Published in final edited form as:

Biochem Biophys Res Commun. 2007 July 13; 358(4): 1128–1135. doi:10.1016/j.bbrc.2007.05.061.

Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction

Carmen Beltrán^{a,*}, Victor D. Vacquier^b, Gary Moy^b, Yanqiu Chen^c, Jochen Buck^c, Lonny R. Levin^c, and Alberto Darszon^a

^aDepartamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos CP 62250, Mexico

^bCenter for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202, USA

^cDepartment of Pharmacology, Weill Medical College of Cornell University, New York, NY 10021, USA

Abstract

cAMP is important in sea urchin sperm signaling, yet the molecular nature of the adenylyl cyclases (ACs) involved remained unknown. These cells were recently shown to contain an ortholog of the mammalian soluble adenylyl cyclase (sAC). Here, we show that sAC is present in the sperm head and as in mammals is stimulated by bicarbonate. The acrosome reaction (AR), a process essential for fertilization, is influenced by the bicarbonate concentration in seawater. By using functional assays and immunofluorescence techniques we document that sea urchin sperm also express orthologs of multiple isoforms of transmembrane ACs (tmACs). Our findings employing selective inhibitors for each class of AC indicate that both sAC and tmACs participate in the sperm acrosome reaction.

Keywords

Acrosome reaction; cAMP; Adenylyl cyclases; Sea urchin; Soluble adenylyl cyclase; Sperm; fertilization

cAMP, an important second messenger in cell signaling, is fundamental in sperm physiology. Sperm must swim, find the egg and undergo the acrosome reaction (AR) to fertilize it [1]. cAMP levels influence sperm ionic fluxes, motility and the AR [2].

Two types of adenylyl cyclases (ACs) are expressed in mammals, transmembrane ACs (tmACs) and soluble AC (sAC) molecularly identified in rat testis [3] and sperm [4]. Restricted to the plasma membrane, tmACs are regulated by heterotrimeric G-proteins and stimulated by forskolin [5]. In contrast, sAC is stimulated by bicarbonate [6] and Ca^{2+} [7,8]. In addition to sAC, mouse [9] and human [10] sperm apparently also contain tmACs.

Why AC activity, cAMP levels, and cyclic AMP-dependent protein kinase (PKA) activity increase [11] during the sea urchin sperm AR is not fully understood. AC activity in these cells is regulated by: PKA [12], pH, intracellular calcium ($[\text{Ca}^{2+}]_i$) [8,13], and indirectly by plasma membrane hyperpolarization [14]. While seemingly sperm AC activity is not

modulated by G-proteins [11], a report in *Paracentrotus lividus* indicates it is [15]. Two egg-jelly layer components: speract, a decapeptide that modulates motility and fucose sulphate polymer, the natural inducer of the AR, cause cAMP elevations. Therefore, AC activation could be involved in these two important sperm processes [1,13,16,17].

Sea urchin sperm were believed to have only one type of AC and no G proteins [11,13]. Recently an ortholog of the mammalian sperm sAC was cloned and sequenced from a sea urchin *Strongylocentrotus purpuratus* testis cDNA library (susAC). Its activity is higher in the presence of Mn^{2+} than Mg^{2+} , and is stimulated by pH [18]. Furthermore, sea urchin sperm posses G-proteins [15,19,20]. Here, we show by using specific activators and inhibitors for transmembrane and soluble ACs that both ACs participate in the sperm AR. Moreover, we document the presence of several tmACs and their differential distribution in sperm by immunolocalization and Western blot experiments.

