ELSEVIER

Biochimica et Biophysica Acta 1466 (2000) 187–204

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

A Ca^{2+}- and voltage-modulated flagellar ion channel is a component of the mechanoshock response in the unicellular green alga *Spermatozopsis similis*

Kerstin Hill [a], Roland Hemmler [a], Peter Kovermann [a], Michael Calenberg [b], Georg Kreimer [b,*], Richard Wagner [a]

[a] *Universität Osnabrück, Fachbereich Biologie/Chemie, Barbarastr. 11, D-49069 Osnabrück, Germany*
[b] *Universität zu Köln, Botanisches Institut, Lehrstuhl I, Gyrhofstr. 15, D-50931 Köln, Germany*

Received 26 August 1999; received in revised form 9 February 2000; accepted 15 March 2000

Abstract

In flagellate green algae, behavioral responses to photo- and mechanoshock are induced by different external stimuli within 10–15 ms. In the accompanying changes in flagella beat, Ca^{2+} has important regulatory roles. Although the axonemal Ca^{2+} responsive elements are well characterized, analyses of flagellar channels involved in Ca^{2+} signalling as well as other ion channels at the single-channel level were not yet conducted in green algae. To gain a further understanding of these important signaling elements in movement responses, intact flagella of *Spermatozopsis similis* were isolated and characterized and the solubilized flagellar membrane proteins were reconstituted into liposomes. We observed three types of channel activity, two of which were weakly anion and cation-selective and in the high-conductance regime typical for porin-like solute channels. The dominating channel activity was a voltage dependent, rectifying, low conductance ($\Lambda = 80$ pS in 50 mM KCl) cation-selective channel modulated by, and highly permeable to, Ca^{2+} ions (SFC1: *Spermatozopsis* flagellar cation channel 1). Depolarizations necessary to activate SFC1 probably only occur in vivo during avoidance reactions of this alga. Ca^{2+}-activation of SFC1 points to a direct link to Ca^{2+}-mediated signaling pathway(s) in the flagella. Both the response to mechanoshock and SFC1 activity were inhibited by Gd^{3+} and Ba^{2+}, thus supporting our assumption that SFC1 represents a major flagellar ion channel involved in this green algal avoidance reaction.

Keywords: Flagellar calcium flux; Flagellar ion channel; Green alga; Shock response; Voltage-modulated channel; Ca^{2+}-modulated channel; *Spermatozopsis similis*

1. Introduction

Flagellate green algae respond to a number of different external stimuli in subtle well-defined ways. Directed responses (e.g. to light) are often peculiar and finally lead to accumulation of cells in areas best matching their individual needs. Depending on the quality and strength of the stimuli, also transient avoidance or shock reactions can be induced. In the case of photoresponses, the signal is processed within 15–50 ms and leads to differential responses of the same effector, i.e. the flagella (for review see [1,2]).

* Corresponding author. Fax: 49-221-470-5181; E-mail: georg.kreimer@uni-koeln.de

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00200-5

In unicellular green algae, such as *Chlamydomonas*, these responses involve peculiar changes in the beating pattern of the flagella. In *Chlamydomonas*, the flagella normally shows an asymmetrical breast stroke-like beat [3]. A temporary change to a symmetrical, undulating waveform, which causes the cell to move backwards, is induced upon stimulation by intense light or mechanical disturbances. *Chlamydomonas* responds to both stimuli by a decrease in its average swimming velocity. *Spermatozopsis* also responds to physiological light shock by decreasing its swimming velocity. However, during the mechanoshock response or non-physiological photoshock, the cells accelerate to velocities >600 μm s^{-1}. The shock responses occur in both algae within 10–50 ms and strictly depend on extracellular Ca^{2+} [4–7]. Analyses of isolated flagellar apparatuses of *Chlamydomonas* and *Spermatozopsis* revealed that the main Ca^{2+}-sensitive elements responsible for the change in flagellar beating are components of the axoneme [8–10]. In addition, the basal bodies of both algae exhibit centrin-mediated reorientation upon changes in the free Ca^{2+} concentration between 10^{-8} and 10^{-6} M. Whereas in *Chlamydomonas*, the reversible reorientation is small ($\approx 20°$), the basal bodies of *Spermatozopsis* are aligned parallel upon an increase in Ca^{2+} [10,11]. This probably facilitates the close alignment of the flagella, apparently a necessary prerequisite for the accelerations observed upon massive stimulation of the cells [6]. Additionally, Ca^{2+} is intricately involved in regulating adaptation to the prevailing light conditions as well as to the strength and sign of phototactic behavior [12]. Ca^{2+}-modulated protein phosphorylation/dephosphorylation and GTPases present in the eyespot region might be involved in these processes (e.g. [13,14]).

Recent electrophysiological analyses by the suction pipette technique, have shown that stimulation by both photoshock and negative pressures lead to inward currents of Ca^{2+} into the flagella. Bright flashes of light first induce photoreceptor currents in the plasma membrane region overlying the carotenoid-rich lipid globules of the eyespot, which are mainly carried by Ca^{2+} [15,16]. When these rhodopsin-activated currents exceed a threshold, two transient Ca^{2+} inward currents in the flagella are triggered in an all-or-nothing manner. The first is the fast flagellar current (F_F), which is followed by a small, slow current (F_S) [17]. Combined analyses of flagella movement and electrical responses on a single cell have demonstrated that a close link exists between these currents and behavioral responses [18]. Effective triggering of flagellar reversal by electrical stimulation is further evidence that these channels are voltage-gated [19]. Recent isolation of mutants in the generation of the all-or-nothing flagellar currents, suggests that the photoshock response is mediated by a multicomponent mechanism clearly distinct from that used for phototaxis [20].

Currents evoked by suction in *Chlamydomonas* differ clearly from photoshock induced F_F and F_S currents in that they are larger and that the generation of pressure induced current spikes is inhibited by Gd^{3+} and Ba^{2+} [7,21]. The F_F and F_S channels are most probably evenly distributed over the entire length of the flagella membrane [22], whereas the distribution of channels opened upon mechanical stimulation is not yet known. Mechanosensitive channels are reported for both flagella and cell body [7,21]. As consistent patch-clamp measurements on whole cells of flagellate green algae were not yet possible, information about these channels at the single-channel level are not yet available.

Changes in intraflagellar Ca^{2+} generally plays a key role in the regulation of flagellar motility, although the actual responses of the axonemes to the same messengers can be opposite in different organisms [1,23,24]. In *Chlamydomonas*, Ca^{2+} fluxes are also intricately involved in mating, excision, and regeneration of flagella [25–27]. Thus, signaling processes during photo- and mechanoshock of green algae are not the only reason for studying the properties of single Ca^{2+}-conducting channels in flagellar membranes. We therefore have incorporated membrane proteins of isolated and purified flagella of *Spermatozopsis* in liposomes and studied the reconstituted channel activities at the single-channel level by the patch-clamp and planar bilayer techniques. Here we characterize a Ca^{2+}-permeable cation channel and combine electrophysiological measurements with behavioral analyses of the avoidance response of *Spermatozopsis*. The results strongly support involvement of this channel in the peculiar avoidance response of this unicellular green alga.

2. Materials and methods

2.1. Organism and culture conditions

Spermatozopsis similis Preisig et Melkonian (strain no. B 1.85) was obtained from the Sammlung von Algenkulturen, Pflanzenphysiologisches Institut, Göttingen, Germany. For isolation of flagella, cultures were grown in a modified Waris solution in 10-l flasks as described [28]. Aliquots of these mass cultures or batch cultures grown in 100-ml Erlenmeyer flasks were used for photo- and mechanoshock assays.

2.2. Isolation of flagella

Usually 10–40 l of late log-phase cultures (cell density: $\sim 5–7 \times 10^6$ cell ml^{-1}) were harvested and concentrated to about 2 l using a Pellicon tangential flow filtration system (Millipore, filter HVLP, pore size 0.45 μm) at low flow rates. Further concentration was achieved through centrifugation (15 min, 610–745×*g*, rotor: GSA, Sorvall). The cells were resuspended in modified Waris solution (without soil extract and vitamins), pelleted as above and resuspended in HMDS solution (10 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 4% sucrose, pH 7.4). All following steps followed the method described by Witman [29] with the following exceptions: the final concentration of dibucaine was reduced to 1 mM, the cells were vigorously pipetted 10–20 times in a 10-ml polystyrene pipette to induce deflagellation, and the purified flagella for biochemical analyses were collected by high-speed centrifugation (45 min, 100 000×*g*). The final flagella pellet was resuspended in HMDS and processed as indicated below. Fixation and processing for whole mount and transmission electron microscopy were done as described [10].

2.3. Preparation of flagella liposomes

Small liposomes were obtained by dissolving 50 mg/ml of purified azolectin (Sigma, type IV S; [30]) in 10 mM MOPS/Tris pH 7 using the microtip of a Branson Sonifier (Danburg/Connecticut). Liposomes were freeze-thawed once. Flagella membranes were solubilized in 80 mM MEGA-9, 50 mM DTT, 20 mM Tricine–Tris (pH 8) for 30 min at room temperature with stirring. The solution was centrifuged (30 min, 14 000 rpm, Eppendorf 5402 centrifuge). The pellet was discharged and protein concentrations were estimated for the supernatant containing the solubilized membrane/matrix proteins by comparing Coomassie brilliant blue stained SDS–PAGE gels of the solubilisate to known standard concentrations of BSA. About 0.3 mg of protein/ml were mixed with preformed liposomes to final concentrations of 0.1 mg protein/10 mg azolectin and 0.1 μg protein/10 mg azolectin. The suspension was freeze-thawed and sonicated in a supersonic bath. After 0.5 h of incubation at room temperature, the suspension was dialyzed for 4 h at room temperature and then overnight against a buffer containing 10 mM MOPS/Tris pH 7 at 4°C and used for bilayer measurements.

2.4. Formation of giant proteoliposomes

Giant vesicles suitable for patch-clamp measurements were obtained by a modified dehydration–rehydration procedure [31]. After thawing, 5–7 ml of the proteoliposomes (about 10 μg protein/ml; 25 mg/ml lipid) were spread on a glass slide and dehydrated at 4°C for 45–60 min in a 500-ml desiccator over dry $CaCl_2$. Afterwards, 10 ml of the electrolyte solution to be used in the patch-clamp measurements were added to the partially dried sample on the slide. To avoid evaporation, the slide was transferred to a Petri dish, the bottom of which was covered with water-saturated paper. After 1 h, giant liposomes with incorporated flagella membranes were observed. These giant flagella membrane/liposome vesicles typically had diameters between 20 and 50 μm. Giant liposomes without fused flagella membranes, when used as controls, did not show any single-channel activity under the applied experimental conditions.

