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The photoreceptor current of the green alga *Chlamydomonas*

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SUMMARY

The unicellular alga *Chlamydomonas* possesses a visual system which guides it to places that are optimal for photosynthetic growth. The rhodopsin, serving as the photoreceptor, conveys light information into a cellular signal. This signal is transmitted via several electrical steps to the flagella, where it modulates the flagellar beating pattern. The first detectable electrical process is the photoreceptor current, which is confined to the eyespot region. It is carried mainly by calcium under physiological conditions. After a flash the current rises with no detectable delay in less than 2 ms to the maximum and decays within tens of ms. The peak amplitude increases with the stimulus intensity over a range of two orders of magnitude, and the decay kinetic is accelerated in parallel. The light sensitivity of the cell is reduced by a factor of 8 when the eye is oriented away from the light source instead of facing it. This gives a measure for the 'contrast' used by the cell during orientation in light. The rhodopsin action spectrum of the photoreceptor current has a maximum at 494 nm, closely matching the absorption maximum of rhodopsin in purified membranes. When the photoreceptor current exceeds a critical level, flagellar currents appear as all-or-non events. Several lines of evidences suggest that these photocurrents are the link between light absorption and alteration of the flagellar beating during a movement response.

1. INTRODUCTION

Chlamydomonas is a biflagellated alga of only 5 to 10 μm across (figure 1). It is a unicellular organism, which not only measures the intensity of the ambient light, as light sensitive bacteria do, but also detects the direction of the light incidence. Thus it can swim towards or away from the light source and consequently find optimal light conditions for photosynthetic growth. For this purpose it possesses a visual system, that absorbs the light and converts the received information into an intracellular signal, which, in turn, modulates the beating pattern of the flagella. As in mammalian visual systems, *Chlamydomonas* can detect single photons (Hegemann & Marwan 1988; Uhl & Hegemann 1990) and adjust to changes in the ambient light level over several orders of magnitude (Foster *et al.* 1984; Morell-Laurens 1987).

The orange eyespot functions as an optical device, forming together with the photoreceptor, which is localized in one of the eyespot overlaying membranes, the intact eye. The optical properties of algal eyes have been analysed in detail by Foster & Smyth (1980) and Foster & Saranak (1989). The eyespot consists of alternating high and low refractive index layers. The high refractive index is realized by packing carotenoids to a high density into vesicles. With a light incidence perpendicular to the surface of the eyespot, the optical path through one layer is roughly a quarter wavelength of 500 nm light. This yields a so called quarter wave stack. Every interface of the stack reflects a certain proportion of an incident light beam. Depending on the light colour and the angle of incidence, reflected waves constructively interfere (mirror optic, figure 2). If the spectral range

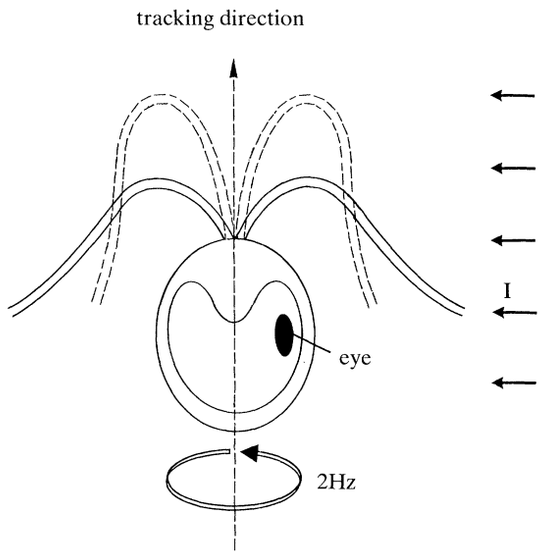

Figure 1. Schematic representation of a *Chlamydomonas* cell swimming with the beating flagella in forward direction. During rotation (counterclockwise) with a frequency of 2 Hz the eye is scanning the environmental light conditions (I), thereby receiving a modulated light signal. The cell changes swimming direction until the modulation amplitude is minimal. This is the case if it swims directly to the light or at higher light levels away from it. The arrow along the cell axis indicates swimming direction.

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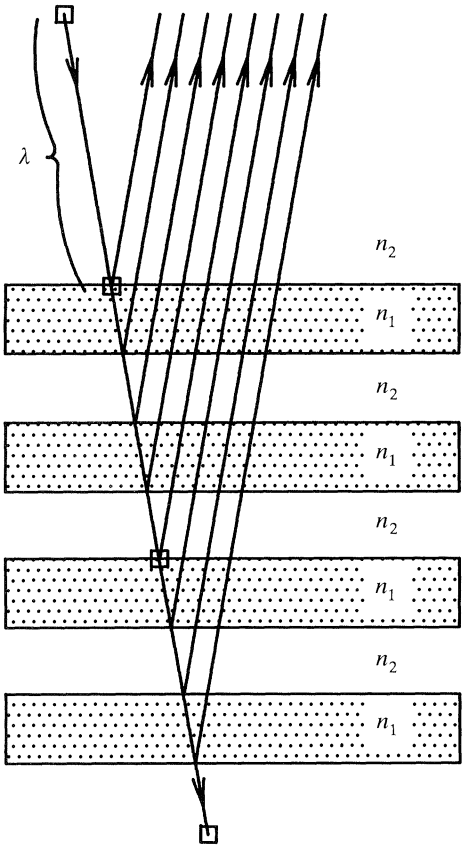

Figure 2. Multilayer reflection as it occurs in the eyes of unicellular flagellates. Constructive interference occurs when the optical path of both high and low refractive index layers (n_1 and n_2) are close to $\lambda/4$ (according to Land (1972), modified).

of constructive interference overlaps with the absorption spectrum of a photoreceptor, located in the eyespot overlaying part of the plasmalemma, the photoreceptor is effectively shielded against light coming from the back side through the cell. In contrast, when light reaches the eye directly, the intensity is enhanced. This gives the eye a monopolar directivity. For non-normal light incidence the wavelength with maximal constructive interference is shifted from 500 nm to shorter wavelength (Land 1972). This type of eyespot is an excellent design for a single celled organism to receive a light signal which is cyclically modulated in intensity during helical swimming. The modulation amplitude is maximal when the cells swim perpendicular to the light incidence, whereas it is minimal when they swim parallel to it. As the cells correct their swimming direction until the modulation amplitude is minimal, they align their swimming direction with the light incidence and thus swim directly to or away from the light source (Foster & Smyth 1980). The correlation between reflectivity and constructive interference to the number of layers has recently been demonstrated experimentally for the unicellular algae *Tetraselmis chui*, *Chlamydomonas eugametos*, and *Hafniomonas reticulata* using confocal laser scanning microscopy (Kreimer & Melkonian 1990).

In *Chlamydomonas* the photoreceptor for behavioural responses to continuous light or light flashes has been identified as a rhodopsin (Foster *et al.* 1984; Hegemann *et al.* 1988; Uhl & Hegemann 1990). This rhodopsin resembles in several respects the sensory rhodopsins from *Halobacteria*. Its absorption spectrum is fine-structured (Beckmann & Hegemann 1991) as in phoborhodopsin, a halobaterial lightsensor (SRII, Takahasi *et al.* 1990). All-trans retinal serves as the chromophoric group, and apparently a 13-*trans* to *cis* isomerization is responsible for rhodopsin activation (Hegemann *et al.* 1991; Lawson *et al.* 1991). All-trans retinal containing rhodopsins are thought to be the ancient type of rhodopsin, whereas in higher animals a new type of 11-*cis*-retinal containing rhodopsins is used for visual processes (Hegemann 1991).

Although G-proteins have been suggested to be present in *Chlamydomonas* (Korolkov *et al.* 1990; Schloss 1990; Hegemann & Harz 1992), no physiological evidence is available for any biochemical step being involved in the signal transduction process from the photoreceptor to the flagella.

