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ORIGINAL ARTICLE

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Photosynthetic control of the plasma membrane H^+-ATPase in *Vallisneria* leaves. I. Regulation of activity during light-induced membrane hyperpolarization

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Abstract In mesophyll cells of the aquatic angiosperm *Vallisneria gigantea* Graebner, red, blue, or blue plus far-red light induced a typical membrane hyperpolarization, whereas far-red light alone had little effect. Both N,N′-dicyclohexylcarbodiimide, a potent inhibitor of H^+-ATPase, and carbonylcyanide *m*-chlorophenylhydrazone, an uncoupler, produced a considerable membrane depolarization in the dark-adapted cells and a complete suppression of the light-induced hyperpolarization. Although 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport, did not affect the membrane potential in darkness, it completely inhibited the light-induced membrane hyperpolarization. In vivo illumination of the leaves with red light caused a substantial decrease in the K_m for ATP, not only of the vanadate-sensitive ATP-hydrolyzing activity in leaf homogenate, but also of the ATP-dependent H^+-transporting activity in plasma membrane (PM) vesicles isolated from the leaves by aqueous polymer two-phase partitioning methods. The effects of red light were negated by the presence of DCMU during illumination. In vivo illumination with far-red light had no effect on the K_m for ATP of H^+-transporting activity. These results strongly suggest that an electrogenic component in the membrane potential of the mesophyll cell is generated by the PM H^+-ATPase, and that photosynthesis-dependent modulation of the enzymatic activity of the PM H^+-ATPase is involved in the light-induced membrane hyperpolarization.

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Keywords Membrane hyperpolarization (light-induced) · Mesophyll cell · Photosynthesis · Plasma membrane H^+-ATPase · Plasma membrane vesicles · *Vallisneria* (PM H^+-ATPase)

Abbreviations BTP: bis(tris[hydroxymethyl]methylamino) propane · CCCP: carbonylcyanide *m*-chlorophenylhydrazone · DCCD: N,N′-dicyclohexylcarbodiimide· DCMU:3-(3′,4′-dichlorophenyl)-1,1-dimethylurea · DM-SO: dimethyl sulfoxide · Pi: inorganic phosphate · PM: plasma membrane

Introduction

Since Nagai and Tazawa (1962) first reported that light-induced membrane hyperpolarization occurred in internodal cells of *Nitella flexilis*, this intriguing light-dependent response has been extensively investigated in several plants by many electrophysiologists. It is well established that the membrane potential of plant cells comprises at least two components: a passive diffusion potential and an electrogenic potential (Kishimoto et al. 1981; Spanswick 1981). An increase in the electrogenic component, whose occurrence depends on the operation of photosynthesis, accounts, at least partly, for the light-induced hyperpolarization of membrane potential (Spanswick 1981). The electrogenic component is ascribable to the activity of a H^+ pump in the plasma membrane (PM H^+ pump; Spanswick 1981; Felle and Bertl 1986).

The plasma membrane (PM) H^+ pump in plant cells plays pivotal roles in a wide spectrum of physiological processes through the generation of a H^+ electrochemical potential gradient across the PM by the active transport of H^+ from the cytoplasm to the outside (Serrano 1989; Michelet and Boutry 1995). The activity of the PM H^+ pump is regulated by various external and internal factors such as phytohormones, fungal toxins, elicitors, and light (Spanswick 1981). The phototropic movement of leaves of *Phaseolus vulgaris* was

induced through inhibition of the activity of the PM H^+ pump in pulvinar motor cells by blue light (Nishizaki 1988). The effect of the blue light was mediated by some unidentified blue-light photoreceptors and not by photosynthesis (Nishizaki 1992; Nishizaki et al. 1997). Stomatal opening is induced by red light as well as by blue light. Acceleration of the activity of the PM H^+ pump of guard cells is closely associated with the effects of red light through a 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU)-sensitive pathway (Serrano et al. 1988; Shimazaki et al. 1989) and of blue light through a DCMU-insensitive pathway (Ogawa et al. 1978; Shimazaki et al. 1986). In leaves of *Pisum sativum*, Stahlberg and Van Volkenburgh (1999) indicated that light-induced cell expansion partly depended on a DCMU-sensitive stimulation of the PM H^+ pump. However, in variegated leaves of *Coleus* × *hybridus*, chlorophyll was required neither for growth of the leaves nor for the stimulation of the PM H^+ pump (Stahlberg et al. 2000). To date, there is no direct evidence that suggests the involvement of phytochrome in regulating the activity of ATP hydrolysis in an isolated PM fraction (Terry et al. 1992).