2.5. Electrophysiological measurements

2.5.1. Patch-clamp measurements

Giant flagella membrane/liposome vesicles were placed in a tissue bath mounted on an Olympus IMT-2 inverted microscope, and the vesicles were viewed using phase contrast optics. Single-channel current recordings using the patch-clamp technique

were performed as described [32]. Holding potentials are always referred to the pipette. GΩ-seals ranging from 5 to 50 GΩ could be achieved by slight suction once the pipette tip was brought into contact with the membrane. Sealing was apparently effected by the protein content of the vesicles (i.e. the lower the protein concentration, the higher the probability of GΩ-seal formation). The currents were amplified using a PATCH-CLAMP L/M-EPC 7 amplifier (List Medical). Current recordings were digitized at a 10 kHz sampling rate using the Axon Digidata 1200 system (Axon Instruments) and stored on a personal computer. For analyses, current recordings were filtered with an 8-pole bessel filter, typically at 1 kHz.

2.5.2. Planar lipid bilayers

Planar lipid bilayers were produced using the painting technique [33]. A solution of purified azolectin (Sigma type IV-S; 50 mg/ml) in *n*-decan (analytical grade, Merck) was applied to a hole (100–500 μm diameter) in a Teflon septum, separating the two bath chambers (total volume $\sim$3 ml). Both chambers were equipped with magnetic stirrers. Bilayer formation was monitored optically and by capacitance measurements. The resulting bilayers had a typical capacitance of $\approx$0.5 mF/cm^2 and a resistance of $>$100 GΩ. The noise was 1 pA (rms) at a 5-kHz bandwidth. After a stable bilayer was formed in symmetrical solutions of 20 mM KCl, 10 mM MOPS/Tris (pH 7.0), the experimental conditions were changed to asymmetric concentrations. Concentrated solutions of KCl and $CaCl_2$ were added to the *cis*-chamber up to final concentrations of 250 mM KCl and 10 mM $CaCl_2$. The proteoliposomes were added to the buffer solution in the *cis*-compartment through the tip of a microloader (Eppendorf) so that the liposomes slowly flowed directly across the bilayer. If necessary, the solution in the *cis*-chamber was stirred to promote fusion. After addition of the flagella liposomes (50–100 mg protein/ml) we usually observed between one and three active channels. The Ag/AgCl electrodes were connected to the chambers through 1 M KCl-agar bridges. The electrode of the *trans*-compartment was directly mounted to the headstage (HS-2A x 10MG) of a current amplifier (GeneClamp 500, Axon Instruments). Reported holding potentials are referred to the *trans* compartment. The amplified currents were typically digitized at 1–2 kHz sampling rate using the Axon Digidata 1200 system (Axon Instruments) and stored on a personal computer. For analyses, a Windows-based analysis software [34] was used in combination with Origin (Microcal Software).

2.6. Mechanoshock and photoshock assay

The photoshock assay takes advantage of the reorientation of the basal bodies in *Spermatozopsis* from an anti-parallel to a parallel configuration upon photo- and mechanoshock [6,10]. All assays were carried out in complete culture medium with samples from log-phase cultures (cell density $\approx 1\times10^5$ cell ml^{-1}). The photoshock assay was carried out as described in [10], except that the algae were equilibrated only for 1 min in the presence or absence of the indicated concentrations of Gd^{3+} and that fixation with Lugol's solution was done 10 s after the photoshock (700 μE m^{-2} s^{-1} of cool white light). For the mechanoshock analyses, 40 μl culture aliquots were incubated for 1–5 min in the presence of the indicated inhibitor concentrations. Mechanoshock was applied by vigorously pipetting the aliquot several times with a 20-μl Eppendorf pipette. After the last agitation, 20 μl of 100 mM EGTA was rapidly added, followed by an addition of 20 μl of Lugol's solution. Analyses of swimming paths and speed were conducted with a commercial movement analysis system (Medea) and the included software package.

3. Results

3.1. Flagella isolation and characterization

Spermatozopsis possess two apical inserted flagella of unequal length with a smooth flagellar surface ($\sim$15–20 and 8–16 μm; [35]). A necklace, present in many cilia and flagella, was revealed by deep-etching between the flagellar proper and the flagellar base/transition regions of the basal bodies. No further particle specializations of the flagellar membrane were evident (data not shown). As *Spermatozopsis* naturally lacks a cell wall, a reduced concentration

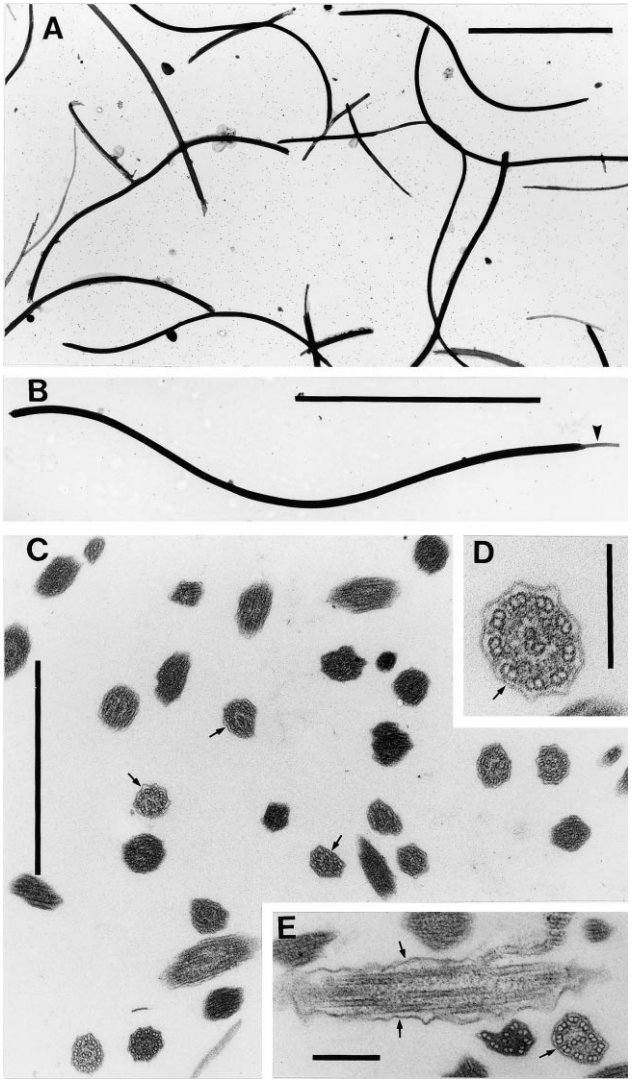


Fig. 1. Electron microscopy of isolated, purified flagella of *Spermatozopsis similis*. (A,B) Whole mount preparations giving an overview (A) and a detail (B) of the preparations. The arrowhead points to the hair-point. (C–E) Thin section analysis of the flagella fraction. (C) Overview; details show a cross section (D) and an oblique longitudinal section (E). Arrows point to the flagellar membrane. Scale bars: A and B, 5 μm; C, 1 μm; and D and E, 200 nm.

of dibucaine (1 mM) was used to avoid damage of the cell bodies and consequent contamination of the flagella fraction by cell debris. However, probably because *Spermatozopsis* neither retracts nor sheds its flagella during the cell cycle, a higher degree of mechanical stress was needed to shear off the flagella. Most of the cells (~90%) were deflagellated and the crescent cell shape was not affected. About 85% of the cells retained flagellar stumps of equal length. The point of breakage appeared to be in the transition region (data not shown).

Isolated flagella appeared largely intact when observed by phase microscopy. However, probably due to the length of the flagella, some broken flagella were always observed. Cell bodies were effectively removed by the subsequent purification procedure and only moderate fraying of the flagella membranes was detected. Electron microscopy shown in Fig. 1A–E confirmed these observations. Neither in whole mount nor in thin section analyses were contaminating cell debris observed. Structural preservation of the isolated flagella was good. Fig. 1B shows that in some cases even the conspicuous flagellar tip is preserved. Most of the flagella retained the flagellar membrane (Fig. 1C–E) and the flagellar matrix, as indicated by the dense staining of the matrix space (Fig. 1C). As described for *Chlamydomonas* [36], the flagella matrix of *Spermatozopsis* appears to be condensed due to membrane shrinkage during the isolation and purification procedure.

3.2. *Different classes of ion channels in liposomes containing reconstituted flagellar membrane proteins*

It was not possible to obtain high-resistance seals by direct patch-clamping of isolated flagella. We therefore reconstituted Mega-9 solubilized flagella membrane proteins using a dialysis technique. Electrophysiological experiments (patch-clamp and planar bilayers) were conducted to characterize ion channel activity in the reconstituted flagellar membranes. Patch-clamp measurements on giant flagella-liposomes yielded, in about 50% of the attempts, high-resistance seals (>5 GΩ), thus allowing for single-channel recordings from the excised patches. Based on the conductances in different symmetrical/asymmetrical buffer systems, three different types of channels were identified in the reconstituted flagellar membrane of *Spermatozopsis*: two large conductance channels, one with a weak cation selectivity and a conductance of $\Lambda = 450$ pS for the fully open state and one weak anion-selective channel with a conductance of $\Lambda = 830$ pS for the fully open channel, and a channel in the lower conductance regime ($\Lambda = 80$ pS), which was also cation-selective with a higher perme-

ability for Ca^{2+} than for K^+ ions. Here we describe the principal channel characteristics of these three different conductances observed in reconstituted flagella membranes, which were apparently identical in patch-clamp measurements after formation of giant liposomes and in planar bilayers after fusion of the proteoliposomes.

