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Effects of Light on the Membrane Potential of Protoplasts from *Samanea saman* Pulvini[1]

Involvement of K^+ Channels and the H^+-ATPase

Hak Yong Kim, Gary G. Coté, and Richard C. Crain*

U-125, Department of Molecular and Cell Biology, The University of Connecticut, Storrs, Connecticut 06269–3125

ABSTRACT

Rhythmic light-sensitive movements of the leaflets of *Samanea saman* depend upon ion fluxes across the plasma membrane of extensor and flexor cells in opposing regions of the leaf-movement organ (pulvinus). We have isolated protoplasts from the extensor and flexor regions of *S. saman* pulvini and have examined the effects of brief 30-second exposures to white, blue, or red light on the relative membrane potential using the fluorescent dye, 3,3′-dipropylthiadicarbocyanine iodide. White and blue light induced transient membrane hyperpolarization of both extensor and flexor protoplasts; red light had no effect. Following white or blue light-induced hyperpolarization, the addition of 200 millimolar K^+ resulted in a rapid depolarization of extensor, but not of flexor protoplasts. In contrast, addition of K^+ following red light or in darkness resulted in a rapid depolarization of flexor, but not of extensor protoplasts. In both flexor and extensor protoplasts, depolarization was completely inhibited by tetraethylammonium, implicating channel-mediated movement of K^+ ions. These results suggest that K^+ channels are closed in extensor plasma membranes and open in flexor plasma membranes in darkness and that white and blue light, but not red light, close the channels in flexor plasma membranes and open them in extensor plasma membranes. Vanadate treatment inhibited hyperpolarization in response to blue or white light, but did not affect K^+-induced depolarization. This suggests that white or blue light-induced hyperpolarization results from activation of the H^+-ATPase, but this hyperpolarization is not the sole factor controlling the opening of K^+ channels.

Leaflet movements of *Samanea saman* are produced by changes in the turgor of extensor and flexor cells in opposing regions of the leaf-movement organ or pulvinus. These changes in turgor are caused by ion fluxes across the plasma membrane, primarily of potassium and chloride (12). These fluxes are regulated by light and the circadian clock (17). Extensor cells take up K^+, Cl^-, and water and swell after exposure to white or blue light, whereas flexor cells take up K^+, Cl^-, and water and swell after exposure to red light followed by darkness (7, 8). Similarly, during circadian-rhythmic leaflet movement, K^+, Cl^-, and water are taken up alternately and rhythmically by flexor and extensor cells (2).

The plasma membrane H^+-ATPase of plant cells has a primary role in coupling ATP hydrolysis to solute movement (22). Outwardly directed H^+ transport by the H^+-ATPase produces both a membrane electrical potential and a pH gradient. The membrane electrical potential drives the uptake of cations, mainly K^+, whereas the pH gradient promotes the uptake of anions such as Cl^- and neutral molecules such as sucrose through H^+-coupled cotransport. For example, during blue light-induced opening of leaf stomata, H^+ extrusion from guard cells creates an electrical gradient that drives K^+ uptake through K^+-selective ion channels (1, 18, 19); stomatal opening is inhibited by the H^+-ATPase inhibitor vanadate (21). Irradiation of isolated *S. saman* pulvini also causes H^+ fluxes, membrane potential changes, and K^+ movement, which regulate pulvinar bending and, hence, mediate light-induced leaflet movement (7, 8). Similarly, opposing changes observed in apoplastic H^+ and K^+ concentrations in the extensor region of the *Phaseolus* pulvinus also support a role for the H^+-ATPase in K^+ uptake during rhythmic leaflet movement (24).

Potassium channels have been described in cells involved in turgor-mediated movements. Voltage-gated K^+ channels activated by membrane depolarization have been observed in patch-clamp studies of plasma membranes of *S. saman* protoplasts (9) and of *Vicia faba* guard cell protoplasts (19, 20). These channels might serve as the pathway for K^+ efflux from shrinking cells. Hyperpolarization-activated K^+ channels have been reported in guard cell protoplasts of *V. faba* (20) and in *S. saman* protoplasts (9). These channels might serve as a pathway for K^+ influx into swelling cells.

We are studying the transduction pathway mediating light-induced changes in K^+ flux. We have examined the effect of light on K^+ channels by measuring the relative membrane potential of *S. saman* protoplasts exposed to light and the sensitivity of this potential to external K^+. We present evidence here that white and blue light induce K^+ channel opening in the plasma membranes of extensor protoplasts while inducing K^+ channel closure in the plasma membranes of flexor protoplasts.

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MATERIALS AND METHODS

Plant Materials

Samanea saman (Jacq.) Merr. trees were grown at 26 ± 1.5°C and 70% relative humidity on a 16-h light:8-h dark cycle (cool white fluorescent light, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Secondary pulvini were taken from the second and third mature leaves at hours 11 to 12 of the light period.

