Photoinduced Electric Currents in Carotenoid-Deficient *Chlamydomonas* Mutants Reconstituted with Retinal and Its Analogs

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ABSTRACT Reconstitution of the photoelectric responses involved in photosensory transduction in "blind" cells of *Chlamydomonas reinhardtii* carotenoid-deficient mutants was studied by means of a recently developed population method. Both the photoreceptor current and the regenerative response can be restored by addition of all-*trans*-retinal, 9-demethyl-retinal, or dimethyl-octatrienal, while the retinal analogs prevented from 13-*cis*/*trans* isomerization, 13-demethyl-retinal and citral, are not effective. Fluence dependence, spectral sensitivity, and effect of hydroxylamine treatment on retinal-induced photoelectric responses are similar to those found earlier in green strains of *Chlamydomonas*, although an alternative mechanism of antenna directivity in white cells of reconstituted "blind" mutants (likely based on the focusing effect of the transparent cell bodies) leads to the reversed sign of phototaxis in mutant cells under the same conditions. The results obtained indicate that both photoreceptor current and and regenerative response are initiated by the same or similar rhodopsins with arhaebacterial-like chromophore(s) and prove directly the earlier suggested identity of the photoreceptor pigment(s) involved in photomotile and photoelectric responses in flagellated algae.

INTRODUCTION

Unicellular flagellates respond to light stimuli by orienting their movement to the light direction under continuous illumination (phototaxis) and/or by photophobic ("stop") responses when a cell is subjected to an abrupt change in light intensity (Diehn et al., 1977). These behavioral responses allow microorganisms to look for and accumulate under optimal light conditions (Nultsch and Hader, 1988).

Electric events on the outer cell membrane seem to be deeply involved in photosensory transduction of flagellated organisms (for review see Sineshchekov and Litvin, 1988; Sineshchekov, 1991a,b). Blue-light-induced electric responses were first found in a single cell of *Haematococcus pluvialis* by means of a specially developed suction pipette technique (Sineshchekov et al., 1978; Litvin et al., 1978). Later an application of the micropipette technique was expanded to *H. lacustris* (Sineshchekov et al., 1991) and a wall-less mutant of *Chlamydomonas reinhardtii* yielding similar results (Sineshchekov, 1988; Harz and Hegemann, 1991).

Using the recently developed method of photoelectric measurements in cell suspension, the typical blue-light-induced electric responses were measured in a number of *C. reinhardtii* strains: wild type, high sensitive, stigma-less (Sineshchekov et al., 1992b; Kreimer et al., 1992) and in several other organisms: *Hafniomonas, Spermatozopsis*, and heterotrophic *Polytomella* (Sineshchekov and Nultsch, 1992). Close similarities found in these objects allow us to assume a basically universal mechanism of photosensory transduction for all the flagellates with an intrachloroplast stigma.

Two basic elements of light-induced electric signals could be clearly distinguished. The first one has no measurable delay and depends on the gradation of light intensity (Sineshchekov et al., 1990). It is caused by a local inward positive current which can be referred to as the photoreceptor current (PRC), because of its localization in the region of the photoreceptor and its spectral sensitivity similar to that of light-induced behavioral responses (Sineshchekov et al., 1978; Litvin et al., 1978; Ristori et al., 1981; Harz and Hegemann, 1991). The PRC is permanent under continuous illumination (Sineshchekov et al., 1978) and depends on the direction of incident light: the signal amplitude was found to be several times larger when the cell was illuminated from the stigma-bearing side (Sineshchekov, 1988, 1991a,b; Harz and Hegemann, 1991). These findings indicate involvement of a direction-sensitive antenna (Foster and Smyth, 1980) in the PRC generation.

Thus, in a freely swimming cell, unilateral illumination would result in a modulation of the membrane potential due to the cell rotation. This modulation has been suggested to trigger the asymmetrical changes of beating of the two flagella observed in the cell upon periodic illumination (Sineshchekov, 1983; Sineshchekov and Litvin, 1988; Ruffer and Nultsch, 1990, 1991), which seem to be the elementary orientation response of phototaxis.

When the flash or step-up light stimulus exceeds a threshold level, the PRC becomes superimposed by a transient regenerative response (RR). This second type of photoinduced electric signals could be related to photophobic motile response. The RR originates from a depolarizing calcium current in the anterior flagella-bearing part of the cell (Sineshchekov, 1983, 1988, 1991a; Harz and Hegemann,

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1991). Both the electric RR and motile photophobic response are all-or-none, appear above similar threshold fluences, and the delay time of both of them is fluence-dependent (Sineshchekov, 1978).

The suggestion that the RR initiates the photophobic response was confirmed by the direct correlation between electric and flagella motile responses found in a cell held on the tip of a measuring micropipette. Typical for photophobic response transition from cilia to flagella type of beating (Schmidt and Eckert, 1976) and the RR were never observed separately (Sineshchekov et al., 1978; Sineshchekov 1991a).

The two photoelectric responses (PRC and RR) seem to be the two steps of the same cascade involved in the mechanism of the sensory transduction in flagellates (Sineshchekov and Litvin, 1988). The reciprocal of the RR delay time is proportional to the amplitude of the PRC, so that the integral under the PRC before the start of the RR is constant for different fluences. However, when the cell membrane is hyperpolarized by photosynthetically active background illumination (Sineshchekov et al., 1976), this integral significantly increases (Sineshchekov, 1988, 1991a,b). These findings gave rise to the conclusion that the RR is triggered when membrane is depolarized by the PRC under the critical level and likely involves the operation of voltage-gated calcium channels. The correspondence of the action spectra of the PRC and the photophobic response (Sineshchekov and Litvin, 1988) favors the suggested relation of these phenomena.

The photoreceptor pigment mediating the photoelectric responses described above has not been yet identified. The overall shape of the action spectrum of the PRC (Litvin et al., 1978; Harz and Hegemann, 1991), partial inhibition of the PRC in hydroxylamine-treated cells (Sineshchekov et al., 1991), estimation of the optical cross-section of the photoreceptor molecules and their orientation within the cell membrane (Sineshchekov, 1991a,b) indicate the involvement of rhodopsin-type pigment(s) in generation of the photoelectric responses, but these data obviously do not allow us to make a final conclusion.

Taking into account the above-mentioned relation of the photoelectric responses to the photomotile responses of the cell, the nature of the pigment mediating the photoelectric responses could be in principle deduced from the results of the behavioral studies; however, this approach is indirect and meets certain difficulties.

The most important information on rhodopsin photoreceptor for behavioral responses were obtained by reconstitution studies in the "blind" cells of *C. reinhardtii* carotenoid-deficient mutants, which restore normal phototaxis upon addition of exogenous retinoid compounds (Foster et al., 1984). However, the results of the behavioral studies carried out with different techniques after that pioneering work indicate that apparently quite different pigments might participate in the photobehavior of *Chlamydomonas*.

On the basis of the single-cell tracking it had been concluded that both phototaxis and photophobic response in *Chlamydomonas* are mediated by a rhodopsin-type pigment(s) which contains all-*trans*-retinal at the ground state and undergoes photoinduced all-*trans*/13-*cis*-isomerization necessary for the photoreceptor signaling (Takahashi et al., 1991; Hegemann et al., 1991; Lawson et al., 1991). These features are characteristic of bacterial rhodopsins rather than for visual pigments from higher animals. On the other hand, in extended time population assays it has been found that retinal analogs prevented from isomerization and even short-chained compounds such as hexanal are also effective in restoration of phototaxis (Foster and Saranak, 1988; Foster et al., 1989).

