

# On the Localization of Voltage-sensitive Calcium Channels in the Flagella of *Chlamydomonas reinhardtii*

Christina Beck and Rainer Uhl

Max-Planck-Institut für Biochemie, D-82152 Martinsried, Federal Republic of Germany

**Abstract.** This study was undertaken to prove that voltage-sensitive calcium channels controlling the photophobic stop response of the unicellular green alga *Chlamydomonas reinhardtii* are exclusively found in the flagellar region of the cell and to answer the question as to their exact localization within the flagellar membrane. The strategy used was to amputate flagella to a variable degree without perturbing the electrical properties of the cell and measure flagellar currents shortly after amputation and during the subsequent regeneration process. Under all conditions, a close correlation was found between current size and flagellar length, strongly suggesting that the channels that

mediate increases in intraflagellar calcium concentration are confined to and distributed over the total flagellar length. Bald mutants yielded tiny flagellar currents, in agreement with the existence of residual flagellar stubs. In the presence of the protein synthesis inhibitor cycloheximide, flagellar length and flagellar currents also recovered in parallel. Recovery came to an earlier end, however, leveling off at a time when in the absence of cycloheximide only half maximal values were achieved. This suggests the existence of a pool of precursors, which permits the maintenance of a constant ratio between voltage-sensitive calcium channels and other intraflagellar proteins.

**C**HLAMYDOMONAS *reinhardtii*, a unicellular green freshwater alga, possesses a photosensory system and two flagella, which enable it to translate information about intensity and direction of the ambient light into defined orientational responses (Bosco and Feinleib, 1979). During normal forward swimming the two flagella move in a breast stroke-like fashion 50 times per second. An apparent asymmetry in the beating pattern leads to a rotation around the longitudinal axis with a frequency of 2 Hz. Thereby, one revolution takes between 20 and 25 strokes, i.e., 500 ms. This allows the alga to screen the ambient light and to correct for changes in its direction (Foster and Smyth, 1980; Ruffer and Nultsch, 1985, 1987, 1990). Depending on the brightness of the actinic light, three classes of behavioral responses can be distinguished: (a) in weak light, *Chlamydomonas* swims towards the light (positive phototaxis); (b) in stronger light, it swims away from it (negative phototaxis); and (c) in very strong light, particularly when there are sudden and pronounced changes in intensity, it stops transiently. In the course of this "stop response," *Chlamydomonas* changes from its normal forward swimming to a phase of slower backward swimming, caused by an undulation movement of the flagella. Afterwards it resumes forward swimming in a new direction. Stop responses start within 50 ms of the stimulus and last for about 300 ms (Uhl and Hegemann, 1990).

The authors' present address is Botanisches Institut der Ludwig Maximilians Universität, Menzinger Strasse 67, D-80638 München, Germany.

There is good evidence that the different flagellar beating patterns are all governed by the internal calcium concentration (Schmidt and Eckert, 1976). This has been concluded from *in vitro* experiments with isolated flagella (Hyams and Borisy, 1978) and the flagella of detergent-extracted cell models (Bessen et al., 1980; Kamiya and Witman, 1984), where it was found that all naturally occurring beating patterns can be mimicked simply by changing the free calcium concentration in the medium. *In vivo* changes in [Ca] can arise from calcium release from internal stores or from a change in the balance between calcium influx through channels in the cell membrane and calcium expulsion through calcium pumps or exchange carriers. While evidence for the former is still lacking, it has been established that rapid light-induced changes in internal calcium can be brought about by two classes of ion channels: one is located in the eyespot region of the cell, where the photoreceptor molecules reside, and the other is confined to the flagellar region. According to the electrical model of Harz and Hegemann (1991) the immediate action of light is to open the channels in the eyespot region, leading to an inward current with concomitant cell depolarization. Current amplitude is graded with photon exposure and when its size exceeds a critical level opens the presumably voltage-sensitive channels in the flagellar region. Under physiological conditions both currents are carried by calcium ions. The threshold for the opening of flagellar channels coincides with the threshold for the stop response, suggesting that the undulation movement of the flagella is caused by a massive local calcium influx (Harz et al. 1992).

So far an exact localization of the voltage-sensitive calcium channels in the flagellar region has not been possible. On the basis of freeze-etch electron microscopic studies it has been suggested that the calcium conductances may reside in the ciliary necklace, with the ciliary necklace particles being the calcium channels (Gilula and Satir, 1972; Fisher et al., 1976). This communication, however, which uses an electrophysiological assay and a gentle procedure for producing healthy cells with flagella that have been amputated to various degrees, comes to a different conclusion. Under all conditions tested the size of the flagellar currents was correlated with the flagellar length, indicating that the voltage-sensitive calcium channels are equally distributed over the total length of both flagella.

## Materials and Methods

### Culturing Vegetative and Gametic Cells

*Chlamydomonas reinhardtii* cells of the cell wall-deficient mutant CW-2 were grown for 10–14 d on high salt acetate medium plates (HSM according to Sueoka et al., 1967, supplemented with 15 mM sodium acetate, 250 mM sorbitol as osmoticum, 0.3% yeast-extract, and 0.9% agar IMA, Nürnberg, Germany) in the presence of continuous white light of 2 Wm<sup>-2</sup>. While vegetative cells and gametes yield photocurrents of similar shape and size, the photosensitivity is higher in the case of gametes (Hegemann, P., personal communication). Therefore, cells were allowed to differentiate into gametes by transferring them from one dish into 10–12 ml nitrogen-deficient minimal medium (NMM) (Hegemann et al., 1988), containing 125 mM sorbitol. After 3 h the cells were pelleted and resuspended into 10–12 ml of NMM without sorbitol. By this time the cells had stabilized to such an extent that only a minor fraction was lysed due to the osmotic shock. Differentiation into flagellated cells was completed after 24 h. Tests for mating competence were not carried out, however. The phototactically most active cells were separated by photoselection in a low salt (freshwater-like) medium containing 3 mM K<sub>2</sub>HPO<sub>4</sub>, 100 μM CaCl<sub>2</sub>, adjusted to pH 6.8 with HCl. They were subsequently resuspended in the same medium and dark adapted for 1 h before an experiment. All incubations were carried out in 50-ml polypropylen tubes at a concentration of 1.5 × 10<sup>8</sup> cells ml<sup>-1</sup>. To allow for optimal aeration, cell suspensions were shaken at 190 rpm on an orbit shaker (Bachofen GmbH, Reutlingen, Germany).