3.3. A weak cation-selective channel permeable to Ca^{2+}

The channel activity described below was observed in about 10% ($n > 100$) of the excised inside-out patches obtained from giant flagella-liposomes. Single-channel recordings in asymmetric Ca^{2+} buffer (50 mM/10 mM $CaCl_2$; bath/pipette) revealed burst-like openings and closures of the channel at positive and negative potentials (Fig. 2A). Direct transitions between the fully closed and different open states were frequently observed (Fig. 2A). At the given time resolution (5 kHz), these would be unlikely to occur with two different independent channels (for discussion see [37]). Thus, the course of the current transients indicates that the smaller amplitudes represent subconductant levels rather than the simultaneous activities of two or more channels within the patches. A detailed analysis revealed mainly three different open-channel amplitudes (see below). The current–voltage relationship of the channel was linear for the three main conductance states (Fig. 2B). In asymmetric Ca^{2+}-buffer, the following slope conductance values for subconductances were obtained (Fig. 2B): $\Lambda_1 = 132 \pm 9.9$ pS, $\Lambda_2 = 252 \pm 10.9$ pS and $\Lambda_3 = 449 \pm 9.1$ pS for the fully open channel. In this asymmetric Ca^{2+}-buffer, we obtained a reversal potential of $E_{rev} = -12$ mV ($E^{2+}_{Ca} = -20$ mV), while in asymmetric K^+-buffer (bath: 50 mM KCl, 5 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.2, pipette: 10 mM KCl, 5 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.2) the determined E_{rev} was -27 mV ($E^{+}_{K} = -41$ mV). In both buffer systems, we found, within error

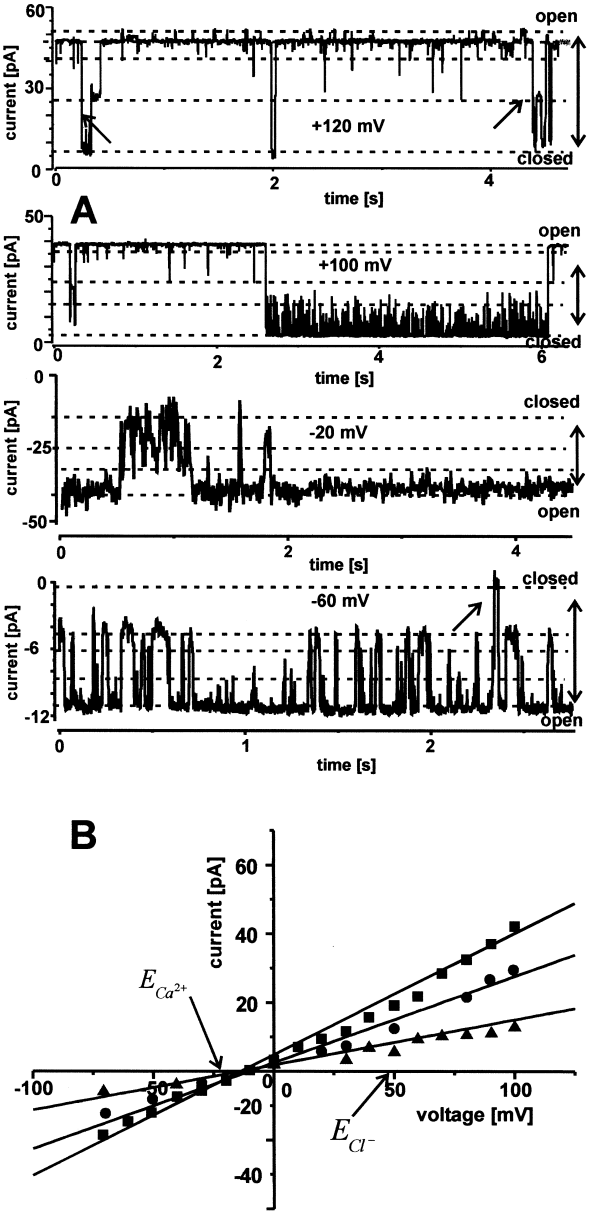


Fig. 2. Flagella membrane proteins reconstituted into liposomes constitute a weak cation-selective high-conductance channel permeable to calcium. Electrophysiological properties were examined by the patch-clamp technique. (A) Subconductance levels of a single-channel obtained from an excised patch are shown at the indicated different voltages. Examples for direct transitions between the different subconductance levels are marked with arrows. The following solutions were used: bath 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7; pipette 50 mM $CaCl_2$, 10 mM MOPS/Tris pH 7. (B) Current–voltage relationship of the three different main conductance levels of a single channel. Experimental conditions are as given in (A). Linear regression of the data revealed the following slope conductances for the three subconductance states: $\Lambda_1 = 132 \pm 9.9$ pS, $\Lambda_2 = 252 \pm 10.9$ pS, and $\Lambda_3 = 449 \pm 9.1$ pS. Theoretical Nernst potentials for 100% selectivity are marked with arrows.

limits, identical reversal potentials for the different open-channel amplitudes supporting our interpretation that these conductances represent subconductant states of a single channel. The reversal potentials may be converted into relative permeabilities by the constant field approach. With the GHK current equation [38], we obtained the following values: $P_K^+ : P_{Ca}^{2+} : P_{Cl}^- = 1 : 0.15 : 0.37$. Therefore, the above-described channel can be considered as a weak cation-selective, high-conductance channel, which is also somewhat permeable to Ca^{2+} ions. In contrast to the Ca^{2+}-permeable channel described below, this channel was not inhibited by Gd^{3+}.

3.4. A weak anion-selective high-conductance channel

This channel activity was only observed in about 5% ($n > 100$) of the excised inside-out patches from giant flagella-liposomes. However, when present, it was the only channel which was mostly open without an externally applied membrane potential. We therefore attempted to characterize this channel in planar bilayers using the osmotically induced fusion of proteoliposomes [39]. Fusion of proteoliposomes to the bilayer is only observed with this technique when liposomes contain a permeant (open) channel. With this technique we were able to obtain the activity of the below described anion-selective channel in more than 70% of the attempts in planar bilayers. After fusion of small flagella-liposomes to the bilayer, single-channel currents with multiple open-channel amplitudes were observed at positive and negative membrane potentials (Fig. 3A). Direct transitions between these conductance states occurred, showing that this channel also exhibits subconductant states. From a detailed analysis of different independent bilayers, the following frequent conductances were obtained (see also Fig. 3B): $\Lambda_1 = 140 \pm 20$ pS ($n = 8$), $\Lambda_2 = 210 \pm 22$ pS ($n = 5$), $\Lambda_3 = 310 \pm 32$ pS ($n = 9$), $\Lambda_4 = 410 \pm 15$ pS ($n = 5$), $\Lambda_5 = 540 \pm 35$ pS ($n = 5$) and $\Lambda = 830 \pm 30$ pS ($n = 5$) for the fully open channel. These results indicate that the channel exhibits complex voltage-dependent gating. The reversal potential was, within error limits, identical for all states. In

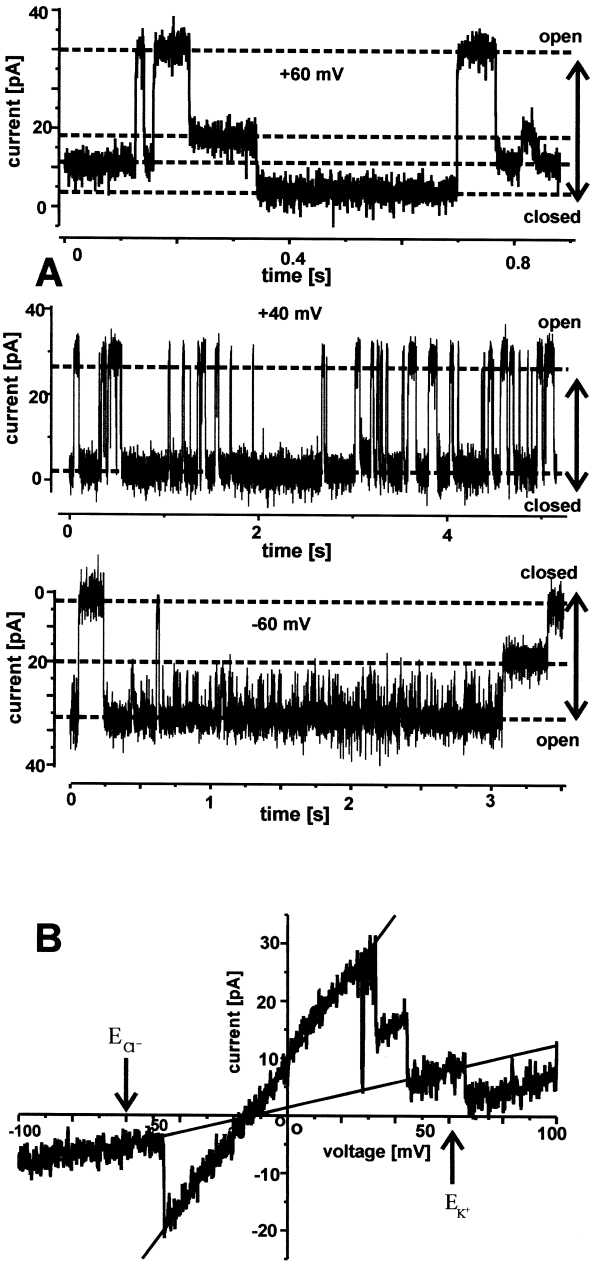


Fig. 3. Flagella membrane proteins reconstituted into liposomes form an anion-selective high-conductance channel. Electrophysiological properties were examined by the bilayer technique. (A) Current traces from a bilayer containing one active channel at different voltages as indicated. Multiple open-channel amplitudes are marked with dashed lines. The *cis* chamber contained 250 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7 and the *trans* compartment contained 20 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7. (B) Current–voltage relationship of the anion-selective channel in response to a voltage sweep ($\Delta U = 20$ mV/s) from $V_h = -100$ mV to $V_h = +100$ mV. The zero current potential is −16 mV. Theoretical Nernst potentials for 100% selectivity are marked with arrows. The buffers used were as in A.

different asymmetric buffer systems (buffer I: *cis*, 250 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7, *trans*, 20 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7; buffer II: *cis*, 250 mM KCl, 250 mM $CaCl_2$, 10 mM MOPS/Tris pH 7, *trans*, 250 mM KCl, 20 mM $CaCl_2$, 10 mM MOPS/Tris pH 7; buffer III: *cis*, 250 mM KCl, 10 mM MOPS/Tris pH 7, *trans*, 20 mM KCl, 10 mM MOPS/Tris pH 7) the following reversal potentials were obtained: $E_{rev}(I) = -19 \pm 3$ mV ($n = 6$), $E_{rev}(II) = -10 \pm 1.5$ mV ($n = 3$), $E_{rev}(III) = -24 \pm 3$ mV ($n = 3$). Conversion of these reversal potentials into relative permeabilities by the GHK-current equation yielded the following values, $P_K^+ : P_{Ca}^{2+} : P_{Cl}^- = 1:1:3$. Thus the channel exhibits a weak anion selectivity while being equally permeable to Ca^{2+} and K^+. From the above results, we can consider this channel as a weak anion-selective channel with a high conductance typical for porin-like channels.

