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Regulation of sperm motility by $PIP_{2(4,5)}$ and actin polymerization

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ABSTRACT

Actin polymerization and development of hyperactivated (HA) motility are two processes that take place during sperm capacitation. In previous studies, we demonstrated that the increase in F-actin during capacitation depends upon inactivation of the actin severing protein, gelsolin, by its binding to phosphatydilinositol-4, 5-bisphosphate (PIP_2). Here, we showed for the first time the involvement of PIP_2/gelsolin in human sperm motility before and during capacitation. Activation of gelsolin by causing its release from PIP_2 inhibited sperm motility, which could be restored by adding PIP_2 to the cells. Reduction of PIP_2 synthesis inhibited actin polymerization and motility, and increasing PIP_2 synthesis enhanced these activities. Furthermore, sperm demonstrating low motility contained low levels of PIP_2 and F-actin. During capacitation there was an increase in PIP_2 and F-actin levels in the sperm head and a decrease in the tail. In sperm with high motility, gelsolin was mainly localized to the sperm head before capacitation, whereas in low motility sperm, most of the gelsolin was localized to the tail before capacitation and translocated to the head during capacitation. We also showed that phosphorylation of gelsolin on tyrosine-438 depends on its binding to PIP_2. Activation of phospholipase C by Ca^{2+}-ionophore or by activating the epidermal-growth-factor-receptor inhibits tyrosine phosphorylation of gelsolin. In conclusion, the data indicate that the increase of PIP_2 and/or F-actin in the head during capacitation enhances gelsolin translocation to the head. As a result the decrease of gelsolin in the tail allows keeping high level of F-actin in the tail, which is essential for the development of HA motility.

Introduction

Ejaculated spermatozoa must undergo a series of biochemical modifications in order to attain the ability to penetrate and fertilize the oocyte. These processes occur in the female reproductive tract and are collectively called capacitation. The capacitated spermatozoon is able to bind to the Zona Pellucida (ZP) of the oocyte and to undergo the acrosome reaction (AR), a process that allows sperm penetration and fertilization of the egg (reviewed in Yanagimachi (1994)).

Capacitation includes multiple physiological and biochemical modifications (reviewed by (Breitbart, 2003; Breitbart and Etkovitz, 2011; Salicioni et al., 2007; Visconti et al., 1995)). 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 (Hernandez-Gonzalez et al., 2006), changes in protein phosphorylation and protein kinase activity (Arcelay et al., 2008; Baldi et al., 2000; Visconti, 2009), and increases in bicarbonate concentration (HCO_3^-), intracellular pH (pHi), Ca^{2+} and cAMP levels.

Additionally, our previous studies showed that actin polymerization occurs during sperm capacitation, and rapid depolymerization occurs prior to the acrosome reaction (Breitbart et al. 2005; Brener et al., 2003; Cohen et al., 2004). Actin is present in the sperm head in acrosomal, equatorial and postacrosomal regions as well as 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 (Itach et al., 2012), and its presence in the head suggests the possible involvement of actin in the sperm acrosome reaction (Finkelstein et al., 2010).

A connection was reported between actin polymerization and cell motility in other cell types (Bernheim-Groswasser et al., 2005; Carlier et al., 2003; Hotulainen and Lappalainen, 2006). Earlier studies in guinea pig sperm showed that F-actin is involved in sperm motility, and random severing of F-actin filaments inhibits flagellar motility (Azamar et al., 2007).

During the capacitation process, sperms change their motility pattern from progressive to HA motility (de Lamirande and Gagnon, 1993; Goodson et al., 2011). HA motility is characterized by an increase in flagellar bending amplitude, and an increase in average lateral head movement (Demott and Suarez, 1992;

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Katz and Vanagimachi, 1980; Suarez et al., 1987). It was shown that the efficiency of penetration of hyper-activated sperm to the ZP is much higher than that of non-hyper-activated sperm (Ho and Suarez, 2001). The HA motility may help spermatozoa swim faster and generate enough force to penetrate cumulus cells and ZP during fertilization (Jin et al., 2007). Inhibition of HA motility prevents fertilization from occurring (Amieux and McKnight, 2002). In addition, our recent study indicated the importance of actin polymerization for the development of HA motility. Sperm motility as well as the development of HA motility during capacitation is also mediated by PLD-dependent actin polymerization (Itach et al., 2012). Phosphatidylinositol 4, 5-bisphosphate (PIP_2), the major polyphosphoinositide in mammalian cells, is required as a cofactor for the activation of PLD in many cell types (Brown et al., 1993; Hodgkin et al., 2000; Liscovitch et al., 1994; Pertile et al., 1995; Schmidt et al., 1996).

PIP_2 is a minor lipid of the inner leaflet of the plasma membrane that controls the activity of numerous proteins and serves as a source of second messengers. Aside from the fundamental signaling role of its derivatives, PIP_2 itself controls the activity of several integral membrane proteins, and affects many proteins associating with the membrane due to PIP_2 binding. Although PIP_2 comprises only 1% of all plasma membrane phospholipids, its extraordinary versatility puts it in the center of plasma membrane dynamics governing motility, cell adhesion, endo- and exocytosis (Di Paolo and De Camilli, 2006; Hokin and Hokin, 1953; Yin and Janmey, 2003). Early studies established the role of PIP_2 as the source of two second messengers in the cell, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Berridge and Irvine, 1984). PIP_2 is important in exocytic and endocytic membrane traffic (Cremona and De Camilli, 2001; Martin, 1998; Ungewickell and Hinrichsen, 2007), ion channel and transporter function (Hilgemann et al., 2001; Suh and Hille, 2005), enzyme activation (McDermott et al., 2004), and protein recruitment (Balla, 1998, 2005; Janmey and Lindberg, 2004; Lemmon, 2003; Takenawa and Itoh, 2006).

PIP_2 serves as an effector of multiple downstream proteins such as MARCKS, gelsolin, PLD, and PI3K. These proteins are present in sperm cells (Etkovitz et al., 2007; Jungnickel et al., 2007) and are involved in regulation of sperm capacitation and/or the acrosome reaction.

Actin polymerization is one of the processes occurring during sperm capacitation, and F-actin breakdown must take place to achieve the acrosome reaction (Brener et al., 2003). The assembly of G-actin to form F-actin, as well as the disassembly of F-actin is highly regulated events (Tanaka et al., 1994; von Bulow et al., 1995, 1997). Gelsolin severs assembled actin filaments, and caps the fast growing plus end of free or newly severed filaments in response to Ca^{2+}, and is inhibited by PIP_2. In a recent study, we showed that the actin severing protein, gelsolin, must be inhibited during capacitation for actin polymerization to occur (Finkelstein et al., 2010).

