Behavioral response of human spermatozoa to a concentration jump of chemoattractants or intracellular cyclic nucleotides

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Introduction

During the last decade, it became clear that mammalian spermatozoa possess the ability to respond chemotactically to substances secreted from the mature oocyte and its surrounding cumulus cells as well as to a number of other substances (Eisenbach and Giojalas, 2006). One of the major questions in sperm chemotaxis is how the cells sense a chemoattractant gradient: do they compare the chemoattractant concentration over time (temporal sensing) or over spatial points (spatial sensing)?

In principle, spermatozoa may either sense a temporal gradient of the chemoattractant, comparing the occupancy of its receptors at different time points [as bacteria do (Macnab and Koshland, 1972)], or they may detect a spatial gradient, comparing the occupancy of receptors at different locations along their body [as leukocytes do (Devreotes and Zigmond, 1988)]. In the case of sea-urchin spermatozoa, Kaupp et al. (2003) found that a sudden temporal increase in the concentration of their chemoattractant (resact) causes a behavioral response even when there is no spatial gradient, suggesting that these spermatozoa sense a temporal gradient. The situation in mammals is not known. The aim of this study was to find out whether human spermatozoa can sense and respond to a temporal chemoattractant gradient.

Materials and Methods

Bourgeonal was obtained from Biomol (Exeter, UK). Caged compounds (7-diethylaminocoumarin-4 yl)methyl (DEACM)-cyclic adenosine monophosphate (cAMP) and DEACM-cyclic guanosine monophosphate (cGMP) were obtained from Biolog (Bremen, Germany). Pluronic F127 was obtained from Molecular Probes (Carlsbad, CA, USA). All other chemicals—progesterone, EGTA, A23187, *Pisum sativum* agglutinin labeled with fluorescein isothiocyanate (FITC-PSA), phosphate-buffered saline—were from Sigma (Rehovot, Israel). The medium BWW was essentially Biggers–Whitten–Whittingham medium—95 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM Na₂HCO₃, 20 mM sodium lactate, 5 mM glucose and 0.25 mM sodium pyruvate, pH 7.4 (Biggers et al., 1971)—supplemented with HEPES (50 mM, pH 7.4) and bovine serum albumin (fraction V; 3 mg/ml). The commercial human tubal fluid (HTF) medium was obtained from Irvine.
Spermatozoa
Ejaculates of normal, healthy donors were allowed to liquefy at room temperature and then treated in one of the following ways (as indicated in the text):

(i) Total sperm population. The semen was washed twice in BWW or HTF by centrifugation at 120 \( \times \) g for 20 min; the spermatozoa in the pellet were resuspended in the corresponding medium and adjusted to a concentration of 10–20 \( \times \) 10⁶ cells/ml.

(ii) Motile subpopulation. Motile human spermatozoa were separated from the seminal plasma by swim-up, using the migration–sedimentation device of Hauser et al. (1992), as described by Sun et al. (2005).

After both preparation procedures, the sperm concentration was adjusted according to the experimental need and the sperm suspension was incubated under an atmosphere of 5% CO₂ at 37°C for 2 h to obtain capacitated spermatozoa (Cohen-Dayag et al., 1995).

(iii) Non-capacitated total population. The semen was washed once by centrifugation at 120 \( \times \) g for 5 min in albumin-free BWW, resuspended in the same medium and adjusted to a sperm concentration of 3–4 \( \times \) 10⁶ cells/ml.

(iv) Non-capacitated motile subpopulation. Semen was divided into two aliquots. For one aliquot, the seminal plasma was separated from the spermatozoa by 10 min centrifugation at 12 000 \( \times \) g and the supernatant was collected. The other aliquot was used for the migration–sedimentation procedure, in which the seminal plasma was added to the albumin-free medium (20% v/v) and this medium substituted for the regular medium.

Evaluation of the capacitation level
Capacitated spermatozoa were identified according to their ability to undergo the acrosome reaction as described by Jaiswal et al. (1999). Acrosome-reacted spermatozoa were identified with FITC-PSA according to Mendoza et al. (1992). Two major fluorescence patterns were identified: one with the acrosome completely fluorescent (acrosome intact) and the other showing only an equatorial fluorescent band (acrosome-reacted). Spermatozoa that were completely fluorescent or transparent (dead cells) were not included in the counting. Therefore, the total number of counted cells (considered as 100%) included live spermatozoa only.