Materials and methods

Gametes and reagents

Strongylocentrotus purpuratus sea urchins were obtained from Marinus (Long Beach, CA) or from Pamanes Inc. (Ensenada, Baja California, México). Spawning was induced by intracoelomic injection of 0.5 mM KCl. Semen was kept undiluted on ice (dry sperm) and used within 24 h. Artificial seawater (ASW) was in mM: 455 NaCl, 10 $CaCl_2$, 10 KCl, 26 $MgCl_2$, 30 $MgSO_4$, 10 Hepes, 2.5 $NaHCO_3$, and 0.1 EDTA (~1000 mOsm, pH 8). Anti-tmAC primary antibodies α -AC (sc-1701), α -AC1 (sc-586), α -AC2 (sc-587), α -AC5/6 (sc-590), α -AC9 (sc-8578) and corresponding peptides were from Santa Cruz Biotechnology (SCB; Santa Cruz, California). Two antisusAC raised in rabbits were used; one against the whole protein (anti-190; [21]), and the other against a susAC peptide mapping at the carboxy terminus, containing a 5-histidine tail (HHHHHVPHFYAL). Alexa fluo 488- or 594-labeled secondary antibodies were from Invitrogen-Molecular probes (Eugene, Oregon). Prestained SDS-PAGE molecular weight standards, Tween 20 and all reagents for SDS-PAGE were from Bio-Rad Laboratories. Two hydroxyestradiol and 17- β estradiol were from Steraloids Inc. (Newport, Rhode Island). KH7 was previously described [22]. From Calbiochem was 2',5'-dideoxyadenosine (2',5'-DDA) (San Diego, California). Protease inhibitors, Forskolin, NaF, bicarbonate ($NaHCO_3$), Brij 78, calmodulin-agarose and all other reagents were from Sigma-Aldrich (St. Louis, Missouri) unless indicated.

PCRs were performed using testis cDNA as template and two oligonucleotide primers. A degenerate sense 5'-TTYGYIGAYATHWSIGG NTT-3' based on rat sAC (*Rattus norvegicus*), NP067716.1 and an anti-sense 5'-ACGGCTGGCTACATTGACAG-3'; exact match from *S. purpuratus* coelomocytes AC R61912 EST 030. A 630-bp PCR product was obtained corresponding to one of the two copies of the mammalian adenylyl/guanylyl cyclase catalytic domain. Screening an *S. purpuratus* testis cDNA Lambda Zap library (Stratagene) with this product yielded two clones containing 26% of the SpAC2 (XP_780688; 479–677) and 25% of the SpAC9 (XP_798394; 509–690) predicted sequences. In addition, we found the predicted sequences for AC1 (SpAC1, XP_787811), AC3 (SpAC3, XM_782404) and AC5 (SpAC5, XP_787809) in the *S. purpuratus* genome. PCR products were TOPO-TA cloned (Invitrogen) and plasmid DNA isolated (Qiagen) and sequenced. BLAST searches with nucleotide (<http://www.ncbi.nlm.nih.gov/blast>) or amino acid sequences were done against the *S. purpuratus* genome database (<http://sugp.caltech.edu/>). Partially purified susAC was obtained using a calmodulin-affinity column as described [21].

Head and flagella membranes were purified as described previously [23]. The crude particulate fraction was obtained at 4 °C as follows. Diluted sperm (1:15 in ASW) free of coelomocytes [18], were pelleted (1500 g/15 min) and resuspended in 10 volumes of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 50 mM benzamidine, 10 µM leupeptin, 10 µM pepstatin A, 10 µg/ml soybean trypsin inhibitor). Cells were broken by three freeze-thaw homogenization cycles and centrifuged at 14,000g 20 min. The supernatant was centrifuged (200,000g/ 2.5 h) and the particulate fraction recovered in the pellet. Protein concentration was measured with the Bradford method modified for membrane proteins [24] with bovine serum albumin (BSA) as standard.

Immunological experiments

Immunolocalization was performed as described [25] except that the sperm (1:2500–5000) and primary antibody (10- to 100-fold; 20–2 µg/ml) dilutions were different. Control experiments for tmACs were performed incubating the primary antibody with the corresponding commercial peptide (1:1–1:5M ratio). The secondary antibody was used for anti-AC (sc-1701) and anti-susAC negative control.

Western immunoblotting

Sperm proteins resolved on 7.5% polyacrylamide minigels under denaturing conditions (SDS-PAGE) were electrotransferred to immobilon-P (Millipore) membranes (0.5 A/45 min in 190 mM glycine, 25 mM Tris base, pH 8.6) in a semi-dry transfer cell (Bio-Rad). After blocking with 5% fat free milk they were incubated with different anti-tmACs (1:25–1:100), anti-susAC peptide (1:1000) and anti-190 (1:5000). The immunodetected proteins were revealed with the ECL chemiluminescence system from Amersham.

