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Electrogenic plasma membrane H^+-ATPase activity using voltage sensitive dyes

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Abstract Fast responding voltage sensitive dyes, RH421 and di-4-ASPBS, were used to study the electrogenic properties of plant plasma membrane proton pumps on sealed plasma membrane vesicles extracted by two-phase partitioning from *Beta vulgaris* and *Avena sativa* cv Swan root material. Fluorescence spectroscopy in the presence of the dye RH421 (10.8 nM) was sufficiently sensitive to detect electrogenic activity of the extracted plant vesicles. The dye detection system could detect inhibition of electrogenic activity of vesicles by vanadate (75 μM) and stimulation by nigericin (0.5 μM). The newly developed dye di-4-ASPBS was less sensitive to detecting the electrogenic proton pump activity. This study represents an important innovation in plant biophysics as this class of fast responding voltage sensitive dyes have never to our knowledge been used to study electrogenic proton pump activity derived from plant membranes and represents a novel approach for carrying out such studies.

Keywords Plant plasma membrane H^+-ATPase · Voltage sensitive dyes · Two-phase partitioning

Introduction

Fast styrylpyridinium dyes have been used as a tool for the investigation of ion transport processes in animal cell membranes, particularly in the electrogenic properties of the animal Na^+, K^+-ATPase (Amoroso et al. 2006; Apell and Diller 2002; Bamberg et al. 2001; Bühler et al. 1991; Clarke et al. 2003; Fedosova et al. 1995; Frank et al. 1996; Humphrey et al. 2002; Kane et al. 1997; Lüpfert et al. 2001; Pedersen et al. 2002; Peinelt and Apell 2004; Schneeberger and Apell 1999). Although ATPase electrogenics in animal systems have been widely resolved using these fast responding voltage sensitive dyes, similar studies on the electrogenic behaviour of the plasma membrane H^+-ATPase of higher plants have not been reported. Rather, the studies that do exist are based predominantly on electrophysiological approaches (Beilby 1984; Kitasato 1968; Saito and Senda 1973, 1974). While electrophysiology has been a useful tool in elucidating information on the electrogenic behaviour of the plant proton pump, this method presents a number of disadvantages that can affect the reliability of measurements (Hope and Walker 1975). In addition, while few studies used slow oxonol dyes in the past to help resolve the electrogenics of ATPases (Bennett and Spanswick 1983; Briskin and Gawienowski 1996), the use of these dye molecules have limitations in that an intact membrane vesicle is required and changes of local membrane potential are only detected on a timescale of seconds to minutes (Plasek and Sigler 1996). Therefore, the current study employed the fast styrylpyridinium dyes RH421 and di-4-ASPBS to determine whether they can be used as an alternative tool in the investigation of electrogenic plant H^+-ATPase transport.

To address this issue, fast styrylpyridinium dyes were used to detect electrogenic plant plasma membranes proton pump activity. The fast dyes offer the advantages of detecting the same electrogenic processes on exceedingly fast ms to ns timescale, as well as being used on both intact vesicles and membrane fragments. Since the response of the plasma membrane proton pump has been characterized using slow dyes and for a number of chemical agents in the past (Marin

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1986; Palmgren 2001; Scherer 1984), it ought to be possible to characterized the H^+-ATPase activity of this proton pump using fast responding voltage sensitive dyes, by using similar means. To our knowledge, this is the first study that has used fast styrylpyridinium dyes such as RH421 and di-4-ASPBS in exploring electrogenic H^+-ATPase activity derived from plant membrane systems. However, fast responding styrylpyridinium dyes have been used to study the H^+-ATPase of the fungus *Neurospora* plasma membrane (Nagel et al. 1991, 1992; Slayman et al. 1989). The chemical structure of the fast responding voltage sensitive dyes RH421 and di-4-ASPBS are presented in Fig. 1 below.