Chemicals and Solutions

For the preparation of protoplasts, the following reagents were used: Gamborg's B-5 (Carolina Biological Supply Co.); macerase (Calbiochem); Onozuka cellulase R-10 (Yakult Honsha Co., Tokyo, Japan); pectolyase Y 23 (Kanematsh-Gosha) and driselase, BSA, Mes, Histopaque, and ascorbic acid (Sigma Chemical Co.). DiS-C_3-(5)[2] was purchased from Aldrich Chemical Co. and DCCD, valinomycin, sodium orthovanadate, DCMU, and TEA chloride were purchased from Sigma Chemical Co.

DiS-C_3-(5) was dissolved in DMSO at 1 mg/mL. Valinomycin (1 mg/mL) and DCCD (10 mM) were dissolved in 95% ethanol. The ethanol concentrations in the assay medium were 0.2% or less. A 10 mM stock solution of vanadate in water was prepared as previously described (3, 21). DCMU and TEA stock solutions were 1 mM and 1 M in water, respectively.

Preparation of Protoplasts

Protoplasts were isolated from extensor and flexor regions of open pulvini of *S. saman* according to the method of Gorton and Satter (4) with two key modifications to achieve 10- to 20-fold higher yields: the cells were maintained in high osmotic pressure medium and were purified using a Histopaque cushion. Briefly, strips of pulvinar tissue were excised from the extensor and flexor regions of 20 pulvini, chopped finely, and placed in 3 mL of preplasmolysis solution (Gamborg's B-5, 0.3 M sorbitol, 50 mM Mes, 0.2% BSA, and 8 mM $CaCl_2$, pH 5.5). The osmotic pressure of the preplasmolysis solution was then raised to 750 to 800 mOsm, as measured with a Wescor 5500 osmometer (Logan, UT) in four steps over a 1-h period with 4 M sorbitol stock solution. Enzyme solution containing 6% (w/v) each of driselase, Onozuka cellulase, and macerase, 0.15% (w/v) pectolyase Y 23, 0.2% BSA, and 1 mM ascorbic acid, was prepared in Gamborg's B-5 and adjusted to pH 5.5 as previously described (4) and added to plasmolyzed tissue. Digestion proceeded for about 1 h at 30°C with gentle rotary shaking at 40 rpm.

Digested pulvinar tissue was collected on 20-μm nylon mesh and washed with B-5 wash solution (Gamborg's B-5, 0.48 M sorbitol, 100 mM KCl, 2 mM $CaCl_2$, and 1 mM ascorbic acid, pH 6.5). Protoplasts were released by incubation of the tissue for 30 min at 30°C in B-5 wash solution with gentle agitation. Debris was removed by filtration of the protoplast suspension, first through a 221-μm nylon mesh to remove coarse debris, and then through a 62-μm nylon mesh. Protoplasts were sedimented from the filtrate at 150*g* for 7 min, most of the B-5 wash solution was removed and the remaining 1 to 1.5 mL of protoplast suspension were layered onto 1.1 mL of Histopaque solution (90% [v/v] Histopaque, 0.4 M sorbitol, and 2 mM $CaCl_2$).

After centrifugation for 10 min at 150*g*, the light green layer at the interface of the B-5 solution and the Histopaque was resuspended in 15 mL of 20 mM Mes (pH 5.5) containing 400 mM sorbitol, 20 mM NaCl, 1 mM $CaCl_2$, and 1 mM KCl. Protoplasts were sedimented by centrifugation at 150*g* for 10 min, the supernatant was decanted, and the cells were resuspended in 0.6 to 1.0 mL of the above solution. Yields of 1.5 to 2.0 × 10^6 extensor and 1.0 to 1.5 × 10^6 flexor protoplasts were routinely obtained from 20 pairs of *S. saman* pulvini.

Light Treatments

Protoplasts were irradiated for 30 s with light from a fiber optic source (Reichert Scientific Instruments). Blue light was obtained with a blue Plexiglas filter with 59% transmittance at a peak wavelength of 470 nm and half bandwidth of 80 nm (8). Red light was obtained with an interference filter with 58% transmittance at a peak wavelength of 660 nm and half bandwidth of 10 nm (No. 53960; Oriel Optic). The photon fluence rates, measured with a L1–170 Quantum/Radiometer/Photometer (Li-Cor Instrument Co.) were 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for white light, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for blue light, and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for red light. Manipulations conducted during the dark period were performed under dim green "safelight." After the light treatment, the protoplasts were gently mixed and changes in fluorescence were measured.