Acrosome reaction, cAMP and adenylyl cyclase determinations

AR was induced with egg-jelly in ASW, pH 8.0, and quantified counting at least 100 sperm under a phase contrast microscope as described [23].

cAMP in whole sperm was determined diluting cells (1:20) in ASW containing 1 mM 1-methyl-3-isobutylxanthine (IBMX) after preincubating at 16 °C in the absence or presence of tmAC or sAC inhibitors. After the times indicated in the figure legends, 20-µl aliquots were transferred to 180 µl ASW, pH 8.0 with or without the AR inducer (Jelly). One minute later, 20 µl of the sperm suspension were used to score the AR, and the rest to quantify cAMP with the cAMP (³H) assay system TRK 432 from Amersham [14]. All cAMP determinations were done by triplicate. Since susAC is also stimulated by alkaline pH [18], pH was carefully maintained at 7.5 in the bicarbonate experiments.

In purified sperm membranes (1–5 µg) or in the crude particulate fraction (5–10 µg) AC activity was determined in 30 µl of the reaction mixture (in mM: 25 Hepes, pH 7.5, 10 MgCl₂, 100 KCl, 1 IBMX and 0.1% Brij 78). Membranes were incubated at 23 °C in the absence or presence of the tmAC inhibitor. After 5 min, a tmAC stimulator was supplemented and 5 min later a tenfold concentrated solution of ATP regenerating system (creatine phosphate, creatine kinase and ATP-Na at final concentrations of 20 mM, 240 U/ml and 2 mM, respectively) was added. The reaction was terminated after 5 min incubation at 23 °C, by adding 25 µl of methanol:chloroform (1:1), vortexing 1 min and boiling for 10 min. cAMP was determined in the supernatant (16,000g, 20 min) as above.

Activity of the partially purified susAC (0.5 µg) was determined as in membranes but in a different buffer (final concentration in mM: 25 Hepes, pH 7.5, 2 EDTA, 7.5 MnCl₂ (or MgCl₂), 40 NaN₃, 5 NaCl, 5 KCl, 1 IBMX and 0.025 µg/ml oligomycin, 1 µg/µl BSA,

0.05% Brij 78) containing or not the AC inhibitor. After 10 min at 23 °C, the reaction was initiated by adding a tenfold concentrated solution of ATP regenerating system as above.

Statistical analysis

Data were averaged from at least three different animals. Bars represent means; $\pm$ standard errors. Microsoft office Excel *t*-test was used to calculate significant differences.

Results and discussion

Localization of susAC

Early work using an antisera raised against the whole 190 kDa sea urchin sperm AC protein, later shown to be susAC [18], indicated it was mainly located in the sperm flagella [21]. However, confocal examination revealed its presence in the head, acrosomal and mitochondrial area as well (Fig. 1A1). These observations were corroborated using a polyclonal antibody prepared against a carboxy terminus susAC-peptide (see Materials and methods; Fig. 1A2). Western blot experiments with both antibodies revealed a 190-kDa band in flagellar membranes. The susAC peptide antibody recognized a doublet (Fig. 1Ba) possibly corresponding to the phosphorylated and unphosphorylated forms of the enzyme [12]. In head membranes this antibody detected faint bands of similar Mr as those in flagella (Fig. 1Ba) plus one of ~75 kDa, as well as other stronger and lower Mr bands possibly of degraded susAC. Similarly, the anti-190 antibody labeled a ~75-kDa protein in head and flagella membranes (Fig. 1Bb). These findings indicate that susAC could be involved in sperm motility and the AR.

susAC is stimulated by bicarbonate and participates in the sperm acrosome reaction

Fig. 2A shows that the Mg^{2+} -dependent activity of a partially purified susAC preparation [21] is stimulated by bicarbonate reaching maximum at 50 mM. As anticipated, flagellar associated susAC [26] was also stimulated ~2-fold by 50 mM bicarbonate (Fig. 2B). Bicarbonate stimulates susAC in a similar fashion as sAC in mammalian sperm [4,22].

As susAC is in the sperm head (Fig. 1), we investigated its role in the AR. Bicarbonate could influence the AR since it is sensitive to DIDS, a Cl^- channel and $Cl^- HCO_3^-$ exchange inhibitor [27] and it regulates susAC. Fig. 2C shows that either decreasing bicarbonate from the normal 2.5 mM to 62 μ M or elevating it to 25 or 50 mM inhibits ~40% of the sperm AR. In addition, Fig. 2D shows that 2-hydroxyestradiol, a selective sAC inhibitor [28] used at 150 μ M, the highest concentration that does not induce by itself the sperm AR (results not shown), partially inhibits the jelly induced AR (~30%) as well as the increase in cAMP levels (~42%). The inactive analog 17- β estradiol served as control. These results suggest that susAC participates in the AR.