Instead, the calcium dependence of light-induced behavioural responses (Stavis & Hirschberg 1973; Nultsch 1979; Schmidt & Eckert 1976; Dolle *et al.* 1986; Hegemann & Bruck 1989) have led to the now widely accepted conclusion that calcium plays an important role for photoreception in *Chlamydomonas* and may function as an intracellular messenger. That the intracellular calcium concentration can directly control the beating pattern of the flagella, has been shown by analysing the flagellar beating of the isolated flagellar apparatus (Hyams & Borisy 1978; Bessen *et al.* 1980) and of permeabilized cells (Kamiya & Witman 1984). Nichols & Rickmanspoel (1978) were able to change the frequency of flagellar beating by mechanical micro-injection of Mg^{2+} and Ca^{2+} into the cytoplasm of intact cells. With inserted microelectrodes, however, cytoplasmic components leaked out of the cells and no stable membrane potential could be attained. The role of calcium found further support by the finding that behavioural light responses are suppressed by chemicals which are known to inactivate calcium currents in mammalian cells. These inhibitors include dihydropyridines, verapamil, ω-conotoxin, amilorides, diltiazem, pimozide, and heavy metals as Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, La^{3+} (Nultsch *et al.* 1986; Hegemann *et al.* 1990; P. Hegemann & R. Uhl, unpublished results). From binding studies with tritiated verapamil, nimodipine, and diltiazem, several binding sites for calcium channel inhibitors have been postulated to be present in the plasmalemma and the flagellar membrane (Dolle 1988; Dolle & Nultsch 1988). Beyond its participation in signal transduction and flagellar beating, calcium also seems to affect adaptation to continuous light or to repetitive flash stimulation (Hegemann & Bruck 1989).

Measurements of light-induced $^{45}Ca^{2+}$ uptake have been precluded in wild-type cells by the cell wall, but have been possible in cell wall-defective 806 strain cells (Hegemann *et al.* 1990). They support the conclusion that light-dependent calcium channels of the plasma membrane are involved in the process of photoreception. Because $^{45}Ca^{2+}$ uptake measurements suffer from low sensitivity and poor time resolution,

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electrical measurements seemed to be necessary in order to understand light-regulated calcium currents in *Chlamydomonas*.

2. MATERIALS AND METHODS

Because the presence of cell wall precludes electrical measurements, the experiments were carried out with the cell wall-deficient mutant CW2. Recently, however, we have found in cooperation with Dr S. Waffenschmidt, University Köln, that electrical measurements are also possible on wild-type cells and probably on any *C. reinhardtii* strain, provided the cell wall has been digested with autolysin.

Chlamydomonas strain CW2 cells were grown on plates of high salt acetate medium (HSA, HSM according to Sueoka *et al.* (1967) supplemented with 15 mM sodium acetate) with 250 mM sorbitol, 0.3% yeast extract, and 0.9% agar (IMA, Nürnberg) in dim white light of about 2 W m^{-2} for 7 to 10 days. Because gametes have the highest light sensitivity, the cells were differentiated into gametes in 8–10 ml nitrogen-deficient minimal liquid medium (NMM) in absence of sorbitol in 50 ml Falcon tubes (1.5×10^7 cells ml^{-1}, 2 W m^{-2} white light, 120 r.p.m.; Hegemann *et al.* 1988). The phototactically most active cells were separated by three rounds of photoselection (Hegemann & Bruck 1989) in NMG^+/K^+ buffer (5 mM HEPES, 10 mM Cl^-, 1 mM K^+, 200 μM BAPTA, 300 μM Ca^{2+}, adjusted with *N*-methyl-glucamine to pH 6.8) and finally resuspended in the same medium. After dark adaptation for more than 1 h the cells were used for electrical measurements. The NMG^+/K^+ buffer was used as electrode and bath electrolyte solution.

In experiments, in which the calcium concentration was varied (figure 10), phosphate buffer (3 mM K_2HPO_4, 200 μM BAPTA, the desired amount of $CaCl_2$, adjusted to pH 6.8 with HCl) was used for photoselection and as bath and electrode electrolyte. The concentration of free Ca^{2+} was calculated according to Tsien (1980). In one experiment the calcium concentration was varied only by factor of ten in order to minimize changes of the cellular surface potential and to avoid steep concentration gradients between inside and outside of the pipette. The cells were photoselected at 1, 10, or 100 μM calcium.

To suck a part of the cell into the pipette without using strong forces, suction pipettes with fairly parallel tips (small cone angle) were pulled in two steps from borosilicate glass capillaries (1.6 mm outer diameter, 0.5 mm walls, Hilgenberg, Malsfeld). Pipettes were broken at the desired width on heated glass (Brown & Flaming 1986) and heat polished (Hamill *et al.* 1981). The final tip diameter was in the range of 2–4 μm.

At the beginning of each experiment the pipette was centred to the axis of the rotary stage by using manipulator T_4 and T_5 shown in figure 3. Cells were sucked into the pipette by low pressure application (Baylor *et al.* 1979) until the resistance reached 200 to 250 MΩ. The low pressure was held constant during the whole measurement. All manipulations were done under microscopic control by using a 40× objective

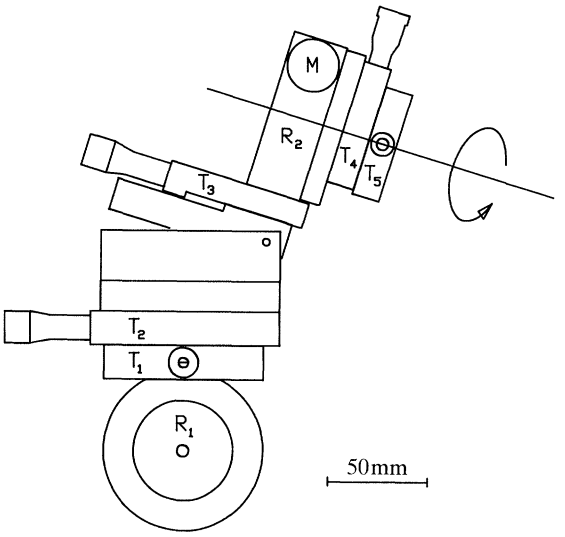

Figure 3. Scheme of the pipette-holding manipulator. It includes five translational (T_1 to T_5) and two rotary elements (R_1 and R_2). M is the motor drive of R_2. Preamplifier and suction pipette are mounted in front of T_5.

(0.75 numeric aperture) and illumination with infrared light (RG 830 filter, Schott, Mainz; not more than 250 W m^{-2}). Cells were stimulated with light flashes of 300 μs duration and $\lambda_{max} = 495$ nm. Light of other quality is indicated in the figures. Wavelengths were selected by interference filters with 5–12 nm half band width (Schott, Mainz). To maintain maximal light responses, the cell was rotated with the rotary stage R_2 (figure 3) under application of green test flashes (greater than or equal to 10 s intervals). The position with maximal response was used for further measurements.

Currents were recorded at constant voltage (0 mV between bath and pipette) with a patch clamp amplifier (EPC-7, List, Darmstadt) and filtered with the 3 kHz low pass bessel filter. Data were recorded and processed on a personal computer with the help of the TL-1-125 labmaster DMA interface and pClamp 5.5.1 software (both from Axon instruments, Foster City). If not otherwise indicated, current records were averaged 10 to 20 times and filtered with a digital Gaussian filter to 500 Hz. The temperature was maintained at 20 ± 1°C.

For changing of the ionic conditions, an application pipette with 60 to 80 μm tip diameter was used, in which two tubules connected to the buffer reservoirs were inserted. The flow was regulated by hydrostatic pressure. The typical delay time was less than 30 s when the cell was placed about 100 μm in front of the application pipette and was exposed to a stream of buffer that was held constant during the whole experiment.