Measurement of Relative Membrane Potential

Changes in protoplast membrane potential were monitored as the change of intensity of fluorescence emission of the membrane potential-sensitive dye, DiS-C_3-(5) (26). The assay medium consisted of 20 mM Mes (pH 5.5), 400 mM sorbitol, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM KCl, and 1 × 10^5 protoplasts/mL. DiS-C_3-(5) was added to the assay medium to a final concentration of 1 μM and the suspension was incubated for 1 h at 25°C to allow stabilization of the fluorescence intensity. Fluorescence intensity was measured with a Perkin Elmer LS-3B fluorescence spectrophotometer. The bandwidths for excitation at 620 nm and emission at 668 nm were 5 and 10 nm, respectively. Although the fluorescence excitation at 620 nm is low intensity (photon fluence 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and the fluorescence emission at 668 nm is lower still, the possibility exists for the stimulation of phytochrome which absorbs maximally at 660 nm. We reduced this possibility by measuring the fluorescence intensity intermittently rather than continuously, mixing the cells gently between fluorescence measurements. Discontinuous illumination also minimizes possible photodamage to cells during fluorescence measurements (27).

[2] Abbreviations: DiS-C_3-(5), 3,3'-dipropylthiadicarbocyanine iodide; DCCD, N,N'-dicyclohexylcarbodiimide; TEA, tetraethylammonium.

RESULTS

Measurement of Changes in Membrane Potential

Changes in membrane potential of *S. saman* protoplasts were measured using the fluorescent dye, DiS-C_3-(5) (26), a permeant cation. Although the exact value of the membrane potential could not be determined in these experiments, changes in membrane potential are indicated by changes in fluorescence intensity that are believed to reflect a rapid potential-dependent redistribution of the dye between cell interior and exterior (28). Hyperpolarization causes a decrease in fluorescence intensity, whereas depolarization causes an increase in fluorescence intensity. This was verified for *S. saman* protoplasts using the K^+ ionophore valinomycin and varying concentrations of KCl (Fig. 1). The addition of valinomycin in the presence of low external KCl caused hyperpolarization that decreased fluorescence intensity, whereas addition of valinomycin in the presence of high external KCl caused depolarization that increased fluorescence intensity.

Light Effects on Membrane Potential

Extensor and flexor protoplasts were isolated in the light and transferred to darkness at the time of normal lights-off in the growth chamber. Measurements were made during a period of 4 h or less commencing when the protoplasts had been in darkness for 5 h. Changes in fluorescence were recorded at 30-s intervals following exposures of 30 s to white or blue light. Within 30 s of the end of the light pulse, both extensor and flexor protoplasts were hyperpolarized, as evidenced by a decrease in fluorescence intensity (Fig. 2). During the following 2 to 2.5 min, some depolarization was observed, although not to the original unstimulated potential. Membrane potential stabilized after 3 min. Addition of 200 mM K^+ at this time induced rapid depolarization of extensor, but not flexor, protoplasts treated with white or blue light (Fig. 2).

The emission spectrum of the DiS-C_3-(5) is similar to that of Chl. However, 0.5 μM DCMU, an inhibitor of photosynthesis, did not affect measured fluorescence (data not shown), indicating that the measured fluorescence changes were not caused by photosynthesis.

Flexor and extensor protoplasts were also exposed to 30 s of red light or kept in constant darkness. No effect on the membrane potential of either extensor or flexor protoplasts was observed (Fig. 3). Subsequent addition of 200 mM K^+ induced rapid depolarization of flexor, but not extensor, protoplasts (Fig. 3).

To demonstrate that failure to observe depolarization upon addition of external K^+ was not due to a K^+ gradient or membrane potential preventing K^+ uptake, we measured membrane potentials of extensor and flexor protoplasts pretreated with 1 μM valinomycin, a K^+ ionophore, 5 min before the light treatment. Valinomycin did not prevent white or blue light-induced hyperpolarization, nor did it itself affect membrane potential in darkness or under red light. Rapid depolarization was observed in both cell types in response to the addition of 200 mM K^+ in the presence of 1 μM valinomycin, regardless of the light treatment (Figs. 2 and 3). The K^+-induced depolarization of extensor protoplasts pretreated with white light or flexor protoplasts in darkness was similar to that induced in the absence of valinomycin (Figs. 2A and 3C). In contrast, K^+-induced depolarization of extensor protoplasts pretreated with blue light or flexor protoplasts pretreated with red light was somewhat larger in the presence of valinomycin (Figs. 2B and 3D). When 1 μM valinomycin was added to extensor or flexor protoplasts just 30 s before addition of 200 mM K^+, the same effects were observed as when valinomycin was added 5 min before the light treatment (data not shown).

Is Light-Induced Hyperpolarization Due to Activation of the H^+-ATPase?