Functional analysis of fibroblasts isolated from gelsolin knockout mice indicated that gelsolin mediates cell migration, since gelsolin deficiency resulted in the formation of excessive stress fibers, defective ruffling and cell motility (Azuma et al., 1998; Witke et al., 1995). In view of the biological activities of gelsolin, these data suggested that it plays an essential role in the regulation of actin polymerization and cycling necessary for cell motility. A correlation between the level of gelsolin expression, cancer, cell migration and organization of the actin cytoskeleton in other cells was shown, as well (Litwin et al., 2009).

Phosphoinositides bind gelsolin and release it from actin filament ends, exposing sites for actin assembly (Janmey et al., 1987). We showed that the release of bound gelsolin from PIP_2 by PBP10, a peptide containing the PIP_2-binding domain of gelsolin, or by activation of phospholipase C, which hydrolyzes PIP_2, causes rapid Ca^{2+}-dependent F-actin depolymerization as well as an enhanced acrosome reaction (Finkelstein et al., 2010).

Even though the role of gelsolin and actin polymerization is well established, the presence and the possible role of this protein in sperm motility are not fully understood. We therefore hypothesized that PIP_2 and gelsolin are involved in regulating sperm motility and in the development of HA motility in sperm capacitation. In support of our hypothesis, we observed that an increase in PIP_2 and F-actin in the sperm head during capacitation leads to the translocation of gelsolin from the tail to the head, allowing an increase in F-actin in the tail, and resulting in the development of HA motility. We further showed that the basal levels of PIP_2 and F-actin and the localization of gelsolin, control cell motility before capacitation, as well as the development of hyper-activated motility during sperm capacitation.

Materials and methods

Materials

PBP10 (Polyphosphoinositide-Binding Peptide, rhodamine B conjugated), U73122, and A23187 were obtained from Cal-Biochem (San Diego, CA). Capacitation medium, F-10 (HAM) nutrient mixture with L-glutamine, was purchased from Biological Industries (Kibbutz Beit Haemek, IL). SU6656 was obtained from Cal-Biochem. Rabbit polyclonal anti-gelsolin and rabbit polyclonal to EGFR (y845) were obtained from Abcam; anti-beta-actin HRP-conjugated and secondary goat anti-rabbit IgG (H+L)–HRP conjugate were obtained from Bio-Rad (Bio-Rad Lab., Richmond, CA, USA). Secondary mouse anti-rabbit Alexa-fluor-568 IgM and anti-PIP_2 (sc-53412) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa-fluor-488 goat anti-rabbit was obtained from Invitrogen (Oregon, USA). Antibody against P-gelsolin Tyr438 was kindly provided by Dr. Joël Vandekerckhove from the Department of Biochemistry, Ghent University (UGent) (Department of Medical Protein Research, Flanders Institute for Biotechnology (VIB) Albert Baertsoenkaai 3B-9000 Gent, Belgium). All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd., Rehovot, Israel) unless otherwise stated.

Sperm preparation

Human semen was liquefied; afterward, the semen was loaded on a gradient (PureCeptyion Lower and Apper Phase Gradient 80% and 40%) and centrifuged for 30 min at 6750 rpm at room temperature. The lower layer containing the sperm was collected and washed twice in HAM F-10, then spun again and allowed to "swim up" after the last wash at 37 °C. The motile cells were collected without the pellet and resuspended in capacitation medium.

Sperm samples were separated to "high" ($>55\%$ motile cells) and "low" ($<40\%$ but $>20\%$ motile cells) motilities according to the preliminary parameters provided by male fertility laboratory of Sheba hospital.

Sperm capacitation

Human sperm (1×10^7 cells/ml) was capacitated by incubation in capacitation media, HAM F-10 supplemented with 3 mg/ml BSA. The cells were incubated in this medium for 3 h at 37 °C in 5% CO_2. The capacitation state of the sperm was confirmed after the 3 h incubation by examining the ability of the sperm to undergo the acrosome reaction.

Assessment of sperm acrosome reaction

Washed cells (1×10^7 cells/ml) were capacitated for 3 h at 37 °C in capacitation medium. The inhibitors indicated were added after 3 h of incubation for 10–20 min and the inducers were then added for another 60 min of incubation. The percentage of acrosome-reacted sperm was determined microscopically on air-dried sperm smears using FITC-conjugated *Pisum sativum* agglutinin (PSA). An aliquot of spermatozoa was smeared on a glass slide and allowed to air-dry. The sperm was then permeabilized by methanol for 15 min at room temperature, washed three times at 5 min intervals with TBS, air dried, and then incubated with FITC-PSA (60 mg/ml) at room temperature in the dark for 60 min, washed twice with H_2O at 5 min intervals, and mounted with FluoroGuard Antifade (Bio-Rad Lab). For each experiment, at least 150 cells per slide on duplicate slides were evaluated (total of 300 cells per experiment). Cells with green staining over the acrosomal cap were considered acrosome intact; those with equatorial green staining or no staining were considered acrosome reacted.

Immunoblot analysis

Sperm lysates were prepared by the addition of lysis buffer that contained 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 6% SDS, protease inhibitor cocktail 1:100 (Cal-Biochem), 50 μM NaF, 50 μM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM Na_3VO_4, to the pellet, and the mixture was vortexed for 10 min at room temperature. Lysates were then centrifuged at 14,000 × g for 5 min at 4 °C, the supernatant was removed, and the protein concentration was determined by the Bradford method (Bradford, 1976). Sample buffer (2 ×) was added to the supernatant and boiled for 5 min. The extracts were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Western blotting was performed (200 mA for 1 h) using a buffer composed of 25 mM Tris (pH 8.2), 192 mM glycine, and 20% methanol. For western blotting, the nitrocellulose membranes were blocked with 5% BSA (for anti-p-gelsolin, anti p-EFGR, tubulin and actin) in Tris-buffered saline (pH 7.6) that contained 0.1% Tween-20 (TBST), for 30 min at room temperature. P-gelsolin and actin were immunodetected using polyclonal anti-p-gelsolin antibody (diluted 1:1000), horseradish peroxidase (HRP)–conjugated anti-actin (1:3000), and polyclonal anti-p-EGFR antibody (diluted 1:3000). The membranes were incubated overnight at 4 °C with the primary antibodies. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with the appropriate secondary antibody diluted at 1:10,000. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham).