As catechol estrogens may have non-specific interactions [29], we also tested KH7, a sAC-specific inhibitor identified from a combinatorial chemical library [22]. KH7 inhibited cAMP production and the AR (Fig. 2E) in a similar manner as that observed with 2-hydroxyestradiol (Fig. 2D). In mammalian sperm KH7 eliminates protein tyrosine phosphorylation during capacitation and motility indicating the participation of the sAC in these processes [22]. In contrast to our results in sea urchin sperm, mouse sAC appears not to be involved in the AR [22].

Our overall results indicate that susAC does participate in the AR, as indicated by the simultaneous inhibition of the AR and cAMP levels by 2-hydroxyestradiol and KH7 and the decreased reaction found upon bicarbonate removal from seawater. Further studies are

necessary to understand if these bicarbonate changes inhibit the AR through their effect on susAC and/or by changing pH_i.

Sea urchin sperm membranes contain tmAC activities

At least nine closely related isoforms of tmACs, AC1–AC9, have been cloned and characterized in mammals. The nine isoforms are activated by G_{αs} and all but AC9 are stimulated by forskolin [5]. The *S. purpuratus* genome predicts that orthologs of mammalian tmACs 1, 2, 3, 5 and 9 are present in this organism (see Materials and methods) besides susAC [18]. To determine if tmACs are functionally present in sea urchin sperm we examined if their AC activity was sensitive to forskolin. Initial experiments using a whole sperm particulate fraction indicated that NaF (~2.9-fold; $p < 0.09$) (Fig. 3A) and also forskolin (modestly, ~1.6-fold; $p < 0.1$) increased their AC activity. As expected, the forskolin induced tmAC stimulation was inhibited by 150 μM 2',5'-DDA, a permeable inhibitor that binds to the P site of tmACs [30]. Forskolin also stimulated AC activity in purified flagellar (Fig. 3B, ~1.6-fold; $p < 0.008$) and head sperm membranes (not shown). The extent of forskolin stimulation depends on the AC activity ratio susAC/tmAC and the specific tmAC isoforms [31] present in sea urchin sperm.

Interestingly, a rabbit antibody made against a conserved region of the nine mammalian tmACs isoforms (anti-AC; sc-1701) stained the whole sea urchin sperm (Fig. 3C). This conserved region (rat AC5 M96159, 1019-GPVVAGVI GARKPQYDIWGNVTNVASRMDSTGV-1051) is found in the *S. purpuratus* tmACs (SptmACs) predicted sequences with high identity (93% for SpAC2 and SpAC5; 81% for spAC1 and SpAC3, and 69% for SpAC9) and similarity (99%, 93% and 81%, respectively; *E* values from $1e^{-8}$ to $5e^{-11}$), supporting the presence of tmACs in this species. The sequence has no similarity with the sAC (AAY164446). Our results support the presence of G-protein regulated tmACs, as reported for *P. lividus* [15].

Several tmACs are differentially distributed in sea urchin sperm besides susAC

To investigate which orthologs of mammalian tmACs are expressed in intact sperm, we tested a battery of commercial polyclonal antibodies made against the amino and carboxy-termini of mammalian tmACs (see Materials and methods). An alignment of the epitopes for anti-AC2 (sc-587) and anti-AC5 (sc-590) antibodies with the predicted sequences of the corresponding SptmACs, AC2 and AC5, showed 61% identity (13/21 residues) and 80% identity (12/15 residues), respectively. Lower identities were obtained with the rest of the antibodies, as indicated by SCB. It is possible that the antibodies are cross-reacting with proteins in sea urchin sperm.

Fig. 4A documents that anti-AC1, -AC2, -AC5/6 (recognizes both AC5 and AC6), and anti-AC9, differentially stain sea urchin sperm. Anti-AC2 and -AC9 label the flagella. These two antibodies plus anti-AC1 and anti-AC5/6 decorate the acrosome region, and anti-AC2, -AC5/6 and -AC9 stain the mitochondrial area. All fluorescence signals were blocked by the corresponding commercial peptides (not shown). However, we were unable to detect AC3 in sperm using 2 different antibodies against it, though it is present in the *S. purpuratus* genome database and in the acrosome of mouse and human sperm [9,10,32].

