Cyclic GMP-specific Phosphodiesterase-5 Regulates Motility of Sea Urchin Spermatozoa

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Motility, chemotaxis, and the acrosome reaction of animal sperm are all regulated by cyclic nucleotides and protein phosphorylation. One of the cyclic AMP-dependent protein kinase (PKA) substrates in sea urchin sperm is a member of the phosphodiesterase (PDE) family. The molecular identity and in vivo function of this PDE remained unknown. Here we cloned and characterized this sea urchin sperm PDE (suPDE5), which is an ortholog of human PDE5. The recombinant catalytic domain of suPDE5 hydrolyzes only cyclic GMP (cGMP) and the activity is pH-dependent. Phospho-suPDE5 localizes mainly to sperm flagella and the phosphorylation increases when sperm contact the jelly layer surrounding eggs. In vitro dephosphorylation of suPDE5 decreases its activity by ~50%. PDE5 inhibitors such as Viagra block the activity of suPDE5 and increase sperm motility. This is the first PDE5 protein to be discovered in animal sperm. The data are consistent with the hypothesis that suPDE5 regulates cGMP levels in sperm, which in turn modulate sperm motility.

INTRODUCTION

Cyclic nucleotide concentrations regulate sea urchin sperm physiology including the activation of motility, chemotaxis toward eggs, and induction of the acrosome reaction (Gardner, 1989; Morisawa, 1994; Darszon *et al.*, 2001, 2005; Neill and Vacquier, 2004). For example, when speract, a sperm-activating decapeptide diffusing from eggs, binds to its receptor on sperm flagella, guanylyl cyclase is activated, causing a rapid transient increase of cGMP (Kaupp *et al.*, 2003). A cGMP-modulated K⁺ channel then opens causing K⁺ efflux and a hyperpolarization of sperm membrane potential (Cook and Babcock, 1993; Galindo *et al.*, 2000). These events trigger Ca²⁺ oscillations in sperm flagella (Wood *et al.*, 2003), which in turn modulate the motility and chemotaxis of sperm swimming toward eggs (Böhmer *et al.*, 2005).

Phosphodiesterases (PDEs) hydrolyze cyclic nucleotides, and together with adenylyl and guanylyl cyclase, which catalyze the formation of cAMP and cGMP, regulate the levels of these second-messengers in cells. The known mammalian PDEs are divided into 11 families, PDE1–11. Some PDEs specifically hydrolyze cAMP (PDEs 4, 7, and 8) or cGMP (PDEs 5, 6, and 9), whereas others hydrolyze both cyclic nucleotides (PDEs 1–3, 10, 11; Fawcett *et al.*, 2000; Soderling and Beavo, 2000). In each PDE family, alternatively spliced, tissue-specific variants have been reported (Beavo, 1995). All PDEs contain a conserved catalytic domain of ~270 amino acids at the carboxy terminus. The

regulatory domains and motifs that vary widely among the PDE families are often found near the amino terminus (Soderling and Beavo, 2000). The catalytic domains of all known mammalian PDEs contain two Zn²⁺-binding motifs (HX₃HX_{24–26}E), which bind Zn²⁺ and Mg²⁺ (Ke, 2004; Zhang *et al.*, 2004). The binding of Zn²⁺ to PDE activates its enzymatic activity (Francis *et al.*, 1994; Percival *et al.*, 1997). The cGMP-specific PDE5 is one of the PDEs that has been intensively studied because of fundamental pharmacological interest. PDE5 inhibitors such as Viagra (Pfizer, New York, NY) are widely used for the treatment of erectile dysfunction (Corbin and Francis, 1999). The domain organization of PDE5 is similar to that of PDEs 2, 6, 10, and 11, in that in addition to the catalytic domain, they all contain two GAF domains that constitute potential allosteric binding sites for cGMP (GAF domains are named after the proteins where they are found: cGMP-binding and stimulated PDEs, *Anabaena* adenylyl cyclases, and *Escherichia coli* FhlA protein; Zoraghi *et al.*, 2004).

Several isotypes of PDEs have been found in animal sperm. For example, inhibitor studies show that PDE1 (calcium/calmodulin-dependent) and PDE4 are in human sperm. Moreover, PDE1 inhibitors stimulate the acrosome reaction, whereas PDE4 inhibitors enhance sperm motility (Fisch *et al.*, 1998). Immunocytochemical data show that PDE1A and PDE3A are localized on different regions of ejaculated human sperm (Lefievre *et al.*, 2002). RT-PCR studies reveal that washed human sperm contain an extended pattern of PDE mRNA transcripts, including those for PDE1–5 and 8 (Richter *et al.*, 1999). Mature mouse sperm also contain several PDE isoforms that are important for capacitation, a series of changes that enable sperm to undergo the AR (Baxendale and Fraser, 2005). In sea urchin sperm, PDE activity is known to be restricted to the flagellum (Sano, 1976; Toowicharanont and Shapiro, 1988). Sea urchin sperm also contain a cGMP-binding cGMP PDE activity (Francis *et al.*, 1980). Until now the molecular identity of this PDE remained uncertain.

