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ATP-dependent proton-pumping activities of zucchini fruit microsomes. A study of tonoplast and plasma membrane activities

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The mesocarp tissue of zucchini (*Cucurbita pepo* L. cv. Black Beauty, zucchini) fruit exhibits ATP-dependent H^+-pumping activities associated with tonoplast (nitrate-sensitive) and plasma membrane (vanadate-sensitive) vesicles. The two activities are easily separated on step gradients with isopycnic densities lower than usually reported (< 20% (w/w) sucrose for tonoplast; 25–35% (w/w) sucrose for plasma membrane). The tonoplast is relatively impermeable to H^+ (the half-time for equilibration of a pH gradient is 23–36 min) compared to plasma membrane (half-time of 4–6 min). Anion permeability was measured by adding ATP in the absence of an accompanying K^+ salt, then measuring the increase in the pH gradient caused by the addition of a K^+ salt. The increase in the pH gradient is presumably due to alleviation of the $\Delta\psi$ component (positive inside) and consequent increase in the ΔpH component (acid inside) of the electrochemical gradient by movement of the anion into the vesicle interior. Cl^- and NO_3^- are permeable, SO_4^{2-} is not. The anion permeabilities of the tonoplast and plasma membrane were similar. This is inconsistent with the marked difference in the H^+ permeabilities, but might be explained by the presence of anion channel(s) associated with tonoplast-derived vesicles.

Introduction

Recent research indicates that there are at least two H^+-pumping activities associated with microsomes from a variety of plant tissues, including oat [1] and soybean [2] roots, radish seedlings [3], beet storage tissue [4] and pumpkin hypocotyls [5]. One activity is located at low densities on sucrose gradients, is inhibited by NO_3^-, and presumably originates from the tonoplast (the vacuolar membrane) [6,7]. The other activity is located at high densities on gradients, is inhibited selectively by vanadate, and presumably originates from the plasma membrane [1–4] (for a review, cf. Ref. 8).

We undertook a preliminary survey of H^+-pumping activities of a number of fruit tissues where the primary cell function is storage. Among the species that we examined, zucchini exhibited the greatest amount of H^+-pumping activity sensitive to vanadate, presumably plasma membrane-derived. As presented here, it is relatively easy to separate this activity from NO_3^--sensitive activity. The ease of purification and the remarkably low permeability to H^+ of these membranes suggest that this system may be readily used for study of transport functions using native vesicles. Because

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; BTP, 1,3-bis(tris(hydroxymethyl)-amino)propane; DCCD, *N*,*N*′-dicyclohexylcarbodiimide.

of its relevance to transport function, we have examined the ATP-dependent H^+-pumping activity in detail, but have ignored for the most part the ATPase activities associated with the H^+-pumping.

Materials and Methods

Microsomal suspension preparation. Zucchini (*Cucurbita pepo* L. cv. Black Beauty, zucchini) seeds (Agway, Syracuse, NY) were germinated and grown under greenhouse conditions. Flowers were pollinated by hand and the developing fruits excised about 11 days later (average fruit weight was 600 g), usually kept overnight at room temperature, and then used.

The peel was removed, as was the inner portion containing the seeds (placental tissue). The remaining mesocarp tissue (for microsomal preparations, the average tissue weight was 80 g; for step-gradient preparations, the average weight was 280 g) was initially cut into small pieces using a food processor (Cuisinart model DLC-10E, Greenwich, CT) and then placed in pre-chilled grinding medium at a 1 g tissue/1 ml grinding medium ratio (a 1 : 2 ratio was used in initial experiments; the change in the ratio had no effect on the H^+-pumping activity). The grinding medium was made either fresh or from stock solutions the day of the experiment, and contained 0.25 M mannitol/25 mM Mes/10 mM EGTA/10 mM $MgSO_4$/2.5 mM dithiothreitol/10% (v/v) glycerol/10% (v/v) methanol; pH adjusted to 7.0 with KOH; 1% (w/v) bovine serum albumin added just prior to homogenization. Homogenization was performed with a polytron (Brinkmann Instruments, Westbury, NY) using two 10-s pulses at setting 10 with a 50-s rest interval, unless otherwise noted.

