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PROTON-PUMPING ADENOSINE TRIPHOSPHATASE IN MEMBRANE VESICLES OF TOBACCO CALLUS

SENSITIVITY TO VANADATE AND K^+

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H^+-pumping adenosinetriphosphatases (ATPases, EC 3.6.1.3) were demonstrated in sealed microsomal vesicles of tobacco callus. Quinacrine fluorescence quenching was induced specifically by MgATP and stimulated by EGTA and Cl^-. Fluorescence quenching reflected a relative measure of pH gradient formation (inside acid), as it could be reversed by gramicidin (an H^+/cation conductor) or 10 mM NH_4Cl (an uncoupler). H^+ pumping was inhibited by tributyltin (an ATPase inhibitor) and sodium vanadate, but it was insensitive to oligomycin or fusicoccin. The vanadate concentration required to inhibit pH gradient formation was similar to that needed to inhibit KCl-stimulated Mg^{2+}-ATPase activity and generation of a membrane potential (measured by ATP-dependent $^{35}SCN^-$ uptake). About 45% of all three activities (ATPase, pH gradient, membrane potential generation) were vanadate-insensitive, supporting the idea that non-mitochondrial membranes of plants have at least two types of electrogenic H^+ pump. A vanadate-insensitive, H^+-pumping ATPase previously shown by methylamine accumulation was characterized to be anion-sensitive and possibly enriched in vacuolar membranes (Churchill, K.A. and Sze, H. (1983) Plant Physiol. 71, 610–617). Yet, pH gradient formation determined by quinacrine fluorescence quenching was decreased by monovalent cations with a sequence K^+,Rb^+,Na^+ > Cs^+,Li^+ > choline, bisTris-propane. Since K^+ stimulated ATPase activity more than Bistris-propane, K^+ appeared to collapse formation of the pH gradient by an H^+/K^+ countertransport. The sensitivity to vanadate and K^+ provides evidence that the plasma-membrane ATPase is an electrogenic H^+ pump.

Introduction

Electrophysiologic experiments with intact plant tissues have suggested that electrogenic H^+ pumps exist at the plasma membrane and the tonoplast (vacuolar membrane). These H^+ pumps are important not only in providing the energy for active transport of inorganic cations, anions, sugars and amino acids [1] but perhaps also in mediating responses of phytohormones [2] and light [3] that regulate plant growth and development.

Although several laboratories have recently demonstrated ATP-driven H^+ pumps in sealed microsomal membranes from higher plant tissues, there are discrepancies regarding the properties and membrane identity of the H^+-pumping ATPase(s). H^+-pumping into vesicles from oat roots [4] or corn coleoptiles [5,6], as determined by

Abbreviations: BTP, bisTris-propane (1,3-bis[tris(hydroxymethyl)methylamino]propane); CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

methylamine accumulation, was stimulated by Cl^-, insensitive to vanadate and localized on low-density vesicles [4–6]. Both groups suggested that H^+ pumping as determined by methylamine uptake originated mainly from vacuolar-type vesicles, since the properties of the H^+ pump were similar to those of the tonoplast ATPase of higher plants. Churchill and Sze [4] have also concluded that methylamine is not a useful ΔpH probe for all membranes, because methylamine can be transported by ammonium porters found on the plasma membrane of fungal and possibly higher plant cells [7]. Thus, useful probes for detecting pH gradients across the plasma membrane appeared to be large organic amines, such as quinacrine.

However, in vitro, H^+ pumping as demonstrated by various laboratories using quinacrine, neutral red or 9-aminoacridine has yielded results similar to those obtained with methylamine. The H^+-pumping ATPase of corn roots [8,9], corn coleoptiles [10] or pea-stem microsomal vesicles [11] was stimulated by Cl^- and insensitive to vanadate. These results are puzzling, as vanadate-inhibited ATPase activity was associated with the microsomal membranes from these tissues [8]. A vanadate-sensitive ATPase is associated with higher plant plasma membrane [12]. In preliminary studies in this laboratory, it was found that H^+ pumping determined by quinacrine fluorescence quenching was inhibited partially by vanadate in microsomal vesicles from oat roots [4].

