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Soluble Adenylyl Cyclase of Sea Urchin Spermatozoa

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Abstract

Fertilization, a key step in sexual reproduction, requires orchestrated changes in cAMP concentrations. It is notable that spermatozoa (sperm) are amongst the cell types with extremely high adenylyl cyclase (AC) activity. As production and consumption of this second messenger need to be locally regulated, the discovery of soluble AC (sAC) has broadened our understanding of how such cells deal with these requirements. In addition, because sAC is directly regulated by HCO_3- it is able to translate CO_2/HCO_3-/pH changes into cAMP levels. Fundamental sperm functions such as maturation, motility regulation and the acrosome reaction are influenced by cAMP; this is especially true for sperm of the sea urchin (SU), an organism that has been a model in the study of fertilization for more than 130 years. Here we summarize the discovery and properties of SU sperm sAC, and discuss its involvement in sperm physiology.

Keywords

Acrosome reaction; adenylyl cyclase; sperm chemotaxis; microdomain; flagellar motility; sea urchin sperm; fertilization

1. Early Work

Sea urchin (SU) spermatozoa (sperm) are highly polarized, terminally differentiated cells that can be obtained in vast quantity at low cost [1]. In addition to the study of their

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biochemistry related to fertilization, these small cells have been important for elucidation of the biophysical mechanism of eukaryotic flagellar motility [2,3], and of flagellar wave form changes underlying sensing and chemo attraction to egg-released peptides [4–7]. (The dimensions of SU sperm are: head ~3 x 1 μm, flagellum ~0.2 x 50 μm. One ml of *S. purpuratus* semen contains 40 billion spermatozoa and 5 ml semen can be obtained from one male.)

The ubiquitous importance of the second messenger cAMP has been established for four decades. Adenylyl cyclase (AC), the enzyme synthesizing cAMP, was first studied in detail in SU sperm by the laboratory of the late David L. Garbers [8–10]. One surprise was that SU sperm have relatively high AC activity. During these early years soluble AC (sAC) [11,12] was still unknown and all ACs were thought to be integral transmembrane proteins (tmACs) [13–15]. When live sperm were treated with soluble SU egg jelly, a 50-fold increase in AC activity occurred. (Egg jelly is the outer investment of the egg containing the high molecular weight fucose sulphate polymer and sperm activating peptides, which trigger the acrosome reaction (AR) and regulate sperm motility.) This AC activation was dependent on extracellular Ca^{2+} and was “particulate”, being released to the low g force supernatant only by detergent treatment of sperm homogenates. In most non-sperm cells the ratio of tmAC activity assayed using MgATP was equal to, or greater than, the activity using MnATP as substrate. However with SU sperm, and sperm of several other animal species, AC activity was found to be several fold higher using MnATP compared to MgATP [9]. When the cAMP concentration increases cAMP-dependent protein kinase (PKA) is activated. Certain proteins in the flagellar axonemal complex become phosphorylated and flagellar motility is initiated and maintained until the sperm fuses with the egg [8,9].

Methods were developed to isolate the plasma membranes of SU sperm, which have a large plasma membrane surface area to volume ratio [1]. The “pH 9” method isolated membranes enriched 6-fold in total AC activity, agreeing well with the enrichment of 4-8-fold in the specific radioactivity of ^{125}I-labeled surface proteins. Triton X-100 extraction of purified SU sperm membranes increased the AC activity by 17-fold, suggesting that much of the AC may be inaccessible to substrate [16]. An alternative isolation method yielded SU sperm membranes enriched 8-fold in AC specific activity [17]. At that time all ACs were still thought to be integral plasma membrane proteins.

1.2. The 1990s

SU sperm AC activity was completely blocked by the calmodulin (CaM) antagonist trifluoperazine [10]. Given this fact, an attempt was made to purify the AC from *Strongylocentrotus purpuratus* sperm using CaM-agarose affinity columns. A 2% Brij-78 detergent extract of these sperm was loaded onto CaM-agarose in the presence of Ca^{2+} and, after extensive washing, the column was eluted with an EGTA buffer. As judged by SDS-PAGE, approximately 20 major proteins eluted from the CaM column. One protein of 190-kDa was electroeluted from gels and a mono-specific rabbit antibody raised to it. The antibody inhibited 94% of the total AC activity in the CaM-eluate of SU sperm, 92% of the AC activity in a detergent extract of domestic horse sperm and 28% of the total AC activity of bovine brain [18]. An affinity column of the antibody removed 95% of the SU AC