The homogenate was filtered through six layers of cheesecloth and the filtrate centrifuged twice (the pellet being discarded each time) at $13\,000 \times g$ for 8 min using a Beckman JA-17 fixed-angle rotor in a preparative centrifuge. The resulting supernatant was centrifuged at $85\,000 \times g$ for 25 min in a Beckman 50.2 Ti fixed-angle rotor. The microsomal pellet was resuspended in about 0.5 ml of suspension medium to make the microsomal suspension. The suspension medium contained 0.25 M mannitol/2.5 mM Mes/2.5 mM dithiothreitol/10% (v/v) glycerol and methanol; the pH was adjusted to 7.0 with BTP.

For step-gradient separations, the microsomal pellet was resuspended in 10 ml of grinding medium. This was underlain with 5 ml each of 20, 25 and 35% (w/w) sucrose solutions and centrifuged at $85\,000 \times g$ for 120 min in a Beckman SW-27 swinging rotor. The interfaces were removed with a bent-tip pasteur pipette and diluted with 10–20 ml of grinding medium, repelleted, and resuspended in 0.5–0.8 ml of grinding medium.

Assays. H^+-pumping was assayed using the fluorescent probe quinacrine as previously described [2]. Briefly, microsomes (usually 0.3 mg of protein) were added to a fluorescent assay buffer (0.25 M sorbitol, 25 mM BTP/Mes (pH 6.5), 5 mM $MgCl_2$, 0.01 mM quinacrine) with added potassium salts (at 50 mM) to a final volume of 1.5 ml. After temperature equilibration at 28°C, H^+ pumping was initiated by the addition of 5 mM Na_2ATP from a 0.5 M stock solution. Changes in fluorescence were followed in a fluorescence spectrophotometer (Perkin-Elmer Model 650-10S). The initial decrease in quinacrine fluorescence was used as a measure of the initial rate of H^+ pumping [9].

ATPase activity was measured as previously described [10] using the Ames assay [11]. Phosphate standards were routinely spiked with the reaction mix (25 mM BTP/Mes (pH 6.5); 5 mM $MgSO_4$; 5 mM ATP (pH 6.5)) since the ATP caused a 20% increase in the slopes of regression lines for the standards. The reaction was initiated by adding about 10 μg of microsomal protein. The mixes were incubatd at 30°C for 30 min, then a killing reagent (containing 2.1% (w/v) sodium lauryl sulfate) [11] was added and A_{820} measured 30 min later.

Protein was assayed according to Bradford [12].

Results and Discussion

We initially examined the effect of modifications of the grinding medium on H^+-pumping activity (Table I). The absence of either glycerol or methanol decreased the amount of H^+-pumping activity. Both appear to protect the intactness of the isolated vesicles; this was found to be true of soybean root microsomes as well [2]. The use of

TABLE I

EFFECT OF GRINDING MEDIUM COMPOSITION ON H^+-PUMPING ACTIVITY OF ZUCCHINI FRUIT MICROSOMES

The data are the mean of two measurements and are shown as % quenching/ min per 0.3 mg protein and % control. The grinding medium and suspension medium are as described in Materials and Methods with changes as shown. For these experiments, the mesocarp tissue from the same fruit was divided into two or three portions, each portion being homogenized in the homogenizing medium with the changes as shown. The microsomal suspensions were prepared at the same time. Resuspension medium also included the changes as shown. The tissue-to-homogenization medium ratio was 1 : 2 and the polytron setting for these experiments was 8.

Changes	Initial rate of quenching	
	%/ min per 0.3 mg protein	% of control
Control	13.3	100
− Glycerol	9.4	71
− Methanol	10.5	79
Control	9.8	100
Sucrose replacing mannitol	8.6	88
Control	10.1	100
Sorbitol replacing mannitol	7.6	75

mannitol as the osmoticum resulted in greater activities than either sucrose or sorbitol.

The effects of NO_3^- and vanadate are shown in Fig. 1. NO^{-3} inhibited a larger proportion of H^+-pumping activity, the amount varying between experiments, than vanadate. A small component of H^+-pumping activity was insensitive to either inhibitor. *N*-Ethylmaleimide, diethylstilbestrol, and DCCD all inhibit with nearly complete inhibition at 100 μM for *N*-ethylmaleimide and diethylstilbestrol, and 200 μM for DCCD.