In this study, we demonstrated that the membrane of mature mesophyll cells of the aquatic monocot *Vallisneria gigantea* hyperpolarized in a photosynthesis-dependent manner, as previously reported for *V. spiralis* (Prins et al. 1980) and *Egeria densa* (Tazawa et al. 1986). In the mesophyll cells of *V. gigantea*, Ca^{2+}-sensitive cytoplasmic streaming is induced, concomitantly with an efflux of Ca^{2+} across the PM, depending on photosynthesis (Takagi et al. 1990). Since both responses were sensitive to vanadate, a general inhibitor of P-type ATPases (Takagi and Nagai 1988), the photosynthesis-dependent membrane hyperpolarization might possibly be involved in the photoinduction of cytoplasmic streaming. An acceleration of the activity of the PM H^+ pump might not be ascribable to a light-induced elevation of the cytoplasmic level of ATP, because a significant difference in the cytoplasmic level of ATP before and after light illumination was not detected in plant cells (Keifer and Spanswick 1979; Kikuyama et al. 1979). Furthermore, no successful study demonstrating that photosynthesis influences the activity of the PM H^+-ATPase, i.e. the activities of ATP hydrolysis and H^+ transport, has been reported in an isolated PM fraction. In *V. gigantea*, we obtained biochemical evidence that strongly suggests that an enzymatic modulation of the activity of the PM H^+-ATPase is involved in the light-induced membrane hyperpolarization.

Materials and methods

Plant material and pretreatment of specimens

Vallisneria gigantea Graebner was cultured, and specimens were prepared, as described by Izutani et al. (1990). Briefly, a leaf segment was cut open in the middle of the mesophyll cell layers, and each specimen was set in a chamber made of an acrylic plate with the mesophyll cell side upward. Specimens thus prepared were kept in complete darkness for 12–18 h in artificial pond water that contained 0.05 mM KCl, 0.2 mM NaCl, 0.1 mM $Ca(NO_3)_2$, 0.1 mM $Mg(NO_3)_2$, and 2 mM Pipes-NaOH at pH 7.0.

Measurements of membrane potential

A glass microelectrode filled with 3 M KCl and connected to an Ag-AgCl electrode was vertically inserted into a dark-adapted mesophyll cell. The membrane potential was measured according to the method of Okazaki et al. (1994). Each specimen was irradiated with monochromatic light as described by Izutani et al. (1990). Metabolic inhibitors were dissolved in dimethyl sulfoxide (DMSO) and then diluted 200 times with artificial pond water. Artificial pond water supplemented with 0.5% DMSO was used as a control.

In vivo illumination of leaves before homogenization

The dark-adapted leaves were irradiated with red (> 580 nm, 1.9 $W \cdot m^{-2}$) or far-red (760 nm, 3.5 $W \cdot m^{-2}$) light for 10 min before homogenization for either measurement of the activity of ATP hydrolysis or isolation of the PM fraction. Red light irradiation was performed with fluorescent lamps (FL20S.PG; National, Kadoma, Japan) covered with three layers of red cellophane. Far-red light irradiation was performed as described by Liu and Iino (1996). The fluence rate of red and far-red light was measured by radiometer (SJI radiometer model 4090; Analytical Technology Inc., Tokyo, Japan). In treatments with DCMU, the leaves were floated in artificial pond water containing 10 μM DCMU 40 min before the start of light irradiation. By measuring chlorophyll fluorescence (van Kooten and Snel 1990), we confirmed that DCMU completely suppressed the photosynthetic electron transport.

