Ca²⁺ spikes in the flagellum control chemotactic behavior of sperm

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The events that occur during chemotaxis of sperm are only partly known. As an essential step toward determining the underlying mechanism, we have recorded Ca²⁺ dynamics in swimming sperm of marine invertebrates. Stimulation of the sea urchin *Arbacia punctulata* by the chemoattractant or by intracellular cGMP evokes Ca²⁺ spikes in the flagellum. A Ca²⁺ spike elicits a turn in the trajectory followed by a period of straight swimming ('turn-and-run'). The train of Ca²⁺ spikes gives rise to repetitive loop-like movements. When sperm swim in a concentration gradient of the attractant, the Ca²⁺ spikes and the stimulus function are synchronized, suggesting that precise timing of Ca²⁺ spikes controls navigation. We identified the peptide asterosap as a chemotactic factor of the starfish *Asterias amurensis*. The Ca²⁺ spikes and swimming behavior of sperm from starfish and sea urchin are similar, implying that the signaling pathway of chemotaxis has been conserved for almost 500 million years.

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Introduction

Eggs attract sperm using chemical factors—a process called chemotaxis (Miller, 1985; Suzuki, 1995; Eisenbach, 2004; Eisenbach *et al.*, 2004). In both mammals and marine invertebrates, the underlying cellular mechanism(s) and the behavioral strategy remain ill defined. Sperm swim on straight trajectories toward the source of chemoattractants and turn when swimming away from the source (Miller, 1985). It is believed that changes in the intracellular Ca²⁺ concentration

([Ca²⁺]_i) control the swimming trajectory (Brokaw *et al.*, 1974; Brokaw, 1979; Ward *et al.*, 1985; Schackmann and Chock, 1986; Cook and Babcock, 1993b; Cook *et al.*, 1994; Kaupp *et al.*, 2003; Matsumoto *et al.*, 2003; Spehr *et al.*, 2003; Wood *et al.*, 2003; Yoshida *et al.*, 2003; Harper *et al.*, 2004; Ishikawa *et al.*, 2004). However, support for this hypothesis is indirect. In sperm, whose cell membrane has been disrupted by detergent, [Ca²⁺] of the medium controls the asymmetry of the flagellar beat. At low [Ca²⁺]_i, flagella beat more symmetrically than at high [Ca²⁺]_i (Brokaw *et al.*, 1974; Brokaw, 1979). Thus, when sperm swim up-gradient on a straight trajectory, [Ca²⁺]_i is expected to be low, and when sperm swim down-gradient, [Ca²⁺]_i should become high (Cook *et al.*, 1994). However, these predictions seem to be incompatible with recent time-resolved studies (Kaupp *et al.*, 2003; Solzin *et al.*, 2004) of Ca²⁺ dynamics in sperm of the sea urchin *Arbacia punctulata*, a model system for sperm chemotaxis. Resact, the chemoattractant of *A. punctulata*, evokes a rapid and transient increase of [Ca²⁺]_i in sperm suspensions (Kaupp *et al.*, 2003; see, however, Nishigaki *et al.*, 2004; Shiba *et al.*, 2005). Stimulation of sperm with resact results in a transient increase of flagellar asymmetry and subsequent reorientation of the swimming direction ('turn'). Because both the Ca²⁺ entry and the motor response occur on a similar time scale, it is plausible that a rise of [Ca²⁺]_i evokes a turn in the swimming trajectory. These results predict that sperm should turn when swimming toward the source of the chemoattractant.

Another puzzle concerns the intracellular messengers involved in the opening of Ca²⁺-permeable channels (Kaupp *et al.*, 2003; Kirkman-Brown *et al.*, 2003; Eisenbach, 2004; Solzin *et al.*, 2004). In one model (reviewed in Darszon *et al.*, 1999, 2001), the increase of intracellular pH and cAMP concentration stimulates Ca²⁺ entry (Cook and Babcock, 1993a; Cook *et al.*, 1994; Darszon *et al.*, 1999, 2001; Wood *et al.*, 2003). In a competing model, cGMP activates Ca²⁺ entry, either directly or indirectly, without an obligatory role for cAMP or protons (Kaupp *et al.*, 2003). The different models may be reconciled by the finding that the Ca²⁺ entry stimulated by resact is composed of an early and a late component (Kaupp *et al.*, 2003) that may be controlled by cGMP and cAMP, respectively.

To gain further insight into the mechanisms that control sperm motility, we developed a sensitive laser-stroboscopic technique that allows the recording of changes in [Ca²⁺]_i in the flagellum of swimming sperm. Moreover, we employed a caged form of resact to establish a defined gradient of resact and to follow the swimming trajectory and changes in [Ca²⁺]_i during chemotaxis. Furthermore, we identified asterosap, a sperm-activating peptide of the starfish *Asterias amurensis*, as a chemoattractant. This enabled us to compare the signaling pathways and chemotactic behavior of two different species. We found that Ca²⁺ responses and swimming behavior of sperm from sea urchin and starfish are similar, suggesting that the signaling pathway and the

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mechanisms underlying chemotaxis have been preserved in species that diverged approximately 500 million years ago.

