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Cooperative Regulation of Cytoplasmic Streaming and Ca^{2+} Fluxes by Pfr and Photosynthesis in *Vallisneria* Mesophyll Cells[1]

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ABSTRACT

In mesophyll cells of *Vallisneria gigantea* Graebner, Ca^{2+} regulates the induction and cessation of cytoplasmic streaming. Streaming is induced when the level of calcium in the cytoplasm is lowered through light-accelerated release of Ca^{2+} from the cells (S Takagi, R Nagai [1988] Plant Physiol 88: 228–232). We have now initiated an investigation on the nature of the photoreceptor(s) that are involved in the regulation of Ca^{2+} movements across the cell membrane and of streaming. Streaming is induced only when phytochrome exists in the phytochrome—far red-absorbing form (Pfr)—and photosynthesis is allowed to take place for at least 4 minutes. The former effect is typically photoreversible by red and far-red light, and phytochrome is spectrophotometrically detectable in the crude extract from the leaves. The latter effect is assessed in terms of the wavelength dependency and the effects of diuron and atrazine, two inhibitors of photosynthesis. A similar requirement for Pfr and photosynthesis is found to be associated with the acceleration of Ca^{2+} efflux in the protoplasts. The results suggest that phytochrome and photosynthetic pigment(s) cooperatively regulate cytoplasmic streaming via modulation of the Ca^{2+} transport in the cell membrane.

The mechanisms that regulate various types of light-dependent intracellular movements have been investigated in detail in several kinds of plant cells (9, 10). It is generally accepted that these types of movement result from a chain of reactions that involve the perception of light, signal transduction, and the generation of motive force. In most of the cases examined to date, B[2] photoreceptor(s) function in the perception of light, and phytochrome is also occasionally involved (10, 25).

In leaf cells of the aquatic angiosperms *Elodea* and *Vallisneria,* rotational streaming of the cytoplasm is induced by external stimuli such as irradiation with visible light or the application of various chemicals (12). We have previously elucidated some details of the mechanism that regulates the induction and cessation of the streaming in mesophyll cells of *Vallisneria gigantea* Graebner as follows: (a) lower concentrations of intracellular Ca^{2+} are favorable for streaming, while higher concentrations have an inhibitory effect (21); (b) continuous irradiation with R induces the streaming most effectively, and the induced streaming ceases upon irradiation with FR (20); (c) the calcium content of the cytoplasm decreases when the cells are irradiated with R but increases when irradiated with FR (20); and (d) Ca^{2+} movements across the cell membrane are involved in the light-dependent changes in the cytoplasmic level of calcium (22). On the basis of these findings, we have concluded that irradiation with R accelerates Ca^{2+} efflux, leading to a decrease in the concentration of Ca^{2+} in the cytoplasm, and that this decrease in the concentration of Ca^{2+} activates the microfilament system (19) that functions in the generation of motive force.

The crucial role of Ca^{2+}, which acts as a mediator somewhere between the reception of stimuli and the resulting responses, has been suggested in a variety of plant cells (11). Furthermore, phytochrome has been suggested to exert its effect via modulation of the Ca^{2+} movements across the cell membrane in the filamentous green alga *Mougeotia* (5), in *Avena* coleoptiles (8), in *Onoclea* spores (23), in *Zea* leaves (4), and in *Triticum* primary leaves (2). However, scarcely any information has been published on the mode of action of phytochrome in the control of the movement of cations (13).

We examined the wavelength dependency of the induction of streaming in *V. gigantea* mesophyll cells in a previous study (20), but we were unable to draw any conclusions about possible photoreceptor(s). Continuous irradiation with R and FR have apparently opposite effects on Ca^{2+} movements across the cell membrane (22) and on streaming (20), whereas neither the Ca^{2+} efflux nor streaming is induced by brief irradiation with R. These observations suggest that two or more photoreceptors, probably phytochrome and some other pigment(s), are involved in the regulation of these phenomena. The present study demonstrates that at least two different photoreceptor systems, namely, phytochrome and photosynthetic pigment(s), act in cooperation to regulate streaming through control of the Ca^{2+} transport in mesophyll cells of *V. gigantea.*

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[2] Abbreviations: B, blue light; R, red light; FR, far-red light; APW, artificial pond water; λ_{max}, the peak wavelength of incident light.

