Photoreceptor Current and Photoorientation in *Chlamydomonas* Mediated by 9-Demethylchlamyrhodopsin

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ABSTRACT Green flagellates possess rhodopsin-like photoreceptors involved in control of their behavior via generation of photocurrents across the plasma membrane. *Chlamydomonas* mutants blocked in retinal biosynthesis are "blind," but they can be rescued by the addition of exogenous retinoids. Photosignaling by chlamyrhodopsin regenerated with 9-demethylretinal was investigated by recording photocurrents from single cells and cell suspensions, and by measuring phototactic orientation. The addition of a saturating concentration of this analog led to reconstitution of all receptor molecules. However, sensitivity of the photoreceptor current in cells reconstituted with the analog was smaller compared with retinal-reconstituted cells, indicating a decreased signaling efficiency of the analog receptor protein. Suppression of the photoreceptor current in double-flash experiments was smaller and its recovery faster with 9-demethylretinal than with retinal, as it would be expected from a decreased PC amplitude in the analog-reconstituted cells. Cells reconstituted with either retinal or the analog displayed negative phototaxis at low light and switched to positive one upon an increase in stimulus intensity, as opposed to the wild type. The reversal of the phototaxis direction in analog-reconstituted cells was shifted to a higher fluence rate compared with cells reconstituted with retinal, which corresponded to the decreased signaling efficiency of 9-demethylchlamyrhodopsin.

INTRODUCTION

Light-induced behavioral responses in *Chlamydomonas* and related species are mediated by rhodopsin photoreceptors (Foster and Smyth, 1980; Foster et al., 1984). Photoexcitation triggers a cascade of rapid electrical events in the plasma membrane of the cell implicated in the signal transduction mechanisms for phototaxis and the photophobic response (for review, see Sineshchekov and Govorunova, 1999, 2001a,b). The rhodopsin-mediated electrical responses can be recorded extracellularly by means of a suction pipette technique (Litvin et al., 1978) or a population assay (Sineshchekov et al., 1992). The first major component of the signaling cascade is an inward photoreceptor current (PC) across the plasma membrane in the eyespot region of the cell (Litvin et al., 1978; Sineshchekov et al., 1990). Gradual changes in PC amplitude presumably control asymmetrical changes in flagellar beating (Sineshchekov, 1991a,b; Rueffer and Nultsch, 1990, 1991), which result in phototaxis. When the intensity and/or duration of the light stimulus exceeds a certain threshold, PC is superimposed by an all-or-nothing regenerative response (Litvin et al., 1978). It is thought to reflect a massive entry of Ca^{2+} ions into the intraflagellar lumen due to activation of voltage-gated ion channels (Sineshchekov, 1991a) that is referred to as the flagellar current (FC) (Harz and Hegemann, 1991). The FC causes a switch from the breaststroke style of flagellar beating to undulation that results in the phobic response in a free-swimming cell (Sineshchekov, 1991a,b; Holland et al., 1997).

Extremely low concentration of the photoreceptor protein in *Chlamydomonas* cells (Foster and Smyth, 1980) in the presence of abundant photosynthetic and eyespot pigments significantly complicates spectroscopy studies in both intact cells and partially purified photoreceptor preparations. A large-scale production of algal rhodopsins by means of genetic engineering is still pending. Therefore, at present in vivo functional tests seem to be the only possibility to probe photoreceptor rhodopsins in green flagellates. Measurement of the PC is the most suitable approach to this goal, because the PC is the earliest so far detected event in the signal transduction chain of phototaxis and the photophobic response.

One of the most promising experimental strategies to study *Chlamydomonas* phototaxis receptors involves the use of "blind" mutants (Foster et al., 1984). These mutants possess opsin but lack retinal due to a defect in the carotenoid biosynthesis pathway, and, consequently, their behavior is not sensitive to light. However, both phototaxis and the photophobic response can be restored in these mutants by the addition of exogenous retinoid compounds. A variety of synthetic retinal analogs with different structural modifications have been tested for their ability to restore photobehavior in "blind" *C. reinhardtii* mutants by means of video microscopy and motion analysis of individual cell tracks (Lawson et al., 1991; Takahashi et al., 1991; Zacks et al., 1993; Sakamoto et al., 1998) and by recording light

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scattering changes in a cell suspension (Hegemann et al., 1991). It has been concluded that chlamyrhodopsin contains all-*trans* retinal in the 6-s-*trans* ring/chain conformation and that photoinduced all-*trans*/13-*cis* isomerization of the chromophore is crucial for the signal transduction (for review, see Spudich et al., 1995), although a different view has been initially proposed (Foster et al., 1984, 1989). Recording rhodopsin-mediated electrical responses from suspensions of reconstituted *C. reinhardtii* cells corroborated the above conclusions and proved that restoration of behavioral responses in "blind" mutants occurred due to specific binding of the exogenous retinoid to chlamyopsin, rather than resulted from an indirect effect of the added substance on the photosignaling system (Sineshchekov et al., 1994). All-*trans*/13-*cis* isomerization is typical for archaeal rhodopsins as opposed to animal visual rhodopsins, which contain 11-*cis* retinal and undergo 11-*cis/trans* isomerization upon photoexcitation (for review, see Spudich et al., 2000).

Incorporation of certain retinal analogs, including pentaenal, induced an apparent diversion of the sign of phototaxis in the FN68 *C. reinhardtii* mutant (Takahashi et al., 1992a). A hypothesis was suggested that this diversion results from a delay in intracellular photosignal transduction that is comparable with the half-period of cellular rotation, i.e., 250 to 500 ms (Takahashi et al., 1992a). Pentaenal and 13-ethylretinal also accelerated the onset of phototaxis and desensitization of the photophobic response in *Chlamydomonas* (Zacks and Spudich, 1994). These analogs are known to extend the lifetimes of the signaling states in archaeal sensory rhodopsins I and II (Yan and Spudich, 1991; Yan et al., 1991, 1993; Takahashi et al., 1992b), so that similar explanation has been suggested for their influence on *Chlamydomonas* photobehavior (Zacks and Spudich, 1994).

Direct measuring of the kinetics of photochemical conversion in chlamyrhodopsin by time-resolved spectroscopy has not yet been possible. Measurement of the recovery of PC after a saturating flash, which is completed within several hundreds of ms, provided an estimate for the upper limit of the duration of the rhodopsin photocycle in green flagellated algae (Sineshchekov 1991a,b; Der et al., 1997). However, it has been recently shown that suppression of PC amplitude observed after a conditioning flash and its subsequent dark recovery are determined by the processes downstream from the rhodopsin photoconversion (Govorunova et al., 1997).