ATP-hydrolyzing activity in a leaf homogenate

Dark-adapted or light-irradiated leaves, 4 g in fresh weight, were cut into small pieces and ground in a mortar and pestle with liquid nitrogen. The leaf powder obtained was suspended in 20 ml of an assay medium that contained 140 mM KCl, 2 mM EDTA-1,3-bis(tris[hydroxymethyl]methyl-amino) propane (BTP), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 21 μM leupeptin, 1 mg ml^{-1} BSA, and 10 mM Mops-BTP at pH 7.0. To 500 μl of the prepared leaf homogenate, we added various concentrations of ATP-BTP with or without vanadate at a final concentration of 500 μM. The reaction was started by the addition of $MgSO_4$ to a final concentration of 20 mM. After incubation for 30 min at 30 °C, released inorganic phosphate (Pi) was determined colorimetrically according to the manufacturer's instructions (Code No. 278-05401; Wako Pure Chemical Industries, Osaka, Japan), which were based on the methods of Taussky and Shorr (1952), with several modifications. The protein content was determined by the method of Bradford (1976), using BSA as the standard.

Because the leaf homogenate contained a non-negligible amount of endogenous ATP, we used a modified form of the Michaelis-Menten equation to correct the concentration of ATP in the assay medium:

$$v = \frac{V_{max}\left([ATP]_{exo}+[ATP]_{endo}\right)}{K_m + \left([ATP]_{exo}+[ATP]_{endo}\right)},$$

where $[ATP]_{exo}$ is the final concentration of ATP added exogenously to the assay medium, $[ATP]_{endo}$ is the concentration of endogenous ATP in the assay medium, and v is the activity of ATP hydrolysis measured at a defined concentration of $[ATP]_{exo}$.

Isolation of the PM fraction

A PM-rich fraction was prepared from the leaves according to Takagi et al. (1988) with several modifications. Healthy leaf

segments were homogenized with a Polytron homogenizer (PT35/2ST"OD"; Kinematica, Lucerne, Switzerland) in a homogenizing medium that contained 300 mM sucrose, 10 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA), 5 mM EDTA, 5 mM $K_2S_2O_5$, 1 mM dithiothreitol (DTT), 10 mg ml^{-1} butylated hydroxytoluene, 1% (w/v) casein, 1.2 μg ml^{-1} aprotinin, 2.5 mg ml^{-1} pepstatin, 20 mg ml^{-1} polyvinylpolypyrrolidone, and 50 mM Mops-KOH at pH 7.6. After differential centrifugation, the resultant pellet was washed with buffer A (250 mM sucrose, 0.1 mM DTT, and 10 mM Mops-KOH at pH 7.6) and designated as a crude microsomal fraction. A PM fraction was isolated from the crude microsomal fraction by aqueous two-phase partitioning (Yoshida et al. 1983) at pH 7.8 using a polymer mixture composed of dextran T500 (Amersham Pharmacia Biotech AB; Uppsala, Sweden) and polyethylene glycol P-3640 (Sigma; St. Louis, Mo., USA). The PM fraction was suspended either in buffer A for marker-enzyme assays or in buffer B (250 mM sucrose, 5 mM EDTA-BTP, and 10 mM Mops-BTP at pH 7.0) for a H^+-transport assay. All the procedures were carried out at 0–4 °C under green safelight (550 nm, 0.15 $W{\cdot}m^{-2}$).

Assays of the activities of marker enzymes

The activity of ATP hydrolysis was measured according to Staal et al. (1991). As the marker for the PM, mitochondrial inner membrane, and tonoplast, the sensitivity of the ATPase activity to 200 μM vanadate, 1 mM NaN_3, and 50 mM KNO_3, respectively, was determined in the presence of 250 μg ml^{-1} Brij 58. The equivalent concentrations of NaCl and KCl were used as substitutes in control experiments. The activity of Triton-stimulated UDP hydrolysis was measured as a marker for Golgi membranes, according to Nagahashi and Nagahashi (1982). Acid phosphatase was assayed in sodium acetate buffer at pH 5.0 with *p*-nitrophenyl phosphate as the substrate (Yoshida et al. 1983). The released Pi was determined by the method of Heinonen and Lahti (1981). As a marker for the endoplasmic reticulum membrane, antimycin A-resistant NADH-Cyt *c* reductase was spectrophotometrically assayed at 25 °C by following the rate of reduction of Cyt *c* at 550 nm (Yoshida 1979). The content of protein was determined by using sodium bicinchoninate (Smith et al. 1985). BSA was used for the standard.

