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Revisiting the Role of H⁺ in Chemotactic Signaling of Sperm

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ABSTRACT Chemotaxis of sperm is an important step toward fertilization. During chemotaxis, sperm change their swimming behavior in a gradient of the chemoattractant that is released by the eggs, and finally sperm accumulate near the eggs. A well established model to study chemotaxis is the sea urchin *Arbacia punctulata*. Resact, the chemoattractant of *Arbacia*, is a peptide that binds to a receptor guanylyl cyclase. The signaling pathway underlying chemotaxis is still poorly understood. Stimulation of sperm with resact induces a variety of cellular events, including a rise in intracellular pH (pH_i) and an influx of Ca²⁺; the Ca²⁺ entry is essential for the chemotactic behavior. Previous studies proposed that the influx of Ca²⁺ is initiated by the rise in pH_i. According to this proposal, a cGMP-induced hyperpolarization activates a voltage-dependent Na⁺/H⁺ exchanger that expels H⁺ from the cell. Because some aspects of the proposed signaling pathway are inconsistent with recent results (Kaupp, U.B., J. Solzin, J.E. Brown, A. Helbig, V. Hagen, M. Beyermann, E. Hildebrand, and I. Weyand. 2003. *Nat. Cell Biol.* 5:109–117), we reexamined the role of protons in chemotaxis of sperm using kinetic measurements of the changes in pH_i and intracellular Ca²⁺ concentration. We show that for physiological concentrations of resact (<25 pM), the influx of Ca²⁺ precedes the rise in pH_i. Moreover, buffering of pH_i completely abolishes the resact-induced pH_i signal, but leaves the Ca²⁺ signal and the chemotactic motor response unaffected. We conclude that an elevation of pH_i is required neither to open Ca²⁺-permeable channels nor to control the chemotactic behavior. Intracellular release of cGMP from a caged compound does not cause an increase in pH_i, indicating that the rise in pH_i is induced by cellular events unrelated to cGMP itself, but probably triggered by the consumption and subsequent replenishment of GTP. These results show that the resact-induced rise in pH_i is not an obligatory step in sperm chemotactic signaling. A rise in pH_i is also not required for peptide-induced Ca²⁺ entry into sperm of the sea urchin *Strongylocentrotus purpuratus*. Speract, a peptide of *S. purpuratus* may act as a chemoattractant as well or may serve functions other than chemotaxis.

KEY WORDS: Ca²⁺ • chemotaxis • cyclic nucleotides • fertilization • kinetics

INTRODUCTION

Protons are considered key players in the physiology of sperm of marine invertebrates (for reviews see Shapiro and Tombes, 1985; Garbers, 1989; Ward and Kopf, 1993; Darszon et al., 1999, 2001). In sea urchin, an increase in the intracellular pH (pH_i) has been proposed to control three distinct cellular events: the initiation of sperm motility, the chemotactic signaling, and the acrosome reaction.

Sperm are stored in a quiescent state in the gonads, but become motile within seconds after spawning into sea water (Gray, 1928; Ohtake, 1976). The transition from quiescence to full motility is initiated by an increase in pH_i (Goldstein, 1979; Christen et al., 1982, 1983; Johnson et al., 1983; Lee et al., 1983).

Eggs attract sperm by releasing peptides (Miller, 1985; Ward et al., 1985). The concentration gradient of a peptide provides cues that lead to an accumulation of sperm near the egg. This process requires the influx of Ca²⁺ ions from the external medium (Ward et al., 1985; Kaupp et al., 2003). Stimulation of sperm with peptides evokes both an increase in pH_i (Hansbrough and Garbers, 1981a; Repaske and Garbers, 1983; Lee and Garbers, 1986; Schackmann and Chock, 1986) and an increase in the concentration of intracellular Ca²⁺ ([Ca²⁺]_i) (Schackmann and Chock, 1986; Cook et al., 1994; Nishigaki et al., 2001; Kaupp et al., 2003; Wood et al., 2003). It has been proposed that the Ca²⁺ influx is initiated by the peptide-stimulated increase in pH_i (Cook and Babcock, 1993a; Cook et al., 1994). The pH_i increase may also serve as feedback mechanism to enhance the inactivation of the peptide receptor (Suzuki et al., 1984; Cook and Babcock, 1993b).

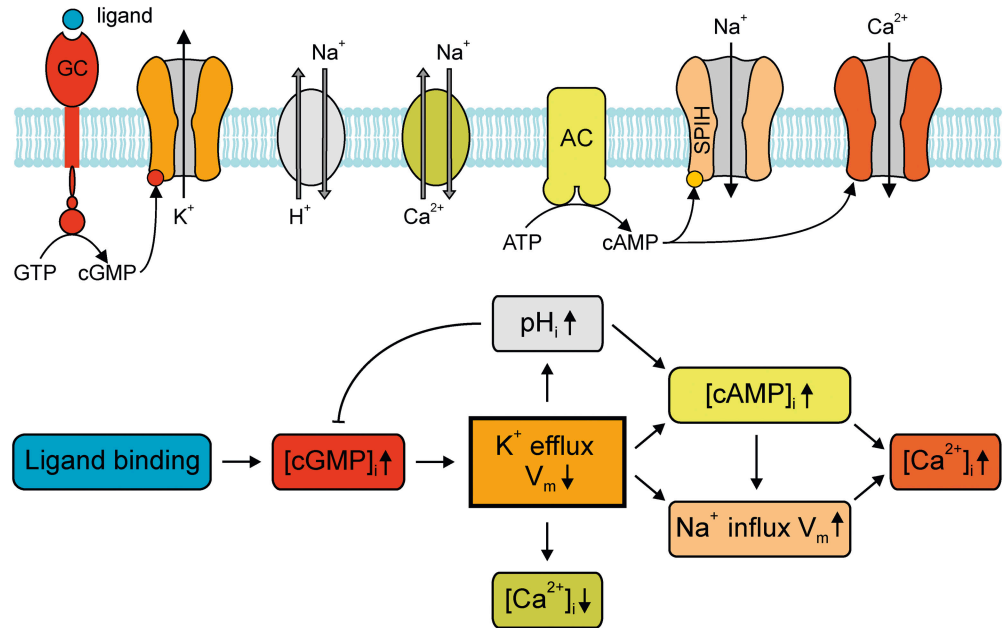
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Abbreviations used in this paper: AM, acetoxymethyl; ASW, artificial sea water; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein.

FIGURE 1. Model of the cellular pathway involved in chemotactic signaling of sperm (from Darszon et al., 1999, 2001). Top, molecular components; bottom, sequence of cellular events. Binding of peptides to a receptor guanylyl cyclase (GC) increases the intracellular cGMP concentration. The sperm hyperpolarizes by opening cGMP-regulated K^+ channels. Two transporters, a Na^+/H^+ and a Na^+/Ca^{2+} exchanger, export protons and Ca^{2+} , respectively, in response to the hyperpolarization; accordingly pH_i rises and $[Ca^{2+}]_i$ falls. The rise in pH_i activates an adenylyl cyclase. The increase in cAMP concentration causes an influx of Ca^{2+} . In principle, cAMP can stimulate the Ca^{2+} entry by two different mechanisms. First, the hyperpolarization and cAMP activate HCN channels (SPIH; Gauss et al., 1998); the ensuing depolarization of the cell activates voltage-dependent Ca^{2+} channels. Second, cAMP could activate a Ca^{2+} channel directly.



Finally, the interaction between factors on the egg coat and specific receptors on the sperm membrane triggers acrosomal exocytosis. A pH_i change appears to be an important mediator of the acrosome reaction (Trimmer et al., 1986; Guerrero and Darszon, 1989; for reviews see Darszon et al., 1999, 2001).