In this study, we examined the influence of 9-demethylretinal (9-dm-retinal) on chlamyrhodopsin signaling. This analog probably constitutes the best-investigated artificial chromophore in both prokaryotic and visual rhodopsins (for review, see Nakanishi and Crouch, 1995; Lou et al., 2000). Binding of 11-*cis* 9-dm-retinal instead of the native chromophore to animal opsins results in significant changes in the pigment properties, which could not only be detected by spectral studies (e.g., Ganter et al., 1989; Han et al., 1997, 1998; Meyer et al., 2000), but also investigated by electrophysiological methods (Corson et al., 1994; Corson and Crouch, 1996). All-*trans* 9-dm-retinal has been shown to restore photobehavior (Hegemann et al., 1991) and photocurrents (Sineshchekov et al., 1994) in *Chlamydomonas* cell suspensions, although no detailed studies on the importance of 9-methyl group of the chromophore for chlamyrhodopsin function has been undertaken so far.

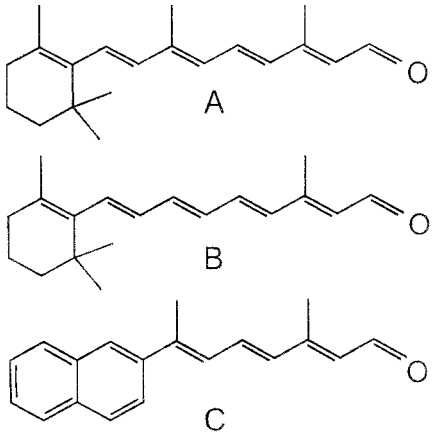

FIGURE 1 Chemical structures of retinoid compounds used in reconstitution experiments. (*A*) All-*trans* retinal; (*B*) 9-dm-retinal; (*C*) naphtylretinal.

PC and photoorientation in *Chlamydomonas* "blind" mutants reconstituted with 9-dm-retinal or retinal were investigated in this study by means of photoelectric recording from cell suspensions to explore the influence of the chromophore structure on the sign of phototaxis. In addition, photocurrents from individual reconstituted cells were examined by the suction pipette technique, the application of which was made possible by creating a hybrid carotenoid- and cell wall-deficient *C. reinhardtii* strain, not available before. Part of this work was presented as a preliminary report (Govorunova et al., 2000b).

MATERIALS AND METHODS

Chemicals

All-*trans* retinal was purchased from Sigma (St. Louis, MO), 9-dm-retinal was synthesized according to Gärtner et al. (1983), and naphtylretinal was synthesized as described in Iwasa et al. (1984). The latter analog was only used as a test chromophore for measuring action spectra of PC in analog-reconstituted cells, because it has been reported to induce a prominent shift of the action spectrum for phototaxis in the FN68 *C. reinhardtii* mutant reconstituted with this analog (Foster et al., 1989) and absorption spectrum in archaeal sensory rhodopsin II (Yan et al., 1991). Retinal and the analogs were purified by high-performance liquid chromatography on the Si60 5 μm Lichrosorb column (Merck, Darmstadt, Germany) using 5% (v/v) ethylacetate in hexane as a solvent and tested by ultraviolet absorption spectroscopy before experiments. Chemical structures of all-*trans* retinal and the analogs used are shown in Fig. 1. Retinoids were added to cells as isopropanol solutions so that the final concentration of isopropanol was less than 0.3%. Other chemicals were of analytical grade.

Strains and culture conditions

Two carotenoid-deficient *C. reinhardtii* strains were used in this study. The N-164 (*lts1-164*) strain (*Chlamydomonas* culture collection at St. Petersburg University, St. Petersburg, Russia) carries a mutation in *lts1* locus (Chemerilova, 1978), allelic to the *lts1-30* mutation of the strain CC2359, which has been extensively used in photobehavior studies (Hegemann et al., 1991; Lawson et al., 1991). Restoration of photoelectric responses in cell suspensions after the addition of exogenous retinal was earlier examined in several *lts1* mutants, including the N-164 and CC2359 strains, and similar results were obtained in all of them (Sineshchekov et al., 1994). However, the N-164 strain was characterized by the fastest growth, which justified its use in population experiments that require relatively large quantities of cells.

A suction pipette technique, which allows recording electrical currents from individual cells (Litvin et al., 1978), can only be applied to flagellates with elastic cell walls, or cell wall-deficient mutants such as *cw* mutants of *C. reinhardii* (Harz and Hegemann, 1991). However, no carotenoid-deficient strain with these properties has been available so far. Therefore, such strain (*cw2 lts1-30*) was produced by a genetical cross of the cell wall-deficient strain *cw2 mt+* and carotenoid-deficient strain CC2359 (*lts1-30 mt−*) (both obtained from *Chlamydomonas* culture collection at Duke University, Durham, NC). Reconstitution experiments carried out by photoelectric recording from cell suspensions have shown that properties of the newly isolated hybrid strain are basically the same as those found in so far tested carotenoid-deficient strains that retain the cell wall, although its slower growth made these measurements time-consuming.

The green 495 strain (*Chlamydomonas* culture collection at St. Petersburg University, St. Petersburg, Russia), which is characterized by high sensitivity of phototaxis (Sineshchekov et al., 1994), was used as a wild-type control for measurements of photoorientation.

Cells were grown in the darkness on agar plates with high salt medium (Harris, 1989) supplemented with 0.3% Bacto tryptone (DIFCO Laboratories, Detroit) at 18°C. The cell wall-deficient hybrid strain *cw2 lts1-30* was grown on agar plates in the presence of 250 mM sorbitol. For a suspension assay, cells were grown in liquid medium of the same composition during 2 or 3 days before measurements because this procedure yielded the largest amount of culture. Cells were converted into pregametes by overnight incubation in the nitrogen-deficient medium (Foster et al., 1984) on a rotary shaker in the dark. Several hours before experiments, the cells were centrifuged and resuspended in the measuring medium containing 1 mM KCl, 0.1 mM $CaCl_2$, 0.081 mM $MgCl_2$, and 5 mM HEPES (pH 6.8) at concentration 0.5 to 1×10^7 cells $\times 10^{-3}$ l.

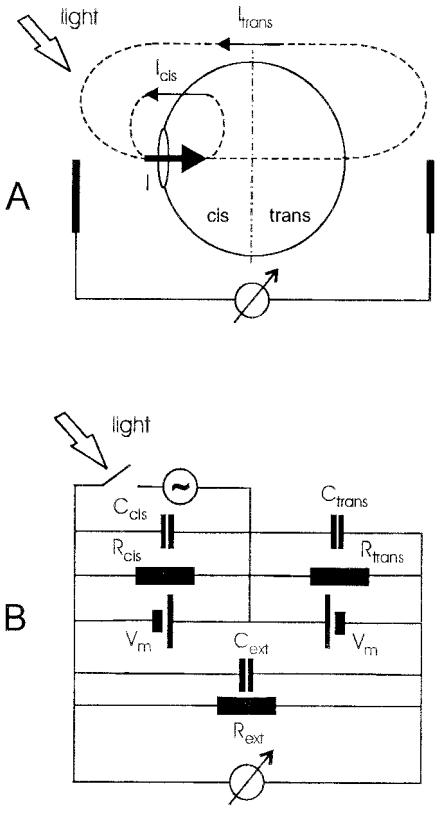

FIGURE 2 (*A*) Basic scheme for extracellular recording of a local transmembrane current; (*B*) equivalent electrical circuit. One-half of the plasma membrane that contains the current source (shown as a small ellipse) is defined as the *cis* half, and the other half of the membrane is defined as the *trans* half. *I*, a photoinduced transmembrane current; I_{cis}, part of the current closing the circuit via the *cis* half of the plasma membrane; I_{trans}, part of the current closing the circuit via the *trans* half of the plasma membrane. C_{cis} and R_{cis}, the capacitance and resistance, respectively, of the *cis* half of the plasma membrane; C_{trans} and R_{trans}, the capacitance and resistance, respectively, of the *trans* half of the plasma membrane; C_{ext} and R_{ext}, the capacitance and resistance, respectively, of the extracellular medium; V_m, the resting membrane potential (Sineshchekov and Govorunova, 2001a).

