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Hypothesis

Photophobic responses and phototaxis in *Chlamydomonas* are triggered by a single rhodopsin photoreceptor

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Abstract

The rhodopsin nature of the photoreceptor for the behavioural light responses in *Chlamydomonas* has originally been revealed by action spectroscopy. Meanwhile most physiological experiments and the identification of *all-trans*-retinal in cell extracts favour that this chlamyrhodopsin contains an *all-trans*-type retinal chromophore with strong similarity to the light sensors SR I and SRII from *Halobacteria*. Reconstitution of retinal-deficient cells with [^{3}H]retinal identified a single retinal protein with a MW of 30,000. Chlamyrhodopsin triggers a photoreceptor current in the eyespot region resulting in direction changes or phototaxis. Furthermore, when the light stimulus oversteps a critical level, two flagellar currents appear, which are the basis for photophobic responses. The physiological, electrophysiological and biochemical experiments suggest that all behavioural responses are triggered by a single rhodopsin-type receptor.

Key words: Rhodopsin; Phototaxis; Blue light receptor; *Chlamydomonas reinhardtii*

1. Introduction

Unicellular algae such as *Chlamydomonas* or its larger relative *Haematococcus* orient their swimming patterns in response to light and thereby find optimal conditions for photosynthetic growth. They exhibit direction changes or stop responses to stimulation with light flashes. Direction changes, occurring preferentially at low flash energies, are caused by a brief alteration of the flagellar beating plane, while the cells stay in the forward swimming mode (Fig. 1A). Under continuous light, cells perform many direction changes that become more frequent but less distinct at higher light intensities. Due to the optical properties of the eyespot the probability of light absorption increases when the eye is facing the light and thereby depends on the orientation of the cell relative to the light source. This is the reason why a sequence of direction changes results in a phototactic net movement [1,2] . At high intensities, the cells swim directly to or away from the light source. On the other hand, flashes of high photon exposure initiate stop responses (also called photophobic response) during which the cells transiently switch from the normal 'breast stroke swimming style' to a reverse 'crawling style' [3]. During reverse swimming the flagella undulate along the cell axis (Fig. 1B). The backward swimming speed is only 20% of that of forward swimming. Thus the response appears as a stop. Such a stop response also occurs when a step up stimulus of high intensity is given. However, the cells immediately adapt and proceed to smooth phototactic swimming.

2. Controversial perspectives about the rhodopsin photoreceptor

Foster and Smyth [1] were the first to postulate that the photoreceptor for behavioural responses in *Chlamydomonas* is rhodopsin. Originally they discussed this hypothesis exclusively on the basis of phototaxis action spectra. Fosters group substantiated the rhodopsin nature by showing that retinal and retinal analogs can restore phototaxis in white strain FN 68 cells, which are almost but not completely blind if grown in darkness. Furthermore they showed that the wavelength of the maximal sensitivity depends on the analog that has been used [4]. This was the first unequivocal identification of a eucaryotic blue light receptor. For a detailed analysis of the chromophoric properties of this rhodopsin Fosters group incorporated more than a hundred retinal analogs in vivo into the *Chlamydomonas* opsin [5–7]. For all these reconstitution experiments a population migration assay (dish test) was used that was described in detail in refs. [4] and [8]. From this large set of reconstitution experiments the authors came to surprising conclusions, which

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initiated a wide confusion among rhodopsin researchers: (i) protonation of the imine N is not required for rhodopsin activation; (ii) no particular regiospecific isomerisation is required; and (iii) the rhodopsin can be activated even when retinal is replaced by hexenal or hexanal, which contain only one C = C double bond or even none at all. They proposed that rhodopsin might be activated by a 'charge redistribution of the excited state chromophore' [7,9].

