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 Ca\(^{2+}\); the Ca\(^{2+}\) entry is essential for the chemotactic behavior. Previous studies proposed that the influx of Ca\(^{2+}\) 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 Ca\(^{2+}\) concentration. We show that for physiological concentrations of resact (<25 pM), the influx of Ca\(^{2+}\) precedes the rise in pH\(_i\). Moreover, buffering of pH\(_i\) completely abolishes the resact-induced pH\(_i\) signal, but leaves the Ca\(^{2+}\) signal and the chemotactic motor response unaffected. We conclude that an elevation of pH\(_i\) is required neither to open Ca\(^{2+}\)-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 Ca\(^{2+}\) 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:** Ca\(^{2+}\) • 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) (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 Ca\(^{2+}\) 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).

Abbreviations used in this paper: AM, acetoxyethyl; ASW, artificial sea water; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein.
Finally, the interaction between factors on the egg coat and specific receptors on the sperm membrane triggers acrosomal exocytosis. A pH increase 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 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 [Ca2+]i, followed by (2) a cAMP-mediated increase in [Ca2+]i—are not supported by a recent study of time-resolved changes in cAMP and [Ca2+]i, 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 pHi is prerequisite both for Ca2+ entry and for the chemotactic behavior. Using rapid-mixing techniques we show that, for physiological concentrations of resact, the chemoattractant of A. punctulata, the Ca2+ signal precedes the pH response. Moreover, a rapid increase in cGMP concentration does not evoke a pH increase. Finally, a pH buffer abolishes the resact-induced change in pHi but leaves the Ca2+ signal and the behavioral response largely unaffected. Collectively, these results do not support the previously proposed role of protons for the rise in [Ca2+]i, and, more generally, for the control of sperm motility during chemotaxis. We also found no evidence that pH changes are involved in the speract-induced Ca2+ entry of sperm from the sea urchin Stronglylocentrotus 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 CaCl2, 9 KCl, 22.94 MgCl2, 25.5 MgSO4, 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 Ca2+ and pH

Changes in pHi and [Ca2+]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 pHi were identical to those used for recording changes in [Ca2+]i (Kaupp et al., 2003), except for the incubation times; BCECF-AM (10 μM) was applied for only 15 min, whereas Fluo-4-AM (10 μM) was applied for approxi-
mately 1 h. Dry sperm was suspended 1:6 (vol/vol) in ASW containing the appropriate dye and Phuronic 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 ~3 × 10^6 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, 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).

In a similar way we measured the effect of imidazole on peptide-induced changes in [Ca^{2+}]. 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 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+}], ≤ 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+}]. After 3–5 min, sperm were stimulated by rapid mixing (1:1) with EGTA-ASW containing 25 pM resact. The final [Ca^{2+}], 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 × 10^6 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 ≤10^{-6} M during the mixing process with EGTA-ASW. Stimulation for different periods of time was set by the flow rate (0.11–5.9 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_2HPO_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 μl of the supernatant was determined by radioimmunoassay (125I-labeled cGMP; IBL) according to the manufacturer's instructions. Calibration curves were obtained by serial dilutions of cGMP standards.

**Caged Compounds and Photoysis**

Experiments using BCCM-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 μM). 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·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 μl) had a diameter of 20 mm and a depth of 60 μm. 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 8/1FM; 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 1:10^1–1:10^5 in ASW containing Phuronic F-127 (0.2%), imidazole (10 mM), and caged resact (1 μM) and incubated for 5 min at 16°C. Phuronic F-127 enhances the fraction of motile cells. Caged resact was photolyzed 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.
Changes in intracellular pH and $[\text{Ca}^{2+}]_i$ in sperm from *A. punctulata* induced by resact. (A) Changes in $\text{pH}_i$ detected by $\Delta F_{518}$ 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 $\text{pH}_i$ 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 $\text{Ca}^{2+}$ and $\text{pH}_i$ signals after stimulation with 6.25 pM resact. (D) Dependence of the delay of the $\text{Ca}^{2+}$ responses (gray trace) and the $\text{pH}_i$ 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 $\text{Ca}^{2+}$ responses are from Kaupp et al. (2003). (E) Effect of imidazole on $\text{pH}_i$ 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 preincubated in ASW/10 mM imidazole and then mixed with ASW (ASW, I)). $F_{518}$ values at $t = 0$ have been set to zero. (F) Effect of imidazole on resact-induced changes in $\text{pH}_i$. 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 $\text{pH}_i$. (G) Effect of imidazole on the resact-induced increase in $[\text{Ca}^{2+}]_i$. Sperm were preincubated in ASW without imidazole (gray traces) or in ASW/10 mM imidazole (black traces). 25 pM resact induced an increase in $[\text{Ca}^{2+}]_i$. 

