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# A sperm-activating peptide controls a cGMP-signaling pathway in starfish sperm☆

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**Abstract**

Peptides released from eggs of marine invertebrates play a central role in fertilization. About 80 different peptides from various phyla have been isolated, however, with one exception, their respective receptors on the sperm surface have not been unequivocally identified and the pertinent signaling pathways remain ill defined. Using rapid mixing techniques and novel membrane-permeable caged compounds of cyclic nucleotides, we show that the sperm-activating peptide asterosap evokes a fast and transient increase of the cGMP concentration in sperm of the starfish *Asterias amurensis*, followed by a transient cGMP-stimulated increase in the $Ca^{2+}$ concentration. In contrast, cAMP levels did not change significantly and the $Ca^{2+}$ response evoked by photolysis of caged cAMP was significantly smaller than that using caged cGMP. By cloning of cDNA and chemical crosslinking, we identified a receptor-type guanylyl cyclase in the sperm flagellum as the asterosap-binding protein. Sperm respond exquisitely sensitive to picomolar concentrations of asterosap, suggesting that the peptide serves a chemosensory function like resact, a peptide involved in chemotaxis of sperm of the sea urchin *Arbacia punctulata*. A unifying principle emerges that chemosensory transduction in sperm of marine invertebrates uses cGMP as the primary messenger, although there may be variations in the detail.



*Keywords:* Caged compounds; Chemotaxis; Cyclic nucleotides; Kinetic measurements; Sperm

## Introduction

Sperm-activating peptides are diffusible components of the jelly coat of eggs from marine invertebrates. About 80 peptides, primarily from 2 different phyla, cnidaria and echinodermata, have been identified (Suzuki, 1995). For example, resact and speract of the sea urchin species *Arbacia punctulata* and *Strongylocentrotus purpuratus*, respectively, are short peptides (Suzuki et al., 1981, 1984; Garbers et al., 1982), whereas asterosap from the starfish *Asterias amurensis* is a large glutamine-rich peptide (Nishigaki et al., 1996). The peptides are either circular (like asterosap and resact) by the formation of an intramolecular disulfide bond (Yoshino et al., 1991; Nishigaki et al., 1996) or linear (like speract) (Suzuki et al., 1981). The peptides affect various sperm functions, including motility, chemotactic behavior, respiration, and the acrosome reaction (Garbers, 1989).

☆ Data deposition: The sequence reported in this paper has been deposited in the GenBank database (Accession No. AB070354).

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Stimulation of sperm with peptides evokes a host of physiological responses, including changes in the concentration of cyclic nucleotides, protein phosphorylation, the flow of $Ca^{2+}$, $Na^{+}$, $K^{+}$, and $H^{+}$ ions across the plasma membrane, and changes in the membrane potential (for review, see Garbers, 1989; Ward and Kopf, 1993; Darszon et al., 1999, 2001). The signaling pathways that underlie these cellular events and the function they serve are not well understood (Kirkman-Brown et al., 2003).

The action of these peptides on sperm physiology has been chiefly studied in sea urchins. Although speract and resact evoke similar physiological responses, the underlying pathways and their specific functions appear to be different. Resact is involved in chemotaxis of *A. punctulata* sperm (Ward et al., 1985), whereas speract has not been shown to be chemotactically active. It may control other features of sperm motility (Cook et al., 1994) or may serve as a cofactor that facilitates the acrosome reaction (Yamaguchi et al., 1988; Shimizu et al., 1990). Moreover, resact binds to a receptor-type guanylyl cyclase (GC) (Shimomura and Garbers, 1986) and thereby stimulates the synthesis of cGMP (Suzuki et al., 1984), whereas the reported receptor for speract is a protein unrelated to GCs (Dangott et al., 1989). These observations seem to testify to a rich diversity of signaling pathways and cellular functions, even within a taxonomic order.

Are these findings restricted to sea urchins or are they of more general significance? To address this question, we studied the asterosap-stimulated signaling pathway in sperm of the starfish *A. amurensis*. Asteroids (starfish) and echinoids (sea urchin) diverged approximately 500 million years ago, whereas the split between *Arbacia* and *Strongylocentrotus* occurred 200 million years ago (Smith, 1988; Wada and Satoh, 1994). We have found that asterosap induced a rapid and transient increase of the cGMP level, whereas the cAMP level did not change significantly. Moreover, we have identified a GC in the sperm flagellum as the asterosap receptor. Picomolar concentrations of asterosap produced a transient increase in the intracellular concentration of $Ca^{2+}$. A similar $Ca^{2+}$ response is observed after the release of cGMP from a caged compound inside sperm. Rapid increases in cGMP and $Ca^{2+}$ have been recently described in sperm of the sea urchin *A. punctulata* (Kaupp et al., 2003). These findings identify cGMP as the primary intracellular messenger, and provide compelling evidence that GCs have been conserved as chemoreceptors of sperm-activating peptides for at least 500 million years.

## Materials and methods

### *Materials*

*A. amurensis* "dry" sperm was collected as described (Matsui et al., 1986). Artificial sea water (ASW) for starfish contained (in mM): 430 NaCl, 9 $CaCl_2$, 9 KCl, 23 $MgCl_2$, 25 $MgSO_4$, and 10 N-2-hydroxyethyl-piperazine-N′-3-propane sulphonic acid (HEPES) (pH 8.2, adjusted with NaOH). *S. purpuratus* "dry" sperm was obtained by injecting 0.5 M KCl into the body cavity of the sea urchin. ASW for sea urchins contained (in mM): 486 NaCl, 10 $CaCl_2$, 10 KCl, 25 $MgCl_2$, 30 $MgSO_4$, 2.5 $NaHCO_3$, 0.1 EDTA, and 10 mM HEPES/NaOH, pH 8.0. The synthetic asterosap isoform P15 (GQTQFGVC*IARVRQQHQGQDEASIFQAILS-QC*QS,intramolecular disulfide bond between two Cys*) was used. Speract was purchased from Peninsula Laboratories.

### *Cloning of guanylyl cyclase*

A λ-ZAPII cDNA library from *A. amurensis* was screened with a cDNA fragment that had been amplified by PCR from testicular first-strand cDNA using two degenerate primer sets (P2: 5′-GA(C/T)AT(A/C/T)GT(A/C/G/T)GG(A/C/G/T)TT(C/T)AC-3′, and P6: 5′-GT(A/G)TT(A/C/G/T)AC(A/C/G/T)GT(A/G)TC(A/C/G/T)CC-3′; gc1: 5-GA(C/T)GT(A/C/G/T)TA(C/T)AA(A/G)GT(A/C/G/T)GAG-3′, and gc2: 5′-A(A/C/G/T)A(A/G)(A/G)CA(A/G)TA(A/C/G/T)C(G/T)(A/C/G/T)GGCAT-3′) that were derived from highly conserved regions of receptor GCs. A full-length cDNA clone (4211 bp) was isolated. We assigned the initiation codon to the ATG at nucleotide position 101, because (1) upstream there is an in-frame stop codon, (2) the ATG is flanked by sequences that conform to Kozak's criteria for a translation initiation site (Kozak, 1981), and (3) the amino acid sequence following this ATG resembles a putative signal peptide. The initiation codon is followed by an open reading frame of 3201 bp.