Functions were fitted to the data using commercial software (Sigma Plot 4.0, Jandel Scientific, Erkrath).

3. PHOTORECEPTOR CURRENTS IN GREEN ALGAE

(*a*) *Appearance in different species*

Rapid light-induced electrical responses have been

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recorded from the giant unicellular alga *Acetabularia* (also a *Chlorophyceae*) many years ago (Schilde 1968). Owing to the macroscopic size intracellular recording has been possible. However, since *Acetabularia* is a seawater alga living in high salt, the results can be compared with those from fresh water algae only with caution. In *Acetabularia* fast green light-induced voltage changes are caused by an inhibition of the electrogenic chloride pump rather than by ion channel activation (Gradmann 1978). As ion channels are discussed in this issue, light responses of *Acetabularia* are not further considered.

Much research on ion channels in plants has been carried out with the giant Characean algae *Nitella* and *Chara* (see reviews by Berestrovsky *et al.* 1987; Katsuhara & Tazawa, this symposium). There calcium channels are involved in the initiation of action potentials in response to various environmental stimuli. The exact mechanism by which channel activation is connected to the initial stimulus, however, remains to be elucidated.

From green algae (*Chlorophyceae*) light-dependent ion currents have been recorded by application of the suction pipette technique, first to *Haematococcus* (Litvin *et al.* 1978) and later also to its smaller relative *Chlamydomonas* (Harz & Hegemann 1991). With *Haematococcus* the potential difference between pipette and the bath electrode was measured, whereas with *Chlamydomonas* the current in the pipette was measured under constant voltage conditions. Because both methods detect the same bioelectric phenomena, the shape of the signals from both algal species is very similar and the differences can be explained by different experimental conditions used. As originally neither the number and distribution of ion channels was known, nor their conductance, a membrane region large enough to yield a macroscopic current was sucked into the pipette. With this method, localized and stable electrophysiological responses can be obtained from cell wall-less *Chlamydomonas* cells (Harz & Hegemann 1991), whereas in case of *Haematococcus* due to the smooth and flexible cell-wall wild-type cells can be used (Litvin *et al.* 1978). By using the suction pipette technique it should be possible to answer several physiological questions in the near future. However, since the conditions for a 'gigaseal' between cell and pipette has not been established yet, real patch clamp, i.e. recording from the 'whole cell configuration' or from an 'exised patch' (Hamill *et al.* 1981) has not been possible. With this obstacle a detailed analysis of the membrane potential and the ion channel properties cannot be accomplished at the moment.

(*b*) *Light dependence*

Stimulation of *Chlamydomonas* cells with bright flashes initiates a sequence of three ion current components (figure 4). The first is localized in the eyespot region, whereas the other two originate from the flagellar region. Owing to the low seal resistance and the low current amplitude, averaged traces are shown in all figures.

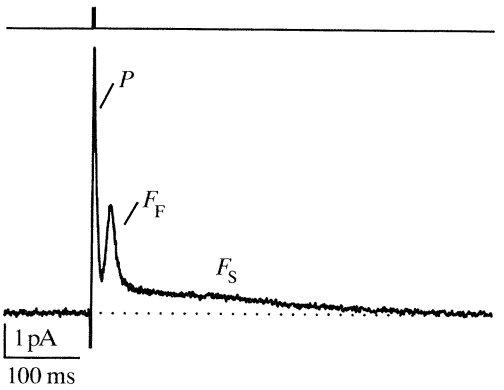

Figure 4. Flash-induced currents from a *Chlamydomonas* strain CW2 cell recorded under a configuration with eyespot and flagella outside the pipette. The photoreceptor current *P* is followed by the fast and slow flagellar currents F_F and F_S.

Since in the first study of electrical effects in *Haematococcus* voltage differences were recorded, the primary electrical event was termed 'primary potential difference' (PPD). However, for expressing its light regulation and its location in the eyespot region the terms 'photoreceptor potential' or 'photoreceptor current' (*P*) seem to be preferable.

The photoreceptor current *P* is transient and appears in *Chlamydomonas* with a delay of less than 500 μs after the flash (figure 5). Due to the relatively long flash duration (300 μs) and the low time resolution (3 kHz), the observed delay has to be regarded as an upper limit.

From *Haematococcus* photoreceptor potentials were recorded with a higher time resolution of 30 μs (Sineshchekov *et al.* 1990). It was found that rise and decay are at least biphasic. The first component of the rise had no measurable delay whereas the second component is triggered after a 120–140 μs lag period. The first component has been interpreted as a charge movement within the photoreceptor molecules com-

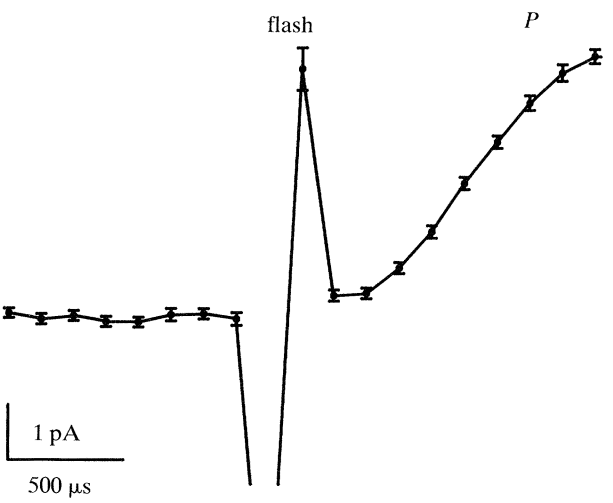

Figure 5. The photoreceptor current rise of one typical cell that has been stimulated with light of a photon exposure of 2.9×10^{19} photons per square metre. Vertical bars at the data points indicate the standard error of the mean (35 recordings). Before the start of the photoreceptor current P an electrical flash artefact is seen.

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parable to the early receptor potential of the visual process in higher animals (Cone 1967), whereas the second is thought to arise from an ionic current through activated channels.

The peak amplitude of the photoreceptor current gradually increases with increasing photon exposure. We stated before that the time between flash and photoreceptor current maximum is constant and independent of the photon exposure (Harz & Hegemann 1991). It is still valid that the photoreceptor current maximum is much more invariant than the delay of the flagellar current maximum discussed below, but an analysis with a higher time resolution revealed that the photoreceptor current maximum shifts to shorter time values by up to about 2 ms when the photon exposure is increased. This shift can only partially be explained by the long flash duration (300 μs) so that cellular processes have to be considered as well.

The dynamic range of the photoreceptor peak current, defined as the linear range on a log scale, extends in *Chlamydomonas* over 1.5 decades of photon exposure. In this range the amplitude rises from 17 to 90% of its maximal value. The shape of the overall stimulus–response curve is roughly sigmoidal on a log scale. For a signalling system, in which signal amplification is constant at all stimulus intensities, the stimulus–response curve can be described by a rectangular hyperbola or Michaelis–Menten equation

$$r/r_{\max} = f/(f+K), \qquad (1)$$

where r (pA) is the response amplitude, $r_{\max}$ is the peak response amplitude at saturating photon exposure, f is the photon exposure (irradiance × time), and K the photon exposure where the peak amplitude is half maximal.