Besides this still confusing discrepancy in the results of behavioral studies, one cannot exclude the existence of several different rhodopsin species mediating cell behavior and photoelectric phenomena (as in *Halobacterium halobium*; Stoeckenius, 1985). That is why the direct investigation of the nature of the photoreceptor pigment(s) involved in the generation of the photoelectric responses is highly desirable.

In the present work we have studied restoration of both photoelectric responses (PRC and RR) in "blind" mutants of *C. reinhardtii* by all-*trans*-retinal and a number of retinal analogs, as well as the effect of hydroxylamine on these responses. Preliminary data concerning carotene and retinal dependence of the photoelectric responses had been reported elsewhere (Sineshchekov, 1991b; Sineshchekov et al., 1992a).

MATERIALS AND METHODS

Photoinduced currents were measured by means of a recently developed population method (Sineshchekov, 1991b; Sineshchekov et al., 1992a,b). In most experiments an improved version of the set-up was used. Cell suspension (0.5 to 1 $\times$ 10^7 cells/ml) in a rectangular cuvette (2 $\times$ 0.8 $\times$ 2 cm) was excited by a flash from the side and the electrical current was measured in the horizontal plane to exclude possible effects of gravi- and aerotactically induced preorientation of the cells.

Two modifications of the method were used. In the first one (unilateral, UL mode) the flash was applied along the line between the electrodes. The electrode remote from the excitation light source was considered as the measuring electrode. In the second configuration of the set-up (preoriented, PO mode) the measuring cuvette was rotated 90° so that the line between the two electrodes was perpendicular to the direction of the excitation flash.

Flash stimuli were provided by a photo-flashgun (Hanimex Pz 1*44, Hong Kong; rise time 0.3 ms) in combination with interference (half-bandwidth 5–11 nm) or broad-band filters. For measurements with the improved time resolution a dye-laser-pumped dye laser (pulse duration 10 ns, maximum pulse energy ~5 $\times$ 10^{-3} J) with coumarin 307 (Lambda Physik, Göttingen, Germany; wavelength, 500 nm) was used.

Continuous (orienting) illumination was produced by a slide projector supplied with anti-heat and interference or broad-band filters. The direction of continuous illumination in the PO mode was the same as the direction of the excitation flash in the UL mode, e.g., the measuring electrode was remote from the light source.

Fluence of excitation flashes and fluence rate of orienting light were attenuated by changing the distance from the photo-flashgun to the sample or by neutral density filters and were measured by a DC1010 light meter (Karl Lambrecht Corporation, Chicago, IL).

Photoelectric signals were amplified by a Keithley 427 current amplifier (Keithley Instruments Inc., Cleveland, OH) or by a laboratory-made high input impedance voltage amplifier (based on an LF 356 IC, rise time of an unfiltered rectangular test signal 1.5 $\times$ 10^{-6} s with 0.5 $\times$ 10^6 Ohm resistance). The signal kinetics was shown not to depend on whether the current or voltage amplifier was used for measurements. In the case of the voltage

amplifier the sample impedance was measured and the output signals were recalculated to units of current.

DMA interface supported by pCLAMP program was used for performance of the experiments and data acquisition, or the signals were digitized and stored in a Thurlby DSA-524 computer-controlled transient recorder (Thurlby Electronics Ltd., Huntington, UK).

The programs pCLAMP-5.5 (Axon Instruments, Inc.) and SPSERV-3.11 (courtesy of Dr. C. Bagyinka, Szeged, Hungary) were used for the analysis of the results.

Carotenoid-deficient "blind" mutants 516/white-3, N164, 494/31, and the green strain 495(+) of *C. reinhardtii* were obtained from Dr. A. S. Chunaev (St. Petersburg, Russia), the "blind" mutant CC2359 was kindly provided by Dr. P. Hegemann and Dr. C. Nonnengasser (Martinsried bei Munchen, Germany).

Growth conditions of the green *Chlamydomonas* strains and formation of gametes can be found in Sineshchekov et al. (1992b). *Chlamydomonas* "blind" mutants were grown in the dark on agar plates of the tryptone-containing medium (Nichelson-Gutrie and Hudock, 1983) at 28°C.

All-*trans*-retinal was purchased from Sigma Chemical Co. (St. Louis, MO). 9- and 13-demethyl retinal, dimethyl-octatrienal, and citral were the kind gift of Dr. P. Hegemann (Martinsried bei Munchen, Germany). 13-*cis*- and 13-*trans*-locked retinal analogs were generously provided by Prof. K. Nakanishi and Dr. F. Derguini (New York, NY). Retinal and analogs were delivered to cells in methanol solution. Final methanol concentration did not exceed 0.5%. All chemicals were of analytical grade. Experiments were carried out at room temperature (22–24°C).

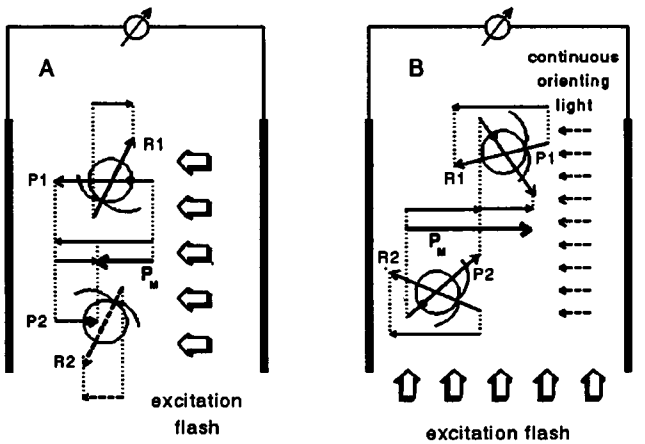

FIGURE 1 Schematic drawing of photoelectric measurements in cell suspensions in two modifications. (*A*) UL mode; (*B*) PO mode. Arrows P1, P2, R1, and R2 represent the photocurrents generated by the individual cells in suspension. P1 and P2 are the photoreceptor currents localized in the stigma region (shown as a solid circle within each cell). R1 and R2 are the RRs localized in the anterior part of the cells. The projections of the individual photocurrents on the measuring direction (the line between the electrodes) are shown by the arrows. Bold arrows (P_M) represent the resultant photoreceptor currents measured in suspension.

RESULTS

Origin and interpretation of photoelectric signals in cell suspensions

Two modifications of the method used give complementary information about both photoelectric responses (PRC and RR), as well as the properties of the directional antenna and photo-orientation of the cells.

In the first one (unilateral, UL mode) the suspension of randomly oriented cells is excited by a flash applied along the line between the measuring electrodes. It has been shown earlier by means of the single-cell recording that directional sensitivity of the photoreceptor apparatus in flagellated algae results in attenuation of the photoreceptor current depending on the light incidence (Sineshchekov, 1988, 1991a,b; Harz and Hegemann, 1991). It means that the amplitudes of the PRC generated by individual cells in suspension depend on their orientation with respect to the excitation flash. Those cells which are oriented by the direction of maximum antenna sensitivity along the line between electrodes toward the flash generate the biggest photoreceptor currents (P1 in Fig. 1 *A*). These local asymmetric currents are not completely compensated by the corresponding smaller currents from the cells of the opposite orientation (P2 in Fig. 1 *A*). The difference in photocurrents (P_M in Fig. 1 *A*) is picked up by the measuring electrodes.