### *Northern blot analysis*

Total RNA (10 μg) from testes and ovaries of *A. amurensis* was separated on a 1% agarose/formaldehyde gel and transferred to a Biodyne B membrane (Pall BioSupport). Samples were cross-linked by UV irradiation and hybridized at 42°C in 50% formamide, 5× SSC, 5× Denhardt's, 1% SDS with a DNA probe comprising nucleotides (nt) 2835-3073. The membrane was washed with 2× SSC/0.5% SDS for 10 min at RT and with 0.1× SSC/0.1% SDS for 15 min at 60°C. The autoradiogram was analyzed with an image analyzer (Fuji BAS 2000 system).

### *Cross-linking experiments*

Sperm membranes were prepared from dry sperm as described (Nishigaki et al., 2000). Cross-linking of proteins with asterosap using sperm membranes was done as described (Nishigaki et al., 2000). Briefly, asterosap was labeled with the photoaffinity cross-linker SASD (sulfosuccinimidyl 2-[*p*-azidosalicylamido]ethyl-1-3′-dithiopropionate; Pierce) and then purified by reverse-phase HPLC. Sperm membranes were incubated with labeled asterosap (10 min on ice) and then irradiated with UV light (10 min on ice). Membrane proteins were separated by a low bisacrylamide (0.13%) SDS–polyac-

rylamide gel (Ward et al., 1983), which facilitates an improved separation of proteins in the $M_r$ range of ~100–150 kDa. The Coomassie-stained asterosap-binding protein was eluted from the gel and its N-terminal amino-acid sequence was determined by using a Shimadzu protein sequencer PPSQ21.

*Western blot analysis and immunohistochemistry*

Polyclonal antibodies were raised in rabbits against the asterosap isoform P15 (anti-asterosap) and against the N-terminal region (amino acid residues 21-168; anti-NTGC) and the C-terminal region (amino acid residues 824-1052; anti-CTGC) of the cloned GC from *A. amurensis*. Anti-asterosap IgG was purified with an Ampure PA kit (Amersham). In ELISA experiments, the antibody reacted with similar affinity to all asterosap isoforms. Both anti-GC antibodies were used as serum.

For Western blot analysis, sperm flagella were separated from heads as described (Vacquier, 1986). Sperm were sheared by using a 22-gauge needle and centrifuged (1000*g*, 5 min, 4°C) to spin down the sperm heads. Sperm flagella were recovered from the supernatant by centrifugation (10,000*g*, 20 min, 4°C). Sperm heads and flagella were washed several times by centrifugation. Purity of preparations was monitored by phase-contrast microscopy. Purified flagella and heads were homogenized in ASW. Proteins were separated on a 10% SDS–polyacrylamide gel and transferred to Immobilon-P PVDF membrane (Millipore). Membranes were blocked in 5% nonfat dry milk/Tris-buffered saline (TBS, pH 7.2), and then incubated with either anti-asterosap IgG (1:1000 dilution) or anti-CTGC (1:300). Membranes were washed with 0.4% Tween 20/TBS, and then incubated with Vectastain *Elite* ABC goat anti-rabbit secondary antibody (1:2000; Vector Laboratory). Immunoreactive bands were detected with a Konica Immunostaining HRP-1000 Kit (Konica, Tokyo). The transfer efficiency was checked by staining the membrane with 0.1% Coomassie Brilliant Blue R250.

For immunocytochemistry, sperm cells were fixed with 3.7% formaldehyde in ASW for 15 min and subsequently washed in PBS. Samples were incubated with the primary antibody anti-CTGC (1:300) for 30 min at room temperature. After several rinses in PBS, samples were incubated with the Alexa Fluor 488-conjugated secondary antibody (1:1000; Molecular Probes). After several rinses in PBS, sections were coverslipped with 50% glycerol/1% propylgallate.

*Quenched-flow measurements*

*A. amurensis* dry sperm was diluted with ASW to a density of 0.64-3.8 $\times$ $10^8$ cells/ml, and *S. purpuratus* dry sperm to a density of 5 $\times$ $10^8$ cells/ml. Sperm suspensions containing more than 90% motile cells were used for the measurements of intracellular concentrations of cGMP and cAMP. Kinetic measurements were performed by using a stopped-flow apparatus (SFM-4; Biologic, France). Sperm suspended in ASW were stimulated by rapid mixing (<1 ms) with ASW (1:1 v/v) containing different concentrations of asterosap (1 nM–1 $\mu$M) and 250 nM speract, respectively. Stimulation times were set by the flow rates (0.11–6.9 ml/s). Flow rates up to 7 ml/s did not result in microscopically detectable damage of sperm cells. The reaction was quenched by rapid mixing with $HClO_4$ (final concentration 0.5 M). Quenched samples were freeze-thawed, sonified for 5 s (Branson B-12, ~90 W), and neutralized by addition of $K_3PO_4$ (final concentration 0.24 M). The $KClO_4$ precipitate and the cell debris were sedimented by centrifugation (15,000*g*, 10 min, 4°C). The cAMP and cGMP concentrations in the supernatant were determined from one and the same sample by radioimmunoassay (RIA) using $^{125}$I cGMP RIA or $^{125}$I cAMP RIA Kits (IBL, Germany). In some experiments, the sperm suspension was incubated with IBMX for at least 5 min before stimulation with asterosap.

*Measurement of changes in intracellular $Ca^{2+}$*

Asterosap-induced changes in the intracellular concentration of $Ca^{2+}$, $[Ca^{2+}]_i$, were measured with the rapid mixing device in the stopped-flow mode using the fluorescent $Ca^{2+}$ indicator Fluo-4 (Molecular Probes, USA). Dry sperm was suspended in ASW loading buffer (1:6 v/v). The loading buffer contained (in mM): 423 NaCl, 9.27 $CaCl_2$, 9 KCl, 22.94 $MgCl_2$, 25.5 $MgSO_4$, 0.1 EDTA, 10 Hepes/NaOH, pH 7.8, 10 $\mu$M Fluo-4AM, and 0.5% Pluronic F127 (Molecular Probes). After 60 min incubation with Fluo-4 AM at 18°C, the sample was diluted 1:20 with ASW, pH 7.8. In the stopped-flow device, the sperm suspension was rapidly mixed with ASW solutions (1:1 v/v) that contained different concentrations of asterosap. The sperm density in the 31-$\mu$l quartz cuvette (FC-15; Bio-Logic) was 1.7-3.3 $\times$ $10^8$ cells/ml.