Fast dyes resemble surfactants (i.e. they are amphiphilic), and contain both a large hydrophobic component and a small hydrophilic component. The hydrophilic region is incorporated into the external surface of the cell membrane, and consists of aromatic rings and a series of conjugated double bonds (Fig. 1). The uncharged amine alkyl chains assist the molecule to insert itself into the membrane. The small hydrophilic component sits in the extracellular solution.

Previous studies have shown that it is possible to obtain plant tissue fractions enriched in ATPase activity (Brightman and Morre 1992; Larsson et al. 1994; Serrano 1984; Yan et al. 1998, 2002). Plant roots have been used for ion transport studies (Larsson et al. 1994; Maathuis 2006; Yan et al. 1998, 2002). Aqueous two-phase partitioning has been shown to be an effective method for the isolation of plasma membranes, which contain the H^+-ATPase (Larsson et al. 1994; Yan et al. 1998, 2002). In this method, the two phases are dextran and polyethylene glycol (PEG). The concentration of each component is optimized to provide the maximum purity of each fraction (Larsson et al. 1994). These two fractions are separated by a clear interface, which usually contain a mixture of both membrane types. To optimise the system for plasma membranes, KI was used as the salt in the homogenising buffer (Giannini and Briskin 1987), and the top layer of the phase system removed and placed in a separate centrifuge tube. Under these conditions, membranes enriched in ATPase proton pumps have been found to partition in the upper phase after being inverted 30× and centrifuged at 720 × *g* for 23 min at 4 °C. In past studies, a number of plants were used in aqueous two-phase isolations.

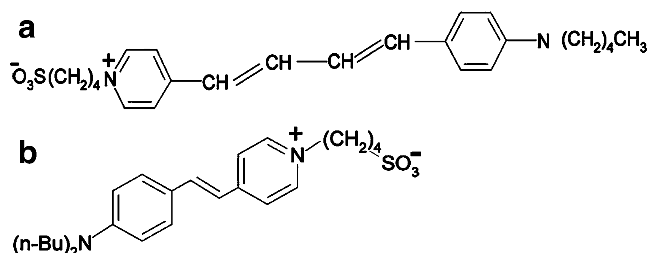

Fig. 1 Structures of the fast responding styrylpyridinium dyes **a** RH421 and **b** di-4-ASPBS

These include *Beta vulgaris* (beetroot) (Lino et al. 1998) and *Avena sativa* (oat) (Astolfi et al. 2003; Berglund et al. 2004; Norberg et al. 1991, 1992; Ratterman and Balke 1987; Roberts et al. 1991; Serrano 1984). This shows that it is possible to use varieties of plant roots in performing such a study. *Beta vulgaris* contains the H^+-ATPase in the plasma membrane and tonoplast, and was used as the initial model system to examine the two-phase partitioning procedure and to characterize the locations of the plasma membrane and tonoplast fractions in the phase system. The tonoplast contains the pigment betalain (Mueller et al. 1997) and as such represents the origin of the red color in beetroot. However, red beets have a low ATPase activity compared with other plant systems, such as oat roots (Serrano 1984), which was the preferred material of this study as in past studies (Astolfi et al. 2003; Berglund et al. 2004; Norberg et al. 1991, 1992; Ratterman and Balke 1987; Roberts et al. 1991; Serrano 1984).

This paper aimed to examine plant ATPase activity of oat roots using the fast responding styrylpyridinium dyes. The advantages of this dye include (a) the ability of detecting and examining the behaviour of electrogenic H^+-ATPase activity and (b) detecting any changes in its behaviour on a very rapid timescale.

Materials and methods

Plant material

Beetroots were purchased from a supermarket and kept moist or planted in soil until required. Oat seeds *(A. sativa* cv Swan), were kindly provided by the Plant Breeding Institute, Cobbity, The University of Sydney, and were germinated overnight in a funnel filled with 5 mM $CaSO_4$ under aerobic conditions. Seeds were then transferred from the funnel and spread over a thin steel mesh wire that were suspended over $CaSO_4$ solution and covered in dark cloth until roots were harvested up to 8 days of growth. Three hundred g fresh weight of beetroot (whole root) and 30–40 g fresh weight of oat root tips were used for each membrane preparation.