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activity from the CaM-eluate. Although the SU sperm AC bound to CaM-agarose, it was not activated by either Ca^{2+} or CaM. Two other CaM antagonists, calmidazolium and chlorpromazine, also blocked the SU sperm AC; the inhibition being reversed by added bovine CaM. Immuno-localization of the 190-kDa protein showed it to be concentrated on the proximal one-third of the flagellum, the region closest to the sperm mitochondrion (SU sperm have a single large mitochondrion that arises from the fusion of several mitochondria during spermatocyte differentiation [19]). The AC activity of the CaM eluate was 42-fold enriched compared to the 40,000 xg supernatant loaded onto CaM-agarose [18].

The great majority of the AC activity described in the above paragraph was undoubtedly SU sperm sAC (SUsAC) and not tmAC. The reasons for this conclusion are the following. The relative molecular mass of SUsAC of 190-kDa agrees with 187-kDa for mammalian sAC. The fact that SU sperm AC has high activity with MnATP as substrate is consistent with it being sAC. In this early paper [18] the strongest evidence for the SU AC being sAC was the fact that, unlike tmAC, all the SU sperm AC activity was particulate and detergent insoluble. Soon afterward it was shown with another species of sea urchin that G proteins activate SU sperm AC activity [20].

The 190-kDa remained in the 170,000 xg detergent supernatant if the membranes were first boiled in SDS and β-mercaptoethanol before ultracentrifugation [16]. The 190-kDa AC was obviously a member of a protein complex that might have functional significance in the axoneme. It could be argued that the apparent concentration of the SU sperm AC in the proximal one-third of the flagellum is an artifact, the antigen moving in the sperm membrane after fixation [18]. However, SU sperm adenylyl kinase, the enzyme catalyzing the reaction, $2ADP \leftrightarrow ATP + AMP$, localizes uniformly along the entire flagellum after identical fixation and immuno-staining procedures as used for SUsAC immuno-localization [21].

When ^{32}P-ATP was added to the EGTA-eluate of the CaM column described above, ~8-10 proteins became radiolabelled. One of the most heavily phosphorylated proteins was the 190-kDa AC. One dimensional peptide mapping showed that 5 to 6 serine residues were phosphorylated by an endogenous PKA. This PKA had a Km for ATP of 5 μM and was maximally stimulated at 4-8 μM cAMP. The PKA inhibitor H89 (IC_{50} = 9 μM) blocked this phosphorylation and bovine catalytic PKA subunit phosphorylated the same sites on the 190-kDa as did the endogenous PKA. Chase experiments with non-radioactive ATP showed that the phosphorylated sites did not turn over. Interestingly, 0.1 μM free Ca^{2+} completely blocked the phosphorylation of the AC, suggesting the activity of a Ca^{2+}-dependent protein phosphatase. Although neither the Km nor the Vmax of the AC activity changed as a result of phosphorylation, the association of the AC with other flagellar proteins important for motility may be affected [22]. Phosphoserine antibodies indicated that the 190-kDa SU AC protein purified from the sperm Brij-78 extract by CaM affinity chromatography, was not phosphorylated. The AC activity assay formulation favored by us is presented in Nomura et al., 2005 [24].

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1.3. Cloning SUsAC

The CaM affinity purified 190-kDa band [18] was excised from SDS-PAGE gels and tryptic peptides sequenced. The coding sequences of these peptides were found in a single open reading frame in the SU genome (http://Echinobase.org). The 5’ and 3’ ends were obtained by RACE and the full-length cDNA and translated protein sequences determined [23]. The SUsAC open reading frame is 5604 nucleotides and the protein 1868 amino acids, yielding a calculated molecular mass of ~211-kDa (Fig. 1). The genomic sequence has 29 introns. The six peptide sequences obtained from the 190-kDa protein excised from gels were present in the protein sequence. Alignment with the human sAC shows the SUsAC has six insertions of 43, 16, 43, 39, 75 and 12 amino acids not found in the human homolog. One dimensional gel peptide mapping indicated five sites for PKA phosphorylation [22]. The sequence shows six PKA sites at residues S^{307}, S^{573}, S^{888}, S^{904}, T^{1256}, and S^{1263}. The T^{1256} residue may not be phosphorylated because phospho-amino acid analysis showed only phosphoserine [22]. Human sAC contains no PKA phosphorylation sites [23].