3.5. A voltage- and Ca^{2+}-modulated flagellar cation channel (SFC1)

The channel described below was the most frequent active channel present in the excised inside-out patches from flagella-liposomes (>60%, $n > 100$), indicating that it might occur with high density within native flagellar membranes. At normal resting potentials observed in unicellular green algae in vivo (about −70 to −135 mV; [40,41]) and low positive holding potentials, the channel was predominantly inactive. Activation of the channel occurred preferentially at high positive holding potentials (Figs. 4A and 5A). Therefore, a current–voltage (*I*/*V*) relation of the open channel could be obtained only at high positive potentials (Fig. 4B). Expanding

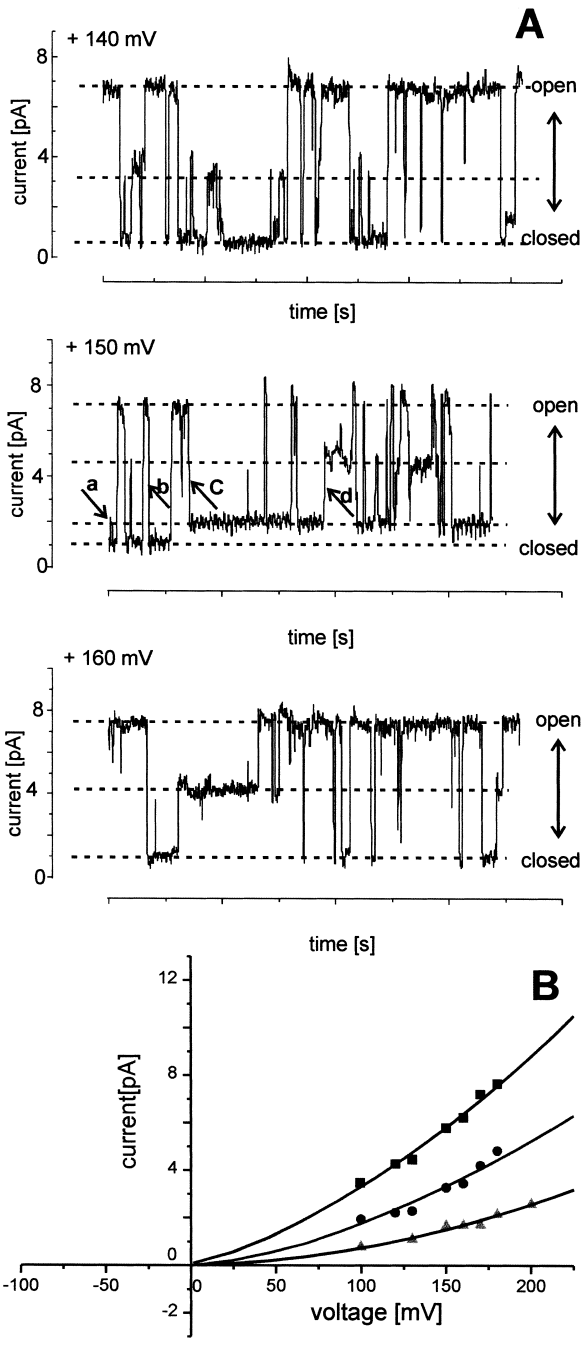


Fig. 4. Flagella proteins reconstituted into liposomes form a voltage-modulated flagellar Ca^{2+} channel, which we named SFC1. Electrophysiological properties were examined using the patch-clamp technique. (A) Single-channel recordings of an excised patch at different voltages as indicated. Subconductance levels could frequently be observed. Examples for transitions between the different open states are indicated by the arrows. The following buffer was used for pipette and bath: 50 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$,10 mM MOPS/Tris pH 7.2. (B) Current–voltage relationship of a channel obtained at high positive potentials. The three main conductance levels could also be observed in the *I*/*V* plot. Because of the non-linear current–voltage relationship, conductances of the different states were determined only from the linear range of the plot at membrane potentials >160 mV. The following conductances were estimated: $\Lambda_1 = 22$, $\Lambda_2 = 50$ pS, and $\Lambda_3 = 80$ pS. The measurements were conducted in symmetrical buffers (as given in A), were the current 0 pA is expected at $V_m = 0$ mV. Therefore the data were extrapolated to 0 mV by an arbitrary second-order polynomial.

the time scale of the current recordings allowed for the discrimination of at least three different open-channel amplitudes, which are also shown in the *I/V* relation (Fig. 5B). Some current traces point to additional substates between the open states 2 and 3. Direct transitions between the fully closed and different open states were frequently observed at the used sampling rate of 5 kHz (Fig. 4A). Since these would unlikely occur with two different independent channels, the course of the current transients indicates that here also the smaller amplitudes represent subconductant levels rather than simultaneous activities of two or more channels in the patches. The observed rectifying *I/V* relation (Figs. 4B and 5A) deviated from that predicted by the constant field approach (GHK-equations) under symmetrical (Fig. 4B) and asymmetrical ionic conditions (not shown). Therefore, conductances of the different states were estimated from the linear portion of the current–voltage relationship at membrane potentials >160 mV (see Fig. 4B). The conductances in symmetrical buffer (50 mM KCl, 2 mM $CaCl_2$ 2 mM $MgCl_2$ 10 mM MOPS/Tris, pH 7.2) were $\Lambda_1 \cong 22$ pS, $\Lambda_2 \cong 50$ pS and $\Lambda_3 \cong 80$ pS for the different substates. The estimated reversal potentials in different asymmetric buffer solutions (buffer I: *cis*, 50 mM KCl, 4 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.2, *trans*, 10 mM KCl, 4 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.2; buffer II, cis: 5 mM KCl, 50 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.2, *trans*, 5 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.2) were $E_{rev}(I) = -11 \pm 3$ mV ($n=3$) and $E_{rev}(II) = -15 \pm 1.5$ mV ($n=3$). These reversal potentials, when converted into relative permeabilities by the GHK-current equation, yielded the following values: $P^{2+}_{Ca} : P^{+}_{K} : P^{-}_{Cl} = 2:1:0.25$. Due to its cation selectivity with a higher permeability for Ca^{2+} ions than for K^+ ions, we named this channel SFC1 (for *Spermatozopsis* flagellar cation channel 1).

3.6. SFC1 is modulated by Ca^{2+}

As gating of cation channels by cytosolic Ca^{2+} occurs in plants and algae (e.g. [42–44]), and intra-

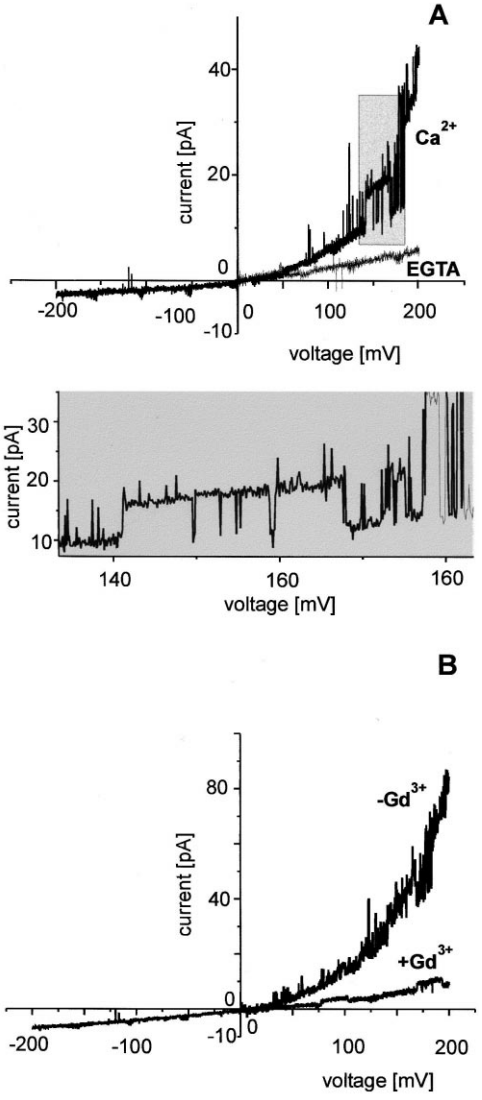
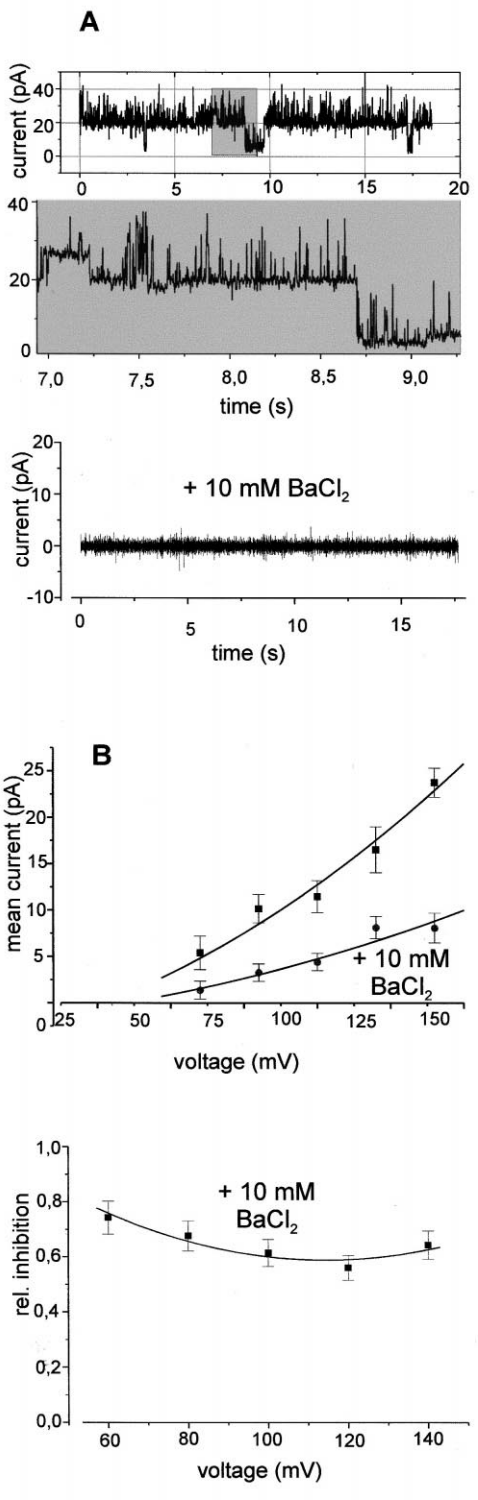


Fig. 5. SFC1 is inhibited by Gd^{3+} and modulated by Ca^{2+}. (A) Current recordings obtained from an excised patch containing several channels in response to voltage sweeps ($\Delta U = 20$ mV/s) from $V_h = -200$ mV to $V_h = +200$ mV. The upper trace shows the current in the presence of $\sim 1 \times 10^{-5}$ M Ca^{2+} and the lower in the presence of EGTA buffer ($\sim 1 \times 10^{-9}$ M Ca^{2+}). The following buffers were used: upper trace, 50 mM KCl, 10 μM $CaCl_2$, 10 mM MOPS/Tris pH 7 in the bath, 250 mM KCl, 10 mM MOPS/Tris pH 7 in the pipette; lower trace, 50 mM KCl, 1 mM EGTA, 10 mM MOPS/Tris pH 7 in the bath, 250 mM KCl, 10 mM MOPS/Tris pH 7 in the pipette. The shaded section is shown enlarged below. (B) Current–voltage relationship obtained from an excised patch containing several channels after an identical voltage sweep as in A. Upper trace, control; lower trace, in the presence of 50 μM $GdCl_3$. The following buffers were used: 50 mM KCl, 10 μM $CaCl_2$, 10 mM MOPS/Tris pH 7 for the bath, 250 mM KCl, 10 mM MOPS/Tris pH 7 for the pipette; lower trace, same buffers, but additionally 50 μM $GdCl_3$ in the bath.

cellular Ca^{2+} is intricately involved in the regulation of flagellar activity, we also analyzed the effect of high and low Ca^{2+} on SFC1. In addition to positive voltage, an increased Ca^{2+} concentration on the bath side was necessary to induce activity of SFC1. This double control is demonstrated in the voltage ramp shown in Fig. 5A. In asymmetrical EGTA-buffered potassium buffer (gradient 5:1; pipette/bath) and low Ca^{2+} ($\sim 10^{-9}$ M), no residual channel activity was observed. Raising the free Ca^{2+} concentration on the bath side to $\sim 1 \times 10^{-5}$ M induced immediately a high activity of SFC1 and a concomitant increase in conductivity. Upon perfusion with potassium buffer adjusted to 10^{-5} M Ca^{2+}, SFC1 activity was recovered instantly, indicating full reversibility of this stimulatory effect. Assuming that membrane proteins often keep their native orientation upon reconstitution [45,79], the bath side can be assigned to be physiologically the flagellar matrix side (see Section 4).

3.7. SFC1 is inhibited by Gd^{3+}

In order to further characterize and correlate SFC1 with known behavioral responses, we have analyzed the effect of inhibitors known to affect flagellar responses in *Chlamydomonas* or *Spermatozopsis*. As shown in Fig. 5B, SFC1 was effectively blocked at low concentrations of Gd^{3+}. Irrespective of the used buffer, the block by 50 μM Gd^{3+} was rapid and completed on average within 2 s. Partial blocking was already observed at concentrations ≥5μM (details not shown). The other Ca^{2+} permeable channel in the flagellar preparation was not affected by these concentrations of Gd^{3+} (see above). The affinity towards Gd^{3+} was high. Complete inhibition of SFC1 was observed even in the presence of a 200-fold excess of Ca^{2+}. The block was irreversible. Reactivation of SFC1 was not achieved even upon intensive perfusion of the bath with Gd^{3+}-free buffer. In *Chlamydomonas*, Gd^{3+} inhibits pressure induced flagellar currents [7] and acid-induced deflagellation in the same concentration range. The latter response is also inhibited by neomycin, an inhibitor of mechano- and voltage-gated ion channels as well as of phospholipase C [46–49]. However, no significant effect of 50 μM neomycin on the activity of SFC1 was observed. Also, diltiazem had no effect on its activity (data not shown).



3.8. SFC1 is inhibited by Ba^{2+}

Ba^{2+} is known to differentially affect whole cell currents induced by either mechanical or photo-stim-

Fig. 6. SFC1 is inhibited by Ba^{2+}. Flagella membrane proteins reconstituted into liposomes were examined by the bilayer technique. (A) Current traces from a bilayer containing two active channels of the SFC1-type before (top), expanded view (middle) and after addition of 10 mM $BaCl_2$ to both bilayer compartments (bottom). The *cis* chamber contained 250 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.0 and the *trans* compartment contained 20 mM KCl, 10 mM $CaCl_2$, 10 mM MOPS/Tris pH 7.0 (holding potential $V_h = 80$ mV). (B) Mean currents of different bilayers containing active SFC1 channels in response to a voltage gate (duration 60 s) in the absence and presence of (*cis*/*trans*) 10 mM $BaCl_2$. Relative inhibition of mean currents after addition of $BaCl_2$ ($n = 3$) are shown in the lower figure. The buffers used were as in A. Mean currents were calculated during the 1-min onset of the given holding potentials.
←

ulation in *Chlamydomonas*. While the flagellar current peak amplitude of F_F is only slightly affected by Ba^{2+}, the F_S current is largely increased [50]. In contrast, both the pressure-induced impulses in *Chlamydomonas* and the avoidance response of *Spermatozopsis* are effectively suppressed by Ba^{2+} [6,7,21]. In contrast to Gd^{3+}, which inhibits the mechanoshock response of *Spermatozopsis* at low concentrations (see below), millimolar concentrations of Ba^{2+} are needed for complete inhibition [6]. In order to gain evidence for the possible involvement of SFC1 in either of these responses, we have analyzed the effect of Ba^{2+} on SFC1 activity. Fig. 6 shows single-channel recordings from a bilayer containing two active SFC1 channels as judged by the typical voltage dependence of this single-channel activity (see also Fig. 5A). In asymmetric buffer solutions of 250/20 mM KCl (*cis*/*trans*), the chord conductance of the fully open SFC1 channel was $\Lambda = 120 \pm 15$ pS, slightly higher than in symmetrical 50 mM KCl solutions on both sides of the membrane (Fig. 6A top and expanded view, middle). In the particular measurement shown in Fig. 6A, the current dropped to almost zero after addition of 10 mM Ba^{2+} to both compartments (Fig. 6A, bottom trace). In different bilayers, average relative current reductions between 75 and 55% were observed after addition of Ba^{2+} (Fig. 6B). Since no changes in the reversal potential were observed after addition of Ba^{2+} to either side of the membrane, these results indicate that Ba^{2+} is to some extent equally permeable from both sides of the membrane, although the channel is blocked to a certain degree by the divalent cation. As Ba^{2+} is also a blocker of potassium channels, we also tested the effects of TEA^+ and apamin, inhibitors of voltage-gated K^+ and Na^+ channels and Ca^{2+}-modulated potassium channels, on SFC1. However, these inhibitors did not affected SFC1 significantly (data not shown).

3.9. Gd^{3+} inhibits the shock response

Spermatozopsis responds to mechanical stimulation and non-physiological light shock by a peculiar avoidance response. During the response, the flagella act as a hydrodynamically coupled pair, allowing for extreme accelerations (velocities of about 60 μm s^{-1} to >600 μm s^{-1}) during the avoidance reactions. This response is initiated in a time window below 18 ms and can be analyzed either directly by video microscopy or via the concomitant Ca^{2+}-dependent reorientation of the basal bodies from the antiparallel to the parallel configuration. The shock response is inhibited by Ba^{2+} or by decreasing the extracellular

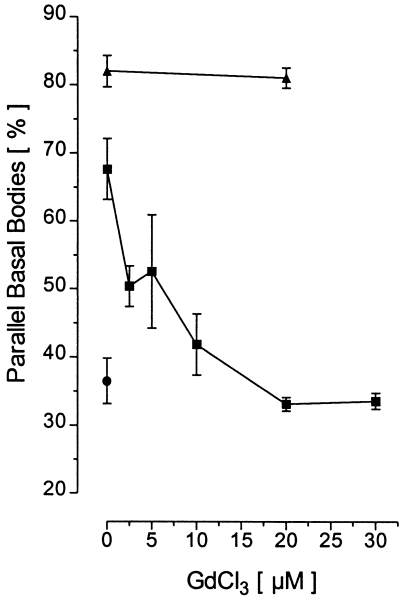


Fig. 7. Mechanoshock-induced basal body reorientation in *Spermatozopsis* cells is inhibited by gadolinium. Squares, cells were preincubated for 5 min with the indicated concentrations of $GdCl_3$ prior to application of a mechanical stress. Controls: triangles, cells were fixed without prior addition of EGTA; circles, no mechanoshock was given to the cells after the 5-min adaptation period. Mean ± S.D. of three to seven independent experiments. Total analyzed cells, 158–372.

free Ca^{2+} concentration [6,10]. The observed voltage dependence of SFC1 strongly suggested that, in vivo, it is most likely activated solely upon strong depolarizations (i.e. during the peculiar avoidance reactions exhibited by *Spermatozopsis*). In order to get a possible link between these behavioral responses and SFC1, we analyzed the effect of Gd^{3+} on both responses.

Dose-dependent inhibition was observed for the avoidance response irrespective of the stimulus used. Already low concentrations of this lanthanide effectively inhibited the reorientation of basal bodies induced by mechanical agitation (Fig. 7). Half-maximal inhibition was observed at $\sim$5–7.5 μM Gd^{3+}. Complete suppression down to the percentage of parallel basal bodies observed in non-stimulated cells was observed between 10 and 20 μM Gd^{3+}. In these concentrations, Gd^{3+} had no inhibitory effect on the reorientation of basal bodies when EGTA was omitted from the fixation (Fig. 7, triangles). Thus, the inhibition can be attributed to a real block of Ca^{2+} entry into the cells and not to a block of Ca^{2+} binding sites in centrin, which is present in the distal connecting fiber responsible for the reorientation of basal bodies [10]. No differences were found in the concentrations of Gd^{3+} necessary to inhibit the mechano- or photoshock-induced reorientation of the basal bodies (not shown). Also, the in vivo effects of Gd^{3+} were rapid. Already after 1 min in the presence of 20 μM Gd^{3+}, the response to mechanical agitation and to extreme light shock was completely abolished. However, longer times (5 min) were routinely used in our analyses, as an extended equilibration time lead to a reduced starting number of cells exhibiting basal bodies in the parallel configuration. These cells were probably already stimulated by the mechanical forces administered by the necessary mixing after application of the Gd^{3+} stock. Reorientation of the basal bodies to the antiparallel configuration can take 3–5 min and occurs independent of the restoration of the normal forward swimming mode, which takes place after a few seconds [10].

The gadolinium effect on the photoshock response was also analyzed at the single cell level by video microscopy. Here it became evident at low concentrations ($\leq$15 μM), that initially the distance the cells jumped backwards rapidly decreased. The cells only exhibited a short stop response, equivalent to the shock response exhibited upon a normal strong step-up stimulus, prior to resuming the normal forward swimming mode. This behavior was comparable to that observed upon reducing the extracellular free Ca^{2+} concentration [6]. As depicted in Fig. 8, the

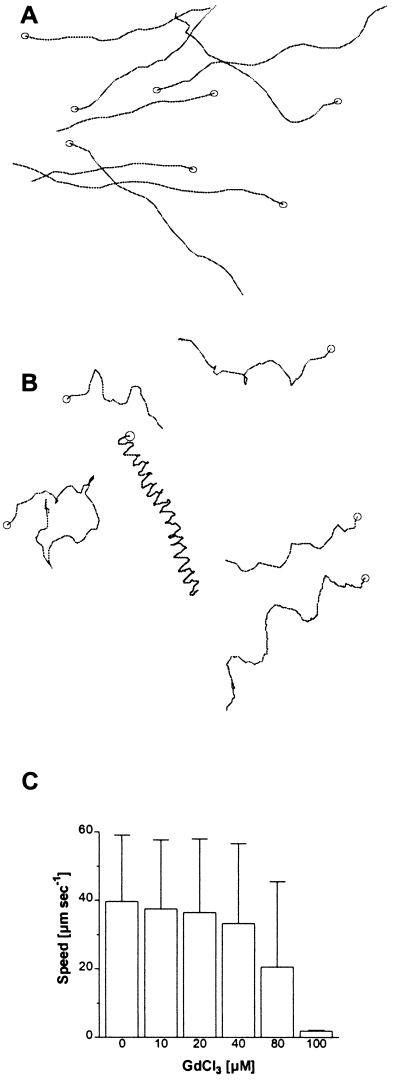


Fig. 8. Effects of gadolinium on the swimming behavior of *Spermatozopsis similis*. (A) Projection of swimming tracks of *Spermatozopsis* in culture medium in the absence of gadolinium. Tracks were captured under red safety light after a 5-min preincubation period in the assay chamber. (B) Projections of swimming tracks in the presence of 40 μM Gd^{3+}. Cells were preincubated for 5 min with gadolinium in the assay chamber prior to the analysis. All other conditions as in A. (C) Effects of increasing concentrations of Gd^{3+} on the average swimming speed. Cells were preincubated for 5 min prior to speed analyses. Mean ± S.D. of three independent experiments. Total analyzed cells, 90–105.

general swimming behavior of *Spermatozopsis* at Gd^{3+} concentrations up to ~30 μM was not affected at the used cell concentrations. Thus, unspecific effects on motility at the effective concentrations can most likely be excluded. However, with increasing Gd^{3+} concentrations, the swimming path of the cells became less straight. At higher concentrations, an increasing abnormal spiraling swimming behavior was observed and the general motility was dramatically affected (Fig. 8B,C).

4. Discussion

Experimental evidence for the presence of voltage-gated Ca^{2+} permeable channels in the flagella membrane of unicellular green algae has until now arisen solely from capacitive current measurements (e.g. [5,7,22]). In order to characterize these channel proteins at the single-channel level, we have applied the following strategy. Intact flagella were isolated from *Spermatozopsis* by adapting the Ca^{2+}/dibucaine method [29] to the needs of this naturally cell-wall-lacking green alga. As direct recordings from the isolated flagella were not successful, solubilized flagellar membrane proteins were reconstituted into liposomes. Consistent with the central role of cation fluxes across flagellar membranes in ciliates [51], we mainly observed activities of cation-selective channels in liposomes containing reconstituted flagellar membrane proteins. However, a voltage-gated weak anion-selective channel with a high conductance was also observed in the flagellar membrane at high frequencies, indicating that it might also occur with high density in native membranes. Considering the central role of Ca^{2+} fluxes in flagellar function and signaling (see Section 1), we focussed our analyses on the major voltage-dependent Ca^{2+}-permeable low conductant channel (SFC1) observed in our preparations.

The SFC1 channel revealed a strongly rectifying current–voltage relationship, showing that the channel was incorporated mainly unidirectional into the bilayer membrane. Assuming that the proteins of the flagellar membrane keep their native orientations, as observed with other membrane proteins during reconstitution [45,79], we can propose that the intra-liposomal side corresponds to the flagella matrix side. Considering further that in vivo the Ca^{2+} concentration in the matrix is orders of magnitudes lower than that outside, the SFC1 channel would transiently catalyze Ca^{2+} influx. This influx requires in vivo transient depolarization of the flagellar membrane. Provided the intra-liposomal side corresponds to the matrix side, this depolarization would correspond in vitro to rectification at higher membrane potentials. This was exactly what we observed in our measurements. Therefore, the asymmetric voltage dependence of SFC1 further supports our proposal. All currently identified green algal flagellar Ca^{2+} currents induced by either photo- or mechanoshock are inwardly rectifying and are triggered by depolarization (see Section 1).

SFC1 activation is observed solely at positive holding potentials in the presence of increased free Ca^{2+} concentrations, suggesting that this channel might be opened in vivo only upon massive depolarizations of the flagellar membrane with a concomitant Ca^{2+} signal from the matrix side. This agrees well with the strict dependence of the shock responses on extracellular Ca^{2+} and with previous suggestions that a larger Ca^{2+} influx occurs during the mechanoshock than during photoshock [6,7]. It has been calculated that depolarizations ≥80 mV can be induced by the photoreceptor currents in *Chlamydomonas* [17,50]. Thus, assuming a similar average steady state free running membrane potential for flagellate green algae at low extracellular K^+ as determined for *Eremosphera* (-85 ± 11 mV; [41]), it is well feasible that the membrane potential could be depolarized close to positive values upon strong stimuli. The channels involved in the shock behavior response in an 'all-or-nothing' manner and appear to be evenly distributed along the entire flagella length [7,17,19,22]. As the dominating reconstituted activity among the cation-selective channels in the flagella, SFC1 might well be a candidate for one of these channel activities observed in whole cell measurements. Activation of the high conductance, voltage-gated anion-selective channel present in high frequencies in the flagellar membrane of *Spermatozopsis* in response to moderate depolarizations, could further facilitate a rapid and massive membrane depolarization. However, the detailed voltage dependence of the reconstituted SFC1 and the anion-selective channel needs to be confirmed by measurements tak-

en under physiologically less disruptive conditions. Important regulatory subunits may well have been lost during proteoliposome formation. Unfortunately, comparison of our SFC1 data to direct patch-clamp analyses of ciliary membranes from other protists is not possible, as these have not yet been analyzed by direct patch-clamp experiments [52].

The three flagellar channels of *Spermatozopsis* characterized so far have relatively large unitary conductances in their fully open states. Also, flagella membranes of wild-type *Chlamydomonas reinhardtii* are dominated by ion channels in this conductance range (G. Pazour, G. Kreimer, R. Wagner and G.B. Witman, unpublished results). Different channels in both the low and high conductance ranges are also known from ciliary membranes of *Paramecium* (1.5–2, 16, 30 and 45 pS; [23,53], *Tetrahymena* (211 pS [54]; 14–20 and 354 pS [55]; 73 pS [56]) and flagella membranes of e.g. sea urchin sperms (22, 46, 82 and 148 pS [57]). Recently, the conductance of mechanosensitive channels in *Chlamydomonas* has been estimated from whole-cell measurements to be 30–50 pS [21]. This closely matches the values observed for SFC1 (22, 50 and 80 pS). A relatively high conductivity of SFC1 might be necessary for a steep increase in the matrix free Ca^{2+} needed for induction of flagellar beat reversal. Flagella Ca^{2+}-binding capacity, however, can be quite considerable as indicated by recent simultaneous analysis of the time course of intraciliary free Ca^{2+} and the early inward Ca^{2+} current induced by a depolarizing voltage step in *Stylonychia* [58]. In this ciliate, upon a 280-ms depolarization, only about 5% of the Ca^{2+} entering the matrix was detected as free Ca^{2+} by the ratiometric Ca^{2+}-sensitive dye fura-2. In other ciliates, smaller depolarizations induce even lower intraciliary Ca^{2+} rises to $\approx 2\times10^{-7}$ M [59]. Many Ca^{2+} channels of higher plants and of mechanogated channels in plants, algae, fungi and animals also fall into the conductivity range observed for SFC1 [44,48,53,60–63].

In which of the known flagella shock responses might SFC1 be involved? Considering its sensitivity towards Gd^{3+} and Ba^{2+}, SFC1 is most likely a component of the signaling system involved in flagellar shock responses evoked by mechanical disturbance of the cells. This conclusion is supported by the observation that both the mechanoshock response of *Spermatozopsis* as well as pressure-induced current impulses originating from the flagella of *Chlamydomonas* are inhibited by Gd^{3+} and Ba^{2+} ([6,7], present study). In contrast, flagellar currents evoked by photoshock are not inhibited by Ba^{2+} (see below). Gd^{3+} blocks mechano- and voltage-sensitive channels and responses in plants and animals [44,48,64]. Although rapid backward jumping of *Spermatozopsis* can also be induced by extreme photoshock [6,10], and Gd^{3+} effectively suppressed this response as well as the accompanying reorientation of basal bodies, involvement of SFC1 in the photoshock pathway is not likely. Firstly, normal stop responses followed by a short period of circling prior to resuming forward swimming in a new direction, were still observed at Gd^{3+} concentrations sufficient to block rapid backwards jumping. Secondly, flagellar currents accompanying the photoshock response in *Chlamydomonas* are slightly (F_F) or even dramatically prolonged (F_S) by Ba^{2+} [50], whereas the mechanoshock response and the generation of pressure-induced impulses is inhibited by Ba^{2+} [6,7]. In addition, low concentrations of Gd^{3+} have no effect on light responses in *Chlamydomonas* [49]. Induction of the peculiar avoidance response of *Spermatozopsis* requires a steep Ca^{2+} gradient and apparently much higher intraflagellar free Ca^{2+} concentrations than reached by a physiological photoshock [6]. In *Chlamydomonas* the pressure-induced Ca^{2+} currents are also significantly larger than those induced by photoshock [7]. Suppression of the light shock triggered response by Gd^{3+} can thus reflect additional interaction of this lanthanide with Ca^{2+}-influx channels on the cell body. This would parallel the situation in *Chlamydomonas*, where Gd^{3+}-sensitive channels are localized on the flagellar membrane and plasma membrane [7,21,49].

Ca^{2+} influx in plants can occur through both highly Ca^{2+}-selective channels and non-selective cation channels [65–67]. SFC1, as many Ca^{2+} channels of higher plant plasma membranes (for review see [63,68]), exhibited only a weak selectivity for Ca^{2+} over K^+ under our measuring conditions. Also in *Chlamydomonas*, the channels involved in generation of F_F currents do not appear to be highly selective for Ca^{2+} and can also be permeated by K^+ [16]. Ca^{2+} channels in the ciliary membrane of *Tetrahymena* species can also be permeated well by K^+

[55,69–71], whereas the two major Ca^{2+} channels in ciliary membranes of *Paramecium* exhibit virtually no permeability towards potassium [53]. However, possible selectivity/regulatory mechanisms might well have been lost or deactivated during our reconstitution procedure. Therefore, the selectivity values reported here for SFC1 certainly reflect only approximations with respect to physiological conditions. Whereas Ca^{2+} suppressed the K^+ conductivity of major flagellar cation channels in *Tetrahymena* [55,70], no significant effect of high concentrations of Ca^{2+} on the conductivity of SFC1 for K^+ was observed. This parallels the findings for the photoinduced F_F current in whole cells of *Chlamydomonas* [16].

SFC1 activity was only observed at increased concentrations of free Ca^{2+} from the assumed matrix side. Ca^{2+} modulation of ion channels is a well-known phenomenon in different systems including plants, yeast, algae and protists. Both channel activation and inactivation have been reported [42–44,50,72–75]. Our data suggest that SFC1 activation in vivo will occur only when depolarization of the flagellar membrane is larger than that observed upon physiological photoshock and is accompanied by an increase in intraflagellar Ca^{2+}. Involvement of at least one additional channel in mechanoshock signaling prior to activation of SFC1 is thus necessary. As SFC1 is probably not the mechanoreceptor channel, activation of mechano- or stretch-activated channels might lead to positive deflections in the membrane potential. Mechanically induced impulses in *Chlamydomonas* probably also result from activation of at least two types of channels, a mechanosensitive and a voltage-gated Ca^{2+} channel [7]. Although Ba^{2+}-carried fluctuating currents can be observed in the flagella of *Chlamydomonas* upon application of pressure, the amplitudes of these currents were largely reduced and no current spikes were generated [7,21]. This indicates that either the mechanosensitive channel is directly inhibited by Ba^{2+} or, as Ba^{2+} usually does not substitute for Ca^{2+} intracellulary, that a second Ca^{2+}- and voltage-gated channel in the large conductance range might be involved in spike generation. If the latter assumption holds true, *Chlamydomonas* flagella might possess a channel with properties similar to SFC1.

In order to prevent increases of intraflagellar Ca^{2+} above critical levels (e.g. axonemes detach from basal bodies at $\approx 10^{-4}$ M [10,76]) and to allow the observed graded responses of *Spermatozopsis* to different stimuli [6], SFC1 as well as the other channels must be tightly controlled and efficiently inactivated. The steep voltage-dependence of SFC1 suggests that it will probably rapidly inactivate upon beginning repolarization of the flagellar membrane. SFC1 activity can also be controlled in parallel by lowering of the Ca^{2+} concentration in the flagellar matrix due to the activity of Ca^{2+} sequestering systems, such as Ca^{2+} ATPase pumps. In *Paramecium*, a complex interplay of different ion channels and inactivation mechanisms (e.g. voltage- and Ca^{2+}-dependent K^+ efflux and inactivation of Ca^{2+} channels by Ca^{2+}) are involved in regulating amplitude and duration of flagella responses [51]. Down-regulation of F_S probably also occurs through Ca^{2+} in the photoshock response of *Chlamydomonas* [50]. Towards this end more detailed future analyses of the Ca^{2+}-dependence of SFC1 activation will be helpful. In addition, as K^+ efflux can be expected upon strong depolarizations [16,40,77], the K^+ conductance of SFC1 also points to a putative concomitant role in flagellar membrane repolarization. Thus, it might additionally have a similar function to Ca^{2+}-activated plant outward rectifying K^+ channels in repolarizing the membrane during the signaling event [74,78]. Such a dual function would represent the most direct control mechanism to prevent an increase of free Ca^{2+} to critical levels. Clearly, more work is needed to understand how the different ion channels are involved in regulating duration and amplitude of Ca^{2+} influx into the flagellar matrix, which is the basis of the stimulus-graded movement responses observed in flagellate green algae. We hope that expansion of the single-channel analyses to *Chlamydomonas* with its great variety of mutants and accessibility to genetic manipulation will help to further unravel ion channel functions within the regulatory network of green algal flagellar signaling.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (G.K. and R.W.).

References

[1] G.B. Witman, *Chlamydomonas* phototaxis, Trends Cell Biol. 3 (1993) 403–408.

[2] G. Kreimer, Cell biology of phototaxis in flagellate algae, Int. Rev. Cytol. 148 (1994) 229–310.

[3] D. Ringo, Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*, J. Cell Biol. 33 (1967) 543–571.

[4] J.A. Schmidt, R. Eckert, Calcium couples flagellar reversal to photostimulation in *Chlamydomonas reinhardtii*, Nature 262 (1976) 713–715.

[5] H. Harz, P. Hegemann, Rhodopsin-regulated calcium currents in *Chlamydomonas*, Nature 351 (1991) 489–491.

[6] G. Kreimer, G.B. Witman, Novel touch-induced, Ca^{2+}-dependent phobic response in a flagellate green alga, Cell Motil. Cytoskeleton 29 (1994) 97–109.

[7] K. Yoshimura, A novel type of mechanoreception by the flagella of *Chlamydomonas*, J. Exp. Biol. 199 (1996) 295–302.

[8] J.S. Hyams, G.G. Borisy, 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 (1978) 235–253.

[9] M. Bessen, R.B. Fay, G.B. Witman, Calcium control of waveforms in isolated flagellar axonemes of *Chlamydomonas*, J. Cell Biol. 86 (1980) 446–455.

[10] G.I. McFadden, D. Schulze, B. Surek, J.L. Salisbury, M. Melkonian, Basal body reorientation mediated by a Ca^{2+}-modulated contractile protein, J. Cell Biol. 105 (1987) 903–912.

[11] M. Hayashi, T. Yagi, K. Yoshimura, R. Kamiya, Real-time observation of Ca^{2+}-induced basal body reorientation in *Chlamydomonas*, Cell Motil. Cytoskeleton 41 (1998) 49–56.

[12] N. Morel-Laurens, Calcium control of phototactic orientation in *Chlamydomonas reinhardtii*: sign and strength of response, Photochem. Photobiol. 45 (1987) 119–128.

[13] L. Linden, G. Kreimer, Calcium modulates rapid protein phosphorylation/dephosphorylation in isolated eyespot apparatuses of the green alga *Spermatozopsis similis*, Planta 197 (1995) 343–351.

[14] M. Calenberg, U. Brohsonn, M. Zedlacher, G. Kreimer, Light- and Ca^{2+}-modulated heterotrimeric GTPases in the eyespot apparatus of a flagellate green alga, Plant Cell 10 (1998) 91–103.

[15] F.F. Litvin, O.A. Sineshchekov, V.A. Sineshchekov, Photoreceptor electric potential in the phototaxis of the alga *Haematococcus pluvialis*, Nature 271 (1978) 476–478.

[16] C. Nonnengässer, E.-M. Holland, H. Harz, P. Hegemann, The nature of rhodopsin-triggered photocurrents in *Chlamydomonas*. II. Influence of monovalent ions, Biophys. J. 70 (1996) 932–938.

[17] H. Harz, C. Nonnengässer, P. Hegemann, The photoreceptor current of the green alga *Chlamydomonas*, Phil Trans. R. Soc. Lond. B Biol. Sci. 338 (1992) 39–52.

[18] E.-M. Holland, H. Harz, R. Uhl, P. Hegemann, Control of phobic responses by rhodopsin-induced photocurrents in *Chlamydomonas*, Biophys. J. 73 (1997) 1395–1401.

[19] K. Yoshimura, C. Shingyoji, K. Takahashi, Conversion of beating mode in *Chlamydomonas* flagella induced by electric stimulation, Cell Motil. Cytoskeleton 36 (1997) 236–245.

[20] A. Matsuda, K. Yoshimura, O.A. Sineshchekov, M. Hirono, R. Kamiya, Isolation and characterization of novel *Chlamydomonas* mutants that display phototaxis but not photophobic response, Cell Motil. Cytoskeleton 41 (1998) 353–362.

[21] K. Yoshimura, Mechanosensitive channels in the cell body of *Chlamydomonas*, J. Membr. Biol. 166 (1998) 149–155.

[22] C. Beck, R. Uhl, On the localisation of voltage sensitive calcium channels in the flagella of *Chlamydomonas*, J. Cell Biol. 125 (1994) 1119–1125.

[23] R.R. Preston, Y. Saimi, Calcium ions and the regulation of motility in *Paramecium*, in: R.A. Bloodgood (Ed.), Ciliary and flagellar membranes, Plenum Press, New York, 1990, pp. 173–200.

[24] C.B. Lindemann, K.S. Kanous, A model for flagellar motility, Int. Rev. Cytol. 173 (1997) 1–72.

[25] U.W. Goodenough, B. Shames, L. Small, T. Saito, R.C. Crain, M.A. Sanders, J.L. Salisbury, The role of calcium in the *Chlamydomonas reinhardtii* mating reaction, J. Cell Biol. 121 (1993) 365–374.

[26] L.M. Quarmby, H.C. Hartzell, Two distinct, calcium-mediated, signal transduction pathways can trigger deflagellation in *Chlamydomonas reinhardtii*, J. Cell Biol. 124 (1994) 807–815.

[27] J.H. Evans, L.R. Keller, Calcium influx signals normal flagellar RNA induction following acid shock of *Chlamydomonas reinhardtii*, Plant Mol. Biol. 33 (1997) 467–481.

[28] G. Kreimer, U. Brohsonn, M. Melkonian, Isolation and partial characterization of the photoreceptive organelle for phototaxis of a flagellate green alga, Eur. J. Cell Biol. 55 (1991) 318–327.

[29] G.B. Witman, Isolation of *Chlamydomonas* flagella and flagellar axonemes, Methods Enzymol. 134 (1986) 280–290.

[30] N.J. Cook, C. Zeilinger, K.W. Koch, U.B. Kaup, Solubilisation and functional reconstitution of a cGMP-dependent cation channel from bovine rod outer segments, J. Biol. Chem. 261 (1986) 17033–17039.

[31] M. Criado, B.U. Keller, A membrane fusion strategy for single-channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes, FEBS Lett. 224 (1987) 172–176.

[32] O.P. Hamill, A. Martiy, E. Neher, B. Sakmann, F.J. Sigworth, Improved patch clamp technique for high-resolution current recording from cells and cell free patches, Pflügers Arch. 391 (1981) 85–100.

[33] P. Mueller, D.O. Rudin, H. Tien, W.C. Wescott, Reconstitution of cell membrane structure in vitro and its transformation into an excitable system, Nature 194 (1962) 979–980.

[34] M. Schwarz, A. Gross, T. Steinkamp, U.I. Flügge, R. Wagner, Ion channel properties of the reconstituted chloroplast triosephosphate/phosphate translocator, Biol. Chem. 269 (1994) 29481–29489.

[35] H.R. Preisig, M. Melkonian, A light and electron microscopical study of the green flagellate *Spermatozopsis similis* spec. nova, Pl. Syst. Evol. 146 (1984) 57–74.
[36] G.B. Witman, K. Carlson, J. Berliner, J.-L. Rosenbaum, *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes, J. Cell Biol. 54 (1972) 507–539.
[37] D.R. Laver, P.W. Gage, Interpretation of substates in ion channels-unipores or multipores, Prog. Biophys. Mol. Biol. 67 (1997) 99–140.
[38] G.J. Allen, D. Sanders, Calcineurin, a type 2B protein phosphatase, modulates the Ca^{2+}-permeable vacuolar ion channel of stomatal guard cells, Plant Cell 7 (1995) 1473–1483.
[39] D.J. Woodbury, J.E. Hall, Role of channels in the fusion of vesicles with a planar bilayer, Biophys. J. 54 (1988) 1053–1063.
[40] B. Malhotra, A.D.M. Glass, Potassium fluxes in *Chlamydomonas reinhardtii*. I. Kinetics and electrical potentials, Plant Physiol. 108 (1995) 1527–1536.
[41] C.S. Bauer, C. Plieth, U.-P. Hansen, B. Sattelmacher, W. Simonis, G. Schönknecht, Repetitive Ca^{2+} spikes in a unicellular alga, FEBS Lett. 405 (1997) 390–393.
[42] O.M. Zherelova, A.A. Kataev, G.N. Berestovsky, Regulation of the calcium channels of the plasmalemma of *Nitellopsis obtusa* by intracellular Calcium, Biophysics 32 (1987) 379–380.
[43] B. Schulz-Lessdorf, R. Hedrich, Protons and calcium modulate SV-type channels in the vacuolar-lysosomal compartment-channel interaction with calmodulin inhibitors, Planta 197 (1995) 655–671.
[44] B. Klüsener, G. Boheim, H. Liá, J. Engelberth, E.W. Weiler, Gadolinium-sensitive, voltage-dependent calcium release channels in the endoplasmic reticulum of a higher plant mechanoreceptor organ, EMBO J. 14 (1995) 2708–2714.
[45] J.-L. Rigaud, B. Pitard, D. Levy, Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins, Biochim. Biophys. Acta 1231 (1995) 223–246.
[46] E. Gabev, J. Kasianowicz, T. Abott, S. McLaughlin, Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP2), Biochim. Biophys. Acta 979 (1989) 905–912.
[47] Y.G. Yueh, R.C. Crain, Deflagellation of *Chlamydomonas reinhardtii* follows a rapid transitory accumulation of inositol 1,4,5-trisphosphate and requires Ca^{2+} entry, J. Cell Biol. 123 (1993) 869–875.
[48] O.P. Hamill, D.W. McBride Jr., The pharmacology of mechanogated membrane ion channels, Pharmacol. Rev. 48 (1996) 231–252.
[49] L.M. Quambry, Ca^{2+} influx activated by low pH in *Chlamydomonas*, J. Gen. Physiol. 108 (1996) 351–361.
[50] E.M. Holland, F.-J. Braun, C. Nonnengässer, H. Harz, P. Hegemann, The nature of rhodopsin-triggered photocurrents in *Chlamydomonas*. I. Kinetics and influence of divalent cations, Biophys. J. 70 (1996) 924–931.
[51] H. Machemer, Electrophysiology, in: H.-D. Görtz (Ed.), *Paramecium*; Springer, Berlin 1988, pp. 185–215.
[52] H. Machemer, Electrophysiology of ciliates, Methods Cell Biol. 47 (1995) 419–424.
[53] B.E. Ehrlich, A. Finkelstein, M. Forte, C. Kung, Voltage-dependent calcium channels from *Paramecium* cilia incorporated into planar lipid bilayers, Science 225 (1984) 427–428.
[54] Y. Oosawa, M. Sokabe, Cation channels from *Tetrahymena* cilia incorporated into planar lipid bilayers, Am. J. Physiol. 244 (1985) C177–179.
[55] Y. Oosawa, M. Sokabe, M. Kasai, A cation channel for K^+ and Ca^{2+} from *Tetrahymena* cilia in planar lipid bilayers, Cell Struct. Funct. 13 (1988) 51–60.
[56] C. Fujiwara-Hirashima, K. Anzai, M. Takahashi, Y. Kirino, A voltage-dependent chloride channel from *Tetrahymena* ciliary membrane incorporated into planar lipid bilayers, Biochim. Biophys. Acta 1280 (1996) 207–216.
[57] A. Lievano, J.A. Sanchez, A. Darszon, Single-channel activity of bilayers derived from sea urchin sperm plasma membranes at the tip of a patch-clamp electrode, Dev. Biol. 112 (1985) 253–257.
[58] H. Machemer, R. Bräucker, S. Machemer-Röhnisch, U. Nagel, D.C. Neugebauer, M. Weskamp, The linking of extrinsic stimuli to behaviour: roles of cilia in ciliates, Eur. J. Protistol. 34 (1998) 254–261.
[59] J. Pernberg, H. Machemer, Fluorometric measurement of intracellular free Ca^{2+}-concentration in the ciliate *Didinium nasutum* using fura-2, Cell Calcium 18 (1995) 484–494.
[60] R.W. Tsien, P.T. Ellinor, W.A. Horne, Molecular diversity of voltage-dependent Ca^{2+} channels, Trends Phys. Sci. 12 (1991) 349–349.
[61] A.M. Hetherington, A. Graziana, C. Mazars, P. Thuleau, R. Ranjeva, The biochemistry and pharmacology of plasma-membrane calcium channels in plants, Philos. Trans. R. Soc. London B 338 (1992) 91–96.
[62] A.R. Taylor, N.F.H. Manison, C. Fernandez, J. Wood, C. Brownlee, Spatial organization of calcium signaling involved in cell volume control in the *Fucus* rhizoid, Plant Cell 8 (1996) 2015–2031.
[63] M. Pinñeros, M. Tester, Calcium channels in higher plant cells: selectivity, regulation and pharmacology, J. Exp. Bot. 48 (1997) 551–577.
[64] J.P. Ding, B.G. Pickard, Mechanosensory calcium-selective cation channels in epidermal cells, Plant J. 3 (1993) 83–110.
[65] J.I. Schroeder, S. Hagiwara, Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of nonselective Ca^{2+} permeable channels, Proc. Natl. Acad. Sci. USA 87 (1990) 9305–9309.
[66] P.J. White, Characterization of a voltage-dependent cation-channel from the plasma membrane of rye (*Secale cereale* L.) roots in planar lipid bilayers, Planta 193 (1994) 186–193.
[67] M. Pinñeros, M. Tester, Characterization of a voltage-dependent Ca^{2+}-selective channel from wheat roots, Planta 195 (1995) 478–488.
[68] M. Tester, Plant ion channels: whole-cell and single-channel studies, New Phytol. 114 (1990) 305–340.
[69] S. Kawahara, Y. Kirino, S. Nagao, Y. Nozawa, Ion permeability of ciliary membrane vesicles isolated from *Tetrahyme-*

na. Single-channel recording study on the membrane reconstituted into a planar lipid bilayer, J. Biochem. 100 (1986) 1569–1573.

[70] C. Fujiwara, K. Anzai, Y. Kirino, S. Nagao, Y. Nozawa, M. Takahashi, Cation channels from ciliary membrane of *Tetrahymena* reconstituted into planar lipid bilayer. Comparision between the channels from the wild *T. termophila* and from its mutant which does not show ciliary reversal, J. Biochem. 104 (1988) 344–348.

[71] Y. Oosawa, Ionic currents of channels that are permeable to monovalent and divalent cations, Biophys. J. 56 (1989) 1217–1223.

[72] Y. Saimi, B. Martinac, Calcium-dependent potassium channel in *Paramecium* studied under patch clamp, J. Membr. Biol. 112 (1989) 79–89.

[73] A. Bertl, D. Gradmann, C.L. Slayman, Calcium- and voltage-dependent ion channels in *Saccharomyces cerevisiae*, Phil. Trans. R. Soc. Lond. B Biol. Sci. 338 (1992) 63–72.

[74] K. Czempinski, S. Zimmermann, T. Ehrhardt, B. Müller-Röber, New structure and function in plant K^+ channels: KCO1, an outward rectifier with a steep Ca^{2+} dependency, EMBO J. 16 (1997) 2565–2575.

[75] F. Saitow, Y. Nakaoka, Y. Oosawa, A calcium-activated, large conductance and non-selective cation channel in *Paramecium* cell, Biochim. Biophys. Acta 1327 (1997) 52–60.

[76] R. Kamiya, G.B. Witman, Submicromolar levels of calcium control the balance of beating between the two flagella in demembranated models of *Chlamydomonas*, J. Cell Biol. 98 (1984) 97–107.

[77] E.G. Govorunova, O.A. Sineshchekov, P. Hegemann, Desensitization and dark recovery of the photoreceptor current in *Chlamydomonas reinhardtii*, Plant Physiol. 115 (1997) 633–642.

[78] K.A. Ketchum, R.J. Poole, Cytosolic calcium regulates a potassium current in corn (*Zea mays*) protoplasts, J. Membr. Biol. 119 (1991) 277–288.

[79] B. Bölter, J. Soll, K. Hill, R. Hemmler, R. Wagner, A rectifying ATP-regulated solute channel in the chloroplastic outer envelope from pea, EMBO J. 18 (1999) 5505–5516.