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A soluble adenylyl cyclase from sea urchin spermatozoa☆

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Abstract

A previously identified, calmodulin-binding, sea urchin sperm flagellar adenylyl cyclase (AC) was cloned and sequenced and found to be a homologue of mammalian sperm soluble adenylyl cyclase (sAC). Compared to the mammalian sAC, the sea urchin sAC (susAC) has several long amino acid insertions, some of which contain protein kinase A phosphorylation sites. The enzymatic activity of susAC shows a steep pH dependency curve, the specific activity doubling when the pH is increased from 7.0 to 7.5. This suggests that like sperm dynein ATPase, the susAC is probably activated by increases in intracellular pH occurring upon spawning into seawater and also when sperm respond to contact with the egg jelly layer. The susAC is strongly activated by manganese, but has low activity in magnesium. Gene database searches identified sAC homologues in species known to have cyclic AMP-dependent sperm motility. This implies (as shown in mouse) that susAC has a role in sperm motility, most probably through axonemal protein phosphorylation or ion channel regulation.

Keywords: Sperm motility; Invertebrate fertilization; Cyclic nucleotides; Flagella; cAMP; Metabolism in sperm

1. Introduction

Spermatozoa are highly polarized, terminally differentiated cells, specialized for motility, chemotaxis towards eggs and receptor-mediated events that regulate ion channel activations triggering acrosomal exocytosis. Sea urchin spermatozoa have been utilized extensively for studying the mechanism of flagellar motility. These cells can be obtained in vast quantities as homogeneous populations. When they are diluted into seawater, a Na^+/H^+ exchanger is activated and the intracellular pH (pHi) increases from ~7.0 to ~7.4. The alkalinization activates the dynein ATPase that hydrolyses ATP to power the flagellum propelling the cell forward. To maintain motility and keep pHi constant, the sperm regulates the activities of ion exchangers and ion channels. When the sperm contacts the egg jelly layer, receptor-mediated signaling pathways trigger increases in intracellular calcium, sodium, cGMP, cAMP, PKA activity and IP_3 reviewed in Garbers (1989), Morisawa (1994), Darszon et al. (2001), Inaba (2003), Neill and Vacquier (2004).

The phosphorylation of axonemal proteins is important for the initiation and maintenance of sperm motility in a variety of species (Walczak and Nelson, 1994). In salmonid fish (Morisawa and Okuno, 1982), sea urchin (Garbers and Kopf, 1980; Bracho et al., 1998), ascidian (Nomura et al., 2000) and mammals (Tash and Bracho, 1998), axonemal phosphorylation is cAMP-dependent. Increases in sperm adenylyl cyclase activity, leading to

Abbreviations: AC, Adenylyl cyclase; sAC, Soluble adenylyl cyclase; *sacs*, sAC genes; susAC, Sea urchin soluble adenylyl cyclase; CaM, Calmodulin; C1, Catalytic domain-1; C2, Catalytic domain-2; DTT, Dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 1-Methyl-3-isobutylxanthine; PKA, Cyclic AMP-dependent protein kinase; pHi, Intracellular pH; PMSF, Phenylmethylsulfonyl fluoride; TPR, Tetratrico peptide repeat.

☆ GenBank accession number of the sea urchin sperm susAC is AY926532.

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cAMP elevation, are thought to be important for both protein phosphorylation by PKA and regulation of sperm ion channels. A sea urchin sperm flagellar pacemaker channel, SPIH, has been shown to be K^+ selective, activated by hyperpolarization and modulated by cAMP (Gauss et al., 1998). SPIH and other cyclic nucleotide-modulated flagellar ion channels could be responsible for periodic oscillations in flagellar Ca^{2+} that are stimulated by the egg peptide speract (Wood et al., 2003). The extreme sensitivity of the chemotactic response of sea urchin sperm to egg peptides is probably mediated by cyclic nucleotide-modulated ion channels (Kaupp et al., 2003). Although large changes in adenylyl cyclase activity correlate with the activation and maintenance of sperm motility, reaction of sperm to egg peptides and the acrosome reaction, the functions of adenylyl cyclases in sperm remain to be defined (Darszon et al., 2001).

Previous studies identified a calmodulin (CaM)-binding sea urchin sperm AC of ~190-kDa. Antisera showed this AC was predominantly located in the proximal half of the sea urchin sperm flagellum (Bookbinder et al., 1990). The antisera depleted AC activity from the CaM-eluate and immunoprecipitated only the 190-kDa band from the CaM-eluate. Because this sperm AC could be resolved by SDS-PAGE of the CaM-eluate, peptide sequences were obtained and the cDNA sequence determined. Sequence analysis shows that this sea urchin sperm AC (susAC) is a homologue of mammalian sperm sAC (Buck et al., 1999) a calcium and bicarbonate-stimulated enzyme (Litvin et al., 2003) required for mouse sperm forward motility (Esposito et al., 2004).