It was suggested that the plasma-membrane ATPase is an electrogenic pump because ionophore-stimulated ATPase activity [13,14] and ATP-dependent SCN^- uptake (membrane potential generation) [15] in microsomal vesicles of tobacco callus were inhibited partially by vanadate. In this study, I demonstrate H^+ pumping in sealed microsomal vesicles from tobacco callus using quinacrine fluorescence quenching. Sealed microsomal vesicles were used because preparations of one membrane type are difficult to obtain, even after separation of membranes by density gradient centrifugation. So the properties of H^+ pumping will serve as indicators of the type of ATPase and the membrane identity. ATP-dependent H^+ pumping is vanadate-sensitive and shows alkali-cation specificity. These results support the previous conclusion that the plasma-membrane ATPase of tobacco callus is an electrogenic H^+ pump [15].

Materials and Methods

Materials. Autonomous tobacco callus tissue obtained from the stem pith of *Nicotiana tabacum* linnaeous cv. Wisconsin no. 38 was used after 1–1.5 months growth on a basal medium without cytokinins or auxins. Quinacrine, oligomycin, gramicidin and CCCP were obtained from Sigma Chemical Co. Tri-*n*-butyltin chloride was purchased from Aldrich. Sodium vanadate (ortho) was supplied by Fisher. Dextran T 70 was obtained from Pharmacia Fine Chemicals. All other chemicals were reagent grade.

Preparation of sealed microsomal vesicles. Sealed microsomal vesicles were obtained from the dextran interface after centrifugation of the crude microsomal fraction over a 10% dextran cushion as previously described [13,14] with minor modifications. BisTris-propane/Hepes buffer and D-mannitol were substituted for Tris-Hepes buffer and sucrose, respectively. The vesicles were relatively free of mitochondria as judged by the low activity of mitochondrial enzymes (cytochrome *c* oxidase and malate dehydrogenase) [13]. Protein concentration was measured by the method of Lowry et al. [16] using bovine serum albumin as the standard.

Quinacrine fluorescence quenching. Quinacrine fluorescence was measured with a Perkin-Elmer MPF-44B or Turner Model 430 spectrofluorimeter at 500 nm after excitation at 420 nm by a procedure described previously [4,8]. The fluorescence of quinacrine is quenched when the amine moves into an acid compartment [17–19]. Either $MgSO_4$ or BTP-Cl was added to a reaction mixture at 25°C to initiate H^+ pumping. Unless otherwise indicated, the complete reaction mixture contained 10 mM BTP-Hepes (pH 7.5), 0.33 mM EGTA, 5 mM $MgSO_4$, 1.5 mM ATP-BTP, 50 mM BTP-Cl and about 150 μg membrane protein in a final volume of 1.5 ml. In the absence of $MgSO_4$ or Cl^-, quinacrine fluorescence intensity was set to 90%.

Although the rate of quinacrine fluorescence quenching per mg vesicle protein varied from day to day, the variation in fluorescence among similarly treated samples from any one preparation

was less than 8%. Thus, activities are expressed relative to a control measured in duplicate on the same day.

$^{35}SCN^-$ uptake. ATP-dependent membrane potential generation (vesicle interior positive) was determined by $^{35}SCN^-$ uptake using a Millipore filtration procedure [15].

ATPase activity. ATPase activity was measured as inorganic phosphate release [13,14,20].

Results

Quinacrine fluorescence quenching as a measure of H^+ pumping

Quinacrine, a fluorescent amine, provides a very convenient and continuous measurement of ΔpH [17]. The fluorescence intensity of amines, such as quinacrine, is quenched as the probe distributes into the intravesicular space according to the existing pH gradient. The principle of weak base distribution is briefly as follows. At any given pH, the charged and uncharged form of a base is in equilibrium with the H^+ concentration. If the uncharged form is freely permeable and the charged form is not, the uncharged probe passes through the membrane to become charged and trapped in the more acidic internal environment. This process continues until the internal charged and uncharged probes attain equilibrium with the pH_i. One plausible explanation for the mechanism of quenching is that the pH gradient results in such a high internal probe concentration that energy transfer between molecules of the same species may cause a decrease in quantum yield [19].

Fig. 1 shows that MgATP induced fluorescence quenching of quinacrine with sealed microsomal vesicles of tobacco callus. The fluorescence intensity of quinacrine decreased with time until a steady state was reached. The quenching could be reversed by gramicidin (a cation/H^+ conductor) (Fig. 1), 10 mM ammonium chloride or 5 μM nigericin in the presence of K^+ (not shown). These results indicated that initially there was net H^+ pumping into the vesicles. At steady state, a relatively constant pH gradient was maintained by continuous inward H^+ pumping balanced by a H^+ leak.

