Control of Phobic Behavioral Responses by Rhodopsin-Induced Photocurrents in *Chlamydomonas*

Eva M. Holland,* Hartmann Harz,# Rainer Uhl,# and Peter Hegemann*
*Institut für Biochemie I, 93040 Regensburg, and #Botanisches Institut der Ludwig-Maximilians-Universität München, 80638 München, Germany

ABSTRACT Both phototactic and photophobic responses of *Chlamydomonas* are mediated by a visual system comprising a rhodopsin photoreceptor. Suction pipette recordings have revealed that flash stimulation causes calcium currents into the eyespot and the flagella. These photocurrents have been suggested to be the trigger for all behavioral light responses of the cell. But this has never been shown experimentally. Here we describe a detection technique that combines electrical and optical measurements from individual algae held in a suction pipette. Thus it is possible to record photocurrents and flagellar beating simultaneously and establish a direct link between the two. We demonstrate that in *Chlamydomonas* only the photoreceptor current in conjuction with a fast flagellar current constitutes the trigger for photophobic responses. Within the time of the action-potential-like flagellar current, the flagella switch from forward to backward swimming, which constitutes the beginning of the photoshock reaction. The switch is accompanied by a complex frequency change and beating pattern modulation. The results are interpreted in terms of a general model for phototransduction in green algae (*Chlorophyceae*).

INTRODUCTION

Chlamydomonas is a green alga ~8 μm in diameter that shows helical swimming with the two flagella in the forward direction. In the course of one helical turn, which takes ~500 ms, the cell rotates once around its longitudinal axis, thus allowing the eyespot to scan the environment for changes in light intensity and quality (Foster and Smyth, 1980). When the ambient light conditions change, the cell turns toward or away from the light source (positive or negative phototaxis), depending on whether the intensity decreases below or increases above the optimal value. At low light levels or in response to weak flashes, individual direction changes can be resolved (Fig. 1 *a*) (Hegemann and Marwan, 1988). Sudden step-up stimuli or intense flashes lead to well-characterized photophobic responses (stop response or photoshock), comprising slow backward swimming for several hundred milliseconds (Fig. 1 *b*) (Schmidt and Eckert, 1976; Hegemann and Bruck, 1989). Previous work on permeabilized cells or isolated flagellar apparatusses has shown that the flagellar beating pattern depends on the Ca^{2+} concentration inside the flagella. This suggests that in living cells a light-induced Ca^{2+} flux across the flagellar membrane may be responsible for beating changes in living cells (Otomo and Brokaw, 1985; Hyams and Borisy, 1978; Bessen et al., 1980; Kamiya and Witman, 1984). Rhodopsin-mediated Ca^{2+} fluxes have been studied by the application of a suction pipette technique to cell wall-deficient cells. The primary electrical event is the so-called photoreceptor current (P-current), which is confined to the eyespot region (Harz and Hegemann, 1991; Harz et al., 1992; Sineshchekov et al., 1990). P-currents follow intensive light stimuli with virtually no delay (< 50 μs), they peak within 1 ms and decay within 20 ms (Sineshchekov et al., 1990; Holland et al., 1996). The short delay between photon absorption and initial rising phase of the P-current has led to the idea that the photoreceptor and P-channel may form a single protein complex (Harz et al., 1992; Holland et al., 1996). When the current-integral of the photoreceptor current exceeds a critical level, two flagellar current components are triggered, a fast one (F_F) and a slow one (F_S). The delay between flash and the F_F-current may be as short as 10 ms and as long as 100 ms, depending on the brightness of the preceeding stimulus. A brighter flash causes a larger P-current amplitude, and the critical depolarization that triggers the F_F-current is reached earlier. Thus, according to the assumption that the cell is isopotential, the communication between eyespot and F_F-channels needs no further transduction element (Harz et al., 1992). At moderate flash exposures, the flagellar current rises slowly, and only when a critical level is exceeded does it self-propagate into an action potential-like F_F-spike. F_S-currents, which always occur in conjunction with F_F-currents, have an amplitude of only a few picoamperes under physiological Ca^{2+} conditions, but they can reach more than 15 pA when Ca^{2+} is replaced by Ba^{2+} (Harz et al., 1992; Holland et al., 1996).

It has been suggested that the above photocurrents trigger the behavioral responses, yet a direct link remains to be established. The flagellar beating of both freely swimming and pipette-held cells has been analyzed carefully by high-speed cinematography (Rüffer and Nultsch, 1987, 1990, 1991, 1995). Unfortunately, however, that work was carried out only under bright light conditions that were necessitated by this technique. The cells were thus highly light adapted, and phobic responses could only be triggered by extraordi-

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Address reprint requests to Dr. Peter Hegemann, Institut für Biochemie I, Universität Regensburg, 93040 Regensburg, Germany. Tel.: 0049-941-943-2814; Fax: 0049-941-943-2936; E-mail: peter.hegemann@biologie.uni-regensburg.de.