Fluorescence was excited by a 150-W Xe/Hg lamp (ALX-220; Bio-Logic) and recorded by a photomultiplier (R-376; Hamamatsu, Bio-Logic). The wavelength of the excitation light was adjusted by a filter set (3 mm KG1, 6 mm GG400, 4 mm FITCA40; Schott, Germany); the emitted fluorescence was passed through another set of filters (6 mm GG495, 4 mm FITCE45; Schott, Germany). The change in photomultiplier output was linear with the changes in fluorescence. Data were acquired with an A/D board (DT2801-A; Bio-Logic) and the BioKine software (version 3.23; Bio-Logic).

For the study of cyclic nucleotide-induced changes in $[Ca^{2+}]_i$, we used novel [6,7-bis (ethoxycarbonylmethoxy)-coumarin-4-yl]methyl-substituted forms of cAMP (BECMCM-caged cAMP) and cGMP (BECMCM-caged cGMP) that rapidly deliver the respective messenger upon irradiation with a flash of UV-light (Kaupp et al., 2003). The BECMCM-caged compounds are cleaved inside the cell by esterases to yield the BCMCM-caged derivatives. The properties of these compounds have been described (Hagen et al., 2001, 2002). For the flash photolysis of caged cyclic

Table 1
Maximal changes in cGMP concentration induced by asterosap

| [asterosap] nM | 0 mM IBMX | | 0.5 mM IBMX | | 2 mM IBMX | |
|---|---|---|---|---|---|---|
| | Mean ± SD | (*n*) | Mean ± SD | (*n*) | Mean | (*n*) |
| 0 | 0.4 ± 0.2 | (7) | 0.6 ± 0.2 | (3) | 0.1 | (2) |
| 1 | 3.4 ± 1.3 | (3) | 6.0 ± 3.6 | (3) | 3.4 | (2) |
| 10 | 5.2 ± 1.3 | (5) | 6.5 ± 3.8 | (3) | 4.9 | (2) |
| 50 | 12.1 ± 5.9 | (3) | 10.1 ± 6.3 | (3) | 9.3 | (2) |
| 100 | 15.4 ± 5.7 | (3) | 15.8 ± 10.5 | (3) | 12.3 | (2) |
| 250 | 31.9 ± 12.6 | (3) | 23.4 ± 13.5 | (3) | 18.1 | (2) |
| 1000 | 40.6 ± 30.9 | (7) | 31.1 ± 16.6 | (3) | 21.5 | (2) |

*Note*. Sperm were stimulated with various asterosap concentrations (1 nM–1 $\mu$M) in the absence or presence of IBMX. Concentrations of cGMP [mean ± SD pmol cGMP/$10^8$ cells ($n$ = number of experiments)] were determined from the maximum values of curves such as those shown in Fig. 1A and B.

nucleotides, sperm were incubated with either BECMCM-caged cGMP or -cAMP (10 $\mu$M), Fluo-4AM (10 $\mu$M), and Pluronic F127 (0.5%) in ASW for 120 min. The cyclic nucleotides were released by a 1.6-ms flash of UV-light from a Xe flash lamp (Strobex 278; Chadwick-Helmuth, USA). The flash was delivered to the cuvette through a liquid light guide and an interposed filter (4 mm DUG11; Schott). The unattenuated flash energy at the cuvette surface was 26.8 mJ $cm^{-2}$ at 150 Ws electrical input. The irradiated area of the cuvette was 0.135 $cm^2$ (1.5 × 9 mm).

## Results

### *Asterosap stimulates rapid synthesis of cGMP*

Peptides from various sea urchin species stimulate the synthesis of both cAMP and cGMP (for review, see Garbers, 1989). We examined the possibility that asterosap activates a cyclic nucleotide-signaling pathway. In order to unequivocally identify the primary messenger, we employed a rapid mixing technique to resolve on a subsecond time scale the changes in the intracellular concentrations of cGMP and cAMP.

The mean resting concentration of cGMP in unstimulated sperm was 0.4 pmol/$10^8$ cells (Table 1).) Asterosap elicited a large and rapid increase in cGMP concentration in a dose-dependent fashion (Fig. 1, and Table 1). The cGMP concentration rose rapidly to a peak in about 400 ms (Fig. 1) and then decayed to a lower plateau within ≤5 s. The decay was observed at all but the lowest concentrations of asterosap. The steady-state level of cGMP was graded with the asterosap stimulus: Higher asterosap concentrations produced higher steady-state levels of cGMP. At 1 $\mu$M asterosap, the cGMP concentration increased by about 100-fold, and even at the lowest concentration tested (1 nM), asterosap produced a significant increase of cGMP (Table 1).

The synthesis by guanylyl cyclase (GC) and the hydrolysis by phosphodiesterase (PDE) determine the intracellular cGMP concentration. A rise of the cGMP concentration, in principle, can be brought about either by stimulation of GC or by inhibition of PDE. To distinguish between these two possibilities, we measured the time course of the asterosap-induced change in cGMP in the presence of 3-isobutyl-l-methylxanthine (IBMX), which inhibits most PDEs (Beavo et al., 1970). IBMX had only a minor effect on the rate of the cGMP increase (compare Fig. 1A and B), whereas it greatly decelerated or entirely abolished the decay from the initial cGMP peak (Fig. 1B). These results demonstrate that asterosap stimulates the synthesis of cGMP and that the transient waveform of the cGMP response is caused by subsequent hydrolysis due to PDE activity.

The resting level of cAMP (5.5 pmol/$10^8$ cells; $n$ = 2) was approximately 14-fold higher than that of cGMP. A high asterosap concentration (1 $\mu$M) did not change the cAMP concentration during a 10-s stimulation period (Fig. 1C). Even in the presence of IBMX, asterosap had no effect on the cAMP level (data not shown). This result demonstrates that asterosap does not directly regulate the synthesis or hydrolysis of cAMP.

Surprisingly, 2 mM IBMX changed the resting levels of cAMP and cGMP in opposite directions: the average cAMP level rose more than sixfold (from 5.5 pmol/$10^8$ cells to 33.5 pmol/$10^8$ cells; $n$ = 2), whereas cGMP levels fell by about fourfold (cp. Table 1). Assuming that the hydrolysis of cAMP and cGMP is equally suppressed by IBMX, these results argue that the activity of adenylyl cyclase (AC) and GC at rest are regulated by different mechanisms. IBMX might stimulate a $Ca^{2+}$ influx, as has been shown for abalone sperm (Kopf et al., 1983, 1984). The ensuing $Ca^{2+}$ increase might stimulate AC activity via a $Ca^{2+}$/calmodulin-dependent mechanism (Kopf and Vacquier, 1984), but might inhibit the GC activity by an unknown mechanism. Alternatively, GC might be inhibited in an unspecific fashion by high IBMX concentrations, as has been observed for the membrane-bound GC from rod photoreceptor cells (Gorczyca et al., 1994). In fact, in the presence of 2 mM IBMX, the asterosap-stimulated increase of cGMP is consistently smaller than in the controls (Table 1).

