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Evidence for an ATP-Dependent Proton Pump on the Golgi of Corn Coleoptiles[1]

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ABSTRACT

Corn (*Zea mays* L. cv Trojan T929) coleoptile membranes were fractionated on sucrose density gradients, and ATP-dependent proton pumping activity was localized by the techniques of [^{14}C]methylamine uptake and quinacrine fluorescence quenching. Two peaks of proton pumping activity were detected: a light peak (1.07 grams/cubic centimeter) corresponding to the previously characterized tonoplast-type H$^+$-ATPase, and a second peak (1.13 grams/cubic centimeter) which coincided with the Golgi markers, latent UDPase, and glucan synthase I. The second peak was lighter than that of the plasma membrane marker, uridine diphosphoglucose-sterol glucosyltransferase (1.16 grams/cubic centimeter) and was not inhibited by vanadate, an inhibitor of the plasma membrane ATPase. The activity was also better correlated with the Golgi cisternae marker, glucan synthase I, than with latent UDPase, a secretory vesicle marker, but a secretory vesicle location cannot be ruled out. The tonoplast-type and Golgi proton pumps were similar in several respects, including a pH optimum at 7.2, stimulation by chloride, inhibition by diethylstilbestrol and *N,N'*-dicyclohexylcarbodiimide (DCCD), insensitivity to oligomycin and azide, and nucleotide specificity for Mg^{2+}-ATP. However, the Golgi H$^+$ pump was much less sensitive to nitrate and iodide, and more sensitive to the anion channel blockers, 4-acetamido-4'-isothiocyano-2,2'-stilbene sulfonic acid (SITS) and 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) than the tonoplast-type H$^+$-pump. The Golgi pump, but not the tonoplast-type pump, was stimulated by valinomycin in the presence of KCl. It is concluded that the Golgi of corn coleoptiles contains a KCl-stimulated H$^+$-ATPase which can acidify the interior of Golgi cisternae and associated vesicles.

In recent years, evidence has accumulated that the PM[3] and tonoplast of plant cells contain H$^+$-ATPases which carry out the electrogenic transport of protons. Such proton pumps apparently serve as primary transport mechanisms, driving the transfer of other solutes across their respective membranes (21). It has been inferred that the PM and tonoplast ATP-dependent proton pumps represent two distinct classes of H$^+$-ATPases based on a number of criteria, including sensitivity to inhibitors, stimulation by ions, and pH optima (21). Little is known about possible transport ATPases on other endomembranes in plant cells. A putative secretory vesicle ATPase with properties similar to the plasma membrane ATPase (*e.g.* vanadate sensitivity) was identified in a membrane vesicle preparation from suspension cultured oat cells (2). Unfortunately, the purity of the preparation was not established using marker enzymes, and it is not clear whether the activity was associated with secretory vesicles or contaminating plasma membranes. No vanadate-sensitive ATPase was found to be associated with the secretory vesicles of pea stem homogenates (22). Nevertheless, an ATP-driven proton pump has been reported to be present on the Golgi of rat and mouse liver (1, 8, 25). In a previous paper, we reported the occurrence of a KCl-stimulated Mg^{2+}-ATPase which appeared to be specifically associated with the Golgi of corn coleoptiles (5). The Golgi ATPase overlapped with the plasma membrane ATPase on sucrose density gradients, but could be resolved as a separate peak by assaying the activity at pH 7.5 instead of 6.5. The two major objectives of the present study were: (a) to determine whether the Golgi ATPase of corn coleoptiles functions as a proton pump; and (b) to compare the properties of the Golgi proton pump with those of the previously characterized tonoplast-type proton pump (10, 11). Because of the lack of an independent marker for the tonoplast, the term 'tonoplast-type' is used throughout this paper (21). However, recent studies with intact vacuoles isolated from corn coleoptiles indicate that the tonoplast-type H$^+$-ATPase purified on sucrose gradients has the same density and properties as the H$^+$-ATPase associated with purified vacuolar membranes (Mandala and Taiz, unpublished data).

MATERIALS AND METHODS

Plant Material. Corn (*Zea mays* L. cv Trojan T929, Pfizer-DeKalb) seeds were soaked 6 to 8 h in distilled H_2O and sown in trays with moist vermiculite. Seedlings were grown in the dark at 20°C with 2 h of dim red light daily to inhibit mesocotyl growth. After 5 to 6 d, coleoptiles (~3 cm) were harvested, debladed, and collected on ice under room lights. Homogenization and subsequent treatments were performed at 0 to 4°C.

Homogenization. Coleoptiles were homogenized and a 1,000*g* supernatant (1KS) was prepared as previously described (5). Briefly, coleoptiles (13 g) were chopped by hand (10 min) with razor blades in 6 ml of homogenization medium (250 mM sucrose, 2 mM EDTA, 1 mM DTT, 0.1% BSA, 50 mM Tris-Mes [pH 7.8], except for Figures 1 and 2, in which BSA was omitted). The tissue was then ground very lightly with a mortar and pestle and strained through nylon. The remaining tissue was lightly reground in an additional 6 ml of homogenization buffer. The final homogenate was filtered through nylon and combined with

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[3] Abbreviations: PM, plasma membrane; GS I, glucan synthase I; IDPase, inosine diphosphatase; MeA, methylamine; UDPase, uridine diphosphatase; UDPG-ST, uridine diphosphoglucose-sterolglucosyltransferase; BTP, bis-tris propane; DES, diethylstilbestrol; KIDA, potassium iminodiacetate; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; SITS, 4-acetamido-4'-isothiocyano-2,2'-stilbene sulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide.

the first homogenate. Unbroken cells, cell wall fragments, starch, and nuclei were removed by a 5-min centrifugation at 1000*g* (Sorvall, SS-34 rotor) and the supernatant (1KS) was collected.

Linear Sucrose Gradients. The 1KS was layered onto a linear gradient consisting of a 2-ml cushion of 45% sucrose (w/w), 20 ml of 15 to 45% sucrose (Fig. 1) or 10 to 40% sucrose (Fig. 4), and 1 ml of a 15 or 10% sucrose overlay. The gradient buffer included 20 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 2.5 mM Tris-Mes (pH 7.5). The gradients were centrifuged at 80,000*g* for 3 h (Beckman L2-65B ultracentrifuge, SW 28 rotor) and fractionated into 16 fractions (1.5 ml).

Nonlinear Sucrose Gradients. The 1KS fraction was layered onto a step gradient consisting of a 3-ml cushion of 35% (w/w) sucrose and 5 ml each of 25, 18, and 10% sucrose (in 0.5 mM EDTA, 20 mM KCl, 1 mM DTT, 2.5 mM Tris-Mes, pH 7.5). The gradients were centrifuged 2 h at 80,000*g*, and the membranes at the different interfaces were collected with a Pasteur pipet. The pellet was discarded.

