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Further Characterization of the Red Beet Plasma Membrane Ca^{2+}-ATPase Using GTP as an Alternative Substrate[1]

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ABSTRACT

The GTP-driven component of Ca^{2+} uptake in red beet (*Beta vulgaris* L.) plasma membrane vesicles was further characterized to confirm its association with the plasma membrane Ca^{2+}-translocating ATPase and assess its utility as a probe for this transport system. Uptake of $^{45}Ca^{2+}$ in the presence of GTP demonstrated similar properties to those previously observed for red beet plasma membrane vesicles utilizing ATP with respect to pH optimum, sensitivity to orthovanadate, dependence on Mg:substrate concentration and dependence on Ca^{2+} concentration. Calcium uptake in the presence of GTP was also strongly inhibited by erythrosin B, a potent inhibitor of the plant plasma membrane Ca^{2+}-ATPase. Furthermore, after treatment with EGTA to remove endogenous calmodulin, the stimulation of $^{45}Ca^{2+}$-uptake by exogenous calmodulin was nearly equivalent in the presence of either ATP or GTP. Taken together these results support the proposal that GTP-driven $^{45}Ca^{2+}$ uptake represents the capacity of the plasma membrane Ca^{2+}-translocating ATPase to utilize this nucleoside triphosphate as an alternative substrate. When plasma membrane vesicles were phosphorylated with [γ-^{32}P]-GTP, a rapidly turning over, 100 kilodalton phosphorylated peptide was observed which contained an acyl-phosphate linkage. While it is proposed that this peptide could represent the catalytic subunit of the plasma membrane Ca^{2+}-ATPase, it is noted that this molecular weight is considerably lower than the 140 kilodalton size generally observed for plasma membrane Ca^{2+}-ATPases present in animal cells.

There is increasing evidence that intracellular Ca^{2+} may play an important regulatory role in the control of plant metabolic events (19 and references therein). It is proposed that transient increases in cytoplasmic Ca^{2+} concentration serve to modulate the activity of key regulatory enzymes such as protein kinases in response to an appropriate stimulus such as hormone binding (26 and references therein). For this regulatory system to operate, plant cells must maintain low cytoplasmic Ca^{2+} levels by transport of this cation out of the cell or into organelles such as the vacuole, ER, and mitochondria (24). Furthermore, a selective permeability increase for Ca^{2+} must occur at these structures upon reception of the appropriate signal (19).

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Previous studies with isolated plant membrane vesicles have allowed the *in vitro* characterization of transport systems involved in mediating Ca^{2+} efflux from the cell or the sequestering of this cation in internal organelles. At the tonoplast, Ca^{2+} uptake has been shown to occur by a Ca^{2+}/H^+ antiport energetically coupled to the proton electrochemical gradient established by the tonoplast ATPase (1, 30). Using fluorescent (16, 20) and radiotracer methods (9, 10), evidence has been presented for an ATP-dependent primary Ca^{2+} transport pump at the ER. This transport pump would mediate Ca^{2+} uptake into the ER and presumably be similar to the Ca^{2+}-ATPases associated with the ER and sarcoplasmic reticulum of animal cells (32 and references therein). From early studies with microsomal membrane fractions (14, 18), it was proposed that an ATP-dependent primary Ca^{2+} transport pump responsible for Ca^{2+} efflux might also be associated with the plasma membrane. Subsequent work with preparations of sealed plasma membrane vesicles has substantiated this proposal (17, 23, 24).

In a previous report from this laboratory (17), ATP-driven $^{45}Ca^{2+}$ uptake was characterized in plasma membrane vesicles isolated from red beet (*Beta vulgaris* L.) storage tissue. The transport of $^{45}Ca^{2+}$ was directly linked to ATP utilization and demonstrated properties which distinguished it from primary Ca^{2+} transport associated with ER vesicles of this tissue (16). In particular, the transport of Ca^{2+} in plasma membrane vesicles had the capacity to utilize GTP as a substrate for driving uptake at 50% of the level observed with ATP. This is also different from what is observed for ATP-driven H^+ transport at the plasma membrane which is substrate specific for ATP (5, 21, 31). In the present communication, GTP-driven Ca^{2+} uptake in plasma membrane vesicles was characterized to confirm its association with plasma membrane Ca^{2+}-ATPase and evaluate its utility as a means to further probe the function of this transport system.

MATERIALS AND METHODS

Plant Material

Red beets (*Beta vulgaris* L., cv Detroit Dark Red) were purchased commercially. The tops of the plants were removed and the storage tissue was stored at 2°C for at least 10 d prior to use to ensure uniformity in membrane isolation (25).

Isolation of Plasma Membrane Vesicles

Plasma membrane vesicles were isolated from red beet storage tissue as previously described (15). Briefly, tissue