Preparing sealed plasma membrane vesicles using two-phase partitioning

The two-phase partitioning procedure was used to prepare plasma membranes fractions enriched for H^+-ATPase. The procedure was carried out as previously published (Larsson 1985; Larsson et al. 1994; vanPutte and Patterson 2003; Widell et al. 1982; Yan et al. 1998, 2002); except for minor variations in the type of buffer used for the homogenization and phase mixtures were made. The homogenization procedure involved blending

the plant roots using a waring blender initially with a series of short bursts (~1–2 s), followed by longer bursts (2×20 s) and then 3×10 s bursts. Discontinuous bending was performed to ensure minimal increases in the vessel's temperature due to the blending process. The plant homogenate was filtered through four layers of Miracloth (Calbiochem, New Jersey, USA) before being further used in the procedure. For the homogenization procedure, 50 mM bis-tris propane (1, 3-bis (tris [hydroxymethyl]-methylamino) propane, BTP, Sigma-Aldrich), pH adjusted to 7.8 using 1.0 M MES2-(N-molpholino) ethanesulfonic acid-4-molpholineethanesulfonic acid buffer (MES, Sigma-Aldrich) was used. The phase system was composed of dextran (6.6%, average molecular weight 400,000–500,000, Sigma-Aldrich), polyethylene glycol (PEG, 6.6%, MW 8,000, Sigma), 330 mM sorbitol or sucrose, 5 mM KCl, 5 mM K_2HPO_4 (pH 7.8), 1 mM DTT and 0.1 mM EDTA (Sigma-Aldrich). These components provide a phase system of 27 g. Nine g resuspension buffer containing the membranes was added to the top layer of the phase system to give a final weight of 36 g. For the phase mixtures, either sorbitol or sucrose was used. Samples were stored in 30% glycerol at −86 °C.

Characterizing isolated membranes

Biuret protein assay

A standard Biuret protein assay based on the Lowry method (Gornall et al. 1949) was used to quantify the protein concentrations of isolated membranes.

Phosphate determinations

A phosphate assay based on ascorbic acid was used to determine the ATPase activity in the isolated membranes (Katewa and Katyare 2003). In this study, it was observed that sorbitol present in the phase system interfered with this phosphate assay. To overcome this problem, an equivalent concentration of solid sucrose was added to the phase mixture in place of sorbitol during membrane extraction. The oat root plasma membrane vesicles isolated using sucrose was subsequently used for phosphate assays. To ensure parity with the fluorometer results, the membranes were extracted using two separate phase systems in parallel, based on either sucrose or sorbitol. The majority of isolations resulted in protein concentrations 6.0–6.5 $mg.mL^{-1}$. However, an isolation performed in sorbitol yielded 25.8 $mg.mL^{-1}$.

ATPase kinetics

ATP saturation curves were constructed using oat root plasma membrane vesicles. For each assay (200 μL final volume), a final amount of 1.3 μg protein was used. Phosphate assays were run in MES buffer (30 mM MES, 100 mM KC1, 1 mM EDTA, 29 mM $MgC1_2$, pH 6.6) and sodium azide (1 mM). The reaction was initiated by adding ATP and stopped after 31 min (no detergent) or 30 min (Triton X, 0.0125%) using 0.6% molybdate ions dissolved in 1.5 M H_2SO_4. The reducing agent was added to each of the samples and left at room temperature (typically 25–27 °C) for an hour to allow the formation of a green complex. Three replicates of each ATP concentration were produced. The ATP concentration was in the range 0–1.5 mM (in the absence of detergent) or 0–2.0 mM (in the presence of 0.0125% (*v*/*v*) Triton X-100 (Sigma, USA)). The absorbance values at 820 nm were read using a UV-Vis spectrophotometer and the results analyzed using a least squares fit to allow K_m and V_{max} values to be determined. In cases where this was impractical, values were read directly from the figure (Fig. 2), and comparisons were made with the value determined using the least squares fit where possible to confirm the reliability of the results estimated from the standard curve.