A mechanism underlying the pH_i increase has been proposed to be a voltage-sensitive Na^+/H^+ exchange (Lee, 1984a,b, 1985; Lee and Garbers, 1986; Babcock et al., 1992; Cook and Babcock, 1993a; Reynaud et al., 1993). Activation of the Na^+/H^+ exchange by a change in membrane voltage has been explained within a model of chemotactic signaling (Fig. 1; Darszon et al., 2001). The peptide binds to a receptor guanylyl cyclase and stimulates the synthesis of cGMP. According to the model, the membrane then hyperpolarizes due to the opening of cGMP-regulated K^+ channels; the hyperpolarization activates the export of protons from the cell. Because two features of the model—(1) an initial decrease in $[Ca^{2+}]_i$ followed by (2) a cAMP-mediated increase in $[Ca^{2+}]_i$ —are not supported by a recent study of time-resolved changes in cAMP and $[Ca^{2+}]_i$ (Kaupp et al., 2003), it became necessary to reexamine the role of protons in chemotaxis of sperm.

We have studied with intact motile sperm of the sea urchin *Arbacia punctulata* whether a rise in pH_i is prerequisite both for Ca^{2+} entry and for the chemotactic behavior. Using rapid-mixing techniques we show that, for physiological concentrations of resact, the chemoattractant of *A. punctulata*, the Ca^{2+} signal precedes the pH_i response. Moreover, a rapid increase in cGMP con-

centration does not evoke a pH_i increase. Finally, a pH_i buffer abolishes the resact-induced change in pH_i but leaves the Ca^{2+} signal and the behavioral response largely unaffected. Collectively, these results do not support the previously proposed role of protons for the rise in $[Ca^{2+}]_i$ and, more generally, for the control of sperm motility during chemotaxis. We also found no evidence that pH_i changes are involved in the speract-induced Ca^{2+} entry of sperm from the sea urchin *Strongylocentrotus purpuratus*, no matter which physiological function speract may subserve.

MATERIALS AND METHODS

Material and Solutions

We obtained “dry” sperm by injecting 0.5 M KCl solution into the body cavity of *A. punctulata*, or by stimulating the animal electrically. Dry sperm of *A. punctulata* and *S. purpuratus* was diluted with artificial sea water (ASW), which contained (in mM) 423 NaCl, 9.27 $CaCl_2$, 9 KCl, 22.94 $MgCl_2$, 25.5 $MgSO_4$, 0.1 EDTA, and 10 HEPES adjusted to pH 7.8 with NaOH. Number of cells in a suspension was determined in a Neubauer cell counter.

Measurements of Changes in Intracellular Ca^{2+} and pH

Changes in pH_i and $[Ca^{2+}]_i$ were monitored with the fluorescent indicator dyes 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein (BCECF) and Fluo-4, respectively (Molecular Probes). Cells have been loaded using the acetoxymethyl (AM) ester derivatives of these indicator dyes (BCECF-AM and Fluo-4-AM). Procedures to record changes in pH_i were identical to those used for recording changes in $[Ca^{2+}]_i$ (Kaupp et al., 2003), except for the incubation times; BCECF-AM (10 μ M) was applied for only 15 min, whereas Fluo-4-AM (10 μ M) was applied for approxi-

mately 1 h. Dry sperm was suspended 1:6 (vol/vol) in ASW containing the appropriate dye and Pluronic F-127 (0.5% vol/vol). After incubation at 17°C, the sample was diluted 1:20 with ASW. Sperm cells were allowed to equilibrate in the new medium for 3–5 min. In the stopped-flow device, the sperm suspension was rapidly mixed (~1 ms) 1:1 (vol/vol) with ASW containing different concentrations of the peptide and/or other substances. Suspensions were mixed with a flow rate of 1 ml/s; at this rate, the dead time was 31 ms. The average sperm density in the cuvette of the stopped-flow device was $\sim 3 \times 10^8$ cells/ml. All experiments were performed at ~18°C. For technical details concerning excitation and emission of fluorescence and signal recording see Kaupp et al. (2003).

We measured the effect of imidazole on the intracellular pH of sperm in two ways: (1) during the uptake of imidazole and (2) during the release of imidazole. First, after loading with BCECF, sperm were diluted 1:20 in ASW. After 3–5 min, the cells were rapidly mixed (1:1) in the stopped-flow device with ASW containing imidazole. The final concentration of imidazole was 10 mM. Second, sperm were diluted 1:20 in ASW containing 10 mM imidazole. After 3–5 min, sperm were rapidly mixed (1:1) with ASW resulting in a final imidazole concentration of ~5 mM.

To measure the effect of imidazole on peptide-induced changes in pH_i , sperm were diluted 1:20 in ASW or ASW containing 10 mM or 20 mM imidazole. After 3–5 min, the suspension was rapidly mixed (1:1) with ASW containing 25 pM peptide or with ASW (control).

In a similar way we measured the effect of imidazole on peptide-induced changes in $[Ca^{2+}]_i$. After loading with Fluo-4, sperm were diluted 1:20 in ASW or ASW containing 10 mM imidazole. After 3–5 min, sperm were rapidly mixed (1:1) with ASW containing 25 pM peptide or with ASW (control).

We measured the resact-induced change in pH_i at low external $[Ca^{2+}]$ in two different ways. For these experiments we used EGTA-ASW (ASW with 11 mM EGTA and all $CaCl_2$ replaced by $MgCl_2$). In the first protocol, *A. punctulata* sperm, after loading with BCECF, were diluted 1:20 in EGTA-ASW (final $[Ca^{2+}]_o \leq 10^{-8}$ M). After incubation for 3–5 min, sperm were rapidly mixed (1:1) with EGTA-ASW containing 25 pM resact. For the second protocol, sperm were diluted 1:20 in ASW maintaining a normal $[Ca^{2+}]_o$. After 3–5 min, sperm were stimulated by rapid mixing (1:1) with EGTA-ASW containing 25 pM resact. The final $[Ca^{2+}]_o$ after the mixing was $< 10^{-6}$ M. Free $[Ca^{2+}]$ in the solutions were calculated with the computer program MaxChelator (Stanford University).

Determination of cGMP Content

The resact-induced change in cGMP of *A. punctulata* sperm at normal and low external $[Ca^{2+}]$ was measured with the quenched-flow technique (SFM-4; Bio-Logic) as previously described (Kaupp et al., 2003). The experiments were performed at 17°C.

The sperm suspension ($1-10 \times 10^8$ cells/ml) was rapidly mixed 1:1 (vol/vol) with either ASW (normal external $[Ca^{2+}]$, control) or EGTA-ASW containing the indicated concentration of resact. External $[Ca^{2+}]$ was lowered to $\leq 10^{-6}$ M during the mixing process with EGTA-ASW. Stimulation for different periods of time was set by the flow rate (0.11–6.9 ml/s).

After stimulation, the sperm suspension was rapidly mixed 1:3 (vol/vol) with $HClO_4$ (0.5 M final concentration) to quench the biochemical reactions. Each sample was then neutralized by adding K_3PO_4 (final concentration 0.24 M). The $KClO_4$ precipitate and cell debris were sedimented by centrifugation for 15 min at 15,000 g and 4°C. The cGMP content in 50–200 μ l of the supernatant was determined by radioimmunoassay (^{125}I -labeled cGMP;

IBL) according to the manufacturer's instructions. Calibration curves were obtained by serial dilutions of cGMP standards.