Fluorescence staining of actin filaments

Sperm cells were spread on microscope slides. After air-drying, sperm was fixed in 2% formaldehyde in TBS for 10 min, placed in 0.2% Triton X-100 in TBS for 30 min, washed three times at 5 min intervals in distilled water, air dried, and then incubated with phalloidin-FITS (4 μM in TBS) for 60 min, washed four times with water at 10 min intervals, and mounted with FluoroGuard Antifade (Bio-Rad).

Immunocytochemistry

For immunocytochemistry, sperm cells were spread on glass slides, air-dried, fixed in formaldehyde (4%) for 10 min, dipped in 0.5% Triton X-100 with 10% BSA in TBS for 60 min, and washed three times at 5 min intervals with PBS. The cells were then incubated for 24 h at 4 °C with rabbit polyclonal anti-gelsolin antibody diluted 1:150 in TBS containing 1% BSA. Next, the slides were washed three times at 5 min intervals with PBS. The bound antibody was detected using Alexa-fluor-568 donkey anti-goat IgG (Invitrogen molecular probes, 1:200 dilution), incubated for 1 h at 37 °C, and followed by washing three times at 5 min intervals with PBS. Next, the slides were stained with DAPI for 3 min, and washed with DDW twice (5 min intervals). Finally, the slides were mounted in FluoroGuard Antifade. Nonspecific staining was determined by incubating the sperm without primary antibody, and no staining was detected.

Microscopy

All images were captured on an Olympus AX70 or AxioimegerZ1 microscope at 400 × magnification. The microscopes were equipped with an Olympus DP50 digital camera and with a Viewfinder Lite ver. 1 software (Pixera Corp., Los Gatos, CA). All fluorescence determinations were performed under nonsaturated conditions. Each experiment and staining were performed on the same day, and sperm pictures 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. The fluorescence intensity was quantified using the MetaMorph Image J software (National Institutes of Health) and the background intensity was subtracted. For F-actin and gelsolin localization in the head, all experiments were carried out in duplicate and at least 100 cells (5–7 pictures) per slide were quantified for fluorescence intensity. The quantification of PIP_2 in the head or tails of sperm was performed using the MetaMorph Image J software (National Institutes of Health).

Motility measurements in CASA device

Calibration of the measurements: the percentage of sperm presenting hyper-activated motility was carefully calculated by eye observation using a 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 a standard count four chamber slide (Leja, Nieuw-Vennet, Netherlands) and analyzed by Computer-Aided Sperm Analysis (CASA) device with an IVOS software (version 12, Hamilton-Thorne Biosciences). Up to 10 sequels, 10 s long were acquired for each sample. Cells were analyzed according to parameters identifying human sperm motility. The proportion of hyper-activated (HA) spermatozoa in each sample was determined using the SORT function of the CASA instrument. In human sperm, HA motility was defined by VCL > 100 μm/s, LIN < 60% and ALH > 5 μm (Almog et al., 2008; Mortimer and Mortimer, 1990).

Determination of cellular levels of PIP_2

Two assays are used: for the first, PBP10-rhodamine (10 μM) was added to pretreated sperm cells, then the cells were spread on microscope slides or placed in triplicate in 96 well black ELISA plates for red fluorescence measurement. PIP_2 expression levels were determined with a microarray scanner (LS Reloaded, Tecan) using a 550 nm laser and 595 nm filter (rhodamine B); the measured ratio is in inverse proportion to PIP_2 levels. Each experiment and staining were performed on the same day, and sperm pictures were photographed within 1 h to reduce fluorescence loss. All images were captured on an Olympus AX70 or used AxioimegerZ1.

The second method was an immunocytochemical assay using anti-PIP_2 antibodies and second antibodies conjugated to FITC. Each experiment and staining were performed on the same day,

and slides were photographed within 24 h to reduce the loss of fluorescence. All images were captured on an Olympus AX70. The fluorescence intensity was quantified using the MetaMorph Image J software (National Institutes of Health) and the background intensity was subtracted.

Statistical analysis

Data are expressed as mean ± SD of at least three experiments for all determinations. Statistical significance was calculated by Student's *t*-test or by ANOVA with Bonferroni's post-hoc comparison test using a SPSS software (Chicago, IL, USA).

Results

Role of PIP_2 in sperm motility

In this study, we wished to investigate the role of sperm PIP_2 in the regulation of sperm total motility and hyper-activated (HA) motility during capacitation. To this end, we first investigated the effect of wortmannin (WT), an inhibitor of PI4K, on total motility of human sperm; this inhibitor was previously shown to inhibit the activity of PI4K in bovine sperm and other cells (Balla et al., 1997; Etkovitz et al., 2007; Powis et al., 1994). Fig. 1A–C shows that WT strongly inhibited human sperm total and hyper-activated motility, and this effect is reversed by addition of external PIP_2. Respectively to the reduction of motility we observed significant reduction of F-actin levels by WT (Fig. 2A and B).

We recently showed that motility of mouse sperm depends on actin polymerization (Itach et al., 2012). Therefore, we next probed the involvement of gelsolin, an actin severing protein, on sperm motility. Gelsolin was activated in two ways: first, we used the peptide PBP10 which is composed of the binding motif of gelsolin to PIP_2 and competes with gelsolin binding to PIP_2, resulting in releasing PIP_2-bound gelsolin and enabling its severing activity on F-actin (Cunningham et al., 2001). In a second approach, PIP_2 levels were reduced by inhibiting PI4K causing the release of gelsolin and its activation. To inhibit gelsolin, we elevated PIP_2 levels using phosphatidic acid (PA), an activator of PI4/5K (Moritz et al., 1992) and spermine, which is also a PI4K activator (Etkovitz et al., 2007), resulting in increased cellular PIP_2 and F-actin levels.