Human sAC is 261 residues shorter than the SUsAC. Where they align, the amino acid identity is only 26%, but in catalytic domains 1 and 2 the percent identities increase to 39% and 43%. Residues 1365-1803 have homology to tetratrico (TPR) domains. (These structural units of a degenerate ~34 amino acid sequence are found in many proteins. They usually occur as tandem repeats of 3-16 residues, which mediate protein-protein interactions, especially in folding. They are often found in mitochondrial import proteins [25].) In SUsAC this domain contains 19 α-helices, eight of which are amphiphilic as shown by wheel diagrams [23]. Helix 4 of 21 residues shows a net positive charge of +3, which would be perfect for binding CaM [24]. As is true for human sAC, no transmembrane segments are predicted in the SUsAC [23].

Although the overall identity between human and SU sAC is only 26%, the recent high-resolution crystal structure of human sAC [25] shows that both the ATP and bicarbonate binding pockets of these two sACs are essentially identical. For ATP binding, the alignment shows that (human numbering): Ala 97, Phe 296, Leu 345, Phe 336, Val 411, Val 406, Phe 338, Arg 176, Asn 412, Arg 416, Ile 48, Asp 47, Asp 99, Ala 97 and Ala 100 are all in identical positions. The exception is human Ala 415 which is Gln in SUsAC. For bicarbonate binding, Lys 95, Met 337, Arg 176, Ala 97, Asp 99 and Lys 95 are also in identical positions in the alignment. There is only one non-identity, where human Val 167 aligns with SUsAC leu. This comparison exemplifies the evolutionary conservation of the sAC protein in deuterostomes.

The facts that the antibody to the 190-kDa CaM-enriched protein detects one band on western blots and that immuno-affinity removes 95% of the AC activity from the Brij-78 extract of sperm, suggest that SUsAC is the major AC of SU sperm. Its primary location in the flagellum is consistent with a role for SUsAC in motility [18]. This SU enzyme shows a steep pH dependency, the activity doubling with a pH increase of 7.0 to 7.5. This intracellular pH (pH_i) change, occurring when sperm are diluted into seawater, is more extreme for flagellar dynein ATPase, where a six-fold increase in activity occurs between pH 7.4 and 7.7 [26]. The pH activation of SUsAC is not affected by GTP, Ca^{2+} or CaM and activity is essentially undetectable in MgATP as compared to the high activity with MnATP

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as substrate. Conversely, mammalian sAC is relatively insensitive to pHs from 7.0 to 8.5 [27,28]. Unlike the mammalian sAC with both a 187-kDa and an alternatively spliced isoform of 50-kDa, western blots of 2-D gels show a single reaction at 190-kDa for the SUsAC. In both SUsAC and mammalian sAC, the tetratrico repeat regions are at the COOH ends of the enzyme. (Figs. 1 & 2) [23].

Gene knockouts of the sAC result in normal male mice with sperm that are immotile and thus sterile. The immotile sperm become motile when exposed to permeable analogs of cAMP [29]. These data are proof that sAC is involved in generating the cAMP required for motility. The inhibition of the SUsAC by the CaM antagonists calmidozolium and chlorpromazine is a direct effect on the enzyme and not on CaM. Thus, although SU sperm contain CaM, there is no direct evidence that it is involved in motility.

1.4. SUsAC Associated Proteins

Given that the SUsAC is concentrated in flagellar axonemes and is particulate, its tight association with other proteins became the next obvious avenue of experimentation. Eukaryotic axonemes are made of 200-400 proteins, few of which have been identified or functionally characterized [30–32]. All sperm that have been studied require cAMP dependent protein kinase (PKA) phosphorylation to initiate and maintain flagellar motility [30,33]. Indeed PKA-substrates are present in the flagella of SU sperm [34]. The SU sperm flagellum is structurally much simpler than the mammalian sperm, and is thus ideal for discovery of SUsAC-associated proteins. Two His-tagged recombinant proteins representing 620 amino acids of the N-terminal region, and 440 residues of the C-terminal region of SUsAC were expressed in bacteria and purified by nickel affinity chromatography. The two recombinant proteins were covalently coupled to Affigel beads to purify specific antisera to each antigen using the whole rabbit antiserum previously raised to the 190-kDa [18]. Both the affinity purified IgGs reacted only with the 190-kDa protein on western immunoblots and showed the majority of the reaction was localized in the sperm flagellum [35].