Assay of the activity of ATP-dependent H^+ transport

The activity of ATP-dependent H^+ transport into inside-out PM vesicles was assayed at 30 °C according to Johansson et al. (1995) with minor modifications. The inside-out PM vesicles were prepared by mixing a small aliquot of the suspended PM fraction in buffer B at 1 mg ml^{-1} of protein with a 1% volume of each of 200 mM ATP-BTP at pH 7.0 and 1,175 mg ml^{-1} Brij 58. The prepared vesicles were added to reaction medium (Johansson et al. 1995) that contained 10 μM quinacrine instead of acridine orange and was supplemented with 500 μg ml^{-1} Brij 58. After incubation for 5 min, the reaction was started by the addition of $MgSO_4$ to a final concentration of 5 mM. The rate of quenching of fluorescence from quinacrine was monitored with a fluorescence spectrophotometer (Model 850; Hitachi, Tokyo, Japan). The excitation and emission wavelengths were 420 and 495 nm, respectively. Green light (550 nm, 0.15 $W{\cdot}m^{-2}$) was used as a safelight throughout the experiments.

We noticed that the kinetic parameters of the activities of ATP hydrolysis and H^+ transport fluctuated substantially with batches of plants, seasons, and so on. Consequently, we always performed each series of comparative experiments in parallel using plants from the same batch. The data obtained were statistically analyzed using *t*-tests.

Results

Light-induced changes in the membrane potential

Table 1 summarizes quantitative results of light-induced changes in the membrane potential of mesophyll cells of *V. gigantea*. Figure 1a shows a typical result obtained by continuous irradiation with red light. In a dark-adapted mesophyll cell, the membrane potential was maintained at around –150 to –200 mV. Upon actinic irradiation, the membrane began to hyperpolarize after a small transient depolarization. Continuous irradiation with either blue light (Fig. 1b) or blue plus far-red light induced similar changes in the membrane potential (Table 1). In contrast, irradiation with far-red light alone had only a very small effect on the membrane potential (Fig. 1c). The effects of far-red light were not evident in the presence of DCMU, an inhibitor of photosynthetic electron transport (Table 1).

When dark-adapted cells were treated with either a potent inhibitor of membrane ATPases (N,N′-

Table 1 Effects of monochromatic light and metabolic inhibitors on the membrane potential in *Vallisneria gigantea* mesophyll cells. E_m (dark): dark-adapted level of membrane potential. E_m (light): maximum hyperpolarized level of membrane potential after light irradiation. Usually, a maximum hyperpolarization was obtained at 12–20 min after the start of light illumination. ΔE_m: E_m(dark)–E_m(light); each value is expressed as the mean ± SE. Red: continuous irradiation with red light (650 nm, 1.9 $W{\cdot}m^{-2}$). Blue: continuous irradiation with blue light (446 nm, 2.7 $W{\cdot}m^{-2}$). Blue + FR: continuous irradiation with blue plus far-red light (729 nm, 2.1 $W{\cdot}m^{-2}$). DMSO: pretreatment with 0.5% DMSO for 1 h before irradiation. DCCD: pretreatment with 25 μM DCCD for 3 h before irradiation. CCCP: pretreatment with 2.0 μM CCCP for 30 min before irradiation. DCMU: pretreatment with 2.0 μM DCMU for 15 min before irradiation. *n*: number of cells measured

Light	Reagent	*n*	E_m (dark)[a]	E_m (light)[a]	ΔE_m[a]
Red	DMSO	6	–177.4 ± 8.3	–234.0 ± 5.5	59.8 ± 7.5
Blue	DMSO	8	–163.4 ± 10.1	–224.1 ± 8.8	60.8 ± 2.0
Blue + FR	DMSO	7	–188.6 ± 7.5	–238.7 ± 21.9	54.0 ± 14.7
FR	DMSO	6	–196.6 ± 11.0	–224.0 ± 13.0	24.7 ± 4.0
Red	DCCD	7	–63.6 ± 5.6	–62.1 ± 6.9	–1.3 ± 1.9
Blue	DCCD	3	–66.0 ± 12.0	–58.0 ± 12.6	–8.0 ± 1.5
Red	CCCP	5	–52.0 ± 2.7	–52.0 ± 2.7	0
Red	DCMU	7	–165.1 ± 18.5	–161.3 ± 19.1	–3.9 ± 0.5
FR	DCMU	3	–147.5 ± 5.5	–147.5 ± 5.5	0