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Abbreviations used: ASW, artificial seawater; cGMP, cyclic guanine monophosphate; EJ, egg jelly; GAF, a conserved domain found in cGMP-binding and stimulated PDEs, *Anabaena* adenylyl cyclases, and *Escherichia coli* FhlA protein; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; pHi, intracellular pH.

Compared with mammalian sperm, sea urchin sperm are morphologically simple and have one-fourth the genome size of mammals. Being deuterostomes, at the base of the line of animal evolution leading to mammals, sea urchins provide ideal model sperm for studying the fundamentals of sperm physiology during animal fertilization. Previously, we reported that a 100-kDa phosphoprotein of these sperm, recognized by a PKA substrate antibody, is a member of the PDE family (Su *et al.*, 2005). Here we identify and characterize this PDE as the first PDE5 protein found in animal sperm. The phosphorylation of suPDE5 increases when sperm contact egg jelly (EJ), a jelly layer composed of polysaccharides, glycoproteins, and peptides surrounding eggs (Hirohashi and Vacquier, 2002). Dephosphorylation of suPDE5 in vitro decreases its activity by ~50%. PDE5 inhibitors block the activity of suPDE5 and enhance sperm motility.

MATERIALS AND METHODS

Cloning and Sequence Analysis

Identification of sperm phosphoproteins by tandem mass spectrometry (MS/MS) resulted in four peptides from the 100-kDa phosphoprotein that matched to PDE5 or 11 (Su *et al.*, 2005). PDE forward (5'-TTCAGGATTCGATCCGTC-CTCTGC-3') and reverse (5'-AGTTGCGTGGTACGAGACATC-3') primers were designed based on the nucleotide sequences taken from the *Strongylocentrotus purpuratus* genome from the Human Genome Sequencing Center at Baylor College of Medicine (<http://www.hgsc.bcm.tmc.edu/projects/seaurchin/>). The full-length cDNA of the 100-kDa PDE (suPDE5) was obtained by PCR using the PDE reverse primer and T7 primer from a sea urchin Lambda ZAP testis cDNA library and by 3' RACE (First Choice RLM kit, Ambion, Austin, TX) using the PDE forward primer and testis cDNA. Sites and domains were identified using the ProfileScan website (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) and the Simple Modular Architecture Tool (SMART; <http://smart.embl-heidelberg.de>). ClustalW (MacVector) was used for alignments.

Phylogenetic Analysis

Complete sequences of suPDE5 and human PDE proteins were used to construct a phylogenetic tree by the neighbor-joining method using PAUP* 4.0b10 (Swofford, 2002). The tree was validated by a bootstrap analysis with 1000 replicates. Protein sequence database accession numbers for human PDE proteins are as follows: PDE1A, BAB20050; PDE1B, AAH32226; PDE1C, Q14123; PDE2A, AAC51320; PDE3A, AAA35912; PDE3B, AAR24292; PDE4A, P27815; PDE4B, NP_002591; PDE4C, Q08493; PDE4D, Q08499; PDE5A, O76074; PDE6A, AAB69155; PDE6B, CAA46932; PDE6C, CAA64079; PDE7A, Q13946; PDE7B, CAH73075; PDE8A, AAC39763; PDE8B, AAN71725; PDE9A, AAC26723; PDE10A, CAI20436; PDE11A, BAB62712. The GenBank accession number of suPDE5 is DQ073640.

Expression and Purification of suPDE5 Catalytic Domain

The cDNA encoding the catalytic domain of suPDE5 (Thr⁵⁴⁰-Ile⁹⁴⁹) was amplified by PCR and cloned into pET15b (Novagen, Madison, WI), which contained an NH₂-terminal His tag. The vector was transformed into Rosetta (DE3) competent cells (Novagen) for expression. After 1 mM IPTG induction for 1 h at 37°C, the bacterial lysate was made as described (Kuwayama *et al.*, 2001) with slight modifications as follows. A 50-ml culture was centrifuged 20 min at 10,000 × g, the pellet was resuspended in 25 ml of ice-cold Tris buffer (20 mM Tris, pH 7.5), and the cells were lysed by sonication with 5 pulses of 3 s each. The resulting bacterial lysate was used for the PDE activity assay.

