Probing visual transduction in a plant cell

Optical recording of rhodopsin-induced structural changes from *Chlamydomonas reinhardtii*

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ABSTRACT Light scattering studies of vertebrate rod cells have greatly aided our understanding of the visual transduction process. This technique has now been successfully applied to study visual transduction in a unicellular alga. Flash-induced light scattering changes have been recorded which are repeatable, graded with photon exposure, and adaptive. They appear on a timescale of 15–1,000 ms and correlate kinetically with flash-induced movement responses. The responsible photoreceptor is a rhodopsin. Evidence is provided for the ability of the organism to count single photons.

INTRODUCTION

Recent interest in *Chlamydomonas* phototaxis has been fueled by reports that these unicellular algae contain a rhodopsin-like visual pigment (1, 2), can detect single photons (3), and adjust to changes in the ambient light level over several orders of magnitude (1, 4, 5). The present study was carried out to substantiate these claims and to elaborate further similarities between the transduction mechanism in the specialized photoreceptor cells of higher animals and unicellular organisms like *Chlamydomonas,* which have diverged from higher eucaryotes almost 1 billion years ago and which have hundreds of other cell functions to take care of within their cell boundaries.

Visual transduction in *Chlamydomonas* requires information transfer over a distance of 5–10 μm, from the site of the photoreceptor molecules in the eyespot-region (6) to the flagella, where an altered beating pattern results 20–100 ms after the stimulus (7, 8). The fast spread of excitation points to an electric propagation rather than a diffusible transmitter (9, 10), but on the basis of behavioral studies alone, our only source of information so far on the transduction mechanism, this has not been verified as yet. We have therefore tried to find an alternative assay which would allow us to measure transduction related processes with high time resolution, and we have found it in form of rapid, flash-induced light-scattering (LS) transients which are a not necessarily direct structural consequence of light-induced ion translocations. While a more detailed examination of the ionic basis of the signals will be given elsewhere, this report shall focus on properties and interpretations which are independent of the molecular and structural origin of the light scattering signals: it will be demonstrated that the signals are fast, graded with photon exposure, regenerative, and adaptive. They have the action spectrum of a rhodopsin and correlate, both with respect to their kinetics and their sensitivity, with rapid behavioral responses. The sensitivity of the technique allows one to visualize actual single quantum events.

MATERIALS AND METHODS

Strain 806 mt$^-$ cells were grown on agar plates for 5 d and differentiated in nitrogen-free liquid minimal medium (NMM: 80 μM $MgSO_4$, 100 μM $CaCl_2$, 3.1 mM K_2HPO_4, 3.4 mM KH_2PO_4, pH = 6.8 plus trace elements) for 24 h at a cell concentration of 7×10^6 cells/ml as previously described (2). Phototactically active cells were photoselected (8) and then dark adapted under optimal aeration for at least 1 h in NMM (pH = 6.0) at a concentration of 1.6×10^6 cells/ml. Fresh aliquots were taken for every set of exposures.

Light scattering responses were recorded in a multiangle flash photolysis apparatus (11) at a cell density of 1.6×10^6 cells/ml. Signals were largest at a scattering angle of 16°, and therefore all signals shown were recorded at this angle. The wavelength of the monitoring beam was 840 nm, far enough in the infrared not to cause significant photoreceptor bleaching or stimulation of photosynthesis.

Action spectra were constructed from stimulus/response curves of dark adapted gametes, using actinic flashes between 400 and 600 nm. The flash duration was $\leq$1 ms and the particular wavelength was selected with narrow-band (8–12 nm), single cavity interference filters which were blocked outside their transmission band from UV-IR (Schott KG, Wiesbaden, FRG). Absolute photon exposures were determined using a calibrated, large area Joulemeter (ED-200; Gentec, Baumann, München, FRG).

Stop-responses were recorded in a computer aided motion analysis system (8).