sections were peeled, cut into cubes, and then vacuum infiltrated for 5 min in ice cold homogenization medium (1.5:1, medium:tissue) containing 250 mM sucrose, 2 mM EDTA, 2 mM Na_2ATP, 1% (w/v) BSA (fraction V powder), 0.5% (w/v) PVP (40,000 mol wt), 0.2 mM PMSF, 15 mM β-mercaptoethanol, 4 mM DTE, 10% (v/v) glycerol, 70 mM Tris-HCl (pH 8.0) and 250 mM KI. DTE, β-mercaptoethanol, ATP, and PMSF were added to the medium just prior to use. Following vacuum infiltration, the tissue was homogenized in the medium using a vegetable juice extractor and the homogenate was filtered through four layers of cheesecloth prior to centrifugation at 13,000*g* (8,500 rpm) for 15 min in a Sorvall GSA rotor. The 13,000*g* pellet was discarded and the supernatant was centrifuged at 80,000*g* (32,000 rpm) in a Beckman type 35 rotor for 30 min. The resulting microsomal pellet was suspended in a suspension buffer consisting of 250 mM sucrose, 10% (v/v) glycerol, 2 mM BTP[4]/Mes (pH 7.2) and 1 mM DTE. The suspended membranes were then layered onto a discontinous sucrose density gradient consisting of 26% (w/w) sucrose layered over 38% (w/w) sucrose. The gradient solutions were buffered with 1 mM Tris/Mes (pH 7.2) and contained 1 mM DTE. The gradients were centrifuged at 100,000*g* (25,000 rpm) for 2 h in a Beckman SW 28 rotor. Membranes recovered at the 26/38% interface were removed using a Pasteur pipet, diluted with suspension buffer to 70 mL and then centrifuged at 80,000*g* (32,000 rpm) for 30 min in a Beckman type 35 rotor. The pellet was suspended at a protein concentration of approximately 4 mg/mL, frozen under liquid nitrogen, and then stored at −80°C until use.

Measurement of Ca^{2+} Transport

Calcium uptake in red beet plasma membrane vesicles was measured at 22°C by a modification of the method of Giannini *et al.* (17). The standard assay contained 250 mM sorbitol, 25 mM BTP/Mes (pH 7.5), 3.75 $MgSO_4$, 100 mM KNO_3, 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$), 0.4 mM NaN_3, 3.75 mM GTP (BTP salt [pH 7.5]) and plasma membrane vesicles (40–45 μg protein) in a 1 mL reaction volume. The reaction was initiated by the addition of plasma membrane vesicles and at the appropriate time, 100 μL aliquots of the reaction solution were collected by filtration on 0.45 μm Metricel filters. The filters were washed four times with 1 mL each of ice-cold wash buffer containing 250 mM sorbitol, 25 mM BTP/Mes (pH 7.5), 3.75 mM $MgSO_4$, 100 mM KNO_3, 15 μM $CaCl_2$, and 0.4 mM NaN_3. Radioactivity associated with the filters was determined by liquid scintillation spectroscopy in 5 mL of scintillation cocktail (Aquasol, New England Nuclear). Prior to use in filtration, the filters were vacuum infiltrated with wash buffer for at least 20 min. Variations in these reaction conditions are indicated in "Results and Discussion."

Measurement of H^+ Transport

Proton transport was measured at 22°C by the quenching of quinacrine flourescence as previously described (15). The standard assay contained 250 mM sorbitol, 25 mM BTP/Mes (pH 6.5), 100 mM KNO_3, 5 μM quinacrine, 3.75 mM $MgSO_4$, 3.75 mM ATP (BTP salt [pH 6.5]) and 50 μg of membrane protein. The reaction was initiated by the addition of ATP and the fluorescence was monitered using a Perkin-Elmer model 230 spectrofluorometer with the excitation monochronometer set at 430 nm and the emission monochronometer set at 500 nm. Any variation in the reaction conditions is indicated in "Results and Discussion."

[4] Abbreviation: BTP, bistris propane.

Phosphorylation

Phosphorylation of the red beet plasma membranes was carried out by a modification of the method of Briskin and Poole (8). The reaction was conducted at ice temperature in a 100 μL volume containing 0.74 mM γ-[^{32}P]GTP (338 mCi/mmol), 0.74 mM $MgSO_4$, 30 μM $CaCl_2$, 30 mM BTP/Mes (pH 7.5) and 140 μg of membrane protein. The reactions were initiated by the addition of membrane protein and then quenched by the addition of 25 mL of ice-cold, 10% TCA containing 40 mM NaH_2PO_4, 5 mM $Na_4P_2O_7$, and 1 mM Na_2ATP. Two mg of carrier BSA (fraction V powder) were added to each quenched sample prior to centrifugation at 27,000*g* (15,000 rpm) for 15 min in a Sorvall SS-34 rotor. The resultant pellets were suspended in 8.5 mL of 30 mN HCl and then centrifugated at 27,000*g* for 15 min. The final pellets were suspended in gel electrophoresis sample buffer containing 1% (w/w) lithium dodecyl sulfate, 50 mM Tris/citrate (pH 2.4), 2% (v/v) β-mercaptoethanol, 4 M urea, 20% (v/v) glycerol, 10 μg/mL pyronin Y, and then incubated at room temperature for 10 min.

Lithium Dodecyl Sulfate PAGE

Lithium dodecyl sulfate gel electrophoresis was carried out by the method of Lichtner and Wolf (22) using 5.6% acrylamide slab gels containing 0.2% (w/w) lithium dodecyl sulfate and buffered to pH 2.4 with 50 mM Tris/citrate. Approximately 140 μg of sample protein was applied per lane and electrophoresis was carried out at 25 mA constant current for 2.5 h at 2°C. The tank buffer contained 50 mM Tris/citrate (pH 2.4) and 0.2% (w/w) lithium dodecyl sulfate. Following electrophoresis, gels were immediately dried on Whatman No. 1 paper and subjected to autoradiography against Kodak XAR-5 x-ray film for 36 h at −80°C with Cronex high plus intensification screens.

For determining mol wt protein standards solubilized and electrophoresed under identical conditions as the phosphorylated samples were used to calibrate the gel system. The gel was stained and destained as described by Briskin and Leonard (6).

Protein Assay

Protein was determined by the method of Bradford (3) using BSA as a protein standard. The Bradford assay reagent was filtered just prior to use.