To determine whether the plasma membrane H^+-ATPase might be involved in the observed hyperpolarization and K^+ ion effects, we measured the effect of vanadate, an inhibitor of the plasma membrane H^+-ATPase, on the fluorescence intensity changes of the extensor and flexor protoplasts in response to white light and K^+ addition. White or blue light-induced hyperpolarization of extensor protoplasts was inhibited about 70 to 90% by 100 μM vanadate (Fig. 4, A and B) and the inhibition was dependent on the concentration of vanadate added (Fig. 5). White light-induced hyperpolarization of flexor protoplasts was also inhibited by vanadate, although not to as great an extent (Fig. 4C). In contrast, K^+-

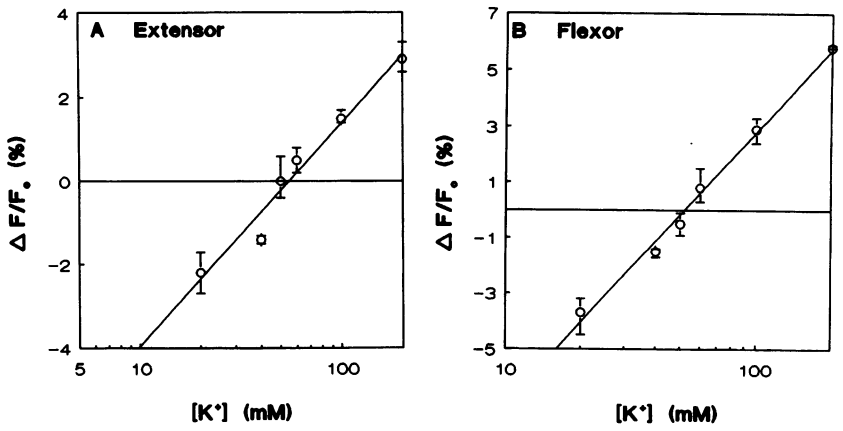

Figure 1. Fluorescence intensity as a function of the log concentration of K^+ in the external medium. Extensor and flexor protoplasts were isolated in the light and transferred to darkness at the normal time of lights-off in the growth chamber. DiS-C_3-(5) was added to protoplasts incubated 4 to 7 h in the dark. After 1 h, different concentrations of KCl with 1 μM valinomycin were added to 1×10^5 protoplasts in 1 mL of buffer. Fluorescence intensity was measured at 30-s intervals and is expressed as the change in fluorescence divided by the initial fluorescence (to normalize for variations between experiments). Error bars indicate the range of values obtained in two or three separate experiments.