Sequential extractions of isolated flagella with 0.1% Triton X-100 containing buffers at various centrifugal speeds and times, and sucrose density gradient ultracentrifugation, showed the SUsAC was particulate, being a complex of many tightly bound proteins. Approximately 45% of the total SUsAC activity remains bound to axonemes after three successive extractions in 0.1% Triton X-100. SDS-PAGE and silver staining of immunoprecipitates prepared using the affinity-purified antibody to SUsAC from Triton X-100 supernatants of sperm flagella revealed approximately 9-12 protein bands. Tandem mass spectrometry of these bands yielded amino acid sequences that were then used to identify the proteins from the SU genome resource (http://Echinobase.org). Three proteins identified using only one peptide sequence were excluded as possible contaminants. Nine proteins were identified from between 2-31 peptide sequences. These proteins were: axonemal dynein heavy chains –7 and –9; α- and β-tubulin; sperm specific Na^{+}/H^{+} exchanger; olfactory cyclic nucleotide gated ion channel; receptor guanylyl cyclase (GC), sperm specific creatine kinase; and cGMP specific phosphodiesterase-5A [35]. It remains unknown which and how many of these proteins interact directly with SUsAC. The two most extreme cases would be that SUsAC binds each of these proteins, or only one of these

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proteins. The macromolecular protein assemblage isolated by CaM affinity chromatography could be an important preparation for future insights into the physiology of axonemal motility.

1.5. SUsAC Association with Micro Domains

After its cloning [36], mammalian sAC has been found in different cell compartments such as the nucleus [37–39], mitochondria and in membrane microdomains, such as caveolae, and lipid rafts, which are also known as the low density-detergent insoluble membrane fraction (LD-DIM) (reviewed in [40]). It is now known that in both nuclei and mitochondria the main source of cAMP synthesis is from sAC.

Sea urchin sperm membrane microdomains are physiologically functional [34], as has been shown in mammals [41–44] and the ascidian *Ciona intestinalis* [45]. Both SUsAC and tmACs, have been found associated with membrane microdomains (reviewed in [46,47]). LD-DIMs were initially isolated using discontinuous sucrose-density gradients from sperm of the SUs Anthocidaris crassispina, Hemicentrotus pulcherrimus and *Strongylocentrotus purpuratus* [48]. In addition to SUsAC, LD-DIM also contain six important proteins, the speract receptor, the receptor for egg jelly-1, a GPI-anchored protein of unknown function and three proteins involved in signal transduction: guanylyl cyclase (GC), the α-subunit of the heterotrimeric G_s protein ($G_{s\alpha}$) and cAMP-dependent protein kinase (PKA) [48]. Speract from the egg jelly of *S. purpuratus* activates GC [4 9, 50] (reviewed in [5,51]). In contrast to mouse and guinea pig sperm [42], SU sperm microdomains do not contain the caveolae marker caveolin (a 21–24 kDa integral membrane protein), although caveolin is present in the whole sperm lysate. As the speract receptor, a GPI-anchored protein and $G_{s\alpha}$ co-immunoprecipitated together, Ohta et al., [52] suggested that this LD-DIM could constitute a signaling complex involved in regulating sperm respiration, motility and the acrosome reaction (AR) [52]. This idea was subsequently proposed for mammalian sperm [53].

Recently, we detected the phosphorylated form of PKA, using one antibody against PKA substrates. The Anti-Phospho-(Ser/Thr) PKA substrate (α-PKAs, cat.9621) was from Cell Signaling Technology, Danvers, MA. and was shown to be monospecific in their catalogue when reacted with human, mouse and rat by standard Western immunoblots. PKA was also identified by proteomic analysis of LD-DIM isolated from *S. purpuratus* sperm in an OptiPrep® density gradient [34]. In the same work we also showed that the *S. purpuratus* LD-DIM contain the receptor for speract and Flotillin-2 [34], a LD-DIM marker protein different from caveolae [54]. This suggest that as in mammals [55], SU sperm also contain different membrane microdomains and that the SUsAC is located in a microdomain containing Flotillin.

SUsAC has also been located in caveolae in membrane microdomains obtained by two other non-detergent methods [56], namely sodium carbonate, pH 11.0 [57] and sonication [55], a physically disruptive procedure that has also been applied to murine sperm to demonstrate the presence of distinct subtypes of membrane rafts [42].