The initial rate of fluorescence quenching in percent was directly correlated with the concentration of membrane protein (Fig. 1, inset). This relationship is to be expected if quenching reflects mainly quinacrine uptake, which is a function of intravesicular volume. The pH gradient (ΔpH) formed was relatively constant for at least 20 min and can be estimated from the following expression:

$$pH_o - pH_i = \log(A_i/A_o) + \log(V_o/V_i)$$

where subscripts i and o refer to internal and external quantities, A is the total amount of the probe and V is the volume. Since the percentage of fluorescence quenching (Q) is a direct measure of dye uptake, $Q/100 - Q$ is equivalent to A_i/A_o. As I did not attempt to calibrate quenching of quinacrine, I will use the initial rate of quenching as a relative measure of the rate of H^+ pumping.

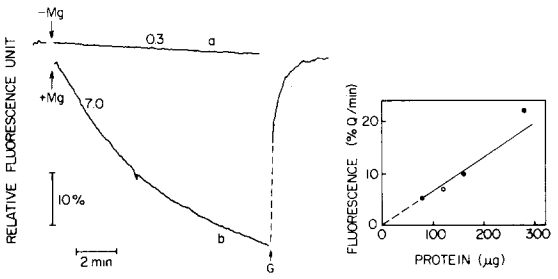


Fig. 1. ATP-induced quinacrine fluorescence quenching in vesicles from tobacco callus. The final reaction mixture (1.5 ml) contained 10 mM Hepes/bisTris-propane (pH 7.5), 0.33 mM EGTA, 50 mM BTP-Cl, 5 μM quinacrine, 1.5 mM ATP-BTP, 180 mM mannitol and 120 μg membrane-vesicle protein. $MgSO_4$ (5 mM) was added to initiate the reaction (trace b). Trace a reflects fluorescence intensity changes in the absence of $MgSO_4$. Numbers represent initial rate of fluorescence quenching expressed as percent per min. Final gramicidin (G) and ethanol concentrations were 2.5 μg/ml and 0.5%, respectively. Inset shows the effect of vesicle protein concentration on ATP induced fluorescence quenching expressed as initial rate. Each point was obtained from one reaction mixture.

General properties of ATP-induced quinacrine quenching

Quinacrine fluorescence quenching was specifically dependent on MgATP (Table I). MgGTP or $MgPP_i$ had no detectable effect. Since ATP concentrations of 1.5 or 3.0 mM induced similar levels of quinacrine fluorescence quenching, 1.5 mM was used in all subsequent experiments.

TABLE I

SUBSTRATE DEPENDENCE OF QUINACRINE FLUORESCENCE QUENCHING IN MICROSOMAL VESICLES OF TOBACCO CALLUS

The reaction mixtures (containing 150 μg vesicle protein) were similar to Fig. 1, except that quinacrine concentration was 2.5 μM and the concentration of ATP, GTP or PP_i was 1.5 mM.

Substrate	Initial rate of quench (%/min)
ATP	0.3
$MgSO_4$	0.2
MgATP	3.3
MgGTP	0.2
$MgPP_i$	0.3

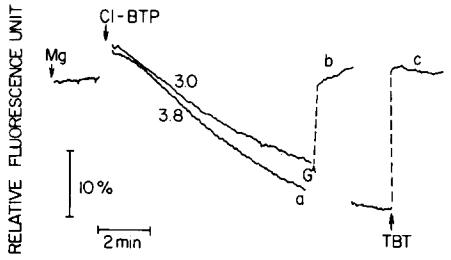


Fig. 3. Effect of oligomycin and tributyltin (TBT) on ATP-dependent quinacrine fluorescence quenching in microsomal vesicles from tobacco callus. Reaction mixtures were similar to those of Fig. 1 except that 50 mM BTP-Cl was added to initiate H^+ pumping. Oligomycin was added to mixture b (to give a final concentration of 5 μg/ml) before H^+ pumping was initiated. Gramicidin was added to give a final concentration of 2.5 μg/ml. Final tributyltin concentration was 5 μM.

EGTA significantly stimulated ATP-induced quinacrine fluorescence quenching in vesicles from tobacco callus (Fig. 2). This result suggested that (i) microsomal membranes contained high levels of calcium, and (ii) calcium inhibited ΔpH formation.