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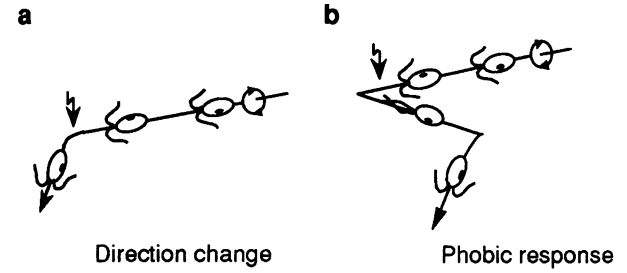

FIGURE 1 Schematic representation of a direction change occurring at low photon exposures (*a*) and of a phobic response after stimulation with a bright flash (*b*).

narily bright stimuli. Photocurrents, on the other hand, can be recorded from dark-adapted cells in response to low or moderate flashes. This communication is an attempt to tie previous observations together by establishing a correlation between photocurrents of various sizes and changes in flagellar beat frequency and beating space. For this purpose we have devised a new detection system that allows us to study the flagellar beating of dark-adapted single cells and to measure photocurrents from the same cell simultaneously. Thus it becomes possible to draw conclusions about how rhodopsin-induced photocurrents control flagellar beating. In this first communication we concentrate on the time period between light absorption and the beginning of a phobic response.

MATERIALS AND METHODS

Optical recording

Cells were visualized with an infrared-sensitive video camera (Ca in Fig. 2) and a 780-nm observation light from a light-emitting diode (LED 7601SG; Hitachi). The beam used for monitoring the flagellar beat was provided by a second LED light source (L-880) radiating at 880 nm (LED 8812SG; Hitachi). The two beams were combined in the condensor with the help of a polarizing beam-splitter cube (PB) (K. Lambrecht Corp.), and they were separated behind the objective (Ob) by means of a custom-made polychroic mirror (DM-2) (Omega Optical, Brattleboro, VT). Thus the observation light reached the camera in the first image plane, and the monitoring light was picked up by two light guides (Fi-1 and Fi-2) of 0.2 mm diameter in a corresponding second image plane in the epifluorescence part of the microscope. The fibers were mounted on micromanipulators such that their relative distance and their absolute position could be varied in two dimensions (Fig. 2 *b*). Moreover, the pair of light guides could be rotated by 360°. For a more detailed analysis, a bundle consisting of 16 fibers was used, instead of only two (Fig. 2 *c*). Optical signals from up to seven fibers could be recorded simultaneously. The light collected by the fibres was measured by hybrid photodetector/amplifier combinations (TSL 250; Texas Instruments). Signals were further amplified and filtered by a Cyberamp 380 (Axon Instruments, Foster City, CA), digitized by an ITC16 DMA interface (Instrutec) and stored, and processed with an Apple Macintosh computer using the AxoData program (Axon Instruments). Slow fluctuations of the flagellar signals were removed by high-pass filtering. The positioning of the two observation spots in the image plane was facilitated because the spots could be visualized directly by backside illumination of the fibers. The wavelength of this third illumination system (760 nm) was chosen such that it was partially reflected and partially transmitted by the dichroic mirror beneath the objective of the inverted microscope.

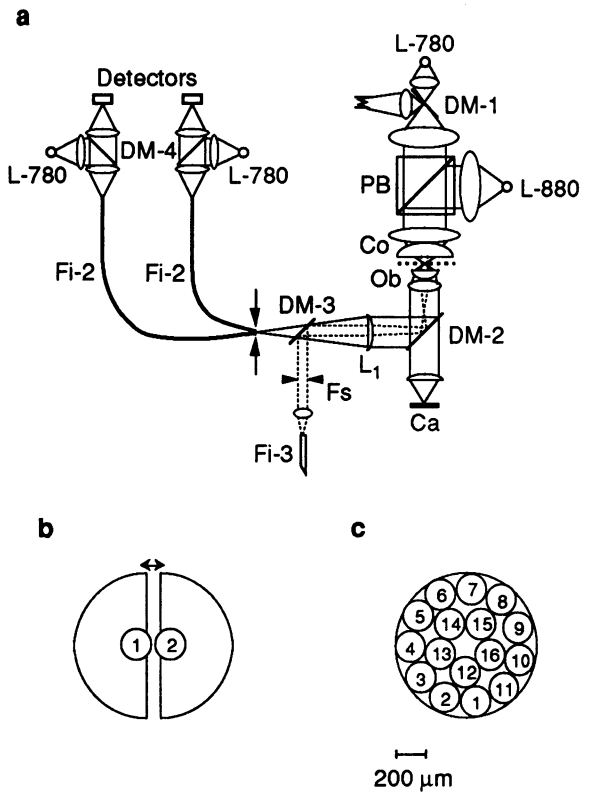

FIGURE 2 (*a*) Optical modification of an inverted microscope for the measurement of flagellar beating activity. L, Light-emitting diodes; DM, dichroic mirrors; Ca, camera; Fi, light guides; Co, condensor (NA = 0.63); Ob, objective 40× (NA = 1.3); FS, field stop; PB, polarizing beamsplitter. Stimulation light (*dashed lines*) was applied via Fi-3. (*b*) Arrangement of two light guides with variable distance. (*c*) Light guide bundle with 16 fibers. Only fibers 1–7 were used for the recording of flagellar beating.