Results

Navigation of *Arbacia* sperm in a resact gradient

We recorded the trajectories before and after stimulation of *A. punctulata* sperm by either extracellular resact or intracellular cGMP. These molecules were released from their caged forms by a flash of UV light. In a shallow observation chamber, sperm swim in circles at the water–glass interface (Miller, 1985; Ward *et al*, 1985; Cook *et al*, 1994; Kaupp *et al*, 2003). Upon release of cGMP, the waveform of the flagellar beat changed, and sperm temporarily left the circular swim-

ming mode (Kaupp *et al*, 2003). The new trajectory was characterized by alternating periods of high curvature ('turns' or 'bends') and low curvature ('straight swimming') (Figure 1A). After several seconds (6.8 ± 1.8 s, $n = 16$ (mean $\pm$ s.d., number of experiments)), sperm resumed their circular swimming mode, probably because the released cGMP has been degraded by phosphodiesterase activity (Kaupp *et al*, 2003). Initially, these circles were larger than before stimulation, but became gradually smaller until the original diameter had been reached. This behavior was faithfully reflected by the changes in the local curvature ($1/r$ (μm^{-1})) of the swimming trajectory (Figure 1A, inset). Shortly after stimulation, the curvature steeply increased and then decreased below the value before stimulation. This

Figure 1 Changes in swimming behavior of *Arbacia* sperm upon photolysis of caged cGMP and caged resact. (A) Swimming trajectory before (blue trace) and after (green trace) release of cGMP from DEACM-caged cGMP (red); the arrowhead indicates the start of the trajectory and small arrows indicate the direction of trajectory. The sections of the trajectory that represent a turn or bend are highlighted in black and by numbered arrows. The interval between consecutive dots is 80 ms. Scale bar, 50 μm . Inset: Changes in the local curvature ($1/r$) of the trajectory after stimulation by cGMP; arrow: UV flash. Negative values of the curvature indicate that the trajectory becomes 'concave' versus 'convex' with respect to the normal of the observation plane. The numbers denote peaks in the curvature and the corresponding sections of the trajectory. The dashed black line highlights the slow component of the decrease of curvature. (B) Trajectories (upper) and curvature (middle) of three sperm cells swimming in a resact gradient that was produced by local UV irradiation (square). Sperm were suspended in artificial sea water (ASW) containing caged resact (50 nM). Scale bar in the upper panel represents 100 μm . Horizontal bars in the middle panels indicate the duration of the UV irradiation. The lowest panel shows the trajectory of sperm no. 1 in the boxed time periods a–c. Numbers 1–3 refer to the peaks in curvature of box b. (C) Changes in swimming speed (black) and curvature (red) upon stimulation with cGMP in the absence of extracellular Ca^{2+} . The decrease in curvature expected from an increase in swimming speed is shown in green. Inset: Trajectory before (blue) and after the flash (green). Scale bar, 50 μm .

spike-like change recurred several times before the curvature gradually returned to prestimulation values. The last stage reflects the transition from larger to smaller circles.

Furthermore, we examined the swimming pattern of sperm in a resact gradient. The gradient was established by diffusion of resact that had been photo-released in a given area of the chamber. After the release of resact, sperm accumulated in the irradiated area, while the surrounding area became depleted of sperm (Supplementary movie S1). Figure 1B (upper panels) shows the trajectories of three cells located outside of the irradiated area. Sperm left their circular trajectory several seconds after irradiation. The delay is qualitatively accounted for by diffusion of released resact. The trajectories were also characterized by consecutive periods of high and low curvature (Figure 1B, middle panel), which produced epicycloid-like movements, that is, cycles whose center moves on a curved or meandering trajectory toward the source. Similar epicycloid movements toward the source have been observed in all 39 analyzed cells. This pattern is characteristic of sperm from many different species (Miller, 1985). When sperm swim in a gradient, the changes in the curvature of trajectories were smaller and less regular than the changes evoked by cGMP. When sperm sensed the gradient, circles first became larger and then smaller again when sperm approached the source (Figure 1B, upper and lower panels). The changes in circle diameter probably reflect adaptation to persistent stimulation. Taking into account the changes in swimming velocity after stimulation (see below), the trajectory can be reconstructed from the curvature profile with high fidelity (not shown).

The swimming pattern can be understood as the result of two different processes, spike-like increases in curvature (turns) superposed on a *slow* continuous decrease of curvature (straighter trajectory) that slowly returns to the prestimulus value. This pattern can be observed both upon activation by cGMP and resact (Figure 1A, inset, and Figure 1B, middle panel, dashed black lines). In the absence of extracellular Ca^{2+} , the spike-like changes in curvature were abolished, whereas the slower continuous changes remained unaffected (Figure 1C). This experiment demonstrates that the curvature spikes require Ca^{2+} , whereas the slower changes do not. Furthermore, motor responses were accompanied by an increase of swimming speed (see, for example, distances between sperm heads before and after the first turn in Figure 2A). The mean speed before and after stimulation was 197 ± 59 and $284 \pm 105 \mu\text{m s}^{-1}$ ($n = 8$), respectively (mean relative increase 1.44; $P < 0.01$; unpaired, two-tailed *t*-test). The increase of swimming speed was largely independent of external Ca^{2+} . The similar time course of both processes suggests that the slow decrease of curvature is caused by speeding of sperm (Figure 1C). In fact, a simple kinematic relation ($1/r = \omega/v$, ω refers to angular frequency and v to speed; Gray, 1955) predicts a decrease in curvature due to an increase in speed (Figure 1C, green trace).

Ca^{2+} spikes in swimming *Arbacia* sperm

We examined the hypothesis that the succession of turns and linear swimming episodes is produced by the resact- and cGMP-induced dynamics of $[\text{Ca}^{2+}]_i$. More specifically, do sperm turn when $[\text{Ca}^{2+}]_i$ is rising, and do trajectories become straighter when $[\text{Ca}^{2+}]_i$ is declining? To this end, we

Figure 2 Ca^{2+} dynamics in swimming *Arbacia* sperm induced by photolysis of caged cGMP. (A) Ca^{2+} fluorescence signals from a single cell along its trajectory after stimulation by cGMP (BECMCM-caged cGMP). The inset shows the trajectory and the direction of movements before (blue trace) and after (green trace) the stimulus (red dot); the arrowhead indicates the start. (B) Changes in fluorescence and curvature before, during, and after the four turns or bends in the trajectory of panel A. Changes in Ca^{2+} fluorescence (blue) and curvature (red) (lower panels) along segments 1–4 shown in the upper panels are also shown. Note the different scales of ordinates. The interval between consecutive images is 60 ms. Scale bars, 50 μm . Colors indicate a linear scale of fluorescence intensity: dark blue, 0 photons pixel^{-1} ; red, 450 photons pixel^{-1} .