All data shown are for representative experiments which have been repeated at least three separate times. Within each experiment the individual data points represent the mean of two determinations.

RESULTS AND DISCUSSION

In our previous studies (17) $^{45}Ca^{2+}$ uptake was examined in red beet plasma membrane vesicles isolated according to the method of Giannini *et al.* (15). This method, which involves the homogenization of plant tissue in a highly protective medium containing 250 mM KI, appears to result in the selective production of sealed plasma membrane vesicles. Characterization of $^{45}Ca^{2+}$ uptake in these vesicles revealed the presence of an ATP dependent, primary Ca^{2+} transport system which had the unique capability of using GTP as an alternative substrate for driving uptake. As this characteristic differed from the endoplasmic reticulum Ca^{2+}-ATPase (16) and plasma membrane H^+-ATPase (7) from this tissue, this could represent a useful means of selectively studying this transport system. Therefore, a more detailed characterization of GTP-driven transport by this system was conducted.

General Characteristics of GTP-driven $^{45}Ca^{2+}$ Uptake in Red Beet Plasma Membrane Vesicles

Consistent with our initial examination of the Ca^{2+} transport system associated with red beet plasma membrane vesicles (17), $^{45}Ca^{2+}$ uptake driven by GTP occurred at a rate approximately 50% of that observed in the presence of ATP when each substrate was present at 3.75 mM (Fig. 1). For both the ATP and GTP driven timecourses of $^{45}Ca^{2+}$ uptake, radiolabel was rapidly discharged by the addition of A23187, indicating that the accumulation of radiolabel in each case represented uptake into plasma membrane vesicles. In contrast, only a low level of radioactivity was associated with the vesicles when these nucleoside phosphates were not present in the uptake assay.

When $^{45}Ca^{2+}$ uptake was examined in the presence of increasing concentrations of Mg:GTP present as the 1:1 molar ratio, radiolabel uptake demonstrated simple saturation type kinetics (Fig. 2A). For each data point, the rate of $^{45}Ca^{2+}$ uptake was determined over the first 5 min of reaction where

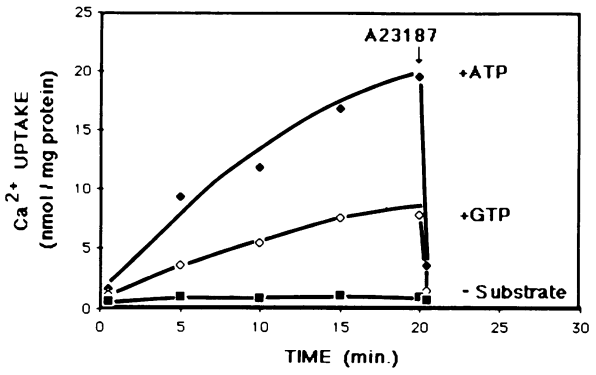

Figure 1. Uptake of $^{45}Ca^{2+}$ by red beet plasma membrane vesicles utilizing either ATP or GTP as substrate. Assays were conducted in the presence of 250 mM sorbitol, 100 mM KNO_3, 0.4 mM NaN_3, 25 mM BTP/Mes (pH 7.5), 3.75 mM $MgSO_4$, 15 μM $CaCl_2$ (2.5 μCi $^{45}Ca^{2+}$ per assay), and 3.75 mM either ATP or GTP. Both ATP and GTP were present as the BTP salt (pH 7.5). Uptake in the absence of nucleoside phosphate is also shown. At the indicated time, 0.4 μg/mL of A23187 was added.

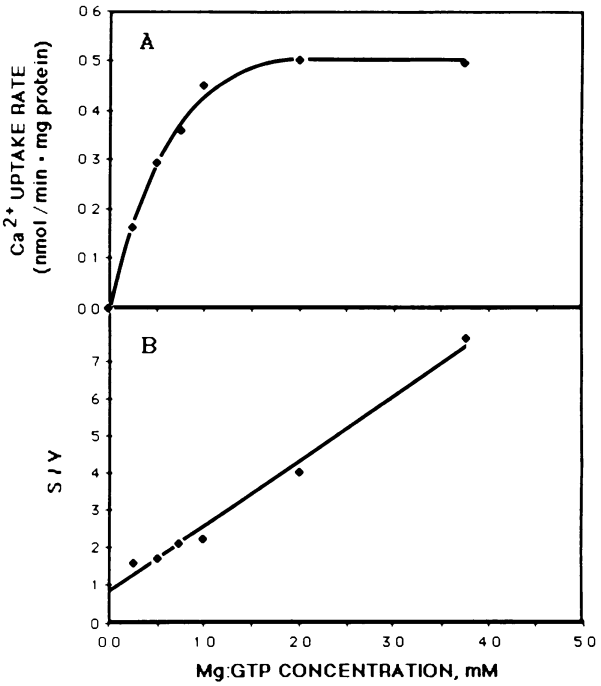

Figure 2. Dependence of $^{45}Ca^{2+}$ uptake on Mg:GTP concentration in plasma membrane vesicles isolated from red beet (panel A). Assays were conducted in the presence of 250 mM sorbitol, 100 mM KNO_3, 0.4 mM NaN_3, 25 mM BTP/Mes (pH 7.5), 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$) and the indicated concentration of Mg:GTP present as the 1:1 molar ratio. The initial rate of uptake was calculated from measurements made during the first 5 min of uptake. In panel B, the data were plotted according to the Hanes-Woolf linear transformation of the Michaelis-Menten equation: $[S]/v = K_m/V_{max} + ([S])1/V_{max}$.

uptake was linear with time. From a transformation of the data using a Hanes-Woolf plot (Fig. 2B), a K_m of 0.45 mM was determined for Mg:GTP. This value is similar to the K_m of 0.37 mM determined for $^{45}Ca^{2+}$ uptake in red beet plasma membrane vesicles when Mg:ATP was used as substrate (17). The finding of a similar K_m for these two substrates that drive transport at different rates could imply a similar affinity for the two nucleoside phosphate compounds by the enzyme but a slower turnover of the enzyme in the presence of GTP. However, further work on the reaction mechanism of this transport enzyme would be required to determine if this does in fact occur.