Western blot experiments with the antibodies that labeled sperm, but anti-AC5/6, detected protein bands in head and flagella sperm membranes (Fig. 4B) with different relative mobility ($M_r \pm$ standard error: AC1, 157 ± 0.38 ; AC2, 113 ± 1.14 ; AC5/6, 250 ± 0 and AC9, 104 ± 1.31 ; $n = 8-17$ SDS-PAGEs). Since most mammalian AC enzymes are glycosylated [33], the apparent M_r could be higher than the real MW. We frequently observed protein degradation in the head membrane fractions (see anti-AC1 in Fig. 4B). Several bands of

lower Mr appear in these membranes when solubilized samples are run after freezing and thawing cycles even though head and flagella membranes contain the same mixture and concentration of proteolytic inhibitors.

Although, anti-AC1 stains just the acrosomal area in intact cells (Fig. 4A), in Westerns it recognizes a protein band in both head and flagellar membranes (Fig. 4B). This antibody may be more sensitive in Western blotting than in immunostaining, though head contamination in flagellar membranes can not be completely discarded. It is likely that anti-AC 5/6 recognizes AC5 in sperm since the sea urchin genome contains AC5 and not AC6. Our findings corroborate the presence of more than one tmAC isoform in sea urchin sperm.

tmAC activity also participate in the acrosome reaction

Sea urchin sperm plasma membranes display tmAC activity and several antibodies against these enzymes decorate the acrosomal area (Fig. 4A). To dissect the participation of tmACs in the AR we used 2',5'-DDA, a selective inhibitor of these enzymes. We first confirmed that the Mn²⁺-dependent activity of the partially purified susAC (see Materials and methods; [21]) is inhibited by 150 µM of 2-hydroxyestradiol but not by the same concentration of either 17-β estradiol, its inactive analog or 2',5'-DDA (Fig. 5A). This activity was confirmed to be susAC by Western blotting (data not shown) and by the ability of 2-hydroxyestradiol to dose-dependently and specifically (17-β estradiol was inert) decrease this activity (Fig. 5B).

When sea urchin sperm were preincubated with 150 µM 2',5'-DDA before and exposed to egg-jelly, the AR and the production of cAMP were inhibited by 49% and 37%, respectively (Fig. 5C). These observations suggest that tmACs also participate in the sea urchin sperm AR. At first hand it could seem puzzling that though the forskolin stimulation is small (~1.6-fold), inhibition of tmACs with 2',5'-DDA significantly decreases the AR. Different tmAC isoforms are differentially stimulated by forskolin and tmACs may play a proportionally more important role in this process than susAC. In mammals, evidence implicates tmACs in the sperm AR [32,34].

Why do sperm need so many ACs? It is likely that the particular location of the various ACs is associated with specific functions, a matter that requires future studies. Our findings indicate that both susAC and tmACs participate in the egg-jelly induced sea urchin sperm AR. In this species, both the natural inducer of the AR and speract, elevate cAMP and cause different [Ca²⁺]_i spatio-temporal changes [1,13]. How do sperm discern between these signals? Complexes formed from different ACs [9,10,26], PDEs [35], AKAPs [36] and ion channels [26] localized in distinct sperm regions [1], may sense and modulate second messengers and ion concentrations to locally determine sperm behavior. Much work is needed to unravel the function the ACs present in the sperm.

Acknowledgments

We thank Xochitl Alvarado and Andres Saralegui for confocal imaging and Claudia Treviño and Pablo Visconti for comments on the manuscript. This work was supported by: DGAPA-UNAM (IN225406-3 to A.D. and IN209002-3, IN225806-3 to C.B.), CONACyT (39908-Q to A.D. and 39939-Q to C.B.) and by the Wellcome Trust and FIRCA to A.D.

Abbreviations

AC	adenylyl cyclase
AR	acrosome reaction

2',5'-DDA	2',5'-dideoxyadenosine
sAC	soluble adenylyl cyclase
SCB	Santa Cruz Biotechnology
susAC	sea urchin sperm soluble adenylyl cyclase
tmAC	transmembrane adenylyl cyclase.