The sign of the signal measured in the UL mode corresponds that of the signal generated by the cells with maximum response (Fig. 1 *A*), but the amplitude of this signal is determined by both the amplitude of the maximum response and by capacity of directional antenna. The role of antenna in formation of the UL mode photoelectric signal in cell suspension recently had been shown directly using a stigmaless mutant of *C. reinhardtii* (Kreimer et al., 1992).

The PRC is localized in the stigma region and its direction corresponds to the direction of maximum antenna sensitivity. Asymmetric currents localized at certain angles from this direction would also contribute to the signal measured in the UL mode, but this contribution would be proportional to their projections in this direction.

Electron micrographs of the cells of the green *Chlamydomonas* strain 495(+) (kindly provided by Dr. G. Kreimer, Koln, Germany) show that the angle between the stigma and flagella-bearing end of a cell is slightly larger than 90°. Since the RR likely appears at the flagella base, one can expect that the projection of this current on the measuring direction and thus its contribution to the signal must be small and having the opposite sign as compared to that of the PRC (Fig. 1 *A*). Indeed, a small but obvious negative wave is present in the signal measured in the UL mode (Fig. 2, *trace 1*). Its kinetics, fluence-dependent delay, strict calcium dependence, and other features strongly support that it reflects the RR generation.

If all the cells in suspension generate the RR (having the same amplitude), these individual photocurrents would compensate each other and no resultant unbalanced signal would be measured in the unoriented mode. However, the probability of the RR is much higher when the antenna is directed toward the excitation flash (Fig. 1 *A*, R1) as compared to the case of the opposite orientation of the cells (Fig. 1 *A*, R2). This difference provides the resultant RR signal measured in suspension in UL mode.

In the second configuration of the set-up (preoriented, PO, mode) the measuring cuvette was turned by 90° so that the line between the two electrodes was perpendicular to the direction of the excitation flash. In this case no flash-induced electric response can be recorded in suspension of randomly

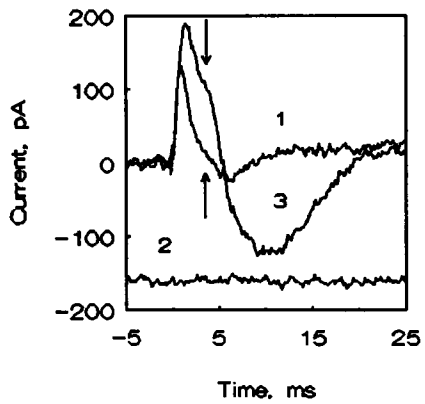

FIGURE 2 Photoelectric signals measured in UL and preoriented PO modes of the experimental set-up in suspension of green cells of the *C. reinhardtii* strain 495(+). Excitation: photo-flashgun with 500-nm broad-band filter, 5×10^{18} photons·m^{-2}. 1, signal in UL mode: the excitation flash applied along the line between the measuring electrodes; 2, no signal, if the flash is applied in the perpendicular direction; 3, signal in PO mode: the flash applied as in 2, but the cell suspension has been preilluminated with 500 nm continuous light (10^{18} photons·m^{-2}·s^{-1}) along the line between the electrodes for 10 s before the excitation. *Arrows* indicate the start of the RR.

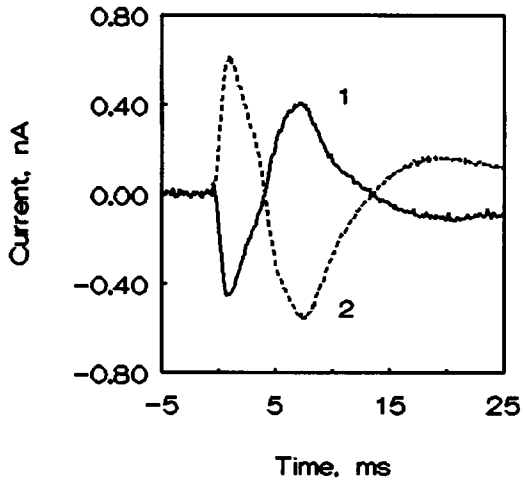

FIGURE 3 Flash-induced electric responses measured in PO mode in suspension of *C. reinhardtii* strain 495(+) cells with positive (1, *solid line*) and negative (2, *dashed line*) phototaxis. Excitation: photo-flashgun with 500-nm broad-band filter, 5×10^{18} photons·m^{-2}. The cell suspension has been preilluminated with 500 nm continuous light (10^{18} photons·m^{-2}·s^{-1}) along the line between the electrodes for 10 s before the excitation. Both signals are measured in the same sample: to induce the negative phototaxis, the suspension of positively phototactic cells was illuminated with red light (wavelength >630 nm, 40 W·m^{-2}) for 15 s.

oriented cells (Fig. 2, *trace 2*), since the projections of all non-compensated currents (which appear due to asymmetric excitation of the sample) on the line between the electrodes are equal to 0.

However, if the cell suspension is illuminated by continuous light along the line between the electrodes that would cause phototactic pre-orientation of the cells in this direction, a flash-induced electric signal appears (Fig. 2, *trace 3*). The signal is composed of both PRC and RR. The ratio between RR and PRC amplitudes substantially increases in the PO mode of measurements. This fact could be predicted taking into account that the RR is localized at the anterior part of the cell and thus its projection on the measuring direction must be maximal when the cell is oriented along the line between electrodes (Fig. 1 *B*).

Only phototactically active short-wavelength continuous light and only in suspension of motile cells results in an appearance of the flash-induced PO mode signal. As it has been shown earlier by means of behavioral assay, illumination with photosynthetically active red light induces negative phototaxis in suspension of positively phototactic *C. reinhardtii* cells of green strains 494(−) and 495(+) (Sineshchekov et al., 1989). In present work we used this phenomenon to investigate the correlation between the sign of phototaxis and the sign of the photoelectric signal in the PO mode. Using a special modification of a cuvette which allows parallel observation of the cell movement due to continuous preorienting light, we have found that the sign of the flash-induced signal is determined by the sign of phototaxis (Fig. 3).

The amplitude of the signal elicited by a standard test flash in the PO mode depends on the duration, spectral range, and fluence rate of the preorienting light in the same way as phototactic orientation of the cells, monitored independently and simultaneously by recording the scattering of the red light (data not shown).

These facts strongly support the above interpretation of the signal measured in the PO mode and show that the amplitude of such a signal upon a standard test flash can be used as an instant probe of the phototactic orientation of cells in suspension (Sineshchekov et al., 1992b).

All-*trans*-retinal-induced photoelectric responses measured in suspension of white cells in comparison with those in green control

No photoelectric response was recorded from gametes of all tested carotenoid-deficient mutants grown in the dark (Fig. 4, *curve 1*). However, an electric signal appears after addition of all-*trans*-retinal to the cell suspension (Fig. 4, *curve 2*). Restoration of the photoelectric signal in the UL mode is completed within 1 min after addition of retinal. In a range of 5–250 nM, both the signal amplitude and kinetics do not depend on retinal concentration (data not shown).

The signal in retinal-reconstituted white cells has the same kinetics as found in green cells, indicating the restoration of both PRC and RR, but the sign of both responses in the UL mode is reversed as compared with the sign in the green cells (compare Fig. 2, *curve 1* and Fig. 4, *curve 2*).