RESULTS AND DISCUSSION

Light-induced light scattering transients from *Chlamydomonas*

Suspensions of dark adapted *Chlamydomonas* cells exhibit transient light scattering changes in response to a

flash of green actinic light (Fig. 1). The signals are graded with photon exposure over a more than 4-log unit dynamic range (Fig. 1 *F*). A sinusoidal response to dim flashes (called a-wave in analogy to the initial event in the light-induced electroretinogram [ERG] of the vertebrate retina, references 12–14), occurring with a delay of 30 ms and reaching its first peak within 140 ms (Fig. 1 *A*), is suspected by an all negative signal component when brighter flashes are used (b-wave, Fig. 1 *B*). With even higher exposures the signal changes its sign again, turning into the positive c-wave, which persists up to the highest photon exposures tested (Fig. 1 *D*). Its delay decreases with increasing exposure to a minimal delay value of 15 ms (Fig. 1 *E*), its amplitude, however, reaches a maximal value at submaximal light levels and starts to decline beyond this point (Fig. 1 *F*). It is this graded light response which is used in the following to probe visual transduction in *Chlamydomonas*.

A rhodopsin is the visual pigment in *Chlamydomonas*

To prove that the above signals are related to photoreception and not to photosynthesis or other possibly light-dependent processes, and to further corroborate the notion that the *Chlamydomonas* visual pigment is a rhodopsin, action spectra were determined for the three signal components (Fig. 2 *A*). All three peaked at the same wavelength, suggesting a common photoreceptor molecule, and the position of the peak (490–500 nm) is in agreement with previously published threshold action spectra for phototaxis (1, 9). The curve-form resembles the absorption spectrum of a rhodopsin from higher eucaryotes (15), but with a slightly lower peak width. This may reflect the fact that not only the absorption maxima of retinal proteins, but also their peak width can be "tuned" over a wide range by changing the intramolecular interactions between retinal and its protein environment (16, 17). It should be kept in mind, however, that the efficiency of the interference reflector in the eye spot (6), which attenuates light from one direction and increases the probability of photon capture for light from the other direction, is certainly wavelength dependent. This would tend to introduce systematical errors in the determination of the photoreceptor absorption spectrum from action spectra unless cells are used which have no such reflector.

Rhodopsins are retinal proteins with a Schiff base linkage between protein moiety and retinal chromophore. Hydroxylamine attacks this bond in a selective way, leading to the formation of retinal-oxime and an inactive apo-protein (18, 19). In most rhodopsins this reaction requires either protein denaturation or partial unfolding, the latter usually occurring in the course of the photocycle. The observation that hydroxylamine increases the photon exposure required for a given LS-response dramatically, but only when the cells had been preincubated in light, not in darkness (Fig. 2 *B*), is further evidence for the retinal-protein nature of the *Chlamydomonas* photoreceptor.

Single photon responses

The wide dynamic range of the optical population response (more than 4 log-units, Fig. 1) does not necessarily imply an equally wide dynamic range for each individual cell: under conditions where the mean number of absorbed photons per cell is <1, a graded population response merely reflects an increasing fraction of cells that have "seen a photon," provided, of course, that the cell can detect single photons. Unambiguous proof that this is the case would require the exact knowledge of the number of rhodopsins per cell, the absorption cross-section of *Chlamydomonas* rhodopsin and the photochemical quantum efficiency. While we don't have precise figures at present, we can make a "worst case" estimate, i.e., take the values from other known rhodopsins which are most unfavorable for our argumentation: (*a*) the number of rhodopsin molecules an eye-spot of 1–1.5 μm diam could maximally accommodate is 10^5. This is the protein density for the two-dimensional bacteriorhodopsin crystal in the purple membrane (20). (*b*) The absorption cross-section of vertebrate rhodopsin is 2×10^{-20} m^2 (21), and (*c*) its quantum efficiency is 0.66 (21). With an orientational efficiency factor of one-third (15), this accommodates the random orientation of the chromophores relative to the plane wave of unpolarized actinic light, but not the shielding due to the interference reflector (6), 2.8×10^{14} photons/m^2 (the lowest exposure we used) would activate, on average, no more than 0.12 rhodopsins per cell. With more realistic estimates for the number of rhodopsins per *Chlamydomonas* cell (i.e., $1–3 \times 10^4$, reference 22), this value drops to 0.012–0.036, suggesting that the whole a-wave region of the light titration is within the "single photon domain" where the probability of double and triple photon hits is negligible. A-wave signals would thus reflect genuine "single quantum responses." Further support for this notion comes from the linear increase in signal amplitude with photon exposure in this range (a photon requirement of two or more would produce a much steeper slope) and the constancy of waveform of the corresponding signals (Fig. 3). Within the framework of our hypothesis, the appearance of a new waveform (b-waves, observable above $3–5 \times 10^{15}$ photons/m^2) would then mark the occurrence of double or triple photon hits. The range