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1.6. SUsAC and Protein Phosphorylation

When SU sperm are either diluted into seawater or exposed to speract, a Na^+/H^+ exchanger is activated, increasing pH_i from ~7.0 to ~7.4. This alkalinization activates the dynein ATPase needed for sperm swimming. This pH change also activates SUsAC [23], increasing cAMP levels, which in turn activates PKA to phosphorylate axonemal proteins needed for initiation and maintenance of sperm motility [9,34,58–64].

Unlike mammalian sperm that can use glycolysis to generate ATP, SU sperm rely solely on mitochondrial oxidative phosphorylation to produce ATP [66]. However, all along the SU sperm flagellum there is an adenylate kinase that can use 2ADP to synthesize ATP and AMP [22]. Sea urchin sperm have only one mitochondrion which synthesizes almost all the ATP of the cell [65]. A pH_i increase is also associated with mitochondrial respiration [26,66,67] and it was proposed that together, cAMP- and pH_i-dependent phosphorylation regulates flagellar motility [30,61]. Immunolocalization and Western blot experiments using antibodies against SUsAC and tmACs from mammals, showed that SUsAC is mainly present in the *S. purpuratus* sperm flagellum, but is also in mitochondrial and acrosomal locations (Fig. 3). A similar distribution is observed for tmAC2 and tmAC9 [68] (Fig. 3). It is not surprising that as in other species [39,69], SUsAC is also present into the sperm mitochondrion. This is consistent with phosphorylation changes found in mitochondrial proteins co-purifying with LD-DIMs isolated from immotile, motile, or speract-stimulated *S. purpuratus* sperm (as detected with anti-PKA or anti-PKC antibodies) [34]. The SU PKA type II regulatory subunit is among the phosphorylated proteins identified in the LD-DIM [34], which unlike human PKA, contains six predicted phosphorylation sites [23]. Although more experiments are needed, these findings, in addition to the fact that a complete CO_2–HCO_3–sAC–cAMP–PKA signaling cascade regulates oxidative phosphorylation [69,70], supports the finding of SUsAC in the sperm mitochondrion.

Because phosphorylation changes regulate the metabolic activity of mitochondria [71], SUsAC may be in the SU sperm mitochondrion. The functional regulation of sAC in the SU mitochondrion is an important area for future study. Speract stimulates sperm respiration, H^+ efflux, increases in Ca^{2+} and elevation of pH_i [8, 51, 72-74]. If SUsAC is also stimulated by these speract-induced changes, this might link the metabolic rate, with ATP generation and motility [73].

2. The SUsAC, Motility Regulation and Chemotaxis

To locate the egg, sperm from many species sense graded chemical cues released by eggs. This phenomenon, known as chemotaxis, has been extensively studied in marine invertebrates that undergo external fertilization. Sea urchin sperm undergo a series of turns interspersed with periods of straight swimming to locate the egg [51 , 74 – 76] . The jelly layer of SU eggs contains sperm-activating peptides that modulate SU sperm motility [77]. Speract, stimulates an *S. purpuratus* flagellar membrane guanylyl cyclase (SU GC) elevating sperm cGMP [50] . cGM P itse lf activates K^+ selective and cyclic nucleotide gated channels (tetraKCNGs) resulting in a membrane potential (E_m) hyperpolarization [78–81]. This E_m change first increases pH_i p o s s i b l y by activating a sperm voltage dependent Na^+/H^+ exchanger (sNHE) [78,82]. It may also remove inactivation from voltage gated Ca^{2+}

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channels (Ca_v) [83,84] enhances Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers (NCKX) [82,85] and stimulates hyperpolarization-activated and cyclic nucleotide-gated channels (spHCN) [86]. Opening of spHCN channels causes Na^+ influx contributing to E_m depolarization and concomitant increases in $[Ca^{2+}]_i$ (reviewed in [87]). The speract induced $[Ca^{2+}]_i$ elevation occurs in a series of oscillations mounted on a sustained increase (reviewed in [5,51]). Each $[Ca^{2+}]_i$ oscillation promotes an asymmetric flagellar beat causing the sperm to turn [88]. Additionally, the pH_i and $[Ca^{2+}]_i$ increases can stimulate SUsAC, elevating cAMP in the flagellum which activates spHCN and also PKA that can phosphorylate SU CG whose activity is regulated by phosphorylation [89]. cGMP levels are decreased by sperm flagellar phosphodiesterase-5A, and inhibition of this enzyme increases cGMP modulating sperm motility [90].

