Shedding light on the role of cAMP in mammalian sperm physiology

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1. Introduction

Mammalian fertilization relies on sperm finding the egg and penetrating the egg vestments. All steps in a sperm's lifetime crucially rely on changes in the second messenger cAMP (cyclic adenosine monophosphate). In recent years, it has become clear that signal transduction in the sperm is not a continuum, but rather organized in subcellular domains, e.g. the sperm head and the sperm flagellum, with the latter being further separated into the midpiece, principal piece, and endpiece. To understand the underlying signaling pathways controlling sperm function in more detail, experimental approaches are needed that allow to study sperm signaling with spatial and temporal precision. Here, we will give a comprehensive overview on cAMP signaling in mammalian sperm, describing the molecular players involved in these pathways and the sperm functions that are controlled by cAMP. Furthermore, we will highlight recent advances in analyzing and manipulating sperm signaling with spatial-temporal precision using light.

2. cAMP signaling in mammalian sperm – cAMP synthesis

cAMP is synthesized by adenylate cyclases (ACs), which catalyze the conversion of ATP into cAMP. In mammals, there are two different types of ACs: the transmembrane ACs (tmACs) and the membrane-associated soluble AC (SACY) (Fig. 1). To date, nine different tmAC isoforms have been identified, displaying different cell-type specific expression patterns and different modes of regulation (Cali et al., 1994; Feinstein et al., 1991; Gao & Gilman, 1991; Katsushika et al., 1992; Krupinski et al., 1989; Watson et al., 1994; Yoshimura & Cooper, 1992). All tmAC isoforms possess the same primary structure, consisting of two transmembrane domains with six transmembrane segments each, and two cytoplasmic catalytic domains. The activity of tmACs is determined by G proteins, which can be circumvented by direct activation through the diterpene forskolin (Dessauer et al., 1997) or its water-soluble analog NKH477 (Hosono et al., 1992) (Fig. 2). The presence of tmACs in mammalian sperm has been controversially discussed (Brenker et al., 2012; Defer et al., 1998; Fraser et al., 2005; Fraser & Duncan, 1993; Leclerc & Kopf, 1995; Livera et al., 2005; Spehr et al., 2003; Strünker et al., 2011; Wertheimer et al., 2013): Some studies failed to report an increase in cAMP levels after stimulating sperm with forskolin or NKH477(Brenker et al., 2012; Hess et al., 2005; Jaiswal & Conti, 2003; Rojas & Bruzzone, 1992; Strünker et al., 2011), whereas others demonstrated elevated cAMP levels after drug stimulation (Baxendale & Fraser, 2005; Livera et al., 2005). Taken into account that forskolin or its analogs have been proven to reliably stimulate tmAC activity in many different cell types (Kamenetsky et al., 2006), lack of forskolin-dependent stimulation of tmAC activity has to be taken seriously. Apart from pharmacological approaches, genetic mouse models have been analyzed to reveal the role of tmAC function for sperm physiology. So far, only AC3 knockout-mice turned out to be infertile: AC3 knockout-sperm were only able to fertilize the egg *in vitro* after removal of the *zona pellucida* (Livera et al., 2005). None of the other knockout mouse-models that have been analyzed so far (AC1, AC5, AC6, AC8) show a fertility defect in male knockout mice (Chien et al., 2010; Iwamoto et al., 2003; Li et al., 2006). We have recently developed a mouse model expressing a cAMP biosensor in sperm flagella (Mukherjee et al., 2016) (Fig. 2). The sensor is equally distributed