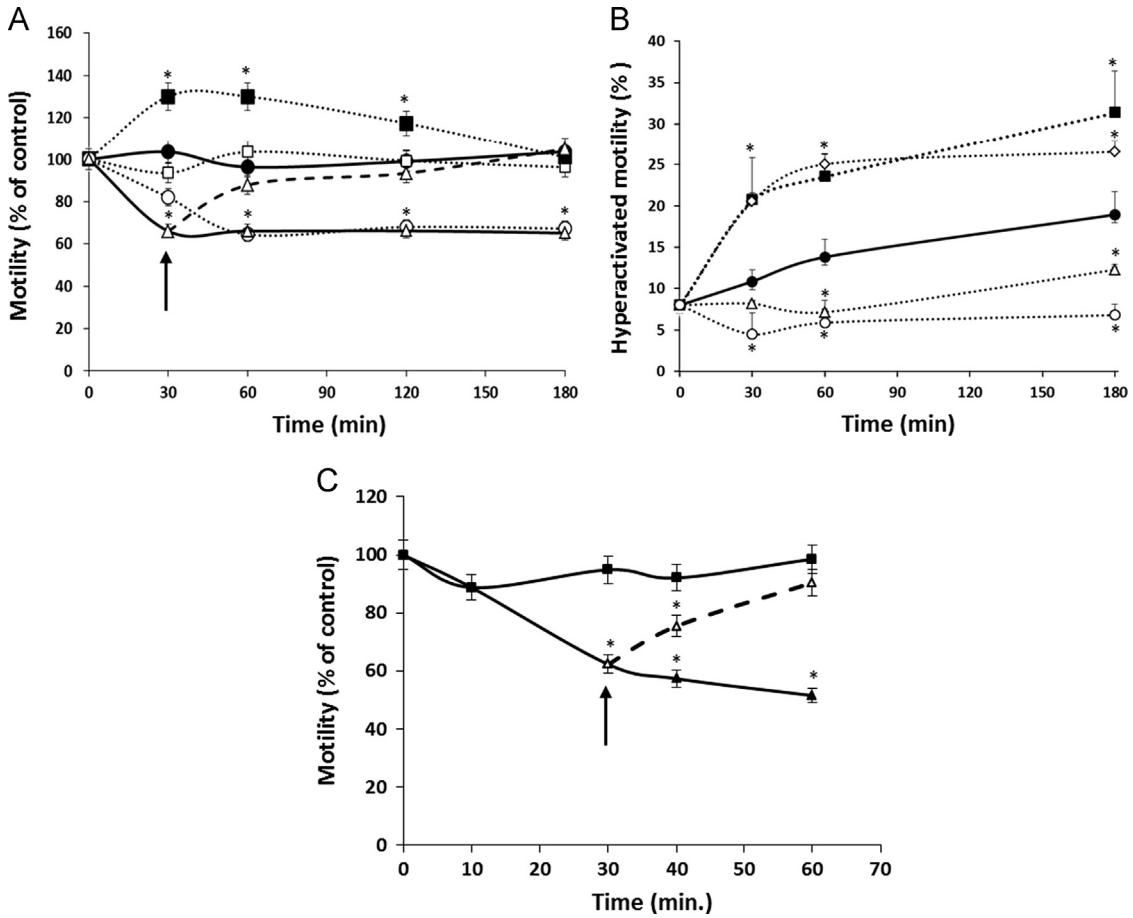

Fig. 1. Effect of spermine, wortmannin, PBP10 and PA on total and hyperactivated sperm motility. (A) Total motility in response to the addition of spermine, wortmannin, PBP10 and PIP_2 to sperm: human spermatozoa incubated in capacitation medium (F-10) with or without PBP10 (1 μM), spermine (10 μM) or wortmannin (10 μM). After 30 min of incubation 5 μM PIP_2 was added to the cells treated with PBP10 (see arrow). Samples were taken at different time, and sperm motility was determined by CASA using the IVOS device. The values represent sperm total motility mean ± SD of duplicates from three experiments. * Represents significant difference compared with control, $P < 0.05$. (●) Control, (–□–) spermine (high motility), (–■–) spermine (low motility), (–○–) wortmannin, (Δ) PBP10, and (–Δ–) PBP10+PIP_2. (B) HA motility in response to PA, spermine, wortmannin, and PBP10. Human spermatozoa incubated in capacitation medium (F-10) with or without PA (3 μg/ml), spermine (10 μM), wortmannin (10 μM) or PBP10 (1 μM). Samples were taken at different time, and sperm hyper-activated motility was determined by CASA using the IVOS device. The values represent the percentage of cell presenting HA motility, the mean ± SD of duplicates from three experiments. * Represents significant difference compared with control, $P < 0.05$. (●) Cont., (–■–) spermine, (–Δ–) PBP10, (–◇–) PA, and (–○–) wortmannin. (C) Total motility in response to the addition of wortmannin and PIP_2 to sperm: human spermatozoa incubated in capacitation medium (F-10) with or without wortmannin (10 μM). After 30 min of incubation 5 μM PIP_2 was added to the cells treated with wortmannin (see arrow). Samples were taken at different time, and sperm motility was determined by CASA using the IVOS device. The values represent the sperm total motility mean ± SD of duplicates from three experiments. ⁎ Represents significant difference compared with control, $P < 0.05$. (■) Control, (▲) wortmannin, and (–Δ–) wortmannin+PIP_2.

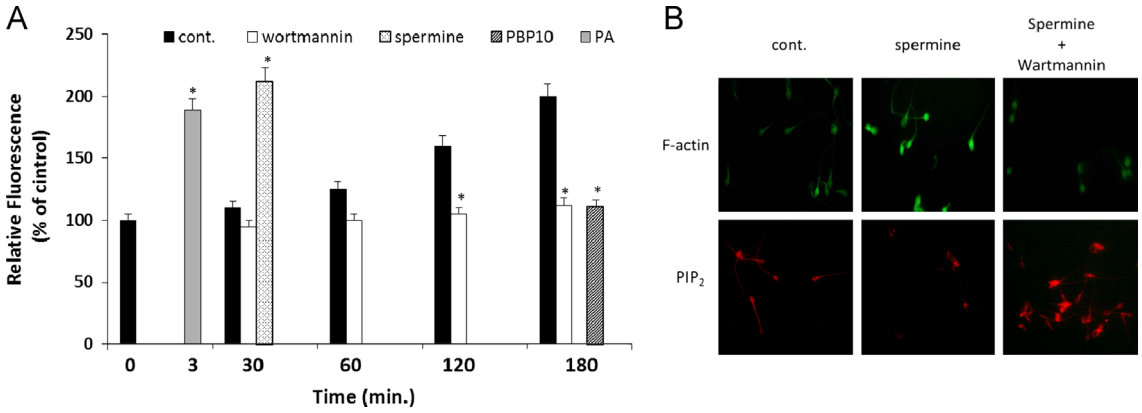

Fig. 2. Effect of spermine, wortmannin, PBP10 and PA on intracellular levels of F-actin and PIP_2. (A) F-actin in response to PA, spermine, PBP10, and wortmannin: human spermatozoa incubated in capacitation medium (F-10) with or without PA (3 μg/ml), spermine (10 μM), or PBP10 (1 μM), or wortmannin (WT, 10 μM). Samples were taken out at different time; the cells were stained with FITC-phalloidin and analyzed for fluorescence intensity in the sperm. The values represent the mean ± SD of duplicates from three experiments. * Represents significant difference compared with zero time control, $P < 0.05$. (B) F-actin and PIP_2 in response to spermine and wortmannin: human spermatozoa incubated in capacitation medium (F-10) with spermine (10 μM) or wortmannin (10 μM). After 30 min samples were examined, the cells were stained with FITC-phalloidin or with PBP10, photographed under a fluorescence microscope to determine fluorescence intensity in the sperm.