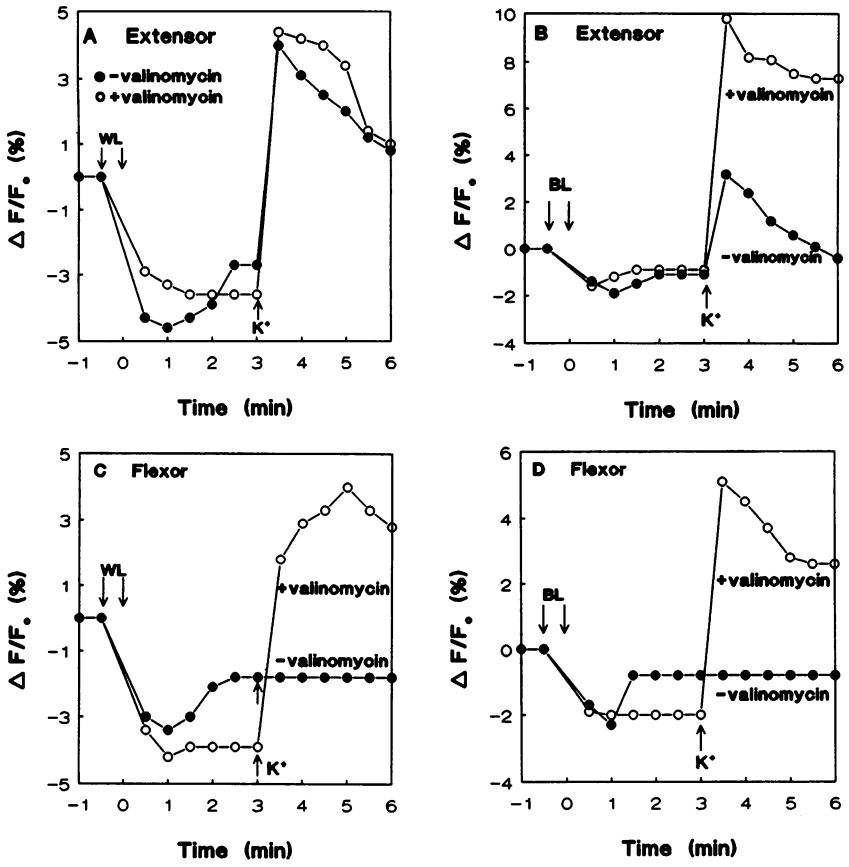

Figure 2. Time course of white and blue light-induced membrane potential changes in *S. saman* protoplasts. Fluorescence intensity changes of 1×10^5 protoplasts/mL, pre-equilibrated with 1 μM DiS-C_3-(5) in the presence (○) and absence (●) of 1 μM valinomycin, were measured. Valinomycin, when present, was added 5 min before irradiation with light. A 30-s pulse of white or blue light was given for the period shown by the arrows. K^+ (200 mM) was added at the time indicated. The upper figures show time courses of (A) white light-induced and (B) blue light-induced membrane potential changes in extensor protoplasts, whereas the lower figures show time courses of (C) white light-induced and (D) blue light-induced membrane potential changes in flexor protoplasts. Data are the mean of three to five experiments; values between experiments varied by less than 15%.

induced depolarization of extensor protoplasts after white or blue light was not inhibited by pretreatment with 100 μM vanadate, although the kinetics of depolarization were somewhat slower than in the absence of vanadate (Fig. 4, A and B). The effects of DCCD, a general inhibitor of H^+-ATPases, on light-induced hyperpolarization of extensor and flexor protoplasts were similar to those of vanadate (data not shown).

Specificity of Ion-Induced Depolarization

To determine whether K^+-induced depolarization was due to K^+ influx through K^+ channels, the effect of TEA, a K^+ channel blocker, on the depolarization of the extensor and flexor protoplasts was measured. When 20 mM TEA was added 30 s before addition of 200 mM K^+, depolarization was completely blocked in extensor protoplasts exposed to white light and in flexor protoplasts in darkness (Fig. 6, A and B). TEA also blocked depolarization of extensor protoplasts exposed to blue light and flexor protoplasts exposed to red light (data not shown). A 30-s treatment with 200 μM Al^{3+}, an inhibitor of inward K^+ channels (19), also completely blocked K^+-induced depolarization of extensor protoplasts irradiated with white light for 30 s or of flexor protoplasts in darkness (data not shown).

Other ions, Na^+, Li^+, and Ca^{2+}, did not depolarize extensor protoplasts treated with white light (Table I). In flexor protoplasts in darkness, Na^+ and Li^+ produced only a small depolarization and Ca^{2+} had no effect (Table I). The depolarizing effect of Rb^+ could not be assessed because 200 mM Rb^+ interacted directly with the dye and increased fluorescent intensity in the absence of protoplasts.

DISCUSSION

Fluorescence as a Measure of Membrane Potential

The use of fluorescent dyes to measure membrane potential has previously been demonstrated for a wide variety of cells (10, 26, 27). Recently, Oren-Shamir *et al.* (10) utilized DiS-C_3-(5) to estimate the membrane potential in *Dunaliella salina* following hypertonic shock and noted a linear relationship between changes in fluorescence intensity and the log of external KCl added simultaneously with valinomycin, results similar to ours (Fig. 1). This supports the premise that relative membrane potential can be measured with this dye. We estimate an internal K^+ concentration of 390 mM in flexor and 325 mM in extensor cells in closed pulvini, based on solute concentrations required to plasmolyze the cells in isolated pulvinar tissue (H.Y. Kim and R.C. Crain, unpublished results) and the fact that most of the osmotic potential in these cells is probably due to K^+ and Cl^- (13). Assuming these values are maintained in isolated protoplasts, we can calculate, using the Nernst equation and the *x*-intercept of Figure 1, a membrane potential of −47 mV for extensor protoplasts and −52 mV for flexor protoplasts. These values are consistent with membrane potentials measured *in situ* in the *S. saman* pulvinus in the dark (11).