along the flagellum, allowing to reliably determine changes in cAMP levels in the different compartments of the flagellum, but not in the sperm head. After stimulation with NKH477 to activate tmACs, no change in cAMP levels were observed, ruling out the presence of tmACs in the sperm flagellum of mice (Mukherjee et al., 2016). In fact, cAMP signaling seems to be compartmentalized with tmACs and the corresponding stimulatory G protein G_s being present in the sperm head, but not in the flagellum (Wertheimer et al., 2013). In contrast to the tmACs, the presence of SACY has been unequivocally confirmed in mammalian sperm. SACY was cloned and purified from 950 rat testis by Levin & Buck (Buck et al., 1999), following up on the discovery of a soluble AC activity in cytosolic extracts from testis (Braun & Dods, 1975; Braun et al., 1977; Neer, 1978). The *SACY* gene encodes for a protein of M_r ~ 187,000 with low specific activity and an alternatively spliced isoform of M_r ~ 50,000 with high specific activity (Buck et al., 1999; Jaiswal & Conti, 2001). The full-length protein consists of two catalytic domains in the N terminus, which display higher homology to cyanobacterial ACs than to the catalytic domains of tmACs (Buck et al., 1999), suggesting a high conservation of SACY throughout evolution. The shorter isoform consists almost exclusively of the two catalytic subunits. The C terminus of the full-length protein contains additional putative regulatory domains, such as an autoinhibitory region (Chaloupka et al., 2006) and canonical P-loop and leucine zipper sequences (Buck et al., 1999). Both isoforms are insensitive to G proteins and forskolin (Buck et al., 1999), but are directly stimulated by bicarbonate (Chen et al., 2000; Garbers et al., 1982; Garty & Salomon, 1987; Visconti et al., 1990) and Ca^{2+} (Garbers et al., 1982; Jaiswal & Conti, 2003; Litvin et al., 2003). Binding of bicarbonate stimulates the enzyme's V_{max} by fostering an allosteric change that closes the active site and rearranges the phosphates in the bound ATP (Litvin et al., 2003; Steegborn et al., 2005). For activation, SACY requires two divalent cations in the catalytic active site to coordinate binding and cyclizing of ATP. *In* vitro, SACY is five to ten times more effective in the presence of Mn^{2+} than Mg^{2+} (Buck et al., 1999; Rojas & Bruzzone, 1992). However, it is not clear whether the physiological intracellular Mn^{2+} concentration would support SACY activity, suggesting that, under physiological conditions, SACY rather binds Mg^{2+}/ATP than Mn^{2+}/ATP. Moreover, in the presence of Ca^{2+}, the affinity to Mg^{2+}/ATP is increased so that physiological cellular ATP is sufficient for SACY activation (Jaiswal & Conti, 2003; Rojas & Bruzzone, 1992). SACY expression accumulates to high levels in developing germ cells (Sinclair et al., 2000), and it is predominantly expressed in the midpiece of mature sperm (Hess et al., 2005). Importantly, bicarbonate-dependent cAMP synthesis through SACY cannot only be recovered in the soluble, but also in the particulate fraction, associated with the plasma membrane (Visconti et al., 1990; Xie & Conti, 2004). In fact, in mature sperm, a two-fold higher SACY activity can be recovered in the particulate compared to the soluble protein fraction (Hess et al., 2005). In sperm, the interaction with the sNHE (see below) is supposed to localize SACY to the plasma membrane ((Wang et al., 2007). Of note, in somatic cells, SACY is mainly found in the soluble fraction (Xie & Conti, 2004).

3. cAMP signaling in mammalian sperm - cAMP hydrolysis

Cyclic nucleotide phosphodiesterases (PDEs) are the enzymes underlying the hydrolysis of cAMP into 5'-adenosine monophosphate (AMP). In mammals, eleven PDE families (PDE1 - PDE11) with multiple, tissue-specific isoforms encoded by more than 20 different genes have been identified. Considering all the splice variants, mammals express more than 100 different PDEs (Conti & Beavo, 2007). PDEs share a conserved catalytic domain proximal to the C terminus, whereas regulatory domains are often located near the N terminus. The regulatory domains include binding sites for cyclic nucleotides, protein-protein interaction domains, and phosphorylation sites. PDE families are grouped according to their specificity to hydrolyze cAMP (PDEs 4, 7, and 8), cyclic guanosine monophosphate (cGMP) (PDEs 5, 6, and 9), or both cAMP and cGMP (PDEs 1, 2, 3, 10, and 11) (Mehats et al., 2002; Soderling & Beavo, 2000). In mammalian sperm, a member of almost every PDE family has been identified by immunocytochemistry (PDE1A, PDE4D, PDE6, PDE8A, PDE10A, PDE11A) (Baxendale & Fraser, 2005) or by pharmacological approaches. For example, in mouse sperm, the majority of PDE activity was attributed to PDE4 and PDE1 using specific inhibitors for PDE4 (rolipram) and PDE1 (MMPX), respectively, (Baxendale & Fraser, 2005). In bovine sperm, PDE activity was mainly attributed to PDE10 and to a lesser extent to PDE3, PDE4, and PDE8 family members (Bergeron et al., 2017; Goupil et al., 2016). Not only cAMP synthesis, but also cAMP degradation through PDEs seems to be compartmentalized in mammalian sperm (Bajpai et al., 2006; Bergeron et al., 2017), underlining the fact that cAMP signaling controls specific sperm functions through an organization into microdomains (Fig. 1).

4. cAMP downstream targets in mammalian sperm

Changes in cAMP signaling are transduced into a downstream response by engaging cAMP target proteins. Most commonly, they belong to the family of cyclic nucleotide-binding proteins containing a cyclic nucleotide-binding domain (CNBD). In the following, the different CNBD-containing cAMP targets in mammalian sperm will be described.

Protein kinase A (PKA)

The principal action of cAMP in sperm is mediated through activation of the cAMP-dependent protein kinase (PKA), a ubiquitously expressed, broad specificity Ser/Thr kinase conserved in all eukaryotes (Burton & McKnight, 2007) (Fig. 1). The inactive PKA holoenzyme is tetrameric, consisting of two regulatory subunits (R) and two catalytic (C) subunits. Mammals harbor five different genes for the catalytic subunit (*PRKACA*, *PRKACB*, *PRKACG*, *PRKX*, and *PRKY*, encoding C_α, C_β, C_γ, PRKX, and PRKY) and four different genes for the regulatory subunit (RI_α, RI_β, RII_α, and RII_β) (Soberg et al., 2013). For many years, PKA activation was thought to rely on two molecules of cAMP cooperatively binding to the CNBDs located at the C terminus of each R subunit, causing dissociation of the holoenzyme into a regulatory subunit homodimer and two free active catalytic subunits (Kim et al., 2007; Kim et al., 2005). However, recent reports demonstrate that catalytically active PKA stays intact as a holoenzyme, which allows the proteins to stay proximal to anchoring sites and substrates (Smith et al., 2017). The catalytic subunit phosphorylates substrate proteins, altering their functional properties. To confine PKA