loaded sperm with the fluorescent Ca^{2+} indicator dye Fluo-4 and with caged cGMP, and simultaneously recorded the swimming trajectory and changes in $[\text{Ca}^{2+}]_i$ after the release

of cGMP. Figure 2 and Supplementary movie S2 show the fluorescence images of a cell along the trajectory before and after stimulation. The flagellum of unstimulated sperm was nonfluorescent, whereas the head was fluorescent. The fluorescence of the head probably results from the Ca^{2+} -filled acrosome and mitochondria. The release of cGMP elicited Ca^{2+} spikes in the flagellum; thus, the flagellum became intermittently visible. In the four segments of the trajectory, where a spike-like increase of $[\text{Ca}^{2+}]_i$ occurred (Figure 2B, upper part of panels 1–4), $[\text{Ca}^{2+}]_i$ began to rise before the curvature increased and before the sperm turned (Figure 2B, lower part of panels 1–4). This result demonstrates that sperm adopt a new swimming direction after a rise of $[\text{Ca}^{2+}]_i$. The Ca^{2+} changes in the flagellum appeared as a train of asymmetrical spikes with the following properties. The delay for the first Ca^{2+} spike was 0.23 ± 0.06 s ($n=8$), which agrees with the delay of Ca^{2+} responses measured in sperm suspensions (Kaupp *et al*, 2003). The time-to-peak of a Ca^{2+} spike (ca. 60–120 ms) was shorter than the decay time (Figure 2B, lower part of panels 1–4, and Figure 3, upper panel), and the half-width was 0.4 ± 0.1 s ($n=8$). The short duration of spikes demonstrates that Ca^{2+} is removed from the cell by a powerful transport system, probably a flagellar $\text{Na}^+/\text{Ca}^{2+}-\text{K}^+$ exchanger (Su and Vacquier, 2002). The cGMP-induced Ca^{2+} spikes were largely similar in amplitude and ceased abruptly (Figure 3, upper panel; see Supplementary Tables S1 and S2); the mean duration of the oscillatory period was 6.6 ± 2.6 s ($n=8$), which matches the mean duration of the period between the flash and before sperm resume a circular swimming trajectory (4.9 ± 1.4 s, $n=7$). During the time period between the spikes, $[\text{Ca}^{2+}]_i$ drops below the detection limit. Because the curvature in-between spikes drops well below the value of unstimulated sperm, it is conceivable that $[\text{Ca}^{2+}]_i$ also drops below the resting value. At low $[\text{Ca}^{2+}]_e$ ($\leq 10^{-7}$ M), Ca^{2+} spikes were abolished.

In some cells, the pattern of the cGMP-induced Ca^{2+} elevations was more complex. For example, a Ca^{2+} spike was missing in a sequel of regularly spaced spikes, spikes were overlapping, or spikes were different in size (Supplementary Figure S1). Even in those examples, the

Figure 3 Ca^{2+} fluctuations in the flagellum (upper) and head (lower) after the release of cGMP (arrow); same cell as shown in Figure 2. Fluorescence intensity is given as photons per flagellum or head.

curvature faithfully reflected the changes in $[\text{Ca}^{2+}]_i$, and the number of Ca^{2+} spikes, whether small or large, correlated with the number of peaks in the curvature (Supplementary Tables S1 and S2).

These figures also illustrate that, while $[\text{Ca}^{2+}]_i$ was still above its resting level, the curvature fell to values that were significantly lower than those in unstimulated sperm, that is, the trajectory became straighter. This observation suggests that the kinetics of the relative changes in $[\text{Ca}^{2+}]_i$ rather than the absolute $[\text{Ca}^{2+}]_i$ controls the curvature. We propose that cGMP elicits two distinct processes that define a motor response unit: a Ca^{2+} -controlled increase of the asymmetry of the flagellar beat ('turn' or 'bend') and an opponent process that causes straight swimming. Although the straightening of the trajectory after a turn seems to be related to the decline of $[\text{Ca}^{2+}]_i$ from its peak value, additional factors may be involved in the control of the trajectory (Figure 1C).

The Ca^{2+} spikes in the flagellum came along with Ca^{2+} changes in the head. However, the relative changes in the head were up to 100-fold smaller than those in the flagellum (Figure 3, compare upper and lower panels). Moreover, $[\text{Ca}^{2+}]_i$ in the head fluctuated without obvious changes in the trajectory; therefore, changes in $[\text{Ca}^{2+}]_i$ in the head are unlikely to control the swimming behavior. The onset of the Ca^{2+} rise of the flagellum preceded the rise of the head by one or two frames, that is, 60–120 ms. Occasionally, Ca^{2+} fluctuations in the head occurred without corresponding changes in the flagellum. In contrast to the flagellum, the Ca^{2+} dynamics in the head was characterized by two components, a steady increase of $[\text{Ca}^{2+}]_i$ superimposed by small Ca^{2+} fluctuations (Figure 3, lower panel). Steady and oscillatory components of changes in $[\text{Ca}^{2+}]_i$ have been observed in the head of immobilized sperm; however, changes in the head were larger than those in the flagellum (Wood *et al*, 2003).

Stimulus function and Ca^{2+} dynamics

We recorded the trajectories and changes in $[\text{Ca}^{2+}]_i$ while sperm were swimming in a resact gradient (Figure 4; see also Supplementary movie S3). Sperm were suspended in caged resact (Kaupp *et al*, 2003); a gradient was established by irradiation with UV light that was not homogeneously distributed. The profile of the gradient was derived from the spatial distribution of the flash energy (Figure 4A). After the gradient had been established, Ca^{2+} spikes appeared and sperm began moving up-gradient (Figure 4B). The pattern of Ca^{2+} spikes of sperm that swim in a gradient was more complex than that stimulated by cGMP (Figure 4C). In particular, the initial burst of larger Ca^{2+} spikes was followed by smaller Ca^{2+} fluctuations. The spikes became smaller when sperm approached the source and when the gradient became shallower. We attribute this difference to the continuous stimulation and the initiation of adaptation.