According to the principle of UL mode measurements described above, the appearance of the signal in white cells after retinal addition implies function of a directional-sensitive antenna which has to be taken into account to understand the origin of the signal. In single-cell experiments it has been found that both PRC an RR always have the same depolarizing direction and can be hardly expected to change

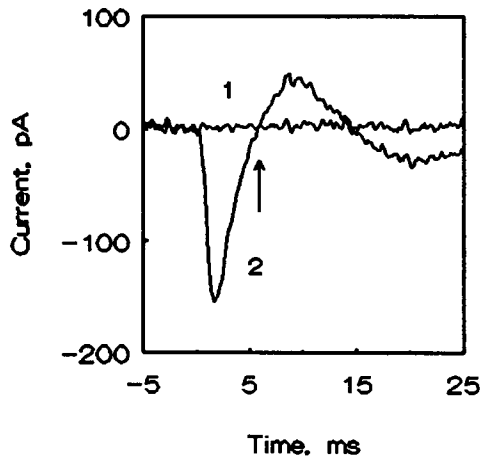

FIGURE 4 Reconstitution of the photoelectric responses in dark-grown gametes of the carotenoid-deficient *C. reinhardtii* strain 516/white-3 with 2×10^{-8} M of all-*trans*-retinal. 1, mutant cells without retinal; 2, the same sample 10 min after retinal addition. Excitation by the laser, 10^{-3} J. *Arrow* indicates the start of the RR.

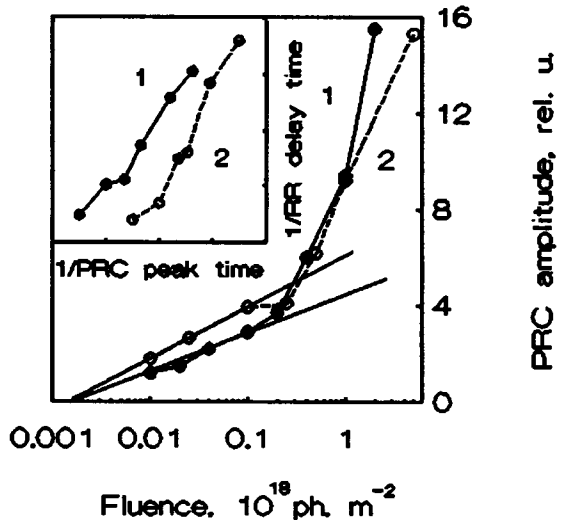

FIGURE 5 Fluence dependence of the photoreceptor current in green and in white reconstituted cells of *C. reinhardtii*. *Inset:* Correlation between the kinetics of two photoelectric responses in green and in white reconstituted cells: *abscissa,* reciprocal of the PRC peak time; *ordinate,* reciprocal of the RR delay time. 1, *solid lines:* green cells, strain 495(+); 2, *dashed lines:* white cells, strain 494/31, reconstituted with 10^{-6} M all-*trans*-retinal. Excitation: photo-flashgun with 500-nm broad-band filter.

sign in reconstituted cells. Thus we conclude that the reason for the reversed sign of the signal measured in suspension of white reconstituted cells must be due to different antenna properties.

The reversed sign of the signal indicates that in a white cell the photoreceptor absorbs more light (and generates a larger signal) when it is turned away from the light source, the situation opposite to that in a green cell. It can be explained by the focusing ("lens") effect of the almost transparent cell body of a mutant cell, instead of shading/reflecting action of the stigma and the chloroplast in a normal green cell. A similar principle of antenna directivity has been suggested for fungal phototropism (Shropshire, 1962).

To gain more information about the retinal-induced signals in white cells as compared with those measured in green cells, we studied fluence dependence and spectral sensitivity of the responses. The fluence-response curve of the PRC amplitude measured in suspension of white cells reconstituted with all-*trans*-retinal is clearly biphasic as it has been recently reported for green cells of *Chlamydomonas* (Sineshchekov et al., 1992b) and reveals the same high- and low-saturating components of the photoreceptor current found in a single cell of *Haematococcus* (Sineshchekov, 1991a).

The first low-saturating component of the PRC in reconstituted white cells is characterized by extremely high sensitivity with threshold fluence very close to the value found in green cells (less than 5×10^{15} photons/m^{-2}, Fig. 5, *curves 1* and *2*). The second component of the PRC in white cells saturates at similar fluence as in green cells (data in preparation).

The similar correlation between two photoelectric responses found in green cells occurs in white reconstituted cells: reciprocal of the RR delay time is proportional to the reciprocal of the PRC peak time (Fig. 5, *inset*). This correlation indicates that both photoresponses in white cells are likely mediated by the same photoreceptor pigment(s), reconstituted by addition of all-*trans*-retinal.

Action spectrum of the PRC generation was constructed on the basis of fluence-response curves using the reciprocals of threshold fluences for different wavelengths. The spectrum for white cells reconstituted with all-*trans*-retinal shows the same main peak (around 500 nm) as for green cells (Fig. 6). However, it is broader and the relative efficiency of long wavelength light is obviously higher than in green cells

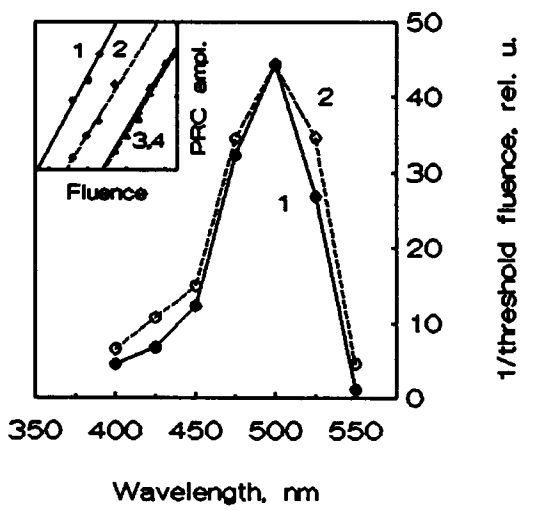

FIGURE 6 Action spectra of the photoreceptor current in green cells (1) and in white cells reconstituted with $5 \cdot 10^{-9}$ M all-*trans*-retinal (2). *Inset:* Relative sensitivity to the blue light of the photoreceptor current in green and white cells. 1, green cells, 500 nm; 2, white cells, 500 nm; 3,4, normalized curves for 550 nm in green and white cells. Excitation: photo-flashgun with corresponding interference filters.

(Fig. 6, *inset*). In the UL mode of photoelectric measurements, stigma plays a crucial role in the formation of the signal enhancing the relative efficiency of the light at the maximum of its absorption/reflection spectrum (see above and Kreimer et al., 1992). According to this, the difference between the action spectra for the PRC generation measured in white and green cells is likely due to the lack of stigmata in cells of the "blind" mutants used.

The above data show that the photoelectric responses measured in suspensions of *C. reinhardii* "blind" mutants after addition of all-*trans*-retinal have similar features (signal kinetics, fluence dependence, and spectral sensitivity) as found in green cells, indicating that the same pigment(s) are involved in signal generation in these two objects.

Effect of hydroxylamine on the photoelectric responses in white reconstituted and green cells

Green cells

Illumination of rhodopsin-type pigments in presence of hydroxylamine results in the cleavage of the chromophore group from the protein moiety, and thus the method of hydroxylamine bleaching is a standard procedure for investigation of different rhodopsins (Oesterhelt et al., 1974, 1986).