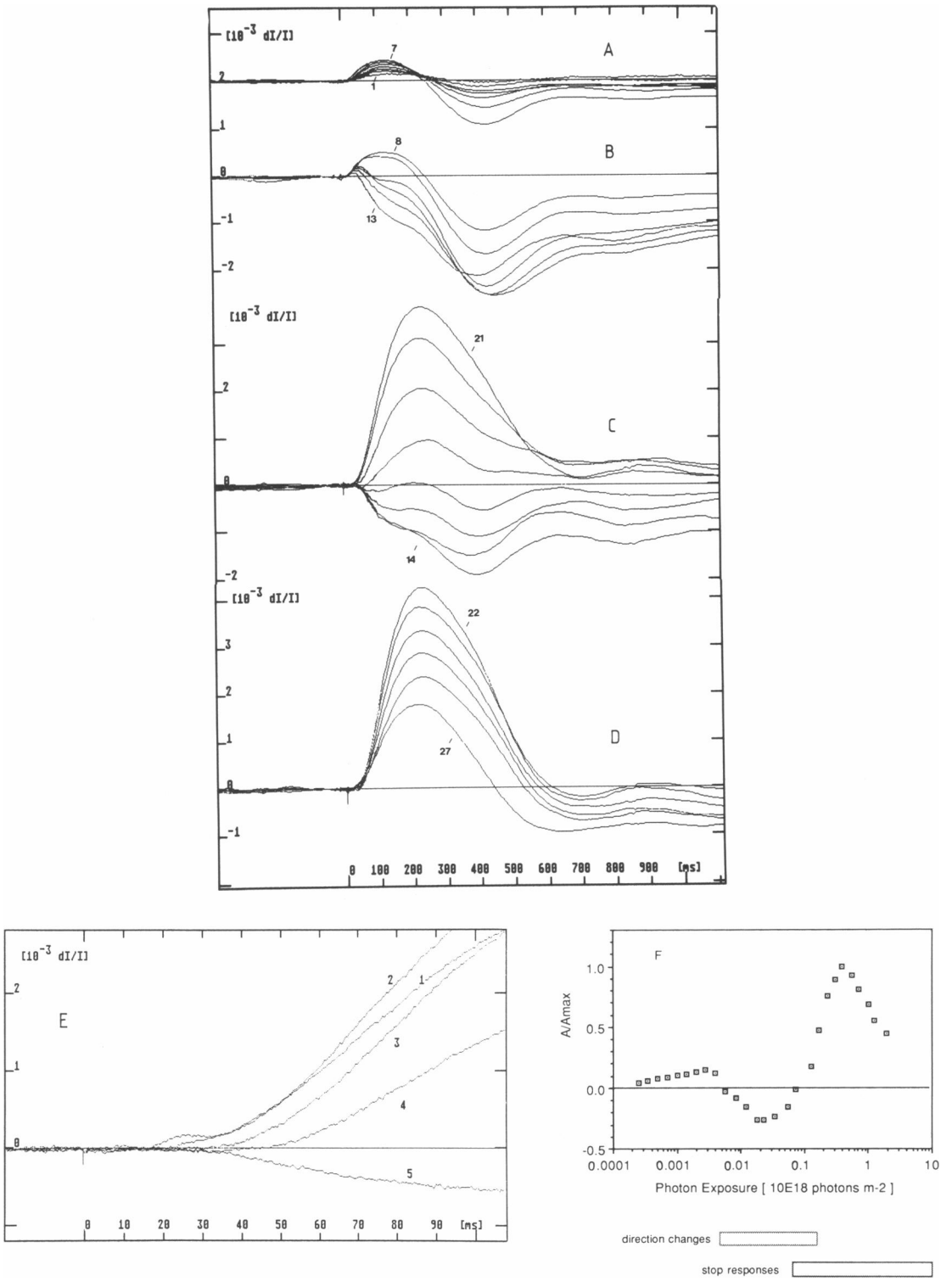


FIGURE 1 Light titration of flash-induced, transient light scattering changes. Photon exposure (0.5 ms duration, 499 ± 4 nm) was increased in steps of 1.4 between 2.8×10^{14} and 1.6×10^{18} photons m^{-2}. (*A*) a-wave dominated responses between 2.8×10^{14} (1) and 1.6×10^{15} photons m^{-2} (7). (*B*) b-wave dominated responses between 3.2×10^{15} (8) and 1.3×10^{16} photons m^{-2} (13). (*C*) c-wave dominated responses between 1.8×10^{16} (14) and 2.8×10^{17} photons m^{-2} (19). (*D*) Between 4.0×10^{17} (24) and 1.6×10^{18} photons m^{-2} (25). In *A* and *B* 10 and in *C* and *D* 2 signals were averaged. (*E*) Initial time-course of the LS-response at photon exposures between 1.6×10^{18} (1) and 3.6×10^{16} (5). The delay decreases from ~40 to 15 ms. (*F*) Response-amplitude (160 ms after the flash) vs. photon exposure.

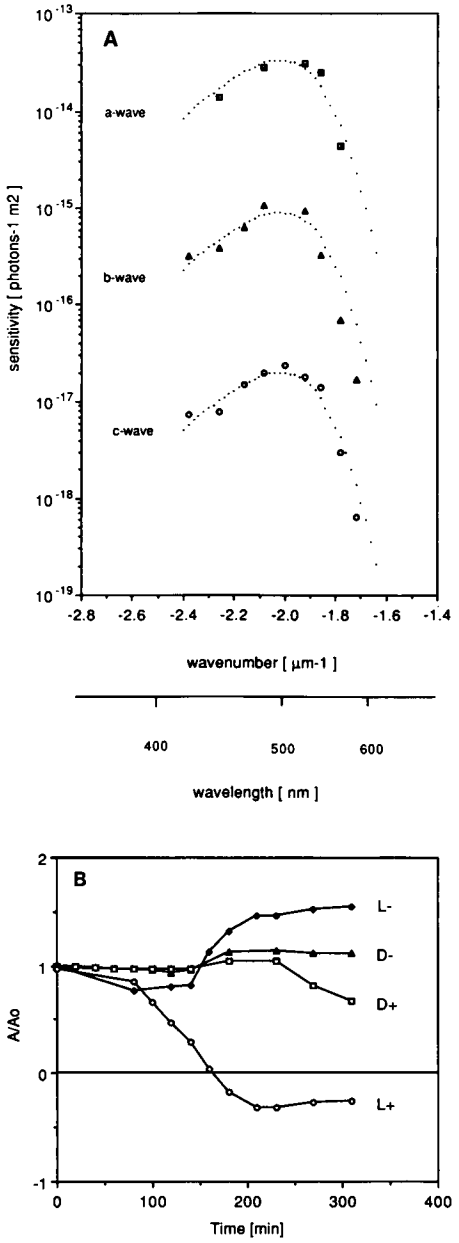


FIGURE 2 (*A*) Action spectrum of the LS-responses. Sensitivity was defined as the reciprocal of the threshold for a-, b-, and c-wave In the case of the a-wave it was determined by extrapolation of the semilogarithmical stimulus-response curves to a response of zero, in the case of b- and c-waves the actual zero-transitions were used.

A plot of log sensititivy vs. wavenumber yields very similar waveforms for all rhodopsins, independent of the spectral position of their absorbance maximum (16, 17, 29, 30). The dashed lines represent the absorption spectrum of bovine rhodopsin, taken from (15) and shifted by 0.04 μm^{-1} to the blue. (*B*) Effect of hydroxylamine on the LS-response. Signal amplitudes in response to flashes of 500 nm (3×10^{17} photons m^{-2}) as a function of preincubation time, both in light (L) and darkness (D), in presence (+) and absence (−) of 2 mM hydroxylamine. The green adapting light (500 nm) had an irradiance of 7.5×10^{18} photons $m^{-2} s^{-1}$. All cells were allowed to dark-adapt for 20 min before the test flash.