A fairly good fit was reached in case of vertebrate photoreceptor currents as shown by Baylor *et al.* (1979). The same was tried for the *Chlamydomonas* photoreceptor peak current but, as seen in figure 6*a*, a Michaelis–Menten curve does not fit the data. A more general expression for the description of stimulus–response curves including cooperative effects is

$$r/r_{\max} = f^n/(f^n+K^n). \qquad (2)$$

However, no value of n was found which gave a satisfactory fit for *Chlamydomonas*. Our explanation was that several (at least two) interfering processes define the current maximum. Under the assumption that an inactivation mechanism sets in at high photon exposure and reduces the amplitude of the current near light saturation but leaves signals at low exposure unaffected, peak currents were normalized to a higher maximal current value. A fit of theoretical curves with a maximum of 33% above the highest measured value is shown in figure 6*b*,*c*. By this means the data could be described quite adequately using equation (2) (figure 6*c*). The n value of 0.7 indicates negative cooperativity, which can again be regarded as a down-regulation mechanism. Other, more complicated models, as have been used for the detailed interpretation of photocurrents from animal photoreceptor cells (Forti *et al.* 1989), might explain the unusual stimulus–response curves of *Chlamydomonas* as well. Better experimental data, i.e. a better seal resistance and a shorter flash, are necessary before a more detailed analysis can be performed.

As already mentioned, in both algal species the decay of the photoreceptor current is accelerated with increasing photon exposure. This contrasts the situation in metazoan visual systems, where photocurrents decay much slower when high flash energies are used (Baylor *et al.* 1979; Stieve *et al.* 1983). The decay of the *Chlamydomonas* photoreceptor current can be described fairly well by a single exponential with a time constant τ that decreases with increasing photon exposure (figure 7). The acceleration of the decay at high photon exposure is again an indication of the contribution of non linear processes.

To explain the processes occurring during rise and decay of the photoreceptor current several parameters may be considered. Negative cooperativity and accelerated decay can be mimicked by the preceding depolarization caused by the photoreceptor current

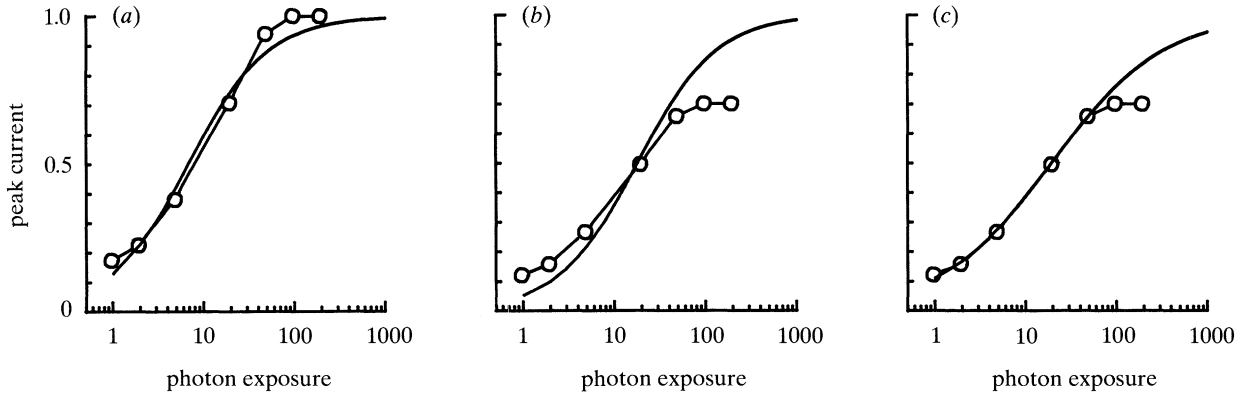

Figure 6. Comparison of the stimulus–response curve for the photoreceptor peak current with calculated saturation functions. In (*a*) the peak currents were normalized to the highest measured value. In (*b*) and (*c*) they were normalised to a 33% higher maximal value. (*a*) and (*b*) show the best fit to a Michaelis–Menten function (equation (1)), and (*c*) to a Hill equation (equation (2)) with a Hill coefficient n of 0.71. The photon exposure is expressed in 10^{18} photons per square metre. Each point represents an average of 15 recordings.

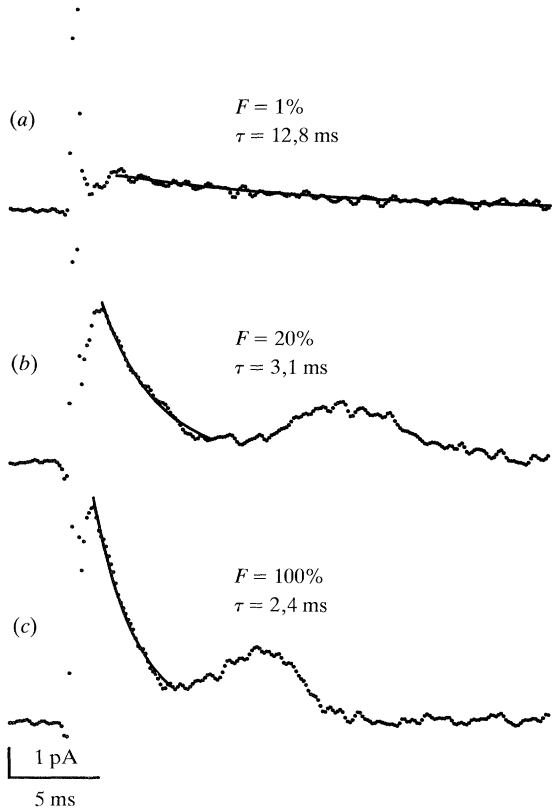

Figure 7. Flash-induced photocurrents at three different photon exposures. 100% flash (F) corresponds to 9.5×10^{19} photons per square metre. The decay was fitted by single exponentials with time constants (τ) given in the figure.

itself. Depolarization of the plasma membrane reduces the driving force for cation influx and in addition it may inactivate the channel (if voltage-sensitive). Alternatively the channel permeating ion might interact directly or via a calcium binding protein with the channel from the cytoplasmic side (ion-sensitive channel). This mechanism is realized, for example, in *Aplysia* neurons and in *Paramecium*, where a local rise of Ca^{2+} is the cause of channel inactivation (Brehm & Eckert 1978; Tillotson 1979). From work with perfused *Nitellopsis*, it has been suggested that in some algae calcium channels are inactivated as the cytoplasmic Ca^{2+} activity increases by only small amounts (Zerelova *et al.* 1987). An allosteric interaction between cation and K^+ channel has been discussed for the K^+-outward rectifier in *Zea* and for the 5 pS rectifier in *Dionaea* (Blatt 1991). In *Chlamydomonas*, however, the decay kinetics change only very little when Ca^{2+} is replaced by Ba^{2+}, which makes inactivation by ions from inside as well as allosteric interaction between ions and channel unlikely. As a third possibility, the ion gradient rapidly decreases due to the small volume between the two eyespot overlaying membranes. This would lead to a decay of the current with the channels still open. In *Haematococcus* two inactivation mechanisms manifest themselves as a biexponential decay of the photoreceptor current (Sineshchekov *et al.* 1990), which has not been found for *Chlamydomonas* yet.

The photon exposure required for a given photoreceptor current depends on the orientation of the eyespot relative to the actinic light. When in *Chlamydomonas* the eyespot points away from the light source and 500 nm light is used for stimulation, the stimulus–response curve is shifted in parallel by a factor of eight to higher photon exposure, compared with a situation where the eyespot faces the light source directly (figure 8). This shift is a measure of the effective front-to-back contrast of the cell at the site of the photoreceptor. Because the extinction of a single cell, which is in the range of 0.3 (at 500 nm), can only explain a shift of the stimulus–response curve by a factor of 2, the observed shift demonstrates the effectiveness of the optical system and verifies that the interference reflector and the photoreceptor form one functional unit. In *Haematococcus*, which has only a monolayered eyespot, operating as a quarter wave plate with low reflection efficiency instead of a quarter wave stack (Land 1972), the stimulus–response curve is only shifted by a factor of 3 (Sineshchekov 1991*b*). As concluded from electron micrographs of the eyespot, in *Chlamydomonas* the mirror optic is optimised for 500 nm at perpendicular light incidence leading to a maximal front-to-back contrast near this wavelength (Foster & Saranak 1989). As for any quarter wave reflector the efficiency should strongly depend on the angle of incidence and the colour of the actinic light. From preliminary electrical measurements with different wavelengths and cell orientations we know that the wavelength dependence of the interference reflector is in fact seen by the photoreceptor (H. Harz & P. Hegemann, unpublished observation). Owing to these properties of the optical system, the orientation of the cell has to be carefully considered before correct action spectroscopy on single cells can be done.