Preincubation of *Chlamydomonas* cells with this agent was shown to reduce the phototactic sensitivity measured in a long-term population assay (Hegemann et al., 1988) and the amplitude of flash-induced light absorbance changes in cell suspension (Uhl and Hegemann, 1990). These data support the conclusion that phototaxis in *Chlamydomonas* is mediated by a retinal-containing protein.

To investigate the nature of the photoreceptor pigment(s) involved in the PRC and the RR generation, we studied the effect of hydroxylamine treatment of the cells on the photoelectric signals. Dark incubation with hydroxylamine in concentrations up to 2 mM for at least 2 h does not inhibit photoelectric responses in *C. reinhardtii* strain 495(+) cells, while prolonged incubation with the agent in the light lowers the amplitude of both PRC and RR until the signal completely disappears (Fig. 7).

The degree of inhibition depends on the photon exposure and concentration of the agent. The increase in hydroxylamine concentration from 0.5 to 2 mM results in a parallel shift of the fluence-response curves of the PRC amplitude, which are almost linear in a double logarithmic plot in a wide range of fluences (Fig. 7, *inset*). Inhibition of the photoelectric responses by hydroxylamine can be partially reversed after intensive washing of cells by repetitive centrifugation and resuspension in a fresh medium. However, the signal reconstitution after washing takes several hours (data not shown).

The observed inhibition of photoelectric responses by hydroxylamine treatment of the cells can be explained by the effect of the agent on either the photoreceptor itself or on the postponed steps in the signal transduction chain. To solve this problem, analysis of the signal kinetics was carried out. It was found that in the presence of hydroxylamine the increase of the photon exposure during the bleaching procedure results not only in the progressive decrease of the PRC amplitude, but also slows down the time course of this response. The correlation between the amplitude and reciprocal of the peak time of the signals measured during hydroxylamine bleaching closely corresponds to that of the signals elicited by the flashes of progressively decreasing fluence in suspension of the untreated cells (Fig. 8, *triangles*).

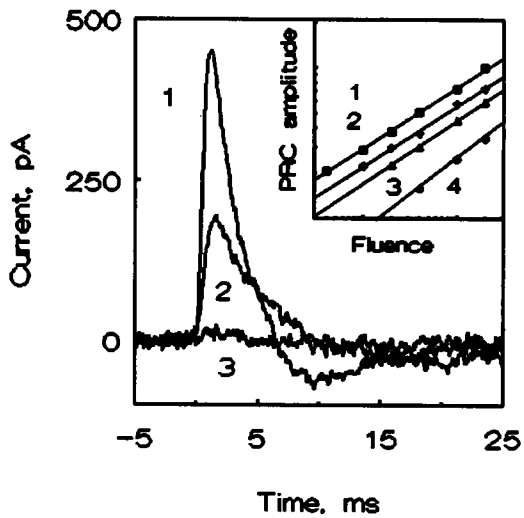

FIGURE 7 Light-dependent inhibition of the photoreceptor current in *C. reinhardtii* strain 495(+) by hydroxylamine. 1, 1 h of incubation with 1 mM hydroxylamine in the dark; 2, the same sample after 10 min of illumination with white light of 5 $W \cdot m^{-2}$; 3, after 10 min of illumination with blue light of 100 $W \cdot m^{-2}$. Excitation by the laser, 5×10^{-3} J. *Inset:* Fluence dependence of the amplitude of the photoreceptor current in *C. reinhardtii* strain 495(+) cells in the presence of hydroxylamine. Identical portions of cell suspension were incubated with the agent for 30 min before measurements under illumination with 20 $W \cdot m^{-2}$ of blue light followed by 30 min of dark adaptation. 1, control (1.5×10^{-3} M KCl); 2–4, hydroxylamine: 2, 10^{-3} M; 3, 1.5×10^{-3} M; 4, 2×10^{-3} M. Excitation: photo-flashgun with 500-nm interference filter.

This simulation of the light-dependent hydroxylamine inhibition by the decrease in the fluence of the excitation flash allows to rule out the possible effect of the agent on later events in the signal transduction chain. Thus we conclude that the hydroxylamine inhibition of the photoreceptor current occurs due to the reduction of the number of active photoreceptor molecules.

The same simulation of the light-dependent effect of hydroxylamine treatment by the decrease of the flash stimulus is found when the dependence of reciprocal of the RR delay time on the PRC amplitude is measured (Fig. 8, *circles*). This fact indicates that both PRC and RR are likely mediated by the same or similar rhodopsin-type pigment(s) and favors the above conclusion that the RR is initiated by the membrane depolarization due to generation of the PRC.

White cells

As in green cells, retinal-induced photoelectric signals in white mutants are sensitive to hydroxylamine treatment of

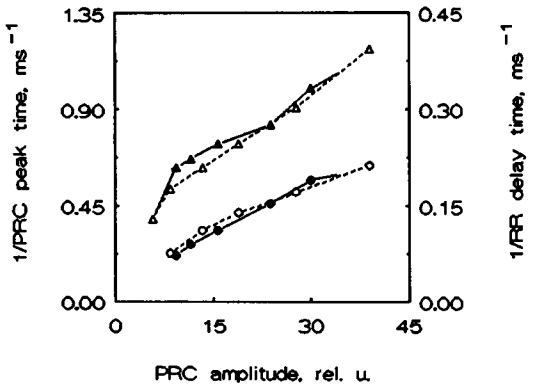

FIGURE 8 Correlation between the amplitude of the PRC and the kinetic parameters of the photoelectric responses: the reciprocal of the PRC peak time (*triangles*) and the reciprocal of the RR delay time (*circles*). *Solid lines, filled symbols:* progressive hydroxylamine inhibition of the photoelectric responses excited by flashes of equal fluence in cell suspension illuminated with continuous blue light at increasing photon exposure. *Dashed lines, open symbols:* photoelectric responses in suspension of non-poisoned cells excited by flashes of decreasing fluence. Excitation by laser.

