REPRODUCTION
REVIEW

Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction

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Abstract

In order to fertilize, the mammalian spermatozoa should reside in the female reproductive tract for several hours, during which they undergo a series of biochemical modifications collectively called capacitation. Only capacitated sperm can undergo the acrosome reaction after binding to the egg zona pellucida, a process which enables sperm to penetrate into the egg and fertilize it. Polymerization of globular (G)-actin to filamentous (F)-actin occurs during capacitation, depending on protein kinase A activation, protein tyrosine phosphorylation, and phospholipase D activation. F-actin formation is important for the translocation of phospholipase C from the cytosol to the sperm plasma membrane during capacitation. Prior to the occurrence of the acrosome reaction, the F-actin should undergo depolymerization, a necessary process which enables the outer acrosomal membrane and the overlying plasma membrane to come into close proximity and fuse. The binding of the capacitated sperm to the zona pellucida induces a fast increase in sperm intracellular calcium, activation of actin severing proteins which break down the actin fibers, and allows the acrosome reaction to take place.
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Introduction

In mammalian species, sperm–egg interaction and mutual activation are mediated by the zona pellucida (ZP), the glycoprotein coat of the egg (reviewed in Wassarman 1988). The spermatozoon binds to the ZP with its plasma membrane intact, via specific receptors that are localized over the anterior head region of the sperm. The binding of the sperm to the ZP stimulates it to undergo an acrosome reaction, which enables the sperm to penetrate and fertilize the egg (reviewed in Yanagimachi 1994).

The binding of the sperm to the egg and the occurrence of the acrosome reaction will take place only if the sperm has previously undergone a poorly defined maturation process in the female reproductive tract, known as capacitation. We recently reviewed the known signal transduction events occurring during capacitation and the acrosome reaction (Breitbart 2003). The possible changes and regulation of the sperm actin cytoskeleton as part of the mechanisms of capacitation and the acrosome reaction are described in this review.

Actin and related proteins in sperm

In spermatogenic cells, actin filaments have been described primarily in the subacrosomal space between the nucleus and the developing acrosome of spermatids of certain mammalian species (Vogl 1989). In mature spermatozoa, however, the structure and location of actin filaments have not been made clear. In most reports, actin seems to be present in its monomeric form, although filamentous (F)-actin has been described in mammalian species as well (Flaherty 1987, Breed & Leigh 1991, Moreno-Fierros *et al.* 1992, Vogl *et al.* 1993, de las Heras *et al.* 1997, Howes *et al.* 2001). In human sperm the regions reported to contain actin include the acrosomal space, the equatorial and post acrosomal regions, and the tail (Clarke *et al.* 1982, Virtanen *et al.* 1984, Ochs & Wolf 1985, Fouquet & Kann 1992). The presence of actin in the tail might be important for the regulation of sperm motility, and its presence in the head suggests a possible involvement in the acrosome reaction. It was reported that actin polymerization is important for initiation of sperm motility during post-testicular maturation (Lin *et al.* 2002). The location of actin in the acrosomal region of several mammalian species including hamster, boar, human, bull, rabbit and guinea-pig (Talbot & Kleve 1978, Camatini *et al.* 1986, Flaherty *et al.* 1988, Olson & Winfrey 1991, Moreno-Fierros *et al.* 1992, Yagi & Paranko 1995) supports its possible role in sperm capacitation and the acrosome reaction. Actin polymerization is necessary for sperm incorporation into the egg cytoplasm (Sanchez-Gutierrez *et al.* 2002) and for sperm nuclei decondensation