### Mechanical Removal of the Flagella

Immediately before an experiment, cells were pelleted and resuspended into 5 ml "quasi-intracellular" medium (3.5 × 10<sup>8</sup> cells ml<sup>-1</sup>), mimicking the proposed intracellular milieu (Nichols and Rikmenspoel, 1978). It contained 10 mM K<sup>+</sup>-ATP, 3 mM MgCl<sub>2</sub>, and 60 mM K<sup>+</sup>, adjusted to pH 6.8 by mixing suitable amounts of 30 mM K<sub>2</sub>HPO<sub>4</sub> and 60 mM KH<sub>2</sub>PO<sub>4</sub>. A free calcium concentration of 100 nM was achieved by titrating 200 μM BAPTA (1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid) with CaCl<sub>2</sub> according to Tsien (1980). Flagella were removed by shearing forces in a 10 ml potter homogenizer. The amputated cells were immediately diluted 10-fold, using a medium which brought the concentration of the various salts back to freshwater conditions. Owing to the experimental procedure the medium contains 100 μM Ca<sup>2+</sup>, 8.5 mM K<sup>+</sup>, 1.5 mM Cl<sup>-</sup>, 1 mM ATP, 300 μM Mg<sup>2+</sup>, and 20 μM BAPTA. The presence of Mg<sup>2+</sup> and ATP has no measurable effect on the electrical currents. To keep the cells dark adapted the whole procedure was performed under red light and at room temperature. Photoreceptor currents with maximal amplitude could be recorded a few minutes following this treatment.

Flagellar regeneration kinetics of a CW-2 population were measured under the same conditions, except that they were not exposed to repetitive actinic flashes. Aliquots were taken at given time intervals and the cells were fixed by the addition of 0.37% formaldehyde (final concentration). Flagellar length was determined using a 100× oil immersion objective (NA = 1.3) and an ocular micrometer. Values from 10–15 cells were averaged for each data point. t = 0 marks the beginning of the amputation. In the case of the long-zero experiments the kinetics of flagellar regeneration were measured from a single cell by frame to frame analysis of a video film.

## Electrical Recording from Deflagellated Cells

Photocurrents were recorded using the suction pipette technique (Litvin et al., 1978; Baylor et al., 1979), as previously applied to *Chlamydomonas* by Harz and Hegemann (1991). In order to suck a substantial fraction of the cell into the pipette in a most gentle manner, pipettes with nearly parallel tip walls were used. They were pulled in two steps from borosilicat capillaries (OD 1.6 mm, wall thickness 0.5 mm, Hilgenberg, Malsfeld, Germany). Since the openings were too small after pulling, the tips were broken with the help of a small, heated glass sphere, and subsequently polished such that an opening of 2–4 μm resulted. Pipette resistances of 60–80 MΩ were obtained. Cells were sucked into the pipette under microscopic control, using infrared illumination from an IR-LED (HLP-40; Hitachi Instrs., Inc., San Jose, CA), emitting 40 mW of radiation at 780 nm), a 40× Plan-Neofluoar objective (Zeiss, Oberkochen, Germany) and an IR-sensitive CCD camera (i2S; Bordeaux-Cedex, France). Suction was applied until about one third of the cell was in the pipette and the resistance had increased to 100–150 MΩ. This was accomplished with the help of a low pressure application after Baylor et al. (1979).

Photostimulation was achieved through the epifluorescence attachment of the inverted microscope, using the objective as condenser system. A xenon-flashlamp (EG&G Electro-Optics, Salem, MA) produced flashes of 40 μs duration, which were filtered through a broadband interference filter transmitting maximally at 500 nm, the absorption maximum of *Chlamydomonas* rhodopsin (Foster et al., 1984; Uhl and Hegemann, 1990; Beckmann and Hegemann, 1991; Harz and Hegemann, 1991).

Electrical signals were recorded with a holding potential of 0 mV, using an EPC-7 patch clamp amplifier (List, Darmstadt, Germany). Signals were passed through a 3-kHz low-pass Bessel filter and fed into the A/D-converter of a TL-1 DMA computer interface, used in a 486 IBM-compatible computer. Data acquisition and analysis was performed under pClamp 5.5.1 (Axon Instruments, Foster City, CA). Single current traces were filtered with a digital gaussian filter to 500 Hz.