Fluorescence measurements of plant H+-ATPase using styrylpyridinium dyes

A number of fluorescence timescan measurements of plant plasma membrane H^+-ATPase isolated from *B. vulgaris* and *A. sativa* were taken. The effects of the inhibitor vanadate (BDH), and the ionophore nigericin (supplied from Sigma-Aldrich and originally from Fluka) and 2, 4-dinitrophenol (DNP) (UNILAB) on plasma membrane H^+-ATPase activity were investigated. The assay buffer contained 30 mM MES, 1 mM EDTA, 25 mM $MgCl_2$, 100 mM KCl, pH 6.6–

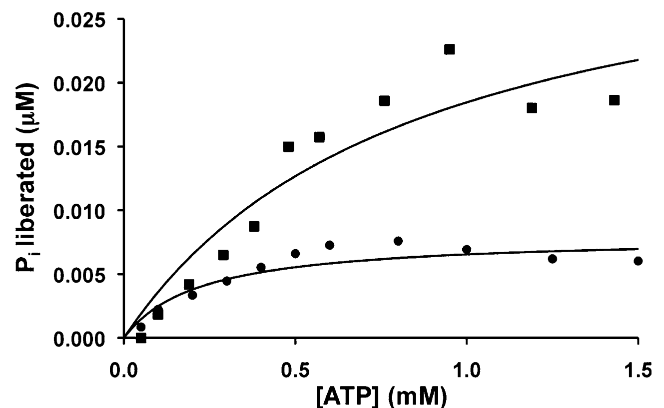

Fig. 2 ATP saturation curves for oat root plasma membrane H^+-ATPase (1.3 μg protein in the cuvette) in the absence (●) and presence (■) of Triton-X (0.0125% (*v*/*v*)). Note that *n* represents the number of data points in each ATP saturation curve, whereas the *r* value represents the Pearson's correlation generated from the least squares fit. These *curves* were produced by using the linear least squares fit

6.8, with or without Brij 58 (0.02%) as indicated in the results. The dyes were used at the final concentrations of 10.8 nM for RH421, and 0.12 μM for di-4-ASPBS. After the fluorescence level stabilized, ATP was added (final concentration 2 mM). Once the signal has restabilized, 50 μM valinomycin, 0.5 μM nigericin or 1 μM DNP (final concentration) was added to observe whether the dyes could detect the ionophore's effect on plant plasma membrane proton pump activity. For measurements produced using *A. sativa* (oat) membranes, the excitation and emission wavelengths were set to 580 nm and 670 nm, respectively for RH421, and 540 nm and 625 nm, respectively for di-4-ASPBS. The excitation and emission filters for both dyes were set to Auto and 550–1,100 nm, respectively, and were performed on the Varian Cary Eclipse fluorometer. Two control measurements for each dye in the absence of membranes were run and averaged for comparative purposes, and used for measurements that utilized the dye di-4-ASPBS. For RH421, a further comparison was made against a theoretically calculated dilution factor based on the subsequent additions of ATP and nigericin to the cuvette relative to the total volume of the cuvette following each particular addition.

For measurements using *B. vulgaris* membranes, the same fluorescence parameters were used, except the filters used were 335–620 nm and open, respectively. Although the dye's fluorescence signal still responds to electrogenic H^+-ATPase activity, under these varied fluorometer conditions, the amplitude of the dye RH421 fluorescence signal was increased.

Results

Detection of plant plasma membrane H+-ATPase using fast styrylpyridinium dyes

An extensive number of fluorescence timescan experiments were performed on beetroot and oat root plasma membrane H^+-ATPase using fast styrylpyridinium dyes. RH795 was unresponsive to electrogenic ATPase activity in trial measurements and was therefore omitted from further study. Typical changes in the fluorescence of RH421 and di-4-ASPBS, as a percentage (dF) in response to dye concentration and changes in the medium, are shown in Fig. 3. There was a fluorescence decrease after adding ATP (dF_{ATP}), of 8.1% (± 4.1%, n=8). The effect was lower with the dye di-4-ASPBS, 5.4% (± 2.3%, n=5), and the difference in the sensitivity of the two dyes was significant as confirmed in a statistical test (Fig. 4) of dF_{ATP} values using a two-tailed t-test (P=0.98, assuming unequal variances). However, while RH421 caused a negative shift, di-4-ASPBS caused a positive shift.