We constructed a photocurrent action spectrum for one special case, namely where the eye directly faces the actinic light. For this purpose photoreceptor currents were recorded from cells with the eyespot

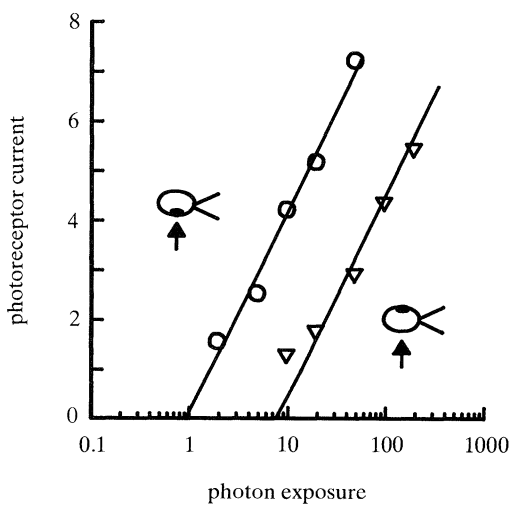

Figure 8. Stimulus–response curves for the photoreceptor peak current (expressed in pA) of one cell with the eye either facing towards the stimulating light (expressed in 10^{18} photons per square metre) (circles) or facing away from it (triangles). From the shift the modulation contrast of the cell at 495 nm can be calculated.

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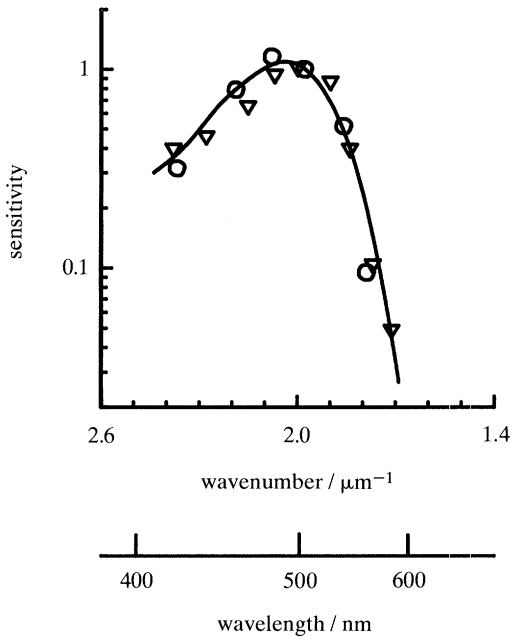

Figure 9. Action spectra of the photoreceptor peak current (circles) and of flash-induced behavioural responses (triangles), the latter measured as light scattering transients (b-wave, taken from Uhl & Hegemann (1990)). The solid line represents the Dartnall standard curve (Knowles & Dartnall 1977).

facing the actinic light under variation of wavelength and photon exposure. Because the cells have to be kept dark-adapted and the eyespot cannot be seen in infrared light, the cells were rotated until the response to test flashes was maximal. At the end of each experiment the position of the eyespot was inspected in white light (bright field). When it was found that the eyespot has not been facing the stimulation light directly, the measurement was discarded. For three cells accurate stimulus–response curves for six wavelengths were obtained. The curves were extrapolated to zero response and the reciprocals of the photon exposures at threshold (i.e. the sensitivities) were plotted versus the wavenumber (figure 9). The resulting action spectrum is a rhodopsin spectrum with the maximum at 494 nm. It closely matches the action spectra for flash induced behavioural responses as they have been previously recorded with a light scattering assay (figure 9; Uhl & Hegemann 1990) and is only a few nm blue-shifted compared with phototaxis action spectra constructed from measurements with retinal supplemented white cells (Foster *et al.* 1984). The photocurrent action spectrum is also similar to the *Chlamydomonas* rhodopsin absorption spectrum detected from purified membrane preparations (Beckmann & Hegemann 1991). The fine structure of the absorption spectrum, however, is not resolved by any of the action spectra.

For *Haematococcus* a photocurrent action spectrum was constructed by measuring the photocurrents in response to flash stimuli of one fixed photon exposure at several wavelengths. The initial rate of the photoreceptor current rise was plotted versus the wavelength of the stimulating light (equal stimulus spectrum). Although the resulting spectrum has an overall rhodopsin shape, it shows six maxima, which are not presently understood (Litvin *et al.* 1978; Sineshchekov 1991*a*).

Recording and interpretation of phototaxis action spectra is more complicated than those of flash-induced responses. One reason is that the difference between photon absorption from both sites of the cell rather than the total amount of absorbed light, determines phototaxis. In addition, in continuous light the cells adapt and may show photoreceptor bleaching or photochromism. All this may change the action spectra in shape and position on a wavenumber scale. In order to minimize these effects, the construction of threshold action spectra is the cleanest possible way to extract information about the photoreceptor nature from phototaxis measurements (for details see Foster & Smyth 1980). Regarding the above-mentioned obstacles, it was surprising to us that the action spectra for photocurrents, for flash induced behavioural responses, and threshold action spectra for phototaxis are in such close agreement.

In the eyespot region of *Haematococcus* in addition to the described potential a second, long-lasting potential difference with low amplitude has been observed. The duration of this current component strictly correlates with the duration of the light (Sineshchekov 1991*a*). The relation to the larger transient photoreceptor potential is not known.

(*c*) *Ion selectivity*

In *Chlamydomonas* two lines of evidence suggested that under physiological conditions the photoreceptor current is carried by calcium (Harz & Hegemann 1991). First, the current disappeared upon addition of the calcium chelator BAPTA. Second, the photoreceptor current is blocked by verapamil (at 600 μM) and pimozide (20 μM), two selective inhibitors for L-type calcium channels of higher animals (Glossmann & Striessnig 1988). As already mentioned, both reagents inhibit movement responses in *Chlamydomonas*. Furthermore, verapamil has been shown to inhibit the uptake of $^{45}Ca^{2+}$ in light (Hegemann *et al.* 1990). Given the above evidence it appeared reasonable to assume that the photoreceptor current is a Ca^{2+} current.

Recently, however, it emerged that the situation is more complicated than originally thought. The current only completely disappears when the extracellular calcium concentration is suddenly changed over a large concentration range as for example from 100 μM to 10 nM or below. This disappearance is transient and after several minutes even in the absence of calcium (very much less than 10 nM) the photoreceptor current recovers up to 30%. When the calcium step-down is smaller (from 1 to 0.1 or from 0.1 to 0.01 μM, figure 10) this transient phase is absent and the residual photoreceptor current can immediately be recorded. When only small changes in the Ca^{2+} concentration are applied, the photoreceptor current can be titrated with calcium. The critical calcium concentration lies in the range of between 1 μM and 100 nM, where the current drops from maximum to below 30%. This is