To purify the expressed catalytic domain of suPDE5, 1 l of IPTG-induced bacterial culture was centrifuged, and the pellet was frozen at -80°C. The pellet was resuspended in 100 ml of ice-cold lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1% NP40, 1 mM benzimidazole, pH 8). After adding 100 mg of lysozyme, the bacteria suspension was stirred at 4°C for 30 min. The lysate was further sonicated 20 bursts and incubated with 10 µg/ml RNase A and 5 µg/ml DNase I for 30 min at 4°C. The clarified cell extract was obtained by centrifugation at 14,000 × g for 30 min and incubated with Ni-NTA resin (QIAGEN, Valencia, CA) overnight at 4°C. The beads were then washed with 100 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8) and the proteins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8). The purified protein was concentrated and the buffer was exchanged with 20 mM Tris pH 7.5 using an Amicon Ultra-15 (Millipore, Bedford, MA) apparatus.

PDE Activity Assay

PDE activity was determined by slight modifications of the previously described method (Sonnenburg *et al.*, 1998). The reaction buffer contained 20 mM Tris (pH 7.5), 15 mM magnesium acetate, 0.2 mg/ml BSA, 2 mM EGTA, and 30 nM [³H]cAMP or [³H]cGMP (Amersham Biosciences, Piscataway, NJ) held at 30°C for 30 min in a final volume of 250 µl. The samples were boiled 2 min, chilled for 3 min, and then incubated with 10 µl of *Crotalus atrox* snake venom (Sigma, St. Louis, MO; 10 mg/ml) for 10 min at 30°C. The samples were then applied to 0.7 ml of DEAE Sephadex A-25 (Amersham Biosciences) and washed with 2 ml of 20 mM Tris, pH 6.8. Twenty milliliters of ACS-II scintillation cocktail (Amersham Biosciences) was added to 900 µl of the effluent and counted for 1 min. The amount of enzyme was adjusted to hydrolyze <30% of the substrate. For kinetic studies, cGMP concentrations varied between 0.1 and 15 µM. For inhibitor studies, 3.33 µM cGMP was used. At this concentration of substrate, the IC₅₀ value approximates the K_i (Hetman *et al.*, 2000). PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX), dipyrindamole, zaprinast, and methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulfate (T-1032) were obtained from Sigma. Viagra tablets (50 mg; a registered trademark of Pfizer Corporation) were commercially available. All inhibitors were dissolved in dimethyl sulfoxide (DMSO).

Gametes

Sea urchin (*S. purpuratus*) gametes were spawned by injection of 0.5 M KCl into adults. Undiluted sperm (4 × 10¹⁰ spermatozoa per ml) were collected and kept on ice for no longer than 24 h. EJ was prepared and quantified as described (Hirohashi and Vacquier, 2002). Speract was from Peninsula Laboratories (Belmont, CA) and dissolved in artificial seawater (ASW; 486 mM NaCl, 10 mM CaCl₂, 10 mM KCl, 27 mM MgCl₂, 29 mM MgSO₄, 2.5 mM NaHCO₃, and 10 mM HEPES, adjusted to pH 8.0 with 1 N NaOH).

Sperm Protein Preparations and Immunoblots

For whole sperm protein preparation, sperm suspensions were precipitated with acetone (80% final concentration), sedimented by centrifugation for 5 min at 21,000 × g and the pellet was dissolved in 10% SDS. For NP40 sperm lysate preparation, sperm suspensions were sedimented by centrifugation for 5 min at 21,000 × g, and the pellet was extracted for 30 min in NP40 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP40, 2 mM EDTA, 50 mM NaF, 1 mM dithiothreitol [DTT], 10 µg/ml aprotinin, 5 µg/ml pepstatin A, 20 µg/ml leupeptin, 1 mM benzimidazole, 0.2 mM sodium vanadate). The NP40-solubilized proteins were obtained after centrifugation at 26,000 × g for 90 min. Isolation of sperm heads from flagella was done as described (Vacquier and Hirohashi, 2004). Sperm proteins were separated on SDS-PAGE gels and transferred to PVDF. The PVDF membranes were probed with the phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology, Beverly, MA; Catalogue number 9621), detected with a HRP-conjugated goat anti-rabbit secondary antibody (following the manufacturer's instructions), and developed with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Western blots were quantified by scanning densitometry using a DuoScan Scanner (AGFA, Orangeburg, NY) and analyzed with NIH Image 1.62 (<http://rsb.info.nih.gov/ni-image/download.html>).