Caged Compounds and Photolysis

Experiments using BECMCM-caged cGMP and cAMP were performed as previously described (Kaupp et al., 2003), except that sperm were incubated with a lower concentration of the caged compounds (30 μ M). The cyclic nucleotides were released by a flash of UV light (duration ~1 ms) from a Xenon flash lamp (JML-C2, Rapp Electronics). The flash was delivered by means of a quartz light guide (Hund) and an interposed filter (1 mm, UV-2; Rapp Electronics). The flash energy at the cuvette surface measured by an energy meter (JM20; Rapp Electronics) was ~ 110 $mJ \cdot cm^{-2}$.

Analysis of the Chemotactic Response

We observed sperm through an inverted microscope (ICM405; Carl Zeiss MicroImaging, Inc.) under dark-field illumination (Neofluar objective, 10/0.3; Carl Zeiss MicroImaging, Inc.). Light from a 100-W halogen lamp was filtered through a heat filter and a long-pass filter (KG3 and OG515; Schott). The observation chamber (volume ~20 μ l) had a diameter of 20 mm and a depth of 60 μ m. The bottom of the chamber was made from a coverslip (0.17 mm); the chamber was covered with a microscope slide (1 mm). The behavior of sperm was recorded by a CCD video camera (CF 8/1FMC; Kappa; frame rate 50 Hz) at exposure times of 1–4 ms, and a digital TV-Card (WinTV-PVR USB/PCI; Hauppauge). Movie files were analyzed and swimming trajectories were constructed by a computer program written in Matlab 6.5 (Mathworks).

Dry sperm was diluted 1:10⁴–1:10⁵ in ASW containing Pluronic F-127 (0.2%), imidazole (10 mM), and caged resact (1 μ M) and incubated for 5 min at 16°C. Pluronic F-127 enhances the fraction of motile cells. Caged resact was photolysed by a UV light flash of 400 ms duration (U-ULH 100-W mercury lamp and U-RFL-T burner; Olympus). The irradiation time was set by an electro-mechanical shutter system (LS6Z2 and VMM-T1; Uniblitz; Vincent Assn.). The flash was directed by a quartz light guide and a dichroic mirror (DCLP 405) through the objective of the microscope. The irradiated area could be restricted by means of a rectangular diaphragm (TILL Photonics). UV irradiation in the absence of caged resact did not elicit any motor responses. All experiments were performed at 17°C.

RESULTS

A Change in pH_i Is Not Required for Ca^{2+} Entry

If an increase in pH_i is prerequisite for Ca^{2+} entry, then the rise in pH_i must precede the rise in $[Ca^{2+}]_i$. We tested this prediction by comparing the kinetics of the changes in pH_i and $[Ca^{2+}]_i$ using the fluorescent indicators BCECF and Fluo-4, respectively.

A. punctulata sperm loaded with the pH indicator BCECF responded with an increase in the fluorescence ΔF_{518} upon stimulation with resact, indicating an intracellular alkalinization (Fig. 2 A). The relative amplitude of the fluorescent pH_i signals ($\Delta F/F_o = 0.09-0.12$) was similar to that in a previous report (Nishigaki et al., 2001). For saturating peptide concentrations, the changes in pH_i ranged between 0.1 and 0.4 units (Repaske and Garbers, 1983; Lee and Garbers, 1986;

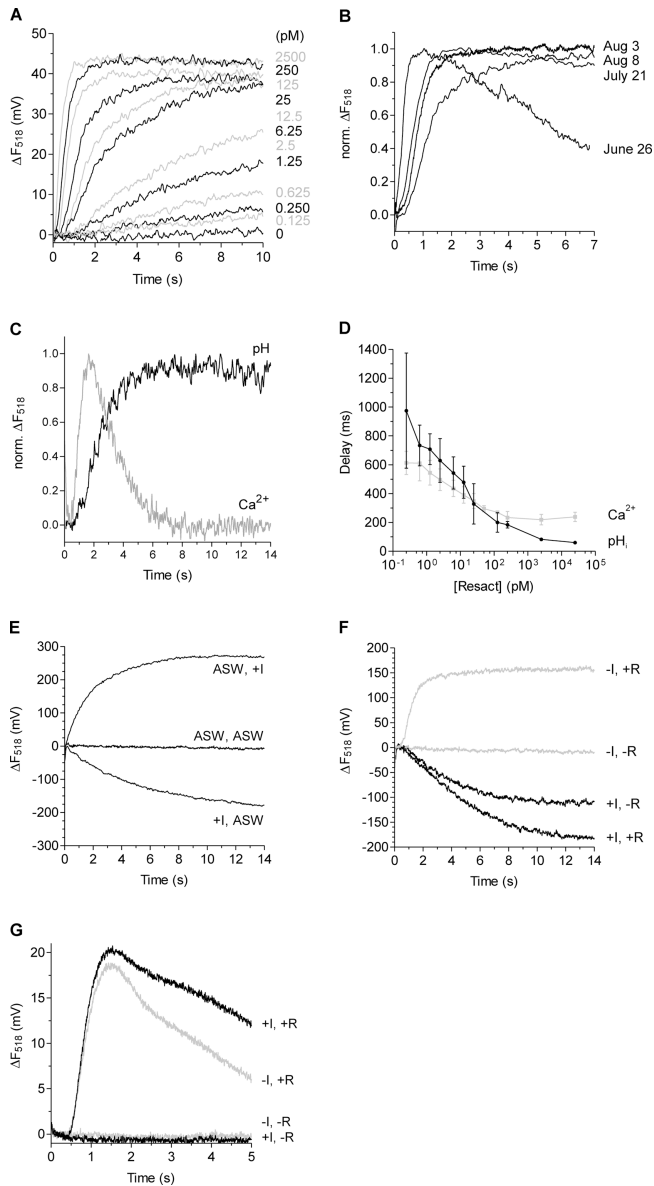


FIGURE 2. Changes in intracellular pH and $[Ca^{2+}]_i$ in sperm from *A. punctulata* induced by resact. (A) Changes in pH_i detected by ΔF_{518} of BCECF. Sperm were stimulated at $t = 0$ with resact concentrations ranging from 0.125 pM to 2.5 nM; each trace represents the average of four recordings. (B) Resact-induced (25 pM) changes in pH_i from four different sperm samples collected at different times during the season. All signals were normalized to the maximum. (C) Comparison of the kinetics of normalized Ca^{2+} and pH_i signals after stimulation with 6.25 pM resact. (D) Dependence of the delay of the Ca^{2+} responses (gray trace) and the pH_i responses (black trace) on the resact concentration. The delay was defined by the intercept between the regression line of the slope of the response rise and the time axis. Vertical bars represent the standard deviation from at least three experiments. The data of the Ca^{2+} responses are from Kaupp et al. (2003). The pH data were collected at the end of the season. (E) Effect of imidazole on pH_i of sperm. The traces represent three different preincubation/mixing conditions. Sperm were preincubated for 5 min in ASW and then mixed with ASW/10 mM imidazole (ASW, +I); as a control, sperm were preincubated in ASW and then mixed with ASW (ASW, ASW); or sperm were

Schackmann and Chock, 1986; Babcock et al., 1992; Reynaud et al., 1993). The kinetics and waveform of the changes in pH_i were dependent on the resact concentration. The delay and rise time shortened with increasing concentrations of resact; however, the waveform of the pH_i signals was quite variable. The pH_i signals displayed either a long delay and a plateau or a short delay and partial relaxation of the signal amplitude (Fig. 2 B). We observed fast and transient signals more often at the beginning of the season (May to June), and slow and nontransient signals more often at the end of the season (August). The reason for this variability is not known. In contrast, Ca^{2+} signals did not exhibit such a variability in their kinetics and waveform (unpublished data).