Experimental and modeling results suggest that the speract-induced $[Ca^{2+}]_i$ increases could open Ca^{2+}-regulated K^+ channels (CaKC) and possibly Cl^- channels (CaCC), which would contribute to hyperpolarize E_m again, removing inactivation from Ca_v and opening of spHCN [91]. This set of changes occurs periodically in a chemotactic gradient resulting in a train of Ca^{2+} increases that repetitively cause asymmetric flagellar bending and concomitant turns and periods of straighter swimming that allow sperm to turn towards the egg.

The first SU egg jelly peptide shown to be chemotactic for sperm was resact, isolated from *Arbacia punctulata* [75]. The responses to this peptide have been extensively studied by the group of Kaupp [51] in parallel to those mentioned above for speract by our laboratory. The findings with resact have established the key participation of cyclic nucleotides and the tetraKCNG and spHCN channels in the chemotactic signaling pathway of SU sperm, and reference to their work is included above. However, we recently found that *Lytechinus pictus* sperm respond chemotactically to speract gradients [76]. In spite of significant advances in our understanding of chemotaxis, the role of SUsAC is still ill defined though it most likely is important in chemotaxis. **Figure 4** illustrates how the circular velocity of SU sperm is reduced by two SUsAC inhibitors (E)-2-(1HBenzo[d]imidazol-2-ylthio)-N'-(5-bromo-2-hydroxybenzylidene) propanehydrazide (KH-7 ([92])) and 2-hydroxyestradiol (2-OHE ([93])).

3. Both SUsAC and tmAC regulate the SU sperm acrosome reaction

Exposure of SU sperm to egg jelly triggers a receptor mediated signaling cascade involving changes in E_m and increases in $[Ca^{2+}]_i$, pH_i, $[Na^+]_i$, cAMP, IP_3 and NAADP levels, as well as activation of PKA, phospholipase D, nitric oxide synthase, phospholipase (PL) D and possibly SU PLCδ (reviewed in [5]). As mentioned earlier, soluble SU egg jelly induces a 50-fold increase in sperm AC activity [8]. Notably, this AC activation, and the acrosome reaction, are both dependent on extracellular Ca^{2+} [10].

Although initially immuno-cytochemical localization showed that SUsAC was restricted to the sperm flagellum [18], confocal imaging using the same antibody prepared against the 190-kDa protein isolated by CaM affinity, and another IgG raised against a SUsAC-peptide sequence, showed reactivity also on the acrosomal region [68].

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Because two SUsAC inhibitors [94], KH-7 (100 μM) and 2-OHE (150 μM), block both the egg jelly induced cAMP synthesis and acrosome reaction (AR) (~80% and 45%, and ~70% and 30% respectively; [68]), it is likely that SUsAC participates in the AR. Furthermore, the presence of 2.5 mM $NaHCO_3$ in sea water is required for the egg jelly triggered AR, suggesting the involvement of SUsAC in the AR [68]. As tmACs are present in the SU sperm head (Fig. 3), and 2', 5'-dideoxyadenosine (2',5'-DDA; 150 μM), an inhibitor of these enzymes [94,95], partially blocks cAMP production and the AR [68], tmACs appear to also participate in the AR, contrary to what is found in mouse sperm [96].

In mammals tmACs (tmAC1-tmAC9) are classified in four groups. Group I (tmAC1, tmAC 3 and tmAC8) are stimulated by Ca^{2+}/Calmodulin (Ca^{2+}/CaM); group II (tmAC2, tmAC4 and tmAC7), are activated by $G_{s\alpha}$ and by PKC mediated phosphorylation; group III (tmAC5 and tmAC6), are inhibited by Ca^{2+} (~1 mM) and by PKA dependent phosphorylation, and group IV (tmAC9) is insensitive to Ca^{2+}, to G proteins and to the diterpene Forskolin [13–15]. Immunolocalization experiments showed that each group of mammalian tmACs is represented in SU sperm by at least one of its members (tmAC1, tmAC2, tmAC5, and tmAC9, which are all present in the SU genome). TmAC1, which is stimulated by Ca^{2+} and CaM, is only present in the acrosomal region of SU sperm (Fig. 3; [68]). Furthermore, antibodies against PKA-substrates as well as PKC-substrates also stain the acrosomal region [34]. These findings suggest the participation of tmACs in the SU sperm AR.

Previously, we showed that *L. pictus* sperm in sea water without K^+ underwent a cAMP increase when hyperpolarized with valinomycin, a K^+ ionophore. This occurs even in the absence of external Ca^{2+} (1.9- and 3.1-fold, respectively) [97]. This, and the results described above indicate that SU sperm have both a Ca^{2+}-dependent and -independent sAC.