### *Cloning of GC from testis cDNA*

The rapid and massive increase of cGMP suggests that asterosap initiates the synthesis of cGMP by binding to a receptor GC. We isolated clone pAA-GC from a testicular cDNA library of *A. amurensis*. The cDNA (4211 bp) codes for a protein of 1067 amino acid (aa) residues (calculated $M_r$ of 119.3 k) that displayed significant sequence similarity to known GCs (Fig. 2). The encoded protein exhibits the characteristic domain organization of receptor GCs from both vertebrates and invertebrates (Wedel and Garbers, 2001): an N-terminal hydrophobic signal peptide (aa residues 1–20), an extracellular ligand-binding domain, a single membrane-spanning segment (aa residues 467–485), an intracellular protein kinase-homology domain (KHD), and a cyclase catalytic domain (CCD) in the C-terminal region (Fig. 2). The GC of *A. amurensis* shows a high degree of sequence similarity with the GC of *S. purpuratus* (76.1%) and *A. punctulata* (67.8%), except for a region in the extracellular domain

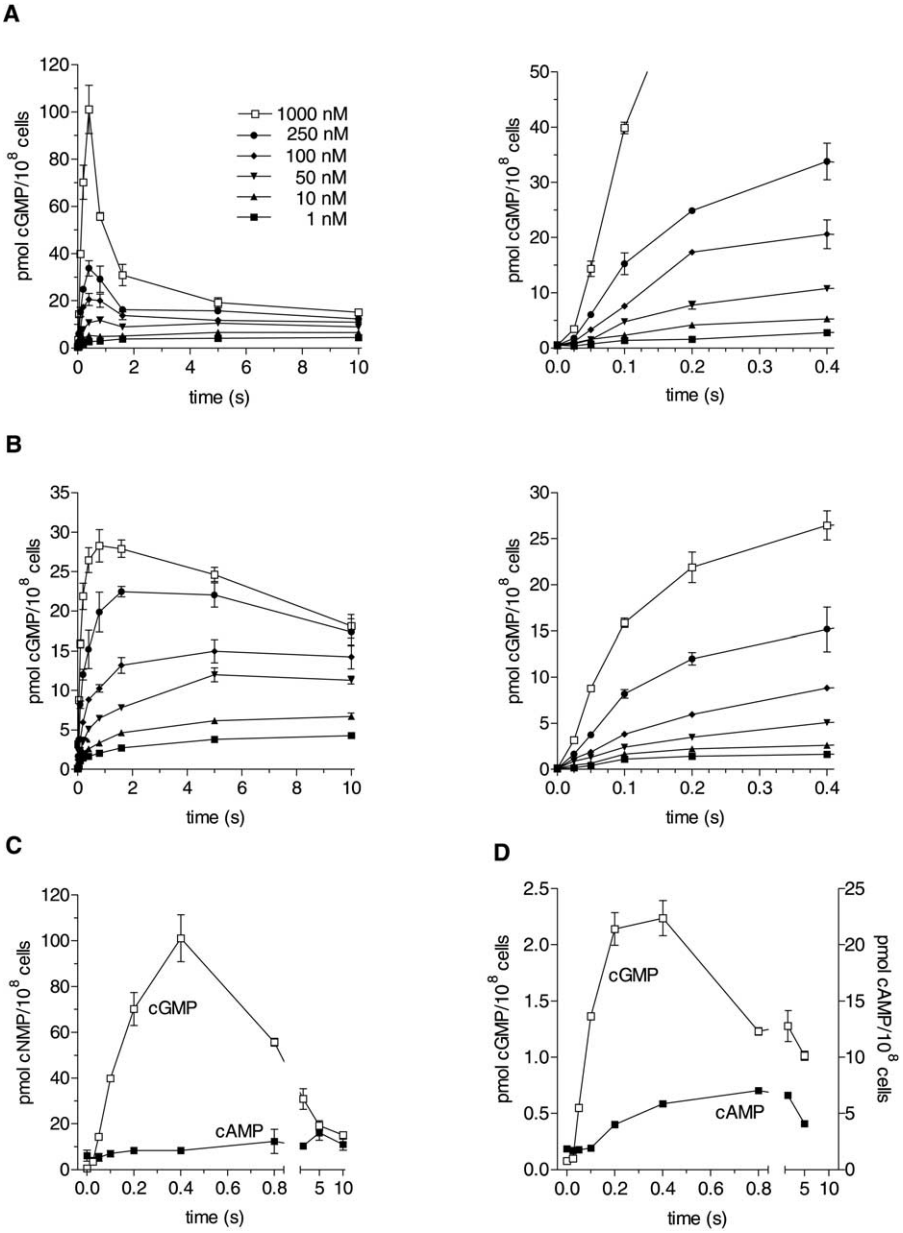


Fig. 1. Peptide-induced changes in cGMP concentration in sperm of *A. amurensis* and *S. purpuratus*. (A) Time course of the change in cGMP induced by asterosap (1 nM–1 $\mu$M). (Right) Same data shown on a faster time scale. (B) Asterosap-induced changes in cGMP concentration in sperm preincubated with 2 mM IBMX. (Right) Same data shown on a faster time scale. Symbols for the asterosap concentrations are the same for (A) and (B). (C) Time course of changes in cGMP and cAMP after stimulation with 1 $\mu$M asterosap. (D) Time course of changes in cGMP and cAMP induced by 250 nM speract in sperm of *S. purpuratus*. Data points represent the mean of triplicates ± SD.

(aa residues ~280–450; see Fig. 2). We speculate that this nonconserved region harbors the binding site for asterosap. Northern blot analysis identified a transcript of ~4.3 kb in male, but not female, gonads (Fig. 3A). The size of the transcript agrees with the size of the cloned cDNA.

