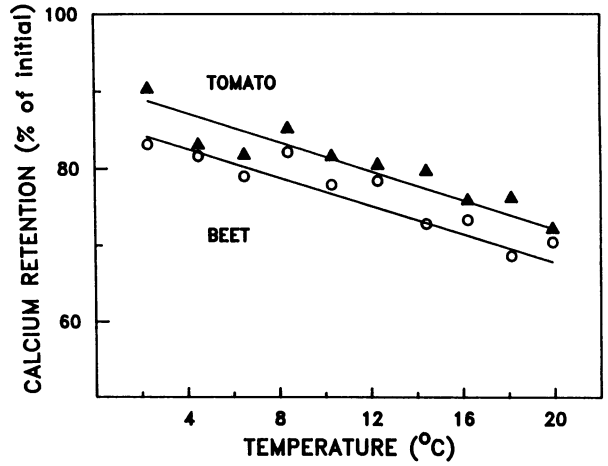

FIG. 9. Temperature-dependence of passive Ca^{2+} efflux from tonoplast-enriched membranes isolated from tomato or red beet. Membrane vesicles were loaded by preincubation in 1 μM $^{45}Ca^{2+}$, then 95 μM EGTA was added to the solution, and the membranes transferred to the indicated temperature. After 20 min the Ca^{2+} retained in membrane vesicles was determined using the filtration assay as described in "Materials and Methods."

system. Nitrate-stimulation of the rate of Ca^{2+} uptake by the high affinity system (see Fig. 1) may result from charge-compensating flux of NO_3^- which would suggest that the putative Ca^{2+}-ATPase catalyzes electrogenic Ca^{2+} influx. Similarly, valinomycin stimulation of low affinity Ca^{2+}/H^+ antiport activity, especially in red beet membranes (Table I) suggests that this system may be electrogenic as well.

The presence of both intracellular Ca^{2+} uptake systems may provide complementary mechanisms that operate in concert to regulate cytoplasmic Ca^{2+} activity. The systems could be likened to mechanisms of coarse and fine controls, with the low affinity, high capacity tonoplast system responsive to relatively large transients in Ca^{2+} activity and the high affinity, low capacity endoplasmic reticulum system responsive to more subtle perturbations in cytoplasmic Ca^{2+} levels.

The temperature dependence of Ca^{2+} uptake in low density beet and tomato membrane vesicles indicated that the apparent activation energy for Ca^{2+} uptake increased in tomato membranes at temperatures below 10 to 12°C (Fig. 4). Thus, in the event of chilling-induced increases in cytoplasmic calcium, restoration of Ca^{2+} homeostasis may be delayed in tomato relative to red beet. To elucidate the mechanism of this low temperature effect on Ca^{2+} uptake in low density tomato membranes we examined the temperature dependence of components of the reaction contributing to Ca^{2+}/H^+ antiport activity in the tonoplast. Temperature did not affect ATPase activity, H^+ transport activity, passive H^+ fluxes or passive Ca^{2+} fluxes to the same extent as the low temperature effect on Ca^{2+} uptake. Considered collectively, these results argue that a general loss of compartmentation resulting from low temperature effects on membrane structure can only account for a minor portion of the inhibition of Ca^{2+} uptake. The results suggest that the major effect of low temperature on Ca^{2+} transport results from an effect of low temperature directly on the Ca^{2+}/H^+ exchange reaction. Three possible explanations for the major effect of low temperature-induced change in activation energy for the Ca^{2+}/H^+ exchange reaction are: (a) structural changes in the membrane microdomain or boundary lipid associated with the carrier complex may have occurred, (b) temperature may directly affect a conformation state of the Ca^{2+}/H^+ exchange protein, or (c) temperature may exert differential effects on rate constants of serial steps

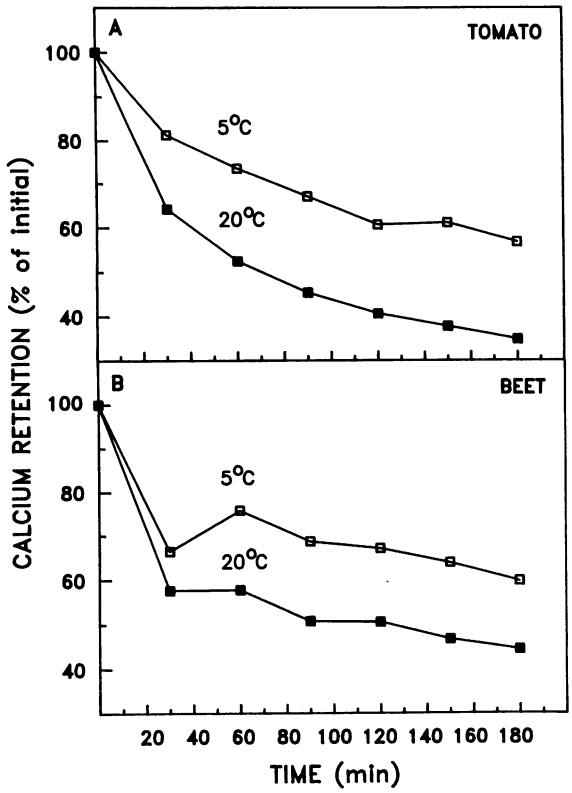

FIG. 10. Time course of Ca^{2+} efflux from tonoplast-enriched membrane vesicles isolated from tomato or red beet. Membrane vesicles were passively loaded by preincubation with 1 μM $^{45}Ca^{2+}$, then 95 μM EGTA was added to the solution, and the test tubes with the membranes were transferred to temperatures of either 5 or 20°C. At the indicated times the Ca^{2+} retained in membrane vesicles was determined using the filtration assay as described in "Materials and Methods."

involved in Ca^{2+}/H^+ exchange and thus alter the rate limiting step of the overall reaction.

The present study does not identify a source for the postulated chilling-induced Ca^{2+} fluxes into the cytoplasm of chilling sensitive cells (14, 21, 22). However, a direct effect of temperature in inducing Ca^{2+} fluxes from either the mitochondria (10) or vacuole now appear to be unlikely sources of a chill-induced Ca^{2+} transient. The plasma membrane remains a candidate since Zocchi and Hanson (23) found that corn root tissue exposed to a chilling shock absorbed appreciably more Ca^{2+} than control roots. The possibility also exists that chilling-induced Ca^{2+} efflux from intracellular stores may be mediated by products of phosphatidylinositol catabolism, such as inositol triphosphate (13). The apparent increase in activation energy for Ca^{2+} transport into intracellular compartments that we observed in tomato could exacerbate a chill-induced increase in cytoplasmic Ca^{2+} by increasing the duration of cytosolic exposure to elevated Ca^{2+} activity.

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