PBP10, causes fast F-actin depolymerization due to the release of bound gelsolin from PIP_2, resulting in its activation as an actin severing protein (Finkelstein et al., 2010). PBP10 inhibited total sperm motility (Fig. 1A) as well as the development of hyper-activated motility during sperm capacitation (Fig. 1B). The inhibitory effect of PBP10 on total motility was almost completely restored by adding exogenous PIP_2 to the cells (Fig. 1A). The polyamine, spermine, activates PI4K in bovine sperm resulting in enhanced actin polymerization (Etkovitz et al., 2007). Spermine increased total motility when the sperm sample exhibited relatively low motility, but not when motility was high (Fig. 1A). However, spermine caused a strong increase in hyper-activated motility (Fig. 1B), conditions under which spermine enhances the cellular levels of F-actin and PIP_2 (Figs. 2A and B, and 3B). The opposite effect, meaning reduction of total motility (Fig. 1A), HA motility (Fig. 1B), F-actin (Fig. 2A and B), and PIP_2 (Figs. 2B and 3B) was achieved by WT, indicating that the levels of PIP_2 regulate actin polymerization and HA motility through gelsolin. The inhibitory effect of WT on sperm motility is almost completely recovered by adding PIP_2 indicating the specificity of WT as an inhibitor of PIP_2 synthesis (Fig. 1C). Addition of PA stimulated HA motility (Fig. 1B) as well as actin polymerization (Fig. 2A) further supporting this pathway. In our previous studies we showed that PA, the product of phospholipase D activity on phosphatidylcholine, causes a very fast increase in F-actin and HA motility in bovine and mouse sperm (Cohen et al., 2004; Itach et al., 2012).

In Fig. 1A we showed that spermine increases total motility only in sperm with relatively low basal motility. We next examined possible explanations for this selective effect. In Fig. 3A, we analyzed the levels of F-actin and HA motility in sperm representing low and high total motility before and after capacitation. In high-motility sperm, we found relatively high levels of F-actin and a high percentage of HA motility before capacitation, and these parameters were enhanced during capacitation (Fig. 3A). In low-motility sperm, lower F-actin and HA motility were found before capacitation, and after capacitation the levels of these parameters were enhanced but did not reach the levels found in the high-motility samples (Fig. 3A). In addition, the levels of PIP_2 in high-motility sperm were much higher in comparison to low-motility sperm before or during capacitation. Moreover, spermine increased PIP_2 levels in sperm representing relatively low motility (Fig. 3C), to level resembling to sperm representing relatively high motility. These data suggest that basal sperm motility is regulated by the levels of PIP_2 in the cells.

Role of gelsolin in sperm motility

In our previous study, we show that in order to increase actin polymerization during sperm capacitation gelsolin is inactivated by its binding to PIP_2. Moreover, we show that gelsolin translocates from the sperm tail to the head during capacitation (Finkelstein et al., 2010). It is likely that there are two reasons for this translocation. First, gelsolin is required in the head to depolymerize F-actin which is a prerequisite for the occurrence of the acrosome reaction (Brener et al., 2003), and second, gelsolin is excluded from the tail to prevent depolymerization of F-actin, which is important for the development of HA motility (Itach et al., 2012). In the present work we attempt to understand the mechanisms that mediate the translocation of gelsolin from the tail to the head during capacitation. Since gelsolin binds to PIP_2 and to F-actin, we assumed that gelsolin translocation to the head depends on an increase in PIP_2 and F-actin in the head during sperm capacitation. Indeed we found that the levels of PIP_2 were enhanced in the sperm head and reduced in the tail during the first hour of capacitation and afterwards a decrease to the basal level is observed (Fig. 4B). Likewise, as expected, F-actin increased mainly in the sperm head during capacitation (Fig. 4B upper pictures). We also observed that the elevation of F-actin occurs simultaneously to the elevation of PIP_2 in the sperm head (Fig. 4B). These results provide additional support to our hypotheses that PIP_2 elevation in the sperm head keeps gelsolin inactive and enables the assembly of F-actin.

Next, we examined the location of gelsolin in the sperm presenting low or high motility. Before capacitation, in sperm with low motility, most of the gelsolin was localized to the tail, while in high motility sperm, a large proportion of gelsolin was already localized to the head (Fig. 5A). After capacitation, gelsolin was localized to the head in low and high motility sperm (Fig. 5A). Treatment with Ca^{2+}-ionophore A23187 (which stimulates PIP_2 hydrolysis by activation of phospholipase C), or with PBP10 (which releases gelsolin from binding to PIP_2), at the end of the capacitation period, induced reverse translocation from the head to the tail (Fig. 5A–C). Inhibition of PIP_2 synthesis by WT prevented the translocation of gelsolin to the head, and enhancing PIP_2 synthesis by spermine or PA significantly increased gelsolin translocation to the head (Fig. 6A–C). In cells presenting high motility, in which gelsolin is already in the head, treatment with WT caused re-translocation of gelsolin from the head to the tail, while in low motility sperm, in which gelsolin is mainly localized to the tail,

