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PKA-dependent phosphorylation of LIMK1 and Cofilin is essential for mouse sperm acrosomal exocytosis

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ABSTRACT

Mammalian sperm must acquire their fertilizing ability after a series of biochemical modifications in the female reproductive tract collectively called capacitation to undergo acrosomal exocytosis, a process that is essential for fertilization. Actin dynamics play a central role in controlling the process of exocytosis in somatic cells as well as in sperm from several mammalian species. In somatic cells, small GTPases of the Rho family are widely known as master regulators of actin dynamics. However, the role of these proteins in sperm has not been studied in detail. In the present work we characterized the participation of small GTPases of the Rho family in the signaling pathway that leads to actin polymerization during mouse sperm capacitation. We observed that most of the proteins of this signaling cascade and their effector proteins are expressed in mouse sperm. The activation of the signaling pathways of cAMP/PKA, RhoA/C and Rac1 is essential for LIMK1 activation by phosphorylation on Threonine 508. Serine 3 of Cofilin is phosphorylated by LIMK1 during capacitation in a transiently manner. Inhibition of LIMK1 by specific inhibitors (BMS-3) resulted in lower levels of actin polymerization during capacitation and a dramatic decrease in the percentage of sperm that undergo acrosomal exocytosis. Thus, we demonstrated for the first time that the master regulators of actin dynamics in somatic cells are present and active in mouse sperm. Combining the results of our present study with other results from the literature, we have proposed a working model regarding how LIMK1 and Cofilin control acrosomal exocytosis in mouse sperm.

1. Introduction

Mammalian sperm are not able to fertilize eggs immediately after ejaculation. They must undergo a series of biochemical modifications in the female reproductive tract collectively called capacitation (Austin, 1951; Chang, 1951). Capacitation prepares the sperm to develop two main features that are essential for fertilization to occur: the capacity to develop hyperactivated motility and the acquisition of the ability to undergo a secretory event known as acrosomal exocytosis. Men or mice carrying mutations affecting the process of acrosomal exocytosis are infertile or display some degree of subfertility (Dam et al., 2007; Kang-Decker et al., 2001; Lin et al., 2007). To penetrate the zona pellucida (ZP), the extracellular matrix surrounding the egg, mammalian sperm must undergo acrosomal exocytosis in an orderly manner (Buffone et al., 2009; Yanagimachi, 1994). In addition, only acrosome-reacted sperm are able to relocalize Izumo1, a protein essential for sperm egg-fusion, to the equatorial segment (Miranda et al., 2009).

One important unresolved question regarding acrosomal exocytosis is how capacitation triggers the priming of the acrosome for exocytosis. Following the tethering/docking of the outer acrosomal membrane and the plasma membrane, the fusion machinery needs to be assembled for exocytosis to occur (Mayorga et al., 2007). Many groups have identified proteins in sperm that have been previously described to participate in exocytosis of secretory cells, such as Rab3A, the SNARE family, α-SNAP, NSF, complexin, the calcium-binding protein synaptotagmin, calmodulin and dynamin among others (De Blas et al., 2005; Hutt et al., 2005; Michaut et al., 2001; Rodríguez et al., 2011; Roggero et al., 2007; Tomes et al., 2005; Yunes et al., 2002; Zhao et al., 2007). In addition, it was recently shown that membrane hyperpolarization that occurs during capacitation is necessary and sufficient to prepare the sperm to undergo acrosomal exocytosis upon appropriate

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stimuli (De La Vega-Beltran et al., 2012). However, how a change in membrane potential or other molecular events that prepare the sperm to undergo exocytosis interplay during capacitation are still unknown. One possible mechanism that could coordinate different aspects of cell signaling in sperm is the regulation of the actin cytoskeleton. In somatic cells, actin dynamics play a central role in controlling the processes of exo/endocytosis (Porat-Shliom et al., 2013). In several mammalian species, actin polymerization occurs during sperm capacitation (Brener et al., 2003; Cabello-Agüeros et al., 2003; Hernández-González et al., 2000). In addition, polymerized actin filaments (F-actin) have been proposed to be severed prior to acrosomal exocytosis (Cabello-Agüeros et al., 2003; Finkelstein et al., 2010; Spungin et al., 1995). The polymerization of F-actin during capacitation occurs not only in the sperm head but also in the flagellum (Itach et al., 2012). The capacitation induced-actin polymerization that takes place in the sperm head may play a role in stabilizing the fusogenic structures observed during capacitation. In other systems, the cortical actin network acts as a dominant negative clamp that blocks constitutive exocytosis (Muallem et al., 1995). Nemoto et al. (2004) revealed that newly fused granules in pancreatic acinar cells are rapidly coated with F-actin which slows the rate of granule fusion without reducing the overall extent of exocytosis. Thus, F-actin stabilizes structures generated by exocytosis and supports the physiological progression of this event.