The kinetics and waveform of the changes in $\text{pH}_i$ were dependent on the resact concentration. The delay and rise time shortened with increasing concentrations of resact; however, the waveform of the $\text{pH}_i$ signals was quite variable. The $\text{pH}_i$ 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, $\text{Ca}^{2+}$ 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 $[\text{Ca}^{2+}]_i$, and the $\text{Ca}^{2+}$ 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 $\text{pH}_i$ signals were longer than those of the $\text{Ca}^{2+}$ signals (Fig. 2, C and D). Only at higher resact concentrations (≥100 pM) was the delay of the $\text{pH}_i$ signal shorter than that of the $\text{Ca}^{2+}$ signal (Fig. 2 D). These results are inconsistent with the idea that, for physiologically relevant concentrations of resact, an increase in $\text{pH}_i$ triggers the $\text{Ca}^{2+}$ entry. We sought independent evidence for this conclusion.

First, we measured the $\text{pH}_i$ response in the presence of imidazole, a membrane-permeant proton buffer. Un-
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 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+/H11001 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, whereas the pH 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 F518 values to zero in Fig. 2 E.

In sperm preincubated with imidazole, the resact-induced alkalinization is entirely abolished; instead, a slow acidification is observed, due to the efflux of imidazole from the cell after mixing of sperm with ASW/resact (Fig. 2 F). The acidification after mixing with ASW/resact is slightly larger than the acidification due to 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 Ca2+ signal largely unaffected (Fig. 2 G). Thus, Ca2+ can enter the cell during acidification.
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$^{2+}$/H$^+$ 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 pM, we examined the possibility that an increase in [Ca$^{2+}$]$_o$ 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 [Ca$^{2+}$]$_o$. The pH$_i$ signal was significantly smaller and slower, whether [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 [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 [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, determined the resact-induced changes in cGMP concentration, [cGMP], at normal and low [Ca$^{2+}$]$_o$. The increase in [cGMP] was greatly attenuated when the extracellular [Ca$^{2+}$] 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 alkalinization. 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 alkalinization do not prevent the motor responses underlying chemotaxis. These results are inconsistent with a vital role of H$^+$ in the chemotactic behavior of sperm.
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 $[\text{Ca}^{2+}]$, and pH 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 pHi 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 Strongylocentrotus sperm saturated at slightly higher concentrations of the peptide (≥25 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 pHi signals were longer than those of the Ca$^{2+}$ signals (Fig. 6, C and D; compare also the time course of Ca$^{2+}$ and pHi signals at low speract concentrations in A and B, respectively). At speract concentrations ≥12.5 pM, the delay of the pHi signal was either equal or shorter than that of the Ca$^{2+}$ signal (Fig. 6 D).

In Strongylocentrotus 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 pHi signals of sperm from Strongylocentrotus and A. punctulata are similar rather than distinct. At low speract concentrations, the Ca$^{2+}$ signal precedes the pHi signal and imidazole abolishes the pHi signal without taking down the Ca$^{2+}$ signal. Thus, like in A. punctulata, a change in pHi is not required for Ca$^{2+}$ entry in Strongylocentrotus.

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 Strongylocentrotus: In Strongylocentrotus, both cAMP and cGMP caused an increase in pHi. However, the pHi 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 pHi would be consistent with the model in Fig. 1; whereas, a cAMP-induced change in pHi 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 pHi has been proposed to play a crucial role for the Ca$^{2+}$ entry that is indispensable for sperm chemotaxis. Our results are not consistent with the involvement of pHi in the control of Ca$^{2+}$ entry and the chemotactic response.

First, at physiological concentrations of resact, the Ca$^{2+}$ signal precedes the pHi 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 pHi occurs sooner than the rise in [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 pHi. The finding that the pHi signal follows the Ca$^{2+}$ signal may imply that the increase in pHi 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 pHi is not required to open Ca$^{2+}$-permeable channels. Third, in the absence of Ca$^{2+}$, the pHi response is abolished or greatly slowed, suggesting that the rise in pHi 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 pHi. Instead, cGMP produced either no pHi signal or a signal that is small compared with the resact-induced pHi signal. In summary, we propose that a change in pHi is not an obligatory step in chemotactic signaling of sea urchin sperm.

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

**Figure 7.** Effect of cyclic nucleotides on [Ca$^{2+}$]$_i$ and pHi in Strongylocentrotus sperm. (A) Ca$^{2+}$ response induced by UV irradiation (arrow) of sperm loaded with caged cGMP or caged cAMP (incubation with 30 μM each). (B) pHi response induced by UV irradiation (arrow) of sperm loaded with caged cGMP or caged cAMP (same concentration as in A).
common cGMP signaling pathway that controls Ca\(^{2+}\) entry. For *A. punctulata* sperm, it has been shown that a rise in [Ca\(^{2+}\)]
, 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: ATP + GDP ↔ GTP + ADP. During this reaction, ATP is consumed. The ATP pool is replenished by the following reaction that is catalyzed by a creatine kinase: creatine+P + ADP + H\(^+\) ↔ creatine + 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+P turnover of 3.5 mM/s and a concomitant increase of pH\(_i\) (van Dorsten et al., 1997).

Whatever the mechanism of alkalinization, 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 speract-induced alkalinization 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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