References

1. Darszon A, Acevedo JJ, Galindo BE, Hernandez-Gonzalez EO, Nishigaki T, Trevino CL, Wood C, Beltran C. Sperm channel diversity and functional multiplicity. *Reproduction*. 2006; 131:977–988. [PubMed: 16735537]
2. Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J.Reprod. Immunol*. 2002; 53:133–150. [PubMed: 11730911]
3. Braun T, Dods RF. Development of a Mn-2+-sensitive, “soluble” adenylate cyclase in rat testis. *Proc. Natl. Acad. Sci. USA*. 1975; 72:1097–1101. [PubMed: 1055368]
4. Buck J, Sinclair ML, Schapal L, Cann MJ, Levin LR. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc. Natl. Acad. Sci. USA*. 1999; 96:79–84. [PubMed: 9874775]
5. Hanoune J, Defer N. Regulation and role of adenylyl cyclase isoforms. *Annu. Rev. Pharmacol. Toxicol*. 2001; 41:145–174. [PubMed: 11264454]
6. Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science*. 2000; 289:625–628. [PubMed: 10915626]
7. Jaiswal BS, Conti M. Calcium regulation of the soluble adenylyl cyclase expressed in mammalian spermatozoa. *Proc. Natl. Acad. Sci. USA*. 2003; 100:10676–10681. [PubMed: 12958208]
8. Litvin TN, Kamenetsky M, Zarifyan A, Buck J, Levin LR. Kinetic properties of “soluble” adenylyl cyclase. Synergism between calcium and bicarbonate. *J. Biol. Chem*. 2003; 278:15922–15926. [PubMed: 12609998]
9. Baxendale RW, Fraser LR. Evidence for multiple distinctly localized adenylyl cyclase isoforms in mammalian spermatozoa. *Mol. Reprod. Dev*. 2003; 66:181–189. [PubMed: 12950106]
10. Spehr M, Schwane K, Riffell JA, Barbour J, Zimmer RK, Neuhaus EM, Hatt H. Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. *J. Biol. Chem*. 2004; 279:40194–40203. [PubMed: 15271985]
11. Garbers DL. Molecular basis of fertilization. *Annu. Rev. Biochem*. 1989; 58:719–742. [PubMed: 2549859]
12. Bookbinder LH, Moy GW, Vacquier VD. In vitro phosphorylation of sea urchin sperm adenylate cyclase by cyclic adenosine monophosphate-dependent protein kinase. *Mol. Reprod. Dev*. 1991; 28:150–157. [PubMed: 2007028]
13. Beltran C, Galindo B, Rodríguez-Miranda E, Sánchez D. Signal transduction mechanism regulating ion fluxes in the sea urchin sperm in signaling systems in the sea urchin. *Signal Transduct*. 2007; 7:103–117.
14. Beltran C, Zapata O, Darszon A. Membrane potential regulates sea urchin sperm adenylylcyclase. *Biochemistry*. 1996; 35:7591–7598. [PubMed: 8652541]
15. Capasso A, Cardinale V, De Petrocellis B, Parisi E. Guanine nucleotide binding proteins activate adenylyl cyclase from sea urchin sperm. *Comp. Biochem. Physiol*. 1990; 978:339–342.
16. Neill AT, Vacquier VD. Ligands and receptors mediating signal transduction in sea urchin spermatozoa. *Reproduction*. 2004; 127:141–149. [PubMed: 15056779]
17. Kaupp UB, Hildebrand E, Weyand I. Sperm chemotaxis in marine invertebrates—molecules and mechanisms. *J. Cell. Physiol*. 2006; 208:487–494. [PubMed: 16619222]