Later, by using a light scattering assay and different motion analysis systems, three groups independently analysed flash-induced behavioural responses by using a mutant named CC2359, which is completely blind under all known conditions. These authors have found that flash induced responses are also rhodopsin mediated [10–13]. Since that time an intensive discussion has begun about the question whether one or several photoreceptors control the behaviour of unicellular algae. The action spectra for flash-induced responses were identical with the threshold action spectra for phototaxis, suggesting that phototaxis and flash-induced responses are mediated by the same or very similar photoreceptors. However, after reconstitution of CC2359 cells with retinal isomers and analog compounds the three groups arrived at conclusions that totally contrast those drawn from the earlier phototaxis experiments. They proposed that (i) the chromophore of the rhodopsin responsible for stop-responses is in an all-trans configuration, (ii) the three double bonds closest to the aldehyde function are of predominant importance for the function, (iii) activation

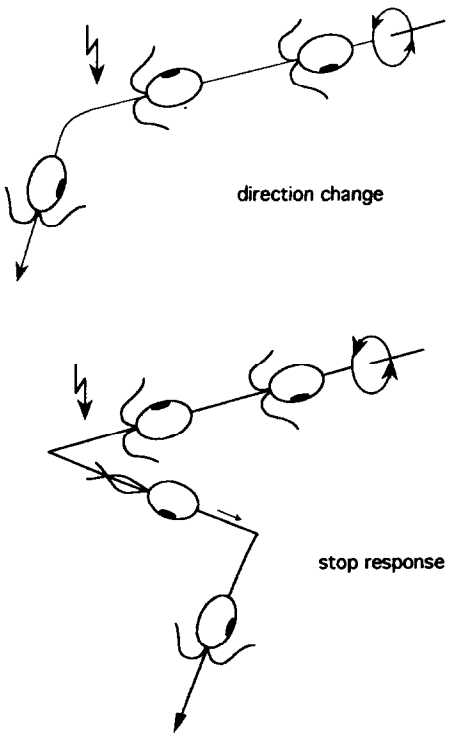

Fig. 1. Schematic representation of the two principle flash-induced behavioural responses.

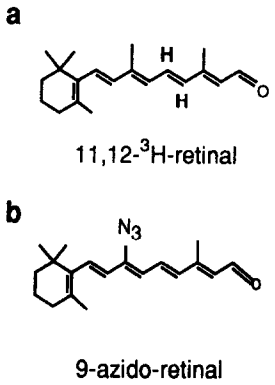

Fig. 2. Structures of 11,12-[^{3}H]retinal (A) and 9-azidoretinal (B).

occurs by an *all-trans* to 13-*cis* isomerisation, and (iv) the 13-methyl group transfers the retinal isomerisation into a conformational change of the protein. Such a chromophore would be closely related to those of the sensory rhodopsins SRI and SRII (phoborhodopsin) from *Halobacteria*. From these results it appeared that phototaxis and flash-induced responses in *Chlamydomonas* were mediated by completely different rhodopsin photoreceptors.

Additional phototaxis experiments were carried out with a phototaxigraph [11] or motion analysis systems [13,14] on both reconstituted strain cells (CC2359 and FN68). The results made it very likely that the photoreceptor responsible for phototaxis, such as the one for flash-induced responses, has an archaebacterial-like chromophore. The only apparent discrepancy between their phototaxis experiments and experiments analysing stop responses was that the retinal regeneration of the stop responses can be inhibited by C13–C14 locked retinals, whereas the retinal regeneration of phototaxis is not inhibited by this compound [14]. Therefore, two photoreceptors, disregarding the strong similarities, have remained under discussion. However, since phototaxis of white cells, that have no pigmented eyespots, shows very shallow stimulus–response curves [4], small changes of the phototactic rate due to small changes of the content of functional rhodopsin might be overlooked. Thus to our judgement the experiments with C13–C14 locked retinal alone do not justify the two-receptor hypothesis.

The modified view about the chlamyrhodopsin chromophore fits into the general understanding of how rhodopsins are activated [15]. Nevertheless the discrepancies to the earlier phototaxis measurements remain to be explained.