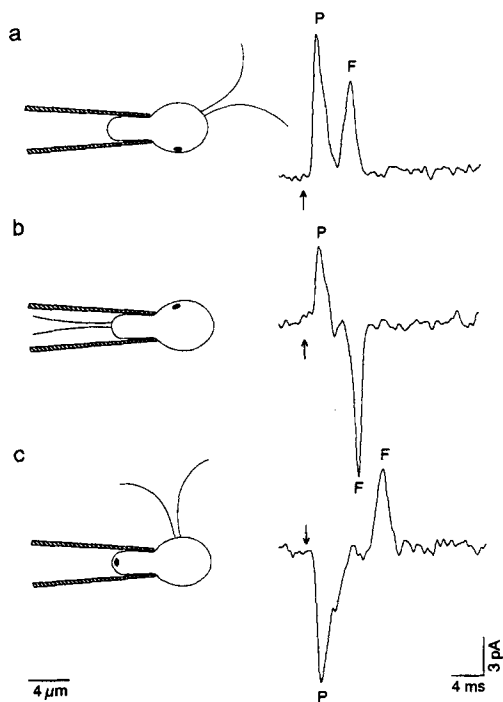
### Autolysin Treatment of Bald Mutants

Bald mutants were cultured in the same way as the CW-2, except that their HSM plates contained no sorbitol. Recording electrical currents from these cells, which possess a cell wall, requires its removal. It was achieved using cell wall-degrading enzyme released by mating gametes (Matsuda et al., 1985) (Jaenicke et al., 1987), kindly provided by Dr. Waffenschmidt (University of Cologne, Germany). Samples of lyophilized enzyme were dissolved in distilled water, bringing the concentration of Ca<sup>2+</sup> and Mg<sup>2+</sup> to 100 μM and 1 mM, respectively, and mixed with 2 vol of the cell suspension. Cycloheximide at 5 μg/ml (final concentration) was supplemented to inhibit the synthesis of new cell wall proteins. After incubation for 1 h at 18°C, the mixture contained cells that had shed main components of their cell wall (Imam and Snell, 1988; Monk, 1988; Waffenschmidt et al., 1988), as judged from the fact that they could be sucked into the pipette without complications, and yielded seal resistances comparable to those obtained with the CW-2 mutant.

## Results and Discussion

### Experimental Strategy

There are two fast, light-dependent calcium currents in *Chlamydomonas*: a primary calcium inward current in the eyespot region, termed photoreceptor current (*P*), and a secondary calcium inward current in the flagellar region, termed flagellar current (*F*). (*P*) follows the stimulus with virtually no delay, while *F* follows *P* with a variable delay of 5–100 ms (data not shown). Both currents are transient. They can be recorded as negative currents when the respective source regions are inside the pipette and as positive currents when the source regions are outside, a configuration which is much more frequently encountered (Fig. 1 a). Mixed constellations, i.e., flagella inside and eyespot outside the pipette (Fig. 1 b) or eyespot inside and flagella outside the pipette (Fig. 1 c), yield current traces with a positive and a negative component. The fact that the time course of both



**Figure 1.** Light-induced photoreceptor (*P*) and flagellar (*F*) currents recorded from single cells of *Chlamydomonas* under different recording configurations (arrows, flash of  $\tau/2 = 40 \mu\text{s}$ ,  $500 \text{ nm}$ ,  $1.5 \times 10^{20} \text{ photons m}^{-2}$ ). (a) Eyespot and flagella outside the pipette. (b) Eyespot outside and flagella inside the pipette. (c) Eyespot inside and flagella outside the pipette.

*P* and *F* is independent of the recording configuration has suggested that the positive currents are capacitative displacement currents, reflecting the release of positive charge from the membrane capacitance as a consequence of the light-induced cell depolarization. Photoreceptor current amplitudes are graded with photon exposure, saturating around  $10^{20} \text{ photons m}^{-2}$ . Flagellar currents, on the other hand, are all-or-none responses which remain unchanged in shape and amplitude, provided the total charge influx through the photoreceptor channels has exceeded a threshold value. Their latency depends on photon exposure and hence the amplitude of the preceding photoreceptor current. It shortens with increasing photon exposure (Harz et al., 1992). Both currents can be maintained for extended periods of time (1–2 h) under conditions where non-deflagellated cells are sucked into the pipette and repetitively light-stimulated. Thereby this electrical assay allows us to localize the voltage-sensitive calcium channels in the flagellar region. Attempts were made as follows to correlate flagellar length with flagellar current size, using cells which had lost their flagella to a variable degree.

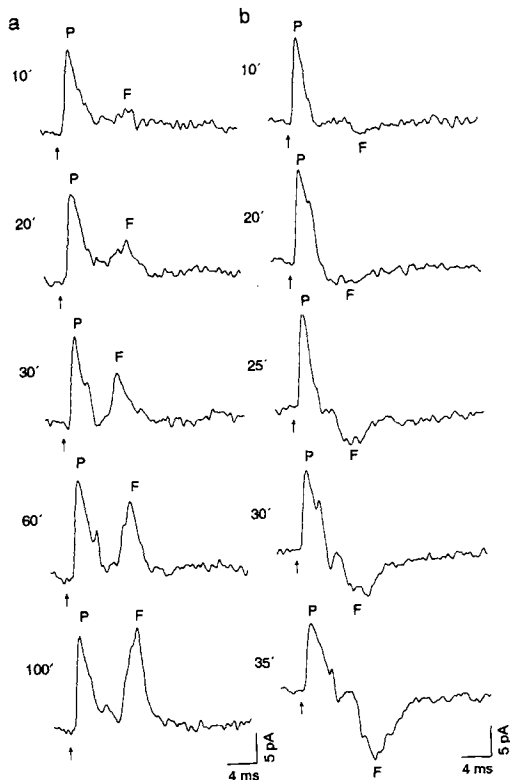
Of the numerous ways to remove *Chlamydomonas* flagella, only mechanical amputation was found a feasible alternative. pH-shock treatment causes the cells to throw off their flagella only at a predetermined breaking point. Moreover, in the case of our experiments it led to a long lasting perturbation of the cell system, resulting in a suppression of both flagellar and photoreceptor current (data not shown). In the time it took to recover maximal photoreceptor currents (40–60 min), the flagella had regained more than 50% of their origi-

nal length precluding a comparison of the regeneration kinetics for morphological and electrical events. As a result of the rapid decrease in pH, calcium ions enter the cell and lead to an internal calcium shock (Huber et al., 1986). This shock has been shown to be responsible for the flagellar autotomy (Sanders and Salisbury, 1989) and is presumably also responsible for the perturbed electrophysiology we observed. For the same reason dibucaine treatment (Thompson et al., 1974) could not be used. Mechanical amputation of the flagella had no such detrimental effects on the photoreceptor current, provided the amputation was carried out in a low-calcium, quasi-intracellular medium. Photoreceptor currents of normal appearance could be recorded as soon as the cells were sucked into the pipette, i.e., within 7–10 min. An additional advantage of the mechanical treatment was that the degree of amputation could be controlled by a variation of the shearing forces.