Immunoprecipitation and In Vitro Dephosphorylation and Phosphorylation

Immunoprecipitation was performed as described (Su *et al.*, 2005) with modifications as follows. Four hundred microliters NP40-solubilized sperm lysate (2 mg/ml) was incubated with 4 µl of the Phospho-(Ser/Thr) PKA substrate antibody and 40 µl of 50% protein A-Sepharose CL4B beads. Precipitated immunocomplexes, bound to the protein A beads, were washed four times in NP40 lysis buffer without phosphatase inhibitors and twice in 20 mM Tris, pH 7.5. For in vitro dephosphorylation, precipitated immunocomplexes were resuspended in 50 µl of reaction buffer (10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, in 20 mM Tris, pH 7.5) and incubated with 5 U calf intestinal alkaline phosphatase (ALP; Boehringer Mannheim, Indianapolis, IN) for 20 min at room temperature. For in vitro phosphorylation, precipitated immunocomplexes were resuspended in 50 µl of reaction buffer (1 mM ATP, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, in 20 mM Tris, pH 7.5) and incubated with 2 U catalytic subunit of PKA from bovine hearts or recombinant human protein (Sigma) for 10 min at room temperature. The samples were then washed twice in 20 mM Tris, pH 7.5, before being subjected to the enzyme activity assay or SDS-PAGE analysis by silver staining.

Sperm Motility Assay and Statistical Analysis

A spectrophotometric method for measuring sperm motility was performed as described (Deana *et al.*, 1986) with modifications as follows. The assessment of sperm motility was carried out by recording the absorbance change at 580 nm caused by downward swimming of sperm into 4% (wt/vol) Ficoll 400,000 (Sigma) dissolved in ASW. Two hundred microliters of sperm suspension in ASW (1:100 dilution of fully concentrated semen, 4 × 10⁸ cells/ml) was layered on top of 800 µl of 4% Ficoll-ASW. Both sperm suspension and

Figure 1. Protein sequence alignment of sea urchin suPDE5, human hPDE5A1, and human hPDE11A4. Residues identical in all three sequences are shown in boldface type and dark shading. Similar residues are boxed. Two GAF domains, one catalytic domain, and two Zn^{2+} -binding motifs are indicated. Four peptide sequences identified by MS/MS are overlaid and indicated as P1, P2, P3, and P4. Asterisks indicate the three putative PKA sites. The four residues that determine the substrate specificity are denoted by downward arrows.

Ficoll-ASW contained DMSO or PDE inhibitors at a final concentration of 1%. Data were compared with the DMSO control using Student's *t* test for paired data, and $p \leq 0.05$ was considered statistically significant.

RESULTS

Sequence and Phylogenetic Analysis

When sea urchin sperm swim in seawater, only one major sperm protein of 100 kDa is recognized by the phospho-(S/T) PKA substrate antibody we use in these studies. MS/MS identified this protein as PDE5 or PDE11 (Su *et al.*,

2005). The sequence of the cDNA encoding this PDE was obtained by PCR from testis cDNA. The ORF is 2847 base pairs and encodes a protein of 949 residues with a calculated molecular mass of ~108 kDa, which shows relatedness to human PDEs 5 and 11 (Figure 1). This sea urchin sperm PDE (suPDE), like human PDE5A1 and PDE11A4, contains two GAF domains at the NH_2 -terminus and a catalytic domain at the COOH -terminus. Two motifs for Zn^{2+} -binding (HX₃HX₂₄₋₂₆E), conserved in the catalytic domains of all PDEs (Francis *et al.*, 1994), are present in this suPDE. All four

Figure 2. Phylogenetic tree of human PDE proteins compared with suPDE5. The tree was constructed by the neighbor-joining method and the values at branch points are bootstrap percentages with 1000 replications. GenBank accession numbers are in *Materials and Methods*. The scale indicates the number of amino acid substitutions. The PDE proteins containing GAF domains are shaded.

peptides of the 100-kDa phosphoprotein identified by MS/MS in the previous study (Su *et al.*, 2005) are found in the suPDE sequence. suPDE also contains three PKA phosphorylation sites. Only one of them, an RRKS¹⁰¹ motif, can be recognized by the commercial PKA substrate antibody. suPDE is equally distant to human PDE5A1 and PDE11A4, with an identity of 37 and 38%, respectively, in the full-length (53 and 56% similarity) and 51 and 54% in the catalytic domain (73 and 74% similarity).