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(Kumakiri *et al.* 2003). The assembly of G-actin to form F-actin is controlled by several actin-binding proteins. The existence of proteins such as calicin (von Bulow *et al.* 1995), the capping proteins CPβ3 (von Bulow *et al.* 1997) and CPα3 (Tanaka *et al.* 1994), destrin, thymosin β10, testis-specific actin capping protein (Howes *et al.* 2001), gelsolin (de las Heras *et al.* 1997), scinderin (Pelletier *et al.* 1999), and the actin-related proteins Arp-T1 and T2 (Heid *et al.* 2002) in mammalian sperm suggests that actin polymerization and depolymerization might be involved in sperm function. In boar and guinea-pig sperm, actin polymerization occurs during capacitation (Castellani-Ceresa *et al.* 1993, Cabello-Agueros *et al.* 2003). Recently, we showed that actin polymerization occurs during capacitation of bull, mouse, human, and ram sperm, whereas F-actin breakdown should occur in order to achieve the acrosome reaction (Brener *et al.* 2003). In human sperm, actin is lost from the acrosomal region following the acrosome reaction (AR) (Liu *et al.* 1999) and blocking actin polymerization inhibited ZP-induced AR (Liu *et al.* 1999, 2002). In addition, inhibition of actin breakdown blocks bovine sperm AR (Spungin *et al.* 1995). Moreover, inhibition of actin polymerization in guinea-pig and human sperm by cytochalosin D, blocks sperm penetration into zona-free hamster eggs (Rogers *et al.* 1989) as well as the *in vitro* fertilization ability of boar (Castellani-Ceresa *et al.* 1993) and mouse (Brener *et al.* 2003) sperm. These evidences suggest that remodeling of actin structure plays an important role in sperm capacitation and the AR.

Protein tyrosine phosphorylation and actin polymerization

It is accepted that protein kinase A (PKA)-dependent tyrosine phosphorylation of several proteins occurs during sperm capacitation (Visconti *et al.* 1995). In human and bovine sperm, reactive oxygen species (ROS) up-regulates protein tyrosine phosphorylation (Aitken *et al.* 1995, Leclerc *et al.* 1997, Rivlin *et al.* 2004), consistent with the suggestion that hydrogen peroxide activates adenylyl cyclase to produce cAMP which activates PKA (Aitken 1997, Rivlin *et al.* 2004).

It is unclear whether a specific ligand induces the signal transduction cascade leading to protein tyrosine phosphorylation in sperm capacitation. One possible ligand is the epidermal growth factor (EGF), which interacts with its receptor (EGFR) identified in the bovine sperm head (Lax *et al.* 1994). EGF stimulates the tyrosine phosphorylation of several sperm proteins (Breitbart *et al.* 1995) and activates phospholipase Cγ (PLCγ) (Spungin *et al.* 1995) and actin polymerization (Brener *et al.* 2003, Cohen *et al.* 2004) in bovine sperm capacitation.

There is a good correlation between actin polymerization and protein tyrosine phosphorylation in bovine and ram sperm capacitation (Brener *et al.* 2003). In bovine sperm, the two processes do not occur in the absence of bicarbonate; both depend on PKA and tyrosine kinase activities, both are enhanced by EGF, hydrogen peroxide, and the tyrosine phosphatase inhibitor vanadate, and both are blocked by glucose (Brener *et al.* 2003). These data suggest that protein tyrosine phosphorylation and actin polymerization are related processes occurring in sperm capacitation. Interestingly, we found that EGF, hydrogen peroxide, or vanadate in the absence of bovine serum albumin (BSA) in the incubation medium, cannot, by themselves, induce capacitation (measured by percent of acrosome reacted cells), although they stimulate tyrosine phosphorylation and actin polymerization (Brener *et al.* 2003). This indicates that these two processes are necessary but insufficient for achieving sperm capacitation. The role of BSA is to increase cholesterol efflux from the plasma membrane, which should occur in order to capacitate the sperm.

We show elsewhere (Cohen *et al.* 2004) and here (Table 1) that actin polymerization in bovine sperm is

Table 1 The effect of various inhibitors on actin polymerization of bovine sperm following incubation with various activators. The numbers represent relative fluorescence intensity ± S.E.M. The fluorescence at zero time (before starting the incubation) was 24 ± 1. Percent inhibition was calculated after subtracting the zero time fluorescence. The various inhibitors were used at concentrations in which they represent specific inhibition.