In correlating flagellar length with channel distribution in the flagella, only a “positive result,” showing unchanged current amplitudes for *P* and *F* after complete deflagellation, would have been easy to interpret. It would have implied that channels were located in the basal region. The “negative result” we found, i.e., the loss of measurable channel activity after deflagellation, is less unequivocal, since changes in the amplitude of the flagellar current do not necessarily reflect changes in the number of corresponding ion channels. For instance, since the current size depends on the membrane area sucked into the pipette and hence the seal resistance, it has to be ensured that this parameter is not changed during the experiment. We tested this by a short 1-mV prepulse immediately before each photostimulation and found essentially unchanged seal resistances in all experiments taken for this study. The driving force, i.e., the electrochemical potential across the membrane, could also account for changes in flagellar current amplitude while the number of flagellar channels remains constant. However, since both photoreceptor current and flagellar current are carried by calcium ions, they share a common driving force. The observation that the amplitude of *P* at saturating light exposure showed only short time fluctuations (less than 20%) around a given mean value, and that the latency of *F*, which depends on the total amount of charge entering the cell during *P*, was not altered, indicates that no systematical changes in driving force had occurred in the course of our experiments.

#### **Correlation between Regeneration of Flagella and Recovery of the Flagellar Current**

No flagellar currents were recorded immediately after removal of flagella, i.e., within 7 min of amputation. Occasionally observed tiny signals with variable delay were likely to reflect individual channel opening events. From 10–120 min there was a gradual increase in current amplitude (Fig. 2), closely matching flagellar regeneration which occurred in parallel for both flagella. Fig. 3 shows this correlation by comparing the increase in the amplitude of flagellar currents from single cells with the average flagellar length of an identically treated cell population. Flagellar regeneration began without delay at an initial rate of  $0.13 \mu\text{m min}^{-1}$ . It gradually declined, accelerated again at  $\sim 60 \text{ min}$ , and subsequently leveled off as the flagella approached their original length. The overall rate of this process was similar to what



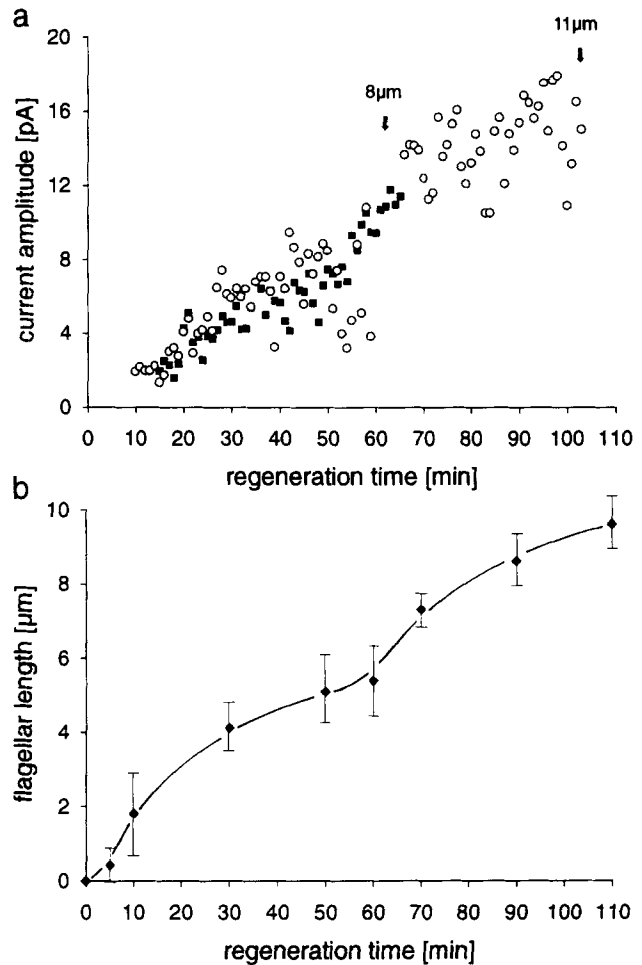
**Figure 2.** Recovery of the flagellar current after deflagellation. Single current traces are shown without averaging (arrows, see Fig. 1 legend). (a) "Eyespot- and flagella-out" configuration. (b) "Eyespot-out and flagella-in" configuration. Note that the recording sequence in *b*, which was measured in the presence of cycloheximide, is much shorter than in *a*, reflecting the shorter time the cell survived in the pipette.

others have found when investigating gametes, but significantly slower than observed for vegetative cells (Lefebvre et al., 1978). The transient increase in regeneration rate at ~60 min was observed in both measurements of flagellar length in a population and of current amplitudes from single cells. It suggests that de novo protein synthesis was delayed. We suppose that it is a genuine phenomenon reflecting transient changes in intracellular calcium levels during deflagellation which are known to affect the timing and extent of flagellar regrowth (Cheshire and Keller, 1991).