Sperm are exquisitely sensitive to resact: binding of a single molecule elicits an increase in $[Ca^{2+}]_i$, and the Ca^{2+} response saturates at 10–25 pM resact (Kaupp et al., 2003). Therefore, we consider resact concentrations ≤ 25 pM as physiological for sperm that have not been exposed to resact before. However, when sperm are swimming up a gradient, they will be exposed to high resact concentrations and may reduce their sensitivity to avoid response saturation. For resact concentrations < 25 pM, the delay and rise time of the pH_i signals were longer than those of the Ca^{2+} signals (Fig. 2, C and D). Only at higher resact concentrations (≥ 100 pM) was the delay of the pH_i signal shorter than that of the Ca^{2+} signal (Fig. 2 D). These results are inconsistent with the idea that, for physiologically relevant concentrations of resact, an increase in pH_i triggers the Ca^{2+} entry. We sought independent evidence for this conclusion.

First, we measured the pH_i response in the presence of imidazole, a membrane-permeant proton buffer. Un-

preincubated in ASW/10 mM imidazole and then mixed with ASW (+I, ASW). F_{518} values at $t = 0$ have been set to zero. (F) Effect of imidazole on resact-induced changes in pH_i . Sperm were preincubated for 5 min in ASW without imidazole (gray traces) or in ASW/10 mM imidazole (black traces). In the absence of imidazole, stimulation with 25 pM resact (-I, +R) induced an intracellular alkalinization. However, in the presence of imidazole, 25 pM resact (+I, +R) induced an acidification rather than an alkalinization. This acidification is due to the twofold dilution of extracellular imidazole by the mixing. As a consequence, imidazole escapes the cell, each molecule leaving a proton behind. Mixing of imidazole-incubated sperm with ASW without resact (+I, -R) resulted also in an acidification. Mixing of sperm with ASW (-I, -R) (control) did not change pH_i . (G) Effect of imidazole on the resact-induced increase in $[Ca^{2+}]_i$. Sperm were preincubated in ASW without imidazole (gray traces) or in ASW/10 mM imidazole (black traces). 25 pM resact induced an increase in $[Ca^{2+}]_i$, both in the absence (-I, +R) as well as in the presence of imidazole (+I, +R). Unstimulated sperm did not show an increase in $[Ca^{2+}]_i$ upon mixing with ASW, neither in the presence (+I, -R) nor in the absence of imidazole (-I, -R).

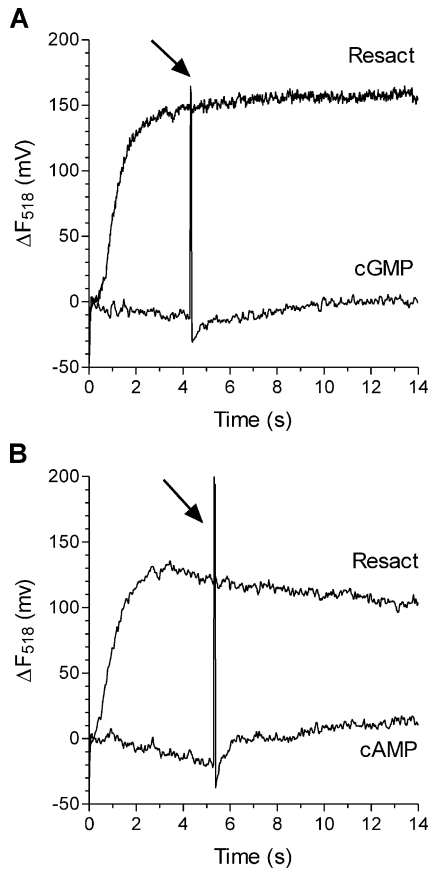


FIGURE 3. Effect of cyclic nucleotides on pH_i in *A. punctulata* sperm. (A) pH_i responses induced by resact (25 pM) or by UV irradiation (arrow) of sperm loaded with caged cGMP (30 μ M). (B) pH_i responses induced by resact (25 pM) or by cAMP released from caged cAMP (30 μ M).

fortunately, imidazole is not a perfect pH buffer tool. It permeates membranes in the neutral form and takes up protons inside the cell; as a result, the cell's interior becomes more alkaline. The pH_i of sea urchin sperm is 6.7–7.9 at rest (Schackmann et al., 1981; Christen et al., 1982; Johnson et al., 1983; Lee et al., 1983) and may rise up to the external pH upon imidazole treatment (if the action of other H^+ transport systems are ignored). When imidazole leaves the cell in its neutral form, the cell's interior acidifies. This is illustrated in Fig. 2 E. Mixing of sperm with ASW containing imidazole (10 mM) increased the pH_i , whereas the pH_i decreased when sperm that had been preincubated in imidazole (10 mM) were mixed with ASW. The initial fluorescence in imidazole-preincubated sperm is higher than in control sperm (ASW incubation); however, for clarity, we have set the initial F_{518} values to zero in Fig. 2 E.

In sperm preincubated with imidazole, the resact-induced alkalization is entirely abolished; instead, a slow acidification is observed, due to the efflux of imidazole from the cell after mixing of sperm with ASW/

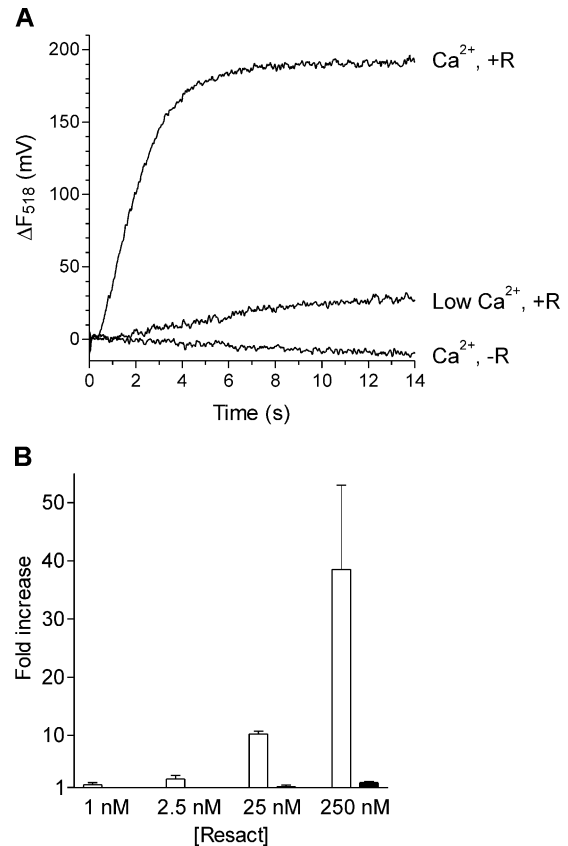


FIGURE 4. Dependence of the pH_i signal and cGMP synthesis on extracellular Ca^{2+} in *A. punctulata* sperm. (A) pH_i signals at normal and low $[Ca^{2+}]_o$. Sperm were preincubated for 5 min in ASW and then mixed with 25 pM resact in EGTA-ASW. The final $[Ca^{2+}]_o$ after mixing was $<10^{-6}$ M. Resact induced a small and slow change in pH_i (low Ca^{2+} , +R). In the controls, sperm were either mixed with 25 pM resact in ASW (Ca^{2+} , +R), or with ASW alone (Ca^{2+} , -R). (B) Relative changes in cGMP concentration upon stimulation of sperm with different concentrations of resact. White bars represent the change in $[cGMP]$ at normal extracellular $[Ca^{2+}]_o$ and black bars at low extracellular $[Ca^{2+}]_o$ ($<10^{-6}$ M). Mean values \pm SD are given for a stimulation period of 200 ms when the cGMP response was maximal at normal extracellular $[Ca^{2+}]_o$ (Kaupp et al., 2003). Data represent the means of at least three experiments (1 nM resact, 2.5 nM resact, $n = 7$; 25 nM resact, $n = 4$; 250 nM resact, $n = 3$). In each experiment triplicate measurements were done.

resact (Fig. 2 F). The acidification after mixing with ASW/resact is slightly larger than the acidification due to the twofold dilution of imidazole in the absence of resact (Fig. 2 F). The additional acidification is readily accounted for by the efflux of imidazole that becomes unprotonated by the resact-induced removal of H^+ from the cytosol. When sperm were stimulated with resact in ASW/10 mM imidazole, the acidification was significantly smaller (unpublished data). In contrast, imidazole left the size and waveform of the Ca^{2+} signal largely unaffected (Fig. 2 G). Thus, Ca^{2+} can enter the cell during acidification.