Soon after the onset of stimulation, changes in $[\text{Ca}^{2+}]_i$, whether small or large, were closely correlated with changes in the curvature (Figure 4C). At a later stage, when sperm had returned to an almost circular trajectory, $[\text{Ca}^{2+}]_i$ continued to spike in most cells, albeit with smaller amplitude; the corresponding changes in curvature became smaller and almost indistinguishable from the noise level. This behavior is particularly pronounced in sperm nos. 4 and 5 (Figure 4C; see also Supplementary Figure S2 for sperm nos. 1–3). The

Figure 4 Ca^{2+} dynamics and trajectories of *Arbacia* sperm cells swimming in a resact gradient. (A) Contour lines of concentrations of the resact gradient established by a flash of UV light. (B) Swimming trajectories of five sperm cells in the resact gradient. Black traces represent trajectories before and colored traces after photolysis of caged resact (150 nM) (red dots). Resact concentration is depicted as a topographical map of the gradient shown in panel A. Adjacent lines represent steps of 2.5 of the relative resact concentration. Scale bar, 50 μm . (C) Changes in $[\text{Ca}^{2+}]_i$ (fluorescence, blue) and curvature (red) during chemotaxis of sperm nos. 4 and 5 in the resact gradient. The trajectory analysis of sperm nos. 1–3 is shown in Supplementary Figure S2. The numbers of the panels refer to the sperm number in panel B. Note the different scaling of the ordinates in the panels. Lower panel: Trajectory of sperm No. 5 during the boxed periods a–c. Numbered arrows indicate the turns during the curvature peaks 1 and 2.

small changes in curvature probably reflect minute adjustments in the diameter and position of the circles. The reduction of the Ca^{2+} spike activity and the minute adjustments probably reflect adaptive processes in the sustained presence of the attractant. Such adaptive mechanisms may also explain why some sperm display changes in curvature without a Ca^{2+} spike and *vice versa*. We suspect that a Ca^{2+} threshold must be reached to elicit a turn and that this threshold is variable.

Due to the loop-like trajectory, sperm swimming in a gradient encounter a more or less periodic change of resact concentration ('stimulus function'). Are the stimulus function and the Ca^{2+} spikes correlated? To answer this question, we performed a crosscorrelation analysis, which reveals the temporal relationship between two time series, for example, the changes in resact concentration and Ca^{2+} spikes. Figure 5A shows the stimulus function and the Ca^{2+} responses along the sperm trajectory in a gradient. The stimulus function first increases instantaneously followed by a periodic increase due to the epicycloid movement of sperm toward the source. It is plausible that our experimental paradigm recapitulates features of the time-dependent pattern of resact concentrations encountered by sperm in their natural habitat, that is, a combination of a sudden increase of

resact due to convections and currents in the sea water and a chemical gradient. The step-like stimulation of sperm with resact (homogeneous irradiation, no gradient) also elicits a train of Ca^{2+} spikes, similar to those elicited by cGMP (Supplementary Table S3). We examined the possibility that the first few Ca^{2+} spikes produced by the step-like increase of resact compromise the analysis. However, the result was largely independent of whether we included or excluded the initial Ca^{2+} spikes (data not shown). Such an outcome is expected if the Ca^{2+} spike frequency and angular frequency of swimming are similar. Future work needs to address this possibility in more detail. The analysis reveals that stimulus function and Ca^{2+} spikes are synchronized (Figure 5B). The phase shift between the crosscorrelation and the autocorrelation of the stimulus function was 280 ± 92 ms ($n = 19$) or $156 \pm 43^\circ$. The delay of the Ca^{2+} increase and, consequently, of the motor responses seems to be a crucial element for the adjustment of the trajectory.

Attraction of *Asterias* sperm by asterosap

We have chosen asterosap, a peptide from the starfish *A. amurensis*, to re-examine the relative importance of cAMP and cGMP for Ca^{2+} entry, because asterosap binds to a receptor GC and activates only a cGMP-signaling

Figure 5 Stimulus function and Ca^{2+} response of *Arbacia* sperm navigating in a resact gradient. (A) Time course of the stimulus function (green) and changes in Ca^{2+} fluorescence (blue). The resact gradient (shown in Figure 4A) was established by a flash of UV light (arrow) resulting in a step in the stimulus function. As a result of the repetitive loop-like movement toward the center of the gradient, the sperm cell is repetitively exposed to increasing and decreasing resact concentrations producing a periodic stimulus function. The Ca^{2+} spikes follow the periodic stimulus function with a delay. (B) Autocorrelation of the stimulus function (green) and crosscorrelation with the train of Ca^{2+} spikes (blue); same experiment as in panel A.

pathway (Matsumoto *et al*, 2003). However, the function of asterosap is not clear. At high concentrations (1 μM), it serves as a cofactor for acrosomal exocytosis (Nishigaki *et al*, 1996); at picomolar concentrations, asterosap stimulates Ca^{2+} entry similar to that observed in sea urchin sperm when stimulated by resact (Kaupp *et al*, 2003; Matsumoto *et al*, 2003; Solzin *et al*, 2004).