Rate-Zonal Centrifugation. The 1KS was layered onto a linear gradient (20 ml, 15 to 35% sucrose in 0.5 mM EDTA, 20 mM KCl, 1 mM DTT, 2.5 mM Tris-Mes, pH 7.5) with a 2-ml cushion of 45% sucrose and a 1-ml overlay of 15% sucrose. The gradients were centrifuged 25 min at 30,000*g* (SW 28 rotor) and fractionated into 16 fractions (1.5 ml).

Dilution of the Fractions. For proton pumping experiments, the gradient fractions were diluted to 10% sucrose (w/w) with 0.5 mM EDTA, 1 mM DTT, 20 mM KCl, and 2.5 mM Tris-Mes (pH 7.5). The diluted fractions were assayed directly or frozen in liquid N_2 and stored at −70°C for up to 2 weeks without loss of activity. Freezing prior to dilution resulted in a loss of activity.

Enzyme Assays. NADPH-Cyt *c* reductase, Cyt *c* oxidase, latent UDPase, UDPG-ST, and GS I activities were determined as previously described (5). ATP hydrolyzing activity was determined by released Pi (5) after a 30-min incubation at 37°C. The reaction mixture contained 3 mM Tris-ATP (plus or minus 3 mM $MgSO_4$), 250 mM sucrose, 50 mM LiCl, 2 μM gramicidin, 1 mM Na-molybdate (to inhibit nonspecific phosphatase), 1 mM NaN_3 (to inhibit mitochondrial ATPase), and 25 mM Tris-Mes, pH 7.2. The activity is expressed as the Mg^{2+}-stimulated activity.

Methylamine Uptake. The uptake of MeA was determined by a Millipore filtration technique (11). At time zero, 20 μl of test solution was added to 100 μl of diluted membranes (in 10% sucrose). The final concentrations were: 3 mM Mg^{2+}-ATP or Mg^{2+}-ADP (control), 18 μM MeA (0.77 μCi/ml/assay, 44 mCi/mmol in ethanol), 20 mM KCl, 10% sucrose, and 12 mM Tris-Mes (pH 7.5). After a 5-min incubation at 30°C, the reaction mixture (110 μl) was filtered on a prewetted 0.45-μm Millipore filter (HATF). The filter was immediately rinsed with 3 ml of cold buffer (20 mM KCl, 10% sucrose, 2.5 mM Tris-Mes, pH 7.5). The entire stop process took less than 15 s. Radioactivity was determined in a liquid scintillation counter. MeA uptake was calculated as the difference in activity between Mg^{2+}-ATP and the Mg^{2+}-ADP control.

Quinacrine Fluorescence Quenching. Membrane vesicles (300–400 μg protein/experiment), the appropriate salts or inhibitors, and 10 μM quinacrine were added to an assay buffer of 25 mM BTP-Mes (pH indicated in text) to a final volume of 0.6 ml. Fluorescence was measured at room temperature with an Hitachi-Perkin Elmer fluorescence spectrophotometer. The excitation wavelength was 420 nm, and the emission wavelength was 495 nm. After temperature equilibration, the reaction was initiated by the addition of Mg^{2+}-ATP. At the end of the experiment, the proton gradient was collapsed by the addition of 3 μl of 1 mM monensin dissolved in ethanol (5 μM, final monensin concentration).

Other Assays. Sucrose concentrations were determined refractometrically. Protein was assayed by the Lowry method (cited in Ref. 5) after a TCA precipitation, and using BSA as a standard.

Chemicals. ATP (disodium salt), valinomycin, DIDS, SITS, and ouabain were purchased from Calbiochem. Sodium azide and 1-amino-2-naphthal-4-sulfonic acid were purchased from Eastman Kodak Co.; sodium lauryl sulfate (SDS) was obtained from Bio-Rad; *N,N'*-dicyclohexylcarbodiimide was obtained from the Aldrich Chemical Co.; and sodium vanadate was from Fisher Scientific Co. All other chemicals were purchased from Sigma Chemical Co. or Mallinckrodt.

RESULTS

Localization of the Golgi ATPase Proton Pump. Two peaks of proton pumping activity (ATP-dependent MeA-uptake) were detected after centrifugation of the 1KS fraction on a near isopycnic linear sucrose gradient: one near the top of the gradient at 1.07 g/cc, less dense than the ER marker, NADPH-Cyt *c* reductase, and a second at 1.13 g/cm^3, between the Golgi marker, latent UDPase, and the PM marker, UDPG-ST (Fig. 1). The distribution of valinomycin-stimulated proton pumping is also shown. Valinomycin stimulated the proton pumping activity of the second peak, but did not affect the activity of the first peak. In the presence of 50 mM nitrate, the lighter peak was completely abolished while the denser peak was only inhibited by about 50% (Fig. 1). On the basis of its low density and marked nitrate sensitivity, we infer that the first peak represents tonoplast vesicles, although this conclusion remains tentative because of the lack of an independent marker for tonoplast membranes (21). The identity of the peak less sensitive to nitrate is not immediately apparent because of its position midway between latent UDPase and UDPG-ST.

Figure 2 illustrates a rate-zonal sucrose gradient of the 1KS

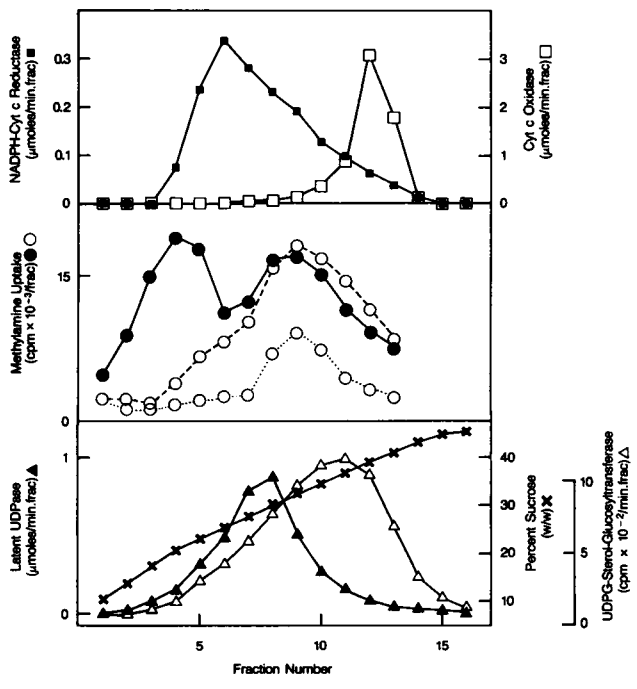

FIG. 1. Linear 15 to 45% (w/w) sucrose gradient of the 1KS fraction of a corn coleoptile homogenate centrifuged for 3 h at 80,000*g*. NADPH-Cyt *c* reductase (■), Cyt *c* oxidase (□), latent UDPase (▲), and percentage of sucrose (×) were analyzed using 50-μl aliquots. UDPG-ST (△) was determined on a 0.2-ml aliquot. MeA uptake was measured in duplicate on 0.1-ml aliquots after dilution of the different fractions to 10% sucrose. Control (●), stimulation by 1 μM valinomycin (O---O) nitrate-insensitive activity (O···O). The plotted values were calculated for each fraction with the dilution factor.