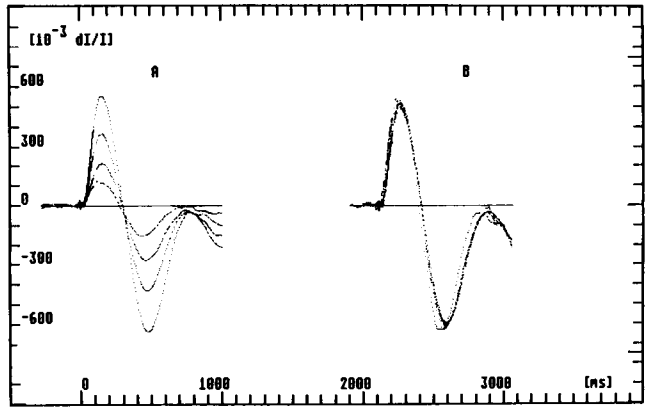


FIGURE 3 LS-responses to very dim flashes (2.8×10^{14} photons m^{-2} – 2.2×10^{15} photons m^{-2}), plotted with the same scale for all signals (*A*) or scaled such that all signals had the same amplitude (*B*). Signals were averaged 10 times.

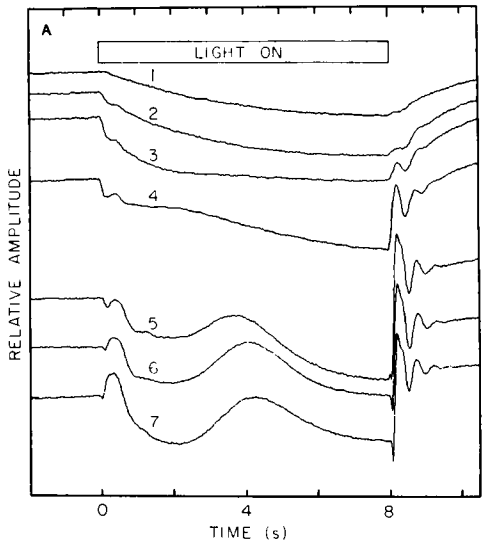
between this exposure and the highest one used in our experiment reflects a genuine dynamic range of *Chlamydomonas* vision of more than 3 log-units, 1 log-unit more than the dynamic range of a dark-adapted vertebrate rod-photoreceptor cell (23).

Mutants reported to have fewer rhodopsins per cell than strain 806 produced similar waveforms but at correspondingly higher photon exposures (data not shown). Light scattering threshold experiments may therefore be used as a single and convenient assay for the average number of receptor molecules per cell.

Adaptation effects

Light pulses of extended duration caused LS-signals with an initial time course similar to flash responses (Fig. 4 *A*). They were superceded by slower processes which lead to a new adaptational state within 8–10 s. "Off-responses" become oscillatory when the "step down" exceeded a critical level and actinic flashes on top of a background produced similar oscillations (Fig. 4 *B*). The threshold for such flash-responses increased with increasing background. A blue background, however, was much less effective in desensitization than orange light of equal "rhodopsin bleaching power" (data not shown), suggesting that the *Chlamydomonas* photoreceptor may undergo a photochromic shift to shorter wavelengths.[1] Such photochromism can seriously distort phototaxis action spectra due to the accumulation of a substantial fraction of the

[1]Alternative explanations like the existence of a second photoreceptor or the influence of the interference reflector in the eyespot appear very unlikely given the shape of the action spectra of the flash responses (Fig. 2).



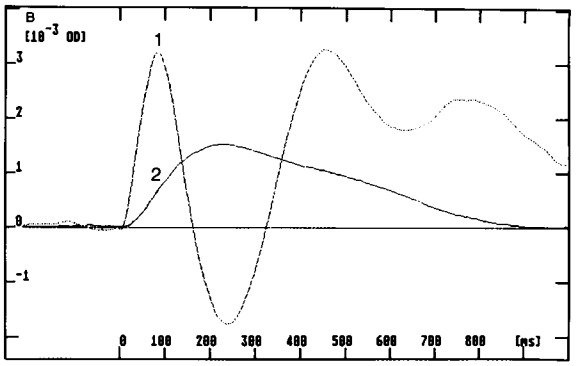


FIGURE 4 (*A*) LS-responses to periods (8 s duration) of constant irradiance (step-up and subsequent step-down). (1) 1.3×10^{16} photons $m^{-2} s^{-1}$. (2) 4.4×10^{16} photons $m^{-2} s^{-1}$. (3) 1.5×10^{17} photons $m^{-2} s^{-1}$. (4) 4.2×10^{17} photons $m^{-2} s^{-1}$. (5) 2.3×10^{18} photons $m^{-2} s^{-1}$. (6) 4.5×10^{18} $m^{-2} s^{-1}$. (7) 9.0×10^{18} photons $m^{-2} s^{-1}$. (*B*) Time course of the LS-response from light adapted cells. A green flash of light (2.0×10^{17} photons m^{-2}) was superimposed on an orange background ($\lambda > 550$ nm, 200 $\mu W/cm^2$) (*trace 1*). In the control experiment (*trace 2*) an identical flash was applied on a dark adapted sample.