MATERIALS AND METHODS

Plant Material

Young plants of *Vallisneria gigantea* Graebner were purchased at a tropical fish store and cultured in water-filled buckets with soil at the bottom. The culture was kept under a 12-h light (2000 lux) and 12-h dark regime at 19 to 20°C. The light source was a bank of 20-W fluorescent lamps (FL20S·PG, National, Kadoma, Japan).

Preparation of Specimens

The pretreatment procedures of specimens for irradiation tests have previously been described in more detail (21). At the end of the light period, a leaf segment was excised from the mother culture and cut into small pieces. A *Vallisneria* leaf is made up of several layers of mesophyll cells surrounded by a layer of epidermal cells. Each piece was further incised in the middle of the mesophyll cell layers and placed alone in a plastic vessel with APW (0.2 mM NaCl, 0.05 mM KCl, 0.1 mM $Mg(NO_3)_2$, 0.1 mM $Ca(NO_3)_2$, and 2 mM Pipes at pH 7.0). After one cycle of the original light-dark regime, each piece was mounted on a glass slide with a cover slip kept in place with a small amount of vaseline at each corner. The glass slide was immersed in a Petri dish filled with fresh APW and kept in the dark for another 12 to 18 h at 19 to 20°C. After these procedures, none of the mesophyll cells in the specimens showed any signs of streaming.

DCMU and atrazine were applied to specimens by three cycles of gentle irrigation of the space between the glass slide and the cover slip, in which the specimen was sandwiched, with APW that contained one or the other of the drugs at a given concentration.

Sources of Monochromatic Light

Such specimen was irradiated with monochromatic light of a given wavelength on the stage of a microscope (Olympus, Tokyo, Japan) through a condenser lens. Monochromatic light of 11 different wavelengths in the visible and far-red regions was provided by a combination of an interference filter (Toshiba, Kawasaki, Japan) and an appropriate cut-off filter (Toshiba, Kawasaki, Japan), as described in Table I. These filters were placed in front of a 30-W tungsten lamp (TB-1, Olympus, Tokyo, Japan). A water-filled glass tank (10 × 10 × 10 cm^3) was placed in the light path to eliminate any effects of heat. The intensity of the light was controlled with neutral density filters (Fuji Photo Film, Tokyo, Japan). In the experiments with background irradiation with B, the specimen was irradiated on the stage from above. B was produced with an interference filter (BP-45, Kenko, Tokyo, Japan) and a cut-off filter (Y-44, Kenko, Tokyo, Japan), and was applied with a fiberscope (FI-150T, Sugiura Lab. Inc., Tokyo, Japan) which was equipped with a 150-W halogen lamp (JCR, Ushio, Tokyo, Japan). The fluence rate of monochromatic light was measured with a silicon photodiode (S1337-1010BQ, Hamamatsu Photonics, Hamamatsu, Japan).

Table I. *Combination of Filters Used to Obtain Monochromatic Light of Various Wavelengths*

λ_{max}	Interference Filter	Half-Bandwidth	Cut-off Filter	Estimated Pfr:Pr(3)
(nm)		*(nm)*		
402.5	KL-40[a]	12.5	L-39[a]	0.46:0.54
423.2	KL-42	14.0	L-39	0.35:0.65
447.8	KL-45	14.0	Y-44	0.35:0.65
465.4	KL-47	15.0	Y-44	0.35:0.65
495.4	KL-50	14.1	Y-49	0.47:0.53
552.0	KL-55	18.0	0-54	0.80:0.20
578.0	KL-58	12.5	0-54	0.80:0.20
595.5	KL-60	16.5	0-59	0.80:0.20
650.0	KL-65	17.5	R-64	0.80:0.20
678.0	KL-68	13.5	R-64	0.58:0.42
729.4	KL-73	14.0	R-69	0:1.0

[a] Toshiba, Kawasaki, Japan.