Mixing assay
An aliquot of a sperm suspension (9 μl of ‘motile subpopulation’) was put on a microscope slide and mixed with 1 μl of either the chemoattractant or, as a negative control, the suspending medium. The sperm movement, observed with a phase contrast microscope (Nikon; \( \times \) 10 objective), was video-recorded and subsequently analyzed by homemade motion analysis software. The experiments were carried out at 37°C on top of a thermostated heating stage (Tokai Hit Co., Japan). In the experiments aimed at determining the requirement for extracellular Ca²⁺, EGTA was added 1 min prior to the experiment to both the sperm suspension and the solution to be mixed with it. In addition, MgCl₂ was added, as appropriate, to keep the ionic strength unchanged.

We studied the response to the human sperm chemotacticants progesterone and bourgeonal. On mixing spermatozoa with either progesterone or bourgeonal, the spermatozoa transiently increased their VCL (hereafter referred to as ‘velocity’) and lateral head displacement, leading to a transient decrease in the linearity of swimming (Fig. 1A–F). At the peak of the response, they exhibited

Figure 1 Response of human spermatozoa to a temporal gradient of progesterone and bourgeonal.
(A) Tracks of spermatozoa before stimulation. (B) Tracks of spermatozoa at the first measurable time point after the addition of progesterone (0.1 μM final concentration). All the tracks are 1 s long. (C, D) Effect of progesterone concentration on the curvilinear (full-path) velocity and the linearity of swimming, respectively. Red, 1 μM; green, 0.1 μM; blue, 10 nM; turquoise, 1 nM; black, negative control (addition of the suspending medium). The results shown are of a typical experiment (out of eight). (E, F) Effect of bourgeonal concentration on the curvilinear velocity and the linearity of swimming, respectively. Red, 100 μM; green, 10 μM; blue, 1 μM; black, suspending medium. The results shown are of a typical experiment (out of four). (G, H) Effect of the free Ca2+ concentration on the sperm response to progesterone (1 μM). The free Ca2+ concentration in the suspending medium was reduced by EGTA and calculated using MaxChelator software (Stanford, USA). Red, 2 \times 10^{-3} M free Ca2+ concentration in the suspending medium; green, 10^{-4} M; blue, 10^{-5} M; cyan, 10^{-6} M;agenta, 10^{-7} M; black, negative control (addition of the suspending medium without progesterone; EGTA absent). The results shown are of a typical experiment (out of three). Each experimental point in Panels C–H represents a kinematic parameter averaged over a 5 s interval. The point t = 0 is defined as the initiation of mixing and the values shown at this point were measured for 5 s just prior to the mixing. The interval t = 0–5 corresponds to the ‘dead’ time of the mixing experiment. To avoid arbitrary fluctuations, all the measured values in Panels C–F were normalized to the control value at t = 0. Each experimental point represents the mean ± SEM of five repetitions, each repetition containing 50–70 tracks.
hyperactivated-like motility (Supplementary data, Movie S1). These results suggest that human spermatozoa can sense and respond to temporal gradients of progesterone and bourgeonal.

To assess the dependence of the behavioral response on the chemoattractant concentration, we analyzed the response in the concentration ranges 1 – 100 nM progesterone (Fig. 1C and D) and 1 – 100 μM bourgeonal (Fig. 1E and F). In the case of progesterone, the threshold of the response appeared to be between 1 and 10 nM, and the adaptation time (i.e., the time until the non-stimulated swimming behavior was restored) seemed to increase with the progesterone concentration, as is the case in the response of bacteria to chemoattractants (Spudich and Koshland, 1975; Segall et al., 1986). In the case of bourgeonal, the threshold was between 1 and 10 μM. With both stimuli, the threshold concentration varied between different ejaculates but remained in the specified region. As expected for temporal assays involving time-dependent changes in the stimulus concentration, these thresholds were orders of magnitude higher than those measured in spatial gradients of progesterone (Teves et al., 2006) and bourgeonal (Spehr et al., 2003) (see Discussion for explanation).

Ca²⁺ influx through gated Ca²⁺ channels was proposed to be one of the molecular events that occur during the chemotactic response of human spermatozoa to bourgeonal (Spehr et al., 2006). To determine whether the behavioral responses to progesterone and bourgeonal require Ca²⁺, we repeated the above measurements in the presence of increasing concentrations of EGTA, yielding extracellular Ca²⁺ concentrations 10⁻⁷ – 10⁻² M. We found that the responses to each of these stimuli required Ca²⁺ concentrations > 10⁻⁸ M (Fig. 1G and H for progesterone; similar results were obtained with bourgeonal—data not shown).