As mentioned in section 1.2, the antibody to the 190-kDa protein isolated by CaM affinity inhibited 94% of the SU sperm total AC activity and an affinity column of this antibody removed 95% of the AC activity from a detergent extract of SU sperm [18]. Using these numbers we estimate that ~5-10% of the total AC activity in SU sperm is due to tmACs.

4. Conclusions and future directions

sAC is present in SU sperm both in the flagellum and head. SUsAC is associated with membrane microdomains, yet we do not know how lipid organization affects its activity. Inhibition experiments indicate SUsAC participates both in motility and in the acrosome reaction. Though we know how SUsAC is activated, we are far from understanding the molecular mechanisms by which it contributes to the regulation of chemotaxis and the acrosome reaction. If SUsAC is present inside the mitochondrion how does it modulate the metabolic and ionic contribution of this organelle to sperm swimming and acrosome reaction?

Because ion transporters and channels are modulated directly or indirectly by cAMP, it is necessary to characterize how pH_i and $[Ca^{2+}]_i$ are regulated by this cyclic nucleotide. How exactly do $[Ca^{2+}]_i$ and pH_i interdependently regulate SUsAC activity? Ionic fluxes (H^+, Ca^{2+}, K^+ and Na^+) and E_m changes are crucial for chemotaxis and the acrosome reaction. In mouse sperm it is known that the sperm specific Na^+/H^+ exchanger and sAC interact [98].

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One future line of research could investigate if other proteins such as CatSper [99,100] and/or other ion transporters form signaling complexes in SU sperm, where they reside and how they function in the cell.

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ABBREVIATIONS

AC	adenylyl cyclase
AR	acrosome reaction
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
LD-DIM	low density detergent-insoluble membranes
PKA	cAMP dependent protein kinase
RACE	rapid amplification of cDNA ends
sAC	soluble adenylyl cyclase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SU	sea urchin
SUsAC	sea urchin soluble adenylyl cyclase
tmAC	transmembrane adenylyl cyclase

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HIGHLIGHTS

* SUsAC associates to membrane micro domains
* SUsAC influences sperm motility through protein phosphorylation
* SUsAC and tmACs regulate the sperm acrosome reaction

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Fig.1. The amino acid sequence of sea urchin soluble adenylyl cyclase (SUsAC) aligned with human sAC (HsAC)

Alignment was performed with Geneious program where identical residues are shaded green and similar ones yellow. Dashes are inserted for optimal alignment. The two catalytic domains (C1, 46-240 and C2, 345-556 amino acids) are marked with single solid black bars. Peptides obtained by amino acid sequencing are marked P1–P6 with overlines. The P-loop (622-629 amino acids) is indicated by a black bracket. The nucleoside triphosphate hydrolase domain is marked by a single dashed underline. The non-regular secondary structure (non-RSS) domain is marked with a double dashed underline and the tetratrico

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peptide repeat-like domain (TPR-like) with a double solid underline. Asterisks mark 3 Methionine residues, which could be translation initiation sites. Six residues marked with arrowheads in the SUsAC show potential PKA phosphorylation sites. Dashed lines above the alignment (H1–H19) indicate positions of predicted α-helices in the TPR-like domains **(Modified from Nomura, *et al*., 2005** [23]).

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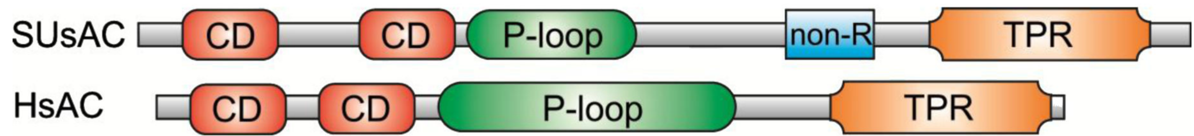

Fig. 2. Domain structures of SUsAC and human sAC predicted by InterProScan

Two catalytic domains (red), P-loop containing nucleoside triphosphate hydrolase (green), non-regular secondary structure (blue) and tetratrico peptide repeat (TPR)-like (orange) domain are predicted (Modified from **Nomura, *et al*., 2005** [23]).