### *The GC is the asterosap receptor*

Previous cross-linking experiments identified a membrane protein with a relative molecular weight ($M_r$) of ~130 kDa as potential receptor for asterosap (Nishigaki et al., 2000). We determined the N-terminal sequence of the asterosap-binding protein and found it to be identical with aa residues 21–50 of the cloned GC, demonstrating that the asterosap-binding protein and the receptor GC are identical. This result also shows that the N-terminal signal peptide is proteolytically cleaved during posttranslational maturation of the polypeptide. A similar proteolytic processing has been reported for the GC of *A. punctulata* (Singh et al., 1988). As expected, the $M_r$ of the asterosap-labeled protein detected by the anti-asterosap antibody and by an antibody directed against the C terminus of GC (anti-CTGC) is iden-

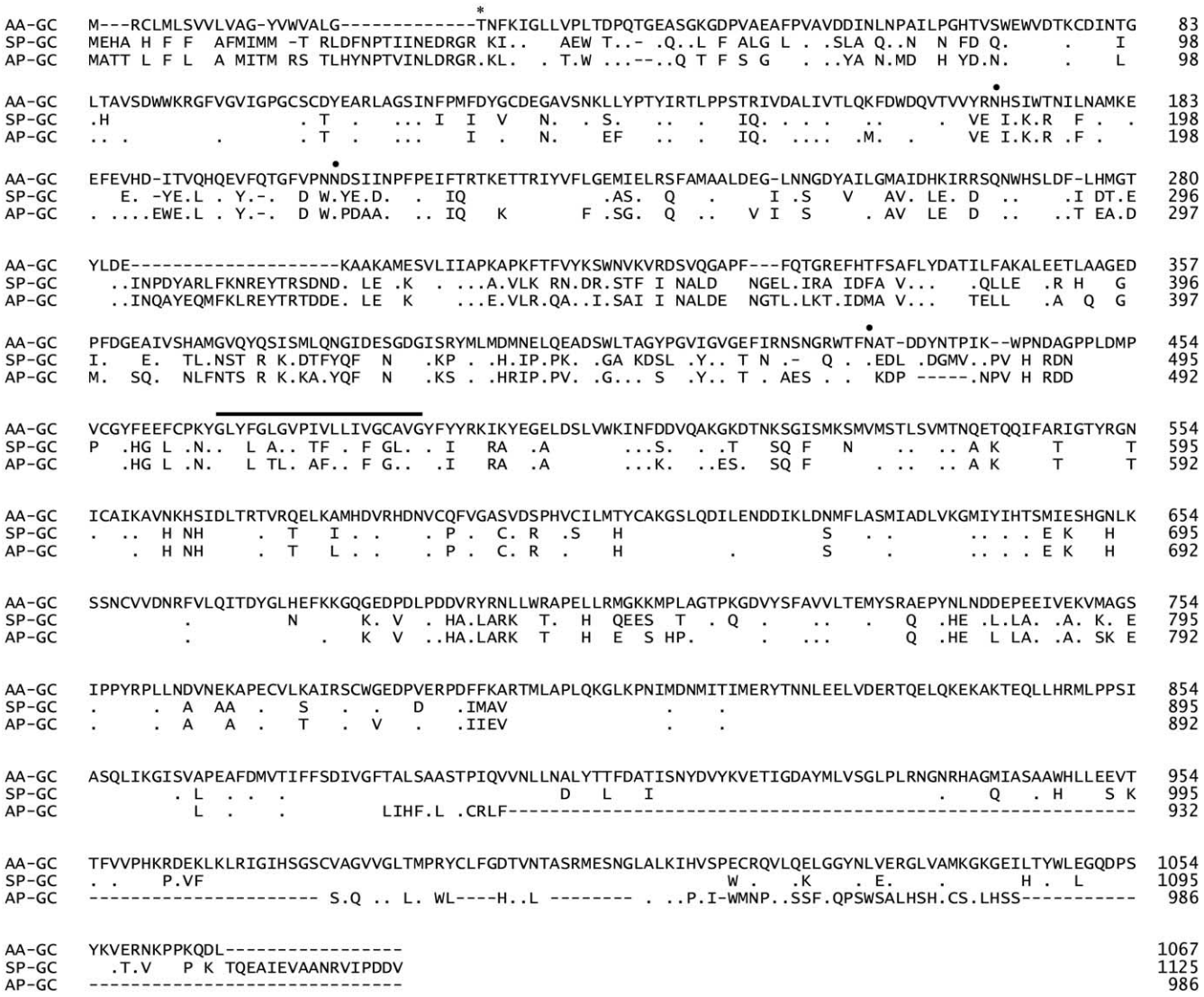

Fig. 2. Comparison of amino acid sequences of receptor-type guanylyl cyclases from invertebrate sperm. Primary structures of GCs from *A. amurensis* (AA-GC), *S. purpuratus* (SP-GC), and *A. punctulata* (AP-GC). Identical residues with respect to the AA-GC sequence are represented by empty spaces, conserved residues by dots, and gaps by dashes. The N-terminal amino acid residue (T 21) of the mature asterosap receptor is indicated by an asterisk and three potential N-linked glycosylation sites by solid dots. The membrane-spanning segment is indicated by a bar. Sequences of SP-GC and AP-GC are from Thorpe and Garbers (1989) and Singh et al. (1988), respectively.

tical (Fig. 3B). The asterosap-labeled protein was not detected after preincubation of the anti-asterosap antibody with the immunizing peptide (data not shown). A second independent antibody, directed against the N terminus of GC (anti-NTGC), and anti-CTGC labeled the same protein (data not shown). The $M_r$ ~125 kDa of the GC determined by SDS–PAGE is in reasonable agreement with the predicted $M_r$ ~117 kDa of the proteolytically processed protein.

### *Localization of GC to the flagellum*

The antibodies anti-CTGC (Fig. 3D) and anti-NTGC (data not shown) both almost exclusively stained the sperm flagellum. The preimmune serum did not recognize sperm (Fig. 3D, lower panels). We confirmed the flagellar localization of the GC by Western blot analysis of membranes from purified sperm flagella and heads. The anti-CTGC antibody recognized a band of $M_r$ ~125 kDa in preparations of whole sperm and flagella, but not in the head fraction of sperm (Fig. 3C).