Cells were stimulated by 10-μs flashes of 500-nm light from a short-arc xenon flash lamp (FXQG-949–1; EG&G, Salem, MA) through the objective, using the epiillumination described in Fig. 2. An unattenuated flash (100%) corresponded to 2×10^{20} photons m^{-2} in the specimen plane. Note that because the eyespot is 45° outside the flagellar beating plane, which had to be placed within the specimen plane in the present experiments, the actinic light reached the eyespot under a corresponding angle.

Electrical recording

Photocurrents were recorded as capacitive displacement currents (Harz et al., 1992), with both eyespot and flagella outside the pipette, using a patch-clamp amplifier (EPC-7; List, Darmstadt, Germany). Raw data were filtered with a 3-kHz low-pass Bessel filter. The holding potential was set to 0 mV. Suction pipettes with a tip diameter in the range of 3–4 μm and an access resistance of 20–50 MΩ were used. Cells were sucked into the pipette until the resistance reached a value between 60 and 150 MΩ.

Cells

Cell-wall-defective *C. reinhardtii* strain CW2 cells were grown and differentiated into gametes overnight. The phototactically most active cells were photoselected and dark-adapted for at least another hour in NMG^+/K^+ buffer (5 mM HEPES, 9 mM HCl, 1 mM KCl, 200 μM K-1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid, adjusted with *N*-methyl-D-glucamine (NMG) to pH 6.8; Holland et al., 1996) plus the

desired concentration of $CaCl_2$ before they were used for optical or electrical measurements. The free Ca^{2+} concentration was 100 μM if not otherwise indicated. It was adjusted and determined as described before (Holland et al., 1996).

RESULTS

To monitor the flagellar beating pattern, algal cells were held in a suction pipette under the microscope and illuminated with infrared light not seen by the rhodopsin photoreceptor. Two quartz light guides were placed in the image plane so that they collected the light from two 3.3-μm spots within the beating space of the two flagella (Figs. 2 and 3 *a*). Photodetectors at the end of these fibers measured a light intensity that was modulated in a multiphasic fashion each time a flagellum passed through the light-barrier system. The exact waveform depended on whether the beating occurred in the focus plane or slightly above or below (Fig. 3 *b*).

In the course of a normal breast stroke, as executed by dark-adapted cells, each of the two flagella passed the detection spot once or twice, depending on the position of the spot relative to the cell body. This is borne out in Fig. 4, where the detection spot was placed in a variety of different positions. In the dark, a detector in position 1 sensed only the power stroke, whereas both the power and recovery strokes could be registered in position 2 (Fig. 4 *a*). During a phobic response (Fig. 4 *b*) the cells switched to a crawl style with flagellar undulation, resulting in a slow backward movement in free-swimming cells (Fig. 1 *b*). This clearly distinct behavior was best sensed in position 3. Positions 1 and 2, on the other hand, became optically "silent" during undulation. In position 4, signals could be recorded from both flagella only during undulation, but not during forward swimming.

The beat frequencies of fully dark-adapted cells ranged from 20 to 65 Hz, the *cis* flagellum always beating 25–30% slower than the *trans* flagellum (Fig. 5 *a*). In contrast, during undulation the flagella were synchronized (Fig. 4 *b*, position 4), with beat frequencies ranging from 50 to 70 Hz (Fig. 5 *b*).

Bright light flashes have been shown to evoke two fast, transient inward calcium currents in *Chlamydomonas* (Fig. 6 *a*). The photoreceptor current (P) is graded with photon exposure and originates in the eye-spot region, the site of the photoreceptors. The fast flagellar current (F_F), on the

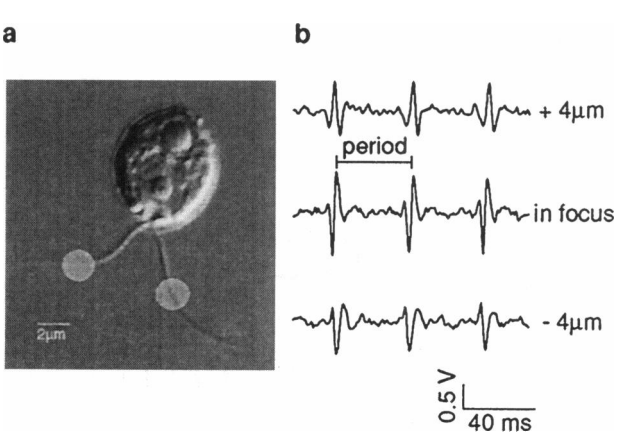

FIGURE 3 (*a*) Nomarsky image of a *Chlamydomonas* cell. The two dots mark the position of the detection spot (fiber) relative to the beating space of the flagella. (*b*) Recording of flagellar beating with the flagella in focus or 4 μm above or below focus.