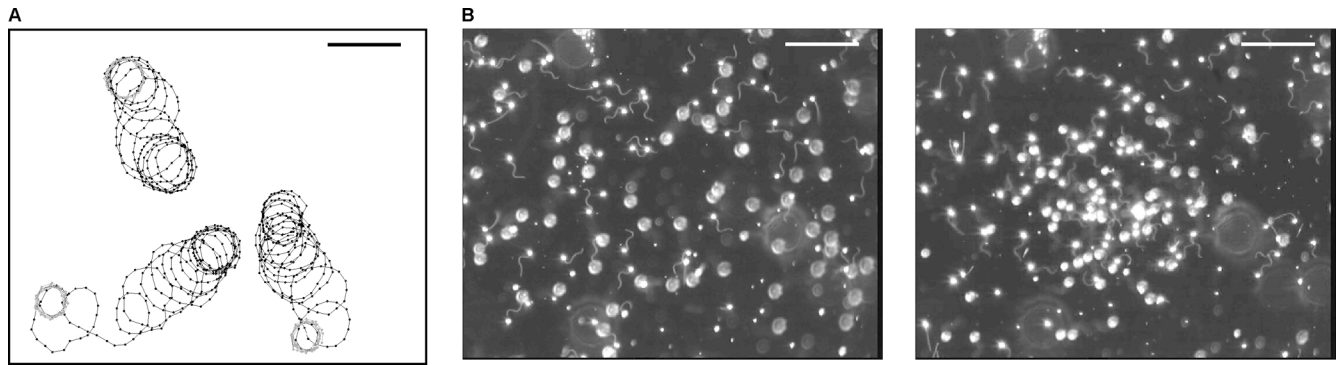


FIGURE 5. Behavior of *A. punctulata* sperm upon stimulation by resact released from caged resact. (A) Swimming trajectories of single sperm cells in a resact gradient produced by a flash of UV light directed to the central area of the visual field. The concentration of caged resact was 1 μM . Gray traces before and black traces after the UV flash. Intervals between consecutive dots, 80 ms. Bar, 100 μm . (B) Accumulation of sperm in the area of released resact in the presence of imidazole (10 mM). Distribution of sperm before photolysis (left) and 26 s after a 400-ms UV flash (right). Bar, 100 μm .

Second, if the pH_i signal is evoked by a cGMP-dependent hyperpolarization (Fig. 1), stimulation by either resact or cGMP should give rise to pH_i signals that are similar in waveform and amplitude. In contrast, cAMP should not produce a pH_i response because, according to the model (Fig. 1), a rise in cAMP is located downstream of the mechanism that causes the pH_i increase. We tested these predictions by comparing the resact-induced pH_i signals with signals evoked by the rapid release of either cGMP or cAMP from caged compounds inside the cell. Release of cyclic nucleotides only produced a small instantaneous decrease of pH_i followed by a slower relaxation (Fig. 3). This decrease is due to the release of protons during photolysis of the caged compounds. A small decrease is also observed in a control solution containing BECMCM-caged cGMP but no sperm (unpublished data). In contrast, both resact and cGMP produced Ca^{2+} responses of similar magnitude and waveform (Kaupp et al., 2003). We conclude from these results that cGMP either does not hyperpolarize the cell, or hyperpolarization does not change pH_i .

Because the Ca^{2+} signal precedes the pH_i signal, at least for resact concentrations up to 25 μM , we examined the possibility that an increase in $[\text{Ca}^{2+}]_i$ is required for the generation of the pH_i signal. To this end, we compared the resact-induced changes in pH_i at normal and low extracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_o$. The pH_i signal was significantly smaller and slower, whether $[\text{Ca}^{2+}]_o$ was lowered several minutes before (unpublished data) or during the stimulation with resact (Fig. 4 A). At high resact concentrations (≥ 2.5 nM) and low $[\text{Ca}^{2+}]_o$, the pH_i signal was also significantly slower (unpublished data). We conclude from this result that the mechanism of the pH_i increase depends on the $[\text{Ca}^{2+}]_o$, irrespective of the waveform of the pH_i signal. Ca^{2+} could affect either the synthesis of cGMP or the H^+ export mechanism. We, therefore, deter-

mined the resact-induced changes in cGMP concentration, $[\text{cGMP}]$, at normal and low $[\text{Ca}^{2+}]_o$. The increase in $[\text{cGMP}]$ was greatly attenuated when the extracellular $[\text{Ca}^{2+}]_o$ was low (Fig. 4 B). It is beyond the scope of this work to study the mechanisms underlying the Ca^{2+} sensitivity. However, the experiments strongly suggest that the smaller and slower pH_i signals result from a lower cGMP synthesis. Taken together, these results argue that a change in pH_i is not required for Ca^{2+} entry and that pH_i has only minor effects on the kinetics and size of the Ca^{2+} response, if any.

A Change in pH_i Is Not Required for Chemotaxis

Unstimulated sperm, in a microscope chamber, swim in regular circles parallel to the surface (Ward et al., 1985; Cook et al., 1994; Kaupp et al., 2003; for review see Miller, 1985). After stimulation with resact, *A. punctulata* sperm undergo turns in their swimming trajectory, followed by periods of smooth swimming (Kaupp et al., 2003). We tested the chemotactic behavior of sperm in the presence of imidazole, which suppressed the resact-induced increase in pH_i . Sperm were bathed in ASW containing caged resact (1 μM) and imidazole (10 mM). In the presence of imidazole, which alkalizes the cell's interior, unstimulated sperm also swam in regular circles (Fig. 5 A, gray traces). A concentration gradient of resact was established by UV irradiation of a central area in the visual field. Within ~ 30 s after the release of resact, sperm accumulated in the irradiated area (Fig. 5, A and B). Motor responses evoked by the release of cGMP from intracellular caged cGMP in the presence or absence of imidazole were similar (unpublished data). Thus, conditions that abolish the resact-induced alkalization do not prevent the motor responses underlying chemotaxis. These results are inconsistent with a vital role of H^+ in the chemotactic behavior of sperm.