To test the hypothesis that asterosap serves as a chemoattractant, we recorded the swimming behavior of sperm near a source of asterosap. Like sea urchin sperm, unstimulated *A. amurensis* sperm swim in circles parallel to the surface of the chamber. The circle diameter was $81.3 \pm 14.8 \mu\text{m}$ ($n = 15$). After a minute ejection (0.1–0.3 μl) from a capillary containing 1 μM asterosap, sperm were attracted to the opening of the capillary (Supplementary movie S4). In most experiments, sperm accumulated in front of the tip within ~ 20 s (Figure 6A). The average number of sperm in a given area near the capillary mouth was 6.8 ± 4.0 ($n = 16$) before the ejection and 14.5 ± 6.9 thereafter, that is, a 2.1-fold increase of sperm density. Cells far from the asterosap source kept their original swimming trajectory for some time, probably because the gradient had to build up by diffusion. Sperm

Figure 6 Swimming behavior of *Asterias* sperm upon stimulation with asterosap. (A) Accumulation of sperm in front of the capillary filled with asterosap. Distribution of sperm before (left) and 17 s after the ejection of 1 μM asterosap (right) is shown. Number of sperm in the dotted square is as follows: $n = 12$, before; $n = 33$, after ejection. The black dashed line represents opening of the capillary. (B) Swimming trajectories of four single sperm cells before (left) the ejection of asterosap and thereafter (right). The interval between consecutive dots is 80 ms. Scale bars, 100 μm . Inset: Trajectory of a single sperm (red).

reoriented their swimming trajectory toward the capillary in epicycloid-like movements (Figure 6B), generated by alternating periods of higher and lower curvature of the trajectory (see below; Figure 7). Thus, the behavior of sperm from the sea urchin *Arbacia* and the starfish *Asterias* was similar in all aspects, including swimming in very narrow circles (not shown) in the presence of asterosap and IBMX, an inhibitor of phosphodiesterases (Kaupp *et al*, 2003). These results show that asterosap is a chemotactically active peptide.

cGMP-induced motor responses of *Asterias* sperm

We studied the changes in swimming behavior brought about by cyclic nucleotides that were released from caged compounds inside *A. amurensis* sperm by a flash of UV light. The motor responses of *Asterias* and *Arbacia* sperm were astonishingly similar. Upon the release of cGMP, the trajectory was characterized by alternating periods of higher and lower curvature; after some seconds, sperm resumed swimming in circles; the circles were initially larger than the circles before stimulation, but became gradually smaller and the curvature gradually returned to prestimulus values (Figure 7A); changes in the local curvature faithfully reflected changes in the swimming trajectory ('turns' and straight swimming; Figure 7B).

The epicycloid-like trajectories of sperm in an asterosap gradient (Figure 6B) were also characterized by consecutive periods of higher and lower curvature; however, the curvature fluctuations were smaller and less regular than those induced by cGMP (compare Figure 7B and C). The steady decrease of the curvature (Figure 7C, arrow) indicates that the circles become larger when sperm are approaching the chemoattractant source.

Figure 7 Swimming behavior of *Asterias* sperm upon stimulation with cyclic nucleotides and asterosap. (A) Swimming trajectory of a single sperm cell upon stimulation with DEACM-cGMP. Scale bar, 100 μm . Inset: Magnification of the area marked by dashed lines. Blue traces, before; green traces, after UV flash; red dot, UV flash. The interval between consecutive dots is 80 ms. (B) Curvature of the trajectory shown in panel A. Arrow, UV flash; numbered arrows, assigned changes in trajectory and curvature. Dashed line highlights the slow decrease of curvature. (C) Curvature of a trajectory in an asterosap gradient. The asterisk indicates time of asterosap ejection and the arrow tonic decrease of the curvature. (D) Curvature of a trajectory after the release of cAMP by a UV flash (arrow). Inset: Trajectory before (blue) and after (green) the UV flash (red).

Under similar experimental conditions, cAMP did not evoke a motor response. Both the diameter and shape of the circles did not change upon the release of cAMP (Figure 7D, inset) and the curvature did not reveal discrete fluctuations above the noise level (Figure 7D). The 'noise' in the curvature is caused by digitalization error and the rolling movement of the head (Gray, 1955). Only after long incubation (4 h) with caged cAMP, motor responses upon the UV flash were detected (not shown). These results (1) identify cGMP as the principal messenger that mediates chemotaxis of starfish sperm and (2) show that the behavioral responses evoked by chemoattractants and cGMP in sperm from sea urchins and starfish are similar.

cGMP-induced Ca^{2+} fluctuations in swimming *Asterias* sperm

Both asterosap and cGMP cause an increase of $[\text{Ca}^{2+}]_i$ (Matsumoto *et al*, 2003). We tested the hypothesis whether the succession of turns and straight swimming, as in *Arbacia*, is produced by cGMP-induced changes in $[\text{Ca}^{2+}]_i$. To this end, we repeated the experiments shown in Figures 2 and 3 with starfish sperm. The results were qualitatively similar (Figure 8).

The Ca^{2+} fluctuations were variable. Three different examples are shown in Figure 9A. Most cells responded with a train of discrete Ca^{2+} spikes. Sometimes, the Ca^{2+} response was composed of overlapping spikes (see Figure 9A, left and right panels). The delay of the evoked Ca^{2+} influx into the flagellum was 0.17 ± 0.04 s, $n = 14$, and the time-to-peak of the first spike was 0.2 ± 0.1 s (Supplementary Table S4). The

Ca^{2+} fluctuations ceased either abruptly (Figure 9A, middle panel) or decreased steadily (right panel). The duration of the Ca^{2+} fluctuations in the flagellum was 2.7 ± 1.3 s (Supplementary Table S4). In most sperm, Ca^{2+} spikes in the flagellum were accompanied by Ca^{2+} changes in the head (Figure 9A, lower panel). Several cells responded with a small gradual increase of $[\text{Ca}^{2+}]_i$ in the head or no change at all. As in sea urchin, the relative changes in fluorescence of the flagellum were significantly larger than those of the head.

As in sea urchins, we examined the hypothesis that the changes in $[\text{Ca}^{2+}]_i$ control the swimming behavior. Therefore, we compared the timing of the Ca^{2+} spikes and the changes in the curvature. In 14 experiments, with one exception, the rise of $[\text{Ca}^{2+}]_i$ preceded the increase of the curvature (Figure 9B). Occasionally, the curvature changed without a Ca^{2+} spike. The number of Ca^{2+} spikes and discrete peaks in the curvature were 7.1 ± 2.5 and 8.0 ± 2.6 , respectively (Supplementary Table S3). Thus, there is a close correspondence between Ca^{2+} spikes and changes in curvature. When $[\text{Ca}^{2+}]_i$ was still well above its resting level, the curvature had decreased to values that were significantly lower than those before stimulation, that is, the trajectory became straighter (Figure 9B). We propose that, as in sea urchin sperm, a motor response unit is composed of two distinct parts: a turn or bend followed by straighter swimming.