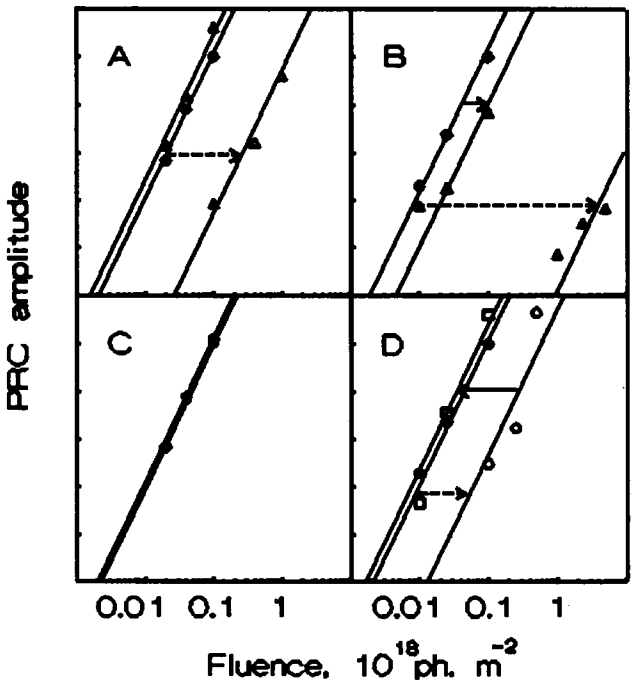

FIGURE 9 Effect of hydroxylamine treatment and illumination on threshold fluence for PRC generation in green strain 495(+) (*A, C*) and white strain 494/31 (*B, D*) of *C. reinhardtii*. (*A*) green cells: ●, control, dark; ▲, hydroxylamine, dark; △, hydroxylamine, light; (*B*) white cells: ●, control, dark; ▲, hydroxylamine, dark; △, hydroxylamine, light; (*C*) green cells: ●, control, dark; ○, control, light; (*D*) white cells: ●, control, dark; ○, control, light; □, dark readaptation of the illuminated control. *Solid arrows* indicate dark effects; *dashed arrows* indicate light-dependent effects. Cells were incubated with 2×10^{-3} M hydroxylamine (or 2×10^{-3} M KCl (green cells) or NaCl (white cells) as control) in the dark for 30 min, than equal portions of cell suspension were either kept in the dark or illuminated using broad-band 500-nm filter (photon radiation 10^{20} photons·m^{-2}·s^{-2}) for 30 min. After that, cells were dark-adapted for 30 min before measurements. The green cells were measured in presence of the agent; the white cells were washed by centrifugation and resuspended in a fresh measuring medium. Excitation: photo-flashgun with broad-band 500-nm filter.

the cells. To estimate the degree of inactivation of the photoreceptor due to hydroxylamine bleaching, we extrapolated the fluence-response curves to zero response to determine the threshold fluence which is supposed to be proportional to the number of still active photoreceptor molecules (Fig. 9).

In green cells incubation with 2 mM hydroxylamine upon illumination results in an ~40-fold increase in threshold fluence, while no inhibition was observed in the dark (Fig. 9 *A*). In white mutant cells the same treatment reveals that the inhibitory effect of hydroxylamine upon illumination is 10-fold higher as compared with that in the green control (Fig. 9 *B*). However, in white cells prolonged (more than 1 h) incubation with hydroxylamine in the dark causes unspecific inhibition, although to a much less extent than in the light. In addition, a reversible inhibitory effect of continuous illumination in the control is found in white cells, which was not observed in green cells (Fig. 9 *C*), but which can cause the increase of threshold fluence for PRC generation in white cells of about 1 order of magnitude (Fig. 9 *D*). This effect can be explained, for instance, by a photodynamic action of added retinal in absence of protecting carotenoids in mutant cells.

Taking into account these two additional contributing effects (unspecific effect of hydroxylamine in the dark and the inhibitory effect of illumination itself), the degree of the photoreceptor pigment inactivation found in white cells after hydroxylamine bleaching is similar to that of typical for the green control. This finding supports the conclusion that retinal-induced photoelectric signals in white cells are mediated by the same photoreceptor pigment as in green cells.

On the basis of the above data concerning all-*trans*-retinal reconstitution and hydroxylamine bleaching of the photoelectric responses in *C. reinhardtii*, we conclude that the photoreceptor pigment(s) mediating both PRC and RR is likely a rhodopsin-type protein.

To elucidate the features of this presumable rhodopsin pigment, we studied reconstitution of the flash-induced electric signals by various retinal analogs.

Reconstitution of the photoelectric responses by retinal analogs

To probe structural requirements of the chromophore involved in restoration of the photoelectric responses in cell suspension, we tested retinal analogs of three different groups.

The first group included analogs locked in either 13-*trans* (Fig. 10, *analog 2*) or 13-*cis* (Fig. 10, *analog 3*) conformation. These analogs were shown to be ineffective in restoration of both phototaxis and photophobic response in *Chlamydomonas* cells of strain CC2359 (Takahashi et al., 1991; Lawson et al., 1991; Zacks et al., 1993).

We found that prolonged incubation with both locked analogs could not restore the photoelectric responses even at the highest fluence tested (Fig. 11, *curve 1*). Preincubation with

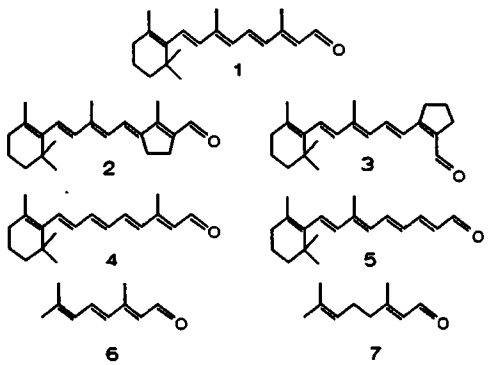

FIGURE 10 Structures of retinoid compounds used. 1, all-*trans*-retinal; 2, 13-*trans*-locked retinal; 3, 13-*cis*-locked retinal; 4, 9-demethyl-retinal; 5, 13-demethyl-retinal; 6, dimethyl-octatrienal; 7, citral.

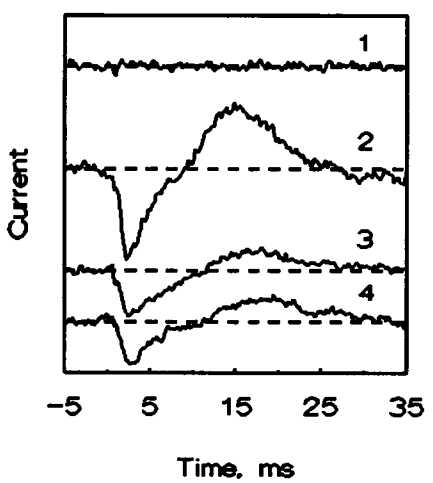

FIGURE 11 Effect of retinal analogs prevented from 13-*cis*/*trans* isomerization on photoelectric responses in cells of *C. reinhardtii* strain CC2359. 1, 1.5×10^{-8} M of 13-*trans*-locked retinal, incubation for 60 min; 2, 10^{-8} M of all-*trans*-retinal; 3, the same as in 2, but added after 40 min of incubation with 1.5×10^{-8} M of 13-*cis*-locked retinal; 4, the same as in 2, but after 40 min of incubation with 1.5×10^{-8} M of 13-*trans*-locked retinal. The signals measured in PO mode after 10 s of illumination with 502 nm light (1.8×10^{18} photons·m^{-2}·s^{-1}). Excitation: photo-flashgun with 500-nm broad-band filter, 4.3×10^{18} photons·m^{-2}.

these analogs inhibits reconstitution of both PRC and RR by all-*trans*-retinal. Preincubation with locked analogs not only decreases the amplitude of the retinal-induced signal, but also slows down the kinetics of the PRC generation (Fig. 11, *curves 3* and *4*).

Analogs of the second group were applied to elucidate the importance of methyl substitutions at positions 9 and 13 for generation of the photoelectric responses. 9-demethyl-retinal (Fig. 10, *analog 4*) is known to restore the normal function of bacteriorhodopsin (Gärtner et al., 1983), but to slow down the photocycle of halobacterial sensory rhodopsin II, thus decreasing the threshold fluence of the photophobic response in this organism (Yan et al., 1991). In *Chlamydomonas* "blind" mutant, this analog was found to restore normal phototbehavior after slow reconstitution (Hegemann et al., 1991).