The uptake of $^{45}Ca^{2+}$ also demonstrated saturation type kinetics when assayed in the presence of increasing concentrations of Ca^{2+} and a Mg:GTP concentration of 3.75 mM (Fig. 3A). The uptake of $^{45}Ca^{2+}$ was linear with time over the first 5 min of reaction for each Ca^{2+} concentration tested and the slope of each timecourse was used to estimate the initial uptake rate. Linear transformation of the data according to a Hanes-Woolf plot (Fig. 3B) revealed a K_m of 7.6 μM for Ca^{2+} which again was similar to the K_m of 6 μM as previously determined for $^{45}Ca^{2+}$ uptake with red beet plasma membrane vesicles when driven by Mg:ATP (17).

As shown in Figure 4, $^{45}Ca^{2+}$ uptake with the red beet vesicles was optimal when the pH of the uptake solution was

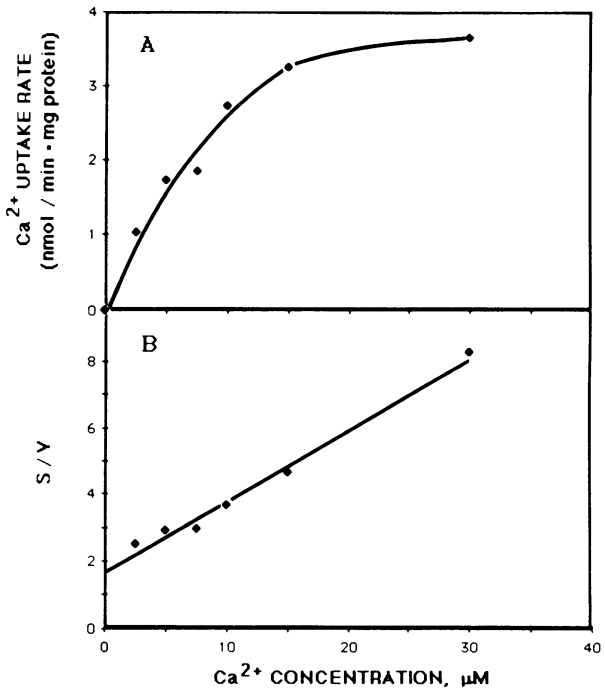

Figure 3. Dependence of GTP-driven $^{45}Ca^{2+}$ uptake on Ca^{2+} concentration in plasma membrane vesicles isolated from red beet (panel A). Assays were conducted in the presence of 250 mM sorbitol, 100 mM KNO_3, 0.4 mM NaN_3, 3.75 mM $MgSO_4$, 3.75 GTP (BTP salt, pH 7.5), 25 mM BTP/Mes (pH 7.5), and the indicated concentration of $CaCl_2$. Each assay contained 2.5 μCi $^{45}Ca^{2+}$. In panel B, the data were plotted according to the Hanes-Woolf linear transformation of the Michaelis-Menten equation (see Fig. 2).

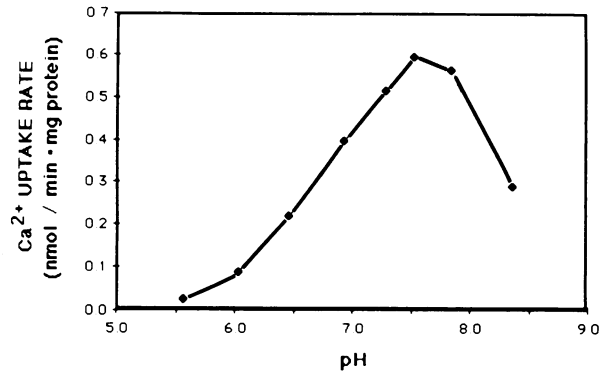

Figure 4. Effect of assay pH on GTP-driven $^{45}Ca^{2+}$ uptake in red beet plasma membrane vesicles. Assays were carried out in the presence of 250 mM sorbitol, 100 mM KNO_3, 0.4 mM NaN_3, 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$ per assay), 3.75 mM $MgSO_4$, 3.75 mM GTP (BTP salt), and 25 mM BTP/Mes to the indicated assay pH. The initial rate of $^{45}Ca^{2+}$ uptake was calculated from measurements made during the first 5 min of uptake.

at 7.5. While this optimal pH for uptake was similar to that observed previously for uptake in the presence of Mg:ATP (17), the profile for Mg:GTP-driven $^{45}Ca^{2+}$ uptake was much sharper in the optimal pH range. In contrast, uptake driven by Mg:ATP demonstrated a broader pH curve where maximal uptake was observed between pH 7.0 to 7.5 (17). This optimum for uptake at pH 7.5 for both ATP and GTP driven Ca^{2+} uptake with red beet vesicles differed from the pH optimum of 6.6 observed for ATP-driven Ca^{2+} uptake in plasma membrane vesicles from radish (27).