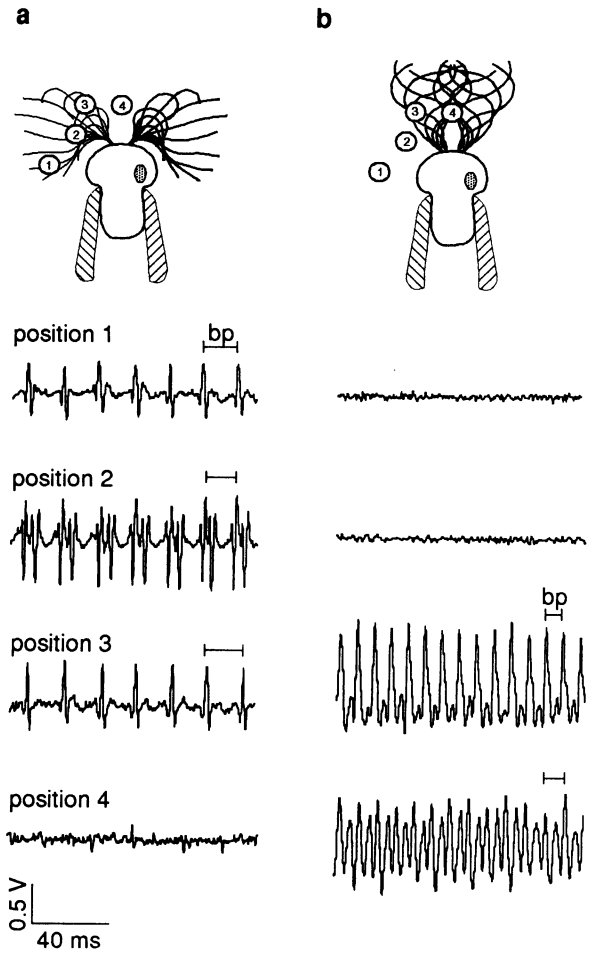

FIGURE 4 The flagellar beating during forward (*a*) and backward (*b*) swimming and recordings from four positions of the flagellar beating space. Note that in *b*, position 4 visualizes the synchronized beating of both flagella. bp, Beat period.

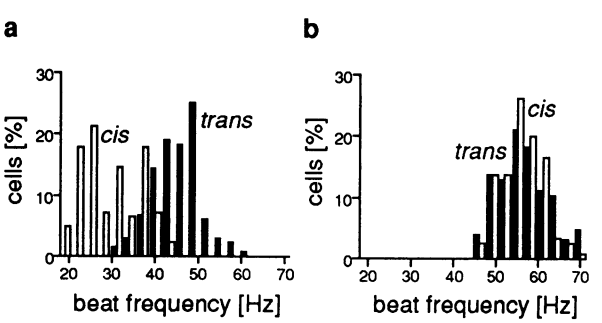

FIGURE 5 Beat frequences of both flagella during forward (*a*) and backward (*b*) swimming at 100 μM Ca^{2+}.