color-shifted intermediate in continuous actinic light. The largely different action spectra derived from behavioral studies at threshold (1) and at high photon irradiance (9) could well be explained by this effect. Short and weak flashes as used in the present study avoid these problems by reducing the probability that a rhodopsin encounters more than one photon in the course of the experiment to virtually zero.

The brightest light used in Fig. 4 *A* could "bleach" more than 90% of the total rhodopsin in 1 s, yet stable LS-responses were obtained from these light-adapted cells for many minutes, indicating that *Chlamydomonas* rhodopsin is rapidly regenerated. This rapidly completed photocycle allows *Chlamydomonas* to cope with much higher irradiances than a vertebrate photoreceptor cell would ever encounter. The latter can afford a much slower regeneration process because its optical apparatus (pupil and lens) has the ability to reduce the ambient photon density by more than 1,000-fold in the plane of the photoreceptors.

Not only is the rhodopsin photocycle rapidly reversible in *Chlamydomonas*, but also the part of the transduction chain which leads to the structural changes causal for the LS-transients. This has been concluded from the observation that repetitive stimulation ($<0.5\ s^{-1}$) with saturating flashes produces nearly identical responses over extended periods of time (data not shown).

Correlation with behavioral responses

Two fast behavioral responses of *Chlamydomonas* have been studied with subsecond time resolution: a change in swimming direction following dim flashes, and a transient stop, including a short period (300–400 ms) of slow backward swimming (7, 8), after brighter flashes. At low exposures, where single photon responses (a-waves) are observed, no behavioral responses were observed. Directional changes became detectable with the transition from a- to b-waves, suggesting that double (or triple) photon hits are necessary for a behavioral response. This appears to be in contrast with a previous report from our laboratory (3) where, based on a Poisson-statistics analysis, it was claimed that directional changes can be triggered by single "effective photons." However, if the LS-assay were to probe an intermediary stage of the transduction chain and if the efficiency of the transmission between this stage and the final behavioral response was <1, a single "efficient" photon could still be sufficient. A more likely explanation would be that the interference reflector in the eyespot, which decreases the probability of photon capture for light coming from one side while increasing it for light from the opposite direction can assume various orientations relative to the incoming light in a statistical single cell experiment. This would tend to distort the Poisson statistics. A contrast ratio of 8 to 10, for instance, would make a process with a photon requirement of two look like a single photon process. A decisive experiment, i.e., measurements of single cell responses with a fixed eyespot orientation relative to the incoming light is currently in preparation.

Stop responses, on the other hand, need much higher exposures than directional changes. Their occurrence was always correlated with a positive LS-signal component (c-wave), and the transition from direction changes to stop responses always coincided with the transition from

b- to c-waves (Fig. 1 *F*). Moreover, the onset of the stop and the rising phase of the c-wave always had the same kinetics (Fig. 5).

In continuous light there was only a transient stop response, followed by a resumption of the original swimming speed within a few seconds. Upon turning the light off, another transient stop occurred (8). Again the LS-response behaved analogously, i.e., each time there was a stop there was also a transient c-wave (Fig. 5). Both processes apparently respond to changes in the ambient light rather than to its steady-state level.

On the electrical basis of the LS-signal

Recently electrical light-responses have been recorded from Haematococcus pluvialis, a unicellular alga related to *Chlamydomonas* (24), and experiments in our laboratory have indicated the existence of similar signals in *Chlamydomonas*. Using the suction pipette technique (23), transient photocurrents were measured between 0.1 and 40 ms after the flash, i.e., within the latency period of the LS-response (H. Harz, unpublished results). This suggests that the light scattering probes stages of the transduction chain which mediate between electrical events and the flagellar effector organ.