		Activator						
Inhibitor	**No activator** (4 h)	% Inhibition	PMA (30 min)	% Inhibition	dbcAMP (10 min)	% Inhibition	PA (3 min)	% Inhibition
None	100 ± 3	–	102 ± 3	–	92 ± 2	–	112 ± 2	–
BAPTA-AM	26 ± 2	97	25 ± 1	99	88 ± 1	6	100 ± 3	14
Genestein	32 ± 1	90	39 ± 1	81	45 ± 1	69	83 ± 3	33
PD98059	30 ± 1	92	28 ± 1	95	30 ± 2	91	89 ± 2	26
Brefeldin	28 ± 2	95	31 ± 2	91	30 ± 1	91	90 ± 2	25
Neomycin	24 ± 1	100	24 ± 2	100	31 ± 1	90	90 ± 2	25

Bovine sperm were incubated for the indicated times in the presence of the PKC stimulator phorbol myristyl acetate (PMA, 100 ng/ml), PKA activator dibutyryl cAMP (dbcAMP, 1 mM) or the PLD product phosphatidic acid (PA) (3 μg/ml). Before this incubation, the cells were incubated for 1 h with the Ca^{2+} chelator BAPTA-AM or for 10 min with the tyrosine kinase inhibitor genestein (20 μg/ml), the MAPKK inhibitor PD98059 (25 μM), the ARF inhibitor brefeldin (10 μM) or the PLC inhibitor neomycin (5 mM). At the end of incubation, samples were stained with FITC-phalloidin as described by us elsewhere (Cohen *et al.* 2004) and the fluorescence intensity in the sperm head was determined quantitatively using the Meta Morph Image J and Adobe Photoshop processing software.

significantly enhanced after short incubation of the cells with dibutyryl-cAMP (dbcAMP) or the phorbol ester phorbol myristyl acetate (PMA), which activate PKA or protein kinase C (PKC) respectively. This F-actin formation is almost completely blocked by the tyrosine kinase inhibitor genestein (Table 1). This suggests that protein tyrosine phosphorylation is involved in PKA- and PKC-induced actin polymerization, although we could not observe any stimulation in tyrosine phosphorylated proteins under these conditions (Rivlin *et al.* 2004). It is possible that the determination of tyrosine phosphorylation using anti-phosphotyrosine is not sensitive enough to detect very small changes in phosphorylation. This point needs further clarification. The suggested activation of the tyrosine kinase is probably needed for the activation of phospholipase D (PLD) (see below), since actin polymerization induced by exogenous phosphatidic acid (PA) (the product of PLD activity) was only slightly inhibited by genestein (Table 1).

In our recent studies, we show that in bicarbonate-deficient medium, hydrogen peroxide could induce protein tyrosine phosphorylation and actin polymerization, two processes which are essential but not sufficient for capacitation (Brener *et al.* 2003, Rivlin *et al.* 2004). We also show that H_2O_2 stimulates sperm adenylyl cyclase (AC) and tyrosine phosphorylation of the 80 KDa protein in addition to other proteins which are tyrosine-phosphorylated under regular capacitation conditions as well (Rivlin *et al.* 2004). The H_2O_2-stimulated phosphorylation of the 80 KDa protein as well the phosphorylation of an 85 KDa protein are both dependent on intracellular Ca^{2+} concentration ($[Ca^{2+}]_I$) (Rivlin *et al.* 2004). Actin polymerization occurred during capacitation (Table 1) or, induced by exogenous H_2O_2 (not shown), was almost completely blocked by chelating intracellular Ca^{2+}. F-actin formation induced by dbcAMP was only slightly inhibited by chelating intracellular Ca^{2+} (Table 1), suggesting that AC activation depends upon $[Ca^{2+}]_i$.

Since tyrosine phosphorylation of the 80 KDa protein and actin polymerization are $[Ca^{2+}]_i$-dependent processes but not in dbcAMP-treated cells, we suggest that H_2O_2 activates a Ca^{2+}-dependent tyrosine kinase in addition to its direct effect on sperm AC. We must emphasize that the bicarbonate-dependent tyrosine phosphorylation of 8 different sperm proteins is not dependent on $[Ca^{2+}]_i$ (Rivlin *et al.* 2004). Thus, the sperm soluble AC, known to be activated by bicarbonate (Chen *et al.* 2000), is relatively insensitive to $[Ca^{2+}]_i$, whereas the tyrosine phosphorylation of the 80 KDa and 85 KDa proteins as well as actin polymerization are $[Ca^{2+}]_i$-dependent processes. This may suggest that actin polymerization depends on Ca^{2+}-dependent tyrosine kinase(s) which leads to the tyrosine phosphorylation of 80 KDa and 85 KDa sperm proteins.

Actin polymerization is regulated by phospholipase D (PLD)

In a recent study, we showed that PLD is involved in bovine sperm actin polymerization during capacitation (Cohen *et al.* 2004). We also demonstrated that the isoform PLD1 is localized mainly in the acrosomal region of bovine sperm (Garbi *et al.* 2000), suggesting its possible involvement in the acrosome reaction. The requirement of PLD activity for F-actin formation is based on the following: first, actin polymerization is significantly inhibited by the PLD inhibitors butan-1-ol and C2-ceramide but not by butan-2-ol; secondly, exogenous PLD or PA stimulates actin polymerization which is not affected by butan-1-ol, and finally, PLD activity is enhanced during capacitation prior to F-actin formation (Cohen *et al.* 2004).

Relatively fast (within 10–20 min) PLD activation and actin polymerization are induced by activating PKA or PKC, and these activities are completely blocked by butan-1-ol, indicating that PLD mediates these activities. Indeed, we show that PLD is activated in sperm by activation of PKA or PKC (Cohen *et al.* 2004). In bovine sperm, PKCα and PLD1 co-exist as a complex, which decomposes after PKC activation (Garbi *et al.* 2000). This complex is decomposed by activating PKC using its direct activator PMA or by activating the lysophosphatidic acid (LPA) receptor (Garbi *et al.* 2000) resulting in PLD activation (Cohen *et al.* 2004).

Inhibition of PKA activity throughout the four hours of sperm capacitation completely blocked actin polymerization, while inhibition of PKC revealed only partial (40%) inhibition (Cohen *et al.* 2004). These findings are in agreement with the notion regarding the obligatory role of PKA in sperm capacitation (Visconti *et al.* 1995). However, we found that activation of sperm PKC induced fast (20–30 min) PKA-independent actin polymerization (Cohen *et al.* 2004). Moreover, when PKA was blocked (using H-89 or bicarbonate-deficient medium), there was a rapid (30 min) increase in F-actin, which was inhibited by PKC inhibition, as well as PKCα activation (Cohen *et al.* 2004). The effect of H-89 or of bicarbonate-deficient medium on actin polymerization was blocked by inhibition of phospholipase C (PLC) activity, suggesting that PKA inhibits PLC activity in bovine sperm (Cohen *et al.* 2004). When PKA is blocked, PLC can be activated leading to PKC and PLD activation and actin polymerization. Neomycin binds to phosphtidilinositol-4,5 biphosphate (PIP_2) (the substrate of PLC and a cofactor for PLD) and inhibits the activity of these two enzymes as well as actin polymerization (Table 1). However, when actin polymerization is induced by PMA, conditions in which there is no need for PLC activity in order to activate PKC, actin polymerization is inhibited by neomycin (Table 1) but not by the PLC-specific inhibitor U73122 (Cohen *et al.* 2004), indicating that neomycin blocks PLD activation by its binding to PIP_2. The fact that neomycin causes only a small inhibition in actin polymerization induced by exogenous PA

(Table 1), supports this notion. PA is the final product of PLD activity, therefore endogenous PLD activity is not needed when exogenous PA is added to the cells. We also show here that inhibition of MAP kinase-kinase (MAPKK) or ADP-ribosylation factor (ARF) revealed high inhibition in actin polymerization induced under capacitation or by PMA or dbcAMP, but relatively low inhibition when exogenous PA is added to the cells (Table 1). This indicates that MAPK and ARF are involved in PLD activation. To summarize this point, we suggest that PLD can be activated by PKC or PKA pathways via MAPK and ARF activation leading to actin polymerization. In addition, PLD can be activated by cAMP independent of PKA, but this activation does not lead to actin polymerization (Cohen *et al.* 2004). A model describing the various pathways is shown in Fig. 1.

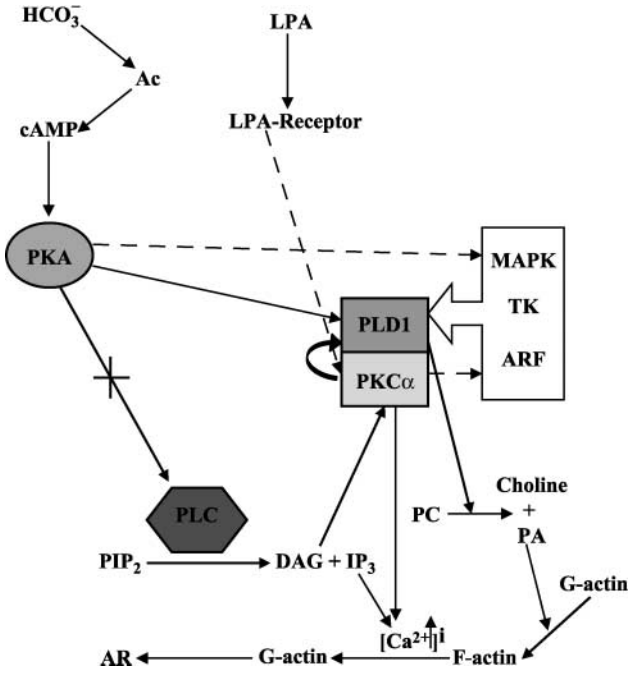

Figure 1 Remodeling of actin in sperm capacitation and the acrosome reaction (AR). G-actin is polymerized to F-actin during sperm capacitation and the fibers should undergo depolymerization in order to accomplish the AR. Actin polymerization depends on PLD activation, which occurs via the HCO_3^-/cAMP/PKA pathway or via the G-protein coupled receptor (GPCR) (LPA-receptor)/PKC pathway. One of the GPCRs in sperm is LPA-receptor which can be activated by LPA, resulting in PKC activation (Garbi *et al.* 2000) and PLD-dependent actin polymerization (Cohen *et al.* 2004). MAP-kinase (MAPK), tyrosine kinase (TK), and ADP-ribosylation factor (ARF) are involved in PLD activation, leading to phosphatidyl-choline (PC) hydrolysis to produce phosphatidic acid (PA), which mediates polymerization of G-actin to F-actin. The binding of capacitated sperm to the egg zona pellucida activates sperm PLC (Tomes *et al.* 1996) to hydrolyze PIP_2 to diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG further activates PKC, and IP_3 activates the Ca^{2+} channel in the outer acrosomal membrane resulting in an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (O'Toole *et al.* 2000). The high increase in $[Ca^{2+}]_i$, activates actin-severing proteins to break down F-actin to G-actin and accomplish the AR.

The crosstalk between PKA and PKC in sperm capacitation

We previously described the gradual binding and activation of PLCγ to the plasma membrane of bovine sperm during capacitation (Spungin & Breitbart 1996). Since PKC activity mediates the acrosome reaction (Breitbart *et al.* 1992), we assume that the described activation of PLCγ would result in PKC activation prior to the acrosome reaction. We showed that activation of PKC during bovine sperm capacitation causes a rapid increase in actin polymerization which is followed by fast depolymerization (Cohen *et al.* 2004), probably due to the increase in $[Ca^{2+}]_i$ (Spungin & Breitbart 1996, Brener *et al.* 2003). However, because high F-actin formation is needed at the end of the capacitation time in order to capacitate the sperm (Brener *et al.* 2003) and this cannot be reached when PKC is highly activated (Cohen *et al.* 2004), capacitation cannot be obtained. Thus, PKC activity should be kept low during sperm capacitation and this is accomplished by activation of PKA which blocks PLC/PKC activities (Cohen *et al.* 2004). Activation of PLC prior to the acrosome reaction would require downregulation of PKA towards the end of the capacitation time. This possibility is supported by others who have shown decreasing activity of adenyl cyclase towards the end of capacitation of mouse and human sperm (Adeoya-Osiguwa & Fraser 2002, Lefievre *et al.* 2002).

It seems that under nonphysiological conditions, activation of PKA or PKC can independently cause PLD activation, leading to actin polymerization (Cohen *et al.* 2004). However, under physiological conditions, the PKA pathway is obligatory for actin polymerization and capacitation, whereas the PKC pathway is important for the acrosome reaction (see model in Fig. 1).

In summary, although PKA or PKC can lead to actin polymerization, a refined balance between the two pathways is required for optimal and sustained activation during sperm capacitation. The activation of PKA would cause inhibition of PLC, and prevent PKC activation during capacitation. It appears that PKA activation promotes capacitation whereas early activation of PKC jeopardizes capacitation. Thus, it would be necessary for inhibition of PKA activity to occur at the end of capacitation in order to achieve PKC activation prior to the acrosome reaction.

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