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REVIEW

Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases

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Mammalian sperm must undergo a series of biochemical and physiological modifications, collectively called capacitation, in the female reproductive tract prior to the acrosome reaction (AR). The mechanisms of these modifications are not well characterized though protein kinases were shown to be involved in the regulation of intracellular Ca^{2+} during both capacitation and the AR. In the present review, we summarize some of the signaling events that are involved in capacitation. During the capacitation process, phosphatidyl-inositol-3-kinase (PI3K) is phosphorylated/activated *via* a protein kinase A (PKA)-dependent cascade, and downregulated by protein kinase C α (PKCα). PKCα is active at the beginning of capacitation, resulting in PI3K inactivation. During capacitation, PKCα as well as PP1γ2 is degraded by a PKA-dependent mechanism, allowing the activation of PI3K. The activation of PKA during capacitation depends mainly on cyclic adenosine monophosphate (cAMP) produced by the bicarbonate-dependent soluble adenylyl cyclase. This activation of PKA leads to an increase in actin polymerization, an essential process for the development of hyperactivated motility, which is necessary for successful fertilization. Actin polymerization is mediated by PIP_2 in two ways: first, PIP_2 acts as a cofactor for phospholipase D (PLD) activation, and second, as a molecule that binds and inhibits actin-severing proteins such as gelsolin. Tyrosine phosphorylation of gelsolin during capacitation by Src family kinase (SFK) is also important for its inactivation. Prior to the AR, gelsolin is released from PIP_2 and undergoes dephosphorylation/activation, resulting in fast F-actin depolymerization, leading to the AR.

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INTRODUCTION

Ejaculated mammalian spermatozoa should reside in the female genital tract for several hours before gaining the ability to fertilize the egg. In humans however, sperm must move out of the seminal plasma immediately after ejaculation and appear in the fallopian tube within minutes. As soon as sperm are moving out of the ejaculate and passing the cervical mucus, they undergo several biochemical changes collectively called capacitation,[1,2] which was first independently reported nearly six decades ago by Austin[3] and Chang.[4] These changes involve molecules absorbing on, or integrating into, the sperm plasma membrane during epididymal maturation. The removal or alteration of these molecules prepares the sperm toward successful binding to the egg and fertilization.

During mammalian fertilization, the capacitated spermatozoon penetrates the cumulus oophrous of the ovum, and then binds to the zona pellucida (ZP) with its plasma membrane intact. After binding to the egg ZP, the spermatozoon undergoes an exocytotic process called the acrosome reaction (AR).[5–8] This event is required for fertilization, because it enables passage of the spermatozoon through the ZP and its subsequent fusion with the egg oolema.

Capacitation includes multiple physiological and biochemical modifications.[5] The biochemical changes associated with the capacitation process include an efflux of cholesterol from the plasma membrane leading to an increase in membrane fluidity and permeability to bicarbonate and calcium ions, hyperpolarization of the plasma membrane,[9] changes in protein phosphorylation and protein kinase activity[10–12] and increases in bicarbonate (HCO_3^-) concentration and intracellular pH, Ca^{2+} and cyclic adenosine monophosphate (cAMP) levels.

Capacitation can be divided into two signaling events: fast and slow.[11] These events take place during the passage of sperm within the female reproductive tract. The fast events include activation of the vigorous and asymmetric movement of the flagella and these happen as soon as the sperm leave the epididymis. The slow events include changes in the pattern of movement (hyperactivation). Protein tyrosine phosphorylation is another landmark of capacitation but occurs during the late stages of capacitation on a different timescale from cAMP/protein kinase A (PKA) activation. The fast event depends on PKA activation mediated by the Ca^{2+} and HCO_3^--dependent soluble adenylyl cyclase. It has been suggested that Ca^{2+} is transported into the cell by the sperm-specific Ca^{2+} channel (CatSper) and HCO_3^- by the Na^+/HCO_3^- cotransporter.[11] The beginning of the slow events of capacitation is marked by the removal of cholesterol from the membrane by bovine serum albumin and the increase in its fluidity.[11]

Eventually, all these changes will lead to the capacitation of the sperm and as a final point, the following events occur: the ability to carry out the AR induced by biological agonist, ZP, or progesterone;

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the ability to produce hyperactivation motility (HAM); exhibition of chemotactic behavior;[13] and the ability to fertilize an oocyte.

PROTEIN TYROSINE PHOSPHORYLATION

Phosphorylation of proteins is a posttranslational modification event that acts as one of the cell's key regulatory mechanisms to control various cellular processes.[14,15] Although both serine/threonine phosphorylation and tyrosine phosphorylation of proteins have been reported in spermatozoa, the tyrosine phosphorylation of a number of protein substrates has been associated with capacitation in the spermatozoa of most mammalian species, and is considered a hallmark event of capacitation.[16–21]

Several tyrosine phosphoproteins in capacitated buffalo and cattle spermatozoa were identified using 2D immunoblotting and mass spectrometry. These include serine/threonine-protein phosphatase PP1γ2 catalytic subunit, the MGC157332 protein, alpha-enolase, 3-oxoacid CoA transferase 2 and actin-like protein 7A.[22]

In human sperm, the identified Tyr-phosphorylated proteins include ion channels, metabolic enzymes and structural proteins (16), CABYR, a calcium-binding protein localized in the principal piece of the tail in association with the fibrous sheath[23] and members of the extracellular signal-regulated kinase family.[24,25] The main Tyr-phosphorylated structural proteins of the fibrous sheath[16] are the family of A-kinase-anchoring proteins and their involvement in motility has been defined.[26]

In order to get a successful capacitation, several molecules are required; these include HCO_3^-, serum albumin and Ca^{2+}. The HCO_3^- enters the sperm through the cotransporter Na^+/HCO_3^-.[27] Physiological levels of HCO_3^- produce a rapid collapse of the asymmetry of the sperm plasma membrane attributable to the activation of enzymes that translocate membrane phospholipids, such as phosphatidylserine and phosphatidylethanolamine,[28] increasing the availability of cholesterol to external acceptors.[11,13] This increase in the HCO_3^- concentration also produces an increase in the intracellular pH and the activation of a unique type of adenylyl cyclase present in the sperm, the soluble adenylyl cyclase which results in increased levels of cAMP and cAMP-dependent PKA activation. The activation of PKA modulates the response of calcium channels such as CatSper, which produces changes in the membrane potential[29] and increases in the intracellular Ca^{2+} concentration. PKA phosphorylates several proteins on Ser and Thr residues, activating, either directly or indirectly, several protein kinases and/or inhibiting protein phosphatases, which will finally produce an increase in the phosphorylation of Tyr residues.[30–34]

It has been shown that PKA inhibition blocks the onset of tyrosine phosphorylation.[20] However, it was recently suggested that the Src family tyrosine kinases (SFKs) mediate the increase in tyrosine phosphorylation in mouse, human and bovine sperm.[35–40]

In murine sperm, PKA coimmunoprecipitates with Src, and this interaction leads to Src phosphorylation.[41] It was shown that the Src kinase family inhibitor, SU6656 inhibits PKA phosphorylation, sperm motility, and *in vitro* fertilization,[34] suggesting that two parallel pathways regulate phosphorylation events leading to capacitation: one requires activation of PKA, and the other requires inactivation of ser/thr phosphatases.

PKA phosphorylates various target proteins that are presumed to initiate several signaling pathways. In sperm exposed to HCO_3^-, cAMP rises to its maximum levels within 60 s, and the increase in PKA-dependent phosphorylation begins within 90 s.[13] This increase in tyrosine phosphorylation is a late event, which depends on the presence of albumin, Ca^{2+} and HCO_3^- in the capacitation medium, and correlates with capacitation.[13] Inhibitors of PKA block sperm capacitation and the associated increase in protein tyrosine phosphorylation.[42]

Whatever the role of Tyr phosphorylation in capacitation, the level of Tyr phosphorylation in human sperm correlates strongly with the sperm-zona-binding capacity[43] and alterations in Tyr phosphorylation have been found in subfertile subjects[44] indicating its physiological role in fertilization.

PROTEIN KINASES A AND C

Mice that lack the unique sperm PKA catalytic subunit Cα2 are infertile despite normal mating behavior, and their sperm exhibits defects which are seen in both early and late capacitation-associated events.[45] Results from mice lacking the atypical HCO_3^--dependent adenylyl cyclase[46,47] have conclusively demonstrated that an HCO_3^--dependent modulation of the cAMP/PKA pathway is involved in the regulation of both fast and slow capacitation-associated processes.

It is well known that PKA is involved in regulation of sperm motility. Activation of the PKA catalytic subunit increases flagellar beat frequency during capacitation.[48] In fact, PKA plays at least two independent roles in the regulation of sperm motility: a 'fast' action is required for the activation of flagellar beat, and a 'slow' action, such as the change in the flagellum waveform symmetry, requires PKA to be active for an extended time period. We recently showed that PKA mediates light-induced hyperactivated motility (HAM) in human sperm.[49] It is currently accepted that capacitated sperm represents HAM. During the capacitation process, sperm change their motility pattern from progressive to HAM.[50,51] HAM is a movement pattern characterized by asymmetrical flagellar beating observed in spermatozoa at the site and time of fertilization in mammals,[52–55] and may be critical to fertilization success.[56] It was shown that hyperactivated sperm penetrate the ZP much more effectively than non-hyperactivated sperm.[57] If HAM is prevented, fertilization cannot occur.[58]

The presence of protein kinase C (PKC) in human,[59] ram[60] and bovine[61] sperm was identified years ago. Functional studies also suggest the involvement of PKC in flagellar motility and AR.[62] PKC exists in 11 isotypes, several of which can be simultaneously present in a single cell. There are three broad categories of PKC based on their requirements for activity, and the structure of their regulatory domains at the NH_2 terminus.[63] First, the conventional PKCs, namely PKCα, βI, βII and γ are dependent on diacylglycerol (DAG), phospholipids and calcium, with their regulatory domains containing a C1 domain, which binds DAG/PMA, and a C2 domain that binds anionic phospholipids in a calcium-dependent manner. Second, the novel PKCs are DAG and phospholipid-dependent, but are calcium-independent, and include PKCδ, ε, μ, η and θ; their regulatory domains contain two C1 and one C2 domains, with the C2 domains lacking the calcium-coordinating acidic residues. The third category is that of the atypical PKCs, which are DAG and calcium-independent and include PKCζ and human PKCι/mouse PKCλ; their regulatory domains lack the calcium-sensitive C2 domain and contain an atypical C1 domain that binds PIP_2 or ceramide.[64] PKCs play a pivotal role in cell signaling, as a serine/threonine kinase, in particular for Ca^{2+}-mobilizing ligands.[65,66] This multi-isoenzyme family is involved in synaptic transmission, memory, learning, cellular growth, differentiation, transformation, metabolism, contraction, regulation of ion channel activity, exocytosis and gene expression. The PKC activators are produced by the various phospholipases. Enhanced phosphoinositide turnover provides Ca^{2+} and DAG *via* activation of phospholipase C (PLC).

The AR requires free extracellular calcium;[67,68] however, activation of PKC induces the AR in a calcium-independent fashion.[69] A calcium-dependent isotype of PLCγ is activated during sperm capacitation,[70] leading to activation of a broad range of PKC isotypes at the

time of capacitation.[8] Activation of sperm PKA leads to inhibition of PKC, probably *via* PLC inhibition.[71]

PKA activates a voltage-dependent Ca^{2+} channel in the outer acrosomal membrane that releases Ca^{2+} from the interior of the acrosome to the cytosol.[72] The rise in Ca^{2+} levels might support PLC activation, followed by formation of IP_3 which further elevate Ca^{2+} levels by mobilizing an acrosomal Ca^{2+} pool, and the formation of DAG, which activates specific PKC isoforms. The sperm plasma membrane contains a Ca^{2+} channel that is activated by PKC.[72,73]

Inhibitors of PKC block motility, while application of a crude PKC agonist increases motility. Studies on human sperm[69] demonstrated that the crude PKC agonist could also induce the AR in the absence of an elevation of intracellular-free calcium as long as the sperm had previously undergone capacitation.

The large number of PKC isotypes, and the expression of most of them in sperm or eggs, suggest that this family of kinases has multiple tasks during gametogenesis, fertilization and early development. Differential regulation of the individual members of this kinase family can occur in three ways. The first is by means of the different cofactor requirements of the three categories of PKC. A second mechanism is the differing substrate specificities of the individual family members. Finally, individual kinases may be regulated by localization or enrichment of the specific isotype at specific locations in the sperm or egg. All these three mechanisms seem to have a role in fine-tuning the function of the kinase.[63] PKC was found to be localized mainly in the equatorial segment of the human sperm.[59,74] In bull sperm, PKC is concentrated mainly in the postacrosomal and upper region of the acrosome.[61] Activation of PKC is associated with translocation of the enzyme from the cytosol to the membrane fraction. Indeed, PKC translocates to sperm plasma membrane after treatment with phorbol ester.[75] Recently, we showed that PKCα undergoes degradation and dephosphorylation during capacitation.[76] This downregulation of PKCα occurs at the same time as the phosphorylation of Tyr467 on phosphatidyl-inositol-3-kinase (PI3K) regulatory subunit p85 is increased.

PI3K

PI3K is primarily responsible for the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) in response to growth factors.[77] This enzyme is implicated in many biological processes, including cell survival, cell growth, cell movement and adhesion, protein synthesis, and cytoskeletal rearrangements. A role for PI3K has been suggested in sperm functions during capacitation and the AR.[38,78,79] PI3K catalytic and regulatory subunits are present in sperm.[79]

It was shown that the PI3K inhibitor, wortmannin (10 nmol l^{-1}), decreases PIP_3 production in bovine sperm.[38] We suggest that PI3K activity during capacitation is activated by PKA and inhibited by PKCα.[38] Inhibition of PKA by H89 blocks the elevation of PI3K phosphorylation. Recently, we demonstrated that PKCα inhibition by itself is insufficient to activate PI3K unless PKA is active. We suggest a dual role for PKA in the regulation of PI3K activity during bovine sperm capacitation. First, PKA mediates PI3K activation, and secondly, PKA mediates PKCα and PP1γ2 degradation, which is necessary for PI3K activation.

A direct role for PKCα in the activation of the S/T phosphatase PP2A, which results in PI3K inhibition through removal of PI3K phosphorylation, was recently shown in epithelial cells.[80] The finding that PKCα and PP1γ2 are degraded during the capacitation process suggested the involvement of degradation system(s) which regulates the cellular levels of these proteins. The two major proteolytic pathways in eukaryotic cells are the ubiquitin/proteasome system and the lysosomal pathway. Since lysosomes do not exist in sperm cells, while proteasomes are present, it is likely that the ubiquitin/proteasome pathway is involved in the degradation of PKCα and/or PP1γ2.[81] Most recently, it was reported that proteasomal activity is important for human sperm capacitation, and that PKA activity regulates the chymotrypsin-like activity of the proteasome during capacitation.[82] Another study revealed that PKA can positively regulate the proteasomal activity by phosphorylation of Rpt6, one of the six AAA-ATPases in the 19S regulatory subunit of the proteasome.[83] We therefore suggest a mechanism of cross-talk between PKC and PKA that regulates PI3K phosphorylation/activation. At the beginning of the capacitation process, PKCα and PP1γ2 are active and present in high levels and inhibit (directly or indirectly) PI3K phosphorylation.[76] With ongoing capacitation, PKA mediates PKCα and PP1γ2 degradation, the inhibition of PI3K by PKCα/PP1γ2 is relaxed and PI3K phosphorylation/activation by PKA can occur.

ACTIN POLYMERIZATION AND DEPOLYMERIZATION

PI3K activation by direct phosphorylation of p85, the regulatory subunit, leads to actin polymerization.[38,84,85] We have previously shown that actin polymerization occurs during sperm capacitation and that F-actin breakdown must take place to achieve the AR.[86] It has been suggested that an increase in F-actin creates a network between the plasma and the outer acrosomal membranes, and the dispersion of F-actin between the two membranes is needed to enable the AR.[86–89]

The presence of actin-binding proteins in mammalian sperm suggests that the assembly of G-actin to form F-actin, as well as the disassembly of F-actin are well-controlled events.[90–92] Gelsolin severs assembled actin filaments, and caps the fast-growing plus end of free or newly severed filaments in response to Ca^{2+}. Phosphoinositides bind gelsolin and release it from actin filament ends, exposing sites for actin assembly.[93,94] We recently showed that gelsolin is inactive during capacitation, and is activated prior to the AR.[70]

The release of bound gelsolin from phosphatidylinositol 4,5-bisphosphate ($PIP_{2(4,5)}$) by PBP10, a peptide containing the PIP_2-binding domain of gelsolin, or by activation of PLC, which hydrolyzes PIP_2, causes rapid Ca^{2+}-dependent F-actin depolymerization as well as an enhanced AR.[70] The activation of PLC, which hydrolyzes $PIP_{2(4,5)}$, releases the bound gelsolin and enables its activity of breaking down F-actin, resulting in the AR. Thus, PLC activity mediates F-actin depolymerization at the end of capacitation, leading to the AR.

We have previously shown that epidermal growth factor receptor (EGFR) is involved in the AR[95] and in actin polymerization during capacitation.[86,96]

We also show that the EGFR is partially activated in sperm incubated under capacitation conditions and is fully activated by adding EGF at the end of the capacitation resulting in the occurance of the AR.[97] In our recent study, we show that α7-nicotinic-acetyl-choline-receptor (α7nAChR) is a potential sperm receptor that can be activated by the egg ZP to induce EGFR-mediated AR.[98] It was also shown by others that EGFR is involved in boar sperm motility[99] and was localized at higher extent to the acrosome region than to the postacrosome and the flagellum.[98] Moreover, EGF signaling was shown to be an important pathway identified in high fertility sperm in a recent comprehensive proteomic analysis.[100]

Previous studies identified Src in human spermatozoa, and it appears to be involved in regulating sperm capacitation, calcium fluxes, tyrosine phosphorylation and the AR.[35] Src and gelsolin coimmunoprecipitate, and Src phosphorylates gelsolin on tyrosine-438 and

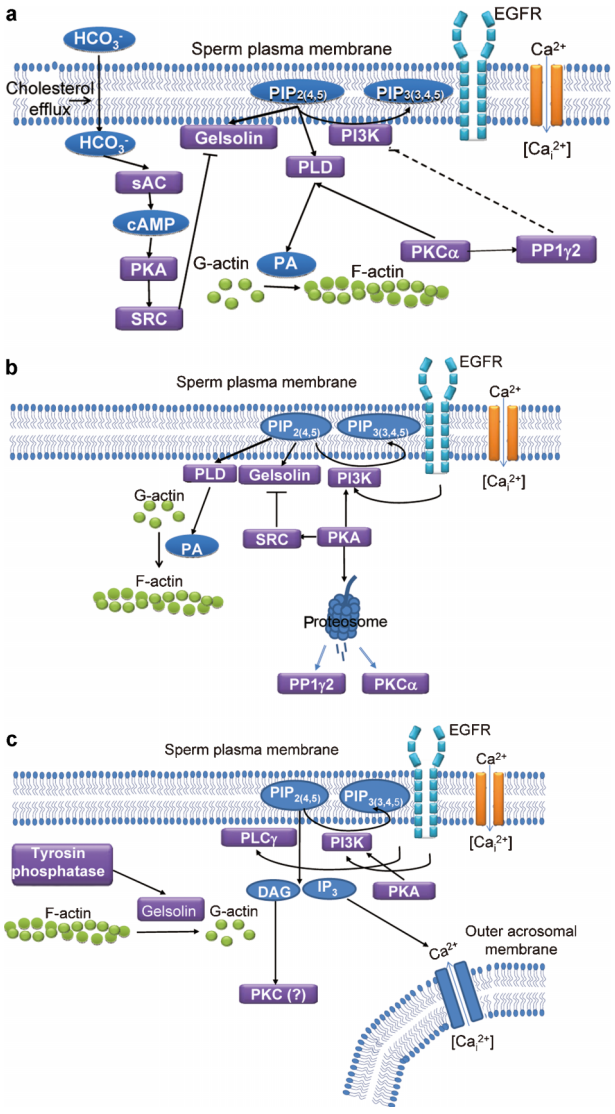

Figure 1 A model describing the involvement of various kinases in sperm capacitation and the AR. The model is composed of three steps: (**a**) Beginning of capacitation. (**b**) Ongoing capacitation. (**c**) The AR. (**a**) Beginning of capacitation. Activation of NBC and CatSper results in fast increase in intracellular HCO_3^- and Ca^{2+} resulting in SACY/PKA activation. Later on efflux of cholesterol from the sperm plasma membrane enhances further bicarbonate permeability into the cell resulting in further activation of SACY, cAMP production and PKA activation. PKA activation leads to Src-mediated gelsolin phosphorylation maintaing PIP_2-bound gelsolin in an inactive state and thereby stabilizing the polymerized actin. At the beginning of capacitation process, PKCα is in its active state which leads to PP1γ2 phosphorylation/activation. PP1γ2 causes PI3K dephosphorylation/inhibition. At this stage, the level of PIP_2 increases, resulting in PLD activation which produces PA leading to actin polymerization. (**b**) Ongoing capacitation. PKA mediates proteasome dependent PKCα and PP1γ2 degradation, leading to PI3K phosphorylation/activation. During capacitation, Ca^{2+} concentration is elevated, EGFR is partially activated resulting in PI3K activation. At this stage, gelsolin still bound to PIP_2 in an inactive state and thereby depolymerization of F actin is prevented. (**c**) The AR. Sperm binding to the egg ZP stimulates relatively high elevation of intracellular Ca^{2+} and EGFR is activated, leading to PLCγ and PI3K activation. PI3K phosphorylation can also occur by PKA activation. PLCγ hydrolyzes PIP_2 resulting in DAG and IP_3 production. DAG activates PKC and IP_3 activates Ca^{2+} efflux from the acrosome. The decrease in PIP_2 levels results in the release of gelsolin to the cytosol following by its dephosphrylation/activation by tyrosine phosphatases leading to F-actin depolimerization and the occurrence of the AR. AR, acrosome reaction; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; NBC, Na^+/HCO_3^- cotransporter; PA, phosphatidic acid; PI3K, phosphatidyl-inositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; SACY, soluble adenylyl cyclase; ZP, zona pellucida.

inhibits its activity.[70] Actin polymerization is blocked by inhibiting the SFKs, suggesting that gelsolin is activated under these conditions.

We suggest that during capacitation, the intracellular Ca^{2+} concentration rises, leading to conformational changes in gelsolin and exposing its F-actin-binding site. As a result, gelsolin is activated and translocated to the head of the sperm.[63] Nevertheless, the elevation of $PIP_{2(4,5)}$ levels and gelsolin phosphorylation maintain gelsolin in an inactive state, allowing actin polymerization to occur. Prior to the AR, the intracellular Ca^{2+} concentration is further elevated: PLC is activated and hydrolyzes $PIP_{2(4,5)}$ resulting in the release of gelsolin to the cytosol. The free gelsolin becomes activated as a result of elevated levels of Ca^{2+} and tyrosine dephosphorylation by tyrosine phosphatases, leading to F-actin dispersion and the AR.[70] This interpretation is consistent with the role of $PIP_{2(4,5)}$ as a cofactor for PLD activation which mediates actin polymerization in capacitation.[71,101]

It has been shown in several cell types that PLD, the enzyme that hydrolyzes phosphatidylcholine to phosphatidic acid and choline,[102] is involved in the regulation of the actin cytoskeleton.[102–104] We showed elsewhere that PLD-dependent actin polymerization is a necessary step in the cascade leading to bull sperm capacitation.[70,71] PLD as well as actin polymerization is also involved in regulating cell motility.[103]

In our recent study, we showed that the development of HAM in mouse sperm during capacitation depends upon actin polymerization.[101] Moreover, we also showed that progressive sperm motility depends on PLD activity as well.[101]

In **Figure 1**, we suggest a model which unifies the different kinase cascades involved in sperm capacitation and the AR.

CONCLUSIONS

During the capacitation process, PI3K is phosphorylated/activated *via* a PKA-dependent cascade, and downregulated by PKCα. PKCα is active at the beginning of capacitation, resulting in PI3K inactivation. During capacitation, PKCα as well as PP1γ2 is degraded by a PKA-dependent mechanism, allowing the activation of PI3K. This activation of PKA leads to an increase in actin polymerization, an essential process for the development of hyperactivated motility, which is necessary for successful fertilization. Actin polymerization is mediated by PIP_2 in two ways: first, PIP_2 acts as a cofactor for PLD activation, and second, as a molecule that binds and inhibits actin-severing proteins such as gelsolin. Tyrosine phosphorylation of gelsolin during capacitation by SFK is also important for its inactivation. Prior to the AR, gelsolin is released from PIP_2 and undergoes dephosphorylation/activation, resulting in fast F-actin depolymerization, leading to the AR.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

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