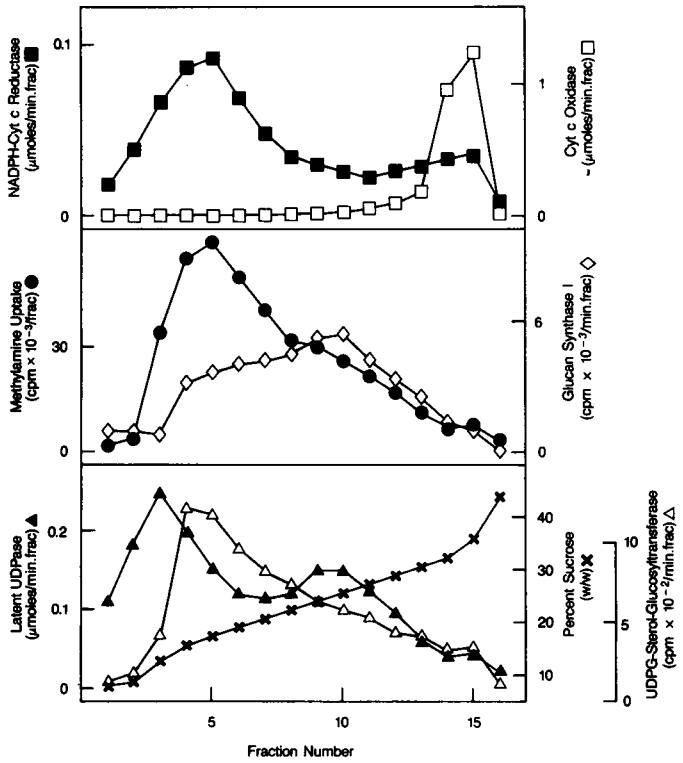

FIG. 2. Rate-zonal sedimentation of the 1KS fraction centrifuged for 25 min at 30,000*g*. NADPH-Cyt *c* reductase (■), Cyt *c* oxidase (□), latent UDPase (▲), percentage of sucrose (×), GS I (◇) (determined on a 0.1-ml aliquot), UDPG-ST (△), and MeA uptake (●) (see Fig. 1).

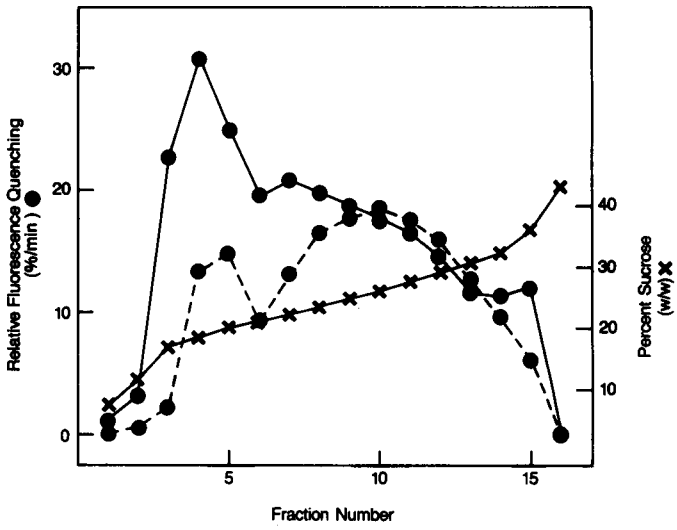

FIG. 3. Rate-zonal sedimentation of the 1KS centrifuged for 25 min at 30,000*g*. BSA (0.1%) was included in the homogenization medium. Relative fluorescence quenching of quinacrine in the presence of 25 mM BTP-Mes (pH 7.2) and 20 mM BTP-Cl. Control (●——●), plus 50 mM KNO_3 (●- - -●), percentage sucrose (×).

fraction. Since the membranes have separated primarily on the basis of size, the Golgi marker, latent UDPase, is found in two peaks as previously described for pea stem homogenates (22), a putative secretory vesicle-enriched zone near the top (fraction 3) and a cisternae-enriched zone further in the gradient (fractions 9–10). GS I, a Golgi cisternae marker, is broadly distributed, but exhibits a peak at fractions 9 to 10, coincident with the heavy peak of latent UDPase. This is consistent with previous results which demonstrated that GS I is associated mainly with the Golgi cisternae, while latent IDPase occurs on both cisternae and secretory vesicles (18, 22). Proton pumping shows a main peak at fraction 5, and a shoulder of activity near fraction 9, overlapping the peak of GS I. The main peak consists of a mixture of membranes, including ER, PM, Golgi, and perhaps, tonoplast. The main peak of proton pumping coincides with the ER and PM markers, but a shoulder of proton pumping activity at fraction 9 overlaps with the main peak of GS I. Since the peaks of proton pumping activity on isopycnic gradients are resolved from the peaks of ER or PM marker activity (Fig. 1), the main peak of proton transport on the rate zonal gradient (Fig. 2) is presumably not associated with the ER or PM. Thus, the first peak may primarily consist of tonoplast-type vesicles. However, GS I has a broad shoulder which overlaps the first peak of proton pumping, indicating the presence of Golgi membranes in this fraction as well. There appears to be a slightly better correlation of proton pumping with GS I than with the upper zone of latent UDPase activity. These results suggest that the Golgi-associated proton pumping activity may be concentrated on the cisternae, although further characterization of the secretory vesicles is required to substantiate this.

Proton pumping was also measured across rate zonal gradients by the quinacrine fluorescence quenching method and the data were plotted as the initial rate of quenching (Fig. 3). Again, the distribution of H^+-transport activity showed a main peak near the top of the gradient and a prominent shoulder at a higher density. The peak at the top of the gradient was partially inhibited by nitrate, suggesting that it contained a mixture of tonoplast vesicles and Golgi membranes, in agreement with the distribution of GS I in Figure 2. Proton pumping in the shoulder region was completely nitrate insensitive, reflecting the fact that the activity was expressed as the initial rate of quenching (Fig. 3). A minor peak of nitrate-sensitive activity in fractions 14 to 15 near the 45% sucrose cushion was probably due to mitochondrial contamination.

