PKA, Germ Cells, and Fertility

Temporal and spatial regulation of PKA activity are essential for vigorous sperm motility and for the resumption of meiosis in oocytes, two events required for successful fertilization. Genetic mutations in mice that affect PKA signaling in germ cells lead to infertility and illustrate the importance of this pathway in mammalian reproduction.

The search for effective alternatives to estrogen- and progesterone-based oral contraceptives has led to increased scrutiny of the final stages of germ cell maturation and the signaling events that are required for fertilization. The maturation of germ cells is tightly regulated, and the second messenger, cAMP, plays a prominent role in mediating this process. Although cAMP can open cyclic-nucleotide gated ion channels (CNGs) and activate the guanine nucleotide exchange factors, Epac1 and Epac2 (14), its major downstream effector in germ cells appears to be PKA. The PKA holoenzyme, compromising two catalytic (C) and two regulatory (R) subunits, is activated when cAMP levels rise following stimulation of Gs protein-coupled receptors and adenylyl cyclase. The phosphorylation of specific substrates by the C subunit of PKA is regulated in part by the subcellular localization of the PKA holoenzyme through the binding of its dimerized R subunits to the scaffolding A kinase-anchoring proteins (AKAPs). AKAPs also interact with other signaling proteins, including other kinases, phosphodiesterases, and protein phosphatases, to form a signaling complex that imparts spatial localization and controls the timing and substrate specificity of protein phosphorylation (45). Previous reviews have considered the role of PKA in germ cell function (4, 12). This review will focus on recent studies of mice with genetic mutations in proteins that are integrally involved in PKA signaling and thus have essential roles in germ cell function and fertility. These studies have supported the conclusion that, in the male germ cell, PKA activity is not essential during the differentiation and development of sperm and that inappropriate PKA activation during these stages can lead to sperm pathology and infertility. However, PKA does play a critical role in sperm capacitation and motility that are required for fertilization. In the female, our recent work demonstrates that the sequestration of PKA to specific AKAPs in the oocyte is a dynamic process that is essential to allow efficient meiotic maturation.

PKA is not Essential for Spermatogenesis and Spermiogenesis

Male germ cell development is supported by PKA activation stimulated by FSH and LH in Sertoli cells and in Leydig cells, respectively. A pathway for the generation of cAMP in immature male germ cells, however, remains unclear. PKA subunits (RIα, RIβ, Ca1, and Ca2) are expressed differentially in spermatogonia, spermatocytes, and spermatids, suggesting that PKA signaling might play a role in mitosis, meiosis, and/or spermiogenesis (FIGURE 1). RIα is expressed throughout male germ cell development, whereas RIα only appears at the late stages in spermatogenesis with protein appearing at the elongating spermatid stage (23). RIα is the predominant functional regulatory subunit in mature sperm as they leave the testis. The major C subunit expressed early in male germ cell development is Ca1. However, later in development, male germ cells begin to express a unique Ca2 (or C7) isoform of the prkaca gene that is distinct from the somatic form, Ca1 (1, 13). In male germ cells, an alternative promoter present in the first intron of the Ca gene becomes transcriptionally active, switching the expression from the Ca1 isoform to the Ca2 isoform during the pachytene stage of spermatogenesis (36). The Ca2 protein contains a distinctive 7 amino acid amino-terminus rather than the 14 amino acid amino-terminus of Ca1 and also lacks the post-translational myristylation found on Ca1. These modifications of the C subunit may alter both the assembly of R subunit-specific holoenzyme and its subcellular distribution. This unique Ca2 is the sole catalytic PKA in mature spermatozoa. We generated Ca-deficient mice to test whether PKA is required in germ cells during development and subsequent maturation events.

Mice with a mutation that disrupts both Ca1 and Ca2 isoforms of the Ca gene have no measurable PKA activity in sperm but only have a slightly reduced sperm count and a fourfold increase in the percentage of sperm with abnormal morphology (38). However, the growth of these mice is retarded, and most (~75%) do not survive the early postnatal period. Therefore, it was surprising to find near normal production of sperm in those males that survived to adulthood. The absence of a dramatic developmental defect in sperm production and differentiation indicates that the presence of PKA activity in developing spermatogonia and spermatids is not essential.

A more specific test of the role of PKA in sperm development from mid-pachytene stage to mature sperm was conducted using the more specific Ca2 knockout mice (32). These mice exhibited completely normal sperm development but had no measurable...
PKA activity in mature sperm. Because Ca2 is only expressed in sperm, the mice were normal in all other respects in contrast to the marked developmental deficits of the complete Ca knockout, supporting our conclusion that PKA is not playing an essential role in sperm development.

**Sperm Lacking PKA Cannot Undergo Capacitation**

Mature sperm from the knockouts lacking all isoforms of Ca had reduced motility, which could not be activated in vitro, but since these animals were runted and affected in many tissues we could not conclude that the lack of motility was a sperm-specific phenotype. The Ca2 knockout gave us an opportunity to investigate this question in an otherwise healthy animal and proved that PKA activity in mature sperm is absolutely required for the series of sperm maturation events that led to the ability to fertilize the egg. These events, which are thought to occur after sperm are deposited into the female reproductive tract, were first noted by Austin (2) and Chang (10) in 1951 and are referred to as capacitation. Sperm acquire optimal fertilizing ability following transit through the epididymis when remodeling of the sperm plasmalemma occurs (46) and capacitation. Sperm motility changes during capacitation from the vigorous motility that occurs just after ejaculation to hyperactivity, a motility pattern that occurs later and is characterized by asymmetric bending or whiplash-like movement of the flagellum (43). Signaling events necessary for capacitation include cholesterol efflux from the sperm plasmalemma, scrambling of phospholipids in the sperm membrane, increases in intracellular calcium, and enhanced protein tyrosine phosphorylation. Bicarbonate, an anion found in the female reproductive tract, is transported into sperm during capacitation and increases production of cAMP by activation of an atypical soluble adenyl cyclase (sAC). Recent work finds that sAC is essential for vigorous sperm motility and fertility (11, 16, 20, 37). Treatment with bicarbonate in vitro rapidly increases flagellar beat frequency and evoked calcium entry (44) and more slowly results in a CAMP-dependent increase in the tyrosine phosphorylation of sperm proteins, including AKAP3, AKAP4, VCP, CABYR, and other substrates, most likely through the downstream activation of tyrosine kinase activity or inhibition of tyrosine phosphatase activity (for review, see Ref. 29). Recent work identifies SRC as a candidate tyrosine kinase stimulated by PKA during capacitation (3).

The Ca2 null males were completely infertile, despite normal mounting behavior and production of copulatory plugs (32). Testis weight and histology and epididymal sperm count also were indistinguishable from normal. Sperm motility parameters, however, were significantly altered. As measured by computer-assisted semen analysis (CASA), the proportion of motile sperm was reduced, particularly within the rapidly moving subpopulation. Stop-motion imaging and waveform analysis revealed rigidity in the proximal 30 μm of the flagellum of Ca2 null sperm with an averaged beat amplitude that was reduced by 60%. These findings demonstrate that Ca2 is required for vigorous sperm motility and is consistent with the hypothesis that PKA-mediated phosphorylation of flagellar proteins enhances ATP-dependent motor activity.

Two downstream actions of bicarbonate were significantly absent in Ca2 null sperm. Although 60 s of bicarbonate treatment increased the beat frequency of wild-type (WT) sperm threefold, Ca2 null sperm were unaffected. Similarly, the rate of depolarization-evoked entry of calcium was raised two- to threefold in WT sperm treated with bicarbonate but was unchanged in Ca2 null sperm. One interpretation is that phosphorylation-dependent facilitation of voltage-dependent Ca2+ channels in sperm plasma membranes by PKA is absent in Ca2 null sperm. Proteins of the CatSper family are expressed exclusively in sperm (26, 33, 35) and have recently been shown to form functional flagellar Ca2+ channel (21). Both CatSper 1 and 2 are required for depolarization-evoked calcium entry and hyperactive motility in sperm (7, 8). Whether Ca2 phosphorylates CatSpers or associated proteins leading to increased Ca2+ entry invites further investigation. Increased phosphotyrosine immunoreactivity in WT sperm after 30 min of bicarbonate treatment was also absent in extractions of Ca2 null sperm, providing evidence that the downstream effects of bicarbonate require PKA-mediated phosphorylation of proteins that regulate protein tyrosine phosphorylation.

**FIGURE 2A** illustrates the role of PKA signaling in sperm motility and CAMP feedback regulation. In this model, bicarbonate initiates a cascade of intracellular events that culminate in increased motility and fertilization competency of sperm. Studies with the Ca2 null sperm demonstrate that PKA is downstream of...
sAC-induced cAMP production. They also show that activation of PKA increases flagellar beat frequency and the tyrosine phosphorylation that prepare the capacitated sperm for fertilization. Co2 null sperm provide an excellent model for study of the sperm proteins that are phosphorylated by PKA and that are engaged in driving the motile apparatus. The Co2 knockout also demonstrates the potential for targeting the sperm-specific PKA with inhibitors to prevent fertilization, providing an alternative to hormonal contraceptives.

Unexpectedly, Co2 null mice revealed a novel role for PKA in negative feedback regulation of cAMP production in sperm. As shown in FIGURE 2B, compared with WT sperm, Co2 null sperm have elevated basal cAMP levels and in the presence of bicarbonate and IBMX, cAMP levels rise much higher in the mutant sperm, and the increase is more sustained. Thus PKA, directly or indirectly, inhibits sAC or stimulates phosphodiesterase (PDE) activity. By manipulating the cAMP second messenger system in WT sperm with bicarbonate, IBMX (a nonspecific PDE inhibitor), or H89 (a specific PKA inhibitor), we found that Co2 probably limits cAMP accumulation by a negative feedback inhibition of sAC activity without the involvement of feedback on IBMX-sensitive PDE activity. The nature of this feedback is unknown and might involve direct phosphorylation of sAC or indirect effects on bicarbonate entry or calcium availability that could regulate the sAC enzyme. Alternatively, sperm express a novel PDE8a that is insensitive to IBMX (39) and might be a possible target involved in PKA negative feedback.

**PKA Activity Can be Toxic to Sperm during Development**

Mice heterozygous for a null mutation in the RIα subunit of PKA (RIα+/–) are severely subfertile and produce a reduced number of sperm that are fragile, most with broken heads and ruptured tails (5). The sperm are less motile and are unable to fertilize eggs. The earliest morphological abnormalities in RIα+/– sperm development occur at the round spermatid stage. Round spermatids from RIα+/– contain clear areas in the nucleus at stage I and premature focal condensation of chromatin at stage IX of spermiogenesis. Loss of RIα when it occurs at a stage of germ cell development before the appearance of RIIα (elongating stage of spermiogenesis; see FIGURE 1) may lead to an excess of unregulated C subunit and toxicity associated with inappropriate PKA activity. To test this hypothesis, RIα+/– mice were crossed with mice engineered to have reduced PKA activity in meiotic and early postmeiotic male germ cells, C2+/– mice. Sperm from RIα+/–, C2+/– adult males were morphologically indistinguishable from WT sperm and were capable of fertilizing eggs. Fertility was restored in RIα+/– males by the genetic rescue with C2+/–, confirming that unregulated PKA activity in meiotic or early postmeiotic male germ cells causes defects in spermatid development.

In a clinical context, haploinsufficiency of the RIα gene (PRKAR1A) in humans occurs in nearly two-thirds of Carney complex (CNC) patients, and male CNC patients have significantly reduced fertility (40, 41). We asked whether male CNC patients, like the RIα+/– mouse, have abnormal sperm morphology and reduced sperm count. Ejaculates from CNC patients contained a high percentage of abnormal sperm with head or tail defects. Nearly half of the patients were azoospermic or oligospermic. These observations suggest that male CNC patients with haploinsufficiency of the PRKAR1A gene have reduced fertility and consequently reduced transmission of the mutation as a result of...
sperm morphology defects and azoospermia. The molecular consequences of unregulated PKA activity in meiotic and early postmeiotic germ cells are unknown but may involve changes in gene expression, nucleosomal protein activity, or protein degradation.

**Does the Subcellular Localization of PKA Play a Role During Capacitation?**

Numerous intracellular responses to elevated cAMP levels have been shown to require PKA signaling that is precisely localized to subcellular compartments through the high-affinity interaction between RII subunits and AKAPs (45). Sperm motility is no exception. Disruption of this interaction by HT31, a synthetic peptide containing the RII-binding domain of a human thyroid AKAP with nanomolar binding affinity for RII (9), has been reported to inhibit bovine sperm motility (42), although stearated HT31 was not an effective inhibitor of mouse sperm capacitation (unpublished observations). Since RIIα, the predominant R subunit found in mature sperm, is firmly attached to the flagellum, RIIα null sperm might be expected to have compromised motility if anchoring is essential. Unexpectedly, RIIα-deficient mice have normal sperm motility and are fertile (6). A compensatory increase in Rα protein levels occurred in the RIIα knockout with only a modest reduction in total PKA activity. However, there were dramatic changes in the localization of PKA catalytic subunit. Indeed, the majority of the holoenzyme was redistributed to the cytoplasmic fraction. Immunoreactive Ca subunit was no longer detectable along the flagellum of RIIα-deficient sperm but instead was found localized to the cytoplasmic droplet. Nevertheless, Rα may compensate in these animals and anchor sufficient levels of PKA to dual AKAPs to permit localized PKA signaling in sperm flagellum.

In a further attempt to disrupt anchored PKA signaling in sperm, we produced mice with a targeted disruption of AKAP1, a highly expressed AKAP in elongating mouse spermatids (24, 34). AKAP1 targets PKA to the mitochondria where it may play a role in germ cell survival by positioning PKA to phosphorylate and inactivate the proapoptotic protein BAD (19). AKAP1 also interacts with an associating partner of Myc-1 (AMY-1) and with the related AMY-1-associating protein that is expressed in testis (AAT-1). Immunocytochemistry indicates that AKAP1, AMY, and AAT-1 all localize to the mitochondria in mouse sperm (17, 47). Although the functions of these proteins have not been elucidated, they may modulate PKA signaling. However, AKAP1 null male mice are fertile with no significant differences in sperm count, motility, or morphology (31). In contrast, a knockout of AKAP4, which is also a major component of the fibrous sheath in sperm, did result in infertility (28) as well as structural changes in the flagellum. From all of these studies, it appears that a functional role for AKAPs at some stage in sperm maturation is likely but that this does not depend on the presence of RIIα.

**PKA Anchoring Impacts Meiotic Maturation in Oocytes**

Oocytes are arrested in the diplotene stage of prophase I at birth and are characterized by an intact germinal vesicle (GV) surrounding the nucleus, partially decondensed chromatin, and microtubule organization resembling a cell in interphase. Resumption of meiosis occurs after follicle maturation and the LH surge and results in GV breakdown (GVBD), chromosome condensation and orientation at the equatorial plate, organization of microtubules in a spindle, exclusion of the first polar body, second meiotic division, and arrest at metaphase II.

Regulation of cAMP levels and PKA activity are essential for the maturation of oocytes. Elevated levels of cAMP in oocytes and activation of PKA maintain meiotic arrest in the later stages of follicle development. Through mechanisms that are not fully understood, the preovulatory surge of LH leads to a decrease in cAMP and PKA activity in oocytes, and meiosis resumes. The mechanism for this paradoxical decrease in cAMP levels following the LH surge is likely to involve the Gα-linked GPR3 receptor on the surface of the oocytes (27). Oocytes from mice lacking GPR3 resume meiosis prematurely, suggesting that a ligand, possibly secreted from granulosa cells, activates GPR3 to maintain the high intra-oocyte levels of cAMP and PKA activity required for meiotic arrest. LH action on the granulosa cells may terminate the production or release of this ligand or induce an antagonist of GPR3.

PKA inhibits the resumption of meiosis through inhibition of maturation promoting factor (MPF). The cell-cycle regulators p34cdc2 (CDK1) and cyclin B1 form a complex (MPF) that initiates GVBD. Inactivation of MPF occurs in part by the phosphorylation of CDK1 by Wee1, and PKA has recently been shown to phosphorylate and activate an oocyte-specific Wee1B, a member of the Wee1 family of Cdk-inhibitory kinases (18). Activation of MPF occurs when CDK1 is dephosphorylated by a dual specificity phosphatase, most likely Cdc25b in mouse (25). PKA directly phosphorylates and inhibits Cdc25c phosphatase activity in Xenopus oocytes (15) and may serve an analogous regulatory role in mammalian oocyte Cdc25b activity. However, other mechanisms of MPF inactivation by PKA are possible. Recently, a novel tyrosine phosphatase, PTPN13, was shown to be required for mouse oocyte maturation, and PTPN13 is directly inhibited by PKA phosphorylation (30).

Spatial control of PKA activity, mediated by AKAP interactions, may be important for oocyte function. Using the peptide inhibitors of PKA/AKAP interactions, we first showed that HT31 when injected into...
oocytes stimulated oocyte maturation even when PKA activity was elevated by high cAMP. This result suggested that PKA must be localized in proximity to its targets such as Wee1B, cdc25b, or PTPN13 to efficiently maintain the arrested state.

The intracellular distribution of the PKA subunits RIα, RIβ, and Ca changes during meiotic maturation, and this relocalization may be mediated by modifications in binding to oocyte AKAPs, such as Ezrin, WAVE1, AKAP140 (a splice variant of AKAP1), and AKAP7γ. Studies with AKAP140 indicate that this AKAP, present throughout meiotic maturation of the oocyte, may play an important role in regulating localized PKA activity. Indeed our studies show that oocytes from AKAP1 knockout (KO) females are defective in their ability to resume meiosis (31).

AKAP1 null females, unlike males, are severely subfertile. The majority of AKAP1 KO oocytes fail to undergo GVBD both in vivo and in vitro and therefore do not mature to metaphase II and become competent for fertilization. This failure to resume meiosis was rescued in vitro by brief inhibition of PKA activity with Rp-cAMPS, suggesting that the defect in AKAP1 KO oocytes is related to excess PKA activity. PKA activity measured in AKAP1 KO oocyte extracts was not different from WT oocytes, however, a difference in the localization of PKA subunits during meiotic maturation was observed. In GV-stage oocytes, PKA subunits (RIα, RIβ, and Ca) do not co-localize with AKAP1 at the mitochondrial membrane in either WT or KO oocytes. However, during the early minutes after meiotic maturation is initiated, RIβ relocated to the mitochondria in WT but not KO oocytes. Therefore, AKAP1 may be required to mediate the redistribution of RI as the oocyte resumes meiotic maturation, and this redistribution removes PKA from targets that might prevent or reverse this maturation process. This model of AKAP action in mouse oocytes is depicted in FIGURE 3 and contrasts with the canonical model in which the major action of the AKAP is to bring kinases together with substrates and other signaling molecules. Instead we propose a scheme in which the AKAP sequesters the kinase away from targets to allow meiotic maturation to proceed without interference. In what type of system would this make sense? Oocyte meiotic maturation is a stoichiastic pathway that is not reversible. Once the biological switch to begin meiotic maturation is thrown, it is imperative that the oocyte make it all the way to metaphase II arrest before fertilization. Sequestering PKA once meiotic maturation is initiated might be a component of this irreversible switch mechanism to ensure reproductive success of the oocyte.

Conclusions

Many laboratories have contributed to our current appreciation of the actions of PKA in the maturing germ cells. We have focused this review on some of the more recent results using PKA and AKAP mouse mutants. It was surprising to us that sperm development, at least from the pachytene stage onward, does not require PKA and is in fact severely compromised when PKA is mis-regulated and activated, as in the case of the RIα heterozygote. The relevance of this to human infertility is clearly seen in RIα heterozygote humans (Carney Complex patients) who display sperm defects and male infertility similar to that observed in mice. We also found it surprising that RIα KO males did not have disrupted sperm capacitation and fertility since all of the remaining PKA activity is type I (RIα) and does not localize specifically to the proximal and principal piece as seen for the type II kinase. Perhaps the low-affinity interaction of type I kinase with AKAPs is still sufficient to keep adequate kinase in proximity to substrates important for capacitation. In contrast, localization to AKAPs does appear to be very important in oocyte meiotic maturation, and our results suggest that AKAPs may be playing both a classic role (bringing kinase close to substrate to facilitate phosphorylation) and a noncanonical role (sequestering kinase to prevent phosphorylation). The time course suggests that the type II PKA switches from a site where it is actively
phosphorylating targets to a sequestration site shortly after oocytes resume meiosis and that this switch is essential for the oocyte to efficiently proceed to the metaphase II arrested state. The role of anchored PKA in human oocyte maturation has not been explored, and no mutations in PKA or AKAPs have been associated with female infertility in humans. However, the highly conserved nature of mammalian oocyte maturation suggests that PKA and AKAP mutations may be likely candidates for unexplained fertility defects that disrupt oocyte maturation.

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References