Evaluation of Effectiveness

Specimens were examined under the microscope with a ×20 objective lens (Plan20, Olympus) and a ×10 ocular lens (P.10, Olympus). About 10 to 20 mesophyll cells could be observed simultaneously in the field of the microscope. The effectiveness of monochromatic light in the induction of streaming was expressed in terms of the ratio of the number of streaming cells (N_x) to the total number of observed cells (N_{total}). The criterion for a streaming cell was that chloroplast(s) exhibited continuous and unidirectional movement for at least 5 s. The apparent latent period between the start of irradiation with R and the commencement of streaming was also estimated with respect to this movement of chloroplast(s).

Preparation of Protoplasts and Assay of Ca^{2+} Flux

As previously described (22), protoplasts were prepared from mesophyll cells of *Vallisneria* by enzymatic digestion and used for spectrophotometric measurements of the Ca^{2+} movements across the cell membrane. Briefly, small pieces of leaf were treated with a solution of Cellulase Onozuka R-10 (Yakult, Tokyo, Japan) and Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) in buffer with NaCl. After washing, the protoplasts were introduced at the bottom of a measuring cuvette with a test solution that contained the Ca^{2+}-sensitive dye murexide at 50 μM. The viability of the protoplasts was confirmed by observing their cytoplasmic streaming induced by light irradiation. Changes in the concentration of Ca^{2+} in the test solution produced by light irradiation were measured as changes in A_{544} of murexide with a two-wavelength/double-beam spectrophotometer (model 356, Hitachi, Tokyo, Japan), the reference beam being set at 500 nm (14). Since the sample chamber of a spectrophotometer was opened during the actinic light irradiation, the measurements were stopped at this time. All the procedures were carried out under dim green safe light at 20 to 21°C.

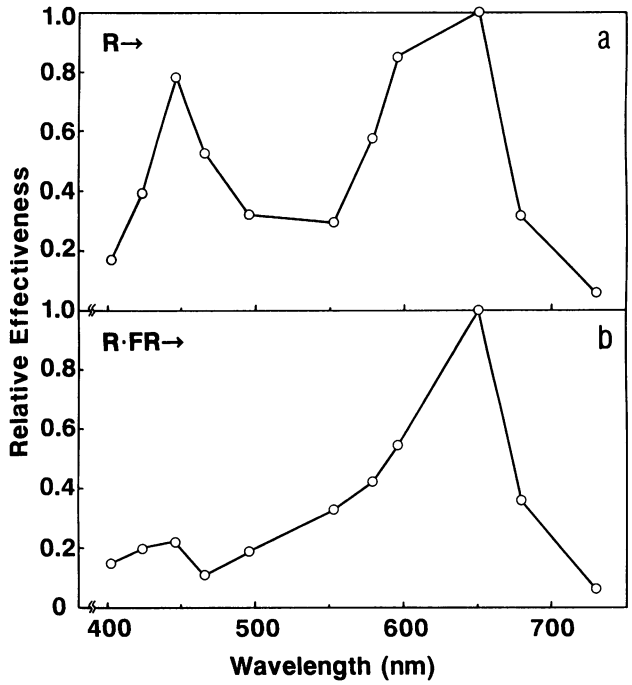

Figure 1. Wavelength dependency of the induction of streaming in *V. gigantea* mesophyll cells. The specimen was continuously irradiated with monochromatic light (0.6×10^{19} photon/s·m^2) either after irradiation with R (650.0 nm, 0.6×10^{19} photon/s·m^2) for 1 min (a) or after alternating irradiation with R and FR (729.4 nm, 0.6×10^{19} photon/s·m^2) for 1 min each (b). The ratios of N_x (the number of streaming cells) to N_{total} (the total number of observed cells) obtained at 1000 s of irradiation with individual monochromatic light were expressed in relative values, the ratio for light of 650 nm being 1.0. Between 50 and 100 cells from 5 to 10 different specimens were examined in each irradiation test.