Temporal behavioral changes stimulated by a step-increase in the intracellular concentration of cyclic nucleotides

To avoid the disadvantage of the mixing approach, which misses the initial behavioral response, we wished to use caged compounds that, upon illumination, would release the chemoattractant instantaneously. However, the currently known chemoattractants, progesterone and bourgeonal, lack the chemical groups (e.g., a hydroxyl group) that would allow fast chemoattractant photorelease from the caged form (J. Corrie, personal communication). Instead, we employed intracellular caged cAMP and cGMP, the rationale being (i) that the chemotactic response of human spermatozoa to bourgeonal or to NO/cGMP pathway-modulating substances may involve changes in the intracellular level of cAMP (Spehr et al., 2004) or cGMP (Miraglia et al., 2007), respectively, and (ii) that, in sea-urchin spermatozoa, photorelease of cGMP evokes a behavioral response similar to that observed upon the photorelease of the chemoattractant resact (Kaupp et al., 2003).

For the measurements, we pre-loaded spermatozoa with the caged form of the cyclic nucleotide in the range of 1 – 100 μM. (Since the efficiency of the cyclic nucleotide photorelease intracellularly from its caged compound is not known, the cyclic nucleotide concentrations, mentioned below, refer to the loading concentrations of the caged form.) Following the photorelease of cAMP or cGMP intracellularly, some of the spermatozoa responded with a change in the swimming behavior, as described below. Here, too, the response was dependent on the presence of extracellular Ca²⁺ (data not shown). The fraction of responsive cells depended on both the identity and concentration of the cyclic nucleotide. Thus, while the threshold of the sperm response to cAMP was 1 μM, the threshold of the response to cGMP was 10 μM. Similarly, the response to cAMP was saturated at ~ 10 μM (where up to 75% of the cells responded) and the response to cGMP was saturated at ≥ 100 μM (≥ 92% of the cells).

About half of the cells responded when the cyclic nucleotide concentration was ~ 5 μM (cAMP) or ~ 45 μM (cGMP). Like any other sperm function, the response also depended on the sperm donor and sample. Since the efficiencies of photorelease of the cyclic nucleotides from their respective caged compounds were shown to be the same (Hagen et al., 2003), the results suggest that the cell is more sensitive to intracellular cAMP than to cGMP.

The behavioral responses usually consisted of a delayed increase in the lateral head displacement and decreased linearity, in most cases transient (Fig. 2 for a few examples). The increased curvature often resulted in changes in the swimming direction (Fig. 2). (To avoid confusion, we emphasize that here, too, we do not deal with changes in the swimming direction relative to the gradient direction because in this study, unlike in chemotaxis assays, there is no chemoattractant gradient over space; the gradient is only over time and equal in all directions.)

Importantly, in spite of the different experimental settings and the different stimuli, the responses to chemoattractants (in the mixing experiments) and cyclic nucleotides (in the photorelease experiments) were quite similar and shared a few common features. Thus, in both cases, the sperm velocity and the amplitude of lateral head displacement increased, and the linearity decreased. Furthermore, in spite of the difference between the measurable time windows in each case and the different stimulus intensity in each case, the results in both approaches were generally consistent, with the response to cyclic nucleotides lasting for ~ 20 s (Fig. 2) and the response to chemoattractants lasting for up to 40 – 50 s (Fig. 1). Moreover, in mixing experiments that yielded long enough tracks allowing track comparison between both approaches, the similarity was evident (Fig. 3). This holds even for the delay. In most cases, the delay in the response to cyclic nucleotides was within the ‘dead time’ preceding the first measurable time point in the mixing experiments. However, in some cases, the delay was clearly observed even in the mixing experiments (e.g., middle track in Fig. 3A).

The responses to the photorelease of cyclic nucleotides varied from cell to cell. We, therefore, divided the responses to four major arbitrary groups according to the extent and rate of changes in the straightness and the amplitude of lateral head displacement (see Materials and Methods for definitions), as changes in these kinematic parameters allowed better discrimination between groups. Prior to stimulation, the straightness of most of the cells was close to 100% and the amplitude of lateral head displacement did not exceed 1 μm.