3. Biochemical evidence for a single rhodopsin

3.1. Identification of retinal

Retinal, the chromophoric group of all rhodopsins, has been identified in *Chlamydomonas* whole cells and in

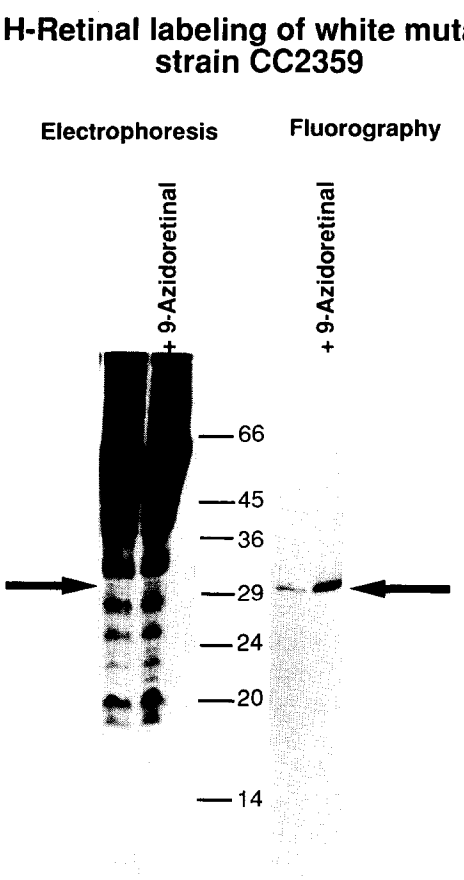

Fig. 3. Demonstration of a single retinal protein in *Chlamydomonas*. A total membrane fraction labeled with [^{3}H]retinal in absence or presence of 9-azidoretinal was seperated by SDS-PAGE. Gels on the left show the Coomassie stains and those on the right side corresponding autoradiographies. 1.7×10^7 cells (strain CC2359) were incubated with 5 nM ^{3}H-labeled *all-trans*-retinal for 1 h, reduced by 1% $NaCNBH_3$ in 100 mM NaOAc pH 5, washed by centrifugation and precipitated with TCA/NH_3 pH 7. The precipitate was resolved in SDS sample buffer and subjected to electrophoresis. Preincubation with 5 μM 9-azidoretinal was done for 1 h prior to incubation with [^{3}H]*all-trans*-retinal. After electrophoresis gels were incubated in EN-^{3}Hance, dryed and exposed to a Kodak-X-OMAT film at −70°C. [^{3}H]retinal was prepared from [^{3}H]retinol (Amersham) by incubation with activated MnO_2 and was purified on an Si-60 HPLC column using 5% ethylacetate in hexan as solvent (v/v).

preparations enriched in putative eyespot membrane fractions [11,16,17]. Initial identification was successfully performed with white cells (FN68), that are grown in darkness but become phototactic after illumination for 20 min. An estimated 30000 *all-trans*-retinal molecules per cell have been found, which corresponds to the lower level of the assumed number for a rhodopsin that mediates phototaxis or stop responses. In green cells, which contain enormous amounts of carotenoids, the analysis is more difficult. However, the dominant isomer in the green cells is also the *all-trans*-retinal, which is contaminated by only small amounts of the 13-*cis* isomer. This result fully supports the *all-trans*-nature of the rhodopsin chromophore.

3.2. *Identification of putative receptor molecules*

Several attempts have been made to further clarify the questions about the algal rhodopsins. One of them was the identification of rhodopsin gene sequences by heterologous hybridisation using opsin genes from vertebrates, invertebrates and halobacteria [18]. Apparently the homology between opsins from different genera is extremely low, as seen in the rhodopsin homologies between halobacteria and animal cells. Therefore it is important that the chlamyrhodopsin protein is identified.