Spectrophotometric Detection of Phytochrome

A crude phytochrome fraction was prepared from light-grown *Vallisneria* leaves by a modification of the method reported previously (1). Leaf tissues (90 g) were first chopped with a razor blade and ground in a Waring blender with 90 mL of 0.1 M potassium phosphate (pH 7.8), that contained 2 mM Na EDTA, 56 mM 2-mercaptoethanol, 1 mM PMSF, and 9 g of moistened insoluble polyvinylpyrrolidone (Polyclar AT, Gokyo-Sangyo, Osaka, Japan). After filtration through four layers of cheesecloth, the filtrate was brought to 0.035% (w/v) polyethyleneimine by the addition of a 10% (w/v) stock solution (18), the pH of which had been adjusted to 7.8 by the addition of HCl. After centrifugation at 24,000*g* for 10 min, 250 g/L ammonium sulfate was added to the supernatant to precipitate the phytochrome. The precipitate was collected by centrifugation at 24,000*g* for 20 min and dissolved in 1 mL of 0.01 M potassium phosphate (pH 7.8) that contained 1 mM Na EDTA and 28 mM 2-mercaptoethanol. The solution was clarified by centrifugation at 31,000*g* for 5 min. All the procedures were carried out at 0 to 4°C under dim green light (1).

Absorption spectra of the crude preparation of phytochrome were determined at 7°C with a dual-wavelength spectrophotometer (model 557, Hitachi, Tokyo, Japan) with slit width of 2 nm, in quartz cuvettes with a 1-cm light path, as described previously (1). Absorption spectra were measured after alternating exposures to irradiation with actinic R and FR, and stored in a desk-top computer (model 9826, Hewlett-Packard, Fort Collins, CO). Difference spectra were calculated on the computer.

RESULTS

Wavelength Dependency of Induction of Streaming

Prior to irradiation with monochromatic light to determine the wavelength dependency of the induction of streaming, specimens were irradiated with either R for 1 min or with R and FR for 1 min each to convert most of the phytochrome in the cells to either the Pfr or the Pr form, respectively. Then each specimen was continuously irradiated with monochromatic light on the stage of a microscope. At every wavelength, the ratio of N_x (the number of streaming cells) to N_{total} (the total number of observed cells) increased with time and exhibited a linear relationship to the logarithm of quantum number for almost the entire first 1000 s of irradiation (data not shown). The ratios of N_x/N_{total} obtained at 1000 s of irradiation were expressed as relative effectiveness of induction of streaming in Figure 1.

It became evident that there were two distinct peaks, a minor one at 450 nm and a major one at 650 nm, when brief irradiation with R was applied before continuous irradiation with monochromatic light (Fig. 1a). However, the minor peak was no longer significant when a brief irradiation with FR was applied immediately after the R irradiation, while the major peak remained unchanged (Fig. 1b).

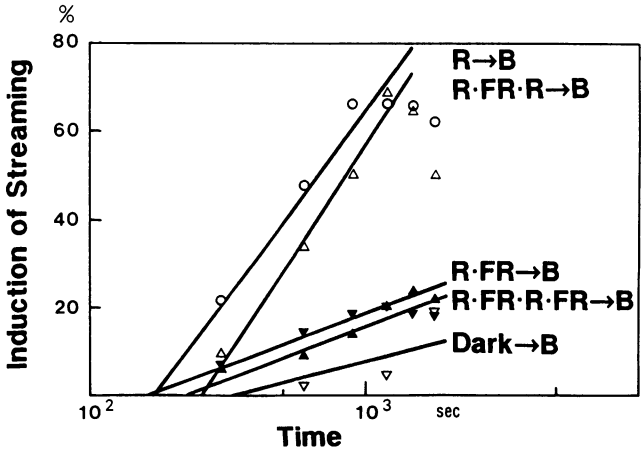

Figure 2. Photoreversible effect of R and FR on the induction of streaming in *V. gigantea* mesophyll cells. Specimens were continuously irradiated with B (447.8 nm, 0.6×10^{19} photon/s·m^2) either immediately after the last dark treatment of the pretreatment procedures or after alternating irradiation with R and FR for 1 min each. N_x was counted at 5-min intervals, and the N_x/N_{total} ratio was plotted as percentage against duration of irradiation with B.