The crystal structures of the catalytic domains of PDE1 (a dual substrate PDE), PDE4 (a cAMP-specific PDE) and PDE5 reveal that nucleotide selectivity is determined by a conserved glutamine and its surrounding residues (Zhang *et al.*, 2004). The three amino acids that surround the conserved Q⁸¹⁷ in the cGMP binding pocket of human PDE5A1 are A⁷⁶⁷Q⁷⁷⁵W⁸⁵³, whereas the corresponding residues in the dual substrate PDE11A4 are A⁸¹⁹S⁸²⁷W⁹⁰⁵ (Figure 1). From the alignment of suPDE to human PDE5A1 and PDE11A4, we identified the conserved Q⁸²² and the three residues that control the substrate specificity in suPDE as follows: A⁷⁷²Q⁷⁸⁰W⁸⁵⁸, which are identical to human PDE5, suggesting that the suPDE is a cGMP-specific PDE.

A phylogenetic tree based on full-length PDE proteins shows that all PDEs that contain GAF domains form a monophyletic clade with suPDE being most similar to human PDE5A1 and PDE11A4. Of the two human proteins, suPDE is closer to human PDE5A1 than to PDE11A4, with a moderate bootstrap value (62%; Figure 2).

Characterization of suPDE5 Activity

The COOH-terminal half of suPDE5, containing the catalytic domain (suPDE-CAT: Thr⁵⁴⁰-Ile⁹⁴⁹), was expressed in bacteria. Lysates from control vector and suPDE-CAT transformed bacteria were assayed for PDE activity with [³H]cAMP or [³H]cGMP as a substrate. suPDE-CAT expres-

Figure 3. Characterization of suPDE5 activity. (A) The control vector (□) or the catalytic domain of suPDE5 (■) expressed in bacteria were lysed and enzyme activity was measured with 30 nM [³H]cAMP or [³H]cGMP. (B) Purified catalytic domain of suPDE5 (PDE-CAT) was used for the PDE assay with 30 nM [³H]cAMP (○) or [³H]cGMP (●) as a substrate. (C) Lineweaver-Burk plot for suPDE-CAT catalyzed cGMP hydrolysis. The reactions were performed with 10 μg of suPDE-CAT and 0.1–15 μM cGMP. (D) An increase in pH stimulates suPDE-CAT activity. Ten micrograms of suPDE-CAT and 30 nM [³H]cGMP were used in the assay. Error bars indicate SEs from at least three experiments.

sion resulted in an increase in cGMP hydrolytic activity from 0.23 to 0.57 pmol/min/mg (Figure 3A). In contrast, the hydrolysis of cAMP decreased from 5.95 to 4.58 pmol/min/mg protein, which might be due to the dilution effect of the endogenous cAMP-specific PDE by addition of the expressed protein. Note that cAMP-PDE activity is 20 times higher than the cGMP-PDE activity in the bacteria strain used in this experiment. The expressed protein was further purified to test its substrate specificity. The purified recombinant suPDE-CAT showed cGMP-specific PDE activity (Figure 3B) with an activity of 2.95 pmol/min/mg. In contrast, suPDE-CAT showed no activity when 30 nM [³H]cAMP was used as a substrate. These results show that suPDE is a cGMP-specific PDE. From the phylogenetic analysis and enzymatic activity, we conclude that this suPDE5 is a homolog of human PDE5.

By using a range of cGMP concentrations (0.1–15 μM), suPDE-CAT hydrolyzed cGMP with a K_m of 10 μM and V_{max} of 1.67 nmol/min/mg (Figure 3C). The K_m value of suPDE5 is comparable to that of PDE5 from bovine lung (5.6 μM; Thomas *et al.*, 1990) and recombinant human PDE5 (6.1 μM; Loughney *et al.*, 1998), which would make the enzyme highly sensitive to small changes in cGMP concentrations. Because sea urchin sperm physiology is tightly controlled by intracellular pH (pHi), the effects of pH on suPDE5 activity were examined. The suPDE-CAT activity shows a steep pH-dependency curve (Figure 3D). At pH 6.75 and 7, suPDE-CAT activity is ~3.48 pmol/min/mg. At pH 7.25 and 7.5, the specific activity increased to 6 pmol/min/mg. At pH 8, activity increased to 9.34 pmol/min/mg.

Figure 4. Localization of suPDE5 in sperm. (A) Sperm heads and flagella were separated by homogenization and differential centrifugation. Four micrograms of head (H) or flagellar (F) proteins were subjected to SDS-PAGE and Western blotting with the PKA substrate antibody. (B) One microgram of sperm head or flagellar lysate and 30 nM [3 H]cGMP were used in the PDE activity assay. Error bars indicate SEs from at least three experiments.