[a]mV

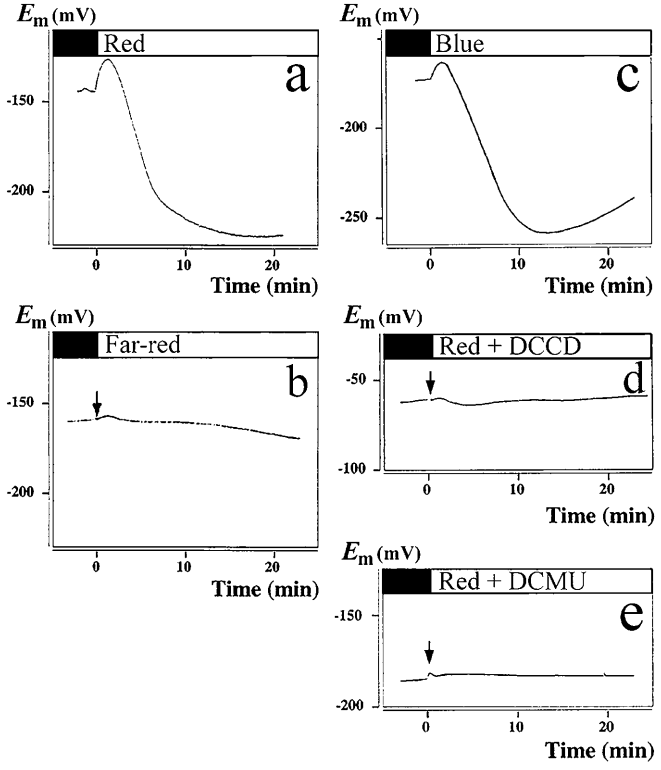

Fig. 1a–e Light-induced changes in the membrane potential in *Vallisneria gigantea* mesophyll cells. Membrane potential (E_m) was measured by using a glass microelectrode. The schedule of light treatments is indicated in the upper panel of each figure. The *black box* in the panel indicates a dark treatment. The effects of red light (650 nm, 1.9 $W \cdot m^{-2}$) (**a**), blue light (451 nm, 2.7 $W \cdot m^{-2}$) (**b**), far-red light (746 nm, 1.6 $W \cdot m^{-2}$) (**c**), red light in the presence of DCCD at 25 μM (**d**), and red light in the presence of DCMU at 2.0 μM (**e**) are shown

dicyclohexylcarbodiimide, DCCD; Fig. 1d) or an uncoupler (carbonylcyanide *m*-chlorophenylhydrazone, CCCP; Table 1) in the dark, the membrane depolarized to about –60 mV. Such cells never showed any significant changes in the membrane potential under subsequent irradiation with red or blue light ($P > 0.05$; Table 1). On the other hand, treatment with DCMU did not affect the dark-adapted level of membrane potential. However, it completely suppressed both a transient membrane depolarization and a subsequent hyperpolarization induced by red (Fig. 1e, Table 1) or blue light (data not shown). These results suggest that, in mesophyll cells of *V. gigantea*, the PM H^+-ATPase provides a H^+ motive force across the PM to generate the inside negative membrane potential, and that the operation of photosynthesis substantially accelerates its activity.

Effects of in vivo illumination on the kinetic parameters of ATP-hydrolyzing activity in leaf homogenate

To examine the mode of light-dependent modulation of the activity of the PM H^+-ATPase, we first determined

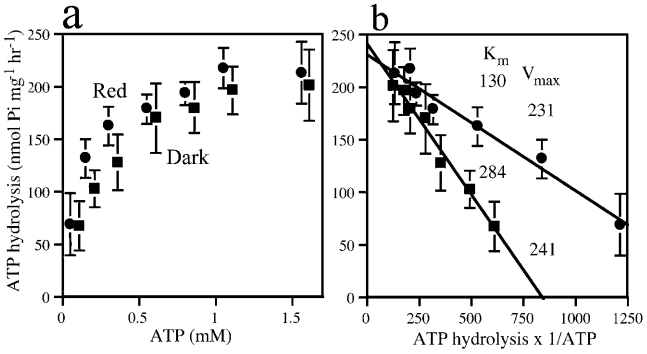

Fig. 2a, b Effects of in vivo illumination with red light on the activity of vanadate-sensitive ATP hydrolysis in a leaf homogenate of *V. gigantea*. The leaf homogenates were prepared from dark-adapted (*squares*) or red-light-irradiated leaves (*circles*). The ATP-hydrolyzing activity is defined as the released Pi per mg protein per hour, and was plotted against the concentration of ATP (**a**). The concentration of ATP in the assay medium was determined as described in Materials and methods. An Eadie-Hofstee plot of the data was also prepared (**b**). K_m for ATP is given as μM. The vertical bar at each point is the SE of six independent experiments