Sperm actin polymerization was reported to depend on phospholipase D (PLD) activity and regulated by cross-talk between protein kinases A (PKA) and C (PKC) (Cohen et al., 2004). In somatic cells, small GTPases of the Rho family are widely known as master regulators of actin dynamics. However, the role of these proteins in sperm has not been studied in detail. In mammals, the Rho family is composed by the small GTPases Rho, Rac and Cdc42 which switch between an active GTP-bound and an inactive GDP-bound form. The cycling of Rho GTPases between these two states is regulated by three sets of proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Some reports have shown the presence of small GTPases in mammalian sperm (Baltiérrez-Hoyos et al., 2012; Ducummon and Berger, 2006; Fiedler et al., 2008; Freeman et al., 2002). In guinea pig sperm, it was suggested that small GTPases RhoA, RhoB and Cdc42 may participate in acrosomal exocytosis (Delgado-Buenrostro et al., 2005). However, their function in mature mouse sperm remains mostly unknown.

Small GTPases activate downstream effector proteins when bound to GTP, thereby stimulating a variety of cellular processes such as morphogenesis, migration, vesicle transport, actin dynamics, etc. Interestingly, the activation of Rho, Rac and Cdc42 and the subsequent signal transduction through specific downstream effectors results in the phosphorylation of LIM-kinases (LIMKs; composed of LIMK1 and LIMK2 in mammals) (Amano et al., 2001; Ohashi et al., 2000). In turn, the phosphorylation of LIMKs results in the activation of Cofilin (Bernstein and Bamburg, 2010). Cofilin is a family of actin-binding proteins that regulate assembly and disassembly of actin filaments. The activity of Cofilin is regulated by several mechanisms, of which phosphorylation has been best characterized. LIMKs specifically phosphorylate Cofilin at Ser-3 and thereby inhibit the actin binding, severing, and depolymerizing activities of Cofilin. Thus, protein kinases and phosphatases related to Cofilin phosphorylation and dephosphorylation at Ser-3 are expected to play a central role in the regulation of actin dynamics.

In the present work, we describe and characterize for the first time the participation of small GTPases of the Rho family in the signaling pathway that leads to actin polymerization during mouse sperm capacitation. We found that modulation of LIMK1 and Cofilin by phosphorylation is essential for controlling the process of capacitation-induced actin polymerization and acrosomal exocytosis.

2. Materials and methods

2.1. Reagents

Chemicals were obtained from the following sources: bovine serum albumin (BSA), progesterone, TRITC-labeled phalloidin, and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO); membrane-permeable Exoenzyme C3 Transferase (C4) was obtained from Cytoskeleton (Denver, CO); Y-27632 was acquired from Cayman Chemicals (Ann Arbor, MI); InSolution RAC1 inhibitor (CAS 1177865-17-6) and H89 from Calbiochem; IPA-3, dbcAMP and IBMX from Sigma and BMS-3 from SynKinase; Anti-phosphotyrosine (pY) monoclonal antibody (clone 4G10) was obtained from Upstate Biotechnology (Lake Placid, NY); anti-RHOA, anti-RHOB, anti-RHOC, anti-ROCK, anti-PAK1, anti-LIMK1, anti-LIMK2, anti-Cofilin, anti-phospho-LIMK1/2 (pLIMK1/2) and anti-phospho- Cofilin (pCofilin) antibodies were purchased from Cell Signaling (Danvers, MA); Anti-β-Tubulin monoclonal antibody was obtained from Sigma. Anti-RAC1 and anti-ACTIN antibodies and propidium iodide were purchased from Santa Cruz (Santa Cruz, CA); Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were purchased from Vector and Cell Signaling respectively.

2.2. Animals

HybridF1 (C57BL/6xBalb/C) mature (10–12 week-old) male mice, as well as mature transgenic [BDF1-Tg (CAG- mtDsRed2, Acr-EGFP) RBGS0020sb] male mice who have sperm with acrosomal vesicles expressing green EGFP fluorescence and midpieces (mitochondria) expressing red Ds-Red2 fluorescence (Hasuwa et al., 2010), were used. Animals were maintained at 23 °C with a 12 h light: 12 h dark cycle. Animal experimental procedures were reviewed and approved by the Ethical Committee of IBYME (CE/003-1/2011). Experiments were performed in strict accordance with the Guide for Care and Use of Laboratory Animals approved by the National Institutes of Health (NIH).

2.3. Sperm capacitation

In all the experiments, mouse sperm were recovered by incising the caudae epididymides in 500 μl of a modified Krebs-Ringer medium (Whitten's-HEPES-buffered (WH) medium) (Moore et al., 1994). This medium, which does not support capacitation, was prepared without bovine serum albumin (BSA) and $NaHCO_3$. After 10 min of incubation at 37 °C, epididymides were removed, and sperm were resuspended to a final maximum concentration of 1×10^7 cells/ml in the appropriate medium depending on the experiment performed. For capacitation, a medium without HEPES containing 5 mg/ml of BSA and 15 mM of $NaHCO_3$ was used, and sperm incubated at 37 °C in an atmosphere of 5% CO_2 in air. The pH was maintained at 7.4. To test the effect of inhibitors on capacitation, sperm were pre-incubated with inhibitors in non-capacitating medium for 10 min prior to the beginning of the capacitating period.

2.4. Extraction of sperm proteins

Sperm were collected by centrifugation for 5 min at 3000 rpm, washed (5 min, 10,000 rpm) in 1 ml of PBS, resuspended in sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% v/v glycerol) and