Photoelectric measurements

Flash-induced electrical currents were recorded extracellularly by means of a suction pipette technique (Litvin et al., 1978) or population assay (Sineshchekov et al., 1992). Both methods allow detecting asymmetric (localized) electrical responses of the cell by measuring the part of the transmembrane current that flows through the external resistance, as it is shown in Fig. 2. Principles of both methods are discussed in more detail by Sineshchekov and Govorunova (2001a).

The suction pipette technique was used for recording the currents from individual cells. Pipettes were pulled out from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) by a two-step procedure using a vertical puller (List Electronics, Darmstadt, Germany) and broken at 5-μm-tip diameter. The pipette served as a salt bridge to the Ag/AgCl electrode connected to the input of a patch-clamp amplifier (EPC-7, List Electronics), whereas the bath electrode was connected to the ground. Sucking of the cell into the tip of the pipette resulted in the electrical insulation of the two parts of the plasma membrane inside and outside the pipette by the glass, so that part of the photoinduced current could be measured by the amplifier. The current directed from the bath to the pipette was considered positive. Further details of suction pipette measurements were as in Govorunova et al., 1997.

In the population assay (Sineshchekov et al., 1992, 1994), flash-induced responses of many synchronously excited cells were measured by a low-noise current preamplifier (Ithaco 564, Ithaca, NY) in series with a CyberAmp 320 signal conditioner (Axon Instruments, Foster City, CA) via a pair of platinum electrodes immersed in a suspension of freely swimming cells. Three modes of the assay were used.

In the unilateral (UL) mode, a suspension of non-oriented cells was excited by a flash applied from the side of the cuvette along the line connecting the two electrodes. The electrode remote from the excitation source was connected to the amplifier input. The cells oriented at the time of the flash with their eyespots toward the light source generate larger PC than those oriented with their eyespots away from it, which gives rise to recording the difference signal. The amplitude of this signal depends on both the amplitude of the transmembrane currents and efficiency of screening, i.e., directional sensitivity of the photoreceptor apparatus.

In the phototactically preoriented (PO) mode, the cells were preoriented with continuous light along the line connecting the electrodes, whereas the excitation flash was applied in the perpendicular direction. Under these

conditions, only the currents from the cells oriented due to phototaxis along the direction of the continuous light contribute to the recorded electrical signal. Magnitude of the signal recorded in the PO mode depends on both the amplitude of the transmembrane currents and the degree of phototactic orientation of the cells. Therefore, measuring of the photoelectric responses in the PO mode allows instant estimation of the degree of photoorientation, provided the amplitude of the transmembrane currents does not change during the experiment or is assessed by recording in the UL mode. The electrode proximal to the source of continuous illumination was connected to the amplifier input. Under these conditions, the positive sign of PC corresponds to positive phototaxis, and the negative sign corresponds to negative phototaxis, because in *C. reinhardtii* the angle between the axis of the swimming helical path and the vector of transmembrane PC is more than 90° (Sineshchekov et al., 1992).

Finally, in the gravitactically preoriented (GO) mode, the responses were measured in a cuvette, where the electrodes were separated in a vertical plane. The upper electrode was connected to the amplifier input. The excitation stimulus was applied in the perpendicular direction, so only the photoinduced currents from the cells oriented by gravitaxis in the vertical plane were detected by the electrodes. Using this version of the suspension method proved to be especially advantageous for "blind" mutants, because their lack of eyespots and photosynthetic pigments significantly reduce directional sensitivity of the photoreceptor apparatus essential for measuring of the signal in the UL mode (Sineshchekov et al., 1994). Therefore, most of the experiments in the current study were carried out by recording from GO cell suspensions. However, long-term measurements (those that required several hours of observations) were performed in the UL mode to avoid possible changes in gravitaxis observed in prolonged experiments (Sineshchekov et al., 2000). Further details of suspension measurements were as in Govorunova et al. (2000a).

Measurement of photoelectric responses by a population assay is less laborious and provides a higher signal-to-noise ratio and reproducibility of the results, as compared with the suction pipette technique. Furthermore, recording by this assay is performed under fully physiological conditions, whereas sucking of the cell into the pipette leads to inevitable deformation of the protoplast and possible activation of stretch-induced ion channels (Yoshimura, 1998). However, using the population assay requires relatively large quantities of culture, and interpretation of the electrical signals recorded from multiple cells is in some cases more difficult than that of single-cell responses. Therefore, both methods were used as complementary in the current study.

The pCLAMP 6.3 software (Axon Instruments) was used for triggering the light stimuli and for data acquisition. Two types of flash excitation stimuli were applied: 1) 10-μs flash produced by a xenon flash lamp (IG and G FXQG-949-1, Polytech, Waldbronn, Germany); 2) 1-ms flash (rise time: ~200 μs) produced by a commercial photoflash (Vivitar, Korea), or by a xenon flash (T.I.L.L. Photonics, Martinsried, Germany). The former source was used for double-flash experiments with short time interval between the two flashes, the latter one was used in experiments that required high stimulus intensities. Continuous-light stimuli were provided by a commercial 50-W Osram halogen lamp. The wavelength of excitation flashes and continuous light was selected by a broad-band K50 filter (Balzers, Vaduz, Liechtenstein) or by interference filters (half-bandwidth: 7 nm) (Schott, Mainz, Germany). Stimulus intensity was attenuated by neutral-density filters and measured by a calibrated photodiode. Evaluation and fitting of the data were performed by Origin 5.0 software (Microcal Software, Northhampton, MA).

RESULTS

Only very small electrical currents could be recorded in carotenoid-deficient cells upon photoexcitation in the absence of exogenous retinoids. The peak amplitude of PC recorded by a suction pipette technique from the newly isolated *cw2 lts1-30* hybrid strain was only 2.3 ± 0.9 pA (mean ± SD, $n = 10$) in the absence of an exogenous chromophore, whereas in the parental *cw2* strain it was 25.2 ± 4.1 pA (mean ± SD, $n = 10$) at the same stimulus intensity. The addition of 9-dm-retinal or retinal to a cell sucked into a pipette (the *cw2 lts1-30* strain) or to a suspension of freely swimming cells (the N-164 strain) resulted in a rapid (less than 1 min) restoration of the response, whereas the addition of an equal volume of isopropanol had no influence on the signal (data not shown).