the kinetic parameters of vanadate-sensitive ATP-hydrolyzing activity in a leaf homogenate prepared from the leaves immediately before and after actinic irradiation. As shown in Fig. 2, red light (of which an irradiation time of 10 min was sufficient to induce membrane hyperpolarization to a saturation level; Fig. 1), produced a considerable decrease in the K_m for ATP of the activity of vanadate-sensitive ATP hydrolysis from 280 to 130 μM. V_{max} did not change substantially. We confirmed that the effect of red light completely disappeared in the homogenate prepared from the leaves irradiated in the presence of DCMU. However, values of K_m for ATP determined in such samples increased considerably to 500–800 μM. This was also true of the homogenate prepared from the control leaves treated with 0.5% DMSO, which was used to dissolve DCMU; the effect of red light on the K_m for ATP became much smaller in the control experiments (data not shown) than that obtained in the absence of DMSO.

Effects of in vivo illumination on the kinetic parameters of H^+-transporting activity in isolated PM vesicles

Because the activity of vanadate-sensitive ATP hydrolysis in leaf homogenate may be due not only to the PM H^+-ATPase, we next attempted to determine the kinetic parameters of the H^+-transporting activity in biochemically isolated PM vesicles. To isolate a PM vesicles with sufficiently high purity and high yield for the transport assays used, we added several modifications to our previous preparation procedures (Takagi et al. 1988), namely, homogenization of the leaves in the presence of casein, supplementation with various types of protease inhibitors, and so on. Table 2 shows the distribution of activities of several marker enzymes in

Table 2 Distribution of marker enzymes in the crude microsome and PM fractions isolated from *V. gigantea* leaves. The activities are given as the means ± SE from two to nine independent experiments. *N.D.* not detected

Enzymes	Crude microsome	PM	Recovery in PM fraction (*n*-fold)
Vanadate-sensitive ATPase[a]	1.16 ± 0	6.74 ± 0.80	5.81
Nitrate-sensitive ATPase[a]	0.18 ± 0	N.D.	0
Azide-sensitive ATPase[a]	0.22 ± 0.06	N.D.	0
Triton-stimulated UDPase[a]	2.90 ± 0.50	N.D.	0
Acid phosphatase[a]	1.36 ± 0.54	N.D.	0
Antimycin A-resistant NADH-Cyt *c* reductase[b]	106.3 ± 6.9	73.9 ± 4.3	0.70

[a] μmol Pi mg^{-1} h^{-1}
[b] nmol Cyt *c* mg^{-1} min^{-1}

the PM and crude microsome fractions. The activity of vanadate-sensitive ATPase was concentrated in the PM fraction about 5- to 6-fold compared with that of the crude microsome fraction. In contrast, most of the other enzyme activities tested were not detected or were only detectable in a small amount in the PM fraction. We succeeded in detecting the activity of ATP-dependent H^+ transport into the PM vesicles only in the presence of a non-ionic surfactant Brij 58 at a saturating concentration in the reaction medium (data not shown). The activity was completely inhibited by DCCD, vanadate, and CCCP (data not shown).