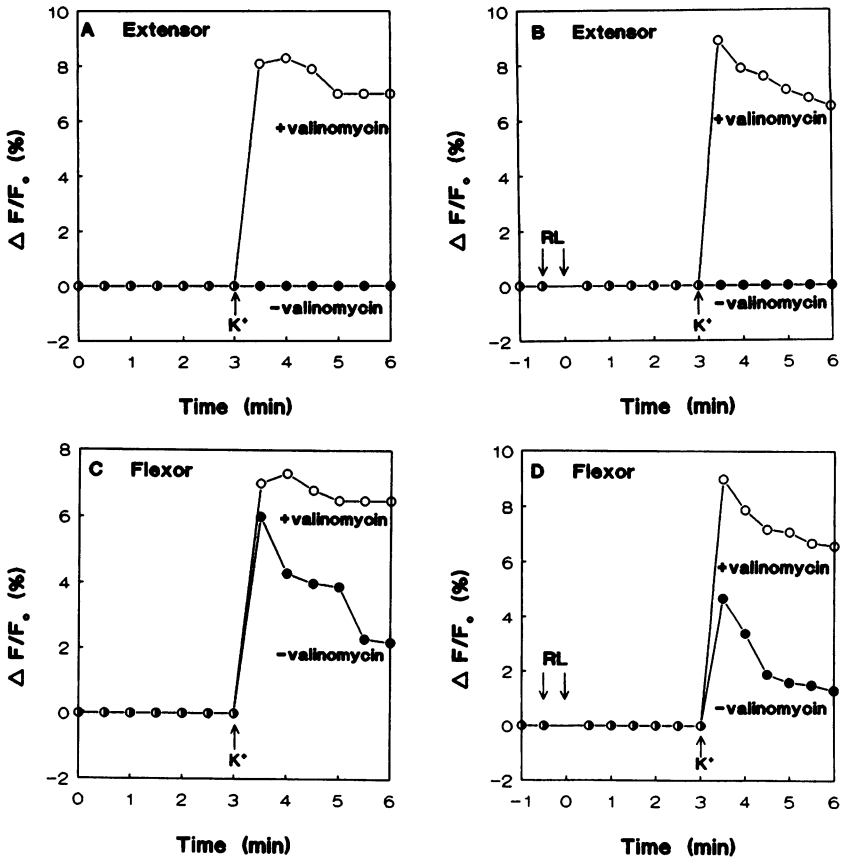

Figure 3. Time course of membrane potential changes in dark control and red light-treated *S. saman* protoplasts with KCl addition in the presence (O) and absence (●) of valinomycin. The upper figures show time courses of membrane potential changes in extensor protoplasts in the dark (A) and following red light (B), whereas the lower figures show time courses of membrane potential changes in flexor protoplasts in the dark (C) and following red light (D). K^+ (200 mM) was added at the time indicated. See legend to Figure 2 for other details. Data are the mean of three to five experiments; values varied by less than 15% between experiments.

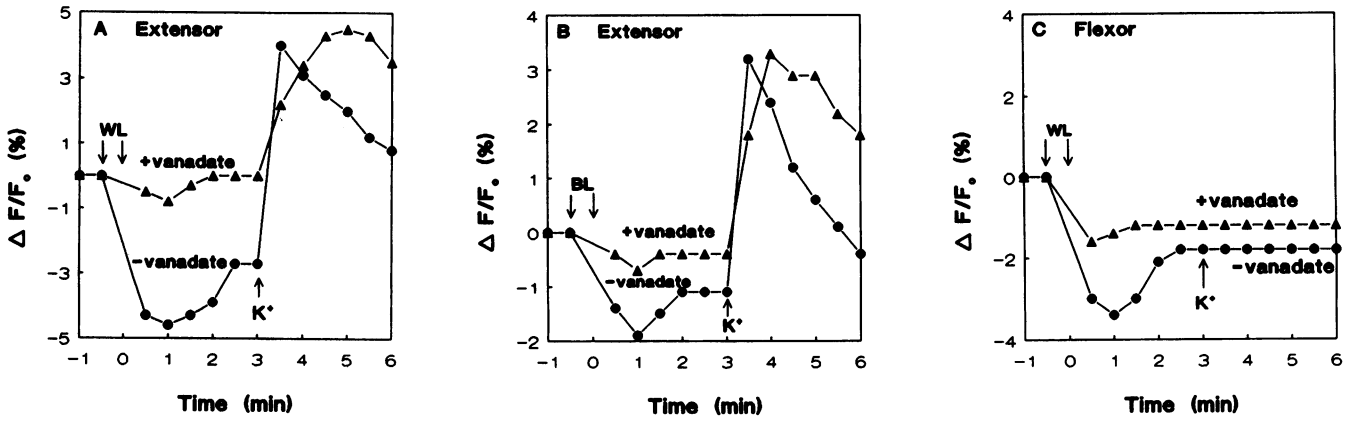

Figure 4. Effect of vanadate on light- and K^+-induced changes in membrane potential of extensor and flexor protoplasts. Extensor (A and B) or flexor (C) protoplasts were treated with white light (A and C) or blue light (B) and with 200 mM K^+ (A, B, and C) as described in the legends to Figures 2 and 3, in the presence (▲) and absence (●) of 100 μM vanadate. Vanadate was added 1 h before irradiation. Values are the average of three experiments, among which there was less than 15% variation.

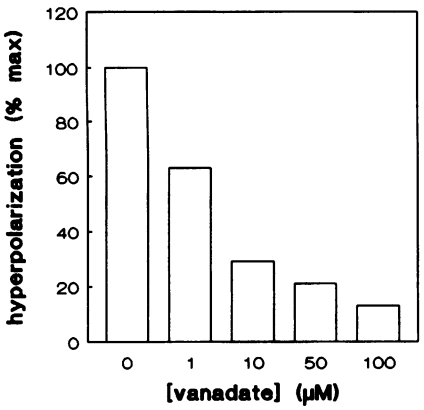

Figure 5. Inhibition of hyperpolarization of extensor protoplasts as a function of vanadate concentration. Extensor protoplasts preincubated with different vanadate concentrations for 1 h were irradiated with white light for 30 s, and the fluorescence intensity was observed at 1 min after white light irradiation. This is the time of maximum reduction in fluorescence intensity in the absence of vanadate. Relative fluorescence change is expressed as a percent of the maximum fluorescence decrease observed in the absence of vanadate.