Effect of ionophores on plant plasma membrane H+-ATPase activity

The effect of the ionophore, nigericin, is shown in Fig. 3a with the dye RH421. Nigericin (0.5 μM) caused the dye's signal to decrease by a further 9% (±2.8%) (n=5). The dye t-tests suggested that this was significant at a P value of 0.9 (two-tailed, ANOVA single factor).

Effect of the uncoupler 2, 4-dinitrophenol (DNP) on plasma membrane H+-ATPase activity

The effects of the ionophore DNP, in its dissociated form (dissociation constant 8.41×10^{-8} M), on the electrogenic H^+-ATPase activity of *B. vulgaris* (beetroot) were studied

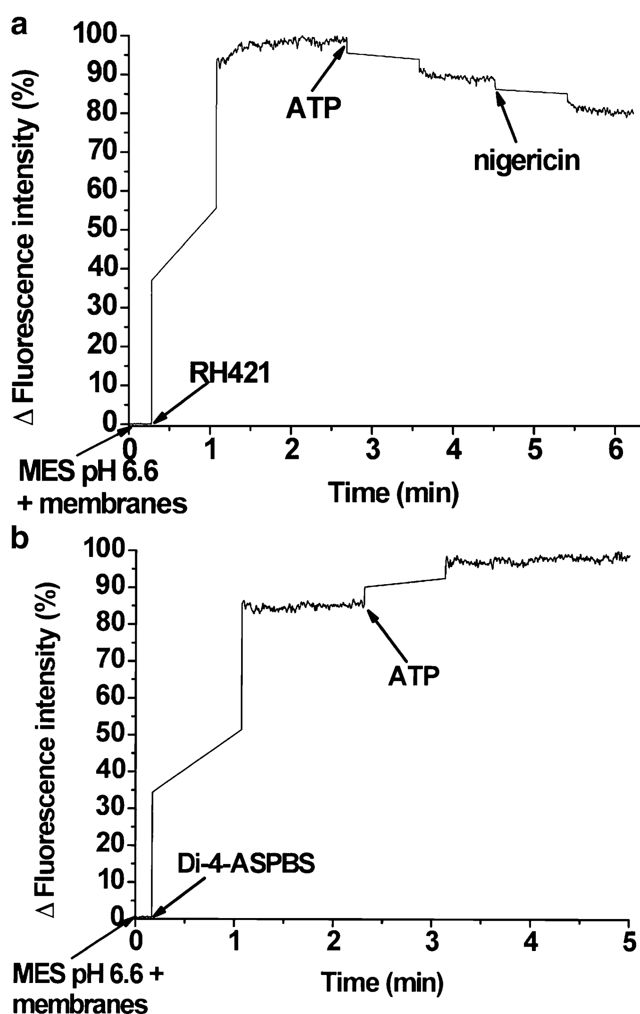

Fig. 3 Fluorescence timescan measurements of **a** the effects of nigericin (0.5 μM final concentration) on oat root plasma membrane H^+-ATPase using the dye RH421, and **b** the potential role of the fast styrylpyridinium dye di-4-ASPBS in the detection of oat root plasma membrane H^+-ATPase activity. The data has been expressed on a percentage scale by setting the maximum fluorescence level of RH42l to 100%