activity to a relevant subset of potential substrates, scaffolding proteins, referred to as A-kinase anchoring proteins (AKAPs), bind to the dimerized regulatory subunits of PKA, targeting the protein to specific subcellular locations (Beene & Scott, 2007; Carnegie et al., 2009). *In vitro,* AKAPs bind to the RII subunit, which allowed to identify two sperm-specific AKAPs, AKAP3 and 4 (Carr et al., 2001; Vijayaraghavan et al., 1999). Loss of AKAP4 in sperm results in infertility with sperm showing morphological defects and reduced sperm motility (Miki et al., 2002). In mammalian sperm, PKA and AKAPs are located in the flagellum (Wertheimer et al., 2013). During sperm development, sperm switch from expressing the somatic Cα1 catalytic subunit of PKA to the unique sperm-specific Cα2 subunit, which contains a distinct N terminus and lacks the post-translational myristoylation found in Cα1 (San Agustin & Witman, 2001). Knocking out both isoforms in mice results in postnatal lethality with only a few knockout mice surviving until adulthood (Skalhegg et al., 2002). Spermatogenesis in these knockout mice progressed normally, but sperm motility is impaired (Skalhegg et al., 2002). Knocking out the sperm-specific Cα2 subunit causes infertility in male mice with no detectable PKA activity in mature sperm (Nolan et al., 2004). The molecular mechanisms underlying this defect will be discussed later in this review. The main regulatory subunit in mature sperm is the RIIα subunit (Burton & McKnight, 2007; Landmark et al., 1993). Loss of RIIα results in a compensatory increase in the RIα subunit (Burton et al., 1999). In turn, RIIα knockout-mice are fertile and do not show any sperm defects (Burton et al., 1999). Apart from creating knockout mice, analyzing PKA function *in vivo* is limited by the lack of highly specific inhibitors that can be used in primary cells or in whole animals. Genetically-modifying the mouse *Prkaca* gene in combination with Cre-mediated recombination *in vivo* allowed to express a PKA C_{α} mutant (C_{α}M120A), containing a mutation in the ATP-binding pocket, while at the same time turning off the expression of the wild-type protein (Morgan et al., 2008). This mutation confers sensitivity to the pyrazolo[3,4-d]pyrimidine inhibitor, 1NM-PP1, which allows to specifically block PKA activity (Morgan et al., 2008). Application of 1NM-PP1 to sperm abolished certain sperm functions, which will be discussed later in this review.

Epac – a guanine-nucleotide exchange factor

The Rap-specific guanine-nucleotide exchange factor Epac (exchange protein directly activated by cAMP) contains a CNBD and a guanine nucleotide-exchange factor (GEF) domain (de Rooij et al., 1998; Kawasaki et al., 1998). Thereby, Epac couples cAMP signaling to the activation of the Rap subfamily of RAS-like small GTPases. So far, two isoforms of Epac, namely Epac1 and Epac2 have been identified (de Rooij et al., 1998; Kawasaki et al., 1998). Epac1 and Epac2 both contain a regulatory and a catalytic domain in their N and C terminus, respectively. The catalytic domain harbors the GEF domain, the regulatory domain contains the CNBD, which, in the absence of cAMP, inhibits the catalytic activity (Rehmann et al., 2008; Rehmann et al., 2006; Rehmann et al., 2003). Epac2 additionally contains a second low-affinity CNBD; however, its function is rather unclear (de Rooij et al., 2000). Experimental evidence suggests that Epac proteins play a role in the sperm head (Branham et al., 2009; Branham et al., 2006; Lucchesi et al., 2016), which we will describe later (Fig. 1).