Group I included spermatozoa that exhibited a slight and continuous increase in amplitude (Fig. 2, Panel I-B) or decrease in linearity (Panel I-C), accompanied by a gradual change in the swimming direction (Panel I-A). In this group, the straightness parameter remained essentially unchanged (having a value of 95 – 100%) (Panel I-C) and we did not notice any consistent trend in the swimming velocity: some cells changed their curvilinear (full-path) velocity in response...
to the cyclic nucleotide photorelease (Panel I-D), while others did not (not shown in the figure). The responses observed in this group were similar to those observed in mixing experiments when the added chemotactrant concentration was low.

Spermatozoa belonging to Group II displayed a gradual but pronounced increase in the amplitude of head displacement (Panel II-B) [reflected also in decreased linearity (Panel II-C)], concomitantly with a pronounced increase in their velocity (VCL; Panel II-D). Although these spermatozoa exhibited marked changes in the swimming direction (Panel II-A), the changes in straightness were moderate (from ~100 to 80%; Panel II-C) and confined to the turning points at the swimming path. The responses observed in this group were similar to those observed in Group III (Panel III-A). This was reflected in a straightness decrease down to 40–60% (Panel III-C). The responses observed in this group were similar to those observed in mixing experiments in some cells immediately after the mixing.

Group IV included spermatozoa whose response was so strong that, following a fast increase in amplitude, their swimming came to a halt for a few seconds (Panel IV-B, C, D), with their flagella essentially arrested in a circular shape (Inset in Panel IV-A) (Supplementary data, Movie S3). Even in the mixing experiments, we occasionally observed the ‘arrest’ response in spite of the relatively long ‘dead time’.

Since spermatozoa belonging to Groups III and IV had smaller progressive motility, we could follow them for longer periods of time before they swam out of the observation field. With these cells, we noticed that some cells exhibited several repetitive phases of response. For example, cells belonging to Group III, became
hyperactivated for 5 ± 2 s (± SEM) after a delay of 1 ± 1 s following
the cyclic nucleotide photorelease, and then again there was a delay of
9 ± 3 s followed by 5 ± 2 s of hyperactivation (14 cells). Cells belong-
ing to Group IV were arrested for the first time for 6 ± 4 s after 1 ±
1 s delay, and then again became arrested (in most cases) or hyperac-
tivated (in some cases) for 3 ± 1 s after a delay of 11 ± 4 s (17 cells).

It is important to note that the borderlines between the four groups
were not always sharp and sometimes cells could belong to either one
of two groups. For example, 3–8% of the cells had characteristics
typical of Groups III and IV together: the cells had as high amplitude
as in tumbling (typical of the Group III) but, periodically, their flagella
became briefly ‘arrested’ (as in the inset of Panel IV-A). The periodical
arrest-states lasted for a fraction of a second each time.

The relative prevalence of each of the four response groups signifi-
cantly depended on the identity of the stimulating cyclic nucleotide
and its concentration (Fig. 4). The largest difference was in Group IV, fluc-
tuating around 20% in the case of cAMP and around 50% in the case of
cGMP. The larger abundance of Group IV in the case of cGMP
appeared to be on account of Groups I and III, which were lower.
This stronger response to cGMP, in spite of being concentration-wise
less effective, may suggest that the two cyclic nucleotides have differ-
et targets (see Discussion).

The delay of the response was characterized by large inter- and
intra-sample variability (Fig. 5). Within this large variability, the delay
was dependent neither on the cyclic nucleotide nor on its concen-
tration, except for the case of 10 μM cAMP where the delay was
higher than at other concentrations (Fig. 5).

Independence of the response
on the capacitated state

It is well established that, in spatial chemoattractant gradients, only
capacitated spermatozoa are chemotactically responsive [for a
review, see Eisenbach (1999)]. This has been shown for human sper-
matzoa with follicular fluid (Cohen-Dayag et al., 1994, 1995), oocyte-
and cumulus-conditioned media (Sun et al., 2005) and the chemoo-
tractants bourgeonal (Spehr et al., 2006) and progesterone (Teves
et al., 2006). Wishing to examine whether this restriction holds for
the temporal responses observed above, we compared the fraction
of capacitated spermatozoa in the samples studied [determined by
the ability of the cells to undergo a Ca2+-ionophore-induced acro-
some reaction, a property that is restricted to capacitated spermato-
"zoo only (Cohen-Dayag and Eisenbach, 1994)] with the fraction of
responsive cells. Although the fraction of capacitated cells in the
samples studied was in the range of 7–30%, the fraction of responsive
cells in both the mixing and photorelease experiments was much
larger. Depending on the magnitude of the stimulus, the fraction of
responsive cells could be as high as 80%, suggesting that the measured
temporal response is not restricted to capacitated spermatozoa only
and is therefore apparently inconsistent with the hypothesized link.
between the temporal and chemotactic responses (however, see Discussion).