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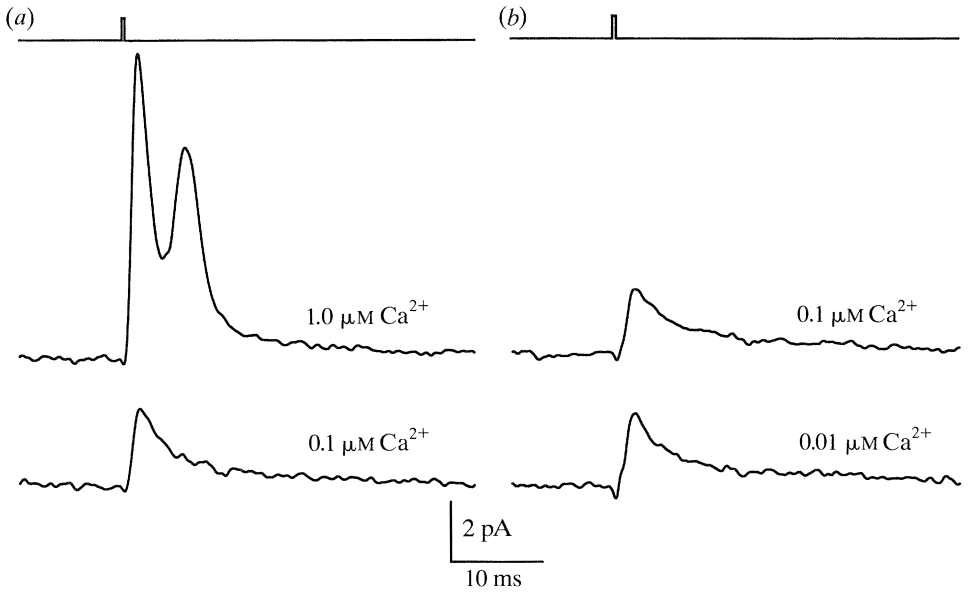

Figure 10. Photocurrents of two different cells at (*a*) 1 and 0.1 μM Ca^{2+} and (*b*) 0.1 and 0.01 μM Ca^{2+}.

the concentration range where the physiological stop response disappears (Hegemann & Bruck 1989). Current and behavioural responses are restored when Ca^{2+} is replenished. The residual current implies that the channel is unspecific, which means that the current can be carried by other ions. The transient disappearance of the photocurrents upon drastic reduction of the Ca^{2+} concentration may originate from an increase of the surface potential and a concomitant decrease of the transmembrane potential (Hille 1984; Eckert & Brehm 1979). Then a voltage-sensitive photoreceptor channel could explain the transient loss of activity.

When Ca^{2+} is replaced by Ba^{2+} (both 100 μM), the kinetics of the photoreceptor current remain almost unchanged and the signal amplitude is only reduced by less than 20% (H. Harz, C. Nonnengäßer, and P. Hegemann unpublished observation). These experiments allow two conclusions: first, the photoreceptor channel can conduct Ba^{2+} and, second, inactivation of the photoreceptor current is more likely to be by the voltage change than by Ca^{2+} binding from inside. Otherwise in case of an ionic inactivation from inside, Ba^{2+} ions, which in other systems substitute well for Ca^{2+} as current carriers but poorly in the inactivation process (Brehm & Eckert 1978; Hagiwara & Ohmori 1982), would have a greater influence on the photoreceptor current. In *Chlamydomonas* a similar inactivation mechanism is likely for the slow flagellar current (discussed below).

Although all experiments point towards a light-induced calcium inward current in the eyespot region, a calcium-activated anion current cannot be strictly ruled out on the basis of suction measurements alone. Originally this idea was considered likely, because in *Characean* algae calcium-activated chloride channels in the plasmalemma play a major role in the amplification of the action potential (Lunevsky *et al.* 1983). In addition, voltage gated Cl^- channels are present in the *characean* tonoplast (Tester *et al.* 1987). However, because external chloride can be substituted for almost any other anion without changing behavioural movement responses (H. Hegemann, unpublished observation), a significant contribution of chloride channels to photoresponses of *Chlamydomonas* has never been rigorously discussed.

4. TRIGGERING OF REMOTE FLAGELLAR CURRENTS

When the photoreceptor current integral exceeds a certain threshold value, a transient inward current in the flagellar region is triggered. It has been termed 'regenerative response' (RR) or more descriptively, a 'fast flagellar current' (F_F) (figure 4). The time interval between flash stimulation and beginning of the flagellar current increases when the photon exposure is lowered (Litvin *et al.* 1978; Harz & Hegemann 1991). The photoreceptor current integral between flash and beginning of the flagellar current is changed only slightly over a wide range of photon exposure (figure 11) and in addition it is insensitive to substitution of Ca^{2+} by Ba^{2+}. Both arguments together suggest that the fast flagellar channel is voltage activated. The amplitude (figure 12) and integral of the flagellar current is almost independent of the photon exposure. The current lasts for only a few ms and its rise and decay have similar kinetics. Its sensitivity to calcium channel inhibitors indicates that in *Chlamydomonas* and *Haematococcus* this flagellar current is also carried by calcium. As for the photoreceptor current, a contribution of Cl^- cannot be strictly excluded but again physiological experiments make this very unlikely. The all-or-none appearance of the flagellar current resembles an action potential, which in contrast to action potentials in many other organisms is not actively propagated over the whole plasma membrane, which is not necessary in an isopotential organism.

In *Chlamydomonas*, the fast flagellar current is superseded by a long-lasting current component (F_S, figure 4). Because its sign reverses depending on the location

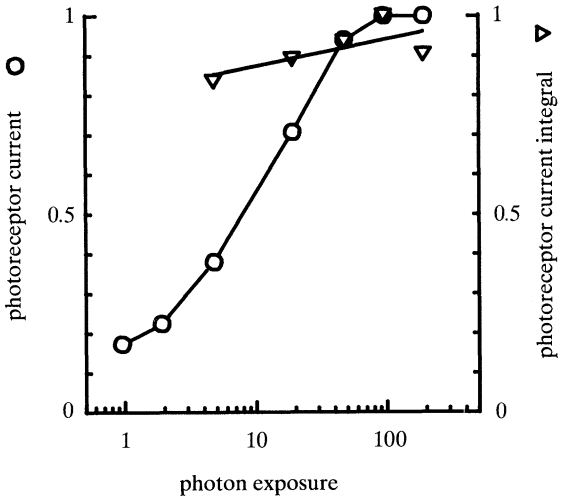

Figure 11. Comparison of the photoreceptor peak current amplitude (circles) with the photoreceptor current integral (triangles) extending from flash until the beginning of the flagellar signal. The data are from the same cell as those in figure 6. The data were normalized to the measured maximal values 6.9 pA and 26.9 fQ. Photon exposure is expressed in 10^{18} photon per square metre.

of the flagella in or outside the pipette (figure 13), it can also be assigned to the flagellar region (discussed in detail below). Its amplitude is very small (less than 1 pA) but it decays within only 300 ms after a flash, which is just the time of slow backward swimming during a 'stop response'. Interestingly, when Ca^{2+} is replaced by Ba^{2+}, F_S persists longer and exhibits a considerably larger amplitude (H. Harz, C. Nonnengäßer and P. Hegemann, unpublished results), thus indicating that Ca^{2+} plays a prominent role in down-regulation of the channel responsible for F_S. In presence of Ba^{2+} it has been demonstrated that F_S shows a similar all-or-none appearance as the fast flagellar current component.

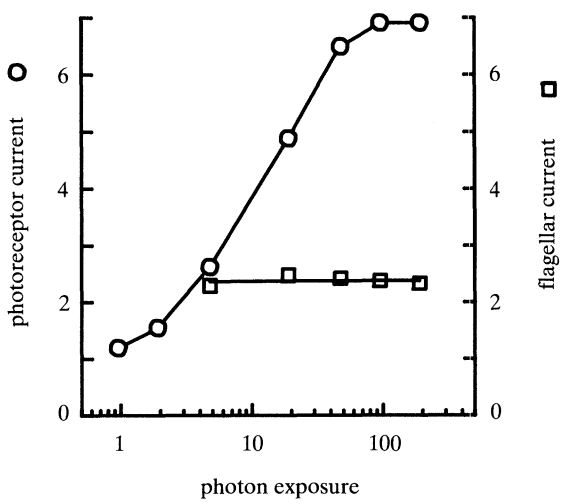

Figure 12. Comparison of the maximal amplitudes of the photoreceptor current (circles, expressed in pA) and of the fast flagellar current (squares, also in pA). At low photon exposure no flagellar current was triggered. Photon exposure is expressed in 10^{18} photons per square metre.