As in sea urchin sperm, stimulation by cGMP also changed the swimming speed (Figure 9C). After the UV flash, the speed first increased within 1 s and then gradually returned to values before stimulation. The mean speed before and

Figure 8 Ca^{2+} signals during swimming of *Asterias* sperm upon stimulation by cGMP. (A) Trajectory before (upper) and after stimulation (lower). Colors indicate a linear scale of the changes in fluorescence: dark blue, 0 photons $pixel^{-1}$; red, 2000 photons $pixel^{-1}$. The interval between images is 60 ms. (B) Single motor response unit (turn and straighter swimming) of three different cells. The trajectory in the lower panel shows two turns. Arrows indicate the swimming direction. The interval between consecutive dots is 60 ms. Scale bars, 50 μm . (C) Normalized fluctuations of curvature and $[Ca^{2+}]_i$ of the single motor response units shown in panel B. Blue, Ca^{2+} fluctuations; red, curvature; dashed lines indicate basic levels before stimulation. Arrows indicate UV flash.

Figure 9 Responses of *Asterias* sperm upon stimulation with cGMP. (A) Ca^{2+} fluctuations in the flagellum (upper) and head (lower) from three different cells. Numbers of photons are given per flagellum or head. During the flash, no measurements of the resting fluorescence in the head were feasible. (B) Curvature and Ca^{2+} fluctuations in the flagellum along the trajectory. Blue, Ca^{2+} fluctuations; red, curvature. Numbers refer to identified 'peaks' of the changes in $[Ca^{2+}]_i$ and curvature. (C) Speed of sperm along the trajectory. Arrows indicate UV flash.

after stimulation was $334 \pm 86 \mu m s^{-1}$ ($n = 14$) and $446 \pm 88 \mu m s^{-1}$, respectively (mean relative increase 1.33; $P < 0.001$).

cAMP did not induce Ca^{2+} fluctuations in starfish sperm (not shown). This result agrees with kinetic studies, where the cAMP-induced Ca^{2+} response measured in a sperm

suspension was about 10 times smaller than the cGMP-induced Ca^{2+} response (Matsumoto *et al*, 2003). Probably the cAMP-induced changes in $[\text{Ca}^{2+}]_i$ are too small to be detected in single sperm and to elicit a motor response.

Discussion

Here, we show that during chemotaxis, the swimming trajectory of sperm is controlled by Ca^{2+} spikes in the flagellum. Ca^{2+} spikes elicit turns followed by periods of straight swimming, which represents a motor response unit ('turn-and-run'); the trajectory is a composite of consecutive response units. When sperm sense a positive gradient of the attractant, the line-up of response units produces directed epicycloid-like movements; that is, the center of the circles moves on a curved or meandering trajectory toward the source.

The most important elements of our model of sperm chemotaxis are shown in Figure 10. We assume that sperm sense the increasing concentration of chemoattractant during a sampling period. After a latency of several hundreds of milliseconds, a Ca^{2+} spike is elicited and gives rise to a transient increase of the flagellar asymmetry and thereby of the curvature. Because of the phase shift between the stimulus function and the Ca^{2+} response, sperm continue to

Figure 10 Model of chemotactic behavior of sperm. In a gradient of chemoattractant (background), a sperm cell (green), swimming on a circular trajectory, detects an increase of the chemoattractant as long as it moves up-gradient (sampling phase; black trace). When swimming down-gradient (yellow circle segment of about 160° (ϕ), corresponding to about 300 ms), the cell suffers a Ca^{2+} influx (spike) and, as a consequence, the curvature of the trajectory transiently increases (red). Thereafter, the curvature decreases below the original value and, again moving up-gradient, the cell encounters the next stimulus. Several repetitions of these events finally direct the sperm to the source.

swim down-gradient for almost half a circle before turning (Figure 10). Thereafter, the flagellar asymmetry and curvature relax below resting levels, whereby the circle becomes displaced toward the source. During this 'run', the next sampling of chemoattractant occurs. This model solves the conundrum why sperm do not turn swimming up-gradient, even though the chemoattractant stimulates an increase of $[\text{Ca}^{2+}]_i$.

An important factor is the relation between the changes in $[\text{Ca}^{2+}]_i$ and the curvature during a turn. If the turn is too short or too long, or if the curvature is either too high or too low, sperm will be heading the wrong direction during the subsequent run. There is no simple relation between the amplitude of Ca^{2+} spikes and the strength of the motor response. Furthermore, there seems no simple relationship between $[\text{Ca}^{2+}]_i$ and the curvature of the trajectory. Instead, the motor response 'adapts' during a train of Ca^{2+} spikes. The locale of adaptation is not known; it may reside at different sites in the cGMP-signaling pathway and in the motor proteins themselves. Adaptation may be set off by Ca^{2+} itself, but mechanisms independent of Ca^{2+} may also contribute. A comprehensive model of chemotaxis must incorporate the interplay between excitation and adaptation while sperm swim in a gradient of the chemoattractant.

An essential feature of bacterial chemotaxis is that cells register both ascending and descending gradients by means of the occupation level of their receptors (Eisenbach *et al*, 2004). It is intriguing that our model does not require the ability to sense a descending gradient. *Arbacia* sperm are exquisitely sensitive; they respond to picomolar concentrations of resact. Given the extremely high binding affinity (K_D of the order of a few pM for the related peptide speract; Nishigaki and Darszon, 2000), binding of resact to the flagellum is essentially irreversible (Kaupp *et al*, 2003). Consequently, resact is not expected to rapidly dissociate from the receptor and, therefore, receptor occupancy cannot be readjusted when sperm swim down-gradient. However, it cannot be excluded that sperm may use a different mechanism to sense descending gradients, for example the rate at which resact is captured by receptors.