Quinacrine fluorescence quenching induced by MgATP was mostly insensitive to oligomycin (Fig. 3). This finding is consistent with the previous conclusion from the laboratory that sealed microsomal vesicles are relatively free of mitochondrial membranes [13,15]. Quenching was reversed immediately by tributyltin (Fig. 3), indicating that tributyltin dissipated a pH gradient by acting as a

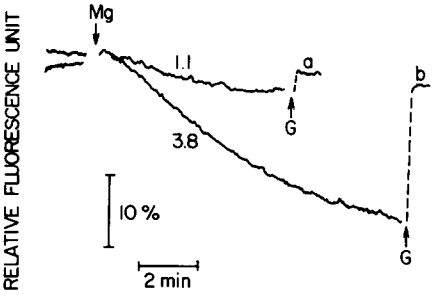


Fig. 2. EGTA-stimulated ATP-dependent quinacrine fluorescence quenching in sealed microsomal vesicles from tobacco callus. Fluorescence quenching was measured in reaction mixtures (containing 125 μg protein) similar to those of Fig. 1 in the absence (a) or presence of 0.33 mM EGTA (b). Gramicidin (G) concentration was 2.5 μg/ml. Data are from one representative experiment out of three. Numbers represent the average value (%Q/min) of the initial rate of fluorescent quenching.

Cl^-/OH^- exchanger [21] in addition to being an ATPase inhibitor [14].

Vanadate sensitivity

Sodium vanadate decreased the rate of quinacrine fluorescence quenching, while sodium phosphate had no effect (Fig. 4A). Inhibition of H^+ pumping could be detected by as little as 25 μM sodium vanadate in the presence of either BTP-Cl (Fig. 4B) or KCl (not shown). The decrease in the rate of quinacrine quenching was dependent on vanadate concentration (Fig. 5a), similar to vanadate inhibition of membrane potential generation (positive inside) and KCl-stimulated Mg-ATPase activity in microsomal vesicles of tobacco callus (Fig. 5b,c) [14,15]. About half of the electrogenic, H^+-pumping ATPase was insensitive to vanadate.

Stimulation by Cl^- and decrease by K^+

Permeant anions, like Cl^-, stimulated ΔpH formation, while impermeant anions, like iminodiacetate, had no effect (Fig. 6). These results are similar to those obtained with oat roots [4] and corn tissues [9]. ΔpH formation was stimulated by increasing concentrations of BTP-Cl (Fig. 7), in part due to Cl^- dissipation of the membrane potential [15]. However, fluorescence quenching was decreased when KCl was substituted for BTP-Cl. At all salt concentrations between about 5 and 50 mM, the initial rate and net fluorescence

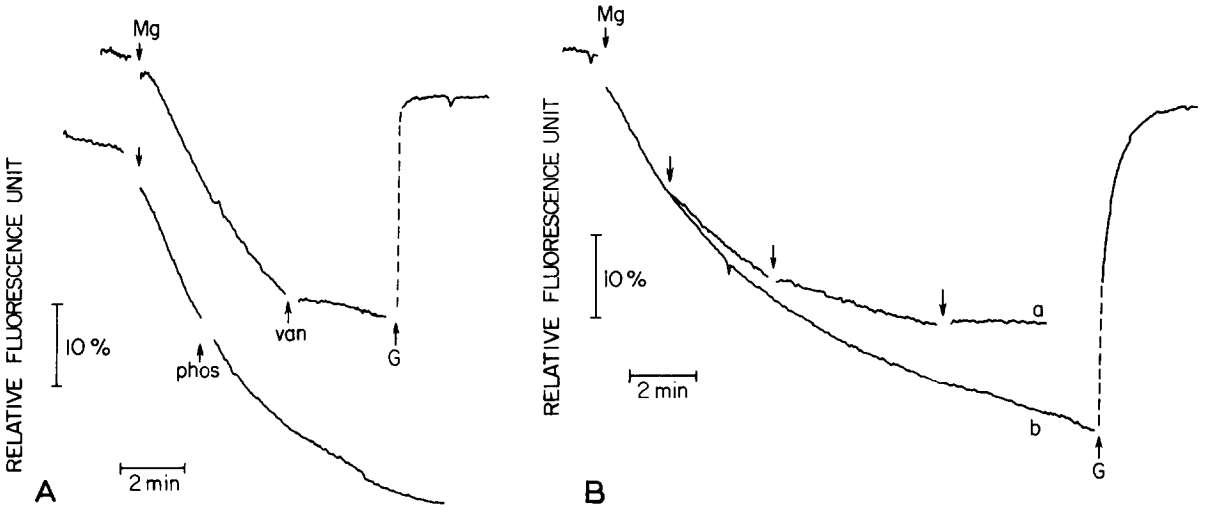
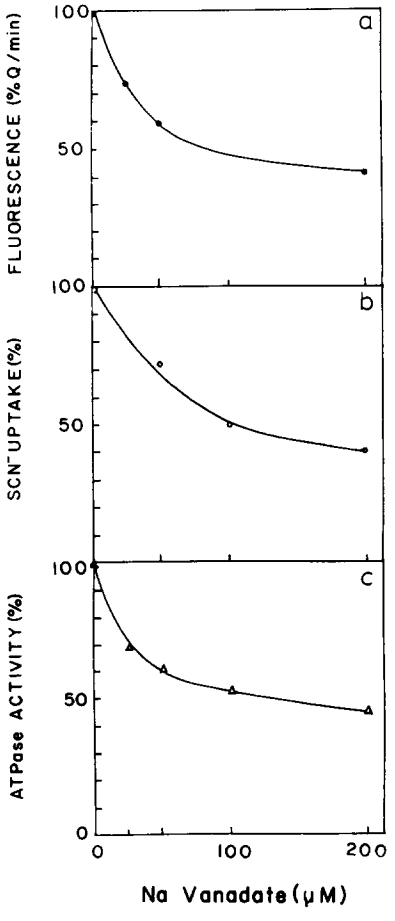