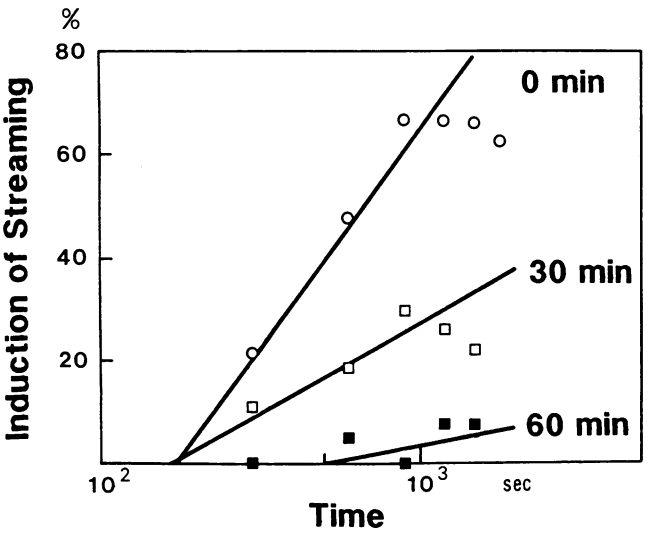

Figure 3. Inhibitory effects of DCMU on the induction of streaming in *V. gigantea* mesophyll cells. Specimens were treated with DCMU at 10^{-7} M in the dark for 0, 30, and 60 min. Then the specimens were continuously irradiated with B after a 1-min irradiation with R. The N_x/N_{total} ratio was plotted against duration of irradiation. N_{total} was 30 to 100.

Reversible Effects of R and FR on Induction of Streaming by B Irradiation

The R-FR photoreversibility of the induction of streaming was examined. Specimens were irradiated with continuous B either immediately after the last 12- to 18-h dark treatment that formed part of the pretreatment procedures or after alternating exposures to irradiation with R and FR. The results in Figure 2 show that streaming was induced in only 7.5% of the dark-adapted specimens by continuous irradiation with B for 1000 s, whereas the induction of streaming by B irradiation took place in 64.5% of the specimens when they were briefly irradiated with R for 1 min prior to the B irradiation. This effect of R was negated by a FR irradiation for 1 min if applied immediately after the R irradiation, and the photoreversibility was repeatedly observed after alternating irradiation with R and FR. In all cases, the induction progressed linearly during the first 1000 s after the start of irradiation with B. Thus, it appears that brief irradiation with R is a crucial prerequisite for the induction of streaming by the B irradiation.

Effects of DCMU and Atrazine on Induction of Streaming

Next we examined whether photosynthesis is involved in the induction of streaming, using two inhibitors. The induction of streaming by continuous irradiation with B, after brief preirradiation with R, was considerably suppressed in the presence of DCMU at 10^{-7} M (Fig. 3). The value of N_x/N_{total} after irradiation for 1000 s with B decreased from 64.5% to 27% after 30 min of treatment with DCMU, and to 3.5% after 60 min. DCMU at concentrations higher than 10^{-7} M inhibited the induction of streaming by continuous irradiation with R, and a second inhibitor of photosynthesis, atrazine, has a similar effect at 50 μM (data not shown). The results suggest that the induction of streaming requires some factor(s) related to photosynthesis.

Effect of Brief R Irradiation during Continuous B Irradiation

Continuous irradiation with B for less than 10 min does not induce streaming (20; Fig. 2). However, when irradiation with R was briefly applied at different times during continuous irradiation with B, streaming was rapidly induced depending upon the timing of the exposure to R. In this case, the minimal required intensity of R to induce streaming was 37 J/m^2 (650 nm, 0.6×10^{19} photon/s·m^2, 20 s). This value is about 9% of that required in the case of continuous R irradiation without the background B irradiation (20). In specimens that had been irradiated with background B for 6 min or longer, the apparent latent period between the start of irradiation with R and the commencement of streaming was 65 to 75 s (Fig. 4). The shorter the time during which cells were exposed to background B, the longer was the latent period. When we simultaneously applied continuous irradiation with B and a brief irradiation with R, 285 s elapsed before streaming was induced. In the case of continuous irradiation with R, the