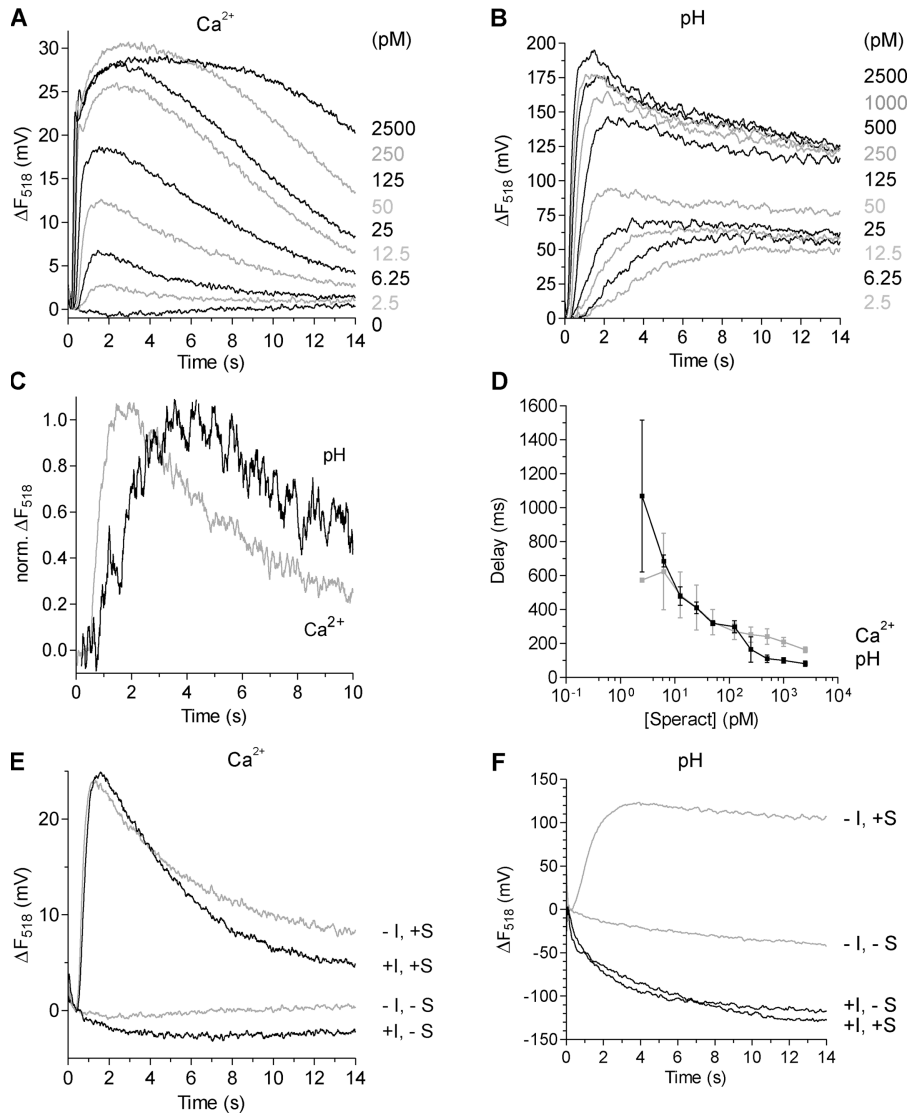


FIGURE 6. Changes in intracellular pH and $[Ca^{2+}]_i$ in *S. purpuratus* sperm induced by speract. (A) Changes in $[Ca^{2+}]_i$ detected by ΔF_{518} of Fluo-4. Sperm were stimulated at $t = 0$ with speract concentrations ranging from 2.5 pM to 2.5 nM; each trace represents the average of three recordings. (B) Changes in pH_i detected by ΔF_{518} of BCECF. Speract concentrations and number of signals for averaging as in panel A. (C) Comparison of the kinetics of normalized Ca^{2+} and pH_i signals after stimulation with 6.25 pM speract. (D) Dependence of the delay of the Ca^{2+} responses (gray trace) and the pH_i responses (black trace) on the speract concentration. Details as in Figure 2 D. (E) Effect of imidazole on speract-induced increase in $[Ca^{2+}]_i$. Sperm were preincubated in ASW without imidazole (gray traces) or in ASW/10 mM imidazole (black traces). 25 pM speract induced an increase in $[Ca^{2+}]_i$, both in the absence (-I, +S) and in the presence of imidazole (+I, +S). Unstimulated sperm did not show an increase in $[Ca^{2+}]_i$ upon mixing with ASW, neither in the presence (+I, -S) nor in the absence of imidazole (-I, -S). (F) Effect of imidazole on speract-induced changes in pH_i . Sperm were preincubated in ASW without imidazole (gray traces) or in ASW/20 mM imidazole (black traces). In the absence of imidazole, stimulation with 25 pM speract (-I, +S) induced an intracellular alkalinization. However, in the presence of imidazole, 25 pM speract (+I, +S) induced an acidification rather than an alkalinization. Mixing of imidazole-incubated sperm with ASW, but without speract (+I, -S) resulted also in an acidification. Mixing of sperm with ASW (-I, -S) (control) did not substantially change pH_i . The peptide-induced pH_i responses of both *S. purpuratus* and *A. punctulata* sperm (Fig. 2 F) are inhibited in the presence of imidazole.

Similarities and Differences Between Species

The model shown in Fig. 1 predominantly rests on experiments with sperm from the sea urchin *S. purpuratus*, whereas the results presented here and in previous work (Kaupp et al., 2003) have been obtained with *A. punctulata*. This raises the issue as to the generality of our conclusions. To examine whether differences between species exist, we have repeated several experiments with sperm of *S. purpuratus*. For two reasons, the comparison with this species is expected to be particularly revealing. First, speract, the peptide of *S. purpuratus*, does not display chemotactic activity (Cook et al.,

1994). Despite this fact, the current model of sperm chemotaxis was readily generalized (Cook et al., 1994). In a capillary assay, we also found no evidence for chemotactic activity of speract (unpublished data). Second, speract reportedly binds to a receptor unrelated to guanylyl cyclase (Dangott and Garbers, 1984; Dangott et al., 1989), yet stimulates a rapid and transient increase of cGMP concentration (Hansbrough and Garbers, 1981a,b; Garbers et al., 1982; Harumi et al., 1992; Cook and Babcock, 1993b; Matsumoto et al., 2003).

In our experiments, speract evoked a rapid increase in both $[Ca^{2+}]_i$ and pH_i with sperm of *S. purpuratus*

(Fig. 6, A and B). Similar signals were observed in sperm from six different animals. The kinetics and waveform of the Ca^{2+} and pH_i signals were similar to those of *Arbacia* sperm (Kaupp et al., 2003). The Ca^{2+} responses at speract concentrations ≥ 25 pM also displayed two kinetic phases, an early and late component. The peptide sensitivities of *Strongylocentrotus* and *Arbacia* sperm are also similar; they respond to concentrations of either speract or resact in the picomolar range. However, the amplitude of the early Ca^{2+} signal from *S. purpuratus* sperm saturated at slightly higher concentrations of the peptide (≥ 50 pM speract) than for *A. punctulata* (~ 25 pM resact; Kaupp et al., 2003).

For speract concentrations ≤ 12.5 pM, the delay and rise time of the pH_i signals were longer than those of the Ca^{2+} signals (Fig. 6, C and D; compare also the time course of Ca^{2+} and pH_i signals at low speract concentrations in A and B, respectively). At speract concentrations ≥ 12.5 pM, the delay of the pH_i signal was either equal or shorter than that of the Ca^{2+} signal (Fig. 6 D).

In *S. purpuratus* sperm preincubated with imidazole, the speract-induced alkalization was entirely abolished; instead a slow acidification was observed (Fig. 6 F), whereas imidazole left the size and waveform of the Ca^{2+} signal largely unaffected (Fig. 6 E). In conclusion, the peptide-stimulated Ca^{2+} and pH_i signals of sperm from *S. purpuratus* and *A. punctulata* are similar rather than distinct. At low speract concentrations, the Ca^{2+} signal precedes the pH_i signal and imidazole abolishes the pH_i signal without taking down the Ca^{2+} signal. Thus, like in *A. punctulata*, a change in pH_i is not required for Ca^{2+} entry in *S. purpuratus*.