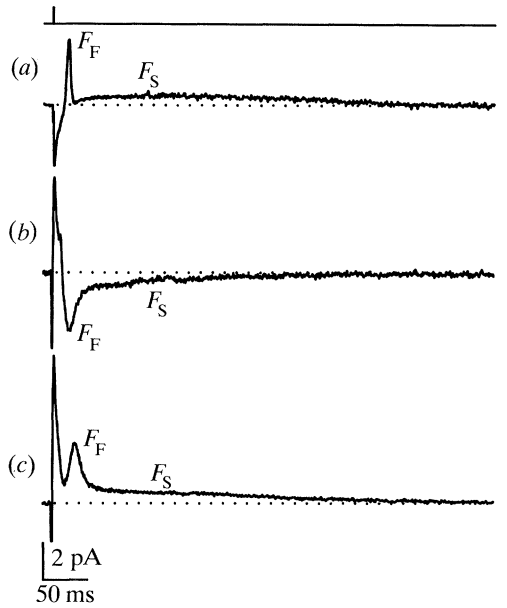

Figure 13. Photocurrents recorded under three different configurations, (*a*) eyespot in the pipette, (*b*) flagella inside the pipette, (*c*) both current source regions outside the pipette. Fast and slow flagellar currents F_F and F_S always had the same sign.

5. ELECTRICAL MODEL

In both alga *Chlamydomonas* and *Haematococcus*, photoreceptor and flagellar currents are detected not only from the source region, i.e. the eyespot or the flagellar region, but from any location on the cell surface. When the source region of the respective current is in the pipette, a transient current out of the pipette into the cell is recorded, whereas when eyespot or flagella are outside the pipette, the signals have the opposite sign. This location-dependent inversion originally led to the assignment of the currents to photoreceptor and flagellar currents (Litvin *et al.* 1978). Delay and kinetics of the corresponding signals are identical under all configurations. This strongly suggests that both positive and negative signals reflect the same light activated currents. If positive signals reflected repolarisation processes, a significant delay between positive and the corresponding negative signals would be expected, reflecting the time for a significant voltage drop and the time for a 'repolarizing' channel to switch from closed to open state. We are convinced that repolarization is slow and not recorded in these experiments.

In contrast, our present interpretation for explaining all experimental data, including sign inversion, is based on a capacitive model, which is illustrated in figure 14 and briefly explained as follows: photoexcitation leads to a transient increase in ion conductivity at the eyespot and subsequently in the flagellar region. When either one of these regions is in the pipette, Ca^{2+} flows from the pipette into the cell. Owing to the capacitance of the plasmalemma, the inward current induces a charge displacement, which in such a small organism can be registered at all sites of the plasma-

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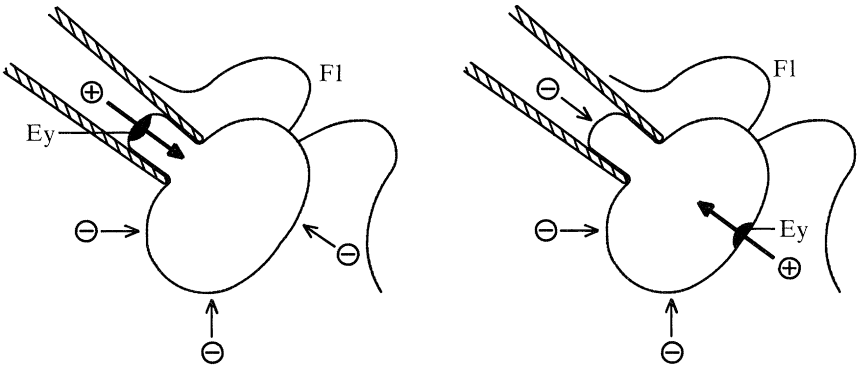

Figure 14. Explanation of the capacitive photoreceptor current. Photoexcitation leads to a cation influx (⊕) at the eyespot region (Ey). The depolarization leads to a compensatory anion (⊖) movement onto the membrane but not across it. This displacement current is the only detected current under eyespot-out conditions.

lemma. The charge displacement can be regarded as an anion movement from the medium onto the cell surface, thus explaining the inverted sign. Under the configuration with eyespot or flagella outside the pipette, only the displacement currents are detectable with a sign of an outward current. The amplitude of the capacitive current should depend on the size of the surface area sucked into the pipette, which, as already mentioned, was in most experiments with *Chlamydomonas* in the range of 30% of the total. However, the pipette resistance and the seal resistance are of the same order, which further reduces the detected current to only 15% of the total. Therefore a measured photoreceptor current of 6 pA reflects a real inward current of 40 pA into the cell. From this argument one would expect that in the 'eyespot in' configuration the measured current would be much larger, because the source region of the current is in the pipette. However, in most cases the eyespot sticks to the pipette wall and is not freely accessible from the pipette, which increases the access resistance from the pipette and lowers the effective seal resistance between bath electrode and eyespot. With a ratio of these two resistances in the range of 2:1, for a 40 pA total photoreceptor current only 13 pA would originate from the amplifier. As 30% of the plasmalemma is in the pipette, under this condition the measured current is reduced by a capacitive current, which has opposite sign. This current component is independent of the location of the eyespot and fed half by the electrode and half from the bath, giving 15% of the total current or in our example 6 pA, which has to be subtracted from the total current. Then only 7 pA should be detected by the amplifier. This argument explains why in most experiments the difference of the current amplitudes under 'eyespot in' and 'out' configuration is only small.

In conclusion the 'capacitive model' qualitatively explains the similarities of the amplitudes and kinetics, and the sign inversion of the photocurrents under the different measuring conditions. The similarity of the amplitudes requires that the cell is more or less 'isopotential', which should be the case for an organism of this small size. Eckert & Naitoh (1970) and Wood (1982) showed even for the much larger *Paramecium* and *Stentor* that a local depolarization expands without significant decrement over the whole cell.

Under conditions where the detected photoreceptor peak current was 6 pA corresponding to a total current of 40 pA, the total current integral is around 160 fQ (10^6 elementary charges). A ball shaped alga with a diameter of 8 μm has a cell surface of 200 μm^2 not including the flagella and a total capacitance of the plasmalemma of 2 pF, based on a specific capacitance of 1 $\mu F\ cm^2$. With $U = Q/C$, 160 fQ total charge would lead to a depolarization of 80 mV, a value which is quite usual in many organisms in response to strong extracellular stimuli.

Most calcium channels from animal tissues and also the few recently found in plant systems have a unitary conductance G in the range of 1 to 25 pS when Ba^{2+} is the charge carrier (Swandulla *et al.* 1989; Tester & Harvey 1989). Assuming a unitary conductance of this order, a resting potential of 100 mV, and ohmic properties, the total photoreceptor peak current of 40 pA could be sustained by only 16 to 400 individual ion channel proteins. An alternative estimation can be made starting from the amount of bleached photoreceptor molecules. With an absorption cross-section of $1.9 \times 20^{-20}\ m^{-2}$ for rhodopsin ($\varepsilon = 50\,000\ M^{-1}\ cm^{-1}$; Beckmann & Hegemann 1991) and a quantum efficiency of 0.67, which is typical for rhodopsins, roughly 12% of the rhodopsins would be bleached by a flash that produces a half maximal photoreceptor current (1×10^{19} photons m^{-2}, figure 11). With an estimated number of 10 000 rhodopsins per cell (which is three times lower than in wild-type) one needs a unitary conductance of only 0.3 pS for a 1:1 stoichiometry between rhodopsin and channel. A low conductance is supported by the finding that the photoreceptor current saturates at very low calcium concentrations of about 1 μM. Clearly the amplification of the transduction chain is low, which sharply contrasts the situation found in animal photoreceptor cells, where a single activated rhodopsin in dark-adapted cells regulates around thousand ion channel proteins (Keiper *et al.* 1984). This high gain is only achieved by sacrificing speed.