18. Nomura M, Beltran C, Darszon A, Vacquier VD. A soluble adenylyl cyclase from sea urchin spermatozoa. *Gene*. 2005; 353:231–238. [PubMed: 15978750]
19. Cuellar-Mata P, Martínez-Cadena G, Castellano L, Aldana-Veloz G, Novoa-Martínez G, Vargas I, Darszon A, García-Soto J. Multiple GTP-binding proteins in sea urchin sperm: evidence for Gs and small G-proteins. *Dev. Growth Differ.* 1995; 37:173–181.
20. Ohta K, Sato C, Matsuda T, Toriyama M, Vacquier VD, Lennarz WJ, Kitajima K. Co-localization of receptor and transducer proteins in the glycosphingolipid-enriched, low density, detergent-insoluble membrane fraction of sea urchin sperm. *Glycoconj. J.* 2000; 17:205–214. [PubMed: 11201792]
21. Bookbinder LH, Moy GW, Vacquier VD. Identification of sea urchin sperm adenylate cyclase. *J. Cell Biol.* 1990; 111:1859–1866. [PubMed: 2121742]
22. Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS, Levin LR, Williams CJ, Buck J, Moss SB. The “soluble” adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev. Cell.* 2005; 9:249–259. [PubMed: 16054031]
23. Darszon A, Labarca P, Beltran C, Garcia-Soto J, Lievano A. Sea urchin sperm: an ion channel reconstitution study case. *Methods: A Companion to Methods Enzymol.* 1994; 6:37–50.
24. Fanger BO. Adaptation of the Bradford protein assay to membrane-bound proteins by solubilizing in glucopyranoside detergents. *Anal. Biochem.* 1987; 162:11–17. [PubMed: 3605581]
25. Granados-Gonzalez G, Mendoza-Lujambio I, Rodriguez E, Galindo BE, Beltran C, Darszon A. Identification of voltage-dependent Ca(2+) channels in sea urchin sperm. *FEBS Lett.* 2005; 579:6667–6672. [PubMed: 16307742]
26. Nomura M, Vacquier VD. Proteins associated with soluble adenylyl cyclase in sea urchin sperm flagella. *Cell Motil. Cytoskeleton.* 2006
27. Morales E, de la Torre L, Moy GW, Vacquier VD, Darszon A. Anion channels in the sea urchin sperm plasma membrane. *Mol. Reprod. Dev.* 1993; 36:174–182. [PubMed: 7504923]
28. Steegborn C, Litvin TN, Hess KC, Capper AB, Taussig R, Buck J, Levin LR, Wu H. A novel mechanism for adenylyl cyclase inhibition from the crystal structure of its complex with catechol estrogen. *J. Biol. Chem.* 2005; 280:31754–31759. [PubMed: 16002394]
29. Zippin JH, Chen Y, Nahirney P, Kamenetsky M, Wuttke MS, Fischman DA, Levin LR, Buck J. Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. *FASEB J.* 2003; 17:82–84. [PubMed: 12475901]
30. Johnson RA, Yeung SM, Stubner D, Bushfield M, Shoshani I. Cation and structural requirements for P site-mediated inhibition of adenylate cyclase. *Mol. Pharmacol.* 1989; 35:681–688. [PubMed: 2498637]
31. Onda T, Hashimoto Y, Nagai M, Kuramochi H, Saito S, Yamazaki H, Toya Y, Sakai I, Homcy CJ, Nishikawa K, Ishikawa Y. Type-specific regulation of adenylyl cyclase. Selective pharmacological stimulation and inhibition of adenylyl cyclase isoforms. *J. Biol. Chem.* 2001; 276:47785–47793. [PubMed: 11602596]
32. Livera G, Xie F, Garcia MA, Jaiswal B, Chen J, Law E, Storm DR, Conti M. Inactivation of the mouse adenylyl cyclase 3 gene disrupts male fertility and spermatozoon function. *Mol. Endocrinol.* 2005; 19:1277–1290. [PubMed: 15705663]
33. Wu GC, Lai HL, Lin YW, Chu YT, Chern Y. N-glycosylation and residues Asn805 and Asn890 are involved in the functional properties of type VI adenylyl cyclase. *J. Biol. Chem.* 2001; 276:35450–35457. [PubMed: 11461898]
34. Xie F, Garcia MA, Carlson AE, Schuh SM, Babcock DF, Jaiswal BS, Gossen JA, Esposito G, van Duin M, Conti M. Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. *Dev. Biol.* 2006; 296:353–362. [PubMed: 16842770]
35. Baxendale RW, Fraser LR. Mammalian sperm phosphodiesterases and their involvement in receptor-mediated cell signaling important for capacitation. *Mol. Reprod. Dev.* 2005; 71:495–508. [PubMed: 15856425]
36. Luconi M, Porazzi I, Ferruzzi P, Marchiani S, Forti G, Baldi E. Tyrosine phosphorylation of the a kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate. *Biol. Reprod.* 2005; 72:22–32. [PubMed: 15342355]

Fig. 1. susAC is also present in the sperm head. (A) An antisera against a susAC peptide (panels 1 and 4) or against the whole susAC (panels 2 and 5 [21]) labels sperm. Negative controls with secondary antibody (panels 3 and 6). Panels 1–3 show fluorescence (confocal microscopy) and 4–6 phase contrast images, respectively. The width of the sperm head is $\sim 1 \mu\text{m}$. In panels 1 and 4, arrows indicate the mitochondria and arrow heads the acrosome. (B) Western blots of head (H) or flagellar (F) sperm membranes using the same antibody (a) as in (A) 1 and 4 or (b) as in (A) 2 and 5. Negative controls with the secondary antibody (not shown).