Effect of Inhibitors on GTP-Driven $^{45}Ca^{2+}$ Uptake

As with $^{45}Ca^{2+}$ uptake measured using Mg:ATP as substrate (17), Mg:GTP-driven uptake of $^{45}Ca^{2+}$ with the red beet vesicles was inhibited by orthovanadate (Fig. 5). This indicates that the enzymatic pathway for GTP utilization (hydrolysis) by this transport system involves the formation of a phosphorylated intermediate which is a general characteristic of the E_1E_2-type ATPases (5 and references therein). As shown in Figure 5, examination of $^{45}Ca^{2+}$ uptake by the vesicles in the presence of increasing concentrations of orthovanadate revealed that 50% inhibition of uptake occurred at an orthovanadate concentration of about 22 μM. This value was similar to the 30 μM orthovanadate concentration required for 50% inhibition of $^{45}Ca^{2+}$ uptake when Mg:ATP was used as substrate (17).

Uptake of $^{45}Ca^{2+}$ by the red beet plasma membrane vesicles was also strongly inhibited by erythrosin B, an iodinated derivative of fluorescein (Fig. 6). Complete inhibition of radiolabel uptake was observed at an erythrosin B concentration as low as 0.5 μM. This was consistent with the previous results of Rasi-Caldogno *et al.* (27) were erythrosin B in the submicromolar concentration range was shown to inhibit ATP-driven Ca^{2+} transport in plasma membrane vesicles from radish seedlings. Although erythrosin B can also inhibit the plasma membrane and tonoplast H^+-ATPases of plant cells, substantial inhibition occurs at a higher concentration range. As shown by Cocucci (13) the plasma membrane and tonoplast ATPases associated with a radish seedling microsomal membrane fraction were inhibited 50 to 85% by erythrosin B when the concentration of this inhibitor ranged from 10 to 100 μM. At the concentration of erythrosin (0.5 μM) where complete inhibition of $^{45}Ca^{2+}$ uptake occurred, only a minor inhibition of ATP-dependent H^+ transport could be observed

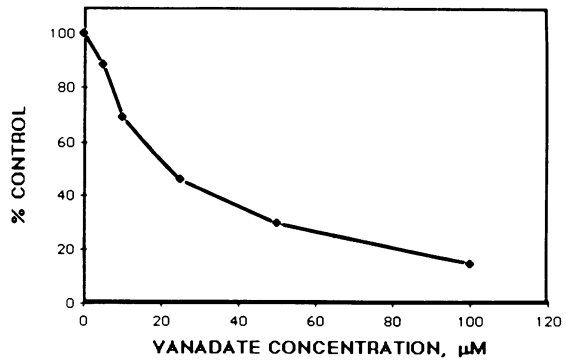

Figure 5. Effect of orthovanadate on GTP-driven $^{45}Ca^{2+}$ uptake in red beet plasma membrane vesicles. Assays were conducted in the presence of 250 mM sorbitol, 100 mM KNO_3, 0.4 mM NaN_3, 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$), 3.75 mM $MgSO_4$, 3.75 GTP (BTP salt [pH 7.5]), 25 mM BTP/Mes (pH 7.5), and the indicated concentration of sodium orthovanadate.

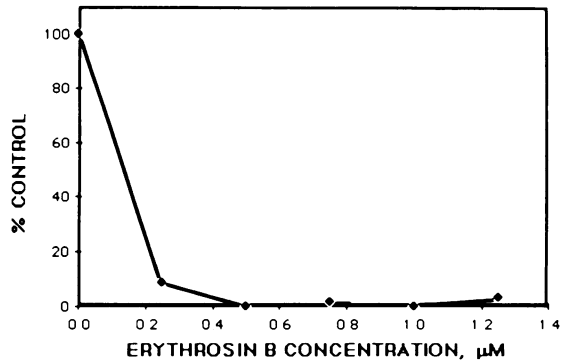

Figure 6. Effect of erythrosin B on GTP-driven $^{45}Ca^{2+}$ uptake in red beet plasma membrane vesicles. Assays were conducted in the presence of 250 mM sorbitol, 100 mM KNO_3, 0.4 mM NaN_3, 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$), 3.75 mM $MgSO_4$, 3.75 GTP (BTP salt [pH 7.5]), 25 mM BTP/Mes (pH 7.5), and the indicated concentration of erythrosin B.