To verify the conclusion that non-capacitated spermatozoa also responded to the concentration jump, we studied spermatozoa that were not allowed to capacitate. Capacitation is initiated once the seminal fluid is removed from the sperm suspension, and albumin (or any other compound which promotes the release of cholesterol from sperm plasma membrane) is present (Visconti et al., 1999). The fraction of capacitated spermatozoa reaches a maximal steady-state level after 1–2 h incubation at 37°C (Giojalas et al., 2004). In view of this, we formed sperm populations poor in capacitated cells by suspending spermatozoa in an albumin-free medium shortly after seminal fluid removal or in an albumin-free medium supplemented with seminal fluid (10–25%; seminal fluid inhibits capacitation for several hours [(Mortimer et al., 1998; Tomes et al., 1998; Suzuki et al., 2002) and our own, unpublished results]. These populations, which contained only ~3% or less capacitated cells (depending on the treatment), swam slower and more linearly than parallel sperm populations incubated in albumin-containing medium. Nevertheless, both types of populations exhibited the same relative chemotactic responses, as they do in bacteria (Khan et al., 1992, 1993), with the criteria being loss of the temporal response either in the presence of chemotaxis inhibitors or with non-chemotactic sperm mutants. Neither such inhibitors nor such mutants are available for mammalian sperm chemotaxis. Therefore, the utmost that can be done is assessment whether the results are consistent with a chemotactic response (see below). Historically, the same was true in other chemotaxis systems, and temporal responses were considered chemotactic much before these criteria were put to the test. This holds, for example, for sea-urchin spermatozoa (Kaupp et al., 2003) [(Kaupp et al., 2006) for a review] and even for the best studied system of chemotaxis, that of bacteria, where the temporal response was considered chemotactic already in the early 1970s (Macnab and Koshland, 1972; Spudich and Koshland, 1975), but the above criteria were first examined two decades later (Khan et al., 1992).

The responses in both approaches appear to be common

An obvious question is whether the responses to chemoattractants and cyclic nucleotides reflect a common response. The probability that they do seems fairly high because, in spite of the different experimental settings and the different identity and magnitude of stimuli in each setting, the changes in behavior shared a few common features. Thus, in both cases, the sperm velocity and the amplitude of lateral head displacement increased. Likewise, the time scales of both

**Figure 5** Concentration-dependent delay of the response to cyclic nucleotides.

(A) Response to cAMP. An asterisk above the box-plot indicates a significantly higher delay than at others concentrations ($P < 0.05$, Kluskal–Wallis test with Dunn’s post-test). (B) Response to cGMP. Each panel includes data obtained from a single sperm donor (out of three). The delay was not dependent on the cGMP concentration ($P > 0.05$, Kluskal–Wallis test). Because the distribution of data points was not normal in each of the panels (according to the Kolmogorov–Smirnov test), the data are shown as boxes representing statistical values. The higher and lower boundaries of the box indicate the 75th and 25th percentiles, respectively; the line within the box marks the median. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively. Experimental points outside the 10th–90th percentiles are shown as black dots. The total number of spermatozoa at each concentration was 24–43.
responses were comparable, with the response to cyclic nucleotides being delayed for ~1 s and lasting for ~20 s and the response to chemoattractants being detectable from the first measurable point (~5 s after mixing) and lasting for up to 40–50 s. The changes in swimming behavior, seen in the tracks of swimming spermatozoa, were similar in both settings (Fig. 3).

Variations in the response

Unlike the mixing experiments, where we could only perform population analysis (due to the need to carry out the measurement in an open drop to allow rapid mixing and to minimize the dead time, resulting in cells swimming in three dimensions and going out of the observation field before sufficiently long tracks could be monitored), in the photorelease experiments (where the cells were viewed between two glasses) we could also carry out single-cell analysis. This enabled us to notice large inter- and intra-sample variations in the response to photorelease of cyclic nucleotides. The intra-sample variations led us to divide the sperm population into four major, arbitrary groups according to the intensity of their responses (Fig. 2). The fact that we observed no significant difference between the abundances of the groups in capacitated and non-capacitated spermatozoa suggests that the multiplicity of response intensities is not a reflection of different sperm ripeness levels. They could, however, be a reflection of different maturation stages of ejaculated spermatozoa (Amann et al., 1993), or just a reflection of intercellular variations in the expression levels of proteins, a common feature of biological systems (Kaern et al., 2005). Large inter-sample variations have frequently been reported for mammalian spermatozoa (Heuchel et al., 1983; Nallella et al., 2004; Keel, 2006), including pronounced variability in the basal levels of cAMP and cGMP between different ejaculates of the same donor as well as between semen samples of different individuals (Willipinski-Stapelfeldt et al., 2004).