Fig. 2.

susAC is stimulated by bicarbonate and participates in the sperm acrosome reaction. Mg^{2+} -dependent AC activity (see Materials and methods): (A) partially purified susAC (0.5 μ g) exposed to different bicarbonate concentrations (representative experiment of three made in triplicate, error bars are within symbols) and (B) flagella membranes (10–5 μ g) with ($n = 6$) or without 50 mM bicarbonate (control $n = 12$). (C) Sea urchin sperm (2.5 μ l of 1:10 dilution in 1 mM Ca^{2+} ASW, pH 8) were transferred to 100 μ l ASW, pH 8, at 16 °C with different $NaHCO_3$ concentrations, containing or not egg-jelly and AR scored after 1 min (see Materials and methods). In (D) sperm were preincubated 30 min with or without 150 μ M 2-hydroxyestradiol (2-OH estradiol) or 17- β estradiol, its negative control. In (E) 50 or 100 μ M of KH7 were tested as in (D). Thereafter AR was induced and the reaction stopped to determine % AR and cAMP concentrations (see Materials and methods). Three to four independent experiments were performed in triplicate.

Fig. 3.

Sea urchin sperm membranes contain tmAC activities. (A) A sperm particulate fraction (5–10 μ g) was preincubated (23 $^{\circ}$ C/5 min) in the absence or presence of 150 μ M 2',5'-DDA. Subsequently, 5 mM NaF ($*p < 0.09$) or 50 μ M forskolin ($**p < 0.1$) was added. (B) Flagella membranes (1–5 μ g) were incubated as in (A) in the absence or presence of forskolin ($*p < 0.008$). In both cases, Mg^{2+} -dependent AC activity was determined (see Materials and methods). Standard errors and number of independent experiments (triplicates) are indicated on the average bars. Significant differences (*, **) are compared with controls. (C) Fixed and permeabilized sperm were exposed to pan anti-tmAC (sc-1701) and anti-rabbit antibodies. Panels show the fluorescence and insets fluorescence and phase contrast overlays.

Fig. 4.

tmACs are distributed differentially in sea urchin sperm. (A) Fixed and permeabilized sperm exposed to antibodies against tmACs: α -AC1 (sc-586), α -AC2 (sc-587), α -AC5/6 (sc-590), α -AC9 (sc-8578). Primary antibodies detected with fluorescent Alexa-labeled secondary antibodies using confocal microscopy. Top and bottom panels from AC1 to AC5/6 show the fluorescence and the overlay of fluorescence with phase contrast, respectively. For AC9 the top panel shows fluorescence and the bottom panel phase contrast. Sperm head width $\sim 1 \mu\text{m}$. (B) Western blots of head (H) or flagella (F) sperm membranes using the same antibodies as in (A). For AC9 Western blot, the SDS-PAGE was run longer and different protein molecular weight standards used (see Materials and methods). In both cases (A,B), the signals were blocked with the corresponding antigenic peptides. $n = 3-10$ experiments.

Fig. 5. tmAC activity also participates in the AR. (A) Mn^{2+} -dependent partially purified susAC (0.5 μ g) is insensitive to 150 μ M 2',5'-DDA or 17- β estradiol but inhibited by 2-OH estradiol. Normalized values of 3–4 independent experiments by triplicate. (B) Concentration dependence of the activity of this preparation to 2-OH estradiol or 17- β estradiol. (C) % AR and cAMP levels of sperm preincubated (30 min) with or without 150 μ M 2',5'-DDA and then exposed to Jelly or not (control). Figure shows normalized values of 3 (cAMP) and 4–7 (% AR) independent experiments made in triplicate determined as described in Materials and methods and Fig. 2D and E.