On the other hand, 13-demethyl-retinal (Fig. 10, *analog 5*) was found to slow down the photocycle and to decrease the transport activity of bacteriorhodopsin (Gärtner et al., 1983). In *Chlamydomonas* this analog did not restore photoinduced light-scattering changes, and preincubation with this analog inhibited the effect of all-*trans*-retinal (Hegemann et al., 1991).

We found that addition of 9-demethyl-retinal results in restoration of both photoelectric responses (PRC and RR) in a suspension of "blind" *Chlamydomonas* mutant within a few minutes (Fig. 12, *trace 3*). Comparing this to the all-*trans*-retinal induced photoresponse (Fig. 12, *trace 4*), practically no changes in signal kinetics, at least at the high fluence of the exciting flash, were found. This result suggests that, unlike in sensory rhodopsin II from *Halobacterium halobium*, 9-demethyl-retinal apparently does not affect the photocycle of *Chlamydomonas* photoreceptor pigment.

Unlike 9-demethyl-retinal, 13-demethyl-retinal is not only functionally ineffective in restoration of photoelectric activity of "blind" cells (Fig. 13, *trace 1*), but also preincubation with this analog inhibits all-*trans*-retinal reconstitution of the photoelectric responses (Fig. 13, *trace 3*), indicating that this analog occupies the chromophore binding site but cannot substitute the native chromophore functionally.

The third group of analogs used included short-chained (C_{10}) compounds: dimethyl-octatrienal, consisting of the retinal backbone from position 8 to the aldehyde group, and citral, lacking the intermediate C=C double bond of the same structure (Fig. 10, *analogs 6* and *7*).

Photoelectric responses measured in the suspension of *Chlamydomonas* "blind" cells after incubation with these analogs are shown in Fig. 12. Citral was found completely ineffective in restoration of photoelectric responses even at the highest fluences of the exciting flash (Fig. 12, *trace 1*).

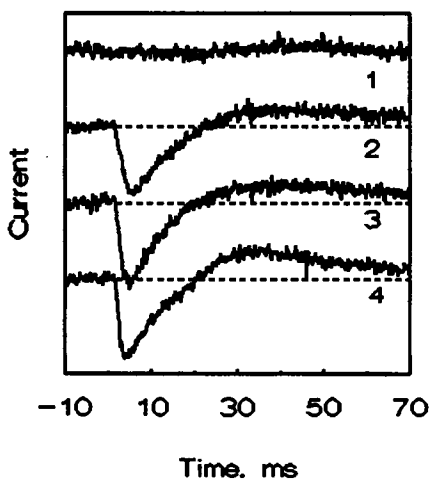

FIGURE 12 Reconstitution of the flash-induced photoelectric responses in cells of *C. reinhardtii* strain CC2359 by 9-demethyl-retinal and short-chained retinal analogs. 1, citral, 10^{-6} M, incubation for 120 min; 2, dimethyl-octatrienal, 10^{-6} M, incubation for 120 min; 3, 9-demethyl-retinal, 9^{-8} M, incubation for 30 min; 4, all-*trans*-retinal, 10^{-6} M, incubation for 10 min. Excitation: photo-flashgun with 500-nm broad-band filter, 1.6×10^{17} photons·m^{-2}.

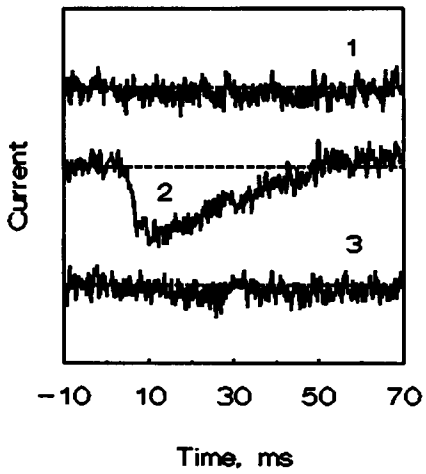

FIGURE 13 Effect of 13-demethyl-retinal on the photoelectric responses in cells of *C. reinhardtii* strain CC2359. 1, 13-demethyl-retinal, 2×10^{-7} M, incubation for 35 min; 2, all-*trans*-retinal, 10^{-6} M, incubation for 35 min; 3, the same, but added after 35 min of incubation with 2×10^{-7} M of 13-demethyl-retinal. Excitation: photo-flashgun with 500-nm interference filter, 2.5×10^{17} photons·m^{-2}.

In suspensions incubated with dimethyl-octatrienal the typical signals were recorded. The kinetics of both PRC and RR of dimethyl-octatrienal induced signal excited by the high-fluence flash was very similar to that of all-*trans*-retinal-induced signal (Fig. 12, *traces 2* and *4*), although threshold fluence for dimethyl-octatrienal-induced signals was found about 100-fold higher (data not shown).

Both dimethyl-octatrienal and 9-demethyl-retinal-induced photoelectric responses were found to be sensitive to unilateral continuous illumination of the cell suspension in the same manner as was found for all-*trans*-retinal (data not shown). This means that in parallel with restoration of the PRC and the RR in analog-doped cells, the ability of phototactic orientation was also restored.

The above data obtained by reconstitution of the photoelectric responses in suspension of *Chlamydomonas* "blind" cells by the retinal analogs 2–7 (Fig. 10) coincide the results of recent reconstitution experiments, when phototaxis of this microorganism was measured by means of single-cell tracking and by recording the photoinduced light-scattering changes (Takahashi et al., 1991; Hegemann et al., 1991; Lawson et al., 1991).

Two conclusions can be drawn from this finding. First, it strongly supports the idea that photoreceptor pigment for the photoelectric responses in green flagellates (PRC and RR) is a rhodopsin-type protein (Sineshchekov, 1991b, Sineshchekov et al., 1991; Harz and Hegemann, 1991). This pigment seems to possess the same chromophore as has been concluded for *Chlamydomonas* rhodopsin from the results of recent behavioral studies (at least three conjugated C=C double bonds within the chromophore moiety and presence of 13-methyl group) and isomerization around C_{13}=C_{14} double bond of the chromophore is likely necessary for the pigment activation.

Second, the results of photoelectric measurements indicate that restoration of phototaxis observed in the recent behavioral studies can be indeed attributed to the reconstitution of the same particular photoreceptor pigment but is not related to possible effects of exogenous compounds on the later events in the signal transduction chain or to the indirect control of phototaxis by another photoreceptor system.

Photo-orientation in reconstituted cells and its relation to the restoration of the photoelectric responses

As described above, photoelectric measurements in the PO mode allow probing the sign and the degree of phototactic orientation of the cells in suspension. In white mutant cells with no added retinal, flash-induced photoelectric signals were not observed in the PO mode nor in the UL mode (Fig. 14, *inset, dashed line*), but within 30 s after all-*trans*-retinal addition, the signal appeared (Fig. 14, *inset, solid lines*). Thus addition of retinal results in restoration of the ability for photo-orientation in white cells in parallel with restoration of the PRC and the RR. The reciprocals of peak times of both PRC and RR are restored within 0.5–1 min (Fig. 14, *solid symbols*). This corresponds to the reconstitution time found in the UL mode measurements. However, the amplitude of the signal in the PO mode still increases for at least 5 min after addition of (Fig. 14, *open symbols*). This increase in the signal amplitude indicates that the time course of restoration of retinal-induced photo-orientation in white cells is slower than the time course of retinal incorporation itself.