The number of molecules that hit an ellipsoid flagellum can be calculated according to (Berg, 1993): $N = L4\pi Dac / \ln(2a/b)$, where L is the Avogadro's number, D the diffusion coefficient of resact, c the resact concentration, and a and b the two axes of the ellipsoid. With $D = 3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $a = 50 \mu\text{m}$, $b = 0.30 \mu\text{m}$, and $c = 1 \text{ pM}$, we obtain $N = 18 \text{ molecules s}^{-1}$. The number of successful binding events will be even smaller. Taking into account the low K_D value and the high density of peptide receptors (several 10 000/flagellum; Shimomura and Garbers, 1986; Shimizu *et al*, 1994; Nishigaki and Darszon, 2000), the binding process is far from chemical equilibrium. Corollaries of this notion are that sperm register relative changes rather than absolute chemoattractant concentrations, and that the sensitivity is limited by the number of molecules that bind to the receptor during a sampling period. Considering that only a fraction of impinging molecules become captured by the receptor during a sampling period of 200–500 ms, a lower limit for the incremental resact concentration that evokes a behavioral response is of the order of a few picomoles.

When spatially unrestricted, sperm swim on a helical trajectory (Crenshaw, 1989). The two-dimensional projection

of a straight helix parallel to its axis yields a circle. The epicycloid-like traces shown in Figures 1B, 4B, and 6B suggest that, in a three-dimensional space, the helix becomes bent and the sperm approaches the egg on a helical trajectory whose axis becomes stepwise directed to the source. Thus, sperm should display true chemotaxis in contrast to bacteria, which undergo a biased random walk (Eisenbach *et al*, 2004). Further insight into the unrestricted swimming behavior of sperm in a well-defined gradient will require three-dimensional tracking or computer simulations.

Another key finding is that cellular signaling and swimming response are conserved among echinoderms. We have shown that starfish sperm reorient their swimming trajectory in a gradient of asterosap and accumulate near the source. Moreover, in a previous study (Matsumoto *et al*, 2003), we have shown that picomolar concentrations of asterosap stimulate Ca^{2+} entry into sperm. Finally, asterosap shares a high degree of sequence similarity with the N-terminal region of a sperm-attracting protein ('startrak'), isolated from immature eggs of the starfish *Pycnopodia helianthoides* (Miller and Vogt, 1995). These findings identify asterosap as a chemoattractant.

Unlike in sea urchin, asterosap does not noticeably increase the cAMP concentration (Matsumoto *et al*, 2003), and cAMP evokes neither a motor response nor Ca^{2+} spikes in the flagellum (Figure 7D). Therefore, cAMP is unlikely to play an essential role in chemotactic signaling of starfish sperm. This result may help to resolve conflicting views of the signaling events in sea urchin sperm. In one model, a rise of cAMP and pH_i activates Ca^{2+} channels (Darszon *et al*, 1999, 2001). In particular, it has been proposed that changes in cAMP concentration produce Ca^{2+} fluctuations in the head of immobilized sperm (Wood *et al*, 2003). We, however, proposed that a rise of cGMP either directly or indirectly stimulates Ca^{2+} entry (Kaupp *et al*, 2003). Apart from some quantitative differences, the signaling events in starfish and sea urchin do not differ substantially. The similarities of (1) the Ca^{2+} fluctuations, (2) the motor response, and (3) the swimming behavior in a gradient of the chemoattractant argue that the chemotactic cGMP-signaling pathway has been conserved in species that diverged approximately 500 million years ago (Smith, 1988; Wada and Satoh, 1994).

A corollary of this conclusion is that cAMP also does not take center stage in chemotaxis of sea urchin sperm. However, the Ca^{2+} flux into *Arbacia* sperm displays two kinetic components—early and late (Kaupp *et al*, 2003). While the initial rapid Ca^{2+} transient is evoked by an increase of cGMP, the messenger that mediates the late Ca^{2+} entry is not known. It has been proposed that the slower and smaller increase of cAMP in *Arbacia* sperm might give rise to the late Ca^{2+} entry (Kaupp *et al*, 2003). The function of the late Ca^{2+} entry is not known. Future work is needed to identify the function of the two Ca^{2+} components, the ion channels involved, and their gating mechanisms.

While our results provide fundamental insights into the chemotaxis of sperm from marine invertebrates, much less is known about chemotaxis in mammals (Eisenbach, 2004; Eisenbach *et al*, 2004). Neither the chemical nature of the chemoattractant nor the receptors have been identified. However, both cyclic nucleotides and Ca^{2+} have been suggested to play a role in the cellular signaling events. Calcium imaging in swimming sperm together with the application of

caged compounds may also shed light on the chemotaxis of mammalian sperm.

Materials and methods

Reagents and solutions

Dry sperm of *A. punctulata* and *A. amurensis* was obtained as described (Matsui *et al*, 1986; Kaupp *et al*, 2003) and diluted with ASW, which contained 423 mM NaCl, 9.27 mM CaCl_2 , 9 mM KCl, 22.94 mM MgCl_2 , 25.5 mM MgSO_4 , 0.1 mM EDTA, and 10 mM HEPES, adjusted to pH 7.8 with NaOH. The synthetic asterosap isoform P15 was used (CQTQFGVC**IARVRQQHQGQDEASIF-QAILSQC**QS, intramolecular disulfide bond between two Cys*) (Nishigaki *et al*, 1996).

The caged compounds [6,7-bis(ethoxycarbonylmethoxy)coumarin-4-yl)methyl-cGMP (BECMCM-caged cGMP) or (7-diethylamino-coumarin-4-yl)methyl cGMP (DEACM-caged cGMP) and resact modified with [S-(4,5-dimethoxy-2-nitrobenzyl)] at the Cys⁸ residue (caged resact) were used as described (Kaupp *et al*, 2003) (see below).

Measurement of changes in $[\text{Ca}^{2+}]_i$

We measured changes in $[\text{Ca}^{2+}]_i$ using the indicator dye Fluo-4 (Molecular Probes, Eugene, OR). Incubation of sperm with Fluo-4AM and caged cGMP was as described (Kaupp *et al*, 2003), except that 20–40 μM Fluo-4AM was used. The sperm density in the observation chamber was adjusted by $1:10^4$ – 10^5 dilution with ASW plus 0.2% Pluronic F127 (Molecular Probes). Single sperm cells were observed through an inverted microscope (IX70, Olympus, Hamburg, Germany). The bottom and top of the chamber were made from coverslips (22 mm $\times$ 22 mm, 0.17 mm); the spacing was fixed by adhesive tape. The chamber had an area of 256 mm² and a depth of approximately 20–30 μm (volume $\sim$ 5–8 μl). The temperature of the chamber was kept at 17°C.

The fluorescence was excited at 488 nm with an Ar-ion laser (Stability 2018, Spectra-Physics, Darmstadt, Germany). CW laser light of 25 mW was fed into the microscope through quartz fiber optics and a dual-port imaging condenser (TILL Photonics, Martinsried, Germany) at the epifluorescence port of the microscope. The fiber optics was vibrated by means of a vortex unit to reduce the spatial coherence of the laser light. A mechanical shutter (Uniblitz LS6, Vincent Associates, Rochester, NY, USA) was used to illuminate the sample in a stroboscopic mode (frequency of 16.6 or 20 Hz); the exposure time was approximately 2 ms. Thereby, sharp images of rapidly moving cells were generated.

The other port of the condenser hosted the quartz fiber optics by which the UV flash for photolysis of caged compounds was delivered ($\sim$ 0.5 ms, UV flash unit; TILL Photonics). Alternatively, photolysis was accomplished using a 100 W mercury lamp and U-RFL-T burner (Olympus, Hamburg, Germany). The irradiation time was set by a shutter (Uniblitz, LS6Z2 and VMM-T1). The UV flash was imaged via the Koehler illumination method. The UV irradiation was reflected and filtered through a short-pass filter (410 nm, TILL Photonics). The irradiated area was adjusted by means of a rectangular or circular diaphragm. In order to establish a concentration gradient of resact in the observation chamber, the optics of the UV part was slightly defocused. The resulting profile of the spatial distribution of the flash energy was measured by means of light scattered at the glass–liquid interface.

Light coming from both condenser ports was reflected via a dichroic mirror (500DLRP, Omega Optical, Brattleboro, VT, USA) into the optical path of the microscope. The fluorescence, collected by an objective (UApo/340 20/0.75; infinity corrected (Olympus)), 0.17 mm coverslip corrected), was passed through the dichroic mirror and an emission filter (535DF30, Omega Optical). Fluorescence was detected with a Peltier-cooled CCD camera (CoolSnap HQ, Roper Scientific, Trenton, NJ, USA), positioned at the intermediate image plane of the microscope's side port. For each experiment, a sequence of 100–300 frames was collected. The frame acquisition frequency of the camera was adjusted to the shutter frequency.

We chose a semiautomatic approach to extract the fluorescence signal from the frames. By generating a digital mask for the head and for the flagellum and multiplying each mask element with the picture element (pixels) of a frame, the fluorescence was

determined in photons per head or flagellum. The digital masks were designed with the help of the coordinates of the head and flagellum: first, the coordinates were defined as the center or the central line of the mask, respectively. Second, the center of the mask was dilated with a star-shaped structuring element. The operations were repeated until the mask fitted the dimensions of head and flagellum.

Recording and analysis of swimming trajectories

We determined the following local parameters along the trajectories: the coordinates of the cell, the swimming speed, and the local curvature of the trajectory. The trajectory was constructed from the coordinates of the center of the head and the set of coordinates along the flagellum. We analyzed only trajectories that met the following conditions: first, sperm coordinates can be assigned unequivocally. Second, sperm swim in the focal plane of the microscope during the entire recording time. Third, sperm show a distinct change in their motility pattern after stimulation.

The mathematical details of the data processing were as follows. The local curvature ($1/r$) was derived from the first and second derivatives of a two-dimensional parametric curve according to $1/r = \dot{x}\ddot{y} - \dot{y}\ddot{x}/(\dot{x}^2 + \dot{y}^2)^{3/2}$ (Bronstein and Semendjajew, 1991). We determined the first and second derivatives by convolution with the symmetric difference operator of the fourth order (Pao, 1999). Because the trajectory is constructed from discrete coordinates, the derivatives are noisy and were smoothed with a Savitzky-Golay filter (Savitzky and Golay, 1964).

If sperm swim clockwise or counter-clockwise with respect to the observation plane, the curvature would be positive or negative, respectively. In order to allow comparison of all trajectories, the coordinates of cells swimming counter-clockwise were mirrored at the y-axis and then translated along the x-axis. If sperm changes its swimming direction from left to right, the curvature changes from positive to negative values.

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Chemotaxis assay of starfish sperm

Astrosap was dissolved in ASW (1 μ M) and injected into the observation chamber by a microliter syringe (701 N Hamilton, Bonaduz, Switzerland) through a plastic capillary that was made from a pipette tip. The injected volume was 0.1–0.3 μ l.

Stimulus function and correlation analysis

Sperm swim in a plane parallel to the bottom or top of the observation chamber. The flagellar beat is restricted to this plane. The stimulus function can be defined as

$$S(t) = \iint_{A_f} c(\vec{P}(t)) dA_f$$

wherein A_f is the envelope area of the flagellar beat and $c(\vec{P}(t))$ is the relative attractant concentration sensed by the flagellum at position $\vec{P}(t)$. To determine the phase shift between the stimulus function and the changes in $[Ca^{2+}]_i$, the crosscorrelation between the stimulus function and the Ca^{2+} signal was compared with the autocorrelation of the stimulus function. To distinguish between the response due to the step increase of resact concentration and the subsequent quasi-oscillating stimulus function, the first Ca^{2+} spike was omitted from the analysis.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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