Localization of suPDE5 in Sperm

To study the localization of phospho-suPDE5, sperm were homogenized and the sperm heads and flagella were separated by differential centrifugation. Immunoblots revealed that phospho-suPDE5 is localized in the flagellum (Figure 4A). PDE activities of sperm heads and flagella were determined using [3 H]cGMP as a substrate (Figure 4B). The specific PDE activity in sperm flagella is 10 times that of heads, which is consistent with the flagellar localization of phospho-suPDE5.

suPDE5 Activity Is Regulated by Phosphorylation

suPDE5 is a PKA substrate in sperm (Su *et al.*, 2005). When sperm contact EJ, the phosphorylation of suPDE5 increases as detected by the PKA substrate antibody (Figure 5A). One minute after sperm were incubated with EJ, the phosphorylation of suPDE5 by PKA increased threefold. We also tested the effects of dephosphorylation on sperm suPDE5 activity. Because suPDE5 is the only phosphoprotein detected by this PKA substrate antibody in sperm not treated with EJ, we purified suPDE5 by immunoprecipitation using this antibody. The immunoprecipitates were then used for dephosphorylation and PDE activity assays. Dephosphorylation of suPDE5 by ALP decreased its activity ~50% (Figure 5B, top panel). The dephosphorylation of suPDE5 was confirmed by the band shift in the silver-stained gel (Figure 5B, bottom panel).

PDE Inhibitors Block suPDE5 Activity

The sensitivities of the bacterial-expressed suPDE-CAT and total sperm PDE activity to a range of PDE inhibitors were examined with cGMP as a substrate at one-third the K_m concentration (the IC_{50} equates to the K_i). The nonselective PDE inhibitor IBMX inhibited suPDE-CAT with an IC_{50} of 32 μ M compared with 3.8 μ M for the whole sperm lysate. The selective PDE5 inhibitors dipyridamole, zaprinast, T-1032, and sildenafil (Viagra), also inhibited suPDE5 with different potencies. The IC_{50} values for these PDE inhibitors are summarized in Table 1. In general, except for dipyridamole, which has a similar IC_{50} for suPDE-CAT and total sperm suPDE, the IC_{50} for suPDE-CAT is 10 times higher than that for total sperm suPDE. Viagra is the most potent inhibitor

Figure 5. Phosphorylation and dephosphorylation of suPDE5. (A) Sperm were incubated with EJ (from 15 s to 2 min), precipitated with 80% acetone, and resuspended in 10% SDS. Four micrograms of protein were loaded in each lane on a 4–15% gradient gel, and Western blotting was performed with the PKA substrate antibody. The intensity of the signal was quantified by scanning densitometry (top panel). Error bars indicate SEs from at least three experiments. The bottom panel represents one of the experiments. (B) Immunoprecipitations of NP40 lysates by the PKA substrate antibody were treated with (+) or without (–) ALP. The samples were subjected to PDE activity assays using 30 nM [3 H]cGMP as a substrate (top) or analyzed by 7.5% SDS-PAGE and silver staining (bottom).

tested, with an IC_{50} of 5 μ M for suPDE-CAT compared with 0.7 μ M for total sperm suPDE activity.

PDE Inhibitors Stimulate Sperm Motility

Sperm motility and chemoattraction to eggs are both regulated by cGMP (Darszon *et al.*, 2001; Kaupp *et al.*, 2003). Therefore, we also tested the effects of PDE inhibitors on sperm motility. The concentrations used for the sperm motility assay were about twice the IC_{50} values found for suPDE-CAT. The downward migration of sperm was measured by recording the relative light scattering increase at 580 nm. Dipyridamole is the most potent sperm motility stimulator (Figure 6A). After 10 min of migration, the absorbance of sperm treated with 50 μ M dipyridamole is six-fold higher than that of the DMSO control, showing that dipyridamole treated sperm swim much faster than control sperm. Other PDE inhibitors also stimulate sperm motility significantly, although the effects are weaker than with dipyridamole (Figure 6B).