Dependence of the currents on the exogenous retinoid concentration was studied by a technically less laborious population assay. Representative signals measured in cell suspensions of the N-164 strain are shown in Fig. 3 *A*. The PC peak amplitude measured in the absence of an exogenous retinoid was 9 ± 1% of that measured in cells supplemented with 100 nM retinal. The dependence of the PC peak amplitude on the retinoid concentration is shown in Fig. 3 *B*. A Michaelis function $R = R_{max} \times C/(EC_{50} + C)$ was fitted to the data, in which R_{max} is the peak amplitude observed at the saturating retinoid concentration, and EC_{50} is the concentration that gives rise to a half-maximum of the response. To take into account a residual amount of endogenous retinal present in carotenoid-deficient cells, C was expressed as $C_{ex} + C_{en}$, in which C_{ex} is a variable concentration of the exogenous chromophore, and C_{en} is a constant endogenous concentration. The value of relative R_{max} obtained from this fit for 9-dm-retinal-reconstituted cells was 55 ± 2%, as compared with 100 ± 1% for cells reconstituted with retinal, and the values of EC_{50} were 1.9 ± 0.6 nM and 1.6 ± 0.2 nM for cells reconstituted with 9-dm-retinal or retinal, respectively. The value for C_{en} calculated from the curve obtained for retinal-reconstituted cells was 0.05 ± 0.04 nM.

The influence of the addition of an excess retinal to cells preincubated with a saturating concentration of 9-dm-retinal was studied to test if reconstitution of chlamyopsin with the analog was complete. Practically no changes in PC were found in a minute range after the addition of 1 μM retinal to cells preincubated with 100 nM 9-dm-retinal (Fig. 3 *C*). This indicated a complete reconstitution of chlamyrhodopsin with 9-dm-retinal and a tight binding of this analog to the apoprotein. A slow increase in the signal amplitude was observed during subsequent incubation of the cell sample on a time scale of hours, until it reached the level of retinal-reconstituted cells after the overnight incubation (Fig. 3 *C*).

Dependence of the photoelectric signals on stimulus intensity was studied by the suction pipette technique (Fig. 4). As it has been shown earlier, the stimulus-response curve of the PC peak amplitude cannot be characterized by fitting a single saturation function unless the existence of a second low-saturating process is assumed (Sineshchekov, 1991a), whereas a combination of two saturation functions results in a fairly good fit (Sineshchekov and Govorunova, 1999, 2001a,b). Therefore, the stimulus-response curves of the PC

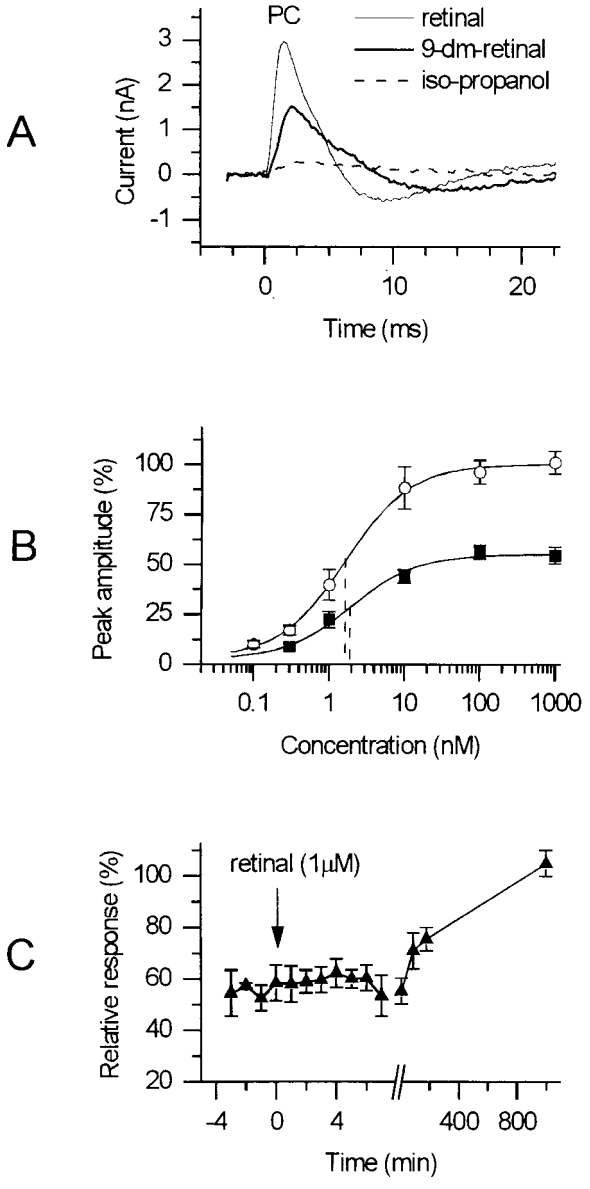

FIGURE 3 (*A*) Flash-induced electrical responses recorded in the GO mode from suspensions of the N-164 *C. reinhardtii* cells preincubated for 3 h with 1 μM retinal, 1 μM 9-dm-retinal, or isopropanol. Excitation is a 1-ms flash, 4.3×10^{19} photons $\times$ m^{-2}. The traces are averages of four single sweeps. (*B*) Dependence of the PC peak amplitude measured in the GO mode in suspensions of the N-164 *C. reinhardtii* cells on the concentration of an exogenous chromophore. Currents were measured at least 10 min after the addition of 9-dm-retinal (*filled squares*) or retinal (*open circles*) to identical cell samples. Each data point represents the mean $\pm$ SE (n = 6–10) of measurements in separate cell samples. Solid lines show a computer fit with a Michaelis function, as described in the text. Dashed lines indicate the EC_{50} values calculated from the fit. (*C*) Influence of an excess retinal on PC peak amplitude recorded in the UL mode from suspensions of the N-164 *C. reinhardtii* cells reconstituted with 9-dm-retinal. Two identical cell samples were preincubated for 2 h with 100 nM of either 9-dm-retinal or retinal, after which 1 μM retinal was added at time 0, as indicated by the arrow. Between measurements, cells were kept in the dark on a rotary shaker. Relative response was calculated by dividing PC peak amplitudes measured in a sample reconstituted with 9-dm-retinal by those in a sample reconstituted with retinal. Each data point represents the mean value $\pm$ SD (n = 4) of independent measurements in separate cell samples.