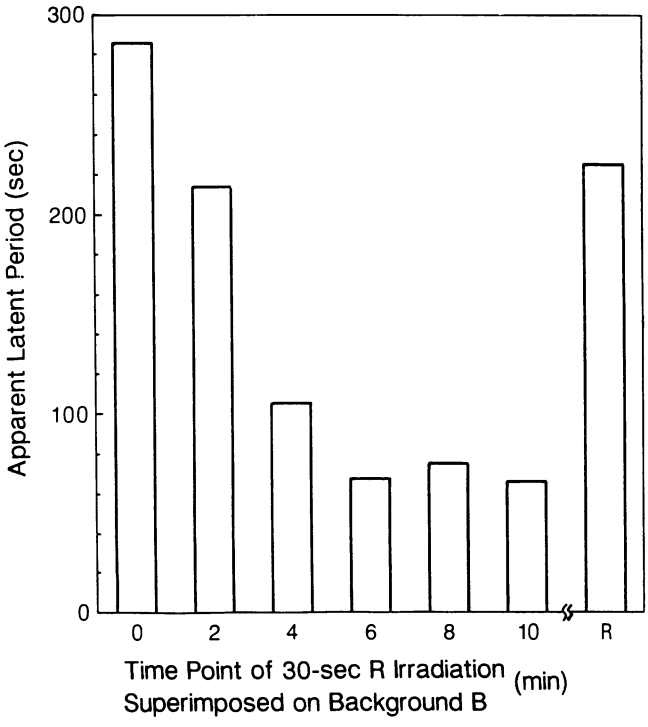

Figure 4. The apparent latent period in the induction of streaming in *V. gigantea* mesophyll cells. Continuous irradiation with B (451.0 nm, 0.6×10^{19} photon/s·m^2), which cannot induce streaming by itself, was applied as background irradiation. 0, 2, 4, 6, 8, and 10 min after the start of irradiation with B, a 30-s irradiation with R (650.0 nm, 0.6×10^{19} photon/s·m^2) was superimposed on the background irradiation. The apparent latent period between the start of R irradiation and the commencement of streaming was examined. R indicates the latent period observed in the case of continuous irradiation with R alone.

apparent latent period was 225 s. It appears that the effect of photosynthetic pigment(s) is separable from that of phytochrome, and that streaming can be induced only in the presence of Pfr.

Ca^{2+} Fluxes under Photocontrol

Brief irradiation with R does not induce the Ca^{2+} efflux in protoplasts isolated from the mesophyll cells of *V. gigantea* (22). We asked whether B and R have an affect on Ca^{2+} fluxes in the protoplasts similar to that observed in the induction of streaming. Figure 5a shows a typical set of results, indicating that the concentration of Ca^{2+} in the test solution increased after a 10-min irradiation with B subsequent to a 1-min irradiation with R. The number of Ca^{2+} released from a protoplast was estimated at 280 fmol. This value is comparable to that examined previously in the case of 10-min irradiation with R (22). The increased concentration of Ca^{2+} fell to the original level within 10 min of further continuous irradiation with FR. Then the protoplasts were treated with DCMU at 10^{-7} M for 60 min in the dark. A brief irradiation with R and a subsequent continuous irradiation with B no longer accelerated the Ca^{2+} efflux.

We reinforced the above-mentioned conclusion with the results of our next experiment. While Ca^{2+} was not released by a 10-min irradiation with B alone, R irradiation for 1 min between a prior 10-min and a subsequent 5-min irradiation with B accelerated the Ca^{2+} efflux (Fig. 5b). It appears, therefore, that Ca^{2+} fluxes across the cell membrane are under the cooperative control of phytochrome and photosynthetic pigment(s).