We have recently shown that a pH 7.5 Mg^{2+}-ATPase is specifically associated with the Golgi of corn coleoptiles (5). We reported that in linear gradients of the 1KS fraction, the pH 7.5 Golgi ATPase is obscured by the greater activity of the PM ATPase (pH 6.5), but can be detected after recentrifugation of a Golgi-rich fraction in a linear isopycnic gradient. The peak of Golgi ATPase can also be distinguished from other ATPases in the 1KS by its differential sensitivity to vanadate and nitrate. As shown in Figure 4, the vanadate-sensitive PM ATPase overlaps with the PM marker, UDPG-ST, on a near isopycnic linear sucrose gradient. Two peaks of vanadate-insensitive ATPase are also present: a light peak in the region of the tonoplast and a second peak coincident with the Golgi markers, latent UDPase and GS I. In the presence of nitrate, the tonoplast ATPase is completely inhibited, while the Golgi ATPase is only partially blocked (Fig. 4). The ATPase results thus are similar to those of proton pumping in Figure 1 but not identical, because H^+ pumping as measured by MeA uptake was less well correlated with the peak of latent UDPase activity. However, the shape of the Golgi ATPase peak in Figure 4 shows a better correlation with GS I than with latent UDPase, supporting the conclusion that the Golgi proton pump is primarily associated with the cisternal membranes.

Characterization Studies. To study the properties of the two populations of proton pumping microsomal membranes, step gradients were prepared which allowed separation of tonoplast-rich (10/18% interface) and Golgi-rich (25/35% interface) fractions (see "Materials and Methods"). A pH curve for proton transport measured by quinacrine fluorescence quenching is shown in Figure 5. Proton pumping activity is expressed both as the initial rate of quenching and the total amount of monensin-reversible quenching after 5 min. Similar curves were obtained

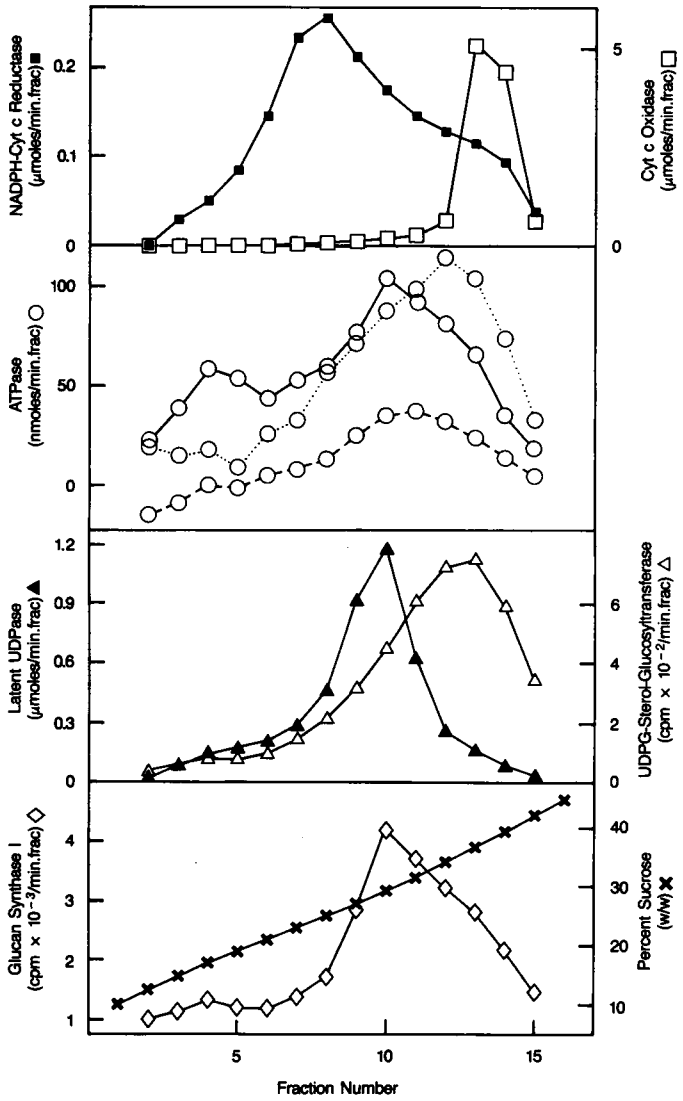

FIG. 4. Linear 10 to 40% sucrose gradient of the 1KS fraction centrifuged for 3 h at 80,000*g*. BSA (0.1%) was included in the homogenization medium. NADPH-Cyt *c* reductase (■), Cyt *c* oxidase (□), latent UDPase (▲), and percentage of sucrose (×) were analyzed with 20-μl aliquots. GS I was determined on 0.1-ml aliquots and UDPG-ST and ATPase activities were determined (in duplicate) on 50-μl aliquots in the presence of 50 mM LiCl and 3 mM ATP:$MgSO_4$. Vandate-insensitive activity (100 μM) (O——O); vanadate-sensitive activity(O···O); KNO_3- and vanadate-insensitive activity (O– – –O).

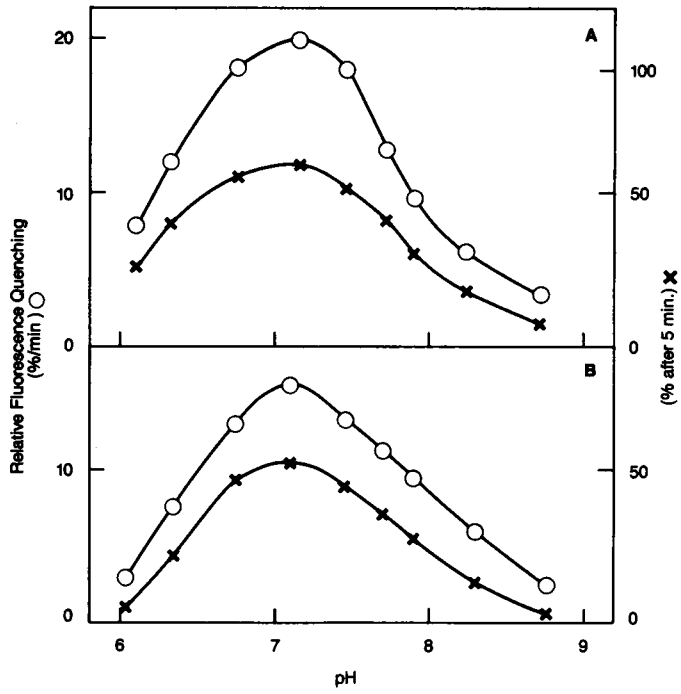

FIG. 5. Effect of pH on the ATP-dependent fluorescence quenching of quinacrine in the presence of 66 mM KCl. A sucrose step gradient was prepared and membrane fractions were collected from the appropriate interfaces. A, 10 to 18% (tonoplast-enriched fraction); B, 25 to 35% (Golgi-enriched fraction). The pH of the medium was adjusted by altering the proportions of BTP and Mes (25 mM, final concentration). The vesicles were incubated 1 h on ice in buffer before starting the reaction. Monensin (5 μM) was added after 5 min to obtain the total quench. Initial rate of quench (O——O), total quench after 5 min (×).

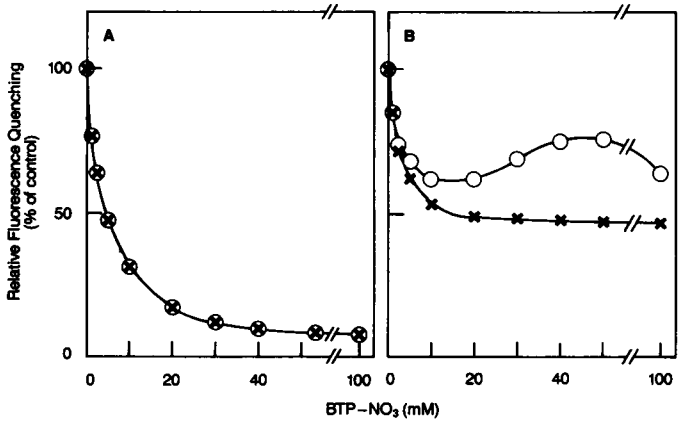

FIG. 6. Inhibition of ATP-dependent quinacrine fluorescence quenching by BTP-NO_3 in the presence of 66 mM KCl. Fractions were obtained from a sucrose step gradient. A, 10 to 18% (tonoplast-enriched fraction); B, 25 to 35% (Golgi-enriched fraction). Initial rate of quench (O——O), controls: A, 17.7%/min; B, 15.2 %/min. Quench after 5 min (×), controls: A, 55%; B, 53.1%. The data were compiled from several experiments, each point being the average of at least two experiments.

for the two fractions, with peaks at pH ~ 7.2. The results were the same when expressed either as the initial rate or the total amount of quench.

The effects of increasing concentrations of nitrate on the activity of the two pumps is shown in Figure 6. The tonoplast-type pump (Fig. 6A) was more sensitive to nitrate than the Golgi pump (Fig. 6B) at every concentration, but particularly at concentrations above 20 mM. The maximum inhibition of the Golgi pump was about 55%, while the tonoplast-type pump was inhibited 90 to 100%. For the tonoplast-type pump, the initial rate and total quench curves were superimposable, whereas the initial rate curve increased relative to the total quench curve above 10 mM in the case of the Golgi pump. This may indicate that nitrate inhibits the tonoplast-type and Golgi proton pumps by different mechanisms.

Figure 7 shows the effects of Mg^{2+}-ATP concentration on proton pumping by the tonoplast- (7A) and Golgi-enriched (7B) fractions in the presence or absence of 5 mM nitrate. Sigmoidal kinetics were observed for both fractions, and K_m values could not be accurately calculated because the double reciprocal plots were not linear. The tonoplast-type proton pump was more sensitive to nitrate than the Golgi pump over the range of substrate concentrations tested, and the discrepancy between the quench rate and total quench curves in the case of the Golgi was

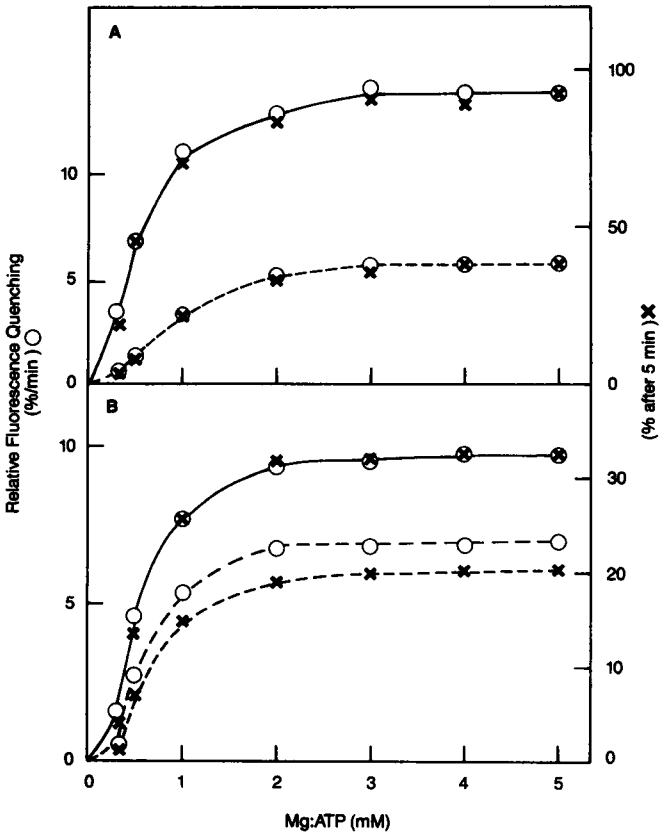

FIG. 7. Effect of Mg^{2+}:ATP concentration on quinacrine fluorescence quenching in the presence or absence of 5 mM KNO_3. Fractions were obtained from a sucrose step gradient and assayed in the presence of 66 mM KCl. A, 10 to 18% (tonoplast-enriched fraction); B, 25 to 35% (Golgi-enriched fraction. Initial rate of quench (○), quench after 5 min (×). Control (——), 5 mM KNO_3 (- - - - -).

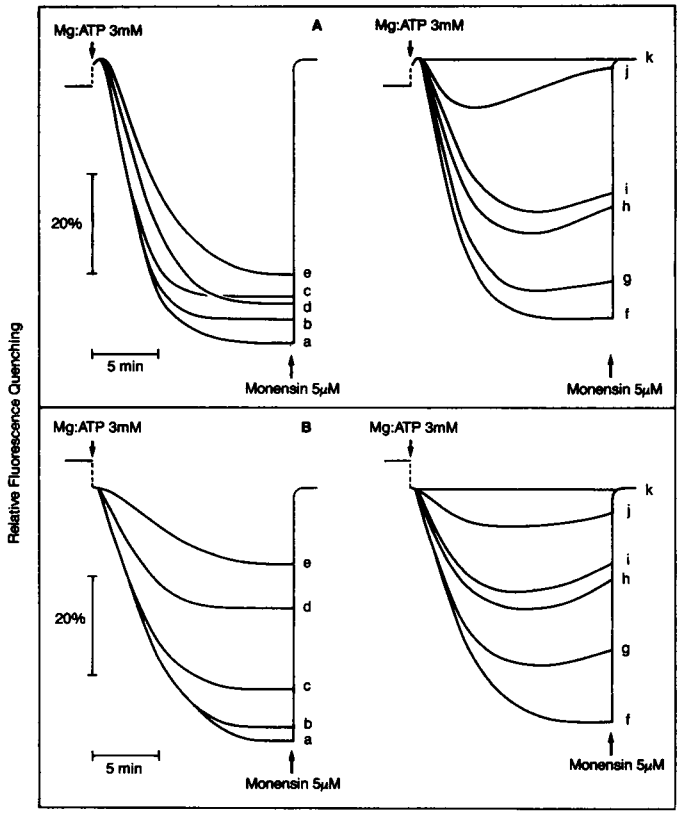

FIG. 8. Effect of inhibitors on ATP-dependent quinacrine fluorescence quenching in the presence of 66 mM KCl. Fractions were obtained from a sucrose step gradient. A, 10 to 18% (tonoplast-enriched fraction); B, 25 to 35% (Golgi-enriched fraction). (a), Control, 100 μM vanadate, 1 mM ouabain; (b), 1 mM Na-molybdate; (c), 1 mM NaN_3; (d), 100 μM SITS; (e), 50 μM DIDS; (f), 1% ethanol; (g), 1% ethanol + 5 μg/ml oligomycin; (h) 1% ethanol + 10 μM DCCD; (i), 1% ethanol + 10 μM DES; (j), 1% ethanol + 50 μM DCCD; (k), 1% ethanol + 50 μM DES.

again apparent.

Figure 8 illustrates typical fluorescence quenching curves for the tonoplast- (8A) and Golgi-enriched (8B) fractions in the presence of various inhibitors. Curves a to e were performed in the absence of ethanol, while curves f to j all contained 1% ethanol, including the control. Monensin (5 μM) was added to collapse the pH gradient at the end of each experiment. Vanadate (100 μM) and ouabain (1 mM) treatments were identical to the controls for both the tonoplast-type (9A) and Golgi (9B) proton pumps. Sodium-molybdate (curve b) and NaN_3 (curve c) had little or no effect on the initial rate, although azide slightly reduced the total quench, possibly due to its ability to transport protons. The anion channel blockers, SITS (curve d) and DIDS (curve e), were more inhibitory to the Golgi proton pump than to the tonoplast-type proton pump at the concentrations tested. Ethanol alone (curve f) slightly reduced the total quench, perhaps indicating increased membrane permeability. Oligomycin (curve g), an inhibitor of mitochondrial ATPases, had no effect on the initial rate of either the tonoplast- or Golgi-enriched fractions, but partially inhibited the total quench. This inhibition only developed after the first 2 min of the reaction, even when a pretreatment was given (data not shown), suggesting an effect on permeability of the membrane to protons rather than an inhibition of the enzyme. DCCD (curves h and j) and DES (curves i and k) inhibited both pumps, particularly at 50 μM.

Although the standard assay mixture contained a background of 16 mM KCl due to the presence of 20 mM KCl in the gradient buffer, it was possible to measure the effects of additional monovalent salts on proton pumping, as shown in Table I. Both the initial rate and total quench data are given, since the two were not always in exact agreement. Chloride and bromide stimulated the H^+-transport activity of the tonoplast- (A) and Golgi-enriched (B) fractions about equally. Iodide partially substituted for chloride in the Golgi, but only slightly stimulated the initial rate, while strongly inhibiting the total quench, in the tonoplast-enriched fraction. Nitrate was also more inhibitory to the tonoplast-type H^+-pump as previously noted, particularly when based on the initial rate of quenching. The slightly greater amount of nitrate inhibition of the initial rate of quenching in the Golgi fraction shown in Figure 6B compared to that shown in Table I we do not consider to be significant. At low nitrate concentrations, initial rate and total quench were inhibited about equally, whereas at high nitrate concentrations (Table I) total quench was inhibited more than the initial rate, *i.e.* higher concentrations seemed to reverse the inhibitory effect of nitrate on the initial rate. The greater inhibition of the total quench by nitrate in Table I than in Figure 6B may reflect the fact that the medium contained 66 mM KCl in Figure 6B and only 16 mM KCl in Table I. Nitrite abolished the activity of both pumps. Fluoride inhibited both pumps as well, but was more effective against the tonoplast-type pump. In addition to the monovalent salts, two other divalent cations were tested for their ability to substitute for magnesium. Manganese partially substituted for magnesium, with the Golgi pump showing higher activity in the presence of Mn^{2+}-ATP than the tonoplast-type pump. Neither fraction utilized Ca^{2+}-ATP as a substrate.

Table I also shows that both LiCl and BTP-Cl were more

Table I. *Effect of Different Salts on the ATP-Dependent Relative Fluorescence Quenching of Quinacrine*

KCl (16 mM) was present as background in all fractions, including the control. When divalent cations were tested, all treatments contained, in addition, 50 mM KCl. Sucrose step gradients were prepared as described in "Materials and Methods." A is 10 to 18% interface, and B is 25 to 35% interface. The values are the average of two to three experiments.

	A		B	
	Initial rate	Quench after 10 min	Initial rate	Quench after 10 min
	%[a] *(%/min)*	%[a] *(%)*	%[a] *(% min)*	%[a] *(%)*
Control ($MgSO_4$, 3 mM)	100 (7.9)	100 (44.3)	100 (6.5)	100 (51.5)
Monovalent				
+ KCl, 50 mM	183	138	171	134
+ NaCl, 50 mM	190	135	186	132
+ LiCl, 50 mM	224	144	203	137
+ BTP-Cl, 50 mM	209	151	173	126
+ KBr, 50 mM	188	115	184	127
+ KI, 50 mM	128	46	177	78
+ KNO_3, 50 mM	9	3	169	38
+ KNO_2, 50 mM	0	0	0	0
+ KIDA, 50 mM	119	106	131	96
+ K_2SO_4, 25 mM	60	49	92	72
+ Na_2SO_4, 25 mM	53	38	104	68
+ NaF, 50 mM	51	17	75	50
Divalent				
+ $MgSO_4$, 3 mM	100	100	100	100
+ $MgCl_2$, 3 mM	106	102	106	100
+ $MnCl_2$, 3 mM	47	49	78	71
+ $CaCl_2$, 3 mM	0	0	0	0

[a] Percentage of the activity of the control.

Table II. *Effect of Valinomycin at Different Concentrations on the ATP-Dependent Relative Fluorescence Quenching of Quinacrine, in the Presence of 66 mM KCl*

A nonlinear sucrose gradient was prepared, and the material at the different interfaces collected. A is 10 to 18% and B is 25 to 35%. Each value is the mean of two experiments.

	A		B	
	Initial rate	Quench after 10 min	Initial rate	Quench after 10 min
	%[a] *(%/min)*	%[a] *(%)*	%[a] *(%/min)*	%[a] *(%)*
Control, 1% ethanol	100 (10.6)	100 (40.5)	100 (13.8)	100 (43.7)
Valinomycin in 1% ethanol				
10^{-8} M	101	98	133	102
10^{-7} M	96	89	196	97
10^{-6} M	76	67	183	83
10^{-5} M	30	14	112	24

[a] Percentage of the activity of the control (1% ethanol).

effective than KCl and NaCl in stimulating the tonoplast-type proton pump. In contrast, KCl and BTP-Cl were equally stimulatory to the Golgi pump. The presence of background levels of KCl in the gradient buffer prevented a determination of the absolute potassium requirements of the two H^+-ATPases. However, the above data indicate that the two may differ in their responses to monovalent cations. As previously shown, valinomycin, a potassium ionophore, specifically promoted the activity of the Golgi pump (Fig. 1). The effects of various concentrations of valinomycin are shown in Table II. At concentrations of 10^{-8} to 10^{-6} M, the ionophore enhanced the initial rate of quenching in the Golgi fraction (B) without affecting the total quench. Tonoplast-type proton pumping was significantly inhibited over the same concentration range (A). At 10^{-5} M, the total quench of both fractions was drastically reduced, perhaps due to a loss of ionophore specificity at the higher concentration. The substrate specificities of the two pumps are shown in Table III. Both pumps were highly specific for Mg^{2+}-ATP. A significant amount of pyrophosphate-driven proton pumping was also observed in both the tonoplast- (A) and Golgi-enriched (B) fractions. evidence will be presented in a separate paper that the pyrophosphatase H^+-pump is a separate enzyme (6).

DISCUSSION

An ATP-Driven H^+-Pump is Associated with the Golgi. We have recently shown that an ATPase with a slightly alkaline pH optimum is associated with gradient purified Golgi membranes of corn coleoptiles (5). The two objectives of the present study were: (a) to determine whether the Golgi ATPase(s) can function as a proton pump; and (b) to compare the properties of the putative Golgi proton pump with those of the previously characterized tonoplast-type proton pump (10, 11). The Golgi of rat

Table III. *Substrate Dependence of Relative Fluorescence Quenching of Quinacrine, in Presence of 66 mM KCl*

A nonlinear sucrose gradient was prepared, and the material at the different interfaces collected. A is 10 to 18% and B is 25 to 35%. Substrate concentrations were 1.5 mM. Initial rate of quench for Mg^{2+}-ATP was 7.75%/min for A and 9.75%/min for B.

Substrate	A (Initial Rate)	B (Initial Rate)
	%[a]	
$MgSO_4$	0	0
ATP	0	0
Mg^{2+}-ATP	100	100
Mg^{2+}-GTP	0	3
Mg^{2+}-CTP	0	0
Mg^{2+}-UTP	0	0
Mg^{2+}-ITP	0	0
Mg^{2+}-ATP	0	2
Mg^{2+}-PPi	41	39

[a] Percentage of activity in presence of Mg^{2+}-ATP.

and mouse liver have already been shown to contain an H^+-ATPase (1, 8, 25), but this is the first study of proton transport in plant Golgi membranes. In addition to ATP-driven H^+-transport, mouse liver Golgi appear to contain a redox system which couples proton transport to NADH oxidation (1). We were unable to detect any NADH-driven proton transport in corn microsomal vesicles by the method of Barr *et al.* (1) (A. Chanson, unpublished data).

Two separate peaks of proton pumping activity were detected in sucrose gradients of microsomal membranes: a light peak with a density similar to that previously reported for the tonoplast (1.07 g/cm^3) (4), and a peak of intermediate density between the ER and the PM (1.13 g/cm^3). The density of the presumed tonoplast vesicles reported here is slightly lighter than the value we previously reported for corn (1.11 g/cm^3) (10), perhaps because a different variety was used in the present study or because pelleting was eliminated prior to gradient centrifugation. Based on a number of criteria, including a low density in sucrose gradients, a slightly alkaline pH optimum, stimulation by chloride, marked inhibition by nitrate, and a lack of inhibition by either the mitochondrial ATPase inhibitors, azide and oligomycin, or the PM-ATPase inhibitor, vanadate, we infer that the light peak represents tonoplast vesicles (4, 10, 11, 16, 21). We propose that the second peak, which was not previously detected, represents Golgi membranes, mainly those of the cisternae, for the reasons outlined below.

On linear sucrose gradients, the peak of the second proton pumping activity occurred at a lighter density than that of the PM marker, UDPG-ST (1.16 g/cm^3). Its distribution was also broader and shifted to a slightly higher density than that of the Golgi marker, latent UDPase (Fig. 1). In contrast, the second peak of vanadate-insensitive ATPase activity showed a better correlation with the peak of latent UDPase. Because of its correlation with latent UDPase and its lack of inhibition by the plasma membrane ATPase inhibitor, vanadate, the second peak appears to be associated with the Golgi. The Golgi ATPase differed from the tonoplast-type ATPase (first peak) in that it was only partially sensitive to nitrate (Fig. 4). Since the properties of the Golgi ATPase (insensitivity to vanadate and partial inhibition by nitrate) are also properties of the second peak of ATP-driven H^+-transport, we tentatively conclude that the two activities represent the same enzyme. The better correlation of latent UDPase with the Golgi ATPase than with the second peak of ATP-driven proton pumping may indicate the presence of additional Golgi ATPase activities not involved in proton transport.

The distributions of both the Golgi ATPase and ATP-dependent proton transport more closely paralleled the distribution of another Golgi marker, GS I, on linear and rate zonal gradients. Thus, latent UDPase and GS I are not interchangeable as Golgi markers. Similar discrepancies in the localization of latent IDPase and GS I have been observed previously (19) and are not unexpected given that the Golgi apparatus has a number of distinct morphological compartments. Several enzymes (7, 20), phospholipids (9), and cholesterol (15) have been shown to be asymmetrically distributed across the Golgi complex. Fragmentation of the Golgi apparatus during isolation separates cisternae from each other and from their associated vesicles. Latent IDPase was previously found to be associated both with cisternae and secretory vesicles of *Pisum* stem Golgi, while GS I was restricted to the cisternal membranes (22). In the present study, rate-zonal gradients of corn coleoptile homogenates resulted in a separation of latent UDPase activity into a major peak near the top of the gradient (putative secretory vesicle fraction) and a smaller peak in the middle of the gradient (cisternae with attached vesicles). The faster sedimenting zone coincided with the peak fractions of GS I as well as a shoulder of proton pumping activity. The broad distribution of GS I in the rate-zonal gradient presumably indicates that the cisternal membrane fragments are heterogeneous in size. If GS I is primarily localized on the Golgi cisternae of corn, the Golgi H^+-ATPase may also be primarily associated with the cisternae.