Table I. *Ion Specificity of Ion-Induced Depolarization in S. saman Protoplasts*

Depolarization was measured as described in Figures 1 and 2 with protoplasts treated with the indicated salts and with white light (WL) or darkness as indicated. White light pulses were for 30 s and were given 2.5 min before salt treatment. Depolarization is expressed as percent of maximum depolarization observed with KCl (set to 100%).

	Depolarization (% of Max)	
	Extensor following WL	Flexor darkness
200 mM KCl	100	100
200 mM NaCl	0	15 ± 4.5
200 mM LiCl	0	10 ± 4.3
20 mM $CaCl_2$	0	0

K^+ Transport in Turgor Regulation

Transport of K^+ plays a central role in the regulation of volume and turgor of plant cells (6, 30). In legumes such as *S. saman* and *Phaseolus*, leaflet movements depend upon differential changes in the volumes of cells in the pulvinus at the base of each leaflet. These changes in cell volume depend in turn on osmotic water uptake or water loss that follows ion influx or efflux from cells in the extensor and flexor regions of the pulvinus (6, 24). Concentrations of K^+, the most abundant ion in the pulvinus, increase approximately fourfold in extensor cells upon leaflet opening (14). Opening and closing of the leaflets and the ion fluxes that cause these movements are rhythmic, being under the control of an endogenous circadian clock (16, 24). Light regulates the circadian clock and also directly affects the leaflet movement. During the dark period of the cycle, white light promotes leaflet opening, with blue light apparently being the most active wavelength (15). During the light period of the cycle, darkness promotes leaflet closure and red light absorbed by phytochrome enhances this dark-induced closure (25).

Based on these effects of light on leaflet movements, we looked for an effect of light on K^+ channels that might carry the ion fluxes that cause this movement. In the presence of the K^+ ionophore valinomycin, the addition of 200 mM K^+ induced rapid depolarization in both cell types regardless of light treatment, consistent with our expectation that this extracellular K^+ concentration would induce a depolarizing inward K^+ current if a K^+ channel or ionophore were available to carry the current. Extensor protoplasts exposed to a brief 30-s pulse of white or blue light, and flexor protoplasts maintained in darkness or exposed to a brief 30-s pulse of red light followed by darkness, exhibited a similar depolarization when 200 mM K^+ was added to the bathing medium in the absence of valinomycin, which suggests that K^+ channels are open in these cells following these treatments. Based on comparison of the degree of depolarization in the presence and absence of valinomycin, blue light appears to be less effective than white light in opening K^+ channels in extensor cells. This might result from the less pronounced hyperpolarization produced by blue light compared to white light, if hyperpolarization triggers K^+ channel opening in these protoplasts. Hyperpolarization has been shown to open K^+ channels in guard cells (12) and *S. saman* protoplasts (9).

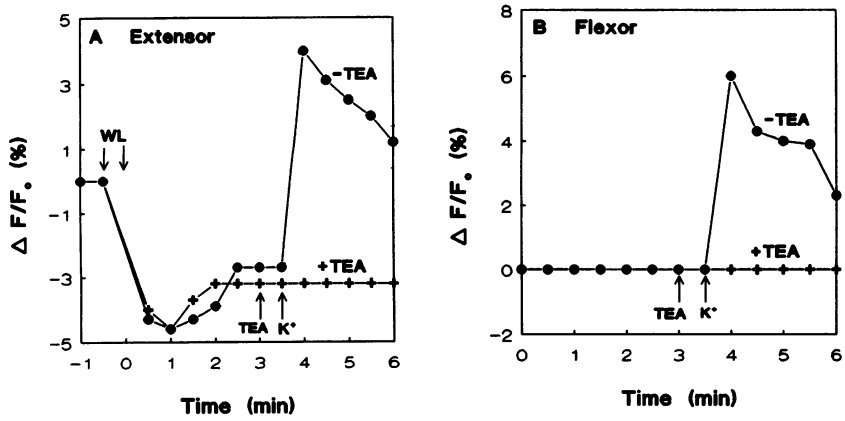

Figure 6. Effect of TEA on the K^+-induced depolarization. Extensor (A) protoplasts (1×10^5/mL) were irradiated for 30 s with white light; flexor (B) protoplasts were kept in darkness. Depolarization was induced by addition of 200 mM K^+ (●); 20 mM TEA, when present (+), was added 30 s before addition of K^+.