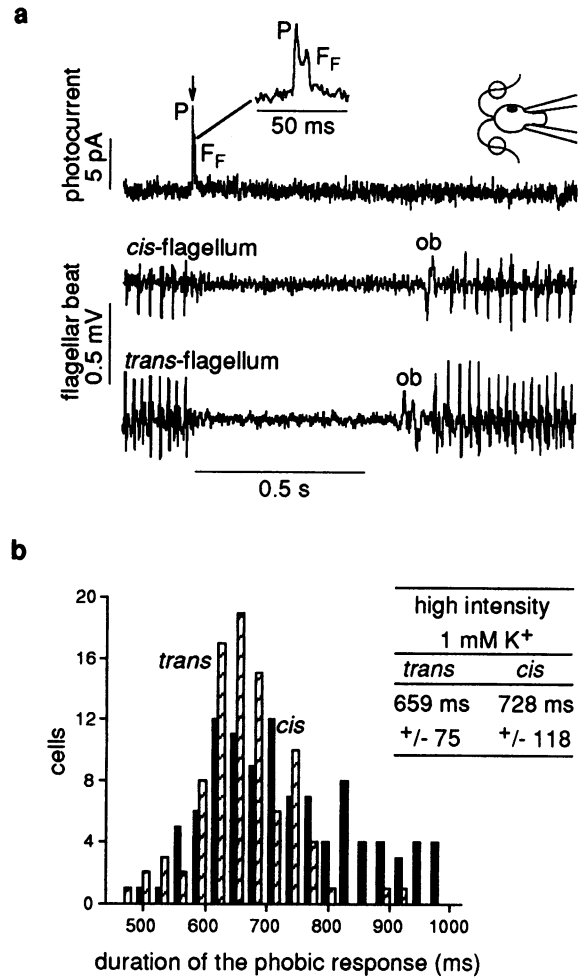

FIGURE 6 (*a*) A photocurrent trace and simultaneous optical beating patterns of both flagella in response to a bright flash (100%). "Overbeats" (ob) with increased beating space appear at the end of the phobic response. The arrow indicates the time of the flash. P, Photoreceptor current; F_F, fast flagellar current. (*b*) Histogram of the stop duration of *cis* and *trans* flagella. Mean values and standard deviation are listed in the inset.

other hand, is an all-or-nothing event, mediated by ion channels, which are presumably voltage gated (Holland et al., 1996) and evenly distributed over the entire length of the flagella (Beck and Uhl, 1994). In all experiments in which optical and electrical signals were recorded simultaneously from a single cell, it was found that the occurrence of the fast flagellar F_F-current was closely linked to the switch of the flagella to undulation movement, which lasted for 500–1000 ms (Fig. 6 *b*). Transition from breast stroke to undulation, as evident from the disappearance of the optical signal in Fig. 6, occurred simultaneously in the two flagella.

At high flash energies, the return from undulation to forward swimming was not a sudden process. The switch occurred within 150 ms, and both flagella slowly resumed the beat frequencies that are typical for forward swimming. Full recovery of the beat required 1 s or more. Before the flagella resumed their normal beating pattern, they usually performed one or two "overbeats," i.e., beats with increased beating volume and duration (Fig. 6). They could be monitored at all four recording spot positions shown in Fig. 4. Overbeats were recorded first by high-speed cinematography (Rüffer and Nultsch, 1995). In our experiments the return to normal swimming was not necessarily synchronized in the two flagella. In most cases (>70%) the *cis* flagella resumed backward swimming later than the *trans* flagella (Fig. 6 *b*). This difference amounted to 20–80 ms under all conditions. It corresponds to two to four extra beats of the *cis* flagellum in undulation mode. These extra beats cause the cell to turn by 30°–60° within the flagellar beating plane (Rüffer and Nultsch, 1995), i.e., around an axis perpendicular to the ordinary axis of rotation. Such a turn allows the cell to assume a new swimming direction after a phobic response, thus avoiding harmful light conditions.

Owing to the fact that F-currents are triggered by the P-current-induced depolarization, dimmer flashes led to an increased delay between flash and F_F current. Particularly

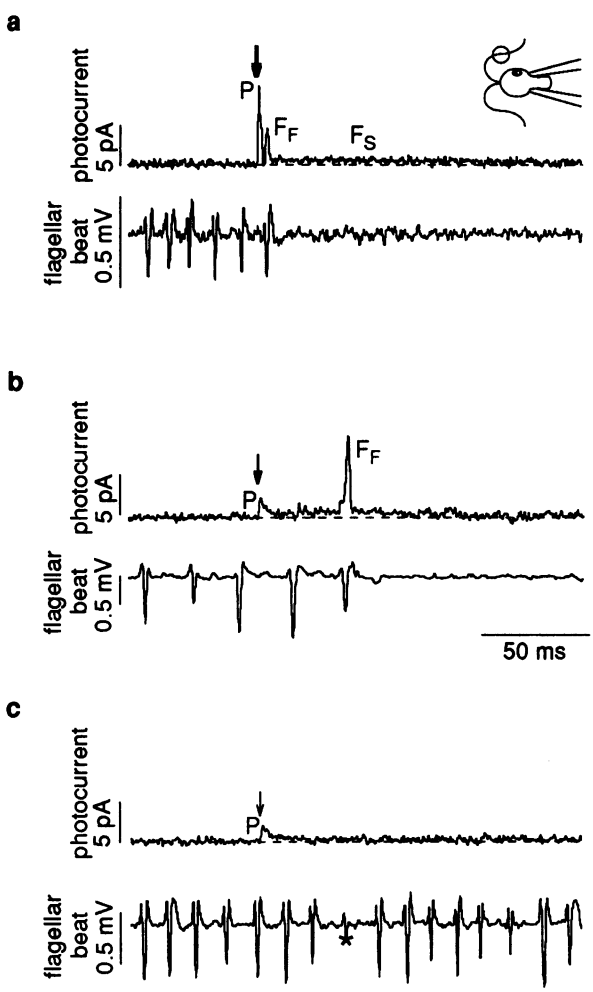

FIGURE 7 Photocurrent and beat response of the *cis* flagellum recorded at position 1, at exposures of 1.4×10^{19} (*a*), 0.7×10^{19} (*b*), and 0.3×10^{19} (*c*) photons m^{-2}. The star marks a transient increase in beating space. F_S, Slow flagellar current.