Release of cGMP from caged cGMP evoked a transient Ca^{2+} signal similar to that evoked by speract; the Ca^{2+} signal produced by cAMP was smaller and characterized by a long plateau (Fig. 7 A). Thus, like in *A. punctulata* sperm, the Ca^{2+} signals evoked by cGMP and the peptide were similar, whereas the cAMP-stimulated signal was distinctively different. We noticed one difference between sperm from *A. punctulata* and *S. purpuratus*: In *S. purpuratus*, both cAMP and cGMP caused an increase in pH_i . However, the pH_i changes were significantly smaller than those evoked by speract (compare Fig. 6 B with Fig. 7 B), whereas the Ca^{2+} signals were of similar size (compare Fig. 6 A with Fig. 7 A). The cGMP-induced increase in pH_i would be consistent with the model in Fig. 1; whereas, a cAMP-induced change in pH_i cannot be reconciled with this model.

DISCUSSION

Protons have been considered among the most important messengers for the control of motility and chemotactic signaling of sperm (for reviews see Garbers, 1989; Darszon et al., 1999, 2001). A peptide-induced rise in pH_i has been proposed to play a crucial role for the

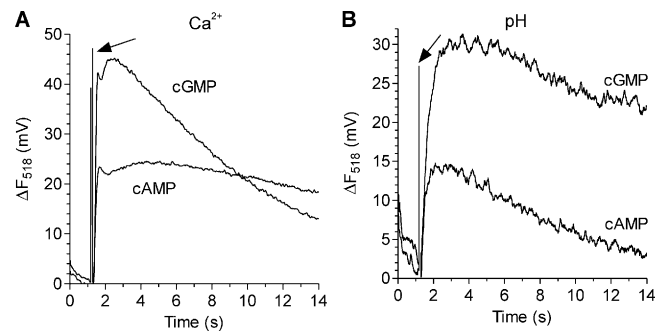


FIGURE 7. Effect of cyclic nucleotides on $[\text{Ca}^{2+}]_i$ and pH_i in *S. purpuratus* sperm. (A) Ca^{2+} response induced by UV irradiation (arrow) of sperm loaded with caged cGMP or caged cAMP (incubation with $30 \mu\text{M}$ each). (B) pH_i response induced by UV irradiation (arrow) of sperm loaded with caged cGMP or caged cAMP (same concentration as in A).

Ca^{2+} entry that is indispensable for sperm chemotaxis. Our results are not consistent with the involvement of pH_i in the control of Ca^{2+} entry and the chemotactic response.

First, at physiological concentrations of resact, the Ca^{2+} signal precedes the pH_i signal. Previous studies used peptide concentrations that were up to four orders of magnitude higher than the resact concentration that saturates the Ca^{2+} response. In these studies, it was noted that the rise in pH_i occurs sooner than the rise in $[\text{Ca}^{2+}]_i$ (Schackmann and Chock, 1986; Nishigaki et al., 2001). Our results confirm these previous findings only for high peptide concentrations. However, for physiologically relevant resact concentrations ≤ 25 pM, the Ca^{2+} signal precedes the increase in pH_i . The finding that the pH_i signal follows the Ca^{2+} signal may imply that the increase in pH_i can occur under depolarizing conditions, i.e., while Ca^{2+} is entering the cell. Second, the Ca^{2+} response persists in the presence of a pH buffer that prevents the resact-induced alkalization. Therefore, a change in pH_i is not required to open Ca^{2+} -permeable channels. Third, in the absence of Ca^{2+} , the pH_i response is abolished or greatly slowed, suggesting that the rise in pH_i is enhanced by Ca^{2+} entry, and not vice versa. Fourth, according to the model shown in Fig. 1, stimulation of sperm with cGMP is expected to hyperpolarize the cell and thereby to increase the pH_i . Instead, cGMP produced either no pH_i signal or a signal that is small compared with the resact-induced pH_i signal. In summary, we propose that a change in pH_i is not an obligatory step in chemotactic signaling of sea urchin sperm.

The physiological reactions of sperm from *S. purpuratus* and *A. punctulata* after stimulation with the respective peptide are rather similar. The waveform and time scale of the Ca^{2+} and pH_i responses as well as the peptide sensitivity are surprisingly uniform. Thus, we conclude that *S. purpuratus* and *A. punctulata* sperm share a

common cGMP signaling pathway that controls Ca^{2+} entry. For *A. punctulata* sperm, it has been shown that a rise in $[\text{Ca}^{2+}]_i$ evokes a chemotactic motor response (Kaupp et al., 2003). By inference, the speract-induced Ca^{2+} entry may also control some aspects of sperm motility in *S. purpuratus*. This change in motility may be related to the swimming behavior during chemotaxis. Whatever the function of speract might be, it is controlled by a cGMP signaling pathway similar to that in *A. punctulata* sperm.

We have not explored the mechanism(s) underlying the pH_i change any further. However, the experiments with caged cGMP and caged cAMP suggest that the reaction that removes protons is located upstream of cGMP; i.e., it is the consumption of GTP that triggers the increase of pH_i rather than the increase in cGMP per se. We propose that protons are consumed in the cytosol during replenishment of the pools for GTP and ATP.

The GTP pool, like in other cells, is probably replenished by the activity of a nucleoside diphosphate kinase (Ogawa et al., 1996) that catalyzes the transphosphorylation: $\text{ATP} + \text{GDP} \leftrightarrow \text{GTP} + \text{ADP}$. During this reaction, ATP is consumed. The ATP pool is replenished by the following reaction that is catalyzed by a creatine kinase: $\text{creatine}\sim\text{P} + \text{ADP} + \text{H}^+ \leftrightarrow \text{creatine} + \text{ATP}$. During this reaction, a proton is consumed, i.e., the cytosol becomes more alkaline. Sea urchin sperm contain a unique membrane-bound form of creatine kinase (Tombes and Shapiro, 1985; van Dorsten et al., 1997; for review see Shapiro and Tombes, 1985). In fact, this enzyme is one of the most abundant proteins in the sperm flagellum and the total creatine content is high (35 mM). Initiation of sea urchin sperm motility produced a creatine $\sim\text{P}$ turnover of 3.5 mM/s and a concomitant increase of pH_i (van Dorsten et al., 1997).

Whatever the mechanism of alkalization, a rise in pH_i is not required for sperm chemotaxis. Procedures that prevent rapid changes in pH_i during peptide stimulation inhibit neither the motor response nor the chemotactic accumulation. More specifically, in the presence of imidazole, the resact-induced alkalization is abolished and, therefore, a change in pH_i is unlikely to account for the Ca^{2+} entry that is required for chemotaxis.