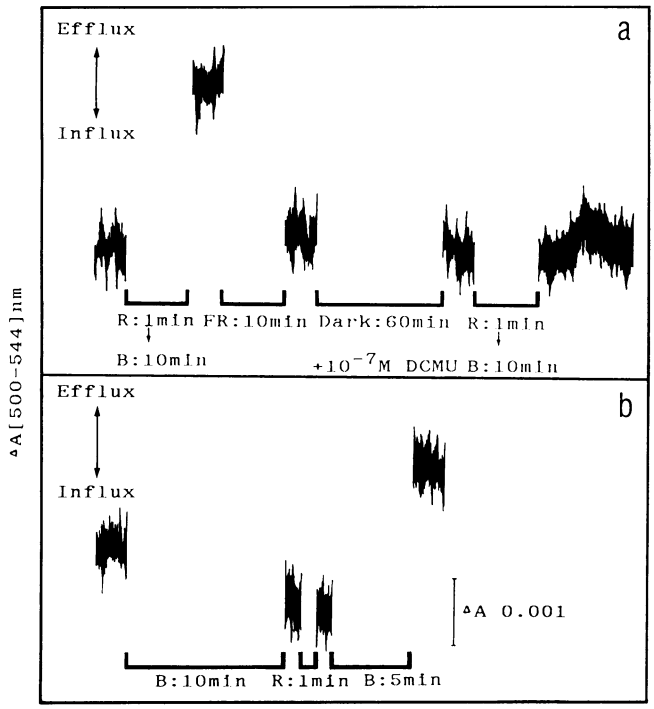

Figure 5. Effects of light irradiation on Ca^{2+} fluxes in protoplasts isolated from *V. gigantea* mesophyll cells. Changes in concentrations of Ca^{2+} in the test solutions bathing the mesophyll protoplasts were measured spectrophotometrically using the Ca^{2+}-sensitive dye murexide. The ordinate is the difference between A_{544} (the measuring wavelength) and A_{500} (the reference wavelength) of murexide. Efflux indicates an increase in the Ca^{2+} concentration and Influx indicates a decrease. a, About 10^4 protoplasts were irradiated with B (447.8 nm, 0.1×10^{19} photon/s·m^2) for 10 min after a 1-min irradiation with R (650.0 nm, 0.1×10^{19} photon/s·m^2). After the spectrophotometric measurement, the same protoplasts were irradiated with FR (729.4 nm, 0.1×10^{19} photon/s·m^2) for 10 min and kept in the dark for 60 min in the presence of 10^{-7} M DCMU. Then the second series of irradiations with R and B was applied. b, Another 10^4 protoplasts were irradiated with B for 10 min, then with R for 1 min, and finally with B for 5 min. The concentration of Ca^{2+} was measured after each irradiation. In both experiments, the total volume of the sample was 0.7 mL. The spectrophotometric measurements were stopped during each actinic light irradiation.

Phytochrome in Vallisneria

We attempted to confirm the presence of phytochrome in the leaves of *V. gigantea*. A crude extract of phytochrome was prepared by ammonium sulfate fractionation after differential centrifugation of the leaf homogenate in the presence of polyethyleneimine. The absorption spectrum of this fraction (Fig. 6a) indicated that most of the Chl was successfully removed. The difference spectrum (Fig. 6b) shows a maximal increase and decrease in absorbance at 665 and 722 nm, respectively. The ratio of the maximum decrease in absorbance to the maximum increase in absorbance was 0.63.

DISCUSSION

The present study clearly demonstrates that the Ca^{2+} efflux across the cell membrane is accelerated as a result of photosynthesis in the presence of Pfr, and that the consequent decrease in the level of calcium in the cytoplasm brings about the induction of streaming in mesophyll cells of *V. gigantea*.

The wavelength dependency of the induction of cytoplasmic streaming determined after alternating irradiation with R and FR (Fig. 1b) is similar to that obtained in an analysis of dark-adapted specimens (20), in which most of the phytochrome is thought to exist as Pr. Light of 650 nm is known to function efficiently in the transformation of Pr to Pfr (3) and is assumed to be absorbed by photosynthetic pigment(s). Hence, these may account for the highest effectiveness of R regardless of the preirradiation treatment. Light of 450 nm and of 680 nm is thought to be most effectively absorbed by photosynthetic pigment(s), but the effectiveness of light at these wavelengths on the induction of streaming was unexpectedly low in the present study (Fig. 1). Continuous irradiation with light at these wavelengths may not result in or maintain the level of Pfr that is required for the induction of streaming (3; Table I). In specimens in which photosynthesis takes place for longer than 6 min, streaming can be induced upon the formation of Pfr with an apparent latent period of as little as 65 to 75 s (Fig. 4). We have thus succeeded in separating the effect of Pfr from that of photosynthetic pigment(s).

Exogenously applied DCMU and atrazine inhibited the induction of streaming (Fig. 3). The acceleration of the Ca^{2+} efflux was not observed in the presence of DCMU (Fig. 5a).