That depolarization is mediated by channels specific for K^+ is supported by our observations that: (a) the depolarization is completely abolished by the K^+ channel blockers, TEA (Fig. 6) or Al^{3+} (data not shown); (b) other cations, Na^+, Li^+, and Ca^{2+}, had little or no effect on the membrane potential; and (c) the choice of counteranion for K^+, glutamate, gluconate, or chloride had no effect on the K^+-induced depolarization (data not shown). The above properties are also characteristic of the K^+ currents described by Schroeder in guard cells (19) and by Moran in *S. saman* protoplasts (9).

Lowen and Satter (8) found that apoplastic K^+ decreases in the extensor region of the pulvinus after white or blue light treatment and in the flexor region of the pulvinus after red light followed by darkness. Our results indicate that K^+ channels are open in extensor protoplasts following white or blue light treatment and in flexor protoplasts in darkness or following red light treatment. These channels, therefore, may function in the uptake of K^+ by swelling cells, which results in the decreased apoplastic K^+ seen by Lowen and Satter (8). Increased apoplastic K^+ is observed in the extensor region after treatment with red light followed by darkness (8) or darkness alone (24), and in the flexor region following white light (8). Our results indicate that inward K^+ channels are closed in these cell types following these light treatments because even at K^+ concentrations that would favor K^+ influx, depolarization did not occur.

The Role of the H^+-ATPase in K^+ Influx

The hyperpolarization after white or blue light irradiation most likely results from activation of the H^+-ATPase. This is supported by the observations that the hyperpolarization is inhibited by vanadate, an inhibitor of P type ATPases, and by DCCD, an inhibitor of H^+-ATPases, and is consistent with the finding that white or blue light stimulates apoplastic acidification in the extensor region of the *S. saman* pulvinus (7). Similarly, blue light stimulates an electrogenic pump, presumably the H^+-ATPase, in guard cells (1). As noted above, hyperpolarization was less pronounced after blue light irradiation than after white light irradiation. This may indicate that light outside the blue region contributes to the activation of hyperpolarization. Photosynthesis powered by red light, for example, might stimulate the H^+-ATPase by increasing the ATP concentration. Alternatively, other light-induced ion movements might also contribute to hyperpolarization, a possibility supported by the observation that vanadate does not totally inhibit hyperpolarization caused by either white or blue light.

Racusen and Satter (11) observed depolarization of flexor tissue when pulvini were transferred to and maintained in white light at approximately the same hour of the circadian cycle as we performed our measurements. This contrasts with the transient hyperpolarization of flexor protoplasts that we observed upon illumination with a 30-s pulse of white light. These apparently contradictory results may result from differences in the behavior of cells *in situ* compared to isolated protoplasts. In addition, several factors differ in the two studies. First, we administered a 30-s pulse of light, whereas Racusen and Satter maintained the pulvini in constant light. Second, their first measurement of membrane potential was made several minutes after initiation of light treatment, a time near the final measurement in our studies, so they might have missed a rapid hyperpolarization. Finally, the Cl^- concentration in the apoplast of the flexor region of a pulvinus in the dark may be much lower than the concentrations in our protoplast incubation medium. Chloride fluxes, which have been suggested to play an important role in stomatal closure (5), could also affect membrane potential of pulvinar cells.

Signal Transduction

The signal transduction events coupling blue light reception to the activation of the H^+-ATPase and to the opening (or closing) of K^+ channels are still not known. Recently, Short and Briggs (23) demonstrated that the phosphorylation of a 120-kD protein was a rapid event following blue light irradiation of pea epicotyls. Blue light activation of a GTP-binding protein also has been demonstrated in the plasma membranes of etiolated peas (29). Whether these events are involved, and if so, how they are involved, in the regulation of the ATPase and of K^+ channels is not known.

It has been proposed that hyperpolarization-gated K^+ channels permit inward-directed K^+ fluxes in both extensor and flexor cells (9, 17). Our data, however, indicate that membrane hyperpolarization caused by activation of the H^+-ATPase is not the sole factor controlling inward K^+ fluxes. Although hyperpolarization of both extensor and flexor protoplasts follows exposure to white or blue light, K^+ channels open in extensor protoplasts and close in flexor protoplasts. Furthermore, preincubation with vanadate inhibits the hyperpolarization in extensor protoplasts, but does not prevent the blue light-induced opening of K^+ channels. If hyperpolarization is not the only mechanism that opens inward K^+ channels, what are the others? How are these mechanisms different in flexor and extensor cells, which respond to the same light signal in opposite ways? Do these mechanisms involve regulation of Ca^{2+}, turnover of inositol phospholipids, or phosphorylation of channels? The availability of isolated protoplasts that respond to light *in vitro* in a manner consistent with their *in vivo* physiological responses should enable us to answer some of these questions.

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