### *Asterosap activates a cGMP-mediated $Ca^{2+}$ response*

Sperm loaded with the fluorescent $Ca^{2+}$ indicator Fluo-4 responded with a transient increase in fluorescence, $\Delta F_{518}$, upon rapid mixing with solutions of various asterosap concentrations (Fig. 4A). After a delay, $[Ca^{2+}]_i$ rose to a peak and then returned to a lower value that was graded with the

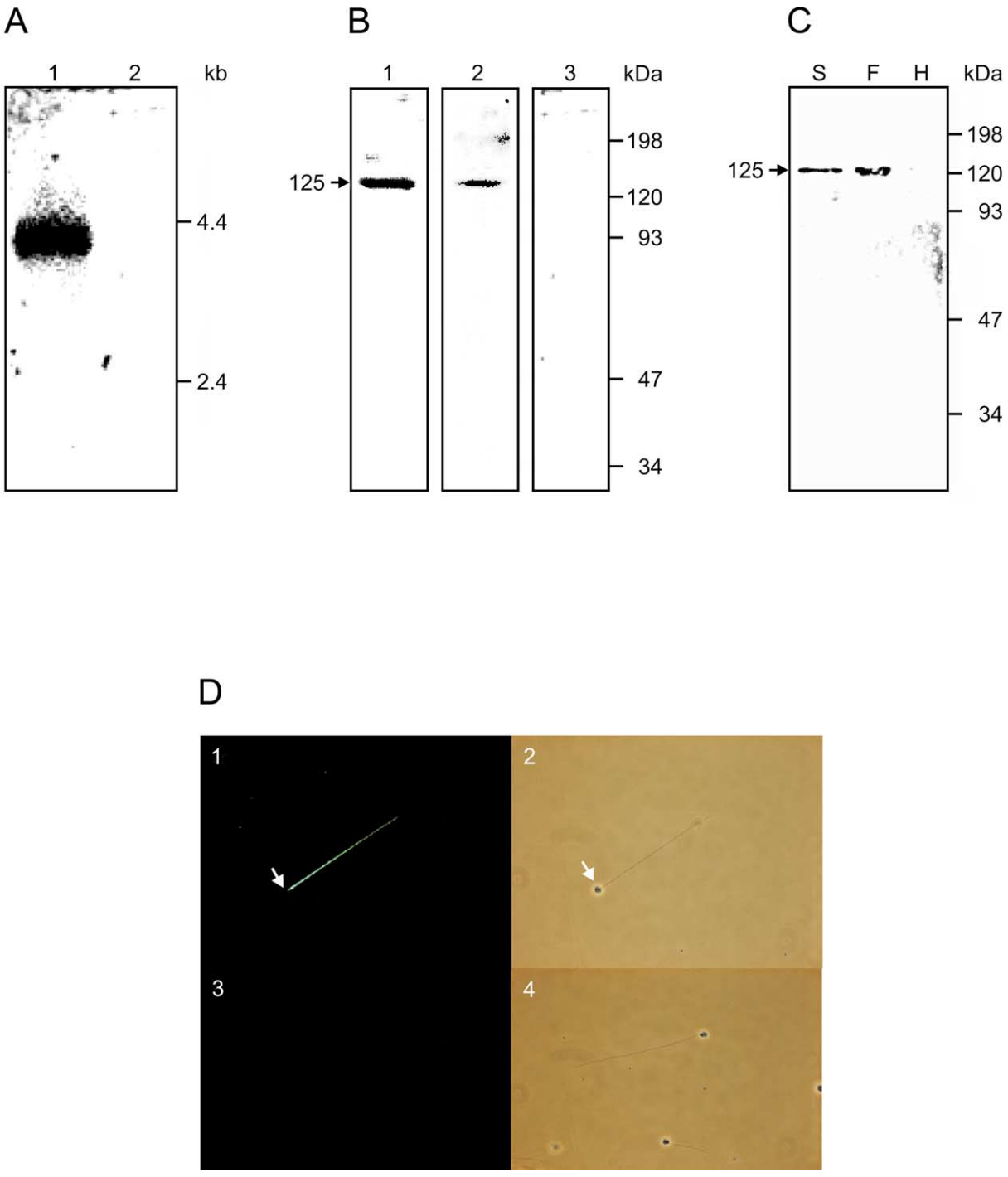


Fig. 3. Site of expression of GC in *A. amurensis*. (A) Northern blot analysis of AA-GC messenger RNA in male gonads (lane 1) and female gonads (lane 2). Total RNA (10 $\mu$g) was used in each lane. Blots were hybridized with a labeled 239-bp cDNA fragment (nt 2835-3073). (B) Western blot analysis of asterosap-labeled flagella. Western blots were incubated either with the anti-CTGC antibody (lane 1), the anti-asterosap antibody (lane 2), or the preimmune serum of the anti-asterosap antibody (lane 3). (C) Western blot analysis of membranes isolated from whole sperm (lane S), flagella (lane F), and heads (lane H). Antiserum anti-CTGC was used. (D) Localization of the GC by immunocytochemistry. Sperm was fixed with formaldehyde and stained with the anti-CTGC antibody and with Alexa Fluor 488-conjugated secondary antibody (1); only the flagellum, but not the head (arrow) is stained. Picture of sperm taken with Nomarski optics (2). The preimmune serum did not stain the flagellum (3); (4) Nomarski optics.

asterosap concentration (Fig. 4A). The $Ca^{2+}$ signals saturated at asterosap concentrations ≥500 nM. The delay (250 ± 41 ms, $n = 4$) was largely independent of the asterosap concentration. The waveform of the asterosap-induced $Ca^{2+}$ signals depended on the asterosap concentration. For asterosap concentrations up to 50 nM, the peak amplitude and the time-to-peak increased with the asterosap concentrations. For asterosap concentrations ≥50 nM, the time-to-

