Developmental Biology 362 (2012) 154–161

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Hyper-activated motility in sperm capacitation is mediated by Phospholipase D-dependent actin polymerization

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ARTICLE INFO

Article history:
Received for publication 19 June 2011
Revised 22 November 2011
Accepted 1 December 2011
Available online 8 December 2011

Keywords:
Capacitaction
Actin polymerization
Phospholipase D
Phosphatidic Acid
Motility
Hyper-activated motility (HAM)

ABSTRACT

In order to fertilize the oocyte, sperm must undergo a series of biochemical changes in the female reproductive tract, known as capacitation. Once capacitated, spermatozoon can bind to the zona pellucida of the egg and undergo the acrosome reaction (AR), a process that enables its penetration and fertilization of the oocyte. Important processes that characterize sperm capacitation are actin polymerization and the development of hyper-activated motility (HAM). Previously, we showed that Phospholipase D (PLD)-dependent actin polymerization occurs during sperm capacitation, however the role of this process in sperm capacitation is not yet known. In the present study, we showed for the first time the involvement of PLD-dependent actin polymerization in sperm motility during mouse and human capacitation. Sperm incubated under capacitation conditions revealed a time dependent increase in actin polymerization and HAM. Inhibition of Phosphatidic Acid (PA) formation by PLD using butan-1-ol, inhibited actin polymerization and motility, as well as in vitro fertilization (IVF) and the ability of the sperm to undergo the AR. The inhibition of sperm HAM by low concentration of butan-1-ol is completely restored by adding PA, further indicating the involvement of PLD in these processes. Furthermore, exogenous PA enhanced rapid actin polymerization that was followed by a rise in the HAM, as well as an increased in IVF rate. In conclusion, our results demonstrate that PLD-dependent actin polymerization is a critical step needed for the development of HAM during mouse and human sperm capacitation.

Introduction

Mammalian spermatozoa are unable to fertilize the oocyte immediately after ejaculation. The spermatozoa must first undergo a series of biochemical transformations in the female reproductive tract collectively called capacitation. Once capacitated, spermatozoa can bind to the Zona Pellucida (ZP) of the oocyte and undergo the acrosome reaction (AR), a process that enables the sperm to penetrate and fertilize the oocyte (reviewed in Yanagimachi, 1994). Recent publication suggests that mouse sperm which undergo AR before contact with the ZP can fertilize the egg (Jin et al., 2011). There is no clearly recognizable marker to define capacitation; however, several intracellular changes are known to occur including an increase in cholesterol efflux, a rise in membrane fluidity, increase in intracellular Ca^{+2} concentration and others (reviewed by Breitbart, 2003 and Visconti et al., 1995).

Two additional processes that occur during sperm capacitation are actin polymerization and changes in swimming pattern. We showed that actin polymerization occurs during capacitation of bull, mouse, human and ram sperm (Brener et al., 2003). Actin polymerization is a process in which units of globular actin (G-actin) connect one another to create filamentous Actin (F-actin). Actin is present in the acrosomal space, the equatorial and post acrosomal regions and in the tail (Clarke et al., 1982; Fouquet and Kann, 1992; Ochs and Wolf, 1985; Virtanen et al., 1984). The presence of actin in the tail might be important for the regulation of sperm motility, and its presence in the head suggests the possible involvement of actin in sperm capacitation and the acrosome reaction.

It has been shown in several cell types that PLD is involved in the regulation of actin cytoskeleton (Cross et al., 1996; Kam and Exton, 2001; Kusner et al., 2002). We showed elsewhere that PLD-dependent actin polymerization is a necessary step in the cascade leading to bull sperm capacitation (Brener et al., 2003; Cohen et al., 2004). PLD is a ubiquitous enzyme widely distributed in various mammalian cells including spermatozoa (Cohen et al., 2004; Garbi et al., 2000). PLD hydrolyzes phosphatidylcholine to Phosphatidic Acid (PA) and choline (Exton, 1999). PA can alter the activities of many enzymes and proteins (Rizzo and Romero, 2002). When primary alcohol is present in the medium, PLD also forms rare phospholipids, such as phosphatidylbutanol (Ptd-But), in a unique reaction referred to as "transphosphatidylation". In this case, PA production is inhibited and the signal transduction through PLD is blocked.

PLD, as well as actin polymerization is also involved in regulating cell motility (Kusner et al., 2002). Previous studies have shown that

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doi:10.1016/j.ydbio.2011.12.002

PLD activity is essential for actin localization and actin-based motility in *Dictyostelium* (Zouwail et al., 2005). In addition, sperm motility is critical to the ability of the sperm to fertilize the oocyte. During the capacitation process, sperm change their motility pattern from progressive to hyper-activated motility (HAM) (de Lamirande and Gagnon, 1993; Goodson et al., 2011). HAM is a movement pattern characterized by asymmetrical flagellar beating observed in spermatozoa at the site and time of fertilization in mammals (Demott and Suarez, 1992; Katz and Vanagimachi, 1980; Suarez et al., 1987), and may be critical to fertilization success (Ho and Suarez, 2001). The HAM may help spermatozoa swim faster and generate enough force to penetrate cumulus cells and ZP during fertilization (Jin et al., 2007). If HAM is prevented, fertilization cannot occur (Amieux and McKnight, 2002).

Even though the role of PLD and motility is well established in somatic cells, the presence and the possible role of this enzyme in sperm motility are not fully understood. Earlier studies in sperm showed that F-actin is involved in sperm motility in guinea pig (Azamar et al., 2007).

The objective of the present study was to investigate the role of PLD-dependent actin polymerization in the development of HAM during mouse and human sperm capacitation.