The effect of oligomycin on H^+ pumping was a small stimulation in one experiment (5 μg/ml oligomycin) and an insignificant inhibition in another (10 μg/ml oligomycin). The effects of both oligomycin and azide on ATPase activity were also small. This indicates that contamination by submitochondrial particles was negligible.

Aging at room temperature after excision affects both the total amount of H^+-pumping activity and the amount of vanadate-sensitive activity. After 2 days aging, both total activity and vanadate-sensitive activity increase (Table II), with a decrease seen after 3 days of aging (data not shown). These results suggest that there is an increase in the amount of vanadate-sensitive (presumably plasma membrane-derived) activity as a response to excision, i.e., long-term removal from the plant.

In preliminary linear sucrose density gradient experiments, we found that NO_3^- inhibition of H^+-pumping activity was located with a peak at 15% (w/w) sucrose, while at higher densities (with

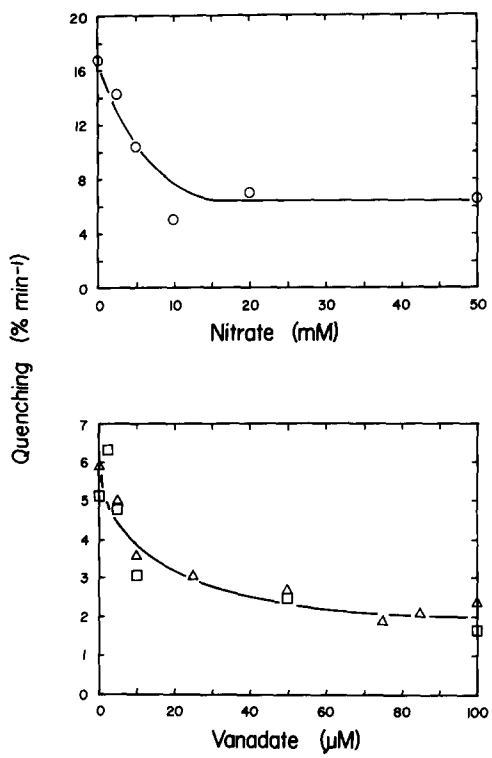

Fig. 1. Effect of NO_3^- and vanadate on H^+-pumping activity of microsomal suspensions. Microsomes were incubated in fluorescence assay buffer as described in Materials and Methods. Quenching was initiated by the addition of 5 mM Na_2ATP. The data are shown as % quenching/ min per 300 μg protein. Nitrate inhibition: 250 μg protein was used. The fluorescence assay buffer included 50 mM KCl and 100 μM vanadate. $MgSO_4$ (5 mM) was used instead of $MgCl_2$. Vanadate inhibition: 300 μg protein was used. The fluorescence assay buffer incuded 50 mM KNO_3 and KCl. The nitrate and vanadate effects were examined using different microsomal preparations; two experiments (one using 5 mM $MgCl_2$ (□), the other $MgSO_4$ (△)) are shown for vanadate inhibition.

TABLE II

EFFECT OF AGING OF ZUCCHINI FRUITS ON H^+-PUMPING ACTIVITY OF THE MICROSOMES

The fruits were excised and held at room temperature for the number of days shown before microsomes were prepared. Data are shown as % quenching/ min per 0.3 mg protein (all assays were done with 0.3 mg protein, except for experiment 1, 2 days aging, which was done with 0.2 mg protein) with % of control in parentheses. ΔVAN is the activity in the presence of KNO_3 minus that in the presence of KNO_3 and vanadate. Potassium salt concentrations were 50 mM and the vanadate concentration was 0.1 mM. In experiment 1, 5 mM $MgSO_4$ was used in the fluorescence assay buffer; in experiment 2, $MgCl_2$ was used.