### Flagellar Currents from Partially Amputated Cells

The clear correlation between flagellar regeneration and recovery of flagellar current would seem to indicate that the channels are evenly distributed along the length of the flagella. An alternative explanation for the observed results could be, however, that stress-induced regulation processes suppress channel activity (Olesen et al., 1988; Morris and Sigurdson, 1989; Watson, 1990) and that this suppression vanishes in parallel with flagellar regrowth. The following experiment makes this assumption very unlikely.

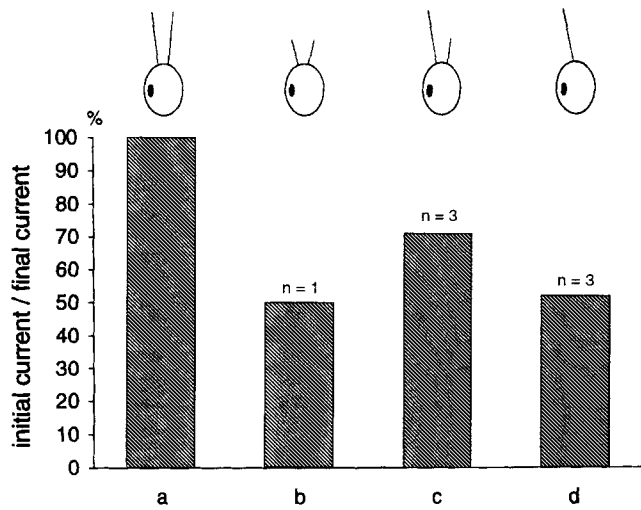
When cells were exposed to weaker shearing forces than previously used, flagella broke off at various positions (Fig. 4), leading to an inhomogeneous population in which some cells had retained both flagella (a), some had lost one half of each flagellum (b), some had lost only half a flagellum (c),



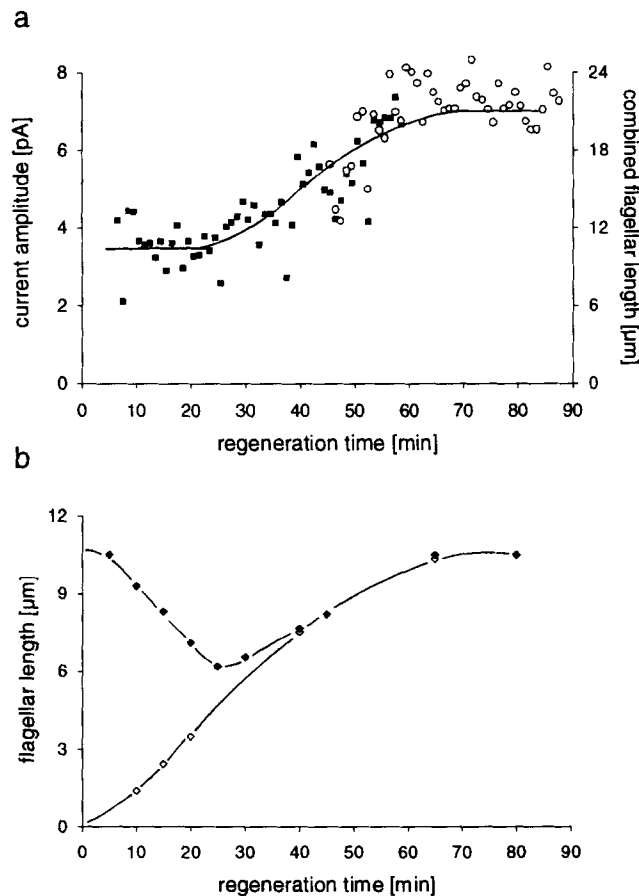
**Figure 3.** (a) Time course of the recovery of the flagellar current in single cells of *Chlamydomonas* after deflagellation. Flagellar current amplitudes were determined every minute. Data from two separate experiments of different duration are plotted together (■ = 65 min; ○ = 103 min). Arrows indicate the end of each experiment and the resulting flagellar length. (b) Time course of flagellar regeneration in a CW-2 population. Each data point represents the average taken from 10–15 cells. Error bars denote standard deviation.

some had lost one but kept the other (d), and some had lost both flagella (see Fig. 3 a). We recorded flagellar currents from the above cell types briefly after amputation treatment and followed their recovery until full regeneration of flagellar length was achieved. Again there was a strong correlation between flagellar length (i.e., the sum of the length of both flagella) and flagellar current amplitude. The ratio between initial current amplitude and final amplitude after complete flagellar regeneration is always the same as the ratio between initial and finally reached flagellar length.

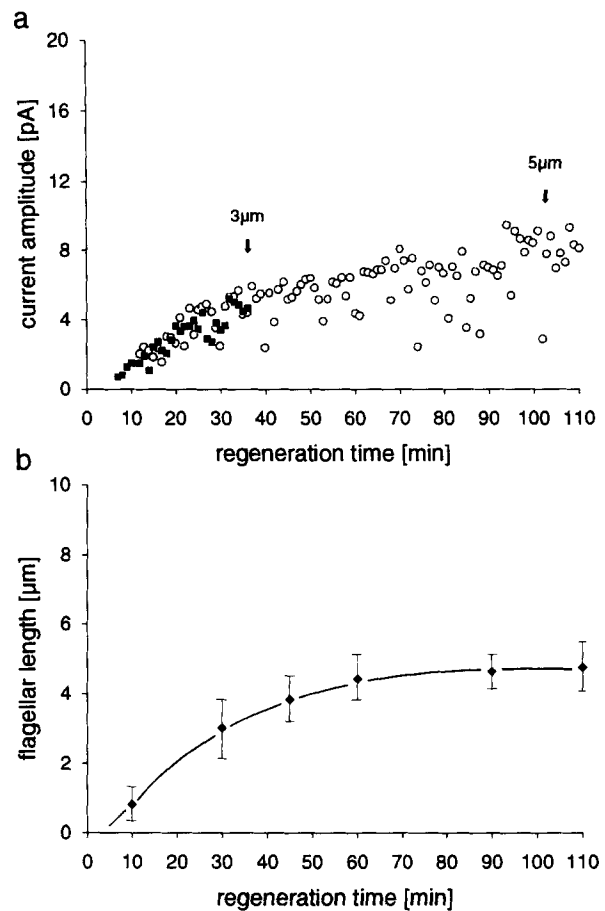
Under conditions where only one flagellum was removed (Fig. 4 d), a peculiar behavior was observed which had been first described by Rosenbaum et al. (1969). Initially the amputated flagellum grew at the expense of the intact one, leading to a concomitant shortening of the formerly long flagellum. When they had reached the same intermediate length, both flagella elongated in parallel to their original length. During the first 25 min the flagellar current remained con-