The stimulus-response dependence of the signal measured in the preoriented mode in white cells closely corresponds to that in green cells. The signal is characterized by the same

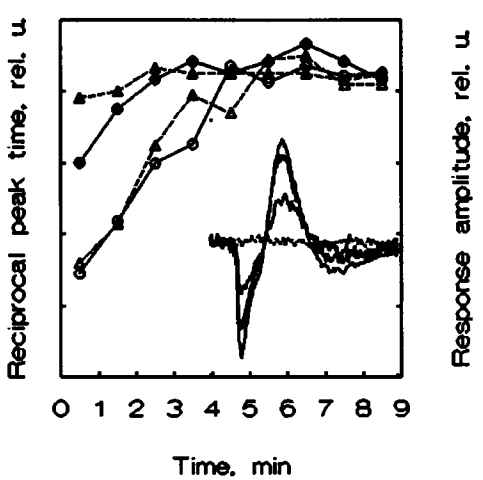

FIGURE 14 Time course of all-*trans*-retinal induced reconstitution of the photoelectric responses and phototaxis in cells of *C. reinhardtii* strain CC2359. *Open symbols,* amplitude; *filled symbols,* reciprocal of the PRC peak time (*circles, solid lines*) and the RR peak time (*triangles, dashed lines*). *Inset:* Photoelectric responses before and 1, 3, and 5 min after addition of retinal. Cells were preoriented for 10 s with 532 nm light of 2.6×10^{18} photons·m^{-2}·s^{-1}. Excitation: photo-flashgun with 500-nm broad-band filter, 4.3×10^{18} photons·m^{-2}.

high sensitivity (threshold for the most effective 500 nm light is around 10^{15} photons/$m^{-2}\cdot s^{-1}$, Fig. 15, *curve 1*) found earlier in green cells (Sineshchekov et al., 1992b). For this wavelength the signal amplitude saturates at the fluence rate of about 10^{17} photons/$m^{-2}\cdot s^{-1}$, the value which is typical for saturation of the phototaxis in retinal-reconstituted cells measured by behavioral assay (Hegemann et al., 1988).

The signals in white reconstituted cells and in green cells, measured under the same preorienting conditions, have similar kinetics, but the sign of both PRC and RR in white cells is again reversed as compared with the sign of these responses in green cells (Fig. 16). The sign of the signal measured in the PO mode is determined by the localization of current generators along the anterior-posterior axis of a cell and by the direction of the photomovement (sign of phototaxis). The reversed sign of the flagellar RR in white cells measured in preoriented mode reveals that these cells have positive phototaxis under conditions when green cells move away from the light source. This situation could be predicted assuming the different mechanism of antenna direction found in white cells. Focusing effect provides the reversal of the direction of maximum antenna direction in white cells (see above).

DISCUSSION

The above data indicate that both the photoreceptor current and the RR in *C. reinhardtii* are initiated by rhodopsin-type pigment molecules. Both responses can be reconstituted in blind mutant cells by the addition of exogenous all-*trans*-retinal in submicromolar concentrations and both show light-dependent sensitivity to hydroxylamine (in green and reconstituted mutant cells) typical for retinal-containing proteins. Generation of the PRC in green and reconstituted *Chlamydomonas* cells shows extremely high ("single quantum") light sensitivity, which is characteristic of the known rhodopsin-mediated photoreactions. Action spectrum of the

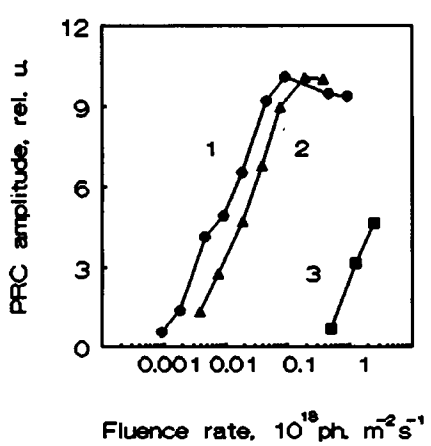

FIGURE 15 Dependence of the PRC measured in PO mode in white cells of *C. reinhardtii* strain N164 reconstituted with all-*trans*-retinal on fluence rate of the orienting illumination of different wavelengths. 1, 500 nm; 2, 450 nm; 3, 600 nm. Excitation: photo-flashgun with 500-nm broad-band filter, 2×10^{18} photons·m^{-2}.

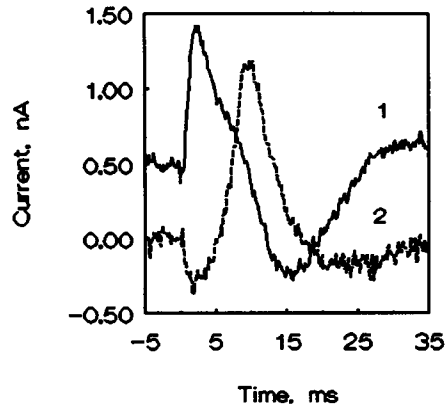

FIGURE 16 Photoelectric responses measured in PO mode under the same conditions in suspension of green and white cells of *C. reinhardtii*. 1, green cells of strain 495(+); 2, white cells of strain 516/white-3 reconstituted with 2×10^{-8} M of all-*trans*-retinal. Cells were preoriented for 10 s with 532 nm light of 2.6×10^{18} photons·$m^{-2}\cdot s^{-1}$. Excitation: photo-flashgun with 500-nm broad-band filter, 4.3×10^{18} photons·m^{-2}.

PRC in white cells reconstituted with all-*trans*-retinal matches approximately the absorption spectra of sensory rhodopsin II from *Halobacterium halobium* (maximum at 487 nm) and *pharaonis* phoborhodopsin from *Natronobacterium pharaonis* (maximum at 500 nm) (Takahashi et al., 1990; Scharf et al., 1992).

The reciprocal of the delay time of the RR correlates with the amplitude of the photoreceptor potential in green cells, in the process of reconstitution of the electric responses in "blind" mutants, and during light-dependent inhibition of the signal by hydroxylamine. This correlation indicates that both responses are driven by the same (or similar) pigment(s) and confirms the results obtained using the single-cell technique of photoelectric measurements showing that the flagellar RR is a direct consequence of the PRC generation and appears when the membrane depolarization exceeds a threshold level. On the other hand, the results of recent behavioral studies suggest the identity of photoreceptor pigments responsible for the photo-orientation of cells and for the photophobic response due to the fact that reconstitution of these two types of the photobehavior in carotenoid-deficient cells show the same relative sensitivity to various retinal analogs (Takahashi et al., 1991).

Reconstituted white cells lack stigmata and bulk chloroplast pigments, therefore participation of these structures in signal formation is excluded. Photo-orientation in this case might occur via a different mechanism based on focusing effect of the transparent cell bodies.

The results obtained by reconstitution of the photoelectric responses with various retinal analogs indicate that a presumable rhodopsin pigment(s) involved in the PRC and the RR generation resembles bacterial type pigments rather than visual rhodopsins from higher animals, since isomerization of all-*trans*-retinal chromophore is necessary for its functioning.

Measurements carried out in preoriented mode show that reconstitution of the PRC and the RR in white cells is accompanied by reconstitution of their ability to photo-

orientation, although the time course of the phototaxis induction is slower than that of retinal incorporation. These findings suggest that in extended time population assays (Foster and Saranak, 1988; Foster et al., 1989) additional mechanisms might control the phototactic rate in reconstituted cells and this might be the reason for reported discrepancies in results of behavioral assays.

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