Days after excision	Initial rate of quenching (%/ min per 0.3 mg protein)				ΔVAN
	+KCl	+VAN	+KNO_3	+VAN+KNO_3	
Expt. 1					
1 day	14.2 (100%)	10.8 (76%)	13.1 (92%)	2.3 (16%)	10.8
2 days	20.9 (100%)	13.4 (66%)	24.5 (117%)	6.8 (32%)	17.7
Expt. 2					
1 day	13.6 (100%)	12.8 (94%)	7.3 (54%)	3.2 (24%)	4.1
2 days	18.9 (100%)	15.3 (81%)	12.8 (68%)	2.5 (13%)	10.3

TABLE III

STEP-GRADIENT LOCALIZATION OF H^+-PUMPING ACTIVITY AND INHIBITOR SENSITIVITY

The step-gradient separations were performed as described in Materials and Methods. The actual amounts of protein used in the quenching experiments were: GM/20, 0.09 mg; 20/25, 0.25 mg; 25/35, 0.175 mg. The quenching and ATPase experiments are from different step-gradient preparations. The effect of inhibitors is shown as % control. The controls contained 50 mM KCl. KNO_3 was present at 50 mM and vanadate (VAN) at 0.1 mM. Microsomes and interface pellets were resuspended in grinding medium (GM).

Interface	Control (%/ min per 0.1 mg protein)	Initial rate of quenching (% of control)		
		+KNO_3	+VAN	+VAN+KNO_3
GM/20%	15.5	22	–	–
20/25%	1.8	64	100	16
25/35%	10.3	114	38	16
Microsomes	6.3	68	81	13
Interface	Control (μmol P_i/h per mg protein)	ATPase activity (% of control)		
		+KNO_3	+VAN	+VAN+KNO_3
GM/20%	8.3	46	43	0
20/25%	6.0	47	37	0
25/35%	17.8	88	48	22

a peak at about 36% (w/w) sucrose), it caused marked stimulation of about 50–70%. Vanadate inhibition of H^+-pumping activity was located at higher densities; the location was broad and varied somewhat (data not shown). To separate the two activities, we used a 20/25/35% (w/w) sucrose step gradient (Table III). Nitrate-sensitive H^+-pumping activity was found at the grinding medium/20% interface, while vanadate-sensitive activity was located at the 25/35% interface. At either interface, the specific activity of H^+ pumping was greater than in the microsomal suspensions, indicating partial purification of both activities. Vanadate-sensitive activity was greatest in the presence of nitrate; this appears to be due to nitrate inhibition of vanadate-insensitive activity, and nitrate stimulation of vanadate-sensitive activity.

ATPase activity was located somewhat differently (Table III). Although NO_3^--sensitive activity coincided with NO_3^- inhibition of H^+-pumping activity, vanadate-sensitive ATPase activity was found at all interfaces; the greatest amount of inhibited activity coincided with the location of vanadate inhibition of H^+-pumping activity.

We have not examined the location of the two H^+-pumping activities relative to biochemical markers for cellular organelles. The difference in the density of the two activities and the inhibitor specificity suggests that the NO_3^--sensitive H^+-pumping activity is derived from the tonoplast [6,7], while the vanadate-sensitive H^+-pumping activity is presumably plasma membrane-derived [1–4]. The densities of these two activities on step gradients is less than those usually reported [1–4].

We have undertaken some characterization of these two H^+-pumping activities. ATP dependence is shown in Fig. 2. The K_m values for ATP are very similar: approx. 0.7 mM for tonoplast and 0.9 mM for plasma membrane.

The tonoplast activity is sensitive to K_2SO_4, while the plasma membrane activity is insensitive