**Figure 4.** Relative flagellar current size after partial amputation. The plot shows the ratio between current amplitudes measured immediately after amputation and current amplitudes produced by the same cell after complete regeneration. Initial flagellar length is illustrated in the top.



**Figure 5.** (a) Time course of the recovery of the flagellar current after amputation of a single flagellum. The plot shows the average taken from two experiments (filled squares) which were finished after 60 min when both flagella had regained their original length and the data points from a third experiment (open circles) which was started when both flagella had reached half of their original length.

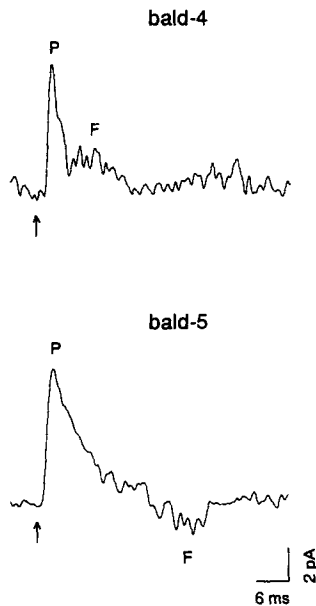


**Figure 6.** (a) Time course of the recovery of the flagellar current after deflagellation and during regeneration in the presence of cycloheximide ( $5 \mu\text{g/ml}$ ). Flagellar current amplitudes were determined every minute. Data from two separate experiments of different duration are plotted together ( $\blacksquare = 35 \text{ min}$ ;  $\circ = 110 \text{ min}$ ). Arrows indicate the end of each experiment and the resulting flagellar length. (b) Effect of cycloheximide ( $5 \mu\text{g/ml}$ ) on flagellar regeneration in a CW-2 population after the amputation of both flagella. Each data point represents the average taken from 10–15 cells. Error bars denote standard deviation.

stant, reflecting the fact that total flagellar length, i.e., the sum of the length of both flagella, was not altered. The increase in total flagellar length after 25 min was then accompanied by a parallel increase in flagellar current amplitude. It reached its maximum simultaneously with the length of both flagella at  $\sim 60 \text{ min}$  (Fig. 5).

When both flagella were removed in the presence of cycloheximide, an inhibitor of protein-synthesis, flagellar regeneration stopped when each flagellum had reached half of its normal length. Flagellar currents again recovered simultaneously with regeneration, stopping at the same time as flagel-

Combined flagellar length was calculated from Fig. 5 b. (b) Flagellar shortening and re-elongation after amputation of one of the two flagella in a single *Chlamydomonas* analyzed from a video film. Closed symbols represent the remaining, open symbols the newly growing flagellum.

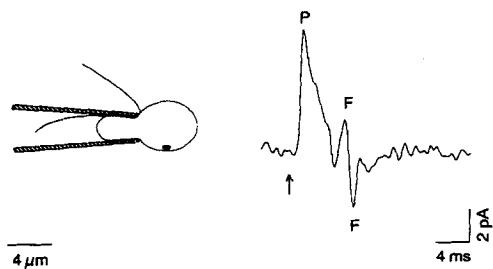


**Figure 7.** Light-induced photoreceptor (*P*) and flagellar (*F*) currents recorded from a bald-4 and a bald-5 mutant. While the eyespot was outside the pipette in both cases, the location of the flagella could not be determined visually. It was presumably outside in bald-4 and inside in bald-5. Arrow: flash of  $\tau/2 = 40 \mu\text{s}$ , 500 nm,  $1.2 \times 10^{21}$  photons  $\text{m}^{-2}$ .

lar regrowth (Fig. 6). Gametic cells produced under our culturing conditions contained high levels of flagellar protein reserves as determined by flagellar regeneration to an extent of 40–50%.

### Flagellar Currents from Bald-Mutants

Further proof for the localization of calcium channels in the flagellar membrane comes from experiments with bald-mutants. Bald-4 and bald-5 lack flagella as judged from their appearance under the light microscope. Basal bodies and transition sites, however, are intact. In these mutants, treated with autolysin in order to facilitate electrical recordings with the suction pipette, photoreceptor currents of regular size but only very small flagellar currents were observed (Fig. 7). They resembled flagellar currents from deflagellated cells 10 min after amputation, when flagellar growth had produced stubs of 1–2  $\mu\text{m}$ . This seems to indicate the existence of small flagellar stubs in bald-mutants, a notion which is further corroborated by electron micrographs showing that all bald-mutants, with the exception of bald-2, actually possess small flagellar stubs of 1–2- $\mu\text{m}$  length (Goodenough, U., personal communication). Possible artifacts, i.e., diminution of flagellar current amplitudes due to the presence of autolysin, were ruled out in control experiments with CW-2,



**Figure 8.** Light-induced (arrows, see Fig. 7 legend) photoreceptor (*P*) and flagellar (*F*) current from a *Chlamydomonas* cell which had one flagellum inside and the other outside the pipette.

where flagellar currents of normal appearance could be recorded.