long F_F-current delays were observed from cells with slow flagellar beating (Fig. 7 *b*). Phobic responses, as monitored optically, coincided with the appearence of F_F-current spikes: whenever there was a F_F-spike, there was a flagellar switch, and both F_F-current and switch occurred with closely correlated delay after the flash (Fig. 7, *a* and *b*). This strict correlation between electrical signals and phobic responses held for more than 1000 traces from 90 different cells. At exposures leading to submaximum P-currents, the appearance of F-currents and consequently also the phobic responses became stochastic events whose likelihood increased with increasing photon exposure. Dim flashes, which led to no visible flagellar currents, caused only brief transient changes in the beating pattern (Fig. 7 *c*). Both amplitude and beat frequency decreased for the duration of one to four beats, i.e., for 20–80 ms. These low-intensity events will be described in detail elsewhere (Holland and Hegemann, manuscript in preparation).

A more detailed correlation between currents and flagellar events became possible when the flagellar beating was recorded with seven detection spots placed as a semicircle in the beating plane of the flagella. A kinetic correlation was greatly facilitated by using moderate photon exposures where the delay between P- and F_F-currents was at maximum. Under these conditions it could be determined how the flagellar switch depends on the position of the flagella at the time of the F_F-current appearence (Fig. 8, *a–c*). In unstimulated cells the power stroke of breast-stroke swimming is recorded in channel 1–3 with the sequence 3, 2, 1, whereas the recovery stroke, which has a narrow beating space very close to the cell body, is invisible when channels 1–7 of the multichannel array (Fig. 2 *c*) are used. All experiments reveal that the flash-induced switch may occur from any phase of the beat cycle. Only the appearance of the F_F-currents and not the position of the flagella determines the time of switching. When the F_F-spike appeared at the end of the power stroke (Fig. 8 *a*) or during the recovery stroke, the beat cycle remained almost unchanged. As in undisturbed cells, the recovery stroke was not detected. The first undulating beat appeared as expected only in channel 4. When the F_F-current spike was fired during the power stroke (Fig. 8, *b* and *c*), the power stroke was interrupted, and the recovery stroke occurred with a larger beating space. Then the recovery stroke was seen in channels 2 and 3 before undulation appeared in channel 4. These flash-induced recovery strokes are easily identified by the altered sequence in channels 2, 3, and 4 or 3 and 4 (*dashed arrows* in Fig. 8 *b* and *c*).