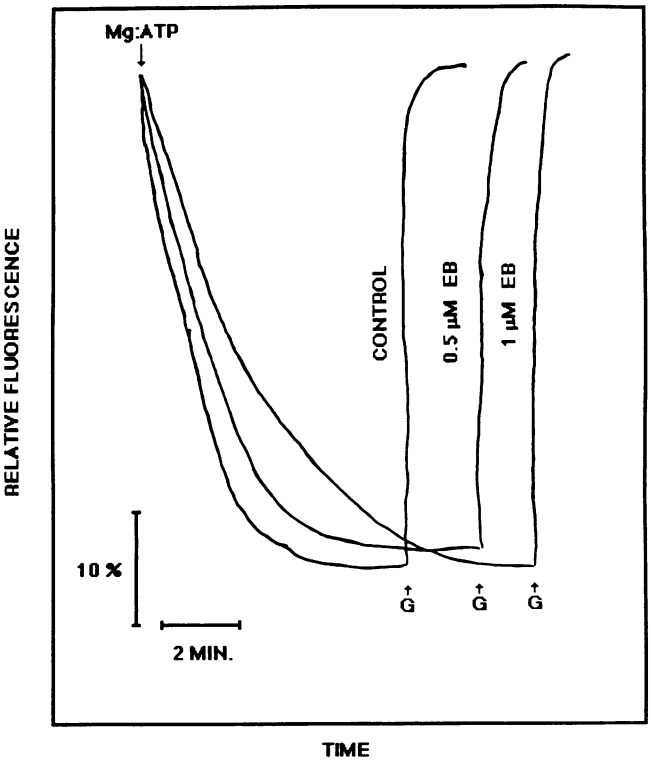

Figure 7. Effect of erythrosin B on ATP-driven H^+-transport in plasma membrane vesicles isolated from red beet. Proton transport was measured by monitoring quinacrine fluorescence quenching as described in "Materials and Methods." The control assay contained 250 mM sorbitol, 25 mM BTP/Mes (pH 6.5), 100 mM KNO_3, 5 μM quinacrine, 3.75 mM $MgSO_4$, 3.75 mM ATP (BTP salt [pH 6.5]), 50 μg of membrane protein, and the indicated concentration of erythrosin B (EB) (when present). As indicated (G), 5 μM gramicidin was added to collapse the pH gradient.

(Fig. 7). Thus, erythosin B at concentrations of 0.5 μM or less could serve as a useful means to selectively inhibit the Ca^{2+} transport system associated with plant plasma membrane fractions. The observation that Ca^{2+} uptake can be completely blocked with only a minor effect on ΔpH using this inhibitor would also support our previous proposal that Ca^{2+} transport in red beet plasma membrane vesicles is mediated by primary transport alone and that a $\Delta\mu H^+$-driven secondary transport system might not be operative in these membranes (see ref. 17 for discussion).

Detection of a Phosphoenzyme Intermediate Using [γ-^{32}P]GTP

The observation that the Ca^{2+}-transport system associated with red beet plasma membrane vesicles could utilize GTP as an alternative substrate for driving transport and that this activity was inhibited by low concentrations of orthovanadate would imply that phosphorylation of the membranes using [γ-^{32}P]GTP might allow identification of a Ca^{2+}-ATPase phosphorylated intermediate on electrophoretic gels. To examine this possibility, plasma membrane vesicle fractions were phos-

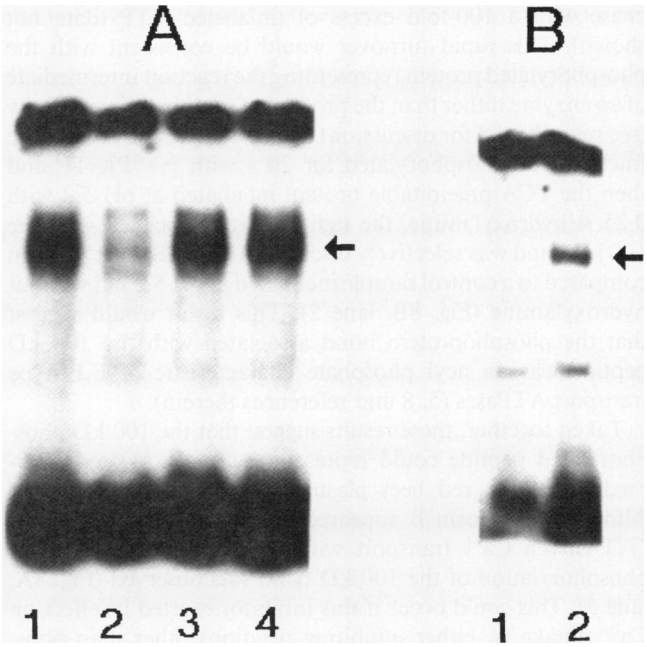

Figure 8. Gel autoradiograph of the phosphorylated protein associated with red beet plasma membrane fractions when incubated with [γ-^{32}P]GTP. Phosphorylation and electrophoresis were carried out as described in "Materials and Methods." The dried gels were placed against x-ray film for 36 h at −80°C with Cronex intensification screens. In gel A the following reaction conditions were used: lane 1, 20 s phosphorylation; lane 2, 20 s phosphorylation followed by a 40 s chase with a 100-fold excess of unlabeled GTP; lane 3, 20 s phosphorylation in the presence of 0.5 μM erythrosin B; lane 4, 20 s phosphorylation. In gel B the following reaction conditions were used: lane 1, 20 s phosphorylation followed by treatment of the TCA precipitated protein with 0.25 M hydroxylamine at pH 5.2 for 30 min at ice temperature prior to the HCl wash; lane 2, 20 s phosphorylation followed by incubation of the TCA precipitated protein at pH 5.2 (50 mM Tris/Mes) for 30 min at ice temperature prior to the HCl wash.

phorylated with [γ-^{32}P]GTP at ice temperature and the phosphorylated protein was analyzed by lithium dodecyl sulfate gel electrophoresis (Fig. 8). This gel system was utilized since it provides optimal conditions (low temperature [pH 2.4]) for electrophoresis of labile acyl phosphate intermediates of transport ATPases (22). The standard procedure of conducting transient ATPase phosphorylation over short time intervals (<60 s) at ice temperature and then trapping the phosphorylated intermediates as TCA precipitable protein was utilized (5, 8).