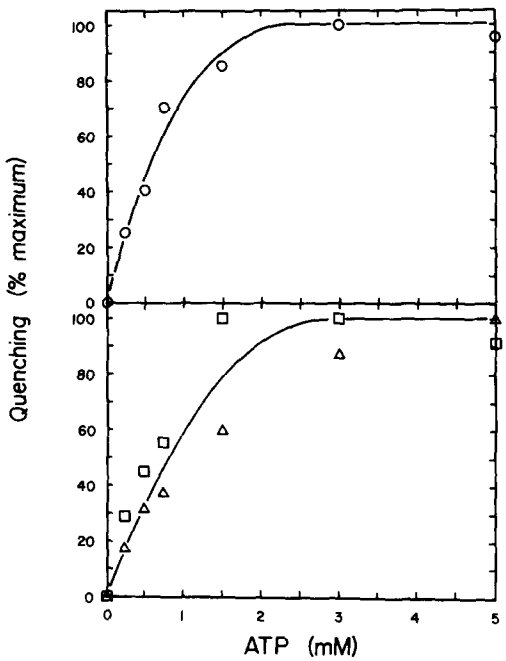

Fig. 2. ATP-dependence of H^+-pumping activity of tonoplast and plasma membrane vesicles. Tonoplast: (upper panel) 50 μg protein from the grinding medium (GM)/20% (w/w) sucrose step interface was incubated in fluorescence assay buffer containing 50 mM KCl and 50 μM vanadate. $MgCl_2$ was kept constant at 5 mM. Maximum activity was 8.5%/ min. Plasma membrane: (lower panel) 200 μg protein (△) or 175 μg protein (□) from 25/35% (w/w) sucrose step gradients was incubated in fluorescence assay buffer containing 50 mM KNO_3. $MgCl_2$ was kept constant at 5 mM. Maximum activities were: △, 9.9%/ min; □, 13.4%/ min.

(Fig. 3). Inhibition of tonoplast H^+-pumping activity by K_2SO_4 was previously reported by DuPont et al. [13]. This inhibition occurs even in the presence of KCl, so it is presumably not a consequence of ability to act as a counterion during initial H^+ pumping. Because of the high concentration required to cause inhibition, it is unlikely that it is of any physiological significance.

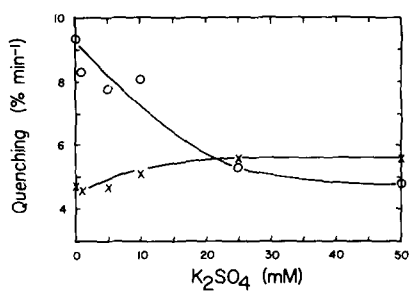

Fig. 3. Effect of K_2SO_4 on H^+-pumping activity of tonoplast and plasma membrane vesicles. Tonoplast (○) (50 μg protein) and plasma membrane (×) (200 μg protein) vesicles were incubated in fluorescence assay buffer containing 50 mM KCl, or 50 mM KNO_3, respectively, with various concentrations of K_2SO_4.

DCCD and diethylstilbestrol inhibit both activities with relatively little difference in the concentration dependence (Fig. 4) and, with respect to DCCD, this result differs from the results of Bowman [14] who found a 10-fold difference in the I_{50} for vacuolar and plasma membrane ATPase in *Neurospora crassa*. Diethylstilbestrol is known to inhibit ATPase activity of both tonoplast and

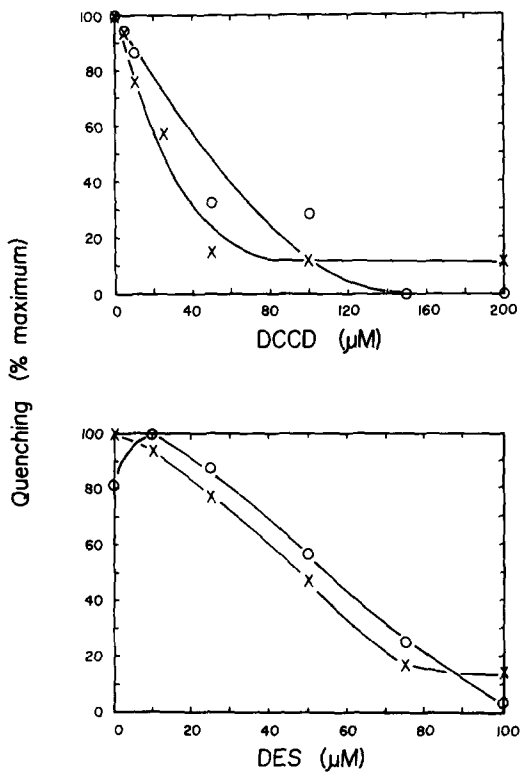

Fig. 4. Effect of DCCD and diethylstilbestrol (DES) on H^+-pumping activity of tonoplast and plasma membrane vesicles. DCCD inhibition: tonoplast (○) (50 μg protein) or plasma membrane (×) (50 μg protein) vesicles were incubated in fluorescence assay buffer containing 50 mM KCl and 50 μM vanadate, or 50 mM KNO_3, respectively. Maximum activities were: tonoplast, 13.5%/ min; plasma membrane, 3.3%/ min. diethylstilbestrol inhibition: tonoplast (○) (50 μg protein) or plasma membrane (×) (200 μg protein) were incubated as above. Maximum activities were: tonoplast, 12.9%/ min; plasma membrane, 6.7%/ min.