Other cAMP target proteins in mammalian sperm

Apart from PKA and Epacs, other CNBD-containing cAMP target-proteins have been identified in mammalian sperm or sperm precursor cells. However, their role in mammalian sperm function is not well understood. Cyclic nucleotide-gated (CNG) channels are nonselective cation channels, which are opened by direct binding of cyclic nucleotides (Kaupp & Seifert, 2002). Of note, a CNG channel was the first ion channel cloned from mammalian testis (Weyand et al., 1994). CNG channels are encoded by six different genes, four α subunits (α1-α4) and two β subunits (β1 and β3). A combination of two α subunits and two β subunits form heterotetrameric CNG channel complexes (Kaupp & Seifert, 2002). However, the role of CNG channels in mammalian sperm physiology is still ill-defined. Although CNG channel subunits were identified in the flagellum of mouse sperm (Wiesner et al., 1998), to date, no sperm function has been attributed to mammalian CNG channels (Biel et al., 1999). The role of CNG channels in controlling sperm function is mainly based on pharmacological studies using membrane-permeable cAMP analogs. Incubation of sperm with 8-Br-cAMP or 8-Br-cAMP evokes a Ca^{2+} influx that has been attributed to CNG channel opening (Kobori et al., 2000). However, we now know that this Ca^{2+} influx is carried by the CatSper channel complex (Ren et al., 2001; Xia et al., 2007; Xia & Ren, 2009) and that 8-Br-cAMP or 8-Br-cAMP directly activates CatSper from the outside (Brenker et al., 2012). Furthermore, photorelease of cAMP from caged cAMP fails to evoke a Ca^{2+} influx (Strünker et al., 2011). Thus, CNG channels do not seem to play a role in mammalian sperm physiology. In sea urchin and zebrafish sperm, the atypical K^+-selective CNGK channels control sperm signaling (Bönigk et al., 2009; Fechner et al., 2015). However, these channels do not seem to be present in mammalian sperm, underlining that CNG channels do not play a major role in mammalian sperm physiology. Another member of the CNBD-containing protein family is the atypical sperm-specific sodium proton exchanger (sNHE, *Slc9a10*), which is expressed in the principal piece of mouse sperm (Wang et al., 2003) (Fig. 1). Apart from the CNBD, the sNHE also contains a putative voltage-sensor motif, similar to the one found in voltage-gated ion channels (Catterall, 2000), indicating that sNHE function could be regulated by cyclic nucleotides and changes in membrane potential V_m (Wang et al., 2003). The physiological function of sNHE in sperm is still enigmatic. Knocking out sNHE in mice renders male mice infertile due to a defect in sperm motility (Wang et al., 2003). However, the sNHE is found in a complex with SACY and the phenotype of sNHE knockout-mice is mainly due to concomitant loss of SACY and not the loss of sNHE (Jansen et al., 2015; Wang et al., 2007). It has been suggested that cAMP controls the intracellular pH of mammalian sperm (pH_i) by controlling sNHE function (Lishko et al., 2012). Future studies will reveal whether the sNHE in fact controls pH_i in mammalian sperm. Last but not least, a new CNBD-containing protein called CRIS (cyclic nucleotide receptor involved in sperm function) has been identified in sperm precursor cells (Krähling et al., 2013). However, CRIS is not expressed in mature sperm and seems to play a predominant role in sperm development (Krähling et al., 2013).

5. The role of cAMP in sperm physiology – sperm capacitation

Sperm physiology crucially relies on cAMP signaling. A deficiency in most of the molecular players in sperm cAMP signaling (see above) results in male sub- or infertility. In the following, we will highlight the main sperm functions that are controlled by cAMP and describe how the cAMP-signaling components integrate into the signaling pathways controlling sperm physiology. When leaving the male reproductive tract after ejaculation, sperm cells are morphologically, but not functionally mature and thus cannot fertilize the egg. To acquire fertilization competence, sperm need to undergo a maturation process called capacitation – a process that was first described in the 1950's by two independent reports (Austin, 1952; Chang, 1951). Increasing evidence points towards a crucial role of cAMP in sperm capacitation by controlling sperm motility and the ability to undergo the acrosome reaction, which we will describe in more detail in the following chapters. Our knowledge about the molecular mechanisms underlying capacitation is based on *in vitro* analysis. Here, capacitation is induced by incubating sperm in medium containing Ca^{2+}, bicarbonate (HCO_3^-), an energy source (e.g. lactate, glucose), and a cholesterol acceptor (e.g. bovine serum albumin, BSA) (Suarez, 2008). For bicarbonate entry, which initiates the signaling cascade, the presence of CFTR (cystic fibrosis transmembrane conductance regulator) is necessary (Hernandez-Gonzalez et al., 2007; Puga Molina et al., 2017) and might be conveyed through SLC26A3 (Chan & Sun, 2014; Chen et al., 2009). In turn, the intracellular cAMP concentration increases through bicarbonate-dependent stimulation of SACY activity in the presence of millimolar extracellular Ca^{2+} (Carlson et al., 2007; Chen et al., 2000; Jaiswal & Conti, 2003; Litvin et al., 2003; Mukherjee et al., 2016; Wennemuth et al., 2003). The kinetics of the bicarbonate-induced cAMP changes vary among species (Battistone et al., 2013; Brenker et al., 2012; Harrison & Miller, 2000; Mukherjee et al., 2016). However, to interpret these results, the different experimental methods used to measure changes in cAMP levels have to be considered. The most frequently applied technique to determine total cAMP levels was based on an ELISA assay (Battistone et al., 2013; Brenker et al., 2012; Harrison, 2004). Recently, the generation of a mouse model expressing a cAMP biosensor in sperm flagella allowed to determine free cAMP levels, i.e. the cAMP that evokes a downstream response, rather than total cAMP levels as measured by an ELISA-based assay (Mukherjee et al., 2016). In fact, comparing the dynamics of the changes in total and free cAMP levels after exposing sperm to capacitating conditions revealed a difference in kinetics: whereas total cAMP levels increased within 1 min and decreased straight after, the levels of free cAMP remained constantly high after 1 min over at least 20 min (Mukherjee et al., 2016). Thus, in future experiments, it is important to take changes in free rather than total cAMP levels into account.