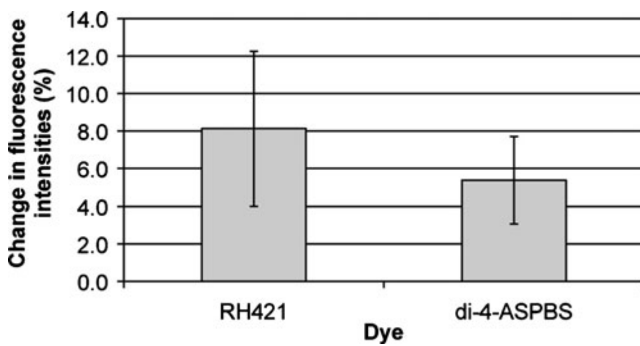

Fig. 4 Percentage change in fluorescence intensity in response to ATPase activity from *A. sativa cv* Swan plasma membranes using the fast styrylpyridinium dyes RH421 (n=8) and di-4-ASPBS (n=5)

using the fast styrylpyridinium dye RH421 (Fig. 5). Addition of DNP (final concentration 1 µM) decreased the dye's fluorescence below the level for dye binding to membrane vesicles (Fig. 5).

Effect of vanadate on plasma membrane H+-ATPase activity

The effects of vanadate on *B. vulgaris* plasma membrane H^+-ATPase activity were investigated as shown in Fig. 6. Vanadate was added to the cuvette either before or after activating ATPase activity. Only when the vanadate was added after activating ATPase activity, at a concentration of 75 µM, was the dye's fluorescence signal decreased (Fig. 6a). The initial value of $dF_{ATP\ (average)}$ was 1.07±0.25, and this decreased to 0.49±0.54 after adding vanadate.

Discussion

Ion pumps have been studied in a range of animal and plant membranes in the past (Bamberg et al. 2001; Briskin and Gawienowski 1996; Bühler et al. 1991; Peinelt and Apell 2004; Tang and Goodenough 2003). In animal tissues fast styrylpyridinium dyes have been developed to study the electrogenic ATP-dependent activities such as the Na^+, K^+-ATPase pumps in animal membranes (Apell and Diller 2002; Bamberg et al. 2001; Bühler et al. 1991; Clarke et al. 2003; Fedosova et al. 1995; Frank et al. 1996; Humphrey et al. 2002; Kane et al. 1997; Lüpfert et al. 2001; Pedersen et al. 2002; Peinelt and Apell 2004; Schneeberger and Apell 1999). These dyes function by binding to the external surface of the membrane and responds to local changes in the elctrogenic activity of ATPase ion pumps that are located in the vicinity of the membrane environment that the dye is attached to (Fedosova et al. 1995; Frank et al. 1996). In past studies of plant tissue oxonol dyes have been used to study similar processes (Blumwald and Poole 1985; Briskin and Gawienowski 1996; Lun'kov et al. 2005; Marin 1986; Pouliquin et al. 1999). However, these dyes are slower

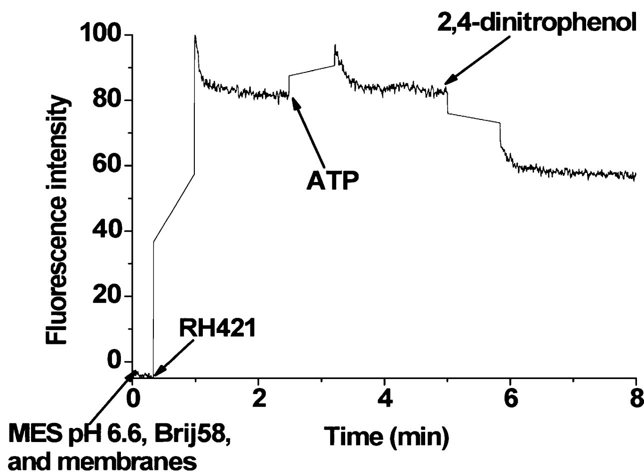

Fig. 5 Effects of 2, 4-dinitrophenol on the electrogenic activity of *B. vulgaris* plasma membrane H^+-ATPase, as detected by the fast styrylpyridinium dye RH421