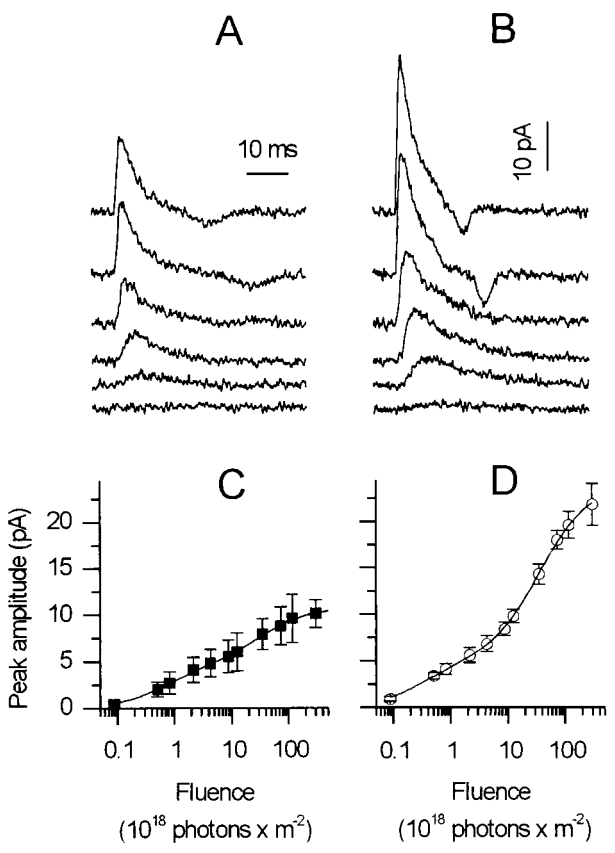

FIGURE 4 Representative single-shot current traces (*A* and *B*) and dependence of PC peak amplitude on flash fluence (*C* and *D*) measured in the individual *cw2 lts1-30 C. reinhardtii* cells reconstituted with 1 μM of either 9-dm-retinal (*A* and *C*) or retinal (*B* and *D*) by the suction pipette technique. The traces were measured at 0.1, 0.2, 0.8, 4.3, 35, and 117 $\times$ 10^{18} photons $\times$ m^{-2} (from below). Each data point in *C* and *D* is the mean $\pm$ SE of measurements in 10 cells. The responsiveness of each cell has been pretested with a single 10-μs flash after which a series of 1-ms flashes of incremental fluence was applied with the time interval of 10 s between them.

peak amplitude measured in cells reconstituted with either 9-dm-retinal or retinal were fitted with a sum of two Michaelis functions, i.e., $R = R_{max} \times I/(I_{50} + I) + R'_{max} \times I/(I'_{50} + I)$ (Fig. 4 *C* and *D*). The parameters of this fit were for 9-dm-retinal-reconstituted cells: $R_{max} = 4.7 \pm 0.3$, $I_{50} = 0.7 \pm 0.1$, $R'_{max} = 6.1 \pm 0.3$, $I'_{50} = 33.4 \pm 6.6$; for retinal-reconstituted cells: $R_{max} = 5.1 \pm 0.3$, $I_{50} = 0.3 \pm 0.1$, $R'_{max} = 18.9 \pm 0.3$, $I'_{50} = 36.1 \pm 2.4$. These parameters were used to estimate sensitivity (Jin et al., 1993), because a signal-to-noise ratio was not high enough to measure incremental responses in a low-intensity range where a linear dependence of the current amplitude on the stimulus intensity is expected. The sensitivity, calculated as R_{max}/I_{50} and R'_{max}/I'_{50}, was, respectively, 2.5 and 2.9 times smaller in cell reconstituted with the analog as compared with retinal.

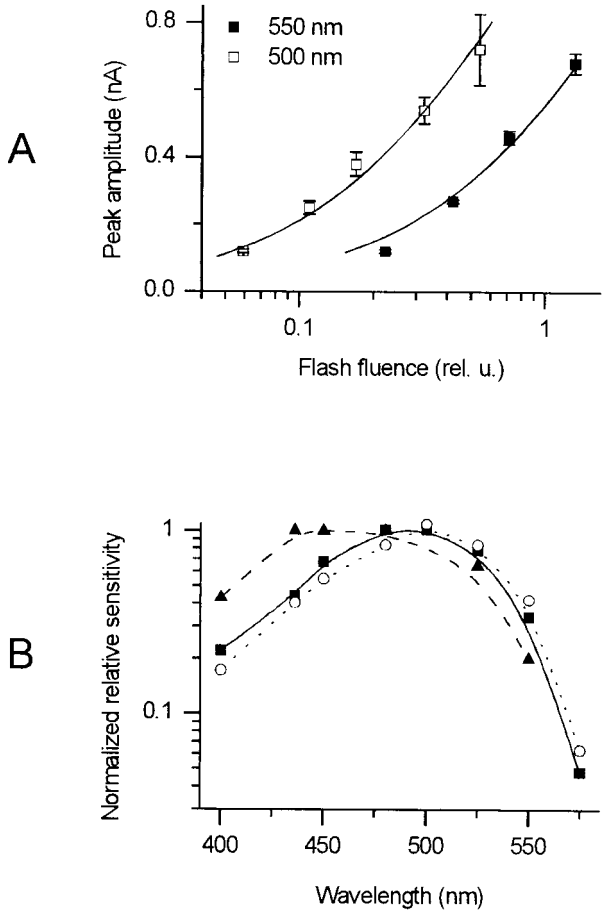

FIGURE 5 (*A*) A representative pair of stimulus-response curves of the PC peak amplitude measured in the GO mode from suspensions of the N-164 *C. reinhardtii* cells reconstituted with 100 nM 9-dm-retinal, as used for construction of the action spectrum. Each data point shows the mean value ± SD of two independent measurements in separate cell samples. Solid lines show a computer fit by a Michaelis function. (*B*) Normalized action spectra for the PC peak amplitude measured in the GO mode from suspensions of the N-164 *C. reinhardtii* cells reconstituted with 100 nM 9-dm-retinal (*closed squares*, *solid line*), retinal (*open circles*, *dotted line*), or naphtylretinal (*solid triangles*, *dashed line*). The lines connecting the data points are computer-generated B-spline lines.

Action spectra for PC peak amplitude were constructed from a series of stimulus-response curves measured in suspensions of reconstituted cells in a range of the low-saturating process where contribution of the high-saturating process is negligible (Fig. 5 *A*). Therefore, data were fitted with a single Michaelis function. For each wavelength, a reference curve for the 500-nm stimulus was measured in the same cell sample to compensate for differences in culture sensitivity on different days of experiment. Relative spectral sensitivity was calculated from the parameters of the fit, as described above. The spectral sensitivity measured in cells reconstituted with 9-dm-retinal revealed a 10-nm shift toward shorter wavelengths compared with that in cells reconstituted with retinal (Fig. 5 *B*). A much larger shift in the same direction (Fig. 5 *B*) was observed in cells reconstituted with naphtylretinal that was used for comparison (for chemical structure see Fig. 1 *C*).

