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Review

Central role of soluble adenylyl cyclase and cAMP in sperm physiology ☆

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ABSTRACT

Cyclic adenosine 3′,5′-monophosphate (cAMP), the first second messenger to be described, plays a central role in cell signaling in a wide variety of cell types. Over the last decades, a wide body of literature addressed the different roles of cAMP in cell physiology, mainly in response to neurotransmitters and hormones. cAMP is synthesized by a wide variety of adenylyl cyclases that can generally be grouped in two types: transmembrane adenylyl cyclase and soluble adenylyl cyclases. In particular, several aspects of sperm physiology are regulated by cAMP produced by a single atypical adenylyl cyclase (Adcy10, aka sAC, SACY). The signature that identifies sAC among other ACs, is their direct stimulation by bicarbonate. The essential nature of cAMP in sperm function has been demonstrated using gain of function as well as loss of function approaches. This review unifies state of the art knowledge of the role of cAMP and those enzymes involved in cAMP signaling pathways required for the acquisition of fertilizing capacity of mammalian sperm. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

1. Introduction

More than 50 years of investigation have passed since the discovery of cyclic adenosine 3′,5′-monophosphate (cAMP). This molecule stands as the first second messenger to be described [1]. Over the last decades, a wide body of literature addressed the different roles of cAMP in cell physiology, mainly in response to neurotransmitters and hormones. Although protein kinase A (PKA) was initially believed to be the sole effector of cAMP, new cAMP targets were later described. Examples of these are the RAP guanine-nucleotide-exchange factor (GEF) EPAC [2,3] and the cyclic-nucleotide-gated ion channels [4] which are also directly responsive to cAMP. Added to the complexity of the pathway, cAMP synthesis is conducted by products of ten distinct genes encoding adenylyl cyclase. Nine of them (Adcy1-9) encode the family of transmembrane adenylyl cyclase (tmAC) isoforms with differential tissue distribution and regulatory responsiveness. All isoforms possess a common predicted topology with high primary structure homology of their catalytic sites. Moreover, they are regulated by G proteins and activated by the diterpene forskolin [5].

In contrast to the tmAC family, a single atypical adenylyl cyclase (Adcy10, aka sAC, SACY) [6] generates multiple isoforms by alternative splicing [7,8]. The signature that identifies sAC among other ACs, is their direct stimulation by bicarbonate [9–12] and as such serves as a CO_2 sensor in many cellular systems [6]. Unlike tmACs, sAC is insensitive to G protein regulation and its enzymatic activity is over ten times more effective in the presence of Mn^{2+}/ATP than in the presence of Mg^{2+}/ATP [12]. sAC was first described as a soluble adenylyl cyclase present in testicular extracts [13]. This property was used to purify sAC from rat testes and to identify it by microsequencing [6]. Not surprisingly, sAC is essential for fertilization; it is abundant in mature sperm and as such is the main source of cAMP in these cells. This review will be centered on the role of cAMP and those enzymes involved in cAMP signaling pathways in mammalian sperm. For more information on other aspects of the molecular basis of sperm function, other recent reviews can be found [14–22].

2. Role of cAMP in sperm function

Cyclic AMP orchestrates different aspects of sperm function required for the acquisition of fertilizing capacity. For a detailed list of principal findings and references, see Table 1. After leaving the testis, mammalian sperm are morphologically mature; however, they are not able to fertilize. Fertilization capacity is acquired after completion of two post-testicular maturational processes. The first one, known as epididymal maturation, is poorly understood at the molecular level.

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Table 1
cAMP in sperm physiology.

Role	Refs.
cAMP regulation of capacitation	[28–34]
cAMP regulation of acrosome reaction	[27,37–41,80]
Membrane lipid remodeling	[28,29,157]
Hyperpolarization of sperm plasma membrane	[30]
Increase in intracellular pH	[30–33]
Increase in intracellular Ca^{2+}	[34,147,148,172–175]
Increase in protein tyrosine phosphorylation	[35]
cAMP role in ATP synthesis	[54]

It involves the activation of signaling pathways and the incorporation of proteins secreted by the epididymal epithelium in the male reproductive tract. During this process, the sperm acquire the ability to move progressively. However, after epididymal maturation, mammalian sperm are still incapable of fertilizing an egg. In the '50s, Chang [23] and Austin [24] independently reported that mammalian sperm should reside in the female tract for an obligatory period of time to acquire 'fertilizing capacity' in a process known as capacitation. This discovery was essential for the development of mammalian *in vitro* fertilization which was achieved by Chang eight years later [25]. Following Chang's [23] and Austin's [24] pioneering work, capacitation was defined as the physiological processes that render the sperm fertile [26].

Cyclic AMP has been reported to be essential for events occurring during capacitation, including activation of motility, changes in the motility pattern known as hyperactivation and for development of the ability to undergo the acrosome reaction. There is clear evidence that many of the cAMP effects on sperm are mediated by activation of PKA. However, other cAMP-binding proteins have been proposed to participate in some of the processes [27]. In addition, cAMP and its targets regulate a variety of capacitation-induced signaling events including: 1) membrane lipid remodeling [28,29]; 2) hyperpolarization of the sperm plasma membrane [30]; 3) increase in intracellular pH [30–33]; 4) increase in intracellular Ca^{2+} [34]; and 5) increase in protein tyrosine phosphorylation [35]. Although one important aspect of the sperm capacitation process is the preparation of the sperm to undergo a physiologically relevant exocytotic reaction known as acrosome reaction, the acrosome reaction *per se* is usually considered outside of the capacitation process [36]. Therefore, the cAMP action on the acrosome reaction will be mentioned independent of capacitation [27,37–41].

Capacitation can be mimicked in simple culture media containing Ca^{2+}, HCO_3^-, energy sources and a cholesterol acceptor that is usually bovine serum albumin. One of the first signaling events observed upon exposing sperm to capacitating conditions is a fast increase in cAMP content [42]. Interestingly, these initial high cAMP levels are rapidly reduced ([43] for human sperm, [44] for boar sperm). Decrease of cAMP levels could be attributed either to hydrolysis by phosphodiesterases (see below) or to cAMP extrusion by specific transporters [45]. As expected, the increase in cAMP immediately activates PKA activity [44]. Although the first attempts to show PKA activation relied on direct PKA measurements [46], more recently, development of antibodies against phosphorylated consensus sequences allowed for more precise kinetic studies. In particular, antibodies against phosphorylated PKA substrates recognizing either RXXpS/pT or RRXpS/pT were instrumental to demonstrate a fast PKA activation [44,47–51]. In all mammalian species studied, PKA is observed to be at maximum levels in less than a minute of exposure to capacitating conditions. This rapid up-regulation of cAMP signaling pathways is induced by HCO_3^- in the capacitation medium, suggesting that it is mediated by sAC [52].

Direct cAMP measurements and Western blots using anti-phospho-PKA substrate antibodies have been used to reveal how the cAMP pathway is regulated. However, these experiments were silent regarding the role of cAMP in sperm function. To study the consequences of cAMP increase and PKA activation in sperm, loss and gain of function strategies were used. Gain of function experiments compared the sperm response in the absence or presence of HCO_3^-, and also employed a combination of permeable cAMP agonists and phosphodiesterase inhibitors to mimic the cAMP response in the absence of an upstream stimulus. On the other hand, loss of function was achieved using pharmacological [35] and genetic knock-out approaches targeting sAC [52–54], the PKA catalytic subunit [49] or the anchoring of PKA to A-kinase anchoring proteins (AKAPs) [55]. Altogether these approaches revealed an essential role of cAMP-signaling pathways, in particular of PKA, in the activation of sperm motility and in the induction of a vigorous asymmetrical movement (*i.e.* hyperactivated motility) necessary for sperm to fertilize. Although the role of cAMP in the acrosome reaction is less conclusive, recent work presented evidence that the GDP/GTP exchanger EPAC, another target of cAMP is present in sperm and is involved in the regulation of the acrosome reaction [37,56]. It is still not clear whether cAMP synthesis in the sperm anterior head is due to the activity of sAC [37] or tmACs [41]. In the following sections, we will discuss individually the role of the enzymes involved in cAMP signaling.

3. G proteins

A positive correlation between cAMP and mammalian sperm motility has been first observed by the group of Henry Lardy in 1971 in bovine samples [57]. These first investigations were followed by active research on the enzymes involved in cAMP synthesis. See Table 2 for a summarized detail of molecules regulating cAMP levels in sperm and corresponding references. Even though cAMP was known to be important for sperm physiology, conflicting reports were published regarding the presence and activity of tmACs in spermatozoa. These enzymes are both positively and negatively regulated by heterotrimeric G proteins, composed of a Gα-subunit and a $G_{\beta}\gamma$-dimer. The Gα-subunit can either stimulate (αs and α_{olf}), or inhibit (αi_1, αi_2 and αi_3) tmACs [58]. Activation of the α subunit promotes the exchange of bound GDP for GTP. Then, GTP binding alters the topology within the Gα-subunit, releasing both an active α-subunit and $_{\beta}\gamma$-dimer from the formerly inactive trimeric G protein. Separately, both the α-subunit and $_{\beta}\gamma$-dimer act on different downstream effectors, such as phosphodiesterases, phospholipases, ion channels and tmACs. Both stimulatory and inhibitory α subunits can be post-translationally modified by bacterial toxins. While all $G\alpha_i$ subunits are ADP ribosylated by pertussis toxin, both $G\alpha_s$ and $G\alpha_{olf}$ can be modified by cholera toxin [59–61]. In both cases, toxin modifications inhibit the respective G protein function and can be followed *in vitro* using the substrate NAD(^{32}P). The differential ADP ribosylation properties of G protein α-subunits have been used over the years to modulate Gαs and Gαi activities and also as a molecular signature for their identification in many cell types [62] including sperm [63,64].

Regarding the presence of G proteins in sperm, few direct experiments have been published. A first report by Hildebrandt et al. failed to detect either Gαs or Gαi by ADP ribosylation studies in total dog sperm extracts using cholera or pertussis toxins respectively [65]. Accordingly, Western blots using antibodies raised against $G\alpha_s$ in bovine and human sperm also failed to find positive evidence [66,67]. However, opposite results can be found in the literature; Kopf et al. [68], using a modification of the ADP-ribosylation methodology, showed that Lubrol PX-extracted 48,000 ×*g* supernatant fractions of sperm from sea urchin, abalone, mouse and human contained substrates for ADP-ribosylation by pertussis toxin. Moreover, the pertussis toxin substrates were of same molecular weight as that reported for $G\alpha_i$ (41 kDa) and could be immunoprecipitated with anti $G\alpha_i$ antibodies [68]. It was argued that the discrepancy with Hildebrandt et al. [65] was as a result of a detergent extraction previous to the ADP-ribosylation, indicating that in the native form, G proteins might be masked.

Table 2
Molecules involved in cAMP regulation.

Molecule		Conclusion	Experimental approach	Refs.
G proteins	Gs	Present	WB	[70]
			CTX and cAMP levels measurements	[71]
			CTX ADP-ribosylation	[95]
			Proteomics	[72]
			ADP-ribosylation, IP, WB, IF, acrosome reaction and $[Ca^{2+}]_i$ levels measurements	[41]
		Absent	CTX ADP-ribosylation	[65]
			WB	[66,67]
	Gi	Present	Lubrol PX extraction and PTX ADP ribosylation, WB, IP	[68,69]
		Absent	PTX ADP-ribosylation	[65]
	Gb	Present	WB	[68,69]
	Gq	Present	Immunodetection	[73,74]
	Gα13 (akaGNA13)	Present	WB	[75]
Soluble adenylyl cyclase		Present	Purification from testis, microsequencing and mass spectrometry and cloning	[6,13,86]
			IP, cyclase activity	[10]
			Loss of function sAC KO and specific inhibitors, gain of function experiments with cAMP permeable analogs	[52,53]
Transmembrane adenylyl cyclases		Present	Enzymatic and immunological assays	[95]
			Mass spectrometry	[72]
			Loss of function AC3 KO	[96]
			FSK stimulation and cAMP levels determination	[41,71,95,96]
			FSK stimulation and AC activity determination, use of GTP analogs	[99,109]
		Absent	FSK stimulation and cAMP levels determination	[11,52,89,104,105,108]
Phosphodiesterases	PDEs 1, 4, 6, 8, 10 and 11	Present	Immunodetection, PDE inhibitors, CTC assay, acrosome reaction measurements	[111–113]
MRP		Present	WB, immunofluorescence, loss of function by MRP inhibitors and extracellular cAMP levels determination	[45]

Abbreviations: WB: Western blot; CTX: cholera toxin; IP: immunoprecipitation; IF: immunofluorescence; IP: immunoprecipitation; FSK: forskolin; CTC: clortetracycline; PTX: Pertussin toxin.

Soon after, Kopf's results were corroborated in other vertebrate and invertebrate species [69]. On the other hand, none of the above presented groups were able to detect $G\alpha_s$ in sperm. It was many years later that Fraser and Adeoya-Osiguwa [70] reported the presence of $G\alpha_s$ by Western blot analysis. A later report [71] showed that the addition of cholera toxin to cell lysates prepared from permeabilized sperm increased cAMP production by 10% suggesting that ADP-ribosylation of $G\alpha_s$ by cholera toxin lead to enhanced stimulation of tmAC activity. The same group detected positive ADP-ribosylation of $G\alpha_s$ by cholera toxin in permeabilized human and mouse sperm, also consistent with the presence of $G\alpha_s$ [71]. Supporting this conclusion, it was recently shown that a cholera toxin ADP-ribosylation substrate protein can be immunoprecipitated from mouse sperm extracts, showing the expected molecular weight for $G\alpha_s$ [41]. Immunofluorescence experiments localized $G\alpha_s$ only to the anterior head of mouse sperm. Moreover, $G\alpha_s$ immunoreactivity was lost in acrosome-reacted sperm suggesting that $G\alpha_s$ resides in the acrosomal region [41]. Unfortunately, in the light of high sequence similarities, the above mentioned techniques did not allow us to discriminate between $G\alpha_s$ and $G\alpha_{olf}$-subunits mainly because: 1) both of them can be ADP-ribosylated in the presence of cholera toxin; 2) they are likely to be recognized by the same antibodies; and 3) once activated, both can stimulate tmACs. Noteworthy, positive data regarding the presence of $G\alpha_s$ in mouse sperm also comes from mass spectrometry analyses of sperm protein extracts [72].

Besides $G\alpha_s$, studies were also conducted to address the presence of G_q, which acts by stimulating phospholipase C_β. Immunodetection analysis showed the localization of G_q to the anterior acrosomal region of mouse [73] and equatorial segment of human sperm [74] suggesting that it might have a role in acrosomal exocytosis. Recently, another Gα member, Gα13 (akaGNA13) has been identified in bovine sperm by Western blot [75]. Their roles in sperm physiology are yet to be determined.

4. cAMP synthesis: soluble and transmembrane adenylyl cyclases

4.1. sAC

Multiple studies have shown that sperm capacitation is a HCO_3^- and Ca^{2+}-dependent process [29,76–80]. The first connection between HCO_3^-, Ca^{2+} and cAMP metabolism was demonstrated by the Garbers group [81]. In this manuscript it was shown that in the presence of HCO_3^-, extracellular Ca^{2+} increases intracellular cAMP within 1 min in guinea pig spermatozoa. When either HCO_3^- or Ca^{2+} was removed from the medium, only a slightly increase of cAMP was observed. Later on, Okamura and collaborators [82] reported that a low molecular weight factor present in boar semen was able to induce pig sperm motility. The low molecular weight molecule was later identified as HCO_3^- and was shown to directly activate adenylyl cyclase activity and consequently increase cAMP levels in sperm [83]. Similar results were later reported in bovine and hamster spermatozoa [84,85]. Independent of these reports, other groups have described the presence of a soluble adenylyl cyclase in testicular extracts [13,86]. This enzyme activity presented certain properties similar to the particulate sperm enzyme. Analogous to the sperm adenylyl cyclase, the soluble testicular enzyme did not appear to be responsive to G proteins and was more active when assayed in the presence of Mn^{2+} than in the presence of Mg^{2+} [86]. Using a biochemical approach, the group of Drs. Lonny Levin and Jochen Buck [6] purified a protein responsible for the soluble cyclase activity from 950 rat testes and microsequenced it by mass spectrometry. The derived peptide sequence was used to build degenerate primers to clone the enzyme. In a follow-up manuscript, they demonstrated that the testicular sAC is present in sperm and that it is responsive to HCO_3^- [10]. The elucidated sequence revealed conservation throughout evolution. Most interestingly, the sAC catalytic domain presents homology to cyanobacterial adenylyl cyclase which is also HCO_3^--dependent [10,32]. Bicarbonate stimulates the enzyme's V_{max} [12] by fostering a conformational rearrangement of the active site, allowing Mg^{2+}–ATP

to properly interact with it [87]. The sensitivity to HCO_3^-, turns sAC into a CO_2 sensor widely distributed in nature. Interestingly, sAC can also function as a pH sensor, due to the effect of pH on the CO_2/HCO_3^- equilibrium [88]. Thus, alkalinization of the intracellular milieu during capacitation might also be expected to impact on sAC activity, through modulation of HCO_3^- levels. As mentioned, cAMP levels peak at 1 min after sperm exposure to HCO_3^- [43,89] and then return to basal levels over the incubation period. A self-regulatory loop has been proposed in which PKA exerts a negative feedback on sAC activity [90–92]. A self-regulatory loop has been proposed in which PKA phosphorylates sAC or an intermediary protein, exerting a negative feedback on its activity. In addition to responding to HCO_3^-, sAC is also a Ca^{2+} sensor. In the presence of Ca^{2+}, the Km for Mg^{2+}–ATP is reduced to levels that are close to the ATP concentration found in cells, turning sAC into a signal transducer highly sensitive to physiologically changes of HCO_3^- [93]. Finally, it is important to consider that although sAC was originally found in the testicular soluble fraction, the HCO_3^- dependent adenylyl cyclase activity in mature sperm is associated with the plasma membrane [85]. More research is needed to understand the nature of this association and how sAC is transferred from the soluble to the particulate fraction during spermiogenesis or epididymal maturation. Alternatively, sAC could be represented by two populations: a soluble and a particulate one.

Although the aforementioned works strongly suggested a role of sAC in sperm motility, loss of function experiments were needed to conclusively demonstrate its role in sperm capacitation. To obliterate sAC function genetic and pharmacological approaches were used. First, elimination of the sAC gene by homologous recombination resulted in a sterile phenotype [52,53]. Sperm from the sAC knock-out were immotile and were unable to undergo the cAMP/PKA-dependent increase in tyrosine phosphorylation associated with the capacitation process [52]. In addition, basal levels of cAMP in $sAC^{-/-}$ sperm were under detection limits when these cells were incubated in capacitation-supporting media containing HCO_3^- [41]. Second, sAC was used as bait in high throughput assays designed to find small molecules which specifically inhibit sAC. The first compound found, KH7, was a competitive inhibitor with Mg^{2+}–ATP and blocked recombinant sAC activity with an IC_{50} of 10 μM [94]. KH7 was then used in sperm and was shown to block cAMP production, the increase in tyrosine phosphorylation and sperm motility [52]. Altogether, these results are consistent with the hypothesis that sAC mediates the HCO_3^--induced increase in cAMP necessary to initiate capacitation. Moreover, in the absence of sAC, sperm have reduced ATP levels, do not undergo hyperactivation and failed to fertilize *in vitro* [52,54].

4.2. tmACs

The presence of tmAC isoforms in sperm has been investigated using enzymatic assays [95], immunological approaches [95], mass spectrometry [72] and genetic knock-outs [96]. Despite these efforts, contrary to sAC, the function as well as the presence of tmACs in mammalian sperm remains controversial. Although some studies presented evidence of their presence in mammalian gametes [41,96–100], others reported negative results [65,89,101–105] (see Brenker et al. [89] for a thorough discussion of these arguments). In many of these studies, the authors used the diterpene, forskolin, which is known to increase cAMP levels in most cell types as a surrogate to identify the presence of tmAC activity. Initially described by Seamon and Daly [106,107], forskolin activates all tmACs except Adcy9. Most importantly, forskolin does not stimulate sAC activity. In mammalian sperm incubated with forskolin, several groups were unable to observe an increase in cAMP [11,52,89,104,105,108]. However, other groups reported a forskolin-dependent elevation of intracellular cAMP concentrations [41,71,95,96]. These last results were in agreement with data from Leclerc and Kopf [99,109] which showed a forskolin effect in adenylyl cyclase activity assayed in membrane fractions of capacitated mouse sperm. Interestingly, this work also showed an effect of both GTP analogs and mastoparan, a wasp venom toxin known to activate G proteins. Considering the abundance of sAC, one possibility is that the effect of forskolin in mammalian sperm is masked by higher levels of endogenous forskolin-independent cAMP synthesis. Considering this possibility, our group recently showed that forskolin increased cAMP levels in sperm from sAC knock-out mice [41].

Despite these data, little is known regarding the function of specific tmACs in sperm. Considering that tmACs are regulated by G proteins, the finding of $G\alpha_s$ only in the sperm acrosome [41] suggests that tmACs have a role in the acrosome reaction. Consistently, incubation with forskolin stimulated the acrosome reaction in capacitated mouse sperm and, in addition, induced an increase in intracellular Ca^{2+} concentration [41]. Because tmACs may have overlapping functions in many cell types, it is difficult to pinpoint the specific isoform involved in a given process. Due to the low abundance of tmACs in sperm and the lack of good specific antibodies, results on specific isoforms in sperm have been inconclusive [95]. Regarding loss of function experiments, in addition to sAC knock-out mice [52], only *Adcy3*-null mice are infertile [96]. Knock-out mice for *Adcy1*, *5*, *6* and *8* are fertile; *Adcy7* knock-out shows complete postnatal lethality [110] and null mice for *Adcy2* and *Adcy9* have not yet been produced. In the case of *Adcy3* null mice, the ability of their sperm to penetrate cumulus-enclosed oocytes *in vitro* is compromised [96].

5. cAMP degradation: phosphodiesterases in sperm physiology

Levels of intracellular cAMP are highly dynamic; its availability depends on the concerted action of both synthesis and degradation. In sperm, the addition of permeable cAMP analogs triggers capacitation-associated events [111]. However, in many cases, depending on the species, no matter how high the concentration of permeable cAMP analogs used, unless phosphodiesterases (PDE) inhibitors are added, no effect is observed. In mammals, 20 genes grouped into 11 PDE families have been described. Each PDE family shows different substrate specificities and pharmacological sensitivities. PDE4, PDE7, and PDE8 family members are more efficient in hydrolyzing cAMP, whereas PDE5, PDE6, and PDE9 are cGMP-specific. On the other hand, PDE1, PDE2, PDE3, PDE10, and PDE11 are active towards both cAMP and cGMP [112]. In sperm, PDE1 was first reported by Wasco and Orr [113] and shown to be stimulated by calmodulin and to associate with the head and tailpieces of demembranated rat caudal epididymal sperm. Using immunological techniques, six additional PDE types have been shown to be present in mammalian sperm (1,4,6,8,10,11) [114]. Inhibition of either PDE1 (by MMPX) or PDE4 (by RS-25344, Rolipram) augmented intracellular levels of cAMP [114]. In human sperm, inhibition of PDE4 by RS-25344 and Rolipram enhanced sperm motility without affecting the acrosome reaction [115]. On the other hand, PDE1 inhibitors selectively stimulated the acrosome reaction [115]. Although more research is needed, these results are consistent with the hypothesis that, in sperm, the molecules involved in cAMP signaling which include adenylyl cyclases, phosphodiesterases and cAMP targets are readily compartmentalized and regulate different sperm processes, some in the head (*e.g.* acrosome reaction); and some in the tail (*e.g.* regulation of motility). This compartmentalization would be possible through local cAMP generation within independently regulated microdomains. It can be hypothesized that a variety of cAMP modules are modulated by AKAPs (see below), which tether not only PKA but many enzymes and substrates to specific cell locations [116]. Among these tethered enzymes, PDEs can function to prevent cAMP diffusion, reinforcing barriers between microdomains. In the light of the restricted cAMP microdomain hypotheses, conclusions from experiments in which sperm are flooded with permeable cAMP agonists should be carefully analyzed.

Table 3
cAMP targets.

Molecule	Assay	Experimental approach	Refs.
PKA	Presence in sperm flagella	WB, IF, PKA activity and cAMP measurements	[41]
	Activation during capacitation	WB	[44,47–51]
	Dependence on sAC	Loss of function sAC KO	[52]
	Role in tyrosine phosphorylation	Loss and gain of function experiments with PKA Inhibitors and cAMP permeable analogs	[35]
	Role in capacitation and sperm motility	Loss of function by pharmacological and genetic knock-out approaches targeting sAC, the PKA catalytic subunit or the anchoring of PKA to AKAPs	[35,49,91,119]
CNGs	Presence in sperm	Cloning, electrophisiological characterization, Ca^{2+} imaging and patch clamp recording	[144,145]
	Role in chemotaxis	Sperm motility recording, fluorescent dyes, Caged compounds and flash photolysis techniques	[142,143]
	Increase $[Ca^{2+}]_i$ in sperm	Ca^{2+} imaging	[146,147]
EPAC	Presence in sperm and involvement in acrosome reaction	Gain of function by EPAC-selective cAMP analogs, acrosome reaction measurements, WB, Immunofluorescence	[27,56,151,152]
NHE	Presence in sperm and role in internal alkalinization and hyperpolarization	Loss of function by NHE-targeted deletion	[31]
		Experiments with pH_i-sensitive fluorescence probes	[21]
	Role in sAC expression during spermiogenesis	Loss of function NHE KO	[32]

Abbreviations: WB: Western blot; IF: immunofluorescence.

6. cAMP-dependent protein kinase (PKA)

The best characterized cAMP target is the holoenzyme PKA, which is essential in both sperm biology and egg biology [90]. Please see Table 3 for a complete list of known cAMP targets in sperm. PKA is a heterotetramer composed of two catalytic subunits (C) containing the enzyme's active site, and two regulatory subunits (R), which block PKA activity when bound to the C subunit. The active catalytic subunit is dissociated as an active kinase upon cAMP binding to R subunits. In mammals, there are four R (*RIα*, *RIβ*, *RIIα* and *RIIβ*) and five C genes (*PRKACA*, *PRKACB*, *PRKACG*, *PRKX*, and *PRKY*). Expression of C genes gives rise to isoforms Cα, Cβ, C , PRKX and PRKY [117]. However, the functions of PRKX, PRKY and C are not known. Interestingly, C is an intronless gene exclusively expressed in the testis [117,118]. Cα and Cβ can combine with the different R subunits to form a variety of PKA holoenzymes. Added to this complexity is the finding that both Cα and Cβ can be found as alternative spliced variants. While Cβ has at least ten different variants, Cα has only two (Cα1 and Cα2).

Because mature sperm are transcriptionally and translationally inactive, gene expression studies are usually conducted in testicular germ cells [90]. Throughout spermatogenesis, only RIα, RIIα, Cα1, Cα2 and C mRNAs are found. Interestingly, while RIα is expressed in all germ cell stages, RIIα expression patterns are restricted to later stages suggesting a role in sperm function. Regarding the C subunits, Cα1 is expressed in spermatogonias which correspond to the mitotic stages of spermatogenesis; however, the Cα1 expression declines in meiotic spermatocytes and is completely replaced by splice variant Cα2 in pachytene spermatocytes and in spermatids. Cα2 contains 7 unique amino acids in the N-terminal domain and lack the myristylation site present in Cα1. It has been proposed that these changes may confer special properties to the PKA holoenzyme in the sperm. Finally, although C mRNA is found in testicular germ cells, protein expression of this mRNA has not been demonstrated yet.

As mentioned above, as soon as the sperm comes in contact with HCO_3^- in the seminal plasma there is a rapid increase in cAMP synthesis mediated by sAC. Design of antibodies against PKA substrate consensus phosphorylation sites has permitted the kinetics of PKA activation in sperm to be monitored. Using these antibodies, it was shown in sperm from different species that PKA activity reaches maximum activity in less than 1 min after exposure to HCO_3^- [43,44,48]. The role of HCO_3^- in this activation strongly suggests that sAC is the adenylyl cyclase involved in this effect. This conclusion is also supported by the lack of effect of forskolin on PKA activation and by the finding that, while $G\alpha_s$ localized to the sperm anterior head, the PKA catalytic subunit is only found in the flagellum [41]. The role of cAMP in the regulation of sperm capacitation has been supported using a battery of inhibitors such as H89, rpScAMP and a permeable stearated version of the peptide protein kinase inhibitor PKI [35,119]. In particular, it is well established that PKA activation is upstream the regulation of other signaling events involved in capacitation (see below). However, although PKA activation is a fast event, it should be noted that some of these PKA-dependent signaling pathways known to be downstream of PKA activation such as the increase in protein tyrosine phosphorylation and the induction of hyperactivated motility are slow processes [120]. The delayed effects of cAMP-dependent pathways suggest that PKA action on this pathway is not direct.

The pharmacological approaches have been later validated using genetic knock-out models. Although elimination of the whole Cα gene by homologous recombination resulted in infertile sperm [121], these mice have many other defects that preclude an accurate assessment of the reproductive phenotype. Considering that Cα2 is the only C subunit present in mature mammalian sperm [91,122], it was possible to obliterate PKA activity specifically from sperm by eliminating the unique N-terminus Cα2 exon by homologous recombination. These mice, containing a functional somatic Cα1, have only a reproductive phenotype. They are sterile and the infertile phenotype is related to defects in sperm motility and capacitation [91,121]. On the other hand, target disruption of the type IIα regulatory subunit (*RIIα*) resulted in a compensatory increase of RIα protein and a dramatic relocalization of PKA such that the majority of the holoenzyme now appears in the soluble fraction and colocalizes to the cytoplasmic droplet. Unexpectedly the *RIIα* null mice are fertile and *RIIα* has no significant effect on sperm motility [123]. Further confirmation of the role of Cα2 in sperm motility was achieved by a pharmacological and genetics combined approach. First described by Bishop et al. [124], this approach consists of a point mutation of the ATP binding domain of a given protein kinase, changing a specific amino acid of the ATP binding site to a smaller one such as alanine or glycine. This mutation makes the respective protein kinase sensitive to bulky ATP competitive inhibitors which otherwise cannot block the wild type kinase activity. Morgan et al. [49] used a knock-in approach to swap the endogenous Cα gene for one in which the "gate-keeper" methionine had been mutated to alanine (called *CαM120A*). Sperm from the mutant Cα but not those from wild type mice were sensitive to the bulky inhibitor NMPP1 [49]. As expected, NMPP1 blocked PKA activation in the mutant sperm; in addition, this compound inhibited the HCO_3^--dependent increase in flagellar beat frequency and the downstream increase in tyrosine phosphorylation [49].

Similar to other protein kinases and phosphoprotein phosphatases, PKA has multiple substrates. This property highlights an important problem in signal transduction: How is the phosphorylated state of a given substrate regulated without affecting the phosphorylation state of other possible substrates? One answer to this question was given by the discovery of proteins that can anchor PKA. These proteins are known as AKAPs (for A Kinase Anchoring Proteins) (for a review see [116] and references therein). AKAPs are a family of proteins that anchor the regulatory subunit of PKA, confining its activity to discrete locations within the cell, which is crucial for successful localized signaling activity of PKA and other enzymes [116,125]. Although AKAPs can bind a series of enzymes allowing scaffolding of many signaling pathways, their discovery was facilitated by the AKAP property of binding RII PKA subunits *in vitro*. Using RII binding assays, two sperm-specific AKAPs were described, AKAP3 [126–128] and AKAP4 [129]. These proteins are distributed throughout the sperm flagellum, and have been found to become phosphorylated during capacitation [129–132]. The role of these phosphorylations has not yet been elucidated but it has been proposed that these post translational modifications may regulate the activity or localization of different signaling complexes needed for sperm capacitation.

AKAPs role in sperm was analyzed using modified permeable peptides such as St-Ht31. This inhibitor peptide is a stearated form of the peptide Ht-31 derived from the human thyroid AKAP and inhibits the interaction between the RII subunit of PKA and AKAP in cell extracts. This peptide has been first used to study PKA signaling in sperm [119,133], and later expanded to other cell types. The stearated peptide HT31 impairs sperm motility and the onset of signaling pathways associated with sperm capacitation [47,119,134]. Interestingly, this blocking peptide produced a stronger inhibition of motility that is obtained solely with the use of PKA inhibitors such as H89. The blocking peptide St-Ht31 was designed to mimic the RII sequence that binds to AKAPs. Homologous RII sequences can be found in other proteins and this AKAP-interacting domain has been named R2D2 [135]. Therefore, the stronger phenotype from St-Ht31 has been attributed to the combined disruption of other R2D2-containing proteins present in sperm cells [126,136]. The role of AKAPs in sperm physiology has been also validated using knockout models. Sperm lacking AKAP4 are infertile and present morphological flagellar defects and reduced progressive motility due to the role of this protein in tail assembly [55]. Sperm numbers were not reduced in male mice lacking AKAP4 but proteins usually associated with the fibrous sheath were absent or substantially reduced in amount. Other cytoskeletal components of the tail appeared fully developed.

7. Other targets of cAMP in spermatozoa

In addition to PKA, cAMP is able to bind and regulate other proteins. These proteins are characterized by a cyclic nucleotide binding domain (CNBD) characterized by depicting a conserved structure which binds cyclic nucleotides and differs in their specificity towards cAMP and cGMP. CNBD-containing proteins are involved in signaling. Some of the best characterized belong to the families of phosphodiesterases, ion channels and G protein exchange factors. CNBD-containing proteins have been identified in mammalian sperm and shown to play a role in the regulation of different aspects of sperm function.

Cyclic nucleotide-gated channels (CNGc) are ion channels whose activity responds to the binding of cyclic nucleotides. These channels result from the combination of two α subunits (CNGα1 to 4) and two β subunits (CNGβ1 to 3), which are differentially expressed in different tissues [137]. These channels have been long known for participating in sensory transduction processes, but their function in other tissues is not well established yet. From a structural point of view, each subunit possesses six transmembrane segments and a single cyclic nucleotide-binding site near the C-terminal region of the protein [138,139]. Binding of cAMP or cGMP to nucleotide binding domain promotes the opening of the CNG channels. Depending on subunit combinations, CNGc displays differential selectivity towards cAMP or cGMP [140] and often carries inward Na^+ and Ca^{2+} currents [141].

The role of these channels in mammalian sperm capacitation is not clear. However, their role in sea urchin sperm chemotaxis is well known [142,143]. Interestingly, the first sperm ion channel cloned from mouse testis was a CNG channel [144]. After this discovery, others have been cloned [145]. The α3 and β1 subunits were localized to the flagellum of mouse spermatozoa [145]. Addition of permeable cGMP analogs to murine sperm results in an increase of intracellular Ca^{2+} [146]. The same effect was observed with permeable cAMP analogs, but with less effectiveness than with cGMP analogs [147]. Even though CNGc were originally proposed to carry Ca^{2+} inward currents upon direct stimulation with cAMP/cGMP [146], it was later shown that sperm lacking the sperm specific Ca^{2+} channel CatSper do not show this response, even though CNGc are still present [148]. Currently the role of CNG channels in sperm physiology is not clear because mice bearing targeted deletion of α3 showed normal fertility rates [149].

Another cAMP effector is the exchange protein directly activated by cAMP (Epac). Epac is a Rap-specific guanine-nucleotide exchange factor that is activated by the interaction of cAMP with the cyclic nucleotide monophosphate-binding domain [2,3]. Epac1 and Epac2, the two isoforms so far described in mammalian cells, possess a regulatory and a catalytic region in the N- and C-terminal segments respectively. The regulatory domain contains the cAMP binding site, which in the absence of cAMP, self inhibits its catalytic activity [150]. In sperm, EPAC has been shown to be present using immunological as well as pharmacological approaches.

Epac1 and Epac2 have been detected by immunoblotting in sperm from different species including human [27,56,151,152]. In addition, immunofluorescence experiments suggested that these proteins localize to the sperm head. In addition, the Epac G protein target Rap1A was also found to be present in sperm [56,151]. Functionally, the Epac-selective 8-(p-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-pCPT-2-O-Me-cAMP) analog is routinely used to stimulate Epac without inducing PKA activation. This cAMP analog induced the acrosome reaction in permeabilized human sperm in a Ca^{2+}-independent manner [27]. These data suggest that extracellular Ca^{2+} entry might be upstream of Epac activation. Moreover, using a streptolysin-permeabilized sperm model, it was shown that addition of Ca^{2+} failed to trigger acrosomal exocytosis when intracellular cAMP was depleted by phosphodiesterase activity or when Epac was sequestered by specific blocking antibodies [27]. Interestingly, the PKA catalytic subunit was not found in the sperm head [41] indicating that the effect of cAMP in the sperm head is PKA-independent and suggesting a role for alternative targets such as Epac in the regulation of the acrosome reaction. A third CNBD protein known to be present in mouse sperm is a specific member of the mammalian NHE superfamily of Na^+/H^+ exchangers, sNHE [31]. This exchanger localized to the principal piece of the sperm flagellum, and is predicted to contain 14 putative transmembrane segments, although its actual topology remains to be determined. One key feature of this exchanger is that close to its intracellular C-terminus, there is a nucleotide-binding domain indicating that modulation by cyclic nucleotides might also be functional [31]. Targeted deletion of *sNHE* gene results in infertility [31]. Homozygous-null males that mated with females, produced vaginal plugs with the same frequency as wild-type males, but did not result in pups. Interestingly, sperm from sNHE null mice lack motility but either permeable cAMP analogs or NH_4Cl rescues this phenotype. Later reports from the same group showed that sNHE is necessary for the full length expression of SACY suggesting that these two proteins may form a protein complex involved in sperm intracellular pH (pH_i) control [32]. Regulation of sperm pH_i is fundamental for motility, capacitation, and acrosome reaction in different mammalian species [30,33,153]. It has been

proposed that cAMP affects the sperm pH_i by controlling the sperm-specific Na^+/H^+ exchanger through its cAMP-binding domain [20].

Finally, a novel CNBD containing protein named CRIS (for cyclic nucleotide receptor involved in sperm function) has been recently identified [154]. CRIS is exclusively expressed in testicular germ cells while it is not present in mature sperm. Interestingly, mouse genetic models lacking CRIS are either infertile or subfertile. While infertility is related to spermatogenic arrest, some of the mice completed this process and contain sperm in their epididymis. These sperm, however, have defects in motility due to problems in Ca^{2+} transport and flagellar bending. At present, the role of cAMP in CRIS regulation has not been established.

8. cAMP-dependent signaling pathways

In the previous sections, we have reviewed the current knowledge on those enzymes that directly participate in cAMP metabolism in sperm. We have also summarized the role of these enzymes and of cAMP in the physiology of the sperm, in particular in sperm capacitation and the acrosome reaction. As mentioned in several sections, sAC is stimulated immediately after the sperm is exposed to HCO_3^- upon ejaculation. In addition, the relevance of sAC, intracellular cAMP concentrations and the consequent PKA activation in the regulation of the sperm fertilizing capacity is well established. However, less is known about those pathways downstream of cAMP and PKA. In the following sections we will address some of these signaling events.

8.1. Lipid remodeling

Similar to what occurs in somatic cells, sperm phospholipids are distributed asymmetrically within the plasma membrane [155] by the concerted activity of several phospholipid transferases: An aminophospholipid transferase that moves phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet [156], a non-specific transferase (floppase) that transfers phospholipids from the inner to the outer leaflet and a scramblase that moves all phospholipids in both directions. The increase of scramblase isoform 1 activity was observed during sperm capacitation associated with phospholipid scrambling [157]. This change in asymmetry is stimulated by HCO_3^- through sAC and the cAMP/PKA pathway, and promotes a fast exposure of phosphatidylethanolamine and phosphatidylserine not related to apoptosis [157]. Regarding the functional implications of this event, it has been proposed that phospholipid scrambling facilitates cholesterol efflux, an event required for capacitation [158,159].

8.2. Increase in tyrosine phosphorylation

Capacitation can be mimicked *in vitro* in a simple medium containing a combination of ions such as Na^+, Cl^-, Ca^{2+} and HCO_3^-, energy substrates such as glucose and a cholesterol acceptor (usually bovine serum albumin). Absence of any of these molecules prevents the sperm from acquiring fertilizing capacity. Omission of any of these molecules also blocks an increase in tyrosine phosphorylation [80,92,160,161]. The capacitation-associated increase in mouse sperm tyrosine phosphorylation is downstream of a cAMP/PKA-dependent pathway [35] and is also the case for capacitation in sperm from other mammalian species including human [130,162–164]. Because forskolin is unable to induce these pathways, it is clear that the only AC involved in the regulation of tyrosine phosphorylation is sAC [41]. Although the role of PKA is upstream this pathway, the mechanism by which PKA activates tyrosine phosphorylation is not known. Considering that PKA is a ser/thr protein kinase, it is clear that a tyrosine kinase should mediate the role of PKA. However, the identity of this tyrosine kinase has not been conclusively elucidated yet. One of the candidates proposed is cSrc [165]. However, sperm from cSrc null mice undergo tyrosine phosphorylation to similar levels than the ones observed in wild type sperm [48,166]. In addition, the cSrc family kinases (SFK) inhibitors SKI606 and SU6656 also blocked phosphorylation of PKA substrates. Interestingly, both PKA and tyrosine phosphorylation pathways were recovered in the presence of 0.1 nM of okadaic acid. This reversal of SFK inhibition by addition of okadaic acid strongly suggests that the role of SFK in capacitation is mediated by down-regulation of a ser/thr phosphatase. Consistently, it has been shown in other cell types that SFKs can inactivate PP2A by tyrosine phosphorylation of its C-terminus domain (TPDYFL) [167–169].

More recently, a second family of tyrosine kinases has been proposed to mediate the role of PKA in sperm tyrosine phosphorylation [170]. The inhibitor PF-431396 has high specificity for members of the focal adhesion kinases, FAK1 and FAK2 (aka PYK2). It was shown that this inhibitor blocks the increase in tyrosine phosphorylation in horse sperm [170]. Moreover, anti-phospho antibodies recognizing activated FAK1 and PYK2 forms detected proteins in capacitated stallion sperm. More research is needed to understand the mechanism of action of these kinases and whether their action is downstream or upstream cAMP-dependent pathways.

8.3. Crosstalk between cAMP and Ca^{2+} pathways

There is a complex interaction between the regulation of cAMP and Ca^{2+}-dependent pathways. In mouse sperm, the cAMP-dependent increase in tyrosine phosphorylation does not occur in Ca^{2+}-free media. However, in human sperm, the tyrosine phosphorylation is up-regulated under these conditions [171]. These different effects of Ca^{2+} in the regulation of cAMP-dependent pathways are not surprising. On one hand, Ca^{2+} is known to activate sAC and consequently positively modulate cAMP synthesis; on the other hand, Ca^{2+} also activates calmodulin-dependent enzymes including PDE1; therefore Ca^{2+} can also stimulate cAMP degradation. Adding to the Ca^{2+} modulation of cAMP synthesis and degradation, it has also been shown that addition of cGMP or cAMP induces a fast increase in intracellular Ca^{2+} [147,148,172–175]. Although this increase was initially believed to be mediated by cyclic nucleotide-dependent channels, it has been recently shown that cAMP and cGMP analogs can directly and promiscuously activate Catsper from outside the cell [89]. The same authors demonstrated that intracellular elevation of cAMP levels using either HCO_3^-, IBMX or caged cAMP compounds is unable to induce intracellular Ca^{2+} elevations [105]. Finally, it is well established using pharmacological and genetic knock-out approaches that Ca^{2+} and cAMP are required for the sperm to undergo hyperactivated motility necessary for spermatozoa to fertilize the egg. Therefore, a crosstalk between these pathways has been proposed. Consistent with a role of cAMP upstream of Ca^{2+} signaling, when intracellular Ca^{2+} is transiently increased by exposing sperm for 10 min to Ca^{2+} ionophore A23187, sperm acquired fertilizing capacity when incubated in either the absence of HCO_3^- or the presence of H89 [34]. These experiments indicate that Ca^{2+} can overcome the need for PKA activation during capacitation. Altogether, results from Brenker et al. [89] indicate that there is no direct role of cAMP in the regulation of intracellular Ca^{2+}. However, similar to the cAMP involvement in tyrosine phosphorylation, Ca^{2+} pathways required for the sperm to achieve fertilizing capacity could be indirectly regulated downstream of cAMP production in a slow time-scale (see above).

8.4. Autocrine and paracrine cAMP signaling

As described throughout this review, many events that occur during capacitation rely on an increase in intracellular cAMP. Interestingly, the expression of members of the multidrug resistance-associated protein (MRP) family has been reported in mammalian sperm [45]. MRPs, also members of the ATP binding cassette (ABC) transporter subfamily C, are known to actively transport cAMP and cGMP and other small

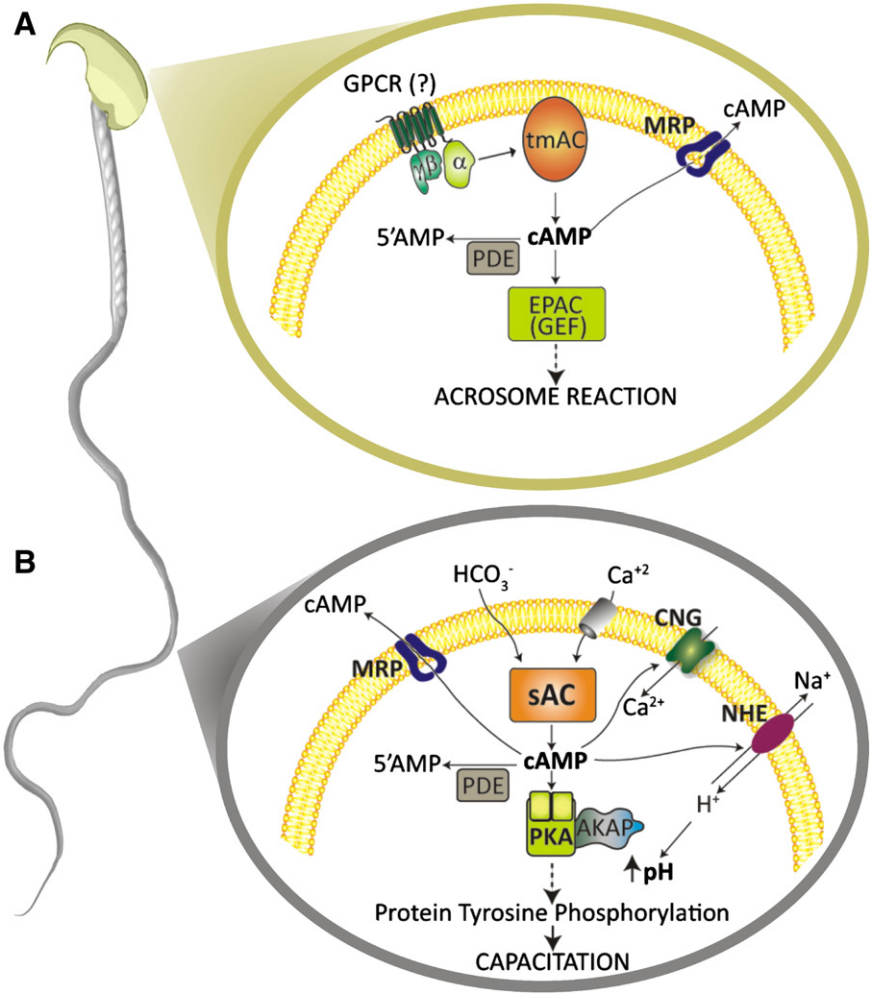

Fig. 1. Model of main pathways of sperm capacitation where cAMP is directly involved. Model of cAMP pathways acting during mammalian sperm capacitation. Two different sources of cAMP orchestrate signaling pathways in the sperm head (A) and in the principal piece (B). In the head, a tmAC increases cAMP levels which act primarily on EPAC, leading to the preparation to undergo the acrosome reaction. In the sperm flagellum, cAMP is increased through the synergic effect of Ca^{2+} and HCO_3^- stimulation of sAC. The increased pool of cAMP acts on PKA, as well as on CNG channels and NHE channels. These molecules impact directly on the tyrosine phosphorylation pathway that correlates to sperm capacitation. See text for details.

non-structurally-related compounds to the extracellular environment. One member of this family, MRP4 is localized to the sperm flagellum and to the post acrosomal region of bovine sperm [45]. In addition, this work showed that cAMP is extruded to the extracellular milieu and that MRP inhibition by probenecid abolished this efflux resulting in the accumulation of intracellular cAMP. Interestingly, these authors presented evidence indicating that extracellular cAMP mediates some of the events associated with sperm capacitation through the activation of adenosine receptors. The action of extracellular cAMP as a modulator of sperm capacitation may suggest a possible paracrine or autocrine modulation of the capacitation process. Further experimentation is necessary to understand this mechanism and its relevance in the physiological context of *in vivo* capacitation.

9. Conclusions

Initiated by the discovery of sperm capacitation more than 50 years ago, investigations in sperm physiology have been fundamental for the advances in reproductive medicine. Despite these advances, many translational challenges remain in the clinic, in male contraception and in the development of reliable *in vitro* fertilization protocols for many mammalian species. The central role of sAC in the fast up-regulation of cAMP and consequently in sperm capacitation is well established. Fig. 1 illustrates the main pathways of sperm capacitation where cAMP is directly involved. As mentioned throughout the review, the essential nature of cAMP in this process has been demonstrated using gain of function as well as loss of function approaches. However, many questions remain unanswered, among them: which is the molecular basis for sAC inactivation by PKA? What is the reason for the significant temporal lag between PKA activation and the onset of other downstream pathways? What are the identities of PKA substrates? How are the cAMP microdomains established and compartmentalized in the sperm? New research using novel technologies and molecular strategies will help answer these questions and understand the molecular mechanisms involved in sperm capacitation.

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