On the basis of a retinal assay, putative eyespot membranes have been enriched and a rhodopsin-like absorption has been identified by difference spectroscopy. In this fraction a 30 kDa retinal binding protein was reconstituted with [^{3}H]retinal [16]. Unfortunately this fraction did not show any flash induced absorption changes so that the retinal protein has not been generally accepted as the photoreceptor. Furthermore, the retinal labeling experiment has been criticised during that time for several reasons. Firstly, retinal labeling of a rhodopsin that has already an intact chromophore is unpredictable. Some species substitute their retinal for [^{3}H]retinal whereas others do not. This is known from *Halobacteria*, where bacteriorhodopsin or halorhodopsin do not exchange retinal, whereas the sensors SRI and SRII do so under certain conditions. Secondly, identification of a retinal protein in a purified membrane fraction does not identify this as the receptor as long as it cannot be shown

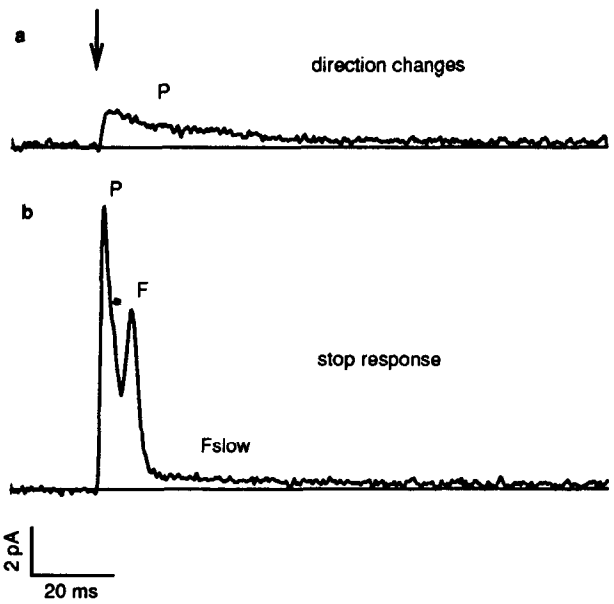

Fig. 4. Photocurrents of a *Chlamydomonas* cell-wall-less cell in response to green flashes of low (a) and high (b) flash energies. A photoreceptor current (P) alone (**a**) leads to direction changes, whereas the appearence of flagellar currents (F) (**b**) induces stop responses. Photocurrents were recorded exactly as described in [20].

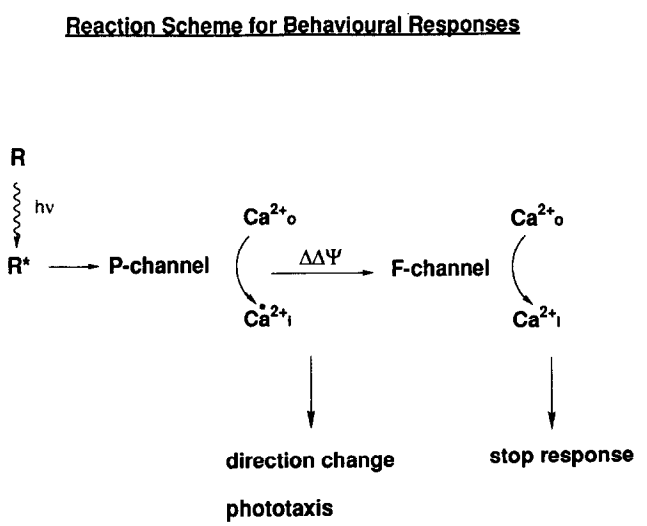

Fig. 5. Suggested reaction scheme for a rhodopsin that triggers all photobehavioural responses. Activation of rhodopsin (R) localised in the eyespot overlaying part of the plasmamembrane is the first step. At low light intensities, activated rhodopsin (R*) initiates opening of the photoreceptor channel (P-channel), which is localised in its close vicinity, if not permanently in direct contact to it. The current hypothesis is that Ca^{2+} entering the cell migrates as a brief Ca^{2+}-wave from the eyespot to the flagella, thus inducing direction changes and phototaxis. When the photoreceptor current reaches a critical level, the depolarisation ($\Delta\Delta\Psi$) activates voltage-dependent flagellar channels. The resulting flagellar Ca^{2+} inward current leads to a transient stop, the latter being mediated by binding of Ca^{2+} to axonemal structures.

that this is the only existing retinal protein in the cell or at least the only one present in expected amounts.