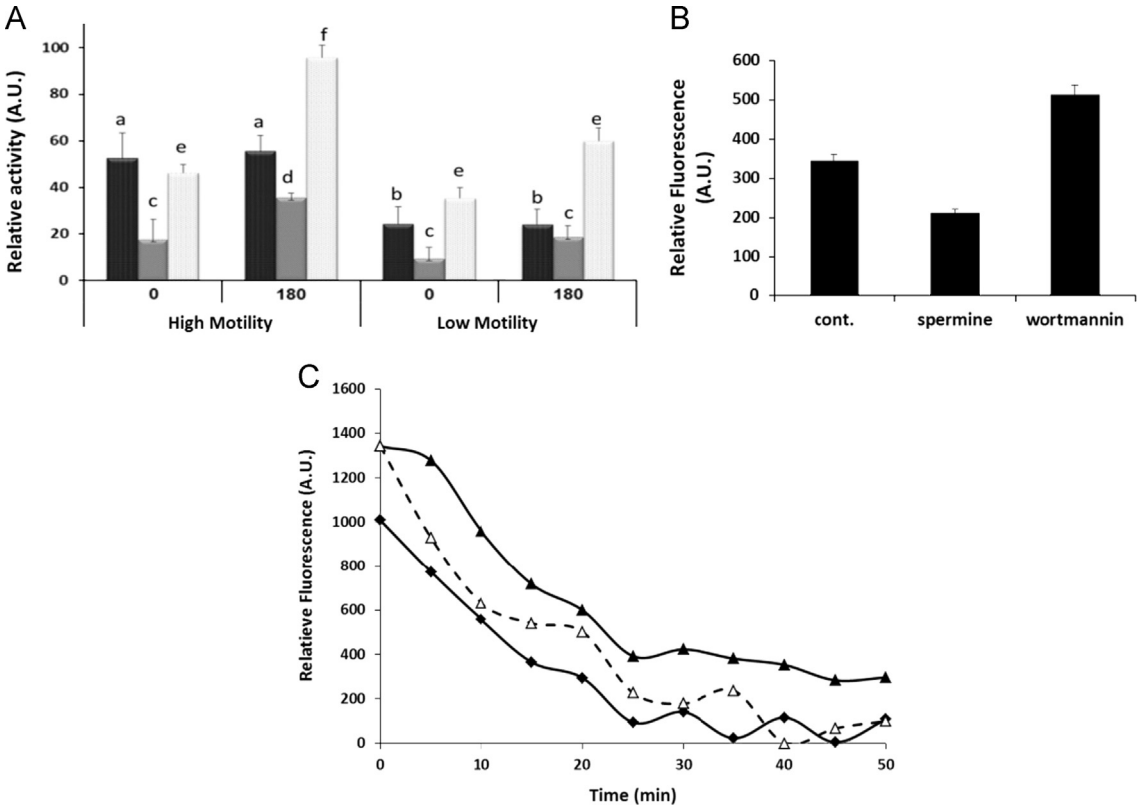

Fig. 3. Total motility, HA motility, F-actin and PIP_2 levels in low and high motility sperm cells. (A) Total motility, HA motility and F-actin levels: human sperm was incubated under capacitation conditions for 3 h. Samples were taken at the beginning of incubation and after 3 h. Sperm motility was determined by CASA using the IVOS device, and cells were stained with FITC-phalloidin, photographed under a fluorescence microscope and analyzed for fluorescence intensity. The values represent the mean ± SD of duplicates from three experiments. (□) F-actin, (▒) hyperactive motility, and (■) total motility. Different letters indicate significance difference in the same parameter, using the Duncan test ($P<0.05$). Groups sharing the same letter do not differ significantly. (B) Effect of spermine or wortmannin on PIP_2 levels determined by PBP10: Human spermatozoa incubated in capacitation medium with spermine (10 μM) or wortmannin (10 μM). Samples were taken out at 30 min, the cells were stained with PBP10–rhodamine-B-labeled (10 μM) in HAM F-10, and fluorescence measurements were performed by spectrofluorometry (EM595 nm, EX 550 nm). The PBP10 fluorescence is inversely proportional to the amount of PIP_2 in the cells. The values represent the mean ± SD of duplicates from three experiments. (C) Kinetics changes of intracellular levels of PIP_2: human sperm with high or low motility was incubated under capacitation conditions in HAM F-10 for 50 min in the presence of PBP10–rhodamine-B-labeled (10 μM), with or without spermine (10 μM), and fluorescence measurements were performed by spectrofluorometry (EM595 nm, EX 550 nm). The PBP10 fluorescence is inversely proportional to the amount of PIP_2 in the cells. The data represent one experiment, typical of three repetitions performed with sperm from three experiments with different donors. (■) High motility, (▲) low motility, and (–Δ–) low motility+spermine.

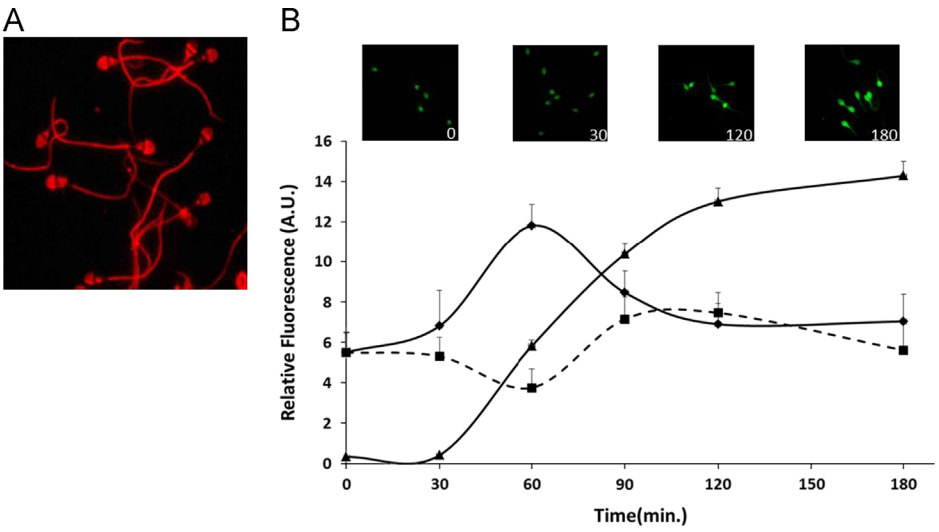

Fig. 4. PIP_2 and F-actin levels during capacitation. (A) PIP_2 staining in the cells: human sperm was incubated under capacitation conditions. At the beginning of incubation the cells were stained with anti-PIP_2 antibody followed by rhodamine RED-X-conjugated antibody, photographed under a fluorescence microscope to determine fluorescence intensity in the sperm. (B) PIP_2 and F-actin staining: human sperm was incubated under capacitation conditions. At various times, samples were taken, and the cells were stained with FITC-phalloidin or anti-PIP_2 antibody followed by rhodamine RED-X-conjugated antibody, photographed under a fluorescence microscope and analyzed for fluorescence intensity in the sperm. (▲) F-actin and (◆) PIP_2 in the sperm head, and (–■–) PIP_2 in the sperm tail. The pictures represent F-actin amount using FITC-phalloidin stain at the different time.