Using the H^+-transporting assay in the inside-out vesicles, we clearly showed, as in the case of vanadate-sensitive ATP-hydrolyzing activity (Fig. 2), that in vivo illumination with red light produced a significant decrease in the K_m for ATP of H^+-transporting activity from approximately 420 to 220 μM ($P < 0.05$; Fig. 3, Expt. 1 in Table 3). Because irradiation with far-red light did not produce any significant changes in the kinetic parameters ($P > 0.05$; Expt. 1 in Table 3), the effect is specific to red light. When the PM vesicles were isolated from the dark-adapted leaves treated with DCMU, the K_m for ATP of H^+-transporting activity increased considerably to 530 μM (Expt. 2 in Table 3), as observed in the case of vanadate-sensitive ATP-hydrolyzing activity examined in the leaf homogenate. When we treated the leaves with 0.5% DMSO, which was used as a solvent for DCMU, similar results were obtained (data not shown). Nevertheless, red light produced a significant decrease in the K_m for ATP ($P < 0.05$; Table 3), and the effect of red light was completely negated by the presence of DCMU during irradiation of the leaves. V_{max} did not change significantly under any light conditions ($P > 0.05$; Table 3).

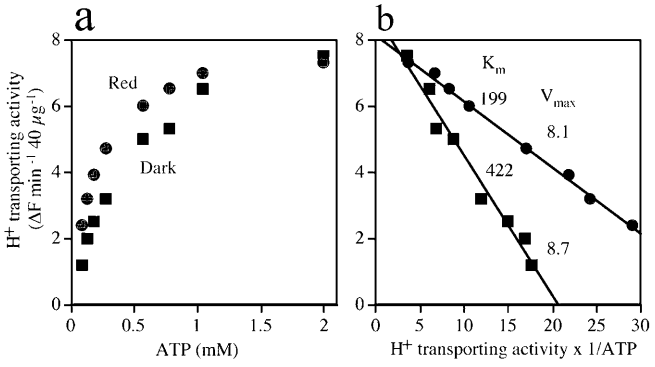

Fig. 3a, b Effects of in vivo illumination with red light on H^+-transporting activity in PM vesicles isolated from *V. gigantea* leaves. PM vesicles were isolated from dark-adapted (*squares*) or red-light-irradiated leaves (*circles*). The activity of ATP-dependent H^+ transport is given as the quenching rate of quinacrine fluorescence per minute per 40 μg of PM protein (ΔF min^{-1} $(40\ \mu g)^{-1}$), and is plotted against the concentration of ATP (**a**). An Eadie-Hofstee plot of the data was also prepared (**b**). K_m for ATP is given as μM

Discussion

Photosynthesis-dependent acceleration of the activity of the PM H^+-ATPase

In mesophyll cells of *V. gigantea*, DCMU completely suppressed the red- or blue-light-induced membrane hyperpolarization (Fig. 1e, Table 1). Although DCMU did not affect the dark-adapted level of the membrane potential, both DCCD and CCCP significantly

Table 3 Effects of red and far-red light and DCMU on the kinetic parameters of ATP-dependent H^+-transporting activity in PM vesicles isolated from *V. gigantea* leaves. The K_m for ATP and V_{max} of ATP-dependent H^+-transporting activity were determined in PM vesicles isolated from dark-adapted (*Dark*), red-light-irradiated (*Red*), and far-red-light-irradiated leaves (*FR*), and expressed as the mean ± SE. In experiment 2, the dark-adapted leaves were incubated in artificial pond water containing either DCMU or DMSO, which was used as a solvent for DCMU, for 40 min before isolation of PM vesicles. V_{max} is given as the quenching rate of quinacrine fluorescence per minute per 40 μg of PM protein (ΔF min^{-1} $(40\ \mu g)^{-1}$). *Significantly different from the data obtained from the dark-adapted leaves ($P < 0.05$)

	n	K_m for ATP (μM)	V_{max} (ΔF min^{-1} $(40\ \mu g)^{-1}$)
Expt. 1			
Dark	6	424.5 ± 22.7	13.4 ± 0.7
Red	4	225.5 ± 27.3*	10.5 ± 0.6
FR	5	378.2 ± 23.6	16.0 ± 1.3
Expt. 2			
Dark + DCMU	6	534.8 ± 22.5	8.3 ± 1.1
Red + DMSO	6	370.2 ± 38.8*	8.2 ± 0.9
Red + DCMU	5	561.0 ± 57.0	10.8 ± 1.0

depolarized the membrane in darkness. Moreover, the light-induced membrane hyperpolarization was never observed in the presence of these chemicals (Fig. 1d, Table 1). As previously demonstrated in characean cells (Nishizaki 1968; Mimura 1995) and in higher-plant cells such as *V. spiralis* (Bentrup et al. 1973; Prins et al. 1980) and *Egeria densa* (Tazawa et al. 1986), a substantial part of the inside negative membrane potential is maintained by an electrogenic process, most probably through the operation of the PM H^+-ATPase; and photosynthesis-dependent membrane hyperpolarization is caused by an acceleration of the electrogenic process.