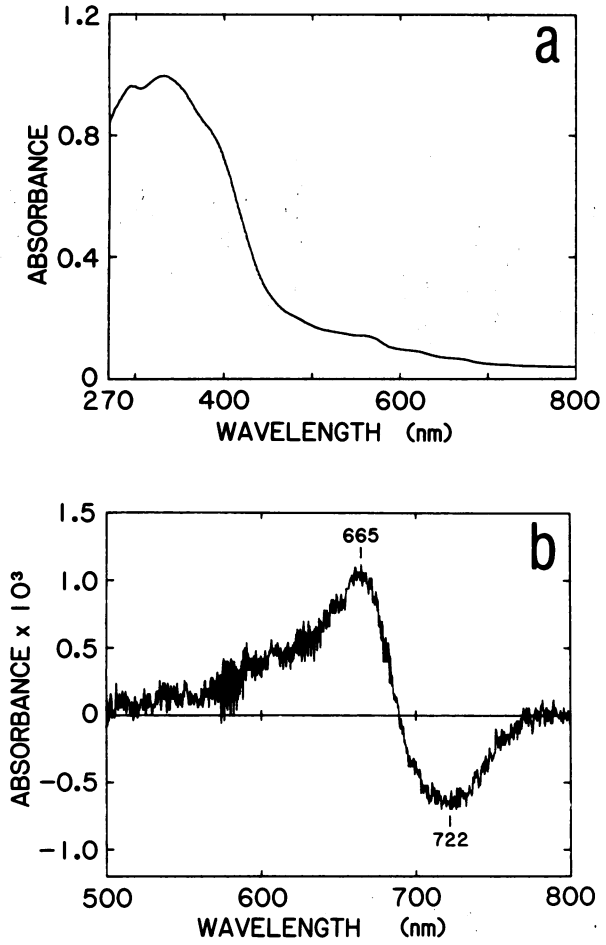

Figure 6. Absorption spectrum (a) and difference spectrum (b) of the crude fraction of phytochrome from leaves of light-grown *V. gigantea*. The crude fraction was prepared by ammonium sulfate fractionation after differential centrifugation of the leaf homogenate in the presence of polyethyleneimine. The difference spectrum was obtained by subtracting the absorption spectrum measured after irradiation with actinic R from that measured after irradiation with actinic FR, and was the average of five sets of measurements from one sample.

Therefore, it appears that photosynthetic pigment(s), rather than B photoreceptor(s) (16, 17), is functioning as one of the photoreceptors. It is well known that both phytochrome and B photoreceptor(s) are involved in several examples of single physiological responses (6, 9, 10, 25). In epidermal cells of *Vallisneria spiralis*, Seitz (15) has examined the mechanism of the orientation movement of chloroplasts and of the induction of cytoplasmic streaming under photocontrol. He proposed that B photoreceptor(s) and photosynthetic pigment(s) are involved in light perception associated with both types of movement. Hitherto, however, no evidence has been presented of the apparent cooperative action of phytochrome and photosynthetic pigment(s) in light-dependent intracellular movement.

We reported previously that EGTA, a chelator of Ca^{2+}, can induce streaming in dark-adapted specimens (19). The level of ATP that is indispensable for the generation of motive force seems to be adequately maintained even in the dark. Acceleration of the Ca^{2+} efflux occurs only when photosynthesis takes place under continuous light irradiation. Because the acceleration is suppressed in the presence of vanadate, an inhibitor of ATPase (22), we can assume that photosynthetic pigment(s) ultimately contributes the supply of energy needed for the Ca^{2+} transport.

We have succeeded in the spectrophotometric detection of phytochrome in extracts of leaves of light-grown *V. gigantea*. The spectral characteristics demonstrated in Figure 6 are consistent with those of large phytochrome (24). The yield of phytochrome in the crude fraction was 1.9×10^{-5} Δ (ΔA)/g fresh weight, which corresponds to 16 ng of large phytochrome/g fresh weight, if we assume that the molar absorption coefficient at 667 nm of large phytochrome Pr is 124,000 (KT Yamamoto, unpublished data). The yield is about a tenth of that from light-grown pea seedlings (1). Phytochrome has been reported to exist in multiple forms: as photolabile and photostable pools at the physiological level; and as genetically distinct molecular species of phytochrome I and phytochrome II at the protein level (1, 7, 18). At present we can answer neither the role of Pfr nor the question as to which pool and which molecular species is functioning in the present case.

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