A flash of the saturating intensity was used as a test flash in double-flash experiments. Application of a conditioning flash shortly before the test flash resulted in suppression of the amplitude of the test-flash-evoked PC as compared with its maximal value obtained when the test flash was used alone. The magnitude of this suppression depended on fluence of the conditioning flash (Fig. 6 *A* and *B*) and the time interval between the two flashes (Fig. 6 *C* and *D*). Generation of the FC after a conditioning flash enhanced suppression of the test-flash evoked PC as it has been found earlier in the wild type (Govorunova et al., 1997). When currents were measured under conditions when no FC was generated in response to the conditioning flash, maximal suppression of the test-flash-evoked PC amplitude was observed within the same time window between 20 and 50 ms in cells reconstituted with either 9-dm-retinal or retinal. However, the amplitude suppression was smaller and slower and the recovery faster in cells reconstituted with 9-dm-retinal than in retinal-reconstituted cells at the same conditioning flash intensity (Fig. 7). When the PC evoked by the conditioning flash in cells reconstituted with the analog was made equal to that measured in retinal-reconstituted cells by decreasing flash intensity in the latter case, the two curves became indistinguishable within the experimental error (Fig. 7).

The influence of the chromophore structure on photoorientation in *Chlamydomonas* was examined by measuring the currents in suspensions of cells preoriented by a continuous light stimulus (i.e., in the PO mode). Photoorientation in wild-type cells was studied by the same assay for comparison (Fig. 8). The sign of the electrical signal measured in the PO mode is determined by the sign of the net phototactic response of the cell suspension, whereas its amplitude depends on both the amplitude of the transmembrane currents and the degree of phototactic orientation of the cells. The dependence of the PC peak amplitude on the fluence rate of the 10-s preorienting light stimulus is shown in Fig. 8 *B*. At lower fluence rates, the N-164 cells reconstituted with either 9-dm-retinal or retinal showed negative phototaxis, whereas wild-type cells had positive one. The absolute magnitude of PC recorded in the PO mode gradually increased until a certain value upon an increase in the fluence rate in all three tested cultures. Further increase in the stimulus intensity led to a gradual decrease of the absolute magnitude of the response up to zero, after which the sign of the current reversed, indicating the reversal of the sign of net phototaxis.

A switch from negative to positive phototaxis was observed in the N-164 cells reconstituted with retinal at approximately the same fluence rate that induced a switch from positive to negative phototaxis in the wild type. The reversal of the phototaxis sign in 9-dm-retinal-reconstituted cells occurred at a severalfold higher fluence rate than in

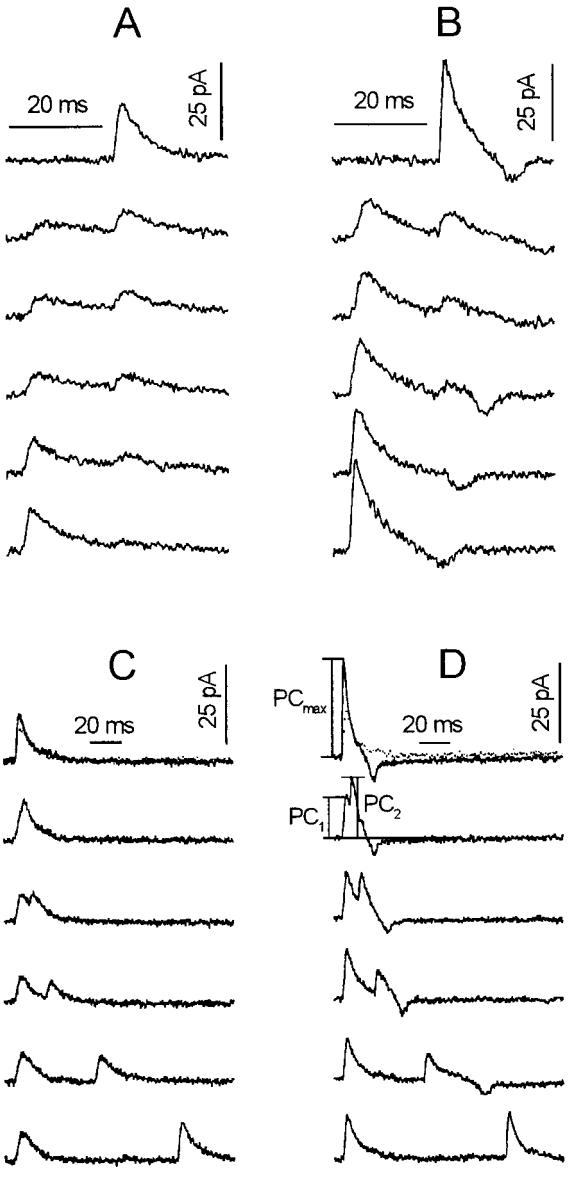

FIGURE 6 Photocurrents recorded by the suction pipette technique from the *cw2 lts1-30 C. reinhardtii* cells reconstituted with 1 μM 9-dm-retinal (*A* and *C*) or retinal (*B* and *D*) upon single- or double-flash excitation with 10-μs flashes. The intensity of the test flash was 3.9×10^{19} photons $\times$ m^{-2}. All traces are single-shot responses recorded in one representative cell in each of the *A* to *D*. (*A* and *B*) Traces were recorded at a fixed interflash interval 20 ms, the intensity of the conditioning flash applied at time 0 was 0, 1.2, 2.4, 5, 10, and 50% of that of the test flash (from above). (*C* and *D*) The dashed traces were recorded in response to a conditioning flash (7.4×10^{18} photons $\times$ m^{-2}) applied alone at time 0. The solid traces were recorded upon double-flash excitation at 0, 5, 10, 20, 50, and 100 ms interval between the flashes (from above). PC_{max} is the maximal peak amplitude of the PC; PC_1 and PC_2 are the peak amplitudes of the PC evoked by the conditioning and the test flash, respectively.

cells reconstituted with retinal, so that the sign of phototaxis in 9-dm-retinal-reconstituted cells was opposite to that in cells reconstituted with retinal in a certain range of stimulus intensities (Fig. 8).

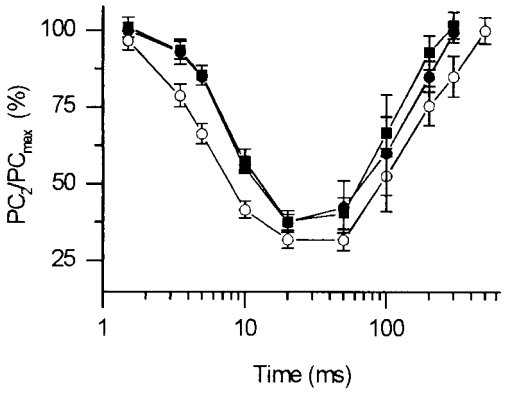

FIGURE 7 Time course of suppression and recovery of PC peak amplitude upon double-flash excitation measured by the suction pipette technique in the *cw2 lts1-30 C. reinhardtii* cells reconstituted with 1 μM 9-dm-retinal (*squares*) or retinal (*circles*). The intensity of the test flash was as in Fig. 6, the intensity of the conditioning flash was 7.4×10^{18} photons $\times$ m^{-2} (*filled squares*, *open circles*), or 3.6×10^{18} photons $\times$ m^{-2} (*filled circles*). Definitions of PC_2 and PC_{max} are as in Fig. 6. Each data point shows the mean $\pm$ SE of measurements in 10 individual cells.