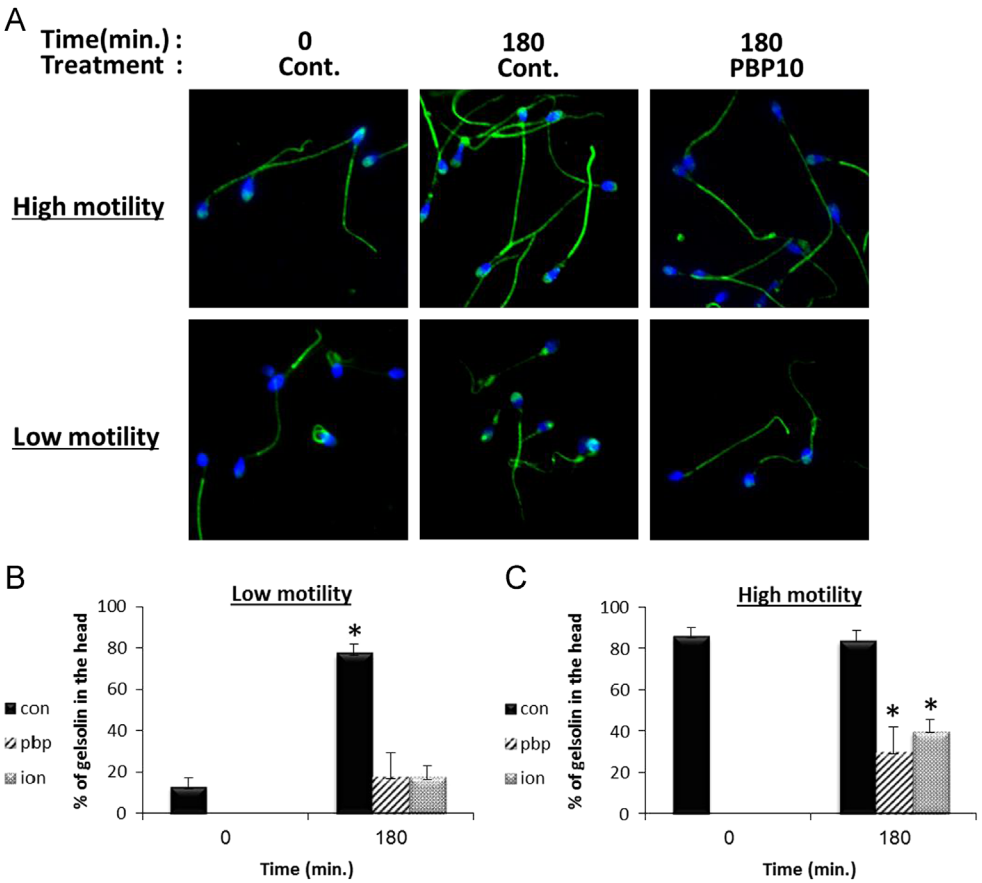

Fig. 5. Gelsolin localization before and after capacitation. (A) Effect of PBP10 on gelsolin staining in whole cells: human sperm was incubated under capacitation conditions for 3 h. PBP10 (1 μM) was added 10 min before the end of the incubation. Samples were taken at zero time and after 180 min of incubation, and the cells were stained with DAPI and with anti-gelsolin antibody followed by Alexa-fluor-488 antibody, photographed under a fluorescence microscope to determine fluorescence intensity in the sperm. The data represent one experiment, typical of at least three repetitions performed with sperm from different donors representing low or high percentage of motile sperm. (B) and (C). Effect of PBP10 and A23187 on gelsolin staining in the head: human sperm with low (B) or high (C) motility was incubated under capacitation conditions in for 3 h. PBP10 (1 μM) or A23187 (10 μM) were added at 170 min for 10 min. Samples were taken at zero time and after 180 min of incubation, and the cells were stained with DAPI and with anti-gelsolin antibody followed by Alexa-fluor-488 antibody. Gelsolin location in the cells was analyzed as described in Materials and methods section, and presented as graphs. The values represent the mean ± SD of duplicates from three experiments. Cont. (■), PBP10 (▨), and A23187 (▩). * Represents significant difference compared with zero time control, $P < 0.05$.

treatment with spermine increased gelsolin translocation to the head. The translocation of gelsolin to sperm head correlates with the increase in PIP_2 levels presented in Figs. 2B and 3A. These data suggest that gelsolin localization in the head depends on PIP_2 levels in the cell head.

We previously showed that gelsolin activity is inhibited by two factors, binding to PIP_2, and phosphorylation on tyrosine using anti-phospho-tyrosine antibodies (Finkelstein et al., 2010). We also presented indirect proof for the role of SRC in gelsolin phosphorylation (Finkelstein et al., 2010). In the present study we used anti-phospho-(tyrosine-438)-gelsolin antibodies to follow gelsolin phosphorylation under various conditions. Gelsolin phosphorylation increased up to 3 h of incubation under capacitation conditions (Fig. 7). In order to support further this suggestion, we first examined SRC activation, by studying its effect on the EGFR, which is phosphorylated by SRC on Tyr845 (Breitbart and Etkovitz, 2011). In addition, we tested the effect of SU6656, an inhibitor of Src-family-kinases (Krapf et al., 2010) on gelsolin phosphorylation. Fig. 7 reveals enhancement of EGFR phosphorylation on Tyr845 and gelsolin phosphorylation on Tyr438 during capacitation and the phosphorylation of both is inhibited by SU6656 (Fig. 7). These results confirm that SRC is responsible for gelsolin phosphorylation on tyrosine-438 during sperm capacitation.

Furthermore, we tested the role of PIP_2 on tyrosine phosphorylation of gelsolin. We found that inhibition of PIP_2 synthesis by WT reduced the degree of phosphorylation while enhancing PIP_2 synthesis by spermine increased gelsolin–tyrosine-phosphorylation (Fig. 8A). Moreover, increasing PIP_2 hydrolysis by activation of phospholipase C, using the Ca^{+2}-ionophore A23187, reduced this phosphorylation. Also, treatment with PBP10 which competes with gelsolin binding to PIP_2, reduced the phosphorylation levels (Fig. 8B). Finally, PA, known to increase PIP_2 levels, enhanced the phosphorylation of gelsolin (Fig. 8C). These data indicate that the level of PIP_2 dictates the degree of phosphorylation of gelsolin. Further support for this conclusion is seen in Fig. 9, in which stimulation of PIP_2 hydrolysis by activation of endogenous phospholipase Cγ using the EGF/EGFR system, caused dephosphorylation of p-gelsolin, which is inhibited by U73122, a known phospholipase C inhibitor. In conclusion our data suggest that PIP_2 levels in cell control gelsolin phosphorylation.