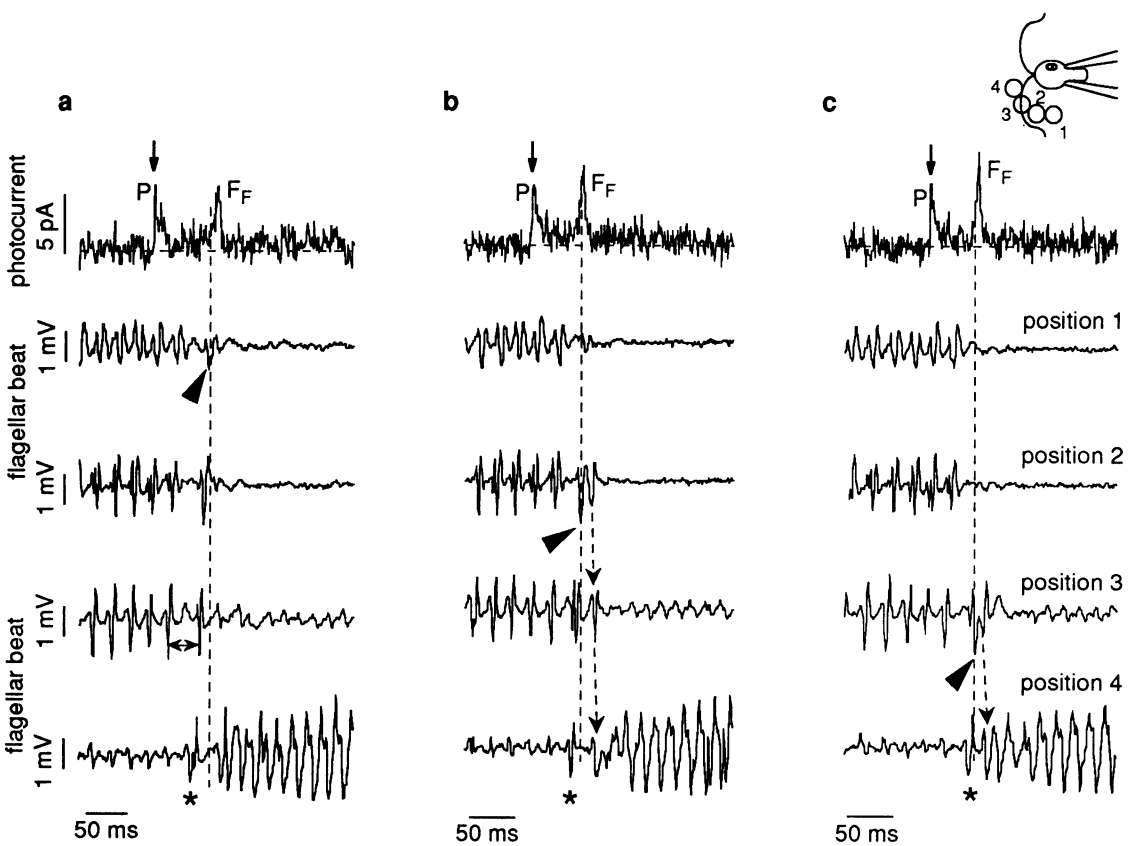

FIGURE 8 Flagellar beating recorded simultaneously from four optical channels in response to moderate flash energies (0.7×10^{19} photons m^{-2}). The star marks the enlarged beat, which often occurs just before the switch. The arrowheads indicate the position of the flagella at the beginning of F_F currents. The dashed arrows mark the reversed optical recording sequence 2, 3, 4 in *b* or 3, 4 in *c*, indicating a recovery stroke with large flagellar beating space. The time of lower beat frequency is indicated by a horizontal double-headed arrow.

Despite the fact that a switch from breast stroke to undulation was inevitably linked to the occurrence of an F_F-current, there were changes in the beat pattern that could definitively be observed before F_F-currents began to rise. At moderate photon exposures, the beat frequency decreased already in the time window between P-current and F_F-current (Fig. 8). As previously mentioned, the flagellar current often exhibited a slow rising phase which, in an self-accelerating fashion, turned into an F_F-current spike (Govorunova and Hegemann, 1997). Fig. 8 demonstrates that, at moderate photon exposures, the lowered beat frequency occurred in conjunction with a larger beating space such that the last beat before the F_F-current appearance could be monitored in all four channels (*stars* in Fig. 8, *a–c*).

Further support for the close link between flagellar current and phobic response came from recordings of spontaneous flagellar currents that occasionally occurred in the dark, preferably after a phobic response. That these spontaneous currents are not P-currents was concluded from experiments in which the eyespot was attached to the pipette wall, a configuration in which the P-currents are almost invisible (Fig. 9). Spontaneous F_F-currents usually consisted of a "current doublet" (Fig. 9, *inset*), presumably because it is the spontaneous firing in one flagellum which, in turn, triggers the voltage-sensitive channels in the other one. These spontaneous F_F-currents invariably led to phobic responses in all experiments. A split of the flagellar current into the contributions of the two flagella was previously recorded from cells that were held with one flagellum within, the other outside the pipette (Beck and Uhl, 1994). The increased probablity of spontaneous F_F-currents subsequent to a stimulus suggests that the cell is still more depolarized than in the dark-adapted state. Similarly, a depolarization of ~40 mV induced by an increase in extracellular K^+ from 1 to 20 mM (Malhotra and Glass, 1995) increased the frequency of spontaneous electric and concomitant behavioral events in darkness.

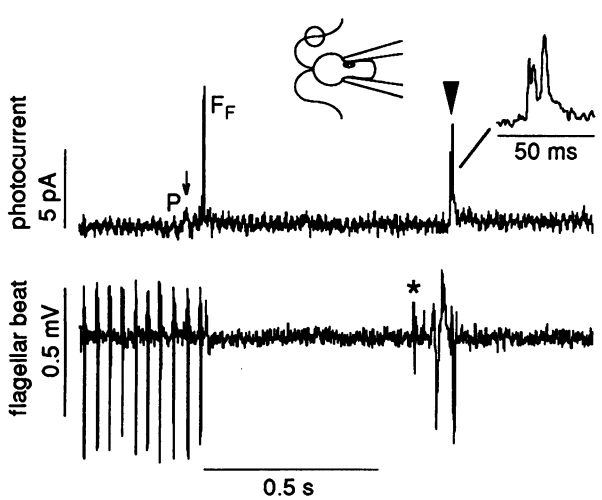

FIGURE 9 Spontaneous flagellar currents (*arrowhead*) after a flash-induced phobic response. A cell was chosen where the eyespot was attached to the pipette wall, a condition in which the P-currents exhibit small amplitudes. The star indicates the end of the light-induced response, i.e., the reversal from backward to forward beating.

DISCUSSION

At elevated photon exposures, suprathreshold P-currents trigger the activation of voltage-dependent F_F-current spikes that have been studied in detail in the past. P-currents as well as the F-currents are mainly carried by Ca^{2+} under physiological conditions (Holland et al., 1996). The Ca^{2+} influx into the flagella during the F-current spike is strong enough to trigger phobic responses. This switch may occur at any position of the flagella during the beat cycle, as has now been shown directly in the experiments presented above. However, whether the Ca^{2+} influx or the membrane potential changes cause the switch has still not been shown directly and is still under debate.