DISCUSSION

Rapid restoration of photoelectric responses in cell suspensions of *C. reinhardtii* carotenoid-deficient mutants upon the addition of 9-dm-retinal was briefly reported in our previous work (Sineshchekov et al., 1994). In this study, a more detailed analysis of 9-dm-retinal-induced photocurrents recorded both in cell suspensions and individual cells was carried out, and the influence of this analog on the sign of phototaxis was examined.

The dependence of PC peak amplitude on concentration of the exogenous chromophore (Fig. 3 *B*) and the absence of a rapid increase in PC amplitude after the addition of an excess retinal on top of a saturating concentration of the analog (Fig. 3 *C*) show that a smaller amplitude of PC in cells preincubated with 9-dm-retinal compared with retinal is not due to incomplete reconstitution of the analog pigment. A slow increase in the PC amplitude observed in 9-dm-retinal-reconstituted cells on a time scale of hours after the addition of an excess retinal likely reflects exchange of the bound chromophore or de novo formation of the apoprotein (opsin).

Binding of all-*trans* 9-dm-retinal to the apoproteins of sensory rhodopsins I and II in *Halobacterium salinarium* increased the sensitivity of photobehavioral responses mediated by these receptors (Yan and Spudich, 1991; Yan et al., 1991). On the contrary, substitution of the native chromophore of vertebrate visual pigment with 11-*cis* 9-dm-retinal dramatically reduced the sensitivity of photoinduced electrical currents (Corson et al., 1994). The sign of the sensitivity change induced by this analog in *Chlamydomonas* revealed by this study is the same as in animal visual cells, but its magnitude is significantly smaller. Such "in-

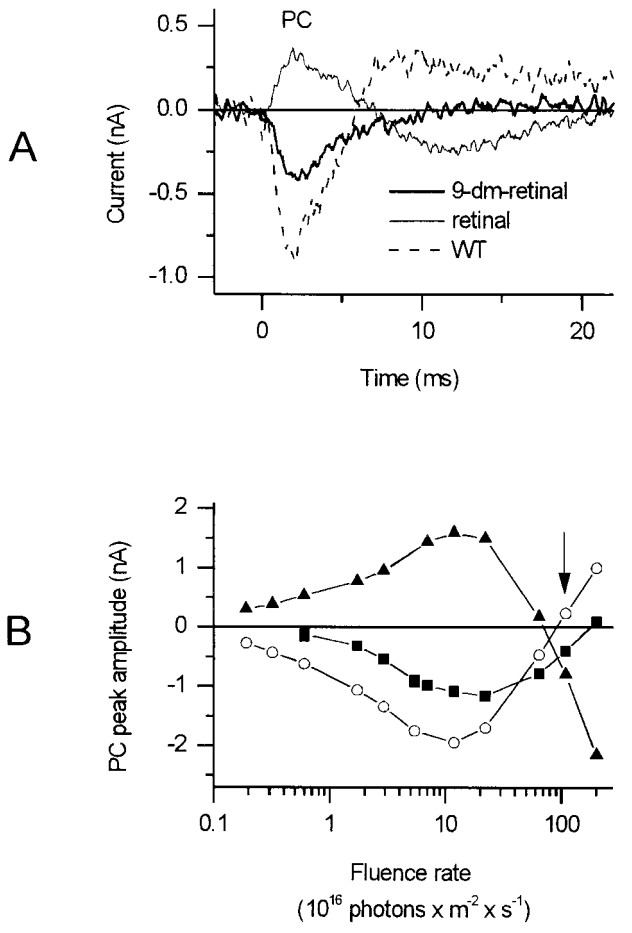

FIGURE 8 (*A*) Photocurrents recorded in the PO mode from *C. reinhardtii* cell suspensions after 10 s of preillumination with 500-nm light (1.1×10^{18} photons $\times$ $m^{-2} \times s^{-1}$). A test flash was 4.3×10^{19} photons $\times$ m^{-2}. Cells of the carotenoid-deficient strain N-164 were preincubated for 2 h with 100 nM 9-dm-retinal or retinal, wild-type cells (WT) of the strain 495 were preincubated for the same time with an equal volume of the solvent (isopropanol). The traces are single-shot responses. (*B*) Dependence of the PC peak amplitude measured in the PO mode in *Chlamydomonas* suspensions on the intensity of a preorienting light stimulus (10 s, 500 nm). Cells were treated as in *A*. Each data point represents the mean value of four independent measurements in separate cell samples. The arrow indicates the fluence rate at which the current traces shown in *A* were recorded.

termediate" effect of 9-demethylated analog on PC and phototaxis could be determined by the structure of chlamyrhodopsin as well as by the specificity of its signaling mechanism.

Many reports pointed out that PC is a complex process, as revealed by analysis of its kinetics (Sineshchekov et al., 1990; Sineshchekov and Govorunova, 1999) and the biphasic dependence of its peak amplitude on stimulus intensity (Sineshchekov 1991a,b; Sineshchekov et al., 1992, 1994). In this study, two phases of the stimulus-response curve were quantitatively characterized in cells reconstituted with 9-dm-retinal or retinal (Fig. 4 *C* and *D*). The origin of the two processes behind the two phases of the curve is not yet clear. A hypothesis has been suggested that they might represent two separate signal transduction pathways mediated by the same receptor (Sineshchekov and Govorunova, 1999, 2001a), or even by different rhodopsin species. Most physiological evidences indicate that *Chlamydomonas* cells contain only one retinal-binding protein that serves as the photoreceptor for phototaxis and the photophobic response (Takahashi et al., 1991). However, results of reconstitution studies in a "blind" *C. reinhardtii* mutant using a C13-*trans*-locked retinal analog pointed to a possibility of separate photoreceptors for these two processes (Zacks et al., 1993), although the data could also be explained in terms of a single receptor and adaptational differences between phototaxis and the photophobic response (Zacks and Spudich, 1994).

The spectral sensitivity of PC measured in *C. reinhardtii* cells reconstituted with 9-dm-retinal was shifted ~10 nm toward shorter wavelengths compared with that in retinal-reconstituted cells (Fig. 5 *B*). This value can be slightly smaller that the real difference between the absorption spectra of the respective regenerated pigments due to the presence of a residual amount of endogenous retinal in carotenoid-deficient cells. The highest sensitivity at 465 nm reported earlier for light-induced behavioral responses in the 9-dm-retinal-reconstituted *C. reinhardtii* mutant (Hegemann et al., 1991) appears to be an overestimation of the spectral shift.