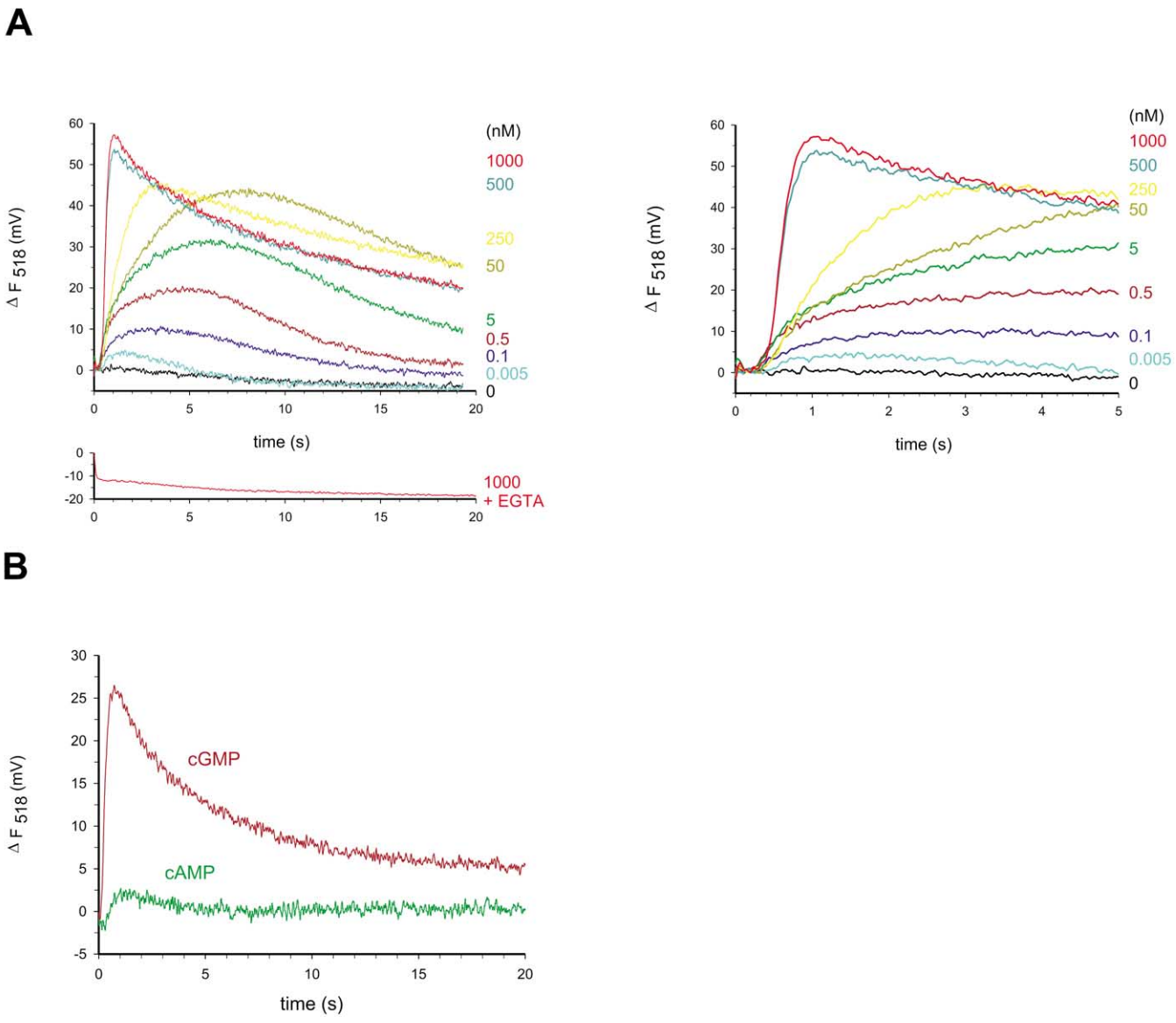


Fig. 4. Changes in $[Ca^{2+}]_i$ induced by asterosap and caged cyclic nucleotides. (A) (Left) Asterosap-induced increase of $F_{518}$ in sperm that were loaded with the $Ca^{2+}$ indicator Fluo-4. (Right) Same signals as in the left panel shown on a faster time scale. (Bottom) Asterosap-induced change in $F_{518}$ in an ASW medium of low $[Ca^{2+}]$ ($<10^{-6}$ M). The asterosap concentrations are indicated at the right hand of traces. (B) Flash-induced changes in $[Ca^{2+}]_i$ in sperm that have been incubated with 10 $\mu$M of either BECMCM-caged cGMP (red trace) or BECMCM-caged cAMP (green trace).

peak became significantly shorter. IBMX (1 mM) largely prevented the relaxation of the $Ca^{2+}$ signal (data not shown), suggesting that recovery from asterosap stimulation requires hydrolysis of cGMP. In the presence of EGTA (11 mM) in ASW ($[Ca^{2+}] < 10^{-6}$ M), the asterosap-stimulated increase of $\Delta F_{518}$ was abolished (Fig. 4A, lower panel); instead, $\Delta F_{518}$ slightly decreased during the stimulation time, probably due to an efflux of $Ca^{2+}$ ions from the cell. These findings demonstrate that $\Delta F_{518}$ reports an increase of $[Ca^{2+}]_i$ due to $Ca^{2+}$ influx from the extracellular medium.

Stimulation of sperm with asterosap may trigger biochemical reactions in addition to cGMP synthesis and an increase in $[Ca^{2+}]_i$ may be triggered by intracellular messengers other than cGMP. In sea urchin sperm, a rise in [cAMP] reportedly opens $Ca^{2+}$ channels (for review, see Darszon et al., 2001). To address this question, we used novel caged forms of cGMP and cAMP. Irradiation of sperm, loaded with caged cGMP, by a short flash of UV-light evoked a transient increase in $[Ca^{2+}]_i$ that is similar in waveform to that evoked by high asterosap concentrations (compare Fig. 4A and B). The delay of the cGMP-induced $Ca^{2+}$ signal (73 ± 17 ms; $n = 9$) was significantly shorter than the delay of the asterosap-induced $Ca^{2+}$ signal (250 ± 41 ms; $n = 4$). The difference in delay can be accounted for by the time required to synthesize a threshold concentration of cGMP.

In sperm loaded with caged cAMP, UV irradiation evoked a $Ca^{2+}$ response that is distinctively different from

that produced by cGMP (Fig. 4B). First, the amplitude of the cAMP-induced $Ca^{2+}$ signal was significantly smaller. Assuming that membrane permeability, absorptivity, and quantum yield of caged cAMP and caged cGMP are similar, these findings suggest that cGMP is about 10-fold more effective in evoking a $Ca^{2+}$ signal than cAMP. Second, the delay of the cAMP-induced $Ca^{2+}$ signal (191 ± 113 ms; $n = 3$) was almost 3 times longer than the delay of the cGMP-induced $Ca^{2+}$ signal (73 ± 17 ms). Third, the time-to-peak of the cAMP-induced $Ca^{2+}$ signal (1147 ± 252 ms; $n = 3$) was longer than the time-to-peak of the cGMP-induced signal (616 ± 117 ms; $n = 9$).

In conclusion, the asterosap-induced $Ca^{2+}$ response is mediated by an increase of cGMP. It is unlikely that cAMP is involved in the generation of the $Ca^{2+}$ response, because asterosap did not significantly enhance the cAMP level during a 10-s stimulation period and because cAMP is significantly less potent in activating the $Ca^{2+}$ entry pathway.

## Discussion

The central discovery of this study is that asterosap, a sperm-activating peptide of the starfish *A. amurensis* activates a cGMP-signaling pathway and thereby increases the intracellular [$Ca^{2+}$]. Sperm-activating peptides evoke a multitude of intracellular events (for review, see Darszon et al., 1999, 2001). Because the precise timing of these events on a physiologically relevant time scale is unknown, the primary signaling events are still enigmatic. In this study, we employed kinetic techniques to unequivocally identify the primary messenger in vivo. We were able to resolve an extremely rapid and large increase in the cGMP concentration: after 25 ms, the cGMP concentration has risen to a roughly sixfold higher level at 1 $\mu$M asterosap. Therefore, it is plausible that the cGMP increase precedes all other intracellular events and represents the first step of asterosap-induced signaling.