Fig. 4. Vanadate inhibition of ATP-dependent quinacrine fluorescence quenching in microsomal vesicles of tobacco callus. Reaction mixtures were as described in Fig. 1. (A) Sodium vanadate (van) or phosphate (phos) was added at the arrow to give a final concentration of 200 μM. (B) Each arrow indicates one addition of 7.5 μl of 20 mM sodium vanadate to reaction mixture a. No addition was made to reaction mixture b. One representative experiment of three.



quenching were lower with K^+ than with BTP^+ (Fig. 7).

To understand the role of cations, the effects of various monovalent cation chlorides on stimulation of quinacrine fluorescence quenching and ATPase activity were examined. Fig. 8 shows that Cl^- salts of organic cations such as BTP or choline were most effective in stimulating ΔpH generation, whereas, Cl^- salts of K^+, Rb^+ or Na^+ were

Fig. 5. Effect of vanadate concentration on pH gradient, membrane potential generation and KCl-stimulated Mg^{2+}-ATPase activity. (a) Vanadate inhibition of ATP-induced quinacrine fluorescence quenching. Data analysed from experiments conducted as described in Fig. 4. 2 min after initiation of H^+ pumping, the rate of fluorescence quenching in the absence of vanadate is set to 100%. The rate of fluorescence quenching after addition of various concentrations of sodium vanadate at 2 min is then determined. Results are the average of 2–3 experiments. (b) Vanadate inhibition of ATP-dependent ^{35}SCN uptake. ^{35}SCN uptake was determined using a reaction mixture (0.5 ml) containing 25 mM Tris-Hepes (pH 6.75), 200 mM sucrose, 0.1 mM $K^{35}SCN$, 10 mM $MgSO_4$, 0.1 mg vesicle protein, sodium vanadate at different concentrations with or without 3 mM ATP. Results are the average of 2–6 experiments. (c) Vanadate inhibition of KCl-stimulated Mg^{2+}-ATPase activity. ATPase activity was measured in 30 mM Tris-Hepes (pH 6.75) with 3 mM $MgSO_4$, 3 mM ATP and various concentrations of vanadate in the presence or absence of 50 mM KCl. Results are the average of 2–4 experiments [14].

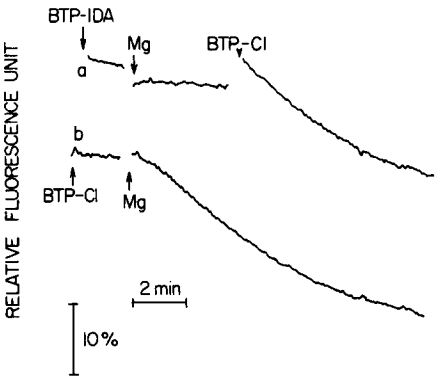


Fig. 6. Effect of impermeant anion (iminodiacetate) on ATP-dependent quinacrine fluorescence quenching in sealed microsomal vesicles of tobacco callus. Reaction mixtures were similar to those of Fig. 1, except that quinacrine concentration was 2.5 μM and 50 mM BTP-iminodiacetate (BTP-IDA) or BTP-Cl was added to reaction mixture a or b, respectively. $MgSO_4$ was used to initiate H^+ pumping. BTP-Cl (150 μl of 0.5 M solution) was added after 4 min to reaction mixture a.