When plasma membrane fractions were phosphorylated for 20 s using [γ-^{32}P]GTP, a major radioactive band having a molecular mass of about 100 kD was consistently observed following gel autoradiography (Fig. 8A, lanes 1 and 4). In addition to this major phosphorylated band at 100 kD, other labeled bands were present which were more variable in labeling intensity from experiment to experiment. Phosphorylation of the 100 kD band demonstrated rapid turnover since the labeling of this band greatly decreased when the 20 s phosphorylation with [γ-^{32}P]GTP was followed by a 40 s chase with a 100-fold excess of unlabeled GTP (Fig. 8A, lane 2). The labeling of this band was also decreased when the 20 s phosphorylation with [γ-^{32}P]GTP was followed by a 40 s chase with a 100-fold excess of unlabeled ATP (data not shown). This rapid turnover would be consistent with the phosphorylated protein representing the reaction intermediate of an enzyme rather than the product of protein kinase activity (see refs. 5 and 8 for discussion). When the plasma membrane fraction was phosphorylated for 20 s with [γ-^{32}P]GTP and then the TCA precipitable protein incubated at pH 5.2 with 0.25 M hydroxylamine, the radioactivity associated with the 100 kD band was selectively discharged (Fig. 8B, lane 1) when compared to a control sample incubated at pH 5.2 but without hydroxylamine (Fig. 8B, lane 2). This result would suggest that the phosphoprotein bond associated with the 100 kD peptide was an acyl phosphate characteristic of E_1E_2-type transport ATPases (5, 8 and references therein).

Taken together, these results suggest that the 100 kD phosphorylated peptide could represent a phosphorylated intermediate of the red beet plasma membrane Ca^{2+}-ATPase. Although erythrosin B appeared to be a potent inhibitor of GTP-driven Ca^{2+} transport with the vesicles, no effect on phosphorylation of the 100 kD band was observed (Fig. 8A, lane 3). This could occur if this inhibitor exerted its effect on Ca^{2+} uptake by either inhibiting reactions other than phosphoenzyme formation or by uncoupling Ca^{2+} transport from nucleoside phosphate hydrolysis.

The association of a 100 kD phosphorylated peptide with the plasma membrane Ca^{2+}-ATPase as the catalytic subunit would be similar to what is observed for other E_1E_2-type transport ATPases such as the plant plasma membrane H^+-ATPase (5 and references therein), the *Neurospora* plasma membrane H^+-ATPase (2), animal cell Na^+,K^+-ATPase (32), and the sarcoplasmic reticulum Ca^{2+}-ATPase (32). However, the Ca^{2+}-ATPases associated with the plasma membrane of animal cells and erythrocytes (12, 29) have catalytic subunits with molecular masses substantially greater than 100 kD (molecular mass approximately 140 kD) and are characteristically stimulated by the Ca^{2+}-binding regulatory protein, calmodulin. As regulation by calmodulin appears to be a key feature of these Ca^{2+}-ATPases with larger catalytic subunit molecular mass and preliminary evidence for calmodulin stimulation of Ca^{2+}-ATPase in plant plasma membrane fractions has been presented (28), the effect of calmodulin on $^{45}Ca^{2+}$ uptake was examined with the red beet plasma membrane vesicles.

Calmodulin Effects on ATP and GTP Driven $^{45}Ca^{2+}$ Uptake in Red Beet Vesicles

When red beet plasma membrane vesicles isolated according to the method of Giannini *et al.* (15) were directly treated with 1 μM calmodulin, no stimulatory effect upon GTP-driven $^{45}Ca^{2+}$ uptake could be observed (Fig. 9A). In addition, no effect on uptake could be observed when assays were conducted using ATP as the substrate for driving transport (data not shown). One possible explanation for this lack of calmodulin effect upon $^{45}Ca^{2+}$ uptake could be the association of endogenous calmodulin with the membrane preparation. As such low levels of this Ca^{2+}-binding protein are required to produce a stimulatory effect, the presence of even small

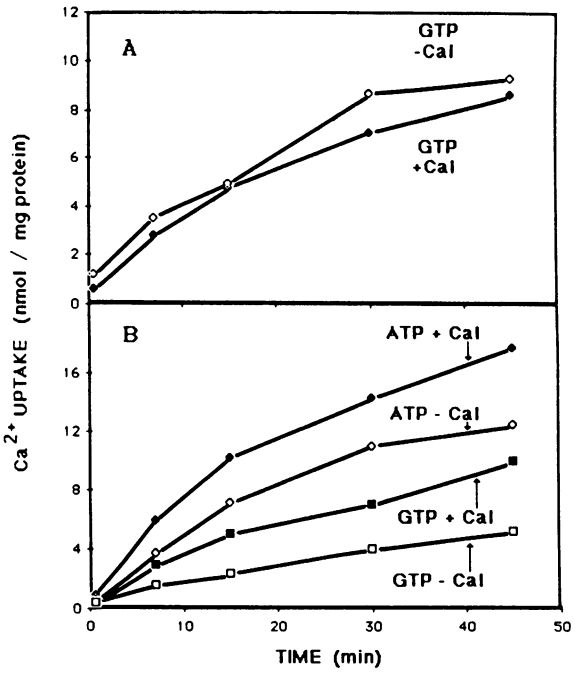

Figure 9. Effect of calmodulin on $^{45}Ca^{2+}$ uptake in red beet plasma membrane vesicles before and after treatment with EGTA. Panel A, $^{45}Ca^{2+}$ uptake with plasma membrane vesicles prior to washing with EGTA. Panel B, $^{45}Ca^{2+}$ uptake in red beet plasma membrane vesicles washed three times with 4 mM EGTA. For each assay, plasma membrane vesicles were preincubated for 30 min in a medium containing 250 mM sorbitol, 25 mM BTP/Mes (pH 7.5), 3.75 mM $MgSO_4$, 100 mM KNO_3, 0.4 mM NaN_3, 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$), and 1 μM calmodulin (when present). Following this incubation, the uptake reactions were initiated by the addition of either ATP or GTP to a final concentration of 3.75 mM. Both substrates were present as BTP salts (pH 7.5).

amounts of endogenous calmodulin could preclude stimulation by the addition of exogenous calmodulin.