DISCUSSION

Changes in cGMP regulate speract-induced sea urchin sperm motility and chemotaxis toward eggs (Garbers, 1989; Darszon *et al.*, 2001, 2005; Neill and Vacquier, 2004). The levels of cGMP are controlled positively by guanylyl cyclase and negatively by PDE. Sea urchin sperm are one of the richest sources of guanylyl cyclase. The enzyme is localized in the flagellum and its activity is controlled by phosphorylation (Ward *et al.*, 1985; Garbers, 1989). Here we describe the cloning and characterization of a sea urchin sperm cGMP-specific PDE (suPDE5). suPDE5 is a member of the PDE5 family because the phylogenetic analysis groups suPDE5 and human PDE5 together (Figure 2), the four amino acids that control substrate specificity are identical to that of PDE5 (Figure 1), and the enzyme hydrolyzes only

Table 1. Inhibitor studies of expressed suPDE-CAT and total sperm PDE on cGMP hydrolyzing activity

Inhibitor	PDE selectivity (IC ₅₀)	IC ₅₀ for suPDE-CAT	IC ₅₀ for sperm PDE
IBMX	Nonselective (2–50 μ M)	32 μ M	3.8 μ M
Dipyridamole	PDE5 (0.9 μ M) PDE6 (0.38 μ M) PDE7 (9.0 μ M) PDE8 (4.5 μ M) PDE10 (1.1 μ M) PDE11 (0.72 μ M)	23 μ M	33 μ M
Zaprinast	PDE5 (0.76 μ M) PDE6 (0.15 μ M) PDE11 (33 μ M)	240 μ M	24 μ M
T-1032	PDE5 (1.0 nM) PDE6 (28 nM)	41 μ M	2.1 μ M
Sildenafil (Viagra)	PDE5 (3.9 nM) PDE11 (>500 nM)	5 μ M	0.7 μ M

cGMP (Figure 3, A and B). We conclude, therefore, that suPDE5 is the ortholog of human PDE5.

When sperm are spawned into seawater, a Na⁺/H⁺ exchanger elevates pHi from ~7.0 to ~7.4. Further alkalization to ~7.5 occurs in response to the egg peptide speract and then to ~7.7 during the acrosome reaction (Darszon *et al.*, 2001). The increase in pHi induced by speract is very rapid and declines sharply thereafter (Nishigaki *et al.*, 2001; Solzin *et al.*, 2004). The down-regulation of cGMP synthesis initiated by increased pHi might be due to the inactivation of guanylyl cyclase and/or increased activity of suPDE5. In *Arbacia punctulata* sperm, cGMP decay after its initial peak elevation by the egg peptide resact is controlled by a PDE activity (Kaupp *et al.*, 2003). Here we show that the activity of bacterial expressed catalytic domain of suPDE5 is regulated by pH (Figure 3D). Although the regulation of the full-length suPDE5 is no doubt far more complicated than the catalytic domain only, the sensitivity of the catalytic domain of suPDE5 to pH (Figure 3D) could account for the regulation of cGMP concentrations by egg peptides. The pHi changes could regulate Zn²⁺ binding to PDE5's two metal binding motifs, which could in turn modulate suPDE5 activity. Interestingly, Zn²⁺ is known to be involved in the pHi regulation of motility and the acrosome reaction of sea urchin sperm (Clapper *et al.*, 1985). In these cells, cGMP-PDE activity is also known to be restricted to the flagellum (Sano, 1976; Toowicharanont and Shapiro, 1988). This localization in flagella (Figure 4) is consistent with previous reports and

further supports suPDE5's role in controlling sperm motility.

suPDE5 was originally identified in sperm as a 100-kDa phosphoprotein (Su *et al.*, 2005). The regulation of mammalian PDE5 by phosphorylation has been previously reported. For example, the phosphorylation of recombinant bovine PDE5 by cGMP-dependent protein kinase (PKG), or the catalytic subunit of PKA, increases its activity 50–70% (Corbin *et al.*, 2000). PDE5 from smooth muscle cells can be phosphorylated and activated by PKA and PKG (Murthy, 2001; Rybalkin *et al.*, 2002). However, sea urchin sperm appear to contain only low levels of PKG (Francis *et al.*, 1980; Garbers and Kopf, 1980). suPDE5 has three PKA phosphorylation sites (Figure 1), at least some of which increase their phosphorylation state when sperm contact EJ. The phosphorylation of suPDE5 and guanylyl cyclase is one mechanism that regulates cGMP concentrations in these cells (Ward *et al.*, 1985). When sperm contact EJ, the phosphorylation of suPDE5, detected by the PKA substrate antibody, is increased threefold (Figure 5A). Because only one of these three PKA sites can be recognized by the PKA substrate antibody, this result suggests that more molecules of suPDE5 protein become phosphorylated at this site (RRKS¹⁰¹). Incubation of sperm with 1 or 0.1 μ M speract for 2 min has no effect on the phosphorylation state of suPDE5 (unpublished data). Other components in EJ could be responsible for triggering the hyperphosphorylation of suPDE5. Addition of 2.5–20 mM NH₄Cl, which will increase