2. Materials and methods

2.1. Obtaining sperm and removal of coelomocytes

Sperm of the sea urchin, *Strongylocentrotus purpuratus*, was spawned by injection of 0.5 M KCl into adults and the undiluted semen collected with a Pasteur pipette and stored in microfuge tubes packed in ice for no longer than 24 h. Each microliter of undiluted semen represents 100 μg of sperm protein and $\sim 4 \times 10^7$ sperm cells. All procedures were at 4 °C. Coelomocytes were removed from the sperm as previously described (Vacquier and Hirohashi, 2004). Briefly, the undiluted semen was suspended in 25 volume Ca^{2+}-free artificial seawater (462 mM NaCl, 9.4 mM KCl, 59.1 mM $MgCl_2$, 10 mM HEPES (pH 8.0) and 5 mM EGTA) and centrifuged at 500 $\times g$ for 10 min. The sperm suspension was decanted into a fresh 50 ml conical tube leaving the coelomocytes as a red pellet. The centrifugation was repeated and the second supernatant decanted into a 50 ml round bottom tube that was centrifuged 15 min at 1500 $\times g$ to sediment the sperm. The sperm pellet, stored on ice, was then resuspended in the desired medium.

2.2. Preparation of the Brij-78 extract of S. purpuratus sperm and calmodulin-agarose chromatography

The Brij-78 extract of sperm was prepared with some modifications to the previous method (Bookbinder et al., 1990). Briefly, after removing coelomocytes and sedimenting the sperm, the 1500 g sperm pellet was resuspended in Buffer A (150 mM NaCl, 1 mM EGTA, 10 mM HEPES (pH 7.5), 1 mM DTT, 2 mM benzamidine-HCl and 1 mM PMSF) and incubated for 30 min on ice. The sperm suspension was then centrifuged 20 min at 10,000 $\times g$ and the pellet resuspended in 10 volume Buffer A containing 2% w/v Brij-78 (Sigma). The detergent extracted sperm suspension was incubated on ice with occasional gentle mixing for 1 h, centrifuged at 40,000 $\times g$ for 1 h and the supernatant saved on ice as the Brij-78 extract. After making the Brij-78 extract 3 mM in $CaCl_2$, it (approximately 2 mg total protein) was applied to a 1 ml CaM-agarose column (Sigma) equilibrated in Buffer B (150 mM NaCl, 2 mM $CaCl_2$, 10 mM HEPES (pH 7.5), 1 mM DTT, 2 mM benzamidine and 1 mM PMSF). After washing with 10-column volume of Buffer B, the CaM-bound proteins were eluted with Buffer C (150 mM NaCl, 2 mM EGTA, 10 mM HEPES (pH 7.5), 1 mM DTT, 2 mM benzamidine and 1 mM PMSF). Fractions with AC activity were pooled, mixed with 1 volume of glycerol and stored at −70 °C. The AC activity was stable for several months. Protein concentrations were measured with the Bradford (BioRad) or Lowry assays using bovine serum albumin as a standard.

2.3. Cloning and analysis of the 190-kDa CaM-binding flagellar susAC

The 190-kDa adenylyl cyclase band was visualized by Coomassie blue staining of SDS-PAGE gels of the CaM-eluate (Bookbinder et al., 1990). The 190-kDa band was excised from gels, equilibrated in distilled water, lyophilized and sent to the Stanford University PAN Facility for sequence determination of tryptic fragments. Oligonucleotide primers were designed and PCR reactions performed by standard methods using a sea urchin testis cDNA Lambda Zap library (Stratagene), or newly made testis cDNA as templates. PCR products were TA cloned (Invitrogen) and plasmid DNA isolated (Qiagen) and sequenced. BLAST searches with amino acid sequences were done against the *S. purpuratus* genome database (http://sugp.Caltech.edu/). The full-length cDNA of the 190-kDa sea urchin sperm AC was obtained by primer walking, 5′ RACE (First Choice RLM kit, Ambion) and PCR of the 3′ terminus using the Lambda ZAP cDNA library as a template.

Alignments of sACs were made with Clustal W. Conserved domain structures were predicted using CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and InterProScan (http://www.ebi.ac.uk/InterProScan/). Cyclic AMP protein kinase-A phosphorylation sites were predicted using ScanSite (http://scansite.mit.edu/motifscan_