### Flagellar Currents of Split Polarity

The conclusion of this communication—the voltage-sensitive calcium channels are evenly distributed over the total length of both flagella—is best exemplified in Fig. 8, where a current trace is shown from a very rare electrical configuration: with one flagellum inside and one flagellum outside the pipette the flagellar current splits into two components of equal size but opposite polarity. Both are all-or-none responses with their latency varying inversely with photon exposure. Therefore, they are typical flagellar currents. The fact that the two current components do not occur simultaneously, in which case they would cancel, could be a consequence of the asymmetric measuring configuration. It could also be a real effect, however, exemplifying the evolutionary and functional difference between the two flagella (Kamiya and Hasegawa, 1987).

### Concluding Remarks

So far an unequivocal localization of voltage-sensitive calcium channels in ciliary membranes has only been possible for Ctenophores. In *Pleurobrachia* they are distributed over most of the length for the comb plate cilia (Moss and Tamm, 1987), while in the macrocilia of *Beroë* they reside exclusively in the basal portion of the ciliary membrane (Tamm, 1988). In *Paramecium* calcium channels are also exclusively in the ciliary membrane (Dunlap, 1977; Machemer and Ogura, 1979), their exact localization, however, remains to be determined. Our findings are therefore the first demonstration of an even channel distribution in flagella of sub-micrometer dimensions. The physiological relevance of this channel distribution appears to be clear. Due to the tiny cross section of the unobstructed intraflagellar aqueous space and due to the presumed presence of cytoplasmic calcium buffers, which would prevent rapid spatial spread of calcium transients, the short delay between light stimulus and the onset of the stop response ( $\sim 20$  ms) (Schmidt and Eckert, 1976), which involves an undulation movement of the whole flagellum, could not be accounted for by calcium diffusion from the basal region to the tip of the flagella. In a system of comparable dimensions, the saccular hair cells, it has been demonstrated that a mobile cytoplasmic calcium buffer present in millimolar concentrations can intercept a significant fraction of calcium ions en route to their targets within a few microseconds (Roberts, 1993).

The observed distribution of calcium channels in the flagella could have another physiological implication: it has been suggested that the regulation of flagellar growth depends on intracellular calcium levels (Johnson and Rosenbaum, 1993). A number of calcium channels evenly distributed in and strictly proportional to the length of the flagella could be involved in the signaling network of the cell controlling flagellar length. However, since growth regulation occurs both in the light and in the dark, one needs to postulate that spontaneous as opposed to light-induced channel opening events play a role in this process. Such spontaneous events have actually been observed, and experiments aimed at testing their significance for flagellar length control are currently under way in our laboratory.