least effective. The increasing order of ΔpH stimulation by cations was K^+, Rb^+, $Na^+ < Cs^+$, $Li^+ <$ choline, BTP. These results did not allow distinction between the specificities of K^+, Rb^+ and Na^+. Although there was no clear selectivity among the alkali cations [14], KCl stimulated Mg^{2+}-ATPase activity more than did BTP-Cl (Table II), choline-Cl or Tris-Cl [14] in the absence of ionophores. These results suggest that K^+ reduced

TABLE II

EFFECT OF KCl OR BTP-Cl ON STIMULATION OF ATPase ACTIVITY IN SEALED MICROSOMAL VESICLES FROM TOBACCO CALLUS

Gramicidin concentration was 2.5 μg/ml. Results are the average of two experiments.

Salt (mM)	Salt-stimulated Mg^{2+}-ATPase activity (μmol P_i/mg protein per h)		
	without gramicidin	with gramicidin	Difference
KCl (50)	3.7	5.4	1.7
BTP-Cl (50)	1.7	3.0	1.3

ΔpH formation not by inhibiting the H^+ pump, but by dissipating the ΔpH.

Effect of NO_3^- and SCN^-

The effect of NO_3^- on quinacrine fluorescence quenching was examined in an attempt to inhibit selectively the vanadate-resistant H^+ pump present in the microsomal vesicles. Nitrate can behave as a permeant anion and is effective in stimulating ΔpH formation in plasma membrane vesicles of *Neurospora* [22]. Nitrate will also inhibit the Cl^--stimulated ATPase and the vanadate-resistant H^+ pump [4]. Fig. 9 shows that nitrate did behave as a permeant anion transiently, but, unlike Cl^-, nitrate

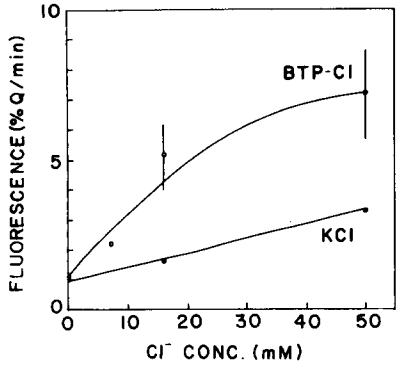


Fig. 7. Effect of BTP-Cl or KCl concentration on ATP-dependent quinacrine fluorescence quenching in microsomal vesicles of tobacco callus. The reaction mixtures are similar to those of Fig. 1, except that various concentrations of BTP-Cl or KCl were added to initiate H^+ pumping. Results are the average of 1–4 experiments. Bars are S.D.

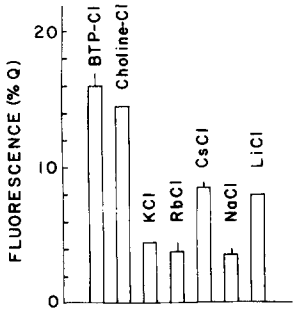


Fig. 8. Effect of cation chloride on ATP-dependent quinacrine fluorescence quenching in microsomal vesicles of tobacco callus. The reaction mixture was similar to those of Fig. 1, except 50 mM of chloride of various cations was added to initiate H^+ pumping. Relative activity of H^+ pumping is expressed as net quenching (in percent) at 5 min. These results are similar to those expressed as initial rates of fluorescence quenching. Results represent the average of two experiments.

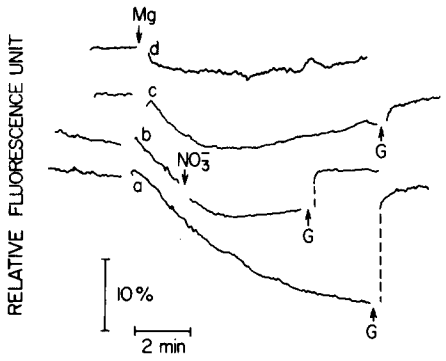


Fig. 9. Effect of nitrate and thiocyanate on ATP-dependent quinacrine fluorescence quenching in sealed microsomal vesicles of tobacco callus. Reaction mixtures a and b were similar to those of Fig. 1. Nitrate (25 μl 0.5 M BTP-NO_3) was added to reaction mixture b 2 min after initiation of H^+ pumping by Mg. Instead of BTP-Cl, 50 mM BTP-NO_3 or BTP-SCN was added to reaction mixtures c or d, respectively. Results are from one experiment. G (gramicidin) was added in ethanol to give a final concentration of 2.5 μg/ml.

also inhibited formation of a ΔpH. If nitrate inhibited only one pump, one would expect to see a decrease of quinacrine fluorescence intensity with time kinetics similar to the control. The amount of quenching would reach a steady state when inward H^+ pumping is equal to outward H^+ leak. Instead, an increase in fluorescence is observed in 3 min in the presence of nitrate. These results indicate that H^+ leak had exceeded inward H^+ pumping, suggesting that nitrate inhibited continued H^+ pumping, rather than specifically inhibiting one pump.