Binding of a 9-demethyl analog instead of the native chromophore leads to a substantial shift of absorption spectra toward shorter wavelengths in animal (Corson et al., 1994), as well as archaeal (Gärtner et al., 1983; Yan and Spudich, 1991; Yan et al., 1991) rhodopsins. However, significant spectral changes observed in 9-demethylrhodopsins can only be explained by an impaired interaction between protein and chromophore upon the removal of the 9-methyl group, because the difference between the absorption maxima of free 9-dm-retinal and retinal in solution is not more than 10 nm. Therefore, a relatively small shift of the action spectrum for PC in *C. reinhardtii* cells reconstituted with 9-dm-retinal compared with retinal apparently indicates that there is no such strong interaction between the 9-methyl group of the chromophore and specific amino acids of the chromophore binding pocket of the apoprotein, as it was shown for this group and tryptophan 182 in bacteriorhodopsin (Yamazaki et al., 1995; Weidlich et al., 1996), or the respective group of 11-*cis* retinal and glycine 121 in animal visual rhodopsin (Han et al., 1997).

The action spectrum for PC measured by our electrophysiological assay in cells reconstituted with naphtylretinal revealed a clear blue shift of ~40 nm (Fig. 5 *B)* compared with that of retinal-reconstituted cells, which is close to the results obtained by a dish test in the *C. reinhardtii* mutant FN68 (Foster et al., 1989). However, incorporation of this analog did not increase the sensitivity of PC in reconstituted

cells comparing with that of retinal, as it was reported by Foster et al. (1989) for phototaxis.

In double flash experiments, the suppression of the PC peak amplitude and its recovery are identical in cells reconstituted with either 9-dm-retinal or retinal when the conditioning-flash-evoked currents are equal (Fig. 7). This observation corroborates the earlier drawn conclusion that the light-induced suppression of PC amplitude is determined by the processes downstream from the photoconversion of the receptor rhodopsin (Govorunova et al., 1997). Membrane depolarization and an increase in intracellular Ca^{2+} concentration can be suggested as possible candidates for these processes. Time-resolved measurements of the membrane potential and intracellular Ca^{2+} concentration, which have not been so far possible in green flagellates, are necessary to directly test these possibilities.

The sign of phototaxis in carotenoid-deficient cells reconstituted with either 9-dm-retinal or retinal changed from negative to positive upon an increase in stimulus intensity. The light dependence of the sign of phototaxis in the wild type was inverse (Fig. 8). These results confirm our earlier observations in carotenoid-deficient cells reconstituted with all-*trans* retinal (Sineshchekov et al., 1994). This phenomenon could be explained by a different optical mechanism for detection of the light direction in reconstituted carotenoid-deficient cells, as compared with the wild type. The sign of the electrical signal measured in the UL mode in reconstituted mutant cells is the opposite to that in wild-type cells (Sineshchekov et al., 1994), which indicates that photoreceptors of reconstituted cells absorb minimal light when facing the light source, and maximum when being turned away from it, whereas in wild-type cells the situation is the opposite. This likely results from focusing of light by the almost transparent cell body ("lens effect") in reconstituted mutant cells, which takes place instead of the combined shading/reflecting action of the eyespot and chloroplast in wild-type cells (Sineshchekov et al., 1994; Sineshchekov and Govorunova, 2001a). A 180° phase shift in illumination of the photoreceptor during helical swimming of the cell leads to the opposite directions of phototaxis in reconstituted carotenoid-deficient mutants and the wild type measured under the same experimental conditions.

The results of this study show that the biochemical machinery responsible for regulation of the phototaxis sign upon an increase in stimulus intensity/duration is retained in the carotenoid-deficient mutants. A switch from positive to negative phototaxis upon an increase in intensity or duration of the light stimulus, similar to that shown in Fig. 8 *B* in wild-type *C. reinhardtii* cells, was frequently observed in earlier studies (Nultsch et al., 1971; Feinleib and Curry, 1971; Uhl and Hegemann, 1990). However, a mechanism for this behavior is not yet fully understood. Experiments in demembranated cell models led to the conclusion that positive phototaxis in *Chlamydomonas* is linked to an increase in intracellular Ca^{2+} concentration, whereas negative one is induced by a decrease in it (Kamiya and Witman, 1984). It has been therefore proposed that a switch from positive to negative phototaxis observed in intact cells upon prolonged illumination can be explained by an adaptation of the cells due to an elevated intracellular Ca^{2+} associated with initial positive phototaxis (Morel-Laurens, 1987).

The fluence rate-response curves of photoorientation measured in mutant cells reconstituted with retinal and in the wild type were strictly symmetrical (Fig. 8). Substitution of 9-dm-retinal instead of the native chromophore shifted both the absolute maximum and the reversal of the phototaxis direction to a higher stimulus intensity (Fig. 8). Such shift of the fluence rate-response curve is likely a direct consequence of a decreased signaling efficiency in 9-dm-retinalchlamyrhodopsin compared with the native pigment or that reconstituted with exogenous retinal.

Takahashi et al. (1992a) proposed that the diversion of the phototaxis sign in the analog-reconstituted cells results from a delay in transduction of the photosignal comparable with the half-period of cellular rotation (250–500 ms). This study shows that the onset of PC in cells reconstituted with 9-dm-retinal does not have such a delay. However, it is not excluded that a slower rise and smaller amplitude of this current compared with that in retinal-reconstituted cells might lead to a delay of a subsequent phase of the signaling cascade, such as changes in the Ca^{2+} concentration within the intraflagellar lumen. These changes are thought to control asymmetric motor responses of the two flagella that drive phototaxis (Kamiya and Witman, 1984).

Preliminary experiments with pentaenal, one of the retinal analogs used in the work of Takahashi et al. (1992a), revealed a similar influence of this analog on PC, as it has been found with 9-dm-retinal (data not shown). Therefore, a decreased signaling efficiency of the analog chlamyrhodopsin can also be suggested as a possible explanation for the diversion of the phototaxis sign observed by these workers in pentaenal-reconstituted cells. However, Takahashi et al. (1992a) used motion analysis to separately measure positive and negative phototaxis in the same cell sample, therefore it is difficult to directly compare their data with the results of our experiments, in which the net photoorientation of the cell population was being instantly assessed by photoelectric recording in the PO mode.

Analysis of photocurrents and photoorientation in 9-dm-retinal- or retinal-reconstituted cells undertaken in the current study corroborates the notion that control of the phototaxis sign in *Chlamydomonas* likely occurs at a postreceptor site of the signal transduction chain (Sineshchekov and Govorunova, 1999). This is in contrast to archaea, in which photoaccumulation and -dispersal of the cells are already determined at the photoreceptor level, either by excitation of different intermediates of the photocycle of the same photoreceptor species, i.e., sensory rhodopsin I, or by excitation of different receptor proteins, i.e., the attractant

form of sensory rhodopsin I or sensory rhodopsin II (Spudich et al., 1995; Hoff et al., 1997).

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