The bicarbonate-dependent increase in cAMP levels after exposing sperm to capacitating conditions immediately activates PKA, leading to downstream protein phosphorylation, which drives the maturation process (Harrison & Miller, 2000; Kaneto et al., 2008; Morgan et al., 2008) (Fig. 2). Mice lacking the sperm-specific Cα2 PKA catalytic subunit are infertile and do not capacitate (Nolan et al., 2004). Downstream of PKA, the main hallmark of capacitation is an increase in protein tyrosine phosphorylation (Visconti et al., 1995). In fact, Cα2 PKA

knockout-mice lack the increase in capacitation-induced protein tyrosine phosphorylation (Nolan et al., 2004). However, PKA-dependent protein phosphorylation is readily observed after an increase in cAMP levels, whereas changes in protein tyrosine phosphorylation are only observed after 30-60 min (Morgan et al., 2008). Indeed, application of the PKA inhibitor 1NM-PP1 to sperm during capacitation revealed that bicarbonate-stimulated PKA activity is required for at least 30 min to initiate downstream protein tyrosine phosphorylation (Morgan et al., 2008). Thus, the action of PKA on the downstream signaling cascade underlying protein tyrosine phosphorylation seems to be rather indirect. Although a number of candidates have been proposed, the tyrosine kinase underlying protein tyrosine phosphorylation during capacitation has been enigmatic. Only recently, the kinase has been identified: the tyrosine kinase FER was identified in a proteomic approach as a target for tyrosine phosphorylation in sperm (Chung et al., 2014) (Fig. 1). In fact, FER is auto-phosphorylated in its activation loop upon capacitation (Alvau et al., 2016; Chung et al., 2014). Most importantly, FER knockout-mice do not show an increase in protein tyrosine phosphorylation under capacitating conditions, but strikingly, male mice were fertile, challenging the current concept of the molecular mechanisms underlying sperm capacitation under physiological conditions (Alvau et al., 2016).

In addition to increasing cAMP levels, incubating sperm under capacitating conditions has also been proposed to result in i) Changes in the lipid content of the plasma membrane, ii) An increase in the intracellular pH, iii) Hyperpolarization of the membrane potential V_m, and iv) An increase in the intracellular Ca^{2+} concentration. In fact, all these changes might be closely related to cAMP. The change in lipid content, when sperm are capacitated *in vitro,* was shown to be mediated by bicarbonate-dependent cholesterol uptake through BSA, whereby the plasma membrane becomes more fluidic (Ehrenwald et al., 1990; Flesch et al., 2001; Gadella & Harrison, 2000; van Gestel et al., 2005). In turn, protein-protein and protein-lipid interactions in the plasma membrane are reorganized, which might alter cellular signaling. The increase in intracellular pH seems to be a pre-requisite for capacitation (Parrish et al., 1989; Vredenburgh-Wilberg & Parrish, 1995). However, the molecules underlying sperm alkalization during capacitation are ill-defined. It has been proposed that the sNHE might underlie the increase in pH in a cAMP-dependent manner (Wang et al., 2007; Wang et al., 2003). However, since the sNHE is found in a complex with SACY and all defects associated with the loss of sNHE in knockout mice have been attributed to the concomitant loss of SACY, the role of sNHE during capacitation is still debated. Other reports demonstrated that a $Na^+/Cl^-/HCO_3^-$-dependent acid-efflux pathway underlies sperm alkalization during capacitation (Zeng et al., 1996). Future studies will have to reveal the molecule(s) underlying an increase in pH_i during capacitation. Further downstream, an increase in cAMP levels and sperm alkalization increases the membrane conductance for both Ca^{2+} and K^+ carried by CatSper and KSper (the latter formed by Slo3), respectively (Brenker et al., 2014; Chavez et al., 2014; Lopez-Gonzalez et al., 2014; Navarro et al., 2007; Ren et al., 2001; Santi et al., 2010; Stival et al., 2015; Zeng et al., 2011). However, it is important to emphasize again that an increase in cAMP does not directly evoke a Ca^{2+} influx (see discussion above). The Ca^{2+} influx and membrane

hyperpolarization evoke other behavioral responses during sperm capacitation, e.g. a change in sperm motility and the acrosome reaction.