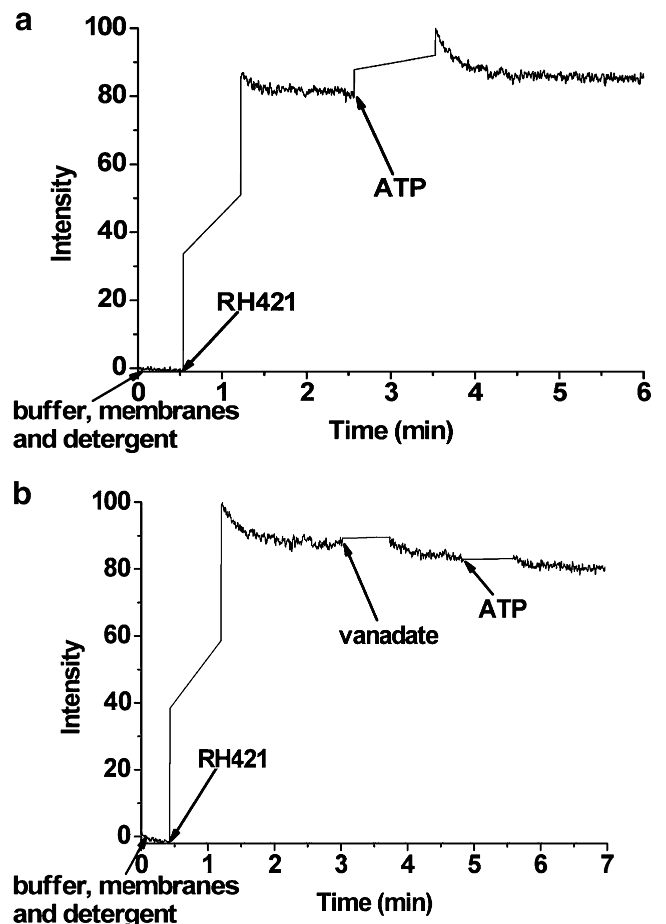

Fig. 6 Detection ability of the fast styrylpyridinium dye RH421 (10.8 nM) in detecting the effects of inhibition of *B. vulgaris* plasma membrane H^+-ATPase using vanadate (75 µM). The dye's fluorescence signal does not change in attempting to activate ATPase activity in the presence of vanadate. **a** shows the change in the dye's signal in response to activating electrogenic H^+-ATPase activity. **b** shows that RH421 is detecting the inhibitory effects of vanadate

responding to electrogenic proton pump activity and can only be used on intact vesicles (Plasek and Sigler 1996). It was therefore of interest in the present study to consider the use of fast styrylpyridinium dyes on plant tissue. To this end beetroot and oat root tissue plasma membranes were investigated. The dyes RH421 and di-4-ASPBS were used.

The dyes respond to electrogenic changes across membranes induced by pump activity, which is energized by ATP. Therefore, in testing the hypothesis that the fluorescence transients, which are observed are indeed the result of H^+-ATPase activity, certain tests can be performed. The activity must be ATP dependent and show the correct characteristics in terms of enzyme kinetics (K_m, V_{max}). As shown in Fig. 2 and Table 1, the kinetic parameters of the isolated plant plasma membrane H^+-ATPase are consistent with the characteristics typical of plant plasma membrane proton pumps as established in past studies (Johansson et al. 1993; Yan et al. 2002). The data presented here shows that the detergent Triton X (0.0125%) has enhanced ATPase activity. Detergent helps to expose the protein to the external buffer conditions (Malyan et al. 1998). The data presented in Fig. 2 are based on a single isolation but were typical of a number of experiments. The proportions of inside-out and right-side-out vesicles for isolation 1 were 40% and 60%, respectively, and for isolation 2, 83% and 17%, respectively, as determined by phosphate assays.