A close correlation between light-induced ion translocations and the subsequent processes monitored by the optical assay is also suggested by the finding that reducing the extracellular calcium concentration leads to a concomittent decrease in signal amplitude and that increasing the photon exposure overcomes this effect. Specific calcium-channel blockers like ω-conotoxin, pimozide, or cobalt mimicked the action of low extracellular calcium or low photon exposure. Moreover, a membrane depolarisation, brought about by increasing the extracellular potassium concentration, attenuated the signal, and potassium-channel blockers like charybdotoxin, quinidine, or apamine did the same. A more detailed descripton of the ionic nature of the signal will be given elsewhere (Hegemann, P., Nonnengässer, C., Kühnle, E., and Uhl, R., manuscript in preparation).

On the structural origin of the LS-signals

Light-induced LS-changes from vertebrate photoreceptor cells exhibit a pronounced angular dependence (11). This has allowed us to identify their structural origin. LS-signals from *Chlamydomonas*, on the other hand, possess no marked angular profile which could aid their interpretation. At present we cannot say to which extent osmotic volume changes, structural changes in the basal body region or in the flagella themselves contribute to the signal. There is no doubt, however, that they probe a later stage of the visual transduction chain with high time

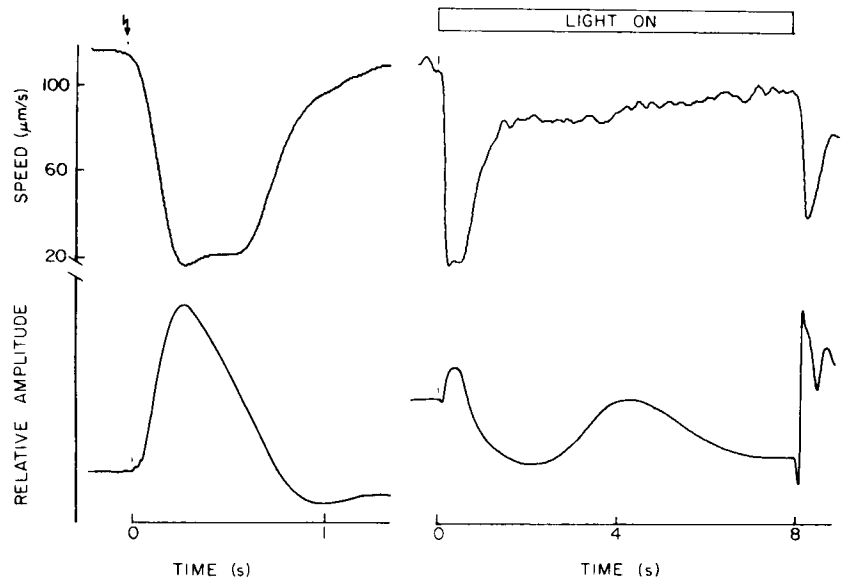


FIGURE 5 Comparison of the kinetics of behavioral (stop-) responses (*upper traces*) and corresponding LS-signals (*lower traces*). The actinic light was a flash (*left panels*) or a continuous light (*right panels*) of the same brightness. The stop responses represent an average of 400 single cell responses. Note that the light scattering response in the bottom right corner has been scaled down 2 times.

resolution and that the conclusions reached in the present study do not depend on a precise knowledge of the structural basis of the assay.

Concluding remarks

Visual perception depends on two principal interactions between light and matter: absorption and scattering, the latter being comprised of refraction, diffraction, and reflection effects. For more than a century absorption has been used extensively for the exploration of the primary processes in visual transduction after the discovery of Boll (25) and Ewald and Kühne (26) that intramolecular processes in the "visual purple" rhodopsin lead to structural changes which change the interaction between the chromophore and its protein environment and hence to an altered absorption profile. Vision related intermolecular processes, on the other hand, have recently been shown to be accompanied by structural changes ivolving extended structural domains, and these lead to an altered diffraction/scattering pattern (recently reviewed in references 27 and 28). Thus, the basis of visual perception (absorption and diffraction) has served as a basis for exploring visual transduction. In particular, the elastic interaction between light and extended structural domains, e.g., light scattering, has become a very convenient, sensitive, and noninvasive diagnostic tool, which allows to probe intracellular processes in real time with millisecond time resolution. This technique is apparently not limited to the study of specialized cells of higher animals, but may also be successfully applied to unicellular organisms as demonstrated above.

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