6. The role of cAMP in sperm physiology – sperm motility

The main signaling pathways controlling the motility of mammalian sperm are regulated by Ca^{2+} and cAMP. The first relay station integrating both Ca^{2+} and cAMP is SACY (Fig. 1). SACY knockout-sperm lack cAMP synthesis, are immotile, and cannot fertilize the egg, resulting in male infertility ((Esposito et al., 2004; Hess et al., 2005; Xie et al., 2006). Incubation with bicarbonate stimulates SACY activity and, in turn, increases the flagellar beat frequency more than 2-fold (Carlson et al., 2007; Wennemuth et al., 2003) (Fig. 1), which is abolished in SACY knockout-sperm (Xie et al., 2006). *In vivo*, this increase in motility occurs when sperm come into contact with reproductive fluids in the male and/or female genital tract, containing high bicarbonate levels (Miki & Clapham, 2013). Applying 1NM-PP1 to sperm revealed that bicarbonate-evoked PKA-dependent protein phosphorylation and an increase in flagellar beat frequency occurs within 90 s (Morgan et al., 2008). The increase in beat frequency does not change the symmetry of the flagellar beat, allowing vigorous swimming on a rather linear trajectory (Wennemuth et al., 2003). In addition, SACY is regulated by Ca^{2+} and reducing the extracellular Ca^{2+} concentration to the low micromolar range abolishes the bicarbonate-dependent stimulation of SACY activity and the increase in flagellar beat frequency (Carlson et al., 2007; Mukherjee et al., 2016). Downstream of cAMP, the main target controlling sperm motility is PKA (Nolan et al., 2004). However, loss of PKA activity does not result in a complete loss of sperm motility: the proportion of motile sperm and the flagellar beat amplitude is reduced, but sperm re not immotile like SACY knockout-sperm. However, in absence of PKA activity, the bicarbonate-dependent increase in sperm motility is fully abolished (Nolan et al., 2004). Hence, PKA predominantly controls the cAMP-dependent increase in flagellar beat frequency. On a molecular level, this is controlled through PKA-dependent phosphorylation of flagellar motor proteins in the axoneme (Lackey & Gray, 2015) (Fig. 1). Although it is widely accepted that PKA-dependent protein phosphorylation controls sperm motility, very little is known about the identities of PKA substrates in sperm. So far, mainly flagellar motor proteins have been identified as PKA targets. One protein that has been identified is FSCB, a calcium-bindin protein that interacts with CABYR, which is involved in fibrous sheath biogenesis (Kaneto et al., 2008; Li et al., 2007; Liu et al., 2011). Other targets are the septin SEPT12 (Shen et al., 2017) and SPIF and TCP11 (Stanger et al., 2016). How these proteins modulate sperm beating, is ill-defined. For other motile cilia, it has been proposed that phosphorylation of dyneins increased the velocity of microtubule gliding across outer arm dynein–coated surfaces and, thereby, increases the beating frequency (Salathe, 2007). In contrast to cAMP, Ca^{2+} influx does not change the flagellar beat frequency, but rather evokes an asymmetric flagellar beat, representing a swimming mode called hyperactivation (Ishijima et al., 2002; Suarez, 2008). Hyperactivation allows sperm to generate the propulsive forces that are needed to penetrate the egg's vestments. Sperm lacking any subunit of CatSper (Fig. 1) do not hyperactivate and cannot fertilize the egg (Chung et al., 2017; Chung et al., 2011). Furthermore, hyperactivated motility is only observed in capacitated sperm and relies on PKA-dependent protein

phosphorylation, since sperm that lack the Cα2 PKA subunit do not show hyperactivated motility (Nolan et al., 2004). However, the cAMP-dependent control of sperm motility can be bypassed by exposing sperm to a Ca^{2+} pulse using the Ca^{2+} ionophore A_{23817} (Navarrete et al., 2016; Tateno et al., 2013). In fact, a short A_{23817} pulse is sufficient to overcome the infertility phenotype of a number of knockout mouse-models, including CatSper1, SACY, and Slo3 (Navarrete et al., 2016). In conclusion, a temporarily elevation of intracellular Ca^{2+} levels might prime the sperm for hyperactivation, bypassing the need for other signaling pathways required to increase Ca^{2+} levels in sperm during capacitation. In fact, in permeabilized sea urchin sperm flagella, it has been shown years ago that addition of ATP and Ca^{2+} is sufficient to induce asymmetric flagellar beating (Brokaw, 1979).

7. The role of cAMP in sperm physiology – acrosome reaction

The acrosome, a Golgi-derived vesicle filled with enzymes, resides as a cap on the sperm head. As a prerequisite for fertilization, the acrosome needs to undergo exocytosis, the so-called acrosome reaction (Austin & Bishop, 1958) (Fig. 1). Only capacitated sperm can undergo the acrosome reaction. However, *in vitro*, not all sperm in a population are capacitated, making it rather difficult to study the acrosome reaction in sperm populations *in vitro* (Yanagimachi, 2011). *In vivo*, one way of visualizing the acrosome reaction is to use transgenic mice, expressing eGFP under the control of the acrosin promoter (Baibakov et al., 2007; Hasuwa et al., 2010; Nakanishi et al., 1999). Using these mice, it was demonstrated that the majority of sperm underwent the acrosome reaction before even contacting the *zona pellucida*, the outer layer of the egg, which has been widely accepted as a physiological stimulus that induces the acrosome reaction (Hirohashi et al., 2011; Jin et al., 2011; La Spina et al., 2016). Thus, it has been questioned whether the acrosome reaction indeed is a prerequisite to penetrate the egg's vestments, although it is required for interaction and membrane fusion of sperm and oocyte during fertilization. On a molecular level, both cAMP and Ca^{2+} are required for the acrosome reaction. Here, the main target for cAMP is Epac (Lucchesi et al., 2016). Epac exchanges GDP from GTP on Rap1, which in turn activates PLCε, producing IP_3 and DAG from PIP_2 (Lucchesi et al., 2016). IP_3 is supposed to mobilize Ca^{2+} from internal stores, which triggers the acrosome reaction (Lucchesi et al., 2016) (Fig. 1). However, so far, genetic mouse models have not revealed a role for PLCε in controlling sperm function. In fact, only PLCδ knockout-mice have been demonstrated to be subfertile because this isoform is supposed to regulate the acrosome reaction (Fukami et al., 2001). Recently, PKA-dependent phosphorylation of proteins that regulate actin dynamics have also been proposed to control the acrosome reaction (Romarowski et al., 2015). Here, PKA-dependent protein phosphorylation determines the activity of the small Rho GTPases RhoA/C and Rac1, which in turn affects the phosphorylation status of LIMK1 and cofilin and controls actin polymerization (Romarowski et al., 2015). Blocking actin polymerization dramatically reduces the ability of sperm to undergo the acrosome reaction (Romarowski et al., 2015). In fact, the role of actin polymerization in controlling the acrosome reaction has already been described years ago (Breitbart & Finkelstein, 2015; Spungin et al., 1995). Furthermore, a change in membrane potential is important for sperm to undergo the acrosome reaction (Arnoult et al., 1999; De La Vega-

Beltran et al., 2012; Zeng et al., 1995). Sperm hyperpolarization, which occurs during capacitation, is sufficient to prepare sperm for the acrosome reaction (De La Vega-Beltran et al., 2012; Zeng et al., 1995). Sperm lacking Slo3 undergo a depolarization rather than hyperpolarization under capacitating conditions, whereby the acrosome reaction is abolished (Santi et al., 2010). It has been proposed that PKA-dependent activation of a member of the Src kinase family, cSrc, regulates Slo3-mediated KSper currents and, thereby, controls the membrane potential of sperm during capacitation (Stival et al., 2015). Blocking cSrc activity decreased Slo3-mediated currents and blocked the acrosome reaction, underlining the fact that a hyperpolarized membrane potential set by the Slo3 channel is required to prepare sperm for the acrosome reaction (Stival et al., 2015).

8. Analyzing and manipulating sperm function by light

The sperm cell itself and the intracellular signaling pathways underlying sperm function are highly compartmentalized. On the signaling level, compartmentalization is achieved by tethering signaling components to certain domains in the sperm head or along the sperm flagellum. For the cAMP signaling cascade, AKAPs cluster proteins in signaling complexes, thereby facilitating signal transduction and limiting cAMP signaling to a specific microdomain (Wertheimer et al., 2013). The importance of localized signaling domains for mammalian sperm function has been demonstrated for the CatSper signaling complex, which forms a Ca^{2+} signaling domain along the flagellum that is organized into four columns (Chung et al., 2014). Loss of one CatSper channel subunit results in loss of the quadrilateral organization and, in turn, impairs tyrosine phosphorylation, capacitation-associated signaling, and sperm motility (Chung et al., 2014). The analysis of signaling complexes in sperm has been mainly hampered by the lack of suitable tools. Most methods that have been used to interrogate sperm signaling events *in vitro* do not provide the spatial and temporal precision, which is needed to analyze the function of subcellular microdomains. To overcome these limitations, optogenetic tools and fluorescent biosensors have recently been employed to modulate and analyze cAMP signaling in mammalian sperm (Jansen et al., 2015; Mukherjee et al., 2016) (Fig. 2). Optogenetics allows to manipulate signaling processes in genetically-engineered cells by light. Different light-activated adenylate cyclases have been described, which differ in their activity, molecular structure, and spectral properties (Iseki et al., 2002; Jansen et al., 2017; Raffelberg et al., 2013; Ryu et al., 2014; Ryu et al., 2010; Stierl et al., 2011). The light-activated adenylate cyclase bPAC (bacterial photoactivated adenylate cyclase) from the bacterium *Beggiatoa* has been used to manipulate cAMP levels in mouse sperm using a transgenic approach (Jansen et al., 2015). Here, cAMP-dependent sperm functions were controlled by light: Stimulation of bPAC evoked phosphorylation of PKA targets, tyrosine phosphorylation, and the capacitation-induced ability of sperm to undergo the acrosome reaction (Jansen et al., 2015). Furthermore, also the flagellar beat frequency, which is controlled by cAMP, could be increased by light-dependent stimulation of bPAC activity. Of note, the light-evoked motility response could be modulated in a graded fashion: the higher the light dose, the faster the flagellar beat. To not only manipulate cAMP levels in sperm with spatial-temporal precision using optogenetics, but to also measure cAMP dynamics in microdomains along the sperm flagellum, a fluorescent

biosensor reporting changes in the intracellular cAMP concentration has been applied in mouse sperm. The mlCNBD-FRET biosensor is based on the CNBD of the MlotiK ion channel (Nimigean et al., 2004) and exhibits exquisite sensitivity for cAMP with a binding affinity in the low nanomolar range (Cukkemane et al., 2007; Mukherjee et al., 2016; Peuker et al., 2013). The expression of mlCNBD-FRET in mouse sperm allowed for the first time to analyze the changes in intracellular cAMP in the freely beating flagellum of mouse sperm and to exclude the presence of tmACs in the flagellum (Mukherjee et al., 2016). In addition, using mlCNBD-FRET, it could be shown that the HCO_3^--induced changes in intracellular cAMP occur with different kinetics in the midpiece and the principal piece (Mukherjee et al., 2016) (Fig. 1). This indicates that SACY stimulation by HCO_3^- does not evoke a uniform cAMP response along the flagellum, but rather reveals that cAMP dynamics are differentially regulated along the sperm flagellum, presumably in a PDE-dependent manner. In addition to the light-activated adenylate cyclases described above, also a photoactivated phosphodiesterase has been engineered (Gasser et al., 2014), which could be used to control cAMP hydrolysis in the flagellum by light (Fig. 2).

9. Concluding remarks

In summary, the cAMP-based optogenetic tool kit has proven its use in sperm, allowing to control sperm function by light. Future applications will help to unravel how cAMP microdomains are regulated and how they control sperm function. Of note, all these tools are genetically-encoded and can therefor only be applied in mouse sperm. However, in the last couple of years, it has been demonstrate that sperm from different species use diverse repertoires of sperm-specific signaling molecules and even closely related protein isoforms feature different properties and serve different functions (Kaupp & Strünker, 2017; Wachten et al., 2017). Thus, each species has to be studied in its own right and caution has to be taken when transferring knowledge from one species to the other.

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Figure legends

Fig. 1 cAMP signaling in mammalian sperm. Different cAMP signaling components and their function in the sperm head and flagellum. The upper sperm image represents a FRET measurement to determine the intracellular cAMP concentration (from low to high) using the mlCNBD-FRET sensor. The dashed arrow pointing towards the acrosome reaction indicates that the acrosome reaction can be evoked by Ca^{2+} influx through a store-depletion operated pathway (O'Toole et al., 2000). For details, see text.

Fig. 2 Manipulating and analyzing cAMP signaling in mammalian sperm. The main cAMP signaling components can be stimulated (Forskolin, NKH477: tmACs) or inhibited (MDL12,330A: tmAC, LRE1, KH7: SACY, IBMX: PDE, H89: PKA) using pharmacological tools. Genetical engineering allows to manipulate cAMP levels by light using optogenetics (bPAC, LAPD) or analyze cAMP dynamics using FRET-based biosensors (mlCNBD-FRET). For details, see text.

Figures

Figure 1

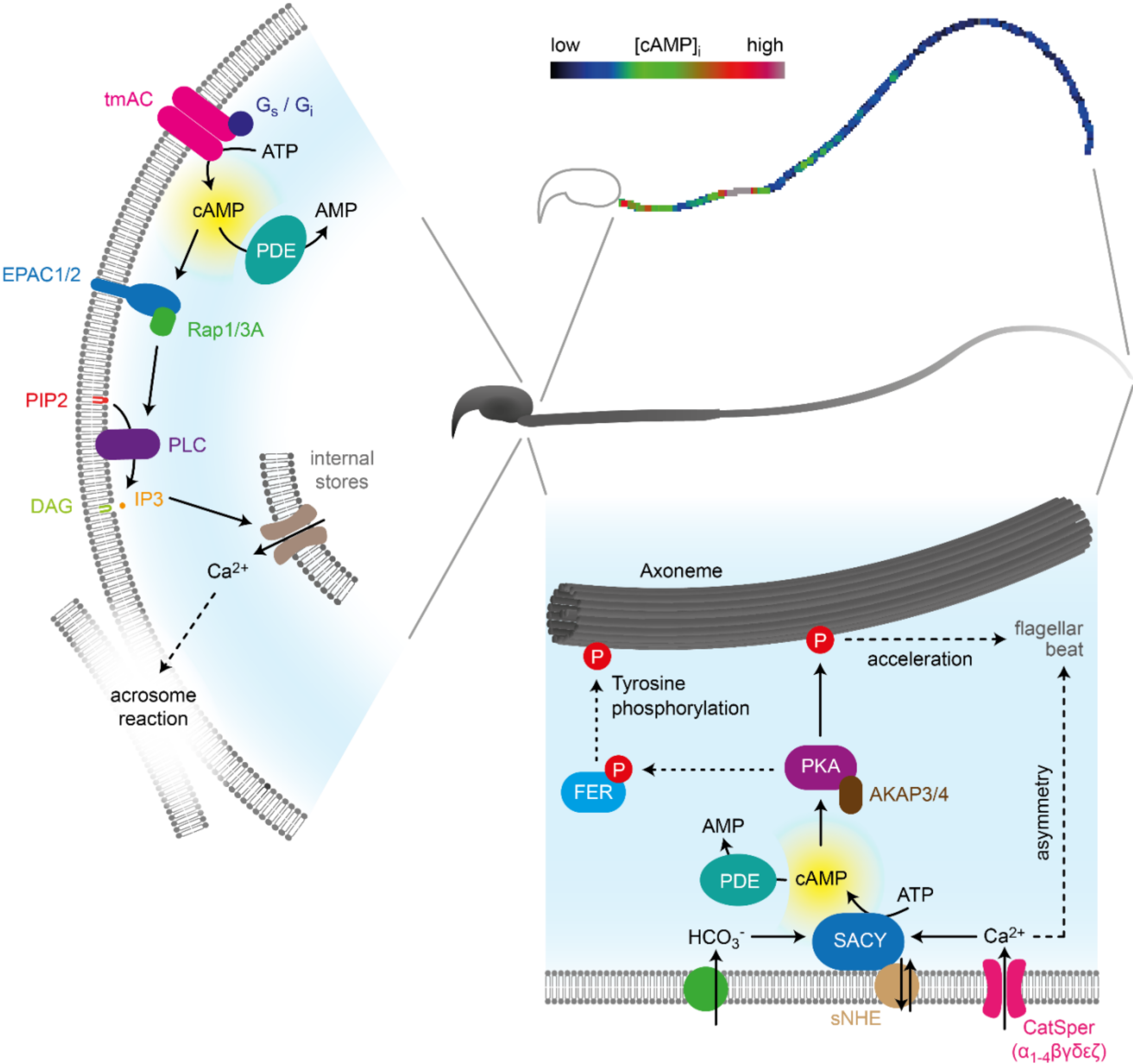

Figure 2