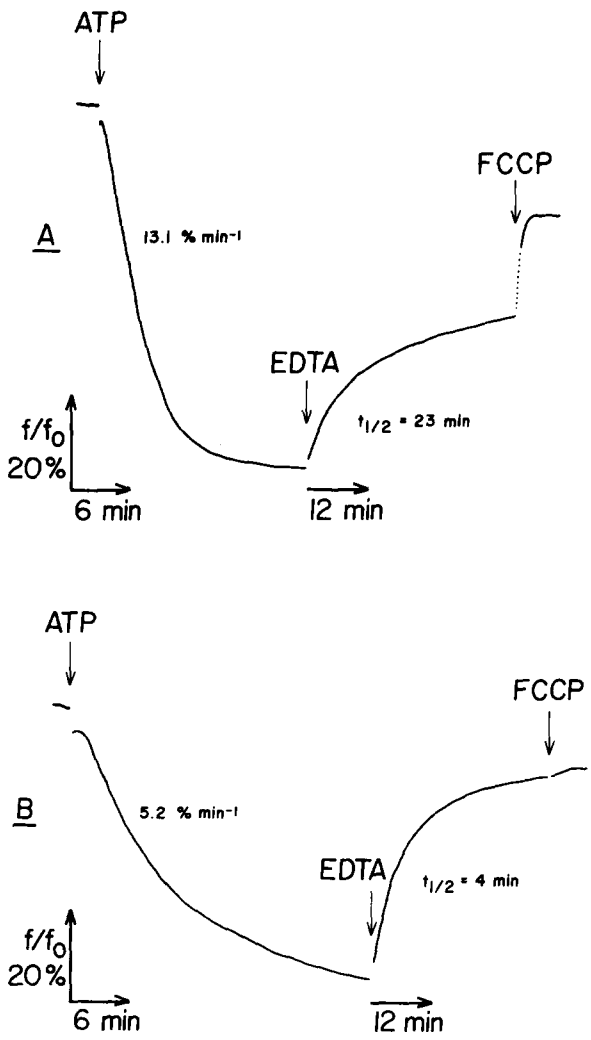

Fig. 5. H^+ permeability of tonoplast and plasma membrane vesicles. Initial rates of quenching (%/ min) and half-times ($t_{1/2}$) are shown. Tonoplast: (A) 50 μg protein was incubated in fluorescence assay buffer containing 50 mM KCl and 50 μM vanadate. $MgCl_2$ was at 5 mM. EDTA (free acid, pH adjusted to 6.5 with BTP) was used at 20 mM to inhibit the H^+-pumping activity (control experiments showed that this level of EDTA caused complete inhibition). *p*-Trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) (6 μM) was used to determine baseline fluorescence so that the half-time could be measured without having an extraordinarily long assay time. Plasma membrane: (B) 200 μg protein was incubated in fluorescence assay buffer with 50 mM KNO_3. $MgCl_2$ was at 2.5 mM. EDTA (as above) was used at 10 mM to inhibit the H^+-pumping activity (control experiments showed it caused complete inhibition at this level).

plasma membrane in higher plants [4].