A labeling experiment with white, retinal-negative cells (strain CC2359) has now been carried out by using *all-trans*11,12-[^{3}H]retinal. Since all behavioural responses are reconstituted in blind cells preferably with *all-trans*-retinal, other isomers have not been tested. [^{3}H]Retinal (Fig. 2A) was added at a concentration to the cell, which was just sufficient to reconstitute all rhodopsin molecules, i.e. 30,000 molecules per cell. After reduction of the retinyllysin Schiff-base linkage with the membrane permeable $CNBH_3^-$ only a single retinal protein was found in the total membrane fraction (Fig. 3). The MW of 30,000 confirms the earlier reconstitution experiments with preparations enriched in putative eyespot membranes. An improvement of the retinal label could be achieved by preincubating the cells with 9-azidoretinal (Figs. 2B and 3). As tested with the help of a light scattering assay [10,11] this analog cannot enter the binding site (data not shown) but apparently prevents nonspecific adhesion or binding of [^{3}H]retinal thus supporting rhodopsin reconstitution. The retinal protein is only a minor membrane component but it is present at quantities of the expected light sensor. This experiment clearly demonstrates that there is only one retinal protein in *Chlamydomonas*, which can serve as the photoreceptor for phototaxis *and* for flash-induced responses.

4. How can one rhodopsin trigger phototaxis and stop responses?

Electrophysiological experiments with suction electrodes have shed light on this question. Flashes of low energy induce Ca-inward currents in the eyespot region (photoreceptor currents, Fig. 4A) [19]. Under these conditions cells perform direction changes. The direct correlation between photocurrents and flagellar beating has recently been achieved by simultaneous detection of electrical events and the flagellar beating frequency of the same cell (Harz et al., in preparation). For direction changes the intracellular signalling system is still unknown, although Ca^{2+} migrating from the eye to the flagellar basal bodies is the most probable mechanism.

The photoreceptor current, at higher flash energies, exceeds a critical threshold level that results in the activation of voltage dependent cation channels in the flagellar membrane (flagellar currents, Fig. 4B). These currents are typical for eucaryotic cilia. When Ca^{2+} is present in the medium, the flagellar currents cause the cells to stop, whereas when Ba^{2+} is present, cells exhibit large flagellar currents that cause prolonged spiralling but no stops ([20] and Harz and Hegemann, unpublished observation). So exactly as it has been concluded from earlier experiments on permeabilized cells or extracted flagella [21,22], Ca^{2+} is the intra flagellar signal transducer that enables backward swimming.

In conclusion, the photoreceptor current alone induces direction changes or phototaxis, whereas the flagellar current is responsible for stop responses (Fig. 5). Regarding the electrophysiological experiments, not only all electrical events can be explained by one receptor, there is not a single observation that points to a second primary photoreceptor so far.

We originally discussed direction changes and stop responses as independent processes [2]. This was based on the observation that for individual cells both responses are stochastic events and give stimulus–response curves that fit nicely to single event Poisson distribution curves. In the light of the recent biochemical and electrophysiological results, we must interpret the stochastic appearence of the stop response by the all-or-nothing characteristic of the flagellar currents.

With this correction in mind, the physiological experiments carried out using the light-scattering assay, the motion analysis system, and the phototaxigraph as well as the biochemical and electrophysiological experiments are all compatible with a one receptor model, which means that all photomovement responses are triggered by a single rhodopsin type photoreceptor.

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