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REFERENCES

- Babcock, D.F., M.M. Bosma, D.E. Battaglia, and A. Darszon. 1992. Early persistent activation of sperm K^+ channels by the egg peptide speract. *Proc. Natl. Acad. Sci. USA*. 89:6001–6005.
- Christen, R., R.W. Schackmann, and B.M. Shapiro. 1982. Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, *Strongylocentrotus purpuratus*. *J. Biol. Chem.* 257: 14881–14890.
- Christen, R., R.W. Schackmann, and B.M. Shapiro. 1983. Metabolism of sea urchin sperm. Interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration. *J. Biol. Chem.* 258:5392–5399.
- Cook, S.P., and D.F. Babcock. 1993a. Activation of Ca^{2+} permeability by cAMP is coordinated through the pH_i increase induced by speract. *J. Biol. Chem.* 268:22408–22413.
- Cook, S.P., and D.F. Babcock. 1993b. Selective modulation by cGMP of the K^+ channel activated by speract. *J. Biol. Chem.* 268: 22402–22407.
- Cook, S.P., C.J. Brokaw, C.H. Muller, and D.F. Babcock. 1994. Sperm chemotaxis: egg peptides control cytosolic calcium to regulate flagellar responses. *Dev. Biol.* 165:10–19.
- Dangott, L.J., and D.L. Garbers. 1984. Identification and partial characterization of the receptor for speract. *J. Biol. Chem.* 259: 13712–13716.
- Dangott, L.J., J.E. Jordan, R.A. Bellet, and D.L. Garbers. 1989. Cloning of the mRNA for the protein that crosslinks to the egg peptide speract. *Proc. Natl. Acad. Sci. USA*. 86:2128–2132.
- Darszon, A., P. Labarca, T. Nishigaki, and F. Espinosa. 1999. Ion channels in sperm physiology. *Physiol. Rev.* 79:481–510.
- Darszon, A., C. Beltrán, R. Felix, T. Nishigaki, and C.L. Treviño. 2001. Ion transport in sperm signaling. *Dev. Biol.* 240:1–14.
- Garbers, D.L. 1989. Molecular basis of fertilization. *Annu. Rev. Biochem.* 58:719–742.
- Garbers, D.L., H.D. Watkins, J.R. Hansbrough, A. Smith, and K.S. Misono. 1982. The amino acid sequence and chemical synthesis of speract and of speract analogues. *J. Biol. Chem.* 257:2734–2737.
- Gauss, R., R. Seifert, and U.B. Kaupp. 1998. Molecular identification of a hyperpolarization-activated channel in sea urchin sperm. *Nature*. 393:583–587.
- Goldstein, S.F. 1979. Starting transients in sea urchin sperm flagella. *J. Cell Biol.* 80:61–68.
- Gray, J. 1928. The effect of dilution on the activity of sea urchin spermatozoa. *Br. J. Exp. Biol.* 5:337–344.
- Guerrero, A., and A. Darszon. 1989. Evidence for the activation of two different Ca^{2+} channels during the egg jelly-induced acrosome reaction of sea urchin sperm. *J. Biol. Chem.* 264:19593–19599.
- Hansbrough, J.R., and D.L. Garbers. 1981a. Sodium-dependent activation of sea urchin spermatozoa by speract and monensin. *J. Biol. Chem.* 256:2235–2241.
- Hansbrough, J.R., and D.L. Garbers. 1981b. Speract - purification and characterization of a peptide associated with eggs that activates spermatozoa. *J. Biol. Chem.* 256:1447–1452.
- Harumi, T., K. Hoshino, and N. Suzuki. 1992. Effects of sperm-activating peptide I on *Hemicentrotus pulcherrimus* spermatozoa in high potassium sea water. *Dev. Growth Differ.* 34:163–172.
- Johnson, C.H., D.L. Clapper, M.M. Winkler, H.C. Lee, and D. Epel. 1983. A volatile inhibitor immobilizes sea urchin sperm in semen by depressing the intracellular pH. *Dev. Biol.* 98:493–501.
- Kaupp, U.B., J. Solzin, J.E. Brown, A. Helbig, V. Hagen, M. Beyer-

- mann, E. Hildebrand, and I. Weyand. 2003. The signal flow controlling chemotaxis of sea urchin sperm. *Nat. Cell Biol.* 5:109–117.
- Lee, H.C. 1984a. A membrane potential-sensitive Na^+ - H^+ exchange system in flagella isolated from sea urchin spermatozoa. *J. Biol. Chem.* 259:15315–15319.
- Lee, H.C. 1984b. Sodium and proton transport in flagella isolated from sea urchin spermatozoa. *J. Biol. Chem.* 259:4957–4963.
- Lee, H.C. 1985. The voltage-sensitive Na^+ / H^+ exchange in sea urchin spermatozoa flagellar membrane vesicles studied with an entrapped pH probe. *J. Biol. Chem.* 260:10794–10799.
- Lee, H.C., and D.L. Garbers. 1986. Modulation of the voltage-sensitive Na^+ / H^+ exchange in sea urchin spermatozoa through membrane potential changes induced by the egg peptide speract. *J. Biol. Chem.* 261:16026–16032.
- Lee, H.C., C. Johnson, and D. Epel. 1983. Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. *Dev. Biol.* 95:31–45.
- Matsumoto, M., J. Solzin, A. Helbig, V. Hagen, S.-I. Ueno, O. Kawase, Y. Maruyama, M. Ogiso, M. Godde, H. Minakata, et al. 2003. A sperm-activating peptide controls a cGMP-signaling pathway in starfish sperm. *Dev. Biol.* 260:314–324.
- Miller, R.L. 1985. Sperm chemo-orientation in the metazoa. In *Biology of Fertilization*, Vol. 2: *Biology of the Sperm*. C.B. Metz and A. Monroy, editors. Academic Press, New York. 275–337.
- Nishigaki, T., F.Z. Zamudio, L.D. Possani, and A. Darszon. 2001. Time-resolved sperm responses to an egg peptide measured by stopped-flow fluorometry. *Biochem. Biophys. Res. Commun.* 284: 531–535.
- Ogawa, K., H. Takai, A. Ogiwara, E. Yokota, T. Shimizu, K. Inaba, and H. Mohri. 1996. Is outer arm dynein intermediate chain 1 multifunctional? *Mol. Biol. Cell.* 7:1895–1907.
- Ohtake, H. 1976. Respiratory behaviour of sea-urchin spermatozoa. I. Effect of pH and egg water on the respiratory rate. *J. Exp. Zool.* 198:303–311.
- Repaske, D.R., and D.L. Garbers. 1983. A hydrogen ion flux mediates stimulation of respiratory activity by speract in sea urchin spermatozoa. *J. Biol. Chem.* 258:6025–6029.
- Reynaud, E., L. de De la Torre, O. Zapata, A. Liévano, and A. Darszon. 1993. Ionic bases of the membrane potential and intracellular pH changes induced by speract in swollen sea urchin sperm. *FEBS Lett.* 329:210–214.
- Schackmann, R.W., and P.B. Chock. 1986. Alteration of intracellular $[\text{Ca}^{2+}]$ in sea urchin sperm by the egg peptide speract. *J. Biol. Chem.* 261:8719–8728.
- Schackmann, R.W., R. Christen, and B.M. Shapiro. 1981. Membrane potential depolarization and increased intracellular pH accompany the acrosome reaction of sea urchin sperm. *Proc. Natl. Acad. Sci. USA.* 78:6066–6070.
- Shapiro, B.M., and R.M. Tombes. 1985. A biochemical pathway for a cellular behaviour: pH_i , phosphorylcreatine shuttles, and sperm motility. *Bioessays.* 3:100–103.
- Suzuki, N., H. Shimomura, E.W. Radany, C.S. Ramarao, G.E. Ward, J.K. Bentley, and D.L. Garbers. 1984. A peptide associated with eggs causes a mobility shift in a major plasma membrane protein of spermatozoa. *J. Biol. Chem.* 259:14874–14879.
- Tombes, R.M., and B.M. Shapiro. 1985. Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. *Cell.* 41:325–334.
- Trimmer, J.S., R.W. Schackmann, and V.D. Vacquier. 1986. Monoclonal antibodies increase intracellular Ca^{2+} in sea urchin spermatozoa. *Proc. Natl. Acad. Sci. USA.* 83:9055–9059.
- van Dorsten, F.A., M. Wyss, T. Wallimann, and K. Nicolay. 1997. Activation of sea-urchin sperm motility is accompanied by an increase in the creatine kinase exchange flux. *Biochem. J.* 325:411–416.
- Ward, C.R., and G.S. Kopf. 1993. Molecular events mediating sperm activation. *Dev. Biol.* 158:9–34.
- Ward, G.E., C.J. Brokaw, D.L. Garbers, and V.D. Vacquier. 1985. Chemotaxis of *Arbacia punctulata* spermatozoa to resact, a peptide from the egg jelly layer. *J. Cell Biol.* 101:2324–2329.
- Wood, C.D., A. Darszon, and M. Whitaker. 2003. Speract induces calcium oscillations in the sperm tail. *J. Cell Biol.* 161:89–101.