Materials and methods

Materials

Butan-1-ol was obtained from Frutarom Industries Ltd. Calcium ionophore A23187 and protease inhibitor cocktail were obtained from Cal-Biochem (San Diego, CA, USA). Capacitation medium, F-10 (Ham's) nutrient mixture with L-glutamine, was purchased from Biological Industries (Kibbutz Beit Haemek, IL).Goat anti PC-PLD1 (C-17) and Donkey anti-goat IgG (H + L)-HRP conjugate were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-goat IgG (H + L)-Alexa Fluor 568 was purchased from Invitrogen (Oregon, USA). Rabbit anti p-PLD1 (Thr 147) was purchase from Cell Signaling. Other chemicals were obtained from Sigma.

Sperm preparation

Sexually mature male mice (C57BlxA.G) were sacrificed by CO_2 asphyxiation. The pair of cauda epididymides and part of the vas deferens were rapidly removed and minced on 0.5 ml HM medium in modified Krebs–Ringer bicarbonate medium (Visconti et al., 1995). The sperm were released from the epididymal lumen for 5 min at 37 °C. The medium was carefully collected and the cells were washed by centrifugation (780 × *g*, 5 min) in the same medium, and then left for "swim-up" for 5 min at 37 °C. The motile fraction was carefully collected, and the washed cells were counted and maintained at 37 °C until use.

Human semen was initially liquefied. The semen was then loaded on a Percoll's gradient (80, 40, and 20%) and centrifuged for 30 min at 6750 rpm at room temperature. The lower layer containing the sperm was collected and washed twice in Ham's F-10 and then recentrifuged and then left for "swim-up" for 5 min at 37 °C. The motile fraction was carefully collected, and the washed cells were counted and maintained at 37 °C until use (only sperm preparations that contained at least 70% motile sperm were used in the experiments).

Sperm capacitation — in vitro

Mouse epididymal sperm ($1*10^7$ cells/ml) were capacitated by incubation in capacitation HMB medium containing: 119.4 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$, 25 mM Hepes (pH 7.4), 25 mM sodium lactate, 5.56 mM Glucose, 0.001% Phenol Red, 10 IU/ml Penicillin, 3 mg/ml BSA. The cells were incubated in this capacitation medium for 90 min at 37 °C with 5% CO_2 (Visconti et al., 1995).

Human sperm ($1*10^7$ cells/ml) were capacitated by incubation in capacitation medium (Ham's F-10) supplemented with 3 mg/ml BSA for 3 h at 37 °C in 5% CO_2 (Finkelstein et al., 2010).

Immunocytochemistry assay

For immunocytochemistry staining, sperm cells were spread on microscope slides, air dried, fixed in formaldehyde (2%) for 10 min, dipped in 0.5% Triton X-100 in TBS for 30 min, and washed three times at 5 min intervals with TBS. Nonspecific reactive sites were blocked with TBS containing 5% BSA for 30 min at room temperature. The cells were then incubated for 2 h with polyclonal anti PC-PLD1 antibody diluted 1:50 in 1% BSA at 37 °C. Next, the slides were washed twice in TBS-T and once (5 min) in TBS. The bound antibody was detected using anti-goat Alexa (1:50 in 1% BSA for 2 h incubation at 37 °C), followed by washing once in TBS-T and twice with H_2O at 5 min intervals and mounting in FluoroGuard Antifade Reagent (BioRad Lab., Richmond, CA). Nonspecific staining was determined by incubating the sperm without primary antibody, and no staining was detected.

Fluorescence staining of actin polymerization

Sperm cells were spread on microscope slides. After air drying, sperm were fixed in formaldehyde (2%) for 10 min, dipped in 0.2% Triton X-100 in TBS for 30 min, washed three times at 5 min intervals in distilled water, air dried, incubated with Phalloidin-FITC (1 μM in TBS) for 45 min, washed six times with H_2O at 5 min intervals, and mounted with FluoroGuard Antifade (BioRad Lab., Richmond, CA).

Microscopy

All images were captured using an Olympus AX70 microscope at a magnification of ×400. The microscope was equipped with Olympus DP50 digital camera and by "Viewfinder Lite" software (version 1 from Pixera Corporation). All fluorescence determinations were done under nonsaturated conditions. Each experiment and staining were done on the same day, and the sperm were photographed within 24 h to reduce the loss of fluorescence. All cell preparations from a single experiment were photographed in the same session and at the same exposure time. The fluorescence intensity was quantified using "Image J" software. For actin polymerization determinations, all experiments were carried out in duplicates and at least 100 cells (5–7 pictures) per slide were quantified for cell fluorescence intensity.

Motility measurements in CASA device

Calibration of the measurements: The percentage of sperm presenting hyper-activated motility was carefully calculated by eye observation using light microscope. The CASA measurements were calibrated according to these determinations.

Sperm cells ($1*10^7$ cells/ml) were incubated in capacitation medium. Samples (5 μl) were placed in standard count four chamber slide (Leja, Nieuw-Vennet, Netherlands) and analyzed by CASA (Computer-Aided Sperm Analysis) device with IVOS software (version 12, Hamilton-Thorne Biosciences). Up to ten sequels, 10 s long, were acquired for each sample. Cells were analyzed according to parameters identifying mouse sperm motility. The proportion of hyper-activated (HAM) spermatozoa in each sample was determined using the SORT function of the CASA instrument. HAM was defined by curvilinear velocity (VCL) > 90 μm/s, linearity (LIN) < 20% and an amplitude of lateral head (ALH) > 7 μm (Mortimer et al., 1998; Nassar et al., 1998). In human sperm, HAM was defined by VCL > 100 μm/s, LIN < 60% and ALH > 5 μm (Almog et al., 2008).