Discussion

In our previous studies, we show that actin polymerization and the development of hyperactive motility are two related processes occurring during sperm capacitation (Brener et al., 2003; Cohen et al., 2004; Itach et al., 2012). This conclusion is supported by others who showed that F-actin is involved in sperm motility, and random severing of F-actin filaments inhibits flagellar motility (Azamar et al.,

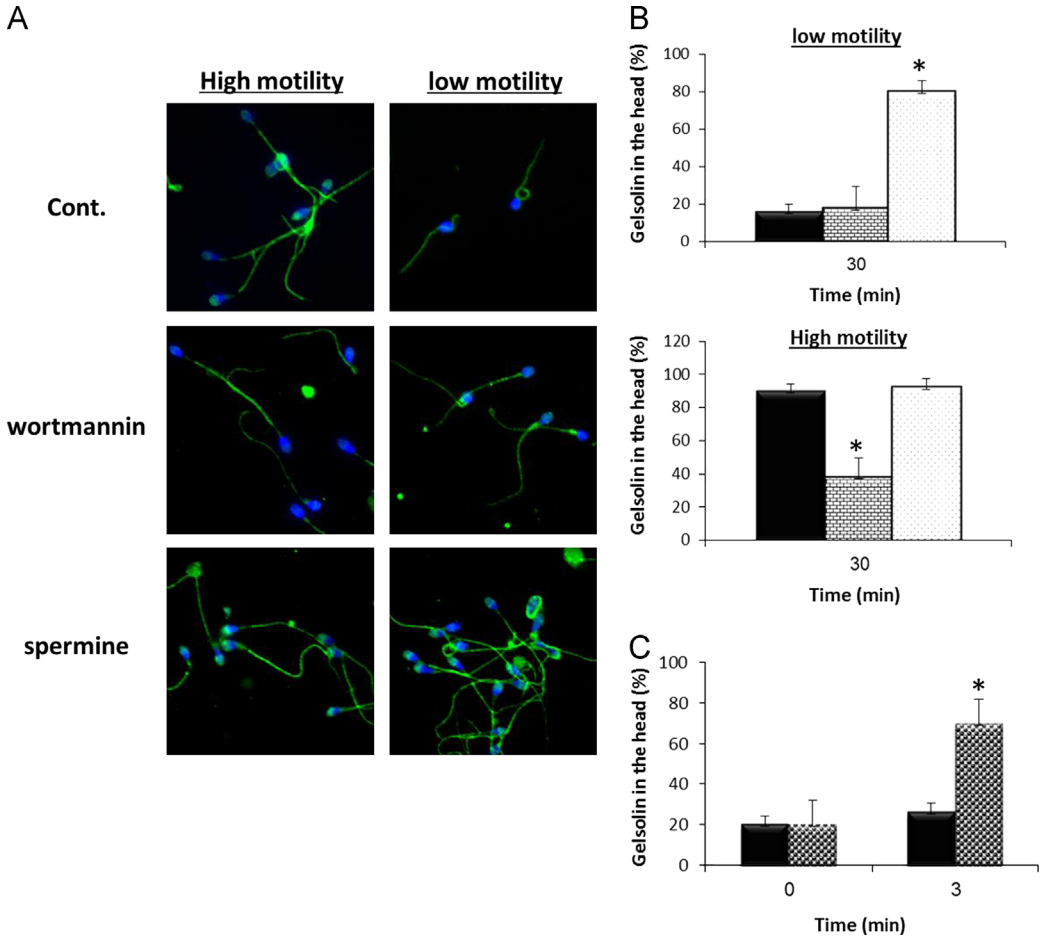

Fig. 6. PIP_2 regulates gelsolin localization in the sperm head. (A) Effect of wortmannin and spermine on gelsolin staining in whole cells: gelsolin staining: human sperm was incubated for 30 min under capacitation conditions in the presence of spermine (10 μM) or wortmannin (10 μM). At the end of the incubation, samples were taken out, and the cells were stained with DAPI and with anti-gelsolin antibody followed by Alexa-fluor-488 antibody, photographed under a fluorescence microscope to determine fluorescence intensity in the sperm. The data represent one experiment, typical of at least three repetitions performed with sperm from different donors representing low or high percentage of motile sperm. (B) Effect of wortmannin and spermine on gelsolin staining in the head: human sperm with low or high motility was incubated under capacitation conditions for 30 min in the presence of spermine (10 μM) or wortmannin (10 μM). At the end of the incubation, samples were taken, and the cells were stained with DAPI and anti-gelsolin antibody followed by Alexa-fluor-488 antibody. The gelsolin location in the cells was analyzed as described in Materials and methods section and presented as graphs. The values represent the mean ± SD of duplicates from three experiments. Cont. (■), wortmannin (▩), and spermine (⁘). * Represents significant difference compared with zero time control, $P < 0.05$. (C) Effect of PA on gelsolin staining in the head: human sperm with low motility was incubated for 3 min under capacitation conditions without or with PA (3 mg/ml). At the end of the incubation, samples were taken, and the cells were stained with DAPI and with anti-gelsolin antibody followed by Alexa-fluor-488 antibody. The gelsolin location in the cells was analyzed as described in Materials and methods section and presented as graphs. The values represent the mean ± SD of duplicates from three experiments. Cont. (■), and PA (▩). * Represents significant difference compared with zero time control, $P < 0.05$.

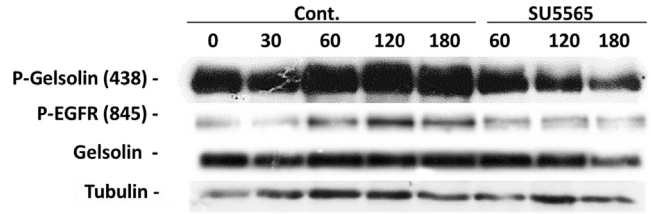

Fig. 7. Tyrosine phosphorylation of gelsolin and EGