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Role of the Plasma Membrane H^+-ATPase in K^+ Transport[1]

Donald P. Briskin* and Margaret C. Gawienowski

Department of Crop Sciences, University of Illinois, Urbana, Illinois 61801

The role of the plant plasma membrane H^+-ATPase in K^+ uptake was examined using red beet (*Beta vulgaris* L.) plasma membrane vesicles and a partially purified preparation of the red beet plasma membrane H^+-ATPase reconstituted in proteoliposomes and planar bilayers. For plasma membrane vesicles, ATP-dependent K^+ efflux was only partially inhibited by 100 μM vanadate or 10 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone. However, full inhibition of ATP-dependent K^+ efflux by these reagents occurred when the red beet plasma membrane H^+-ATPase was partially purified and reconstituted in proteoliposomes. When reconstituted in a planar bilayer membrane, the current/voltage relationship for the plasma membrane H^+-ATPase showed little effect of K^+ gradients imposed across the bilayer membrane. When taken together, the results of this study demonstrate that the plant plasma membrane H^+-ATPase does not mediate direct K^+ transport chemically linked to ATP hydrolysis. Rather, this enzyme provides a driving force for cellular K^+ uptake by secondary mechanisms, such as K^+ channels or H^+/K^+ symporters. Although the presence of a small, protonophore-insensitive component of ATP-dependent K^+ transport in a plasma membrane fraction might be mediated by an ATP-activated K^+ channel, the possibility of direct K^+ transport by other ATPases (i.e. K^+-ATPases) associated with either the plasma membrane or other cellular membranes cannot be ruled out.

The plant plasma membrane H^+-ATPase links ATP hydrolysis to the extrusion of protons from the cytoplasm to the cell exterior (Briskin, 1990; Michelet and Boutry, 1995). This provides a driving force for solute transport at the plasma membrane, consisting of an acid-exterior pH gradient and interior-negative electrical potential difference (Briskin and Hanson, 1992, and refs. therein). Furthermore, the plasma membrane H^+-ATPase has a major role in a number of important physiological processes, including cell elongation (Rayle and Cleland, 1992), stomatal movements (Assmann, 1993), and cellular responses to a number of factors, including plant growth regulators, light, and fungal toxins (Briskin, 1990; Palmgren, 1991, and refs. therein; Briskin and Hanson, 1992).

A characteristic property of the plant plasma membrane H^+-ATPase is the stimulation of its activity by K^+ (Briskin, 1990; Briskin and Hanson, 1992, and refs. therein). In early studies of the plasma membrane H^+-ATPase, it was suggested that this K^+ stimulation of activity might reflect the direct transport of this cation by the enzyme (Leonard, 1982, and refs. therein). For membrane fractions isolated from barley, oats, maize, and wheat, the relative level of K^+ stimulation of ATPase correlated with the relative rates of K^+ ($^{86}Rb^+$) uptake into root tissue of these species (Fisher et al., 1970). For both oats and maize, K^+ stimulation of ATPase activity in plasma membrane fractions displayed a complex kinetic profile similar to that observed for K^+ ($^{86}Rb^+$) uptake into root tissue (Leonard, 1982; Briskin and Hanson, 1992, and refs. therein). Thus, it was proposed that the plasma membrane H^+-ATPase might conduct an H^+/K^+ exchange that would contribute to K^+ uptake into the plant cell (Briskin and Hanson, 1992). This transport mechanism was thought to be important for K^+ uptake when external K^+ concentrations were low (<1 mM), and the observed uptake could not be accounted for by electrophoretic movement involving uniports or channels (Cheeseman and Hanson, 1979, 1980).

However, other data have appeared inconsistent with the plasma membrane H^+-ATPase mediating direct K^+ transport. Clearly, the level of K^+ stimulation of ATP hydrolytic activity is relatively small compared with that observed for other ATPases that transport this cation directly (Serrano, 1990). Furthermore, K^+ is not absolutely required for ATP hydrolysis (Briskin and Hanson, 1992, and refs. therein) or H^+ transport (Serrano, 1990, and refs. therein) by the plasma membrane H^+-ATPase. Moreover, the rapid stimulation of ATP-dependent, H^+ transport with purified plasma membrane H^+-ATPase in reconstituted proteoliposomes would be consistent with K^+ acting on the cytoplasmic face of the enzyme in the stimulation of activity (Gibrat et al., 1990; Serrano, 1990). With the identification of a plasma membrane H^+/K^+ symporter that could account for K^+ accumulation at low K^+ concentrations (Maathuis and Sanders, 1994; Schachtman and Schroeder, 1994), the need to invoke direct ATPase-mediated K^+ transport was no longer necessary.

Although recent advances have been made in the understanding of secondary transport mechanisms for K^+ uptake in plant cells (Bentrup, 1990; Maathuis and Sanders, 1994; Schachtman and Schroeder, 1994), the possible involvement of the plasma membrane H^+-ATPase in K^+ uptake has not been fully resolved. Indeed, direct K^+ transport by the plant plasma membrane H^+-ATPase could remain a possibility if such a coupling of H^+ and K^+ fluxes was not obligatory for activity and accounted for perhaps a portion

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* Corresponding author; e-mail dbriskin@uiuc.edu; fax 1–217–244–1115 or 1–217–333–4777.

Abbreviations: BTP, bis tris propane; $\Delta\mu H^+$, proton-electrochemical gradient; $\Delta\Psi$, membrane electrical potential; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; PBFI, K^+-binding benzofuran isophthalate.

of the total K^+ uptake. In this communication, the involvement of the plant plasma membrane H^+-ATPase in direct K^+ transport was examined using red beet (*Beta vulgaris* L.) plasma membrane vesicles and partially purified red beet plasma membrane H^+-ATPase reconstituted in either proteoliposomes or a planar bilayer.

MATERIALS AND METHODS

Red beets (*Beta vulgaris* L, var Detroit Dark Red) were purchased commercially. The leafy tops of the plants were removed, and the storage tissue was placed in moist vermiculite and maintained at 4°C for at least 2 weeks to ensure uniformity in membrane isolation (Poole et al., 1984).

Preparation of Red Beet Plasma Membrane Fractions and Partially Purified Plasma Membrane H^+-ATPase Reconstituted in Proteoliposomes

Plasma membrane fractions were prepared from red beet storage tissue as described by Gildensoph and Briskin (1990). Plasma membrane vesicles were recovered from discontinuous Suc density gradients and collected by centrifugation at 100,000*g* for 1 h. The red beet plasma membrane H^+-ATPase was partially purified from the plasma membrane fraction by the method of Singh et al. (1987). This method involves an initial extraction of the plasma membrane fraction with 0.1% deoxycholate and then solubilization of the H^+-ATPase using 0.1% Zwittergent 3–14 (Calbiochem). The solubilized plasma membrane H^+-ATPase was reconstituted into preformed liposomes, and the detergent was removed by chromatography on Sephadex G-200 (Pharmacia). The liposomes were produced from soybean phospholipids (Asolectin), which had been further purified by solvent partitioning (Singh et al., 1987). Following elution in the void volume from the Sephadex G-200 column, the proteoliposomes were collected by centrifugation at 250,000*g* for 30 min in a Beckman TL-100 ultracentrifuge and suspended in 250 mM Suc, 2 mM BTP/Mes, pH 7.2, 10% glycerol, and 2 mM DTT to a protein concentration of about 5 mg/mL.

Measurement of K^+ Transport in Plasma Membrane Vesicles and Reconstituted Proteoliposomes

K^+ transport in plasma membrane vesicles and proteoliposomes was measured using the K^+-sensing fluorescent dye PBFI, obtained from Molecular Probes (Eugene, OR). The dye (100 μM PBFI) was loaded into membrane vesicles and proteoliposomes using a freeze/thaw procedure (Giannini and Briskin, 1988) in the presence of 250 mM sorbitol, 2.5 mM BTP/Mes, pH 6.5, and 1 mM KCl. The transport assay was conducted in a 1-mL reaction volume containing 250 mM sorbitol, 30 mM BTP/Mes, pH 6.5, 3 mM $MgSO_4$, 3 mM ATP, and 1 mM KCl with modifications as indicated in the figure legends. PBFI fluorescence was measured with the excitation and emission set at 343 and 500 nm, respectively. The dye fluorescence was calibrated by the addition of KCl to an assay containing vesicles (or proteoliposomes) preloaded with dye in the presence of 5 μM nigericin and 5 μM tributyltin.

Optical Measurement of the Membrane Potential

The production of an interior-positive $\Delta\Psi$ was measured by the fluorescence quenching of oxonol V under conditions identical to those used for the measurement of K^+ transport (Giannini and Briskin, 1987). The dye was present at 15 μM, and the excitation and emission monochronometers were set at 590 and 650 nm, respectively.

Determination of Membrane Vesicle and Proteoliposome Orientation

The orientation (sidedness) of plasma membrane vesicles and proteoliposomes with respect to the H^+-ATPase catalytic site was determined by the detergent latency of ATP hydrolytic activity as described by Palmgren et al. (1990). The ATPase assay medium contained 25 mM Tris/Mes, pH 6.5, 50 mM KCl, 3 mM $MgSO_4$, 3 mM ATP (Tris salt, pH 6.5), and 0.02% (w/v) Brij 58 (when present). The assay was conducted at 25°C, and the released Pi was determined by the method of Ames (1966).

Incorporation of the Plasma Membrane H^+-ATPase into a Planar Bilayer Membrane and Measurement of ATP-Dependent Electrical Current

The production of planar bilayer membranes and reconstitution of the plasma membrane H^+-ATPase were conducted according to the method of Briskin et al. (1995). Soybean phospholipids were purified as described by Singh et al. (1987), dissolved in *n*-decane at a concentration of about 25 mg/mL, and then used to generate a planar bilayer membrane across a 0.25-mm aperture. The thinning of the lipid to form a bilayer membrane was monitored by the measurement of membrane capacitance as described by Alvarez (1986). Fusion of the H^+-ATPase (in proteoliposomes) with the planar bilayer was conducted in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, 10 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT (both chambers). Ten microliters of H^+-ATPase-containing liposomes (about 40 μg of protein) in 250 mM Suc, 1 mM BTP/Mes, pH 7.2, and 1 mM DTT were slowly injected in one chamber adjacent to the bilayer (*cis* chamber), and mixing was applied for about 20 min using a magnetic stir bar. Following injection of the H^+-ATPase preparation, the extent of fusion with the planar bilayer was monitored by the decrease in membrane capacitance as described by Hirata et al. (1986). When membrane capacitance reached a minimum level (about 35–50 pF below the initial value), the solution on the *trans* chamber was transiently lowered below the aperture and then raised to its original level to promote liposome fusion with the bilayer (Hirata et al., 1986). The solution in the *cis* chamber was then exchanged with fresh assay solution to remove residual, nonfused liposomes.

The electrical current generated by the reconstituted plasma membrane H^+-ATPase was measured in the presence of 50 mM Suc, 25 mM BTP (pH 7.5 *cis*; pH 5.0 *trans*), 2 mM $MgCl_2$, 0.5 mM ATP (BTP salt, pH 7.5, *cis* side only), 20

mM ADP, 40 mM Pi, and gradients of KCl across the bilayer membrane (10 mM *cis*/10 mM *trans*; 10 mM *cis*/50 mM *trans*; 50 mM *cis*/10 mM *trans*). When present, Na_3VO_4 was at 100 μM. The electrical potential of the *trans* chamber was held at virtual ground, and command voltages were fed to the *cis* chamber via a similar electrode. The output from the I-V converter was amplified, conditioned using a low-pass Butterworth filter, digitized, and then passed to a Macintosh computer.

Protein Determination

Protein was determined by the method of Bradford (1976) using BSA as a standard. The Bradford color reagent was filtered immediately prior to use.

RESULTS

Use of PBFI as a Fluorescent Probe for K^+ and Orientation of Membrane Preparations

To examine K^+ transport in red beet plasma membrane vesicles and plasma membrane H^+-ATPase reconstituted in proteoliposomes, a K^+-sensing fluorescent probe (PBFI) was used. As shown in previous studies (Jezek et al., 1990; Ros et al., 1995), this hydrophilic dye (when present at about 60–100 μM) provides a sensitive measure of internal K^+ concentration changes in membrane vesicles that are useful in K^+ transport measurements. Once entrapped in membrane vesicles or proteoliposomes, PBFI provides a continuous signal for internal K^+ concentration changes, which is a particular advantage over the use of radiotracers (i.e. $^{42}K^+$ or $^{86}Rb^+$) in studies in which K^+ efflux from the vesicles is examined. In a radiotracer-based assay, radiolabel would be preloaded into vesicles and the ATP-dependent loss of label from the vesicle would be determined. We have previously found this approach unsatisfactory for measurement of K^+ transport with red beet plasma membrane vesicles, since background radiotracer efflux in the absence of ATP can be substantial and additional radiolabel loss from the vesicles can occur during the rapid filtration associated with sample collection (J.L. Giannini and D.P. Briskin, unpublished results; Giannini et al., 1987).

To load PBFI into red beet plasma membrane vesicles and H^+-ATPase proteoliposomes, a freeze/thaw procedure was used (Giannini and Briskin, 1988). The fluorescence response of entrapped PBFI to internal K^+ changes was then examined by the method of Jezek et al. (1990). With this approach, the internal K^+ concentration is adjusted by treating PBFI-loaded plasma membrane vesicles or reconstituted proteoliposomes with KCl solutions containing nigericin and tributyltin. Because nigericin and tributyltin mediate H^+/K^+ and Cl^-/OH^- exchange, respectively, equilibration of KCl across the vesicle (or proteoliposome) membrane is ensured. As shown in Figure 1, PBFI fluorescence increased with increasing K^+ concentration, and the fluorescence response of PBFI to internal K^+ appeared linear for both plasma membrane vesicles and H^+-ATPase proteoliposomes. However, one limitation associated with the use of PBFI in K^+ transport experiments

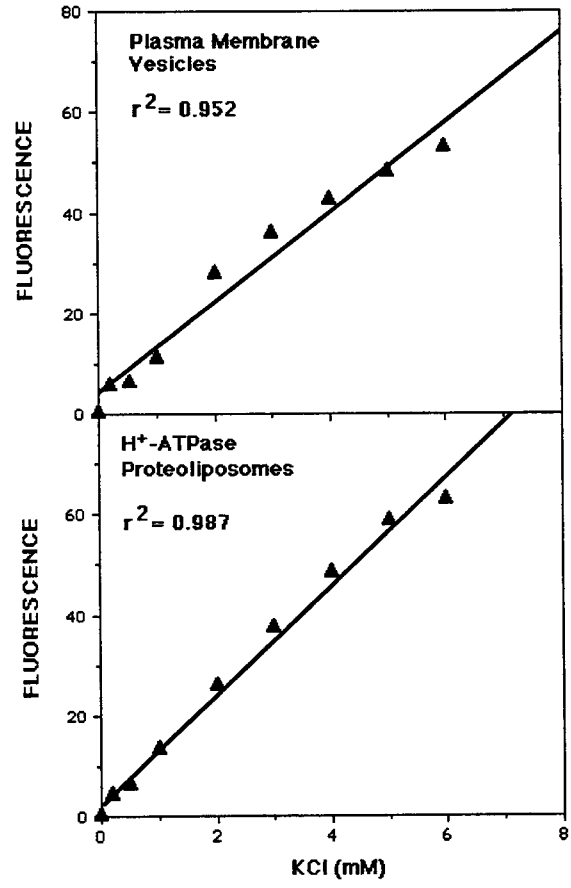

Figure 1. Fluorescence response of entrapped PBFI to changes in K^+ concentration. Plasma membrane vesicles and reconstituted proteoliposomes were loaded with 100 μM PBFI as described in "Materials and Methods," but without KCl in the loading solution. Increasing concentrations of KCl were then added to the external solution, with equilibration to the vesicle (or proteoliposome) interior being maintained using 5 μM nigericin and 5 μM tributyltin. The fluorescence of PBFI was monitored with the excitation at 343 nm and the emission at 500 nm.

with vesicles or proteoliposomes is its low sensitivity at K^+ concentrations much less than 1 mM. For this reason, all subsequent experiments were conducted with 1 mM KCl present on each side of the vesicle or proteoliposome membrane. Although this concentration is at the upper end of the concentration range at which ATPase-mediated K^+ transport had been assumed to occur (Cheeseman and Hanson, 1979), more recent studies have suggested that active K^+ uptake in plants may be mediated by high-affinity transport systems operating in the micromolar range (<100 μM) and passive K^+ uptake may be mediated by low-affinity transport systems operating in the millimolar range (>1 mM) (Maathuis and Sanders, 1994). Therefore, under the conditions of this study, it is likely that both high-affinity and low-affinity-type K^+ transport systems could be operative if present in plasma membrane vesicles or proteoliposomes.

The orientation of red beet plasma membrane vesicles and reconstituted proteoliposomes was examined follow-

ing the freeze/thaw treatment used to entrap PBFI. Based on the latency of ATP hydrolytic activity with 0.02% (w/v) Brij 58 (Palmgren et al., 1990), plasma membrane vesicles appeared to have an approximately random distribution of inside-out and rightside-out vesicles (Table I). On the other hand, the reconstituted proteoliposomes appear to be preferentially oriented with the H^+-ATPase active site on the exterior surface of the liposomes. It should be noted that our method used for reconstitution of the red beet plasma membrane H^+-ATPase involves incorporation of solubilized protein into preformed liposomes. As discussed by Jain and Zakim (1987), reconstitution into preformed liposomes may result in a preferential orientation of the membrane protein, and this preferential orientation for the red beet plasma membrane H^+-ATPase appears to be retained following freezing and thawing of the proteoliposomes. Based on these estimates of sidedness, it is apparent that a substantial proportion of the vesicles or proteoliposomes should be active (i.e. energized by ATP) during K^+ transport assays involving entrapped PBFI.

K^+ Transport in Red Beet Plasma Membrane Vesicles and Plasma Membrane H^+-ATPase Reconstituted in Proteoliposomes

When transport assays were conducted with 1 mM KCl present on each side of the membrane, the addition of 3 mM ATP resulted in a decrease in entrapped PBFI dye fluorescence for both plasma membrane vesicles and H^+-ATPase reconstituted in proteoliposomes (Fig. 2). For red beet plasma membrane vesicles, the decrease in PBFI fluorescence in the presence of ATP was about 2- to 3.5-fold higher than that observed for assays conducted in the absence of ATP. For the plasma membrane H^+-ATPase reconstituted in proteoliposomes, the decrease in PBFI dye fluorescence was about 2- to 2.5-fold higher than that observed in the absence of ATP. This ATP-dependent decrease in PBFI dye fluorescence, which could be reversed by the addition of 5 μM valinomycin, would be consistent with K^+ transport out of the vesicle (or proteoliposome) to the external solution.

For red beet plasma membrane vesicles, ATP-dependent K^+ transport could be inhibited by 100 μM vanadate or 10 μM FCCP (Table II). However, neither of these chemical agents could inhibit K^+ transport back to the level observed in the absence of ATP. Because 100 μM vanadate would be expected to fully inhibit the plasma membrane H^+-ATPase (Briskin and Hanson, 1992; Briskin et al., 1995), this result would imply that some of the observed ATP-dependent K^+ transport may be energized by a vanadate-insensitive ATPase distinct from the plasma membrane H^+-ATPase. Alternatively, an ATP-dependent, vanadate-insensitive K^+ flux also might result from the presence of ATP-stimulated K^+ channels. Indeed, ATP-stimulated, inwardly rectifying K^+ channels have been demonstrated for the plasma membrane using a patch clamp (Spalding and Goldsmith, 1993; Wu and Assmann, 1995) and could mediate K^+ efflux from the vesicles if a driving force were present. Partial inhibition of K^+ efflux by 10 μM FCCP would also suggest that, although most of the ATP-dependent K^+ transport was linked to $\Delta\mu H^+$, a minor component of K^+ transport may be directly linked to ATP hydrolysis via a separate K^+-ATPase or, alternatively, result from the action of ATP-stimulated K^+ channels. These different modes of K^+ transport might result from different transport systems being associated with the plasma membrane, or from vesicles from other cellular membranes being present in the red beet plasma membrane fraction as contaminants.

To ascertain the role of the plasma membrane H^+-ATPase in K^+ transport, the characteristics of ATP-

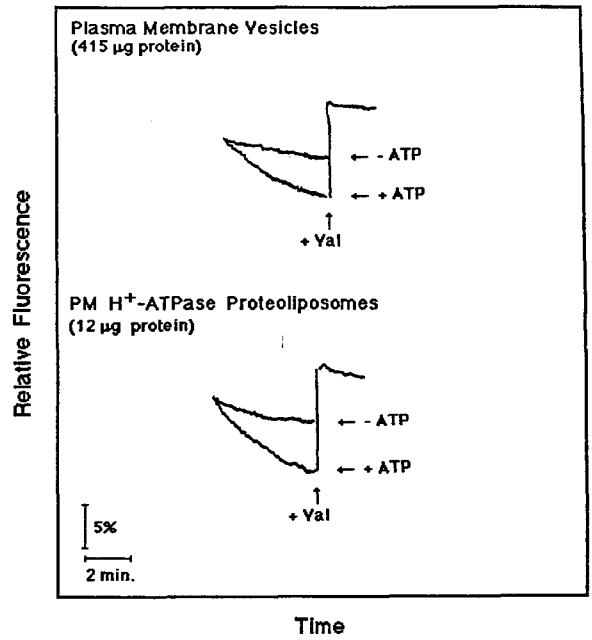

Figure 2. Time course of K^+ transport by red beet plasma membrane vesicles and plasma membrane H^+-ATPase reconstituted in proteoliposomes. PBFI was loaded into plasma membrane vesicles and reconstituted proteoliposomes as described in "Materials and Methods." K^+ transport was measured in the presence of 250 mM sorbitol, 30 mM BTP/Mes, pH 6.5, 3 mM $MgSO_4$, 1 mM KCl (initial concentration on both sides of the membrane), and 3 mM ATP (BTP salt, pH 6.5, when present). At the indicated time, 5 μM valinomycin (+Val) was added to collapse the K^+ gradient. The PBFI fluorescence was monitored with the excitation at 343 nm and the emission at 500 nm.

Table I. *Sidedness of plasma membrane vesicles and reconstituted plasma membrane H^+-ATPase proteoliposomes prepared from red beet storage tissue*

The ATP hydrolytic activity associated with plasma membrane (PM) vesicles and reconstituted plasma membrane H^+-ATPase proteoliposomes was assayed as described in "Materials and Methods" in the presence or absence of 0.02% (w/v) Brij 58. The percentage of inside-out vesicles (or proteoliposomes) was determined as (activity in the absence of detergent)/(activity in the presence of detergent) × 100.

Preparation	ATP Hydrolytic Activity		
	−Brij 58	+Brij 58	Inside-out
	$\mu mol\ Pi\ h^{-1}\ mg^{-1}$ protein		%
PM vesicles	13.45	25.32	53.12
Reconstituted PM H^+-ATPase	140.13	195.46	71.69

dependent K^+ efflux were further examined for proteoliposomes containing the partially purified enzyme. As shown in Table II, the level of ATP-dependent K^+ transport was enriched for this preparation on a protein basis relative to red beet plasma membrane vesicles. However, treatment with either 100 μM vanadate or 10 μM FCCP now resulted in full inhibition of ATP-dependent K^+ transport back to the level observed in the absence of ATP. This would indicate that for this more enriched preparation of the enzyme all of the ATP-dependent K^+ transport appeared energized by the plasma membrane H^+-ATPase, and energy coupling was entirely indirect via the $\Delta\mu H^+$ established by this enzyme.

Such an indirect coupling of the H^+-ATPase to K^+ transport was consistent with the relationship between $\Delta\Psi$ and K^+ efflux for reconstituted H^+-ATPase proteoliposomes (Fig. 3). When ATP-dependent K^+ transport was examined for proteoliposomes preloaded with either 1 mM KCl or 1 mM KSCN (with the same salt on the exterior), a lower rate of K^+ efflux was observed when SCN^- was present. As measured using oxonol V fluorescence, this lower rate of ATP-dependent K^+ efflux coincided with a smaller $\Delta\Psi$ (positive-interior) across the proteoliposome membrane. At this concentration, SCN^- as a permeant anion, can partially collapse $\Delta\Psi$ generated by the plasma membrane H^+-ATPase (Kaestner and Sze, 1985), which appears to serve as a driving force for K^+ efflux. In contrast, if K^+ transport were directly mediated by the plasma membrane H^+-ATPase, ATP-dependent K^+ transport might be expected to be either insensitive or accelerated by a decrease in $\Delta\Psi$.

Because the collapse of the H^+-ATPase-generated $\Delta\Psi$ using a permeant anion leads to a corresponding increase in the pH gradient (data not shown; also see Giannini and Briskin, 1987), a substantial decrease in ATP-dependent K^+ efflux by SCN^- would be further evidence against a H^+/K^+ symporter mediating K^+ efflux in the proteoliposomes under these conditions. Such a protein might be co-purified with the plasma membrane H^+-ATPase and co-reconstituted in proteoliposomes, and our results would be more consistent with ATP-dependent K^+ efflux being mediated by either a K^+ uniport or a channel mechanism utilizing the interior-positive $\Delta\Psi$.

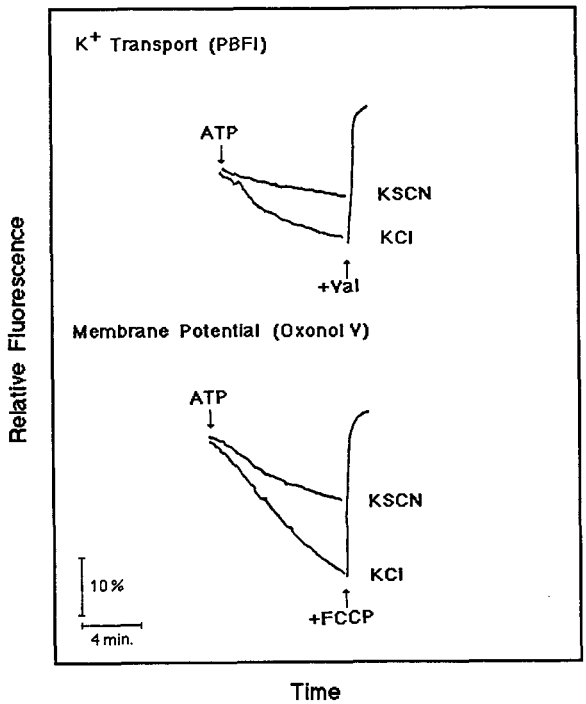

Figure 3. Effect of SCN^- on ATP-dependent K^+ transport and membrane potential production by reconstituted proteoliposomes. PBFI was loaded into proteoliposomes containing the partially purified plasma membrane H^+-ATPase as described in "Materials and Methods." K^+ transport was measured in the presence of 250 mM sorbitol, 30 mM BTP/Mes, pH 6.5, 3 mM $MgSO_4$, 1 mM KCl or KSCN (initial concentration on both sides of the membrane), and 3 mM ATP (BTP salt, pH 6.5). At the indicated time, 5 μM valinomycin (+Val) was added to collapse the K^+ gradient. The PBFI fluorescence was monitored with the excitation at 343 nm and the emission at 500 nm. Under identical reaction conditions, the production of an interior-positive $\Delta\Psi$ was measured using 15 μM oxonol V. Oxonol V fluorescence was determined with the excitation at 590 nm and the emission at 650. At the indicated time, 5 μM FCCP was added to collapse the potential.

Table II. *Properties of K^+ transport associated with plasma membrane vesicles and reconstituted plasma membrane H^+-ATPase*

K^+ transport was assayed in the presence of 250 mM sorbitol, 30 mM BTP/Mes, pH 6.5, 3 mM $MgSO_4$, 1 mM KCl, and 3 mM ATP (BTP salt, pH 6.5, when present). When added, Na_3VO_4 and FCCP were present at concentrations of 100 and 10 μM, respectively. Both plasma membrane vesicles and reconstituted proteoliposomes were preloaded with 100 μM PBFI and 1 mM KCl as described in "Materials and Methods." The initial rate of PBFI fluorescence decrease (K^+ efflux) was monitored with the excitation at 343 nm and the emission at 500 nm.

Preparation and Treatment	K^+ Transport	Fold Increase above −ATP Rate
	% fluorescence min^{-1} mg^{-1} protein	
Plasma membrane vesicles		
−ATP	1.08	1.00
+ATP	3.86	3.57
+ATP + vanadate	2.42	2.24
+ATP + FCCP	1.51	1.40
Reconstituted plasma membrane H^+-ATPase		
−ATP	82.92	1.00
+ATP	208.33	2.51
+ATP + vanadate	87.74	1.06
+ATP + FCCP	88.31	1.07

Thermodynamic Analysis for Direct K^+ Transport with the Plasma Membrane H^+-ATPase Reconstituted in a Planar Bilayer Membrane

As an independent means to assess the role of the red beet plasma membrane H^+-ATPase in energizing K^+ transport, the enzyme was reconstituted in a planar bilayer membrane as described previously (Briskin et al., 1995). Since the enzyme preparation incorporated into the bilayer was the partially purified/reconstituted fraction, bilayer studies would be directly comparable to those in which K^+

transport was measured using PBFI. With this bilayer system, the effect of H^+ and K^+ gradients on the electrical current generated by the red beet plasma membrane H^+-ATPase could be examined. If the H^+-ATPase were to be involved in direct K^+ transport (i.e. H^+/K^+ exchange), the free energy of ATP hydrolysis (ΔG_{ATP}) would be expended in establishing not only $\Delta\mu H^+$ but a K^+ electrochemical gradient ($\Delta\mu K^+$) as well:

$$\Delta G_{ATP} \geq n\Delta\mu H^+ - m\Delta\mu K^+ \quad (1)$$

where n and m represent the stoichiometries for H^+ and K^+ transport, respectively, and the arithmetic difference denotes the opposing orientation of these gradients. As such, the reversal potential (E_{rev}) for the plasma membrane H^+-ATPase would be expected to depend on both H^+ and K^+ electrochemical gradients present across the bilayer membrane:

$$E_{rev} = 1/(n - m) \times (\Delta G_{ATP}/F - nE_{H^+} + mE_{K^+}) \quad (2)$$

where F is the Faraday constant and E_{H^+} and E_{K^+} are equilibrium potentials ($E_j = RT/F \times \ln[C_j^{cis}/C_j^{trans}]$) for H^+ and K^+, respectively (Läuger, 1991).

For the partially purified red beet plasma membrane H^+-ATPase, the effect of voltage on current production (I/V curve) was examined in the presence of an opposing 2.5-unit pH gradient and different K^+ gradients across the bilayer membrane. The presence of an opposing pH gradient is required to observe a substantial effect of voltage on pump current within the range of voltages (<200 mV) that can be maintained across the bilayer membrane (Briskin et al., 1995). For each condition, I/V curves were generated in the presence or absence of 100 μM vanadate. Because this concentration of vanadate has been shown to fully inhibit ATP-dependent current production by the plasma membrane H^+-ATPase (Briskin et al., 1995), a difference curve produced from these data should represent current production by the H^+ pump.

From a comparison of the I/V curves measured in the presence or absence of vanadate, it is apparent that the vanadate-sensitive current ("difference current") was the dominate feature of the overall current measured under each condition (Fig. 4). This current reflects the flow of positive charge from the *cis* to the *trans* side of the membrane associated with H^+ transport by the plasma membrane H^+-ATPase (Briskin et al., 1995). However, when the plasma membrane H^+-ATPase was inhibited by vanadate, a much smaller current was observed, which demonstrated a linear dependence on the voltage applied across the bilayer. This "leak" current that was observed when the plasma membrane H^+-ATPase was inhibited by vanadate should represent the sum of all passive ion fluxes driven by the imposed electrical difference in each case.

When difference curves were determined, it was apparent that the presence of different K^+ gradients had very little effect on the measured reversal potential for the red beet plasma membrane H^+-ATPase (Fig. 4). As shown in Table III, this is in contrast to what might be expected for a direct coupling of K^+ transport to ATP hydrolysis by the H^+-ATPase. Here, hypothetical reversal potentials were estimated for different K^+ transport stoichiometries under conditions consistent with those for Figure 4. Because the H^+/ATP transport stoichiometry for the plasma membrane H^+-ATPase has previously been shown to be about 1 (Brauer et al., 1989; Briskin and Reynolds-Niesman, 1991) and ATP-dependent transport by this enzyme is electro-

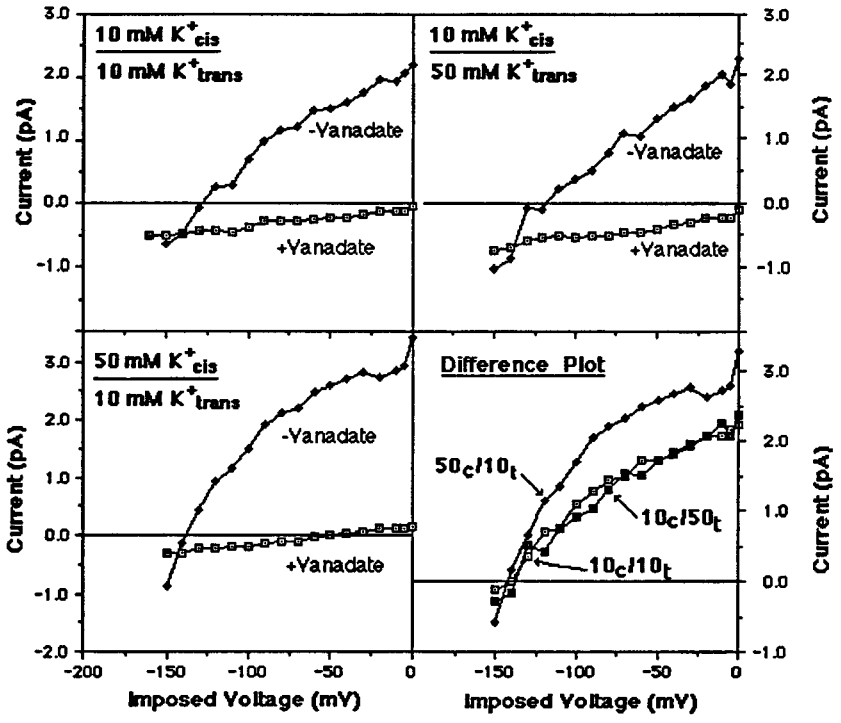

Figure 4. Effect of voltage on electric current production by the plasma membrane H^+-ATPase in the presence of K^+ gradients across a bilayer membrane. The plasma membrane H^+-ATPase was partially purified and reconstituted into a planar bilayer membrane as described in "Materials and Methods." Current production was measured in the presence of 50 mM Suc, 25 mM BTP/Mes (pH 7.5 *cis* side; pH 5.0 *trans* side), 2 mM $MgSO_4$, 0.5 mM ATP (BTP salt, pH 7.5 *cis* side only), 20 mM ADP, 40 mM Pi, and gradients of KCl across the bilayer as indicated. When present, Na_3VO_4 was at 100 μM. In the lower right panel, difference plots are shown for each K^+ gradient across the bilayer, and the reversal potential represents the point at which each plot crosses the *x* axis. c, *cis*; t, *trans*.

Table III. *Hypothetical reversal potentials for K^+ transport in the presence of transmembrane K^+ gradients*

Reversal potentials were estimated for various coupling ratios of K^+ transport with H^+ transport stoichiometry fixed at 1 H^+/ATP. The reversal potential values were estimated using Equation 2 under conditions consistent with the experiments performed in Figure 4. These include ΔG_{ATP} = −7.36 kcal/mol, an opposing 2.5-unit pH gradient, and *trans*-bilayer K^+ concentrations (*cis*/*trans*; c, cis; t, *trans*) of either 50/10 mM, 10/50 mM, or 10/10 mM.

H^+:K^+ Coupling Ratio	Reversal Potential in the Presence of *Trans*-Bilayer K^+		
	50 mM_c/10 mM_t	10 mM_c/50 mM_t	10 mM_c/10 mM_t
		mV	
1:0.75	−561	−807	−684
1:0.50	−301	−383	−342
1:0.25	−214	−241	−227
1:0.00	−170	−170	−170

genic (Briskin and Hanson, 1992; Briskin et al., 1995), only fractional transport stoichiometries for K^+ (i.e. $n > m$ in Eq. 2) were considered. With such fractional stoichiometries for H^+/K^+ counterflux, differences in reversal potential in the presence of 5-fold K^+ gradients of differing orientation would be observed even when the stoichiometry values for coupling were low (e.g. H^+:K^+ = 1:0.25). Furthermore, direct coupling of K^+ transport by the H^+-ATPase would increase the expected reversal potential beyond a range that could be measured using the bilayer system (i.e. ±200 mV). When taken together, these results would be inconsistent with the red beet plasma membrane H^+-ATPase mediating direct K^+ transport and would support an indirect role for the enzyme in providing a driving force for K^+ uptake via ATP-dependent $\Delta\mu H^+$ generation. In this respect it should be noted that, although our measured reversal potentials (about −150 mV) for primary H^+ transport by the H^+-ATPase (i.e. H^+:K^+ = 1:0) are lower than those calculated in Table III (reversal potential, −170 mV), these measured values would still be consistent with a unitary stoichiometry for coupling H^+ transport to ATP hydrolysis (i.e. H^+/ATP = 1.07).

DISCUSSION

Although it has been proposed that the plant plasma membrane H^+-ATPase might directly mediate K^+ influx in addition to H^+ efflux (Briskin and Hanson, 1992, and refs. therein), the results of this study are not consistent with such a mechanism for the uptake of this cation. When the red beet plasma membrane H^+-ATPase was partially purified and reconstituted into proteoliposomes, ATP-dependent K^+ efflux that was fully inhibited by either 100 μM vanadate or 10 μM FCCP was observed (Table II).

Although inhibition by vanadate indicates that the plasma membrane H^+-ATPase was providing the driving force for K^+ transport, full inhibition by the protonophore FCCP would indicate that the linkage to ATP hydrolysis was indirect via the $\Delta\mu H^+$ established by the H^+ pump. An indirect linkage via $\Delta\mu H^+$ was consistent with the observed decrease in ATP-dependent K^+ efflux, which occurred when the $\Delta\Psi$ was decreased in the presence of SCN^- (Fig. 3). The lack of a direct linkage of ATP hydrolysis to K^+ transport was further supported by measurements of the electric current generated by the plasma membrane H^+-ATPase when partially purified and reconstituted into a planar bilayer membrane. Imposition of 5-fold K^+ gradients of either orientation across the bilayer had little effect on the reversal potential for the plasma membrane H^+-ATPase operating against an opposing 2.5-unit pH gradient (Fig. 4).

Several early observations regarding K^+ uptake in plant tissue have been previously suggested to support a direct role for the plasma membrane H^+-ATPase in mediating influx of this cation (Lucas and Kochian, 1988; for a review, see Briskin and Hanson, 1992). At low K^+ concentrations in the apoplast (<1 mM), K^+ uptake into cells is active because it occurs against an electrochemical gradient (Cheeseman and Hanson, 1979, 1980; Maathuis and Sanders, 1994). Since only a K^+ uniport or channel mechanism for uptake was considered in earlier studies (Cheeseman and Hanson, 1979), it was concluded that active K^+ uptake might represent a direct linkage of chemical energy (i.e. ATP) to transport via an ATPase.

Because the properties of H^+ efflux were often observed to correlate with those of K^+ influx when measured using bulk solution techniques (Poole, 1974; Behl and Raschke, 1987), it was argued that the plasma membrane H^+-ATPase might be responsible for mediating active K^+ uptake in coupled H^+/K^+ exchange (for a discussion, see Kochian et al., 1989). However, a more detailed analysis of K^+ and H^+ fluxes measured using K^+- and pH-sensitive microelectrodes by Kochian et al. (1989) clearly demonstrated a lack of correlation between H^+ efflux and K^+ influx. Furthermore, the subsequent identification of a plant plasma membrane H^+/K^+ symporter (Maathuis and Sanders, 1994; Schachtman and Schroeder, 1994, and refs. therein) as a high-affinity K^+ uptake system eliminated the need to postulate a direct linkage to ATP hydrolysis as the mechanism for active K^+ uptake. As recently shown by Maathuis and Sanders (1994), the H^+/K^+ symport mechanism can account for active K^+ accumulation in plant cells at low K^+ concentrations (<100 μM), given the $\Delta\mu H^+$ present at the plasma membrane as a driving force. Furthermore, in whole-cell patch-clamp measurements conducted by these authors, cytoplasmic ATP had no effect on K^+-dependent currents representative of high-affinity K^+ uptake, implying the lack of any direct linkage to ATP hydrolysis, as would be expected for a K^+-transporting ATPase.

The observed stimulation of the ATP hydrolytic activity of the plasma membrane H^+-ATPase by K^+ has also been considered as evidence for a direct role in K^+ uptake (Leonard, 1982; Briskin and Hanson, 1992, and refs. therein). In this respect, an analogy has been made to other eukaryotic transport ATPases, such as the animal cell Na^+,K^+-ATPase and gastric mucosal H^+,K^+-ATPase in which K^+ stimulation of ATP hydrolytic activity is associated with direct K^+ transport (Tonomura, 1986, and refs. therein). It should be noted that some properties of K^+

stimulation for the plasma membrane H^+-ATPase do appear to correlate with those for K^+ uptake (e.g. kinetics and species dependence—see Briskin and Hanson, 1992, and refs. therein), and mechanistic studies have suggested that K^+ stimulation is due to acceleration of reaction steps (e.g. phosphoenzyme turnover) that could possibly be associated with K^+ transport (Briskin, 1990; Briskin and Hanson, 1992, for discussion). However, the overall level of K^+ stimulation for the plant plasma membrane H^+-ATPase is small when compared with other K^+-transporting P-type ATPases (Serrano, 1990, and refs. therein). Furthermore, the observation of K^+ stimulation for the enzyme activity of a primary transport system is not always indicative of a direct role in K^+ transport. For example, although the ATPase activity of the animal sarcoplasmic reticulum Ca^{2+}-ATPase is stimulated by monovalent cations including K^+, this clearly does not reflect direct K^+ transport by this enzyme (for a discussion, see Tonomura, 1986). As with the plant plasma membrane H^+-ATPase (Briskin, 1990, and refs. therein), this K^+ stimulation of the sarcoplasmic reticulum Ca^{2+}-ATPase occurs with an increase in phosphoenzyme turnover in the enzyme reaction mechanism (Tonomura, 1986, and refs. therein).

Recent studies also have shown that, although the H^+-pumping pyrophosphatase of the plant tonoplast is stimulated by K^+ (Davies et al., 1992), a role in direct K^+ transport may be unlikely (Sato et al., 1994; Ros et al., 1995). Our results also demonstrate that, although the plasma membrane H^+-ATPase is stimulated by K^+, this does not necessarily reflect direct K^+ transport by this enzyme. Rather, the stimulatory effect of K^+ may reflect the role of this monovalent cation as an effector or modulator in the enzyme mechanism. Such a proposal would be consistent with the observation that the site on the plasma membrane H^+-ATPase responsible for K^+ stimulation is associated with the cytoplasm-facing side of the protein (Gibrat et al., 1990).

K^+ efflux in the reconstituted H^+-ATPase preparation is strongly dependent on the positive-interior membrane potential generated by the H^+ pump. As discussed earlier (see "Results"), such a strong dependence on the membrane potential in our studies would be consistent with a $\Delta\Psi$-dependent uniport or channel mechanism for K^+ efflux. Such a transport mechanism could represent a protein from the plasma membrane fraction that is co-purified with the H^+-ATPase and provides a conductance pathway for $\Delta\Psi$-driven K^+ efflux in reconstituted proteoliposomes. In this respect, previous patch-clamp studies have shown that inward-rectifying K^+ channels at the plant plasma membrane can remain active over many minutes and could account for substantial K^+ flux under the conditions of these experiments in which K^+ concentrations were about 1 mM (Schroeder et al., 1994, and refs. therein).

Alternatively, this transport path for K^+ may be associated with the plasma membrane H^+-ATPase, which has been suggested for the fungal plasma membrane H^+-ATPase. Using a highly purified preparation of the *Schizosaccharomyces pombe* plasma membrane H^+-ATPase reconstituted in liposomes, Villalobo (1982) demonstrated the presence of a K^+ channel that mediated $\Delta\Psi$-driven K^+ efflux. In addition, Ramirez et al. (1989) showed that for yeast (*Saccharomyces cerevisiae*) a specific mutant in the plasma membrane H^+-ATPase gene (PMA1) altered the properties of a K^+ channel by making channel opening dependent on intracellular ATP. From these patch-clamp studies, these authors concluded that either the K^+ channel was associated with the plasma membrane H^+-ATPase protein or it represented another protein in the plasma membrane that was closely associated with the plasma membrane H^+-ATPase.

In contrast to what was observed for the partially purified plasma membrane H^+-ATPase preparation, a minor component of protonophore-insensitive, ATP-dependent K^+ transport was observed for the red beet plasma membrane fraction. Although an ATP-activated K^+ channel might account for this flux, the presence of a separate K^+-transporting ATPase associated with either the plasma membrane or another cellular membrane present in the plasma membrane fraction cannot be ruled out. Based on the lack of sensitivity of K^+ uptake to changes in external pH, Kochian et al. (1989) suggested the possibility of a plasma membrane-associated K^+-ATPase separate from the plasma membrane H^+-ATPase. Further studies will be conducted to determine whether this protonophore-insensitive K^+ flux represents the activity of a separate K^+-ATPase and to identify its cellular membrane location.

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