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## References

- Baylor, D. A., T. D. Lamb, and K. -W. Yau. 1979. The membrane current of single rod outer segments. *J. Physiol. (Lond.)* 288:589-611.
- Beckmann, M., and P. Hegemann. 1991. *In vitro* identification of rhodopsin in the green alga *Chlamydomonas*. *Biochemistry* 30:3692-3697.
- Bessen, M., R. B. Fay, and G. B. Witman. 1980. Calcium control of waveform in isolated flagellar axonemes of *Chlamydomonas*. *J. Cell Biol.* 86:446-455.
- Boskov, J. S., and M. E. Feinleib. 1979. Phototactic response of *Chlamydomonas* to flashes of light II. response of individual cells. *Photochem. Photobiol.* 30:499-505.
- Cheshire, J. L., and L. R. Keller. 1991. Uncoupling of *Chlamydomonas* flagellar gene expression and outgrowth from flagellar excision by manipulation of  $Ca^{2+}$ . *J. Cell Biol.* 115:1651-1659.
- Dunlap, K. 1977. Localization of calcium channels in *Paramecium caudatum*. *J. Physiol. (Lond.)* 271:119-133.
- Fisher, G., E. S. Kaneshiro, and P. D. Peters. 1976. Divalent cation affinity sites in *Paramecium aurelia*. *J. Cell Biol.* 69:429-442.
- Foster, K. W., and R. D. Smyth. 1980. Light antennas in phototactic algae. *Microbiol. Rev.* 44:572-636.
- Foster, K. W., J. Saranak, N. Patel, G. Zarilli, M. Okabe, T. Kline, and K. Nakanishi. 1984. A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eucaryote *Chlamydomonas*. *Nature (Lond.)* 311:756-759.
- Gilula, N. B., and P. Satir. 1972. The ciliary necklace. *J. Cell Biol.* 53:494-509.
- Harz, H., and P. Hegemann. 1991. Rhodopsin-regulated calcium currents in *Chlamydomonas*. *Nature (Lond.)* 351:489-491.
- Harz, H., C. Nonnengässer, and P. Hegemann. 1992. The photoreceptor current of the green alga *Chlamydomonas*. *Phil. Trans. R. Soc. Lond. B.* 338:39-52.
- Hegemann, P., U. Hegemann, and K. W. Foster. 1988. Reversible bleaching of *Chlamydomonas reinhardtii* rhodopsin *in vivo*. *Photochem. Photobiol.* 48:123-128.
- Huber, M. E., W. G. Wright, and R. A. Lewin. 1986. Divalent cations and flagellar autotomy in *Chlamydomonas reinhardtii*. *Phycologia* 25:408-411.
- Hyams, J. S., and G. G. Borisy. 1978. Isolated flagellar apparatus of *Chlamydomonas*: characterization of forward swimming and alteration of waveform and reversal of motion by calcium ions *in vitro*. *J. Cell Sci.* 33:235-253.
- Imam, S. H., and W. J. Snell. 1988. The *Chlamydomonas* cell wall degrading enzyme, lysin, acts on two substrates within the framework of the wall. *J. Cell Biol.* 106:2211-2221.
- Jaenicke, L., W. Kuhne, R. Spessert, U. Wahle, and S. Waffenschmidt. 1987. Cell-wall lytic enzymes (autolysins) of *Chlamydomonas reinhardtii* are (hydroxy) proline-specific proteases. *Eur. J. Biochem.* 170:485-491.
- Johnson, K. A., and J. L. Rosenbaum. 1993. Flagellar regeneration in *Chlamydomonas*: a model system for studying organelle assembly. *Trends Cell Biol.* 3:156-161.
- Kamiya, R., and G. B. Witman. 1984. Submicromolar levels of calcium control the balance of beating between the two flagella in demembrated models of *Chlamydomonas*. *J. Cell Biol.* 98:97-107.
- Kamiya, R., and E. Hasegawa. 1987. Intrinsic difference in beat frequency between the two flagella of *Chlamydomonas reinhardtii*. *Exp. Cell Res.* 173:299-304.
- Lefebvre, P. A., S. A. Nordstrom, J. E. Moulder, and J. L. Rosenbaum. 1978. Flagellar elongation and shortening in *Chlamydomonas*. *J. Cell Biol.* 78:8-27.
- Litvin, F. F., O. A. Sineshchekov, and V. A. Sineshchekov. 1978. Photoreceptor electric potential in the phototaxis of the alga *Haematococcus pluvialis*. *Nature (Lond.)* 271:476-478.
- Machemer, H., and A. Ogura. 1979. Ionic conductances of membranes in ciliated and deciliated *Paramecium*. *J. of Physiol. (Lond.)* 296:49-60.
- Matsuda, Y., T. Saito, T. Yamaguchi, and H. Kawase. 1985. Cell wall lytic enzyme released by mating gametes of *Chlamydomonas reinhardtii* is a metalloprotease and digests the sodium perchlorate-insoluble component of the cell wall. *J. Biol. Chem.* 260:6373-6377.
- Monk, B. C. 1988. The cell wall of *Chlamydomonas reinhardtii* gametes: composition, structure and autolysin-mediated shedding and dissolution. *Planta (Heidelb.)* 176:441-450.
- Morris, C. E., and W. J. Sigurdson. 1989. Stretch-inactivated ion channels coexist with stretch-activated ion channels. *Science (Wash. DC)* 243:807-809.
- Moss, A. G., and S. L. Tamm. 1987. A calcium regenerative potential controlling ciliary reversal is propagated along the length of ctenophore comb plates. *Proc. Natl. Acad. Sci. USA.* 84:6476-6480.
- Nichols, K. M., and R. Rikmenspoel. 1978. Control of flagellar motion in *Chlamydomonas* and *Euglena* by mechanical microinjection of  $Mg^{2+}$  and  $Ca^{2+}$  and by electric current injection. *J. Cell Sci.* 29:233-247.
- Olesen, S. P., D. E. Clapham, and P. F. Davies. 1988. Haemodynamic shear stress activates a  $K^{+}$  current in vascular endothelial cells. *Nature (Lond.)* 331:168-170.
- Roberts, W. M. 1993. Spatial calcium buffering in saccular hair cells. *Nature (Lond.)* 363:74-76.
- Rosenbaum, J. L., J. E. Moulder, and D. L. Ringo. 1969. Flagellar elongation and shortening in *Chlamydomonas*. *J. Cell Biol.* 41:600-619.
- Rüffer, U., and W. Nultsch. 1985. High-speed cinematographic analysis of the movement of *Chlamydomonas*. *Cell Motil. Cytoskeleton.* 5:251-263.
- Rüffer, U., and W. Nultsch. 1987. Comparison of the beating of cis- and trans-flagella of *Chlamydomonas* cells held on micropipettes. *Cell Motil. Cytoskeleton.* 7:87-93.
- Rüffer, U., and W. Nultsch. 1990. Flagellar photoreponses of *Chlamydomonas* cells held on micropipettes: I. change in flagellar beat frequency. *Cell Motil. Cytoskeleton.* 15:162-167.
- Sanders, M. A., and J. L. Salisbury. 1989. Centrin-mediated microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 108:1751-1760.
- Schmidt, J. A., and R. Eckert. 1976. Calcium couples flagellar reversal to photostimulation in *Chlamydomonas reinhardtii*. *Nature (Lond.)* 262:713-715.
- Sueoka, N., K. S. Chiang, and J. R. Kates. 1987. Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardtii*. *J. Mol. Biol.* 25:47-66.
- Tamm, S. L. 1988. Ionophoretic localization of Ca-sensitive sites controlling activation of ciliary beating in macrocilia of *Beroë*: the ciliary rete. *Cell Motil. Cytoskeleton.* 11:126-138.
- Thompson, G. A., L. C. Baugh, and L. F. Walker. 1974. Nonlethal deciliation of *Tetrahymena* by a local anesthetic and its utility as a tool for studying cilia regeneration. *J. Cell Biol.* 61:253-257.
- Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19:2396-2404.
- Uhl, R., and P. Hegemann. 1990. Probing visual transduction in a plant cell. Optical recording of rhodopsin-induced structural changes from *Chlamydomonas reinhardtii*. *Biophys. J.* 58:1295-1302.
- Waffenschmidt, S., R. Spessert, and L. Jaenicke. 1988. Oligosaccharide side chains of wall molecules are essential for cell-wall lysis in *Chlamydomonas reinhardtii*. *Planta (Heidelb.)* 175:513-519.
- Watson, P. A. 1990. Direct stimulation of adenylate cyclase by mechanical forces in S49 mouse lymphoma cells during hyposmotic swelling. *J. Biol. Chem.* 265:6569-6575.