Indirect support for the existence of a Golgi proton pump comes from studies with monensin, an ionophore which collapses Na^+/H^+ gradients. Monensin induces the swelling of Golgi cisternae in algal (12) and plant (3, 13) cells. Boss *et al.* (3) have postulated the presence of a proton pump on the Golgi of carrot cells, based on ultrastructural studies of monensin-induced swelling of the cisternae on the maturing (*trans*) face. R. G. W. Anderson (personal communication) has recently used the pH probe, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP), which accumulates in acidic compartments within cells and which can be visualized by electron microscopy, to demonstrate that the cisternae and vesicles of human fibroblast Golgi are indeed acidic compartments, with the maturing face being the most acidic. This is consistent with our results and those of Boss *et al.* (3) and leads to the hypothesis that the Golgi H^+-ATPase of corn coleoptiles may be most active on the cisternal membranes of the maturing face.

Characterization of the Golgi Proton Pump. *Inhibitors.* The two proton pumps were similar in their responses to a variety of inhibitors. Both were strongly inhibited by DES and the proton channel blocker, DCCD, and were insensitive to ouabain, vanadate, molybdate, azide, and oligomycin. Discrepancies were observed between the initial rates of H^+ transport and the total monensin-reversible pH gradient. For example, oligomycin had little or no effect on the initial rate of either the tonoplast-type or Golgi proton pump, but consistently reduced the total quench of both. Since reductions in the total quench could be caused by membrane leakiness rather than inhibition of the H^+-ATPase, more weight was placed on the initial rate in our interpretations. One apparent difference between the tonoplast-type and Golgi proton pumps was the greater sensitivity of the Golgi pump to DIDS and SITS, which act as anion channel blockers in animal cells. However, the specificity of DIDS and SITS has not been established in plant cells. Vanadate inhibition is a property of eukaryotic plasma membrane ATPases and other ATPases having a phosphorylated intermediate (21). Insensitivity to vanadate implies that the Golgi and tonoplast-type H^+-ATPases, like the F_0F_1-type H^+-ATPases of mitochondria and chloroplasts, do not have phosphorylated intermediates. There is little information available in the animal literature on the nature of the Golgi ATPase. West and Clegg (24) have demonstrated the presence of a transiently phosphorylated protein in Golgi preparations from

rat mammary glands. They suggested that this rapidly labeled protein, with a mol wt of 70,000 D, may represent a phosphoenzyme intermediate of a Golgi Ca^{2+}-ATPase. However, the enzyme itself has not yet been purified and the identity of the phosphoprotein is uncertain. To our knowledge, the vanadate sensitivity of the Golgi H^+-ATPase of animal cells has not yet been determined.

Ions. KCl was included in the homogenization and gradient buffers because its presence enhanced the recovery of Golgi proton pump activity. The enhancement appears to be due to stabilization rather than to activation, since addition of KCl to membranes prepared in its absence did not fully restore activity (data not shown). BSA in the homogenization medium also appeared to preferentially increase proton pumping by the Golgi. These observations suggest that the Golgi proton pump may be more labile than the tonoplast-type pump.

Despite the presence of 16 mM KCl in the final reaction mixture, H^+ transport was increased by additional 50 mM monovalent salts. Both the tonoplast-type and Golgi H^+ pumps were stimulated by chloride. When the membranes were isolated in the absence of KCl, proton transport in both fractions (highly reduced in the case of the Golgi) was strictly dependent on the presence of chloride, *i.e.* K-Mes was completely inactive, even in the presence of valinomycin (data not shown).

Chloride stimulation of the tonoplast-type electrogenic proton pump appears to be mainly due to a direct stimulation of the enzyme (21). Chloride uptake is also driven by the proton pump, however, thus contributing to the maintenance of electrical neutrality (11). The Golgi pump appears to be electrogenic, since ATP-dependent thiocyanate uptake was detected in the Golgi fraction (data not shown). Thus, chloride could be taken up as a counterion during electrogenic proton pumping by Golgi membranes. Direct activation of the Golgi H^+-ATPase by chloride is also likely since potassium and valinomycin, which should collapse the electrical gradient, did not eliminate the stimulation by chloride. There are two possible explanations for the stimulation of the Golgi pump by valinomycin. The Golgi pump might be highly electrogenic, resulting in an inhibition of pump activity even in the presence of chloride. Valinomycin would collapse the electrical gradient by facilitating potassium efflux from the vesicles, thus relieving the inhibition of the pump (21). (Note that the vesicles already contain potassium from the isolation and gradient buffers.) Alternatively, the Golgi pump may operate via an H^+/K^+-exchange mechanism (either electrogenic or electroneutral). Once the internal potassium is depleted, the rate would be limited by the rate of potassium uptake, which is stimulated by valinomycin. At the present time, it is not possible to choose between these alternatives. However, it is significant that the tonoplast-type pump does not exhibit the same stimulation by valinomycin. Thus, either the tonoplast-type H^+-ATPase is less inhibited by the electrical gradient or less of an electrical gradient develops due to the higher permeability to counterions. Valinomycin stimulation of proton transport has also been observed in reconstituted plasma membrane ATPase preparations which, unlike the Golgi proton pump, is promoted by nitrate and inhibited by vanadate (14, 23).

The tonoplast-type proton pump was more sensitive to inhibition by fluoride, iodide, and nitrate than the Golgi pump. The inhibition by nitrate is specific for ATP-driven proton transport, since pyrophosphate-driven proton transport in the same vesicle fraction is promoted by nitrate (6). This suggests a direct effect of nitrate on the enzyme rather than an indirect effect on proton permeability. There are several possible explanations for the partial inhibition of the Golgi proton pumping activity by nitrate: (a) the nitrate-sensitive portion of the activity might represent tonoplast contamination; (b) two types of H^+-transport ATPases might be present on the Golgi, one nitrate-sensitive and the other nitrate-insensitive (or even nitrate-stimulated); and (c) a single Golgi H^+-ATPase might be partially (~50%) inhibited by nitrate. While the data do not yet allow us to choose among these alternatives, the fact that nitrate has little effect on the initial rate of fluorescence quenching tends to support the third alternative. However, other explanations are possible. In any case, at least a portion of the Golgi-associated H^+-ATPase activity differs from the tonoplast H^+-ATPase in its response to nitrate and is therefore likely to be a separate enzyme.

In summary, we have demonstrated ATP-dependent proton transport on sucrose gradients of corn microsomal membranes at a density similar to that of the previously described Golgi ATPase (5). While the identification is still tentative, the properties of the Golgi H^+-pump, including vanadate insensitivity and partial insensitivity to nitrate, are sufficiently distinct from those of the tonoplast and plasma membrane proton pumps to effectively rule them out as major contaminants. There is an apparent closer correlation of the Golgi proton pumping activity with GS I, a marker for Golgi cisternae, than with latent UDPase, a marker for secretory vesicles. However, the precise nature and distribution of the Golgi proton pump(s) may be tissue specific and developmentally regulated.

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