To examine these light-mediated processes more directly, we determined the kinetic parameters of the activity of vanadate-sensitive ATP hydrolysis in the leaf homogenate. In vivo illumination of the leaves with red light produced an appreciable decrease in the K_m for ATP of ATP-hydrolyzing activity (Fig. 2). Furthermore, the decrease in K_m for ATP was similarly induced by red light in the ATP-dependent H^+-transporting activity in the highly purified PM vesicles (Fig. 3, Table 3). The effects of red light disappeared in the presence of DCMU during in vivo illumination. Because we demonstrated the presence of a PM H^+-ATPase equipped with the C-terminal autoinhibitory domain in the leaves of *V. gigantea* (Harada et al., in press), to our knowledge, this is the first successful demonstration of photosynthesis-dependent changes in the kinetic parameters of PM H^+-ATPase activity. Our results may provide some enzymological basis for a light-induced membrane hyperpolarization in plant cells, a process that has been analyzed solely by electrophysiology to date.

In kinetic analyses of the ATP-hydrolyzing activity and the H^+-transporting activity, DMSO increased the K_m for ATP (compare Expt. 1 with Expt. 2 in Table 3). Örvar et al. (2000) reported that DMSO reduces the membrane fluidity. Because PM H^+-ATPase activity is affected by the composition of phospholipids and free unsaturated fatty acids in the membrane (Kasamo and Nouchi 1987; Kasamo 1990), which might influence the membrane fluidity, it is possible that DMSO affects the kinetic parameters of the PM H^+-ATPase by changing the membrane fluidity. In addition, a considerable difference in the values of K_m for ATP between the ATP-hydrolyzing activity in leaf homogenate (Fig. 2) and the H^+-transporting activity in PM vesicles (Fig. 3, Table 3) was observed. A large part of the difference would be ascribable to seasonal changes and batches of plants. The difference might also be caused by incorrect estimation of the concentration of endogenous ATP in the ATPase assay medium.

Lack of light-dependent changes in V_{max} of PM H^+-ATPase activity

We could not detect light-dependent changes in the V_{max} of activities of ATP hydrolysis (Fig. 2) and H^+ transport (Fig. 3, Table 3). Olsson et al. (1995) suggested that the C-terminal autoinhibitory domain of the PM H^+-ATPase might have multiple regulatory sites. Regenberg et al. (1995) examined this possibility by a C-terminal deletion analysis of the PM H^+-ATPase of *Arabidopsis* AHA2 carried out in a yeast expression system. The removal of the final 38 C-terminal amino acid residues produced only a decrease in the K_m for ATP; an increase in the V_{max} was brought about when an additional 12 residues were removed from the C-terminus. It is possible that, in mesophyll cells of *V. gigantea*, photosynthesis-dependent modification of the PM H^+-ATPase is localized in the most C-terminal region in the autoinhibitory domain. In vitro treatment of the isolated PM fraction with auxin induced similar changes in the kinetic parameters of ATP-hydrolyzing activity or H^+-transporting activity, namely, a decrease in the K_m for ATP alone and no change in the V_{max} in hypocotyl hooks of *Cucurbita pepo* (Scherer 1984), roots of *Pisum sativum* (Gabathuler and Cleland 1985), and leaves of *Nicotiana tabacum* (Santoni et al. 1991).

Separately, technical limitations in detecting possible changes in the V_{max} of activities of ATP hydrolysis and H^+ transport should be considered. Changes in the V_{max} of both activities of the mesophyll PM H^+-ATPase might be masked by the activity of other isoforms of the PM H^+-ATPase. Because the leaves of *V. gigantea* contain several kinds of isoform of PM H^+-ATPase (Harada et al., in press), the leaf homogenate and the PM vesicles isolated from the leaves are ''hybrid'' fractions. To characterize the light-responsive PM H^+-ATPase in more detail, we are isolating the PM H^+-ATPase gene(s) specifically expressed in the mesophyll cells of *V. gigantea*.

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