Figure 6. PDE inhibitors stimulate sperm motility. (A) Time-course traces of absorbance increases produced by downward migration of sperm in a Ficoll-containing medium with 1% DMSO (○) or 50 μ M dipyridamole (●). (B) The absorbance of DMSO or inhibitor-treated samples after sperm migrated for 10 min in the Ficoll medium. The concentrations of inhibitors used in the assay were T-1032 (100 μ M), zaprinast (500 μ M), Viagra (10 μ M), IBMX (100 μ M), and dipyridamole (50 μ M). Error bars indicate SEs from at least three experiments. * $p \leq 0.05$; ** $p \leq 0.005$.

intracellular pH, does not induce the hyperphosphorylation of suPDE5 (unpublished data). Although dephosphorylation by calf ALP (CAP) is not restricted to only PKA phosphorylation sites, we found that dephosphorylation of suPDE5 by CAP caused the diagnostic gel mobility shift and decreased its activity by ~50% (Figure 5B). In vitro phosphorylation of immunoprecipitated suPDE5 by the catalytic subunit of PKA failed to increase its enzymatic activity (unpublished data).

suPDE5 is sensitive to the nonselective inhibitor IBMX with an IC_{50} of 32 μ M. Total sperm PDE activity is also inhibited by IBMX with a 10-fold lower IC_{50} of 3.8 μ M. For all the inhibitors tested, except dipyrindamole, which has a similar IC_{50} for suPDE5 and total sperm PDE, the IC_{50} for total sperm PDE is also 10-fold lower than that for suPDE5. The simplest explanation for these results is that the native form of suPDE5 is more sensitive to PDE inhibitors than the bacterial expressed suPDE5-CAT. Other PDEs in sperm might also be sensitive to these inhibitors. Except for IBMX, the IC_{50} values for the PDE inhibitors are much higher than those for mammalian PDE5 (Table 1). This is not uncommon for many sea urchin enzymes, which usually need higher concentrations of inhibitors to block their activities. For example, sea urchin sperm require 5 mM ouabain to block the Na^+/K^+ ATPase activity, which is ~1000 fold higher than for mammals (Gatti and Christen, 1985).

Irradiation of sperm loaded with caged cGMP induces a transient increase in flagellar asymmetry, causing turns or tumbling, followed by swimming in straighter trajectories (Kaupp *et al.*, 2003; Wood *et al.*, 2005; Böhmer *et al.*, 2005). Therefore, sperm motility enhanced by PDE inhibitors (Figure 6) could result from the accumulation of cGMP, causing the cell to swim a straighter trajectory. In terms of the IC_{50} values, Viagra is the most potent in vitro inhibitor in blocking suPDE5 activity (Figure 5), but it only has moderate effects on sperm motility (Figure 6B). This could be due to its lower permeability to sea urchin sperm compared with the other inhibitors tested.

Mammalian sperm motility is also regulated by PDE. Human sperm motility is selectively enhanced by the PDE4 inhibitor, rolipram, suggesting that PDE4, a cAMP-specific PDE, contributes to motility regulation (Fisch *et al.*, 1998). High concentrations of the PDE5 inhibitor sildenafil (100 μ M Viagra) triggers human sperm motility (Lefièvre *et al.*, 2000). Clinical investigations studying sperm function in men with normal erectile function also show that Viagra increases sperm swimming velocity (du Plessis *et al.*, 2004).

The balance between guanylyl cyclase and cGMP-specific PDE controls cGMP levels in sperm. The rapid, dynamic regulation of suPDE5 by pH and/or phosphorylation modulates cGMP concentrations during fertilization. We hypothesize that motile sea urchin sperm have moderate suPDE5 activity because of the hypophosphorylation state of the enzyme at relatively low pH. The active suPDE5 maintains low cGMP levels in swimming sperm before fertilization. When sperm swim into EJ, the initial activation of guanylyl cyclase increases cGMP concentrations in seconds. The subsequent inactivation of guanylyl cyclase by its dephosphorylation and the hyperactivation of suPDE5 caused by increasing pH and hyperphosphorylation result in a rapid fall of cGMP concentrations. These dynamic changes in cGMP concentrations in turn regulate sperm motility.

Although RT-PCR studies show that washed human sperm contain PDE5 mRNA transcripts (Richter *et al.*, 1999), PDE5 protein, the target of Viagra, has never been found in mature sperm. In this study we discovered the first PDE5 protein in animal sperm, in this case, sea urchin sperm. The

discoveries regarding the molecular regulation of sea urchin sperm will be applicable to mammalian sperm. Given that Viagra is very potent in blocking the activity of PDE5 and is used widely in the treatment of erectile dysfunction, the effects of Viagra on human sperm quality, motility, and embryogenesis have to be considered seriously.

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