SCN^-, a permeant anion, prevented quinacrine fluorescence quenching at 17 or 50 mM (Fig. 9), consistent with the idea that SCN^- is a chaotropic agent that decreases ATPase activity [23].

Effect of fusicoccin

Fusicoccin, a fungal toxin, stimulates acid secretion from intact plant tissues and may act by enhancing the activity of an H^+-pumping ATPase of the plasma membrane [24]. However, I found no effect of fusicoccin on the initial rate of H^+ pumping into vesicles or the maintenance of a pH gradient (data not shown). Abscisic acid, a plant-growth regulator, also had no effect on ATP-induced quinacrine fluorescence quenching (not shown).

Discussion

Evidence for an electrogenic, H^+-pumping ATPase of the plasma membrane

Direct evidence for H^+-pumping ATPases in sealed microsomal vesicles of tobacco callus is demonstrated using quinacrine fluorescence quenching as a ΔpH probe. Two major properties of the H^+ pump support our previous conclusion that a plasma-membrane ATPase is an electrogenic H^+ pump [15].

H^+ pumping is inhibited by vanadate (Fig. 4), an inhibitor of the KCl-stimulated Mg^{2+}-ATPase of plant plasma membrane [12,25]. Inhibition of H^+ pumping is dependent on vanadate concentration, similar to the concentration kinetics of vanadate inhibition of both KCl-stimulated Mg^{2+}-ATPase activity [14] and ATP-dependent membrane potential generation (Fig. 5) [15]. These results provide strong evidence that KCl-stimulated Mg^{2+}-ATPase activity, membrane potential generation and ΔpH formation are manifestations of an electrogenic, H^+-pumping ATPase.

The rate of net H^+ pumping is dependent on the cation present. The increasing order of effectiveness in stimulating ΔpH formation by monovalent cations is K^+, Rb^+, $Na^+ < Cs^+$, $Li^+ <$ BTP, choline (Fig. 7). These effects probably occurred on the plasma membrane, because this sequence is strikingly similar to that of cation stimulation of a plasma membrane ATPase activity from oat roots [23,26]. The order of decreasing potency for stimulation of plasma membrane Mg^{2+}-ATPase is $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$, Tris, choline [23,26]. Furthermore, pH gradient formation in vesicles enriched in a vanadate-insensitive, H^+-pumping ATPase showed little or no cation specificity [4,8].

I conclude from these results and previous studies [14,15] that the sealed microsomal vesicles consist in part of plasma membrane. If one assumes that the plasma membrane ATPase is completely inhibited by 200 μM vanadate [14], about 50–60% of the H^+ pumping activity in the sealed microsomal fraction could originate from inside-out vesicles of the plasma membrane.

Role of K^+

K^+ causes dissipation of a pH gradient by

H^+/K^+ countertransport with H^+ efflux and K^+ uptake into the vesicles. The following data support this conclusion: (i) BTP-Cl generates a larger ΔpH than does KCl (Fig. 7). Similarly, potassium iminodiacetate partially reverses quinacrine fluorescence quenching stimulated by BTP-Cl (not shown); (ii) K^+ stimulates ATPase activity more than does BTP^+ (Table II), consistent with the idea that dissipation of an electrochemical gradient of H^+ will stimulate ATP hydrolysis and H^+ pumping [13,14]; (iii) K^+ does not alter membrane potential generation (inside positive) [15]; and (iv) in the presence of K^+, valinomycin (K^+ carrier) plus CCCP (H^+ conductor) stimulate ATPase more than either ionophore alone, suggesting the presence of an electrochemical gradient of K^+ [14]. The interpretation of an H^+/K^+ countertransport mechanism is predicted by the chemiosmotic hypothesis whereby membrane potential, inside positive, would drive uptake of anions (e.g., Cl^-) [4] and ΔpH, inside acid, would drive uptake of cations.