As shown in Fig. 3, the H^+-ATPase responded to the addition of ATP as demonstrated by the changes in the dye's fluorescence pattern on the fluorometer traces. These changes occurred in the presence of ATP for both the dyes RH421 and di-4-ASPBS. Since an electrogenic activity is involved, uncouplers should also abolish the fluorescence transients as observed in Fig. 5. It is possible that DNP was interfering with the integrity of the vesicles, as this uncoupler contains a basic group and can behave as a weak acid in solution. It is possible that this event damaged the vesicles, affecting the amplitude of the dye's signal. It has been reported that dissociated DNP does not affect lipid structure, but is metabolized through plant roots when the external pH is 5 or 7 (Jackson and St. John 1982). Based on this evidence, it is possible that the DNP promoted the influx of ions from the external buffer solution into the vesicle, across the H^+-ATPase, or potential unidentified ion channels. This additional influx of ions would change the nature of the local electric field strength that has already been formed by the proton pump, causing the substantial decrease in the dye's fluorescence signal. Nevertheless, the overall conclusion concerning the utility of the dyes is still valid and further studies would be needed to clarify the actual mechanism.

Vanadate is a powerful specific inhibitor of P-type ATPase pumps and was shown to have a consistent inhibitory effect (Fig. 6a) with a sensitivity similar to that found in animal membranes (Humphrey et al. 2002). To confirm that H^+-ATPase inhibition was taking place, vanadate was added to the cuvette before activating ATPase activity (Fig. 6b). After adding ATP, minimal changes to the fluorescence level were observed. This confirms the actions of vanadate on the proton pump that is being detected by the fast styrylpyridinium dye RH421. The inhibitory effect of vanadate on the plasma membrane proton pump was increased when vanadate was added to the cuvette before H^+-ATPase activity was activated. When the protein was already active in the presence of ATP, vanadate may compete with the ATP for the protein's active site. Vanadate binds to the phosphorylation site of plasma membrane ATPases (Palmgren 2001; Sharma and Davidson 2000), and is the mechanism by which vanadate inhibits the protein's activity. Vanadate becomes a negatively charged molecule when dissolved in solution, and is a competitive inhibitor, binding to one section of the ATP binding site of the protein. The fast styrylpyridinium dye RH421 successfully detected the processes of ATP activation and inhibition from *B. vulgaris* plant plasma membrane H^+-ATPase. We conclude in general that the changes in fluorescence signal shown are consistent with the fast styrylpyridinium dyes detecting H^+-ATPase activity.

This is the first study to our knowledge that has used fast styrylpyridinium dyes such as RH421 and di-4-ASPBS to observe the electrogenics of plant plasma membrane H^+-ATPase activity.

Future studies

The current study successfully used fast voltage sensitive styrylpyridinium dyes to detect the electrogenic proton

Table 1 K_m and V_{max} values for ATPase from two plasma membrane preparations of *A. sativa*. ATPase activity was determined in the presence and absence of Triton-X (0.0125%). Values determined using Least Squares Method (Ritchie and Prvan 1996).

Detergent used	K_m (mM)	V_{max} (μmol P_i. μg^{-1} protein.s^{-1})
No detergent—mixture of right-side-out and inside-out vesicles	0.221±0.078	0.008±0.001
	0.156±0.048	0.017±0.002
Triton X—all vesicles right-side out	0.841±0.1807	0.034±0.002
	0.259±0.056	0.020±0.002

pump activity from higher plant plasma membranes. This approach has the potential to be a breakthrough in plant biophysics studies in that fast styrylpyridinium dyes such as RH421 and di-4-ASPBS have never to our knowledge been used to study electrogenic ion transport processes in ion transporting proteins derived from plant membranes. As there is a lack of knowledge on the kinetic properties of the individual steps of the plant plasma membrane H^+-ATPase reaction cycle, these dyes could be used to study the different steps of the cycle, and to confirm which steps are electrogenic and non-electrogenic, given that the dyes do not respond to the non-electrogenic steps of the corresponding Na^+, K^+-ATPase reaction cycle (Bamberg et al. 2001). This approach could be used to study F-type ATPases in thylakoid membranes, provided there is no interference that is caused by the chlorophyll present in these membranes. The method of fluorescence spectroscopy can be used as an alternative or complementary tool to conventional electrophysiology.

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