The H^+ permeability of the tonoplast and plasma membrane vesicles was measured by creating a pH gradient by adding ATP, then inhibiting the enzyme by chelating the Mg^{2+} present with EDTA (Fig. 5). The decrease in the pH gradient is best described as a first-order reaction, as shown previously [15], so that the half-time is inversely proportional to the H^+ permeability: $t_{1/2} = 0.693\ (V/A)\ (1/P_H)$, where V and A are the volume and surface area, respectively, of the vesicles, and P_H is the permeability to H^+. This equation does not take into account the internal buffering capacity of the vesicles. However, if it is assumed that a constant fraction, a, of the intracellular protons are mobile [16], the expression for the half-time becomes $t_{1/2} = 0.693\ (V/A)\ (1/aP_H)$ and the half-time remains inversely proportional to P_H. The tonoplast vesicles exhibit a low permeability

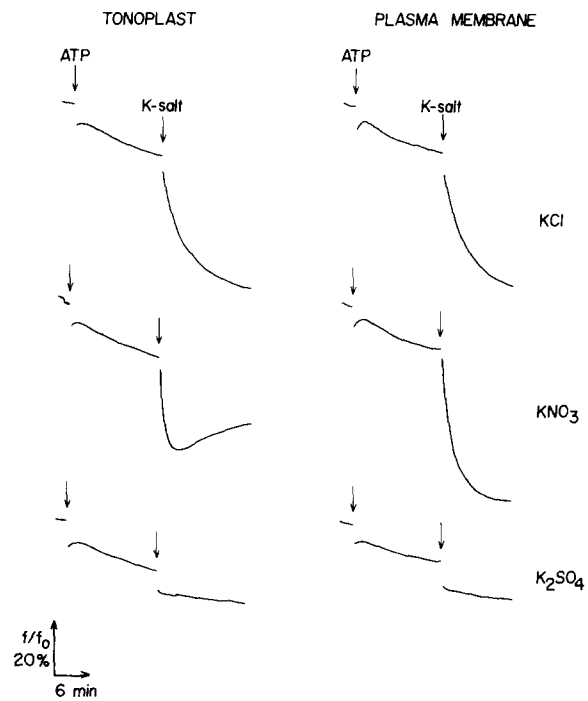

Fig. 6. Anion-induced quenching. Tonoplast (50 μg protein) or plasma membrane (200 μg protein) were incubated in fluorescence asay buffer containing 5 mM $MgSO_4$ instead of $MgCl_2$. No potassium salt was initially present. ATP (5 mM) was added to initiate quenching, and 12 min later, 50 mM potassium salt was added from a 1 M stock solution (KCl and KNO_3) or 0.5 M stock solution (K_2SO_4).

to H^+ ($t_{1/2}$ values ranged from 23 to 36 min), while the plasma membrane vesicles exhibit a high permeability ($t_{1/2}$ of 4–6 min) similar to that of soybean root microsomal vesicles [15] and corn root microsomal vesicles [17]. This difference in the $t_{1/2}$ may also be due to differences in the V/A ratio, but this would require at least a 4-fold difference in the radii of the tonoplast versus plasma membrane vesicles, which we consider unlikely.

We also examined the anion permeability of the membranes by initially adding ATP in the absence of a potassium salt, then adding 50 mM potassium salt 12 min later when fluorescence quenching had approached a steady state (Fig. 6). The added anion alleviates the $\Delta\psi$ component (positive inside) of the proton electrochemical gradient, allowing an increase in the ΔpH component (acid inside) [15]. Cl^- and NO_3^- are effective counterions; the inhibition of the tonoplast H^+-pumping activity by NO_3^- causes an apparent H^+ leak after the initial NO_3^--induced quenching. SO_4^{2-} is ineffective as a counterion. The anion-induced quenching rates are the same for either tonoplast or plasma membrane vesicles even though the difference in H^+ permeability suggests that the anion permeability of the tonoplast vesicle membrane should be less than that for the plasma membrane vesicles. It is possible that there is an anion channel associated with the tonoplast vesicles which would contribute to overall anion permeability [9,15]; such an anion channel would explain this disparity.

In conclusion, the zucchini fruit tissue has considerable amounts of plasma membrane H^+-pumping activity which is easily separable from tonoplast activity. The microsomes exhibit a low permeability to H^+, with the tonoplast vesicle membranes being most impermeable. This makes the zucchini system a good one for studies of transport using either tonoplast or plasma membrane vesicles. Furthermore, excision of the fruit and subsequent aging causes an increase in the plasma membrane activity which may be useful in studies of the path of synthesis and genetic regulation of transport proteins.

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