There are striking similarities between the peptide-activated signaling pathways in sea urchin and starfish. First, the basal levels of cAMP and cGMP in unstimulated *A. amurensis* sperm are similar to those reported for sea urchin sperm. Second, the main features of the peptide-induced rise of cGMP are similar. In sperm of both sea urchin and starfish, the rise of cGMP is rapid, large, and transient (Harumi et al., 1992; Kaupp et al., 2003; and Fig. 1). Previous studies in sea urchin sperm reported significantly smaller peptide-induced elevations of the cGMP level (Hansbrough and Garbers, 1981a,b; Garbers et al., 1982; Suzuki et al., 1984, 1988; Shimomura and Garbers, 1986; Cook and Babcock, 1993b). The quantitative differences are readily accounted for by the rapid and transient nature of the cGMP response. In previous studies, samples have been quenched ≥5 s after the stimulation by peptides; as a result, the peak increase of cGMP has escaped detection in most studies due to insufficient time resolution (see, however, Harumi et al., 1992). Third, the cAMP increase is smaller, slower, and less sensitive than the cGMP response (sea urchin; Kaupp et al., 2003) or entirely absent (starfish; this manuscript). Fourth, peptides evoke a $Ca^{2+}$ entry in sperm of sea urchin (Schackmann and Chock, 1986; Cook and Babcock, 1993a) and starfish that is rapid and transient (Nishigaki et al., 2001; Kaupp et al., 2003; and Fig. 4). Finally, the rapid release from caged compounds of either cAMP or cGMP evokes the entry of $Ca^{2+}$ into the cell. The delay and waveform of the cyclic nucleotide-stimulated $Ca^{2+}$ responses in starfish and sea urchin sperm are of similar magnitude (Kaupp et al., 2003; and Fig. 4). In both species, the cAMP-evoked $Ca^{2+}$ responses are slower and smaller than the cGMP-evoked $Ca^{2+}$ responses.

Either cAMP and cGMP activate one and the same $Ca^{2+}$ conductance, though with different kinetics and sensitivities, or cAMP activates a different $Ca^{2+}$ conductance. We also cannot exclude that the cAMP- and cGMP-activated $Ca^{2+}$ influx might occur in different regions of the cell. A $Ca^{2+}$ conductance stimulated by both cGMP and cAMP is reminiscent of cyclic nucleotide-gated (CNG) channels (for review, see Kaupp and Seifert, 2002). As attractive as this idea might be, the long delay of the $Ca^{2+}$ response argues against the involvement of CNG channels, which display little delay when activated by the use of caged cyclic nucleotides (Karpen et al., 1988; Hagen et al., 1996; Dzeja et al., 1999). However, we cannot rule out that the activation of putative CNG channels in invertebrate sperm is inherently slower than that studied in mammalian cells or that the CNG channels might be located in a subcellular domain with restricted diffusion (Rich et al., 2000, 2001). Alternatively, $Ca^{2+}$ entry might involve other channels that require further step(s) to become activated. What might be the nature of the intervening steps? The opening of the $Ca^{2+}$-entry channels might involve cyclic nucleotide-dependent phosphorylation or additional unknown protein(s).

Resact acts as a potent chemoattractant for *A. punctulata* sperm (Ward et al., 1985). We speculate that asterosap, like resact, serves as a chemoattractant as well. This hypothesis is supported by several lines of evidence. First, *Asterias* sperm respond to asterosap with tumbling episodes and swimming in narrow circles when the hydrolysis of cyclic nucleotides is prevented by preincubation of sperm with IBMX (E. Hildebrand, unpublished observations). A similar behavior pattern is induced by the chemoattractant resact and IBMX in *A. punctulata* sperm (Kaupp et al., 2003). Second, asterosap shares a high degree of sequence similarity with the N-terminal end of a sperm-attracting 13-kDa protein ("startrak") from the starfish *Pycnopodia helianthoides* (Miller and Vogt, 1995). "Startrak" was purified from immature eggs and presumably represents an unprocessed prepolypeptide. In fact, the analysis of cDNA encoding asterosap shows that asterosap precursors are large prepolypeptides that comprise several isoforms of asterosap

(Matsumoto et al., 1999). Third, the asterosap receptor is localized exclusively on the flagellum, suggesting that asterosap controls sperm motility. In fact, asterosap accelerates the swimming speed of sperm at nanomolar concentrations in acidified artificial sea water (ASW) (Nishigaki et al., 1996). Fourth, asterosap promotes $Ca^{2+}$ entry that, in sea urchin, is required for the chemotactic response (Ward et al., 1985; Kaupp et al., 2003). Finally, sperm are exquisitely sensitive: picomolar concentrations of asterosap are sufficient to evoke a $Ca^{2+}$ response. However, asterosap, at high concentrations, may also serve as a cofactor that facilitates the acrosome reaction induced by ARIS (acrosome reaction-inducing substance), a high-molecular weight glycoconjugate of the egg jelly (Nishigaki et al., 1996).

The similarities between *A. amurensis* and *A. punctulata* sperm support the idea that chemosensory signaling in sperm has been conserved for almost 500 million years. In light of these parallels, it seems odd that the receptor for speract of *S. purpuratus* sperm is a protein unrelated to GCs (Dangott et al., 1989). In fact, this observation would seem to underpin diversity rather than conservation of chemosensory signaling in sperm. To address this issue, we measured the speract-induced rise of cGMP in *S. purpuratus* sperm on a subsecond time scale. As in *A. amurensis* sperm, the changes in cGMP stimulated by speract were extremely fast, large, and transient (Fig. 1D). This result argues that a rise in cGMP also represents the initial peptide-stimulated event in *S. purpuratus* sperm just as in *A. punctulata* sperm (Kaupp et al., 2003). Functional expression of the cloned receptor GC from *S. purpuratus* sperm could provide definitive answers. Unfortunately, attempts failed to functionally express GC from invertebrate sperm in heterologous expression systems (for review, see Yuen and Garbers, 1992).

Chemosensory signaling using cGMP as primary messenger and GCs as receptor is not restricted to sperm. In the genome of *Caenorhabditis elegans*, 29 different genes of receptor GCs have been identified, of which several have been localized to sensory neurons (Yu et al., 1997). Olfaction and odor discrimination in *C. elegans* are mediated by GCs (L'Etoile and Bargmann, 2000; Birnby et al., 2000). Furthermore, in the main olfactory epithelium of rodents, a subset of olfactory neurons selectively utilizes cGMP-signaling components, namely a receptor-type GC (subtype D) (Fülle et al., 1995; Juilfs et al., 1997), a cGMP-stimulated PDE (PDE2) (Juilfs et al., 1997), and a cGMP-selective cyclic nucleotide-gated channel (Meyer et al., 2000). Although, the "odorant," i.e., the ligand(s) of GC-D, are not known, there is initial evidence that this subset of olfactory neurons control some feature of reproductive behavior. Thus, chemosensory signaling by means of cGMP and GCs appears to be a phylogenetically ancient system that has been conserved across various phyla and cellular systems.

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