In *Paramecium*, the best characterized flagellar current is a voltage-dependent Ca^{2+} current that is activated upon a primary depolarization and delivers a short (several milliseconds) but strong Ca^{2+} impulse triggering ciliary reversal as well as its own inactivation (reviewed by Andrivon, 1988). This current resembles the algal F_F-current. The second *Paramecium* Ca^{2+} channel is a slow voltage-inactivated current that is responsible for maintaining the high internal Ca^{2+} level resulting from the first impulse. Such a slow F_S-current is also present in *Chlamydomonas*. It lasts for some hundred milliseconds at physiological Ca^{2+} and is greatly enhanced in amplitude and duration when Ca^{2+} is replaced by Ba^{2+}. As expected, such an enhanced Ba^{2+} influx also causes an extended stop duration (data not shown).

Ca^{2+} changes during flagellar responses

The dependence of eucaryotic flagellar beating modes on the Ca^{2+} level has been studied in detergent-extracted models of *Paramecium* and *Chlamydomonas* after reactivation of the axonemes with Mg^{2+} and ATP (Hyams and Borisy, 1978; Bessen et al., 1980; Kamiya and Witman, 1984; Otomo and Brokaw, 1985; Andrivon, 1988). The experiments presented above suggest that the role of Ca^{2+} in a living cell is at least qualitatively similar. As long as the content of free Ca^{2+} is maintained below 6×10^{-7} M, in both systems the axonemes beat like the flagella of forward-swimming cells. (Slight differences in the Ca^{2+} values are very likely due to the difficulty of adjusting precise Ca^{2+} concentrations.) When the concentration is raised above 6×10^{-7} M, flagellar reversal occurs and the models swim backward. We calculated before that during a F_F-current the total Ca^{2+} concentration increases to 10^{-4}-10^{-3} M (Harz et al., 1992). Even under the assumption that most of this Ca^{2+} is protein bound, we assume that a concentration of 10^{-6} M is reached before the F_F-current reaches the peak. Below the

threshold for F_F currents, less extreme responses of the two flagella may be controlled by submicromolar Ca^{2+} changes. In reactivated cell models at 10^{-8} M Ca^{2+}, the axonemes were active, as in unstimulated cells, allowing the conclusion that 10^{-8} M is the steady-state Ca^{2+} concentration of a living *Chlamydomonas* cell. In *Chlamydomonas* models at 10^{-7} to 10^{-6} M Ca^{2+}, the amplitude of the *cis* axoneme was selectively reduced. Because in pipette-held cells flashes of low energy briefly reduce the activity of the flagella with some preference for the *cis*, we conclude that this is caused by a Ca^{2+} rise between 10^{-7} M and 6×10^{-7} M. Total inactivation of the *cis* flagellum, which leads to circular swimming in cell models, was never seen at any Ca^{2+} concentration in living cells. Thus this formerly observed total inactivation is likely to result from a prolonged exposure of the axonemes to high Ca^{2+} levels, which does not occur under physiological conditions in living cells.

Beat frequency changes

More complicated to establish is a quantitative relation between the internal Ca^{2+} level and the flagellar beat frequency increase during a phobic response. In models of *Paramecium*, the frequency slowly increases when the Ca^{2+} increases from 10^{-8} to 2×10^{-7} M. The frequency then drops until the Ca^{2+} concentration is 6×10^{-7} M before ciliary reversal occurs. After the switch to backward beating, the frequency reaches its maximum at 10^{-6} M Ca^{2+} (Andrivon, 1988). In line with these earlier observations, the frequency drop seen in living *Chlamydomonas* cells within the time range between P-current and the F_F-current appearances is again interpreted as a Ca^{2+} rise to 6×10^{-7} M, starting from a level between between 10^{-8} M and 2×10^{-7} M. As seen from the different frequencies at rest (Fig. 5 *a*), this starting Ca^{2+} level might be slightly different in in the two flagella. After the switch to undulation, the frequency increase in both flagella to the same maximum (Fig. 5 *b*) is understood as a parallel approach of the intraflagellar Ca^{2+} to the final concentration of above 10^{-6} M in both flagella.

The length of a phobic response and recovery of normal swimming

The end of the phobic response and reestablishment of steady-state conditions first require the arrest of the inward Ca^{2+} current by inactivation of the two Ca^{2+} channels. The fast F_F-current is inactivated several milliseconds after its activation, either spontaneously or by the drop of the membrane potential to less negative values (Harz et al., 1992; Holland et al., 1996). In contrast, the slow F_S-current is inactivated by Ca^{2+} from the intramembrane site (Holland et al., 1996). This latter type of feedback mechanism helps the cell to cope with a wide range of ionic conditions and to keep the duration of a phobic response constant.

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