I suggest that the H^+/K^+ countertransport in a vesicle could be a function of a plasma membrane protein, perhaps the ATPase. In intact plant or yeast cells [27], H^+ efflux is often closely connected to K^+ influx [1]. The driving force for potassium accumulation can be attributed to the membrane potential (negative inside the cell) created by an H^+-extrusion pump. When the external potassium concentration is increased, the membrane potential, $\Delta\psi$, becomes smaller, this decrease being accompanied by an increase in ΔpH [27]. These results suggest that H^+ and K^+ fluxes are regulated by the electrochemical gradients of K^+ and H^+, respectively. In an inside-out plasma membrane vesicle, H^+ pumping into vesicles generates a membrane potential, positive inside. The driving force for K^+ uptake becomes dependent on the pH gradient (acid inside) rather than the membrane potential. K^+ could accumulate in the vesicles in exchange for H^+ efflux. This mechanism would be analogous to H^+ uptake accompanied by K^+ efflux in an intact cell, a process shown in yeast cells with protonophores [28].

The specificity for K^+ over Li^+, Cs^+ or BTP^+ would suggest that K^+ is transported via a specific protein rather than via a lipid pathway alone. Whether the H^+/K^+ countertransport is mediated by one plasma-membrane ATPase or a separate protein has yet to be determined. Although stimulation of the plasma membrane ATPase by K^+ does not imply any direct transport function for the enzyme, K^+ directly affects the enzyme mechanism by increasing dephosphorylation of a phosphorylated enzyme intermediate [29]. Electrogenic H^+ transport and H^+/K^+ countertransport could be mediated by one ATPase regulated by the electrochemical gradient of H^+ and K^+ [30].

Two types of electrogenic, H^+-pumping ATPase

Tobacco callus has two types of H^+-pumping ATPase in the sealed microsomal vesicles. This conclusion is mainly based on: (i) partial inhibition by vanadate of ATPase activity [14], membrane potential generation [15] and ΔpH formation (Fig. 5); (ii) separation of vanadate-sensitive and vanadate-resistant ATPases and H^+-pumping activities by density gradients [31,32]; and (iii) association of Cl^- and K^+ sensitivity with the vanadate-resistant and vanadate-inhibited ATPase, respectively [33]. The results with tobacco callus are qualitatively similar to those found with oat roots [31,4], where it was suggested that the vanadate-sensitive and vanadate-resistant ATPases are enriched on the plasma and vacuolar membranes, respectively. The main difference is that the microsomal membranes from tobacco callus have a higher fraction of vanadate-sensitive, H^+-pumping ATPase than do oat roots.

Although, studies in this laboratory with tobacco callus or oat roots have consistently revealed two types of electrogenic H^+ pump [4,14,15], most other reports have demonstrated only a vanadate-resistant H^+ pump [5,6,8–11]. I suggest that this discrepancy could be a function of the developmental stage of the plant tissue or the ability of specific membranes to form sealed vesicles. The sealed vesicles from oat roots retained a Donnan potential, suggesting that most of the membranes were intact 'provacuoles' and not vesiculation products of broken membranes [4]. If plasma membranes containing vanadate-sensitive ATPase failed to vesiculate tightly after homogenization, then vanadate-sensitive ATPase activity would still be detected in the absence of vanadate-inhibited H^+ pumping. This situation

could be prevalent in growing monocot roots, such as corn, where H^+ pumping in microsomal vesicles is vanadate-resistant [5,6,8], although the ATPase activity is partially vanadate-inhibited [8]. Dicot tissues may be more enriched in plasma membrane vesicles with H^+-pumping ATPase and ionophore-stimulated ATPase activities [34].

A direct approach to studying the properties of a specific H^+-pumping ATPase would be to solubilize, purify and reconstitute the ATPase and H^+-pumping activities in artificial lipid membranes. Although this approach is now routinely used for studying transport proteins from various organisms [35], transport ATPases from higher plants have not been reconstituted until recently. In a preliminary report, Vara and Serrano [36] solubilized and reconstituted a partially purified ATPase from a plasma membrane-enriched fraction of oat roots. Though the enzyme was vanadate-sensitive, it was not shown whether H^+ pumping (determined by 9-amino-6-chloro-2-methoxyacridine fluorescence quenching) was inhibited by vanadate. The effect of alkali cations on H^+ pumping was not determined. The H^+-pumping properties of a purified and reconstituted plasma membrane ATPase need to be determined to verify the results and conclusions reached in this paper with native membranes, i.e., (i) H^+-pumping ATPase of the plasma membrane from higher plant is vanadate-sensitive, and (ii) K^+ preferentially dissipates the pH gradient formed by the plasma membrane ATPase because of an H^+/K^+ countertransport.

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