Figure 15 shows a simplified electric equivalent circuit for the proposed primary events of photoreception in *Chlamydomonas*.

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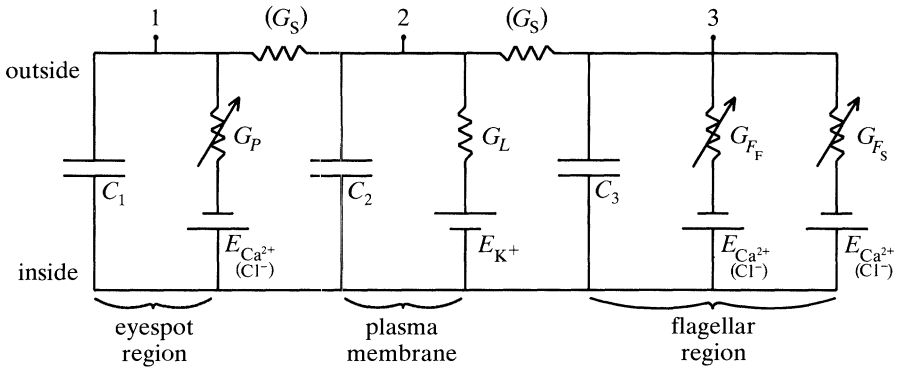

Figure 15. Simplified electric equivalent circuit for measuring photoreceptor and two flagellar currents. G_P, G_{F_F}, G_{F_S}, G_L are the photoreceptor, the two flagellar and the leak conductances, respectively. C_{1-3} represent parts of the plasma membrane capacitance, and E is the electromotive force for Ca^{2+} and K^+. The equivalent circuit considers only the major conductances although all channels might have only a low ion specificity and might conduct other ions (for example, Cl^-). The numbers 1, 2 and 3 represent the principal measuring positions with their respective pipette seal conductances (G_S) With eye and flagella outside the pipette, the current is measured at position 2 with position 1 and 3 set on ground. If eye or flagella are in the pipette, current is detected at the positions 1 or 3 respectively. The other positions were set on ground.

6. DISCUSSION

Although a direct link between electrical phenomena and behavioural light responses still needs to be established – e.g. by simultaneous recording of electrical processes and flagellar beating from the same cell – it appears beyond doubt that the photocurrents are part of the rhodopsin-controlled signal transduction chain that regulates the behaviour of the cells in light. However, because the measured photocurrents are high intensity responses, they should predominantly reflect processes that are related to stop responses. At lower photon exposure, where cells perform direction changes, photoreceptor currents are small and flagellar currents are not detectable.

The close link between photocurrents and photobehaviour is supported by the following observations.

1. All substances that suppress the photocurrents, i.e. low calcium, pimozide or verapamil, also suppress the behavioural responses.

2. The action spectra for the photoreceptor current and for behavioural responses are very similar with respect to shape and maxima.

3. The photocurrents originate from two regions that have been thought to be most essential for photobehaviour, i.e. the eyespot as the assumed site of the photoreceptor and the flagella as the effector organ.

4. The all-or-none appearance and the variable delay of the flagellar current closely resemble well known characteristics of the stop response.

5. The slow flagellar current F_S has a similar duration as the stop response.

6. The dependence of the photoreceptor current on the direction of the light incidence makes it an attractive candidate for the postulated signal, which is modulated during rotation of the cell and finally causes the cell to orient in light.

From the above results the simplified scheme in figure 16 was derived, which includes the major components of the signal transduction chain regulating behavioural light responses in *Chlamydomonas* and *Haematococcus*. It omits all regulation and repolarization processes.

With the identification of the photoreceptor as a rhodopsin, the analysis of rhodopsin regulated currents and a detailed description of the behavioural responses, *Chlamydomonas* has become one of the few model systems in the plant world, in which signalling can be documented from the stimulus via the receptor and transduction processes to the physiological response. Although much more data are available about other signalling systems in plant cells, as for example phytochrome controlled processes, in most cases major steps of the signal transduction process are still missing. The advantages of photoreception in *Chlamy-*

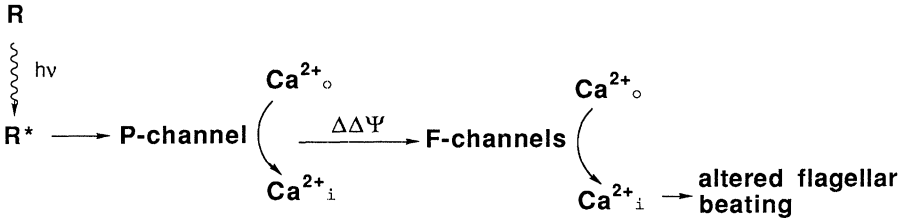

Figure 16. Schematic representation of the known elements involved in photoreception of *Chlamydomonas* and other related algae. R, R* represent inactive and light activated rhodopsin, P- and F-channels the photoreceptor and the flagellar channels, Ca^{2+}_o and Ca^{2+}_i extracellular and cytoplasmic Ca^{2+} concentration, and $\Delta\Delta\psi$ the change of the membrane potential.

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domonas are that the system lacks too many branches and is fast. Therefore it is probably easier to analyse than signalling in higher plants. Moreover, single celled algae allow both recording of behavioural responses and electrical processes from a whole living organism.

The postulated existence of calcium channels in plant cells is not new. Calcium channels have been suggested to play numerous roles in plants, i.e. polar growth, light- and hormone-regulated growth and development, gas exchange by guard cells, secretion and photomovement (Leonard & Hepler 1990). Voltage dependent calcium entrance has been directly documented in *Chara* (Williamson & Ashley 1982), as have abscisic acid-activated calcium channels in stomatal closure of guard cells. The latter has been demonstrated by application of the patch-clamp technique in conjunction with Ca^{2+} dyes (Schroeder & Hagiwara 1989).

The very fast activation of the photoreceptor channel constitutes a principal difference between the algal and metazoan visual systems. In animal photoreceptors light affects an ion channel via several biochemical amplification steps (reviewed by Stryer 1988) leading to a delay between light and beginning of the hyperpolarisation in the range of 5 to 50 ms (Baylor *et al.* 1979). In the *Limulus* lateral eye the mean latency for a quantal response is even 185 ms (Wong *et al.* 1980). Thus the control of the photoreceptor channel in algal and animal vision seems to be quite different. From hormone controlled processes it is well known that sensors can be remote, as in the muscarinic acetylcholine receptor, or intrinsic, as for the nicotinic acetylcholine receptor, the GABA or the glycine receptor. Recently it was shown on guard cells of *Vicia faba* that the Cl^- channel is directly gated by extracellular growth hormones (Marten *et al.* 1991), providing convincing evidence that ion channels with intrinsic sensors also exist in plants. So far such close receptor-channel coupling has not been found for a light sensitive system. Activation of the photoreceptor channels in single celled algae appears in a time range where in other systems channels are directly activated by a voltage jump (Chiu 1977), by application of acetylcholine (Liu & Dilger 1991), or in case of stretch activated channels from hair cells by stimulation with a piezoelectrically activated probe (Corey & Hudspeth 1983). By analogy we assume that in *Chlamydomonas* as well as in *Haematococcus* there must exist a close coupling between photoreceptor and photoreceptor channel with no chemical steps in between. Furthermore, there is no necessity for amplification, because a 1:1 coupling stoichiometry is sufficient to explain all data. We favour the idea that rhodopsin and the photoreceptor channel form one protein complex.

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