A treatment which can be used to reduce the level of endogenous calmodulin associated with cell membrane fractions is to wash the membranes several times with a Ca^{2+} chelator such as EGTA (see ref. 29 for discussion). As calmodulin binding requires association with free Ca^{2+}, the removal of this cation allows calmodulin to dissociate from the membranes and be released to the high speed supernatant. When the red beet plasma membrane fractions were washed three times with 4 mM EGTA, a consistent stimulation of $^{45}Ca^{2+}$ uptake by calmodulin could now be observed which occurred when either ATP or GTP was used as the substrate for driving radiolabel uptake. Therefore, these results would suggest that our initial inability to observe calmodulin stimulation of uptake might be related to the presence of sufficient levels of endogenous calmodulin to preclude an effect by the addition of exogenous calmodulin. However, it does raise questions as to whether the Ca^{2+}-transporting ATPase of these membranes is similar to the analogous transport enzyme associated with the animal cell plasma membrane because of the lower molecular weight of the phosphorylated peptide presumed to be the catalytic subunit of this transport enzyme.

GENERAL DISCUSSION

In this communication, $^{45}Ca^{2+}$ uptake associated with red beet plasma membrane vesicles was further characterized using GTP as the substrate for driving transport. For a number of the characteristics which were examined, uptake utilizing GTP demonstrated properties similar to those previously observed when ATP was used as substrate (17). These included the kinetic relationship between $^{45}Ca^{2+}$ uptake rate and Mg:substrate concentration (Fig. 2) or Ca^{2+} concentration (Fig. 3), the pH optimum for $^{45}Ca^{2+}$ uptake (Fig. 4), and the sensitivity of $^{45}Ca^{2+}$ uptake to low concentrations of orthovanadate (Fig. 5). Uptake of $^{45}Ca^{2+}$ was also inhibited by submicromolar concentrations of erythrosin B (Fig. 6) which was consistent with what was previously observed for ATP-driven Ca^{2+} transport mediated by the Ca^{2+}-ATPase associated with radish plasma membrane vesicles (27). When the red beet membrane vesicles were treated in a manner which has been shown to remove endogenous calmodulin (EGTA washing), $^{45}Ca^{2+}$ uptake in the presence of either ATP or GTP was stimulated in an equivalent manner by the addition of exogenous calmodulin (Fig. 9). Calmodulin stimulation of Ca^{2+}-ATPase activity has recently been shown in a highly purified plasma membrane fraction produced from maize leaves (28). Taken together, these results would support the concept that this GTP-driven component of $^{45}Ca^{2+}$ uptake represents the activity of a plasma membrane Ca^{2+}-ATPase which can use this nucleoside triphosphate as an alternative substrate for driving transport.

As this ability to utilize GTP differs from other plant E_1E_2-type ATPases which have been characterized thus far (*i.e.* plasma membrane H^+-ATPase, endoplasmic reticulum Ca^{2+}-ATPase) and the enzyme mechanism for GTP utilization would appear to involve a phosphoenzyme intermediate (*i.e.* orthovanadate inhibition), phosphorylation using [γ-^{32}P]GTP could provide a means to identify the catalytic subunit of this enzyme on electrophoretic gels. When red beet plasma membrane vesicles were phosphorylated with [γ-^{32}P]GTP under conditions used to detect such phosphoenzyme intermediates and then analyzed by dodecyl sulfate PAGE, a rapidly turning over phosphorylated peptide was observed which had a molecular mass of about 100 kD (Fig. 8). The protein phosphate bond in this 100 kD phosphorylated peptide was an acyl phosphate type which is a common feature of the phosphorylated intermediates associated with E_1E_2 transport ATPases (5 and references therein). While these results would suggest that the 100 kD peptide could represent the catalytic subunit of the plasma membrane Ca^{2+}-ATPase, this does differ from what has been generally observed for the calmodulin-stimulated, Ca^{2+}-ATPases associated with animal plasma membrane where a molecular mass of about 140 to 150 kD has been observed for the catalytic subunit (11, 12, 29). Furthermore, recent studies by Briars *et al.* (4) have suggested that a 140 kD peptide might represent the catalytic subunit of a calmodulin-stimulated, Ca^{2+}-ATPase purified from maize coleoptiles. In this work, Ca^{2+}-ATPase solubilized from a microsomal fraction was purified by calmodulin affinity chromatography and then tested for cross-reactivity on Western blots with antibodies to purified erthrocyte Ca^{2+}-ATPase. Immunostaining revealed cross-reactivity with a 140 kD peptide, similar to the molecular mass observed for the erythrocyte Ca^{2+}-ATPase. As studies involving limited proteolysis of the erythrocyte Ca^{2+}-ATPase have revealed that proteolytic fragments with molecular masses in the 71 to 90 kD range can retain the capacity for ATPase activity, Ca^{2+}-transport and calmodulin stimulation (11, 12), one explanation for our results could be that the phosphorylated peptide associated with red beet membranes might represent a proteolytic fragment of the Ca^{2+}-ATPase. However, inclusion of a protease inhibitor such as PMSF throughout the isolation was found to have no effect upon the molecular size of the rapidly turning over peptide phosphorylated by [γ-^{32}P]GTP (LE Williams, unpublished results). Further studies are currently underway to determine if an alternative means of identifying the catalytic subunit of the red beet Ca^{2+}-ATPase, using labeled protein modification reagents or affinity probes, can be developed to clarify this result.

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