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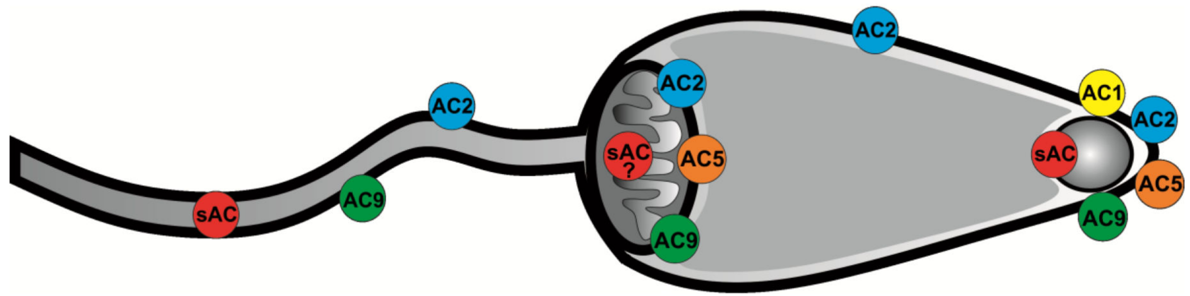

Fig. 3. Location of sAC and tmACs in sea urchin sperm

sAC, AC2 and AC9 are distributed along SU sperm. AC1 is only found in the acrosomal area, while AC5 is in the acrosomal and mitochondrial area. AC1, C2, AC5 and AC9 are tmACs (Based on [68]).

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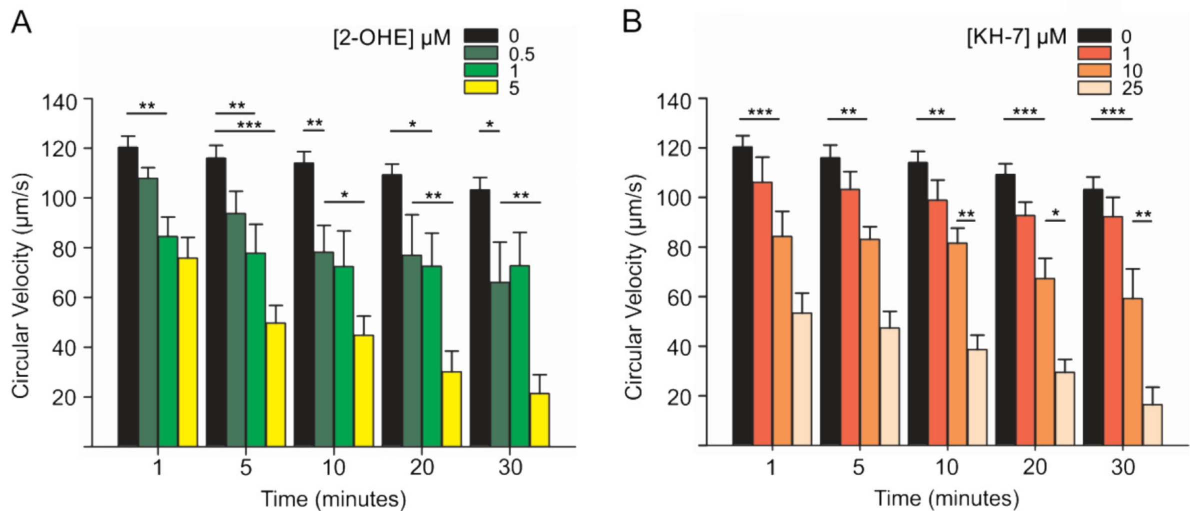

Fig. 4. SUsAC influences sperm motility

Diluted *S. purpuratus* sperm (~2 x 10^6 ml^{-1} in artificial sea water (in mM: 486 NaCl, 10 $CaCl_2$, 26 $MgCl_2$, 10 KCl, 30 $MgSO_4$, 10 HEPES, 2.5 $NaHCO_3$ and 0.1 EDTA-2Na ~1000 mOsm, pH 8) in a Petri dish (treated with 0.1% (w/v) poly-HEME in ethanol) were maintained at 14°C on a microscope stage (Eclipse TE300; Nikon) for 0, 1, 5, 10, 20 or 30 min. in the presence or absence of the indicated concentrations of sAC inhibitors: (A) 2-OHE or (B) KH-7. Circular velocity under dark-field illumination was recorded on an EMCCD Andor. Images were collected with the Andor iQ 1.8 software at 5 fps. Control sperm (0) contain the same volume of dimethyl sulfoxide (<0.1% v/v; solvent for inhibitors). Values are means ± s.e.m. of n=4-8 experiments made in duplicate from different animals. *** (P 0.001); ** (P 0.01); * (P 0.05).