Relation of the results to chemotaxis

A major question of this study is whether the temporal responses observed in this study are related to chemotaxis. Indeed, as mentioned above, in the absence of chemotaxis inhibitors and mutants this question cannot be answered directly. Nevertheless, the answer to this question appears positive because a number of observations, outlined below, are consistent with chemotaxis and none seem to contradict it.

Supporting evidence

(i) Transient nature of the response. Every chemotactic response to a step change in a stimulant concentration is transient due to adaptation (Springer et al., 1979). The temporal responses to progesterone and bourgeonal (Fig. 1) as well as to cAMP and cGMP (Fig. 2) were transient.

(ii) Duration and extent of the response. The duration and extent of chemotactic temporal responses are dependent on the stimulus magnitude, i.e. on the added stimulant concentration, the higher the concentration—the stronger and longer the response (Spudich and Koshland, 1975; Khan et al., 1993). The response to the chemoattractants was indeed longer at higher chemoattractant concentrations (Fig. 1), and the responses to both the chemoattractants and cyclic nucleotides were more intense when the stimulant concentrations were higher.

(iii) Components of the response. The response of human spermatozoa to a spatial chemoattractant gradient is composed of three elements: a directional response (chemotaxis), enhanced velocity (chemokinesis) and eventually hyperactivation (Ralt et al., 1994). All these elements were transiently observed in the temporal responses (Figs 1 and 2).

(iv) Ca2+ dependence. One of the molecular events thought to occur during the chemotactic response of human spermatozoa to bourgeonal (Spehr et al., 2006), and progesterone (Publicover et al., 2008) is Ca2+ influx through gated Ca2+ channels. It is, therefore, expected that any chemotactic response would be Ca2+-dependent. The response to each of the stimuli was dependent on extracellular Ca2+.

(v) Commonality. In the well-characterized system of sea-urchin spermatozoa, the first two steps of the response to the chemoattractant, resact, consist of a delay followed by turns or tumbling (Kaupp et al., 2003). This is very similar to the response observed with human spermatozoa (Figs 1–3). Note, though, that the basic swimming mode of sea-urchin spermatozoa is different from that of human spermatozoa in that they swim in circles rather than straight forward. Consequently, in sea-urchin spermatozoa, the first two steps of response are followed by a step of swimming in a rather straight line (Kaupp et al., 2003).

Apparent difficulties and other options

(i) Chemoattractant concentrations higher than in chemotaxis assays. The chemoattractants concentrations used in the mixing experiments were orders of magnitude higher than those used in chemotaxis assays. For example, in the mixing experiments (termed also ‘temporal assays’), the progesterone concentration was in the range 10−9–10−6 M (Fig. 1), whereas the peak responses to progesterone in chemotaxis assays involving spatial gradients (termed also ‘spatial assays’) are in the range 10−12–10−10 M and at 10−6 M (Teves et al., 2006). This means that, excluding the 10−6 M concentration, we observed a response to progesterone concentrations that are ineffective as chemoattractants in spatial assays. This is a well-known phenomenon in chemotaxis, resulting from the basic difference between temporal and spatial assays. First, the concentration dependence in temporal assays is a conventional saturation curve; in contrast, in spatial assays of chemotaxis, it is a peak dependence, resulting from the fact that saturated receptors cannot sense the chemoattractant gradient and, consequently, the cells do not respond to the gradient when the chemoattractant concentration is too high (Eisenbach, 2004a). This may well lead to a situation in which a temporal response is observed with a chemoattractant concentration that is chemotactically ineffective because of being too high. Second, the threshold concentration (i.e. the minimal concentration needed for detecting a response) in temporal assays is always higher than the threshold in spatial assays, meaning that temporal assays require larger concentration changes to make the response detectable (Macnab and Koshland, 1972; Brown and Berg, 1974; Segal et al., 1986; Khan et al., 1993; Kaupp et al., 2003). This is because in temporal assays, where the concentration of the stimulant changes all at once, the response is
too subtle to be detected when the concentration change is as small as in spatial chemotaxis assays. In contrast, in spatial gradients the subtle response is integrated over time and becomes detectable even when the change in stimulant concentration is small (Block et al., 1982). Thus, the higher concentrations that had to be used in the mixing experiments are consistent with a chemotactic response.

(ii) **Capacitation level.** It is well established that only capacitated spermatozoa are chemotactically responsive in spatial assays (Cohen-Dayag et al., 1995; Eisenbach, 1999; Fabro et al., 2002). Therefore, the lack of correlation between the fraction of capacitated spermatozoa and the fraction of spermatozoa that behaviorally responded to the stimulus, either a chemoattractant or a cyclic nucleotide, appears to question the suggestion that the temporal responses reflect chemotactic responses. However, capacitation is a multi-process event, with many of the processes occurring at the sperm surface level (for a review, see Jaiswal and Eisenbach (2002)). It is not known which of these processes is obligatory for the occurrence of chemotaxis and what makes a capacitated cell chemotactically responsive. It is reasonable that the main difference between capacitated and non-capacitated spermatozoa with respect to chemotaxis is their sensitivity to chemoattractants, with the threshold detection level being significantly lower in capacitated cells (i.e. having higher sensitivity). Thus, according to this rational assumption, a response can be observed in temporal assays even with non-capacitated spermatozoa because the changes in stimulant concentrations in these assays are sufficiently high to stimulate even these low-sensitive cells.

(iii) **Hyperactivation response.** Since progesterone is known to trigger human spermatozoa to become hyperactivated (Uhler et al., 1992), one could argue that the results reflect this well described effect rather than a chemotactic response. We believe that there is no conflict between the two. First, the time scale between the published measurements of hyperactivation in response to progesterone and our measurements were different, 10 min in the former (Uhler et al., 1992), and up to 40 s in the latter (Fig. 1). However, this difference in the duration of the response between the two studies could potentially be explained by the difference in the experimental setup (tube in Uhler et al., an open drop in the present study) or by the existence of two phases of response: a strong, fast and transient phase of response detected in the time scale of this study, and a weaker, prolonged phase of response measured at later time points. Second, hyperactivation is not an independent phenomenon; it is a term reflecting a certain swimming mode (Yanagimachi, 1994; Ho and Suarez, 2001). As explained below, it is quite possible that very brief episodes of hyperactivation-like turns are part of the chemotactic response (Ralt et al., 1994) just like episodes of tumbling are part of the chemotactic response of *Escherichia coli* (non-stimulated *E. coli* tumble occasionally; the tumbling frequency changes in response to a stimulus (Macnab and Koshland, 1972)).

In conclusion, the results are well consistent with the possibility that the changes in swimming behavior in the temporal assays are expressions of chemotaxis.

**A model: manifestation of the temporal response in a spatial gradient**

The consistency of the results with a chemotactic response suggests that human spermatozoa, like bacteria (Macnab and Koshland, 1972) and sea-urchin spermatozoa (Kaupp et al., 2003), detect temporal changes even when they are in a spatial gradient. In other words, they respond to time-dependent changes in receptor occupancy rather than to the occupancy of receptors at different ends of the cell. On the basis of this suggestive conclusion and the characteristics of the temporal response, we propose the following model of what is happening in a spatial chemoattractant gradient (Fig. 6).

According to the model, the response characteristics observed in temporal gradients also occur in spatial gradients: an excitation phase, composed of a delay (i.e. no change in the motility parameters) and a subsequent turn, and an adaptation phase during which the cell ceases to respond to the chemoattractant even though it is still present (Figs 1 and 2). When a spermatozoon swims up a spatial gradient (the positive gradient in Fig. 6), the turning step is repressed. When it swims down a gradient (the negative gradient in Fig. 6) or in no gradient, the cell turns. In the latter case, if not re-stimulated,
the cell eventually adapts and resumes its original swimming mode. It should be noted that the turns would be more subtle and rare than in the temporal assays because of the much weaker and more gradual stimulation.

An important feature of the model is that cessation of sensing an increase in the chemoattractant concentration is sufficient to stimulate a turn. This is because some chemoattractants have very low $K_d$ values [e.g. resact in the case of sea-urchin spermatozoa (Kaupp et al., 2003) and progesterone in the case of mammalian spermatozoa (Eisenbach and Giojalas, 2006)] and may bind irreversibly to their cognate receptors, suggesting that spermatozoa cannot sense decreasing gradients of these chemoattractants.

This model, though speculative, is consistent with all the available information about sperm behavior in a chemoattractant gradient. Thus, in all species with external fertilization whose sperm behavior has been investigated in a spatial chemoattractant gradient [sea-urchin (Böhmaer et al., 2005), starfish (Böhmaer et al., 2005), ascidians (Yoshida et al., 1993) and mollusks (Miller, 1977)], the spermatozoa exhibit turns when moving away from the chemoattractant source. Human spermatozoa, too, have been shown to exhibit turns while moving away from the bourgeon source (Spehr et al., 2004).

Comparison between the responses to cAMP and cGMP

On the one hand, the responses to both cyclic nucleotides shared common features (e.g. similar groups of response patterns and similar delays). On the other hand, there were differences related to the sensitivities and intensities of the responses. Thus, the response to cAMP was an order of magnitude more sensitive to the cyclic-nucleotide concentration than the response to cGMP. In contrast, the response to cGMP was more intense, reflected in a higher fraction of responsive cells and in a higher fraction of cells exhibiting the ‘arrest’ pattern (Fig. 4; Supplementary data, Movies S2 versus S3). These observations are consistent with a possibility, raised earlier (Eisenbach, 2004b), that mammalian spermatozoa may possess several signaling pathways for chemotaxis, of which one involves cAMP modulation and the other, cGMP signaling. The opposing dependences of the sensitivity and intensity of the response on the identity of the cyclic nucleotide raise the possibility that cAMP and cGMP may have different targets. Our observations that the responses to both cyclic nucleotides were Ca$^{2+}$-dependent further support that the changes in the level of cAMP or cGMP precede the Ca$^{2+}$-requiring step. It may thus be that the cyclic nucleotides do not affect flagellar bending directly but rather causes (directly or indirectly) changes in the intracellular Ca$^{2+}$ concentration, which affect flagellar bending (Eisenbach, 2004b), or that Ca$^{2+}$ is required for an enzymatic activity that modulate (directly or indirectly) the bending.

Implications of the cyclic nucleotide-stimulated response for motility in general

cAMP has long been recognized as an important second messenger in the regulation of sperm motility (Tash and Means, 1982, 1988; Harrison, 2003). The function of cGMP in this capacity is still unclear. While all published studies have demonstrated long-term effects of cAMP or cGMP (timescale of minutes to hours), our current study demonstrates effects of these cyclic nucleotides on motility in a timescale of seconds. Another difference between our current study and published ones is the transient nature of the hyperactivated motility observed in our study in up to 70% of the cells, compared with a continuous response to cAMP and a smaller fraction of responsive cells (~15% in human (Calogero et al., 1998)). These differences raise at least two possibilities. One is that cyclic nucleotides fulfill regulatory roles in mammalian sperm motility at a number of levels or in a number of processes. Another possibility is that the time scale dissimilarity between the experiments reflects different rate-limiting steps in their setups. For example, in the published experiments, the intracellular cyclic nucleotide concentration was changed by using phosphodiesterase inhibitors or by employing permeant analogs of cyclic nucleotides or demembranated cells. It is not unlikely that in those experiments, the rate-limiting step of the observed changes in motility was the penetration of the compound into the cell, a step absent in our experiments, or that the slow penetration allowed compensating/adaptation processes to take place.

Generally speaking, the responses observed in the current study on a time scale of seconds, had a similar trend to those observed in published studies on longer time scales. These include an increase in hyperactivated motility when the intracellular cAMP level rises in boar (Harayama and Miyake, 2006), hamster (White and Aitken, 1989), bovine (Bajpai et al., 2006), primate (Yeung et al., 1999; Baumber and Meyers, 2006) and human (Calogero et al., 1998) spermatozoa. Unlike cAMP, the published information on the influence of cGMP on mammalian sperm motility is scarce and inconclusive (Cuadra et al., 2000; Lefevere et al., 2000; du Plessis et al., 2004). In contrast, cGMP effects on sperm motility are well documented for marine species such as sea-urchin and starfish, where this cyclic nucleotide was found to be involved in regulation of motility during sperm chemotaxis (Cook and Babcock, 1993; Kaupp et al., 2003; Matsumoto et al., 2003). In these species, consistent with our observations, a step-increase in the intracellular cGMP concentration causes a transient increase in flagellar asymmetry but with a much shorter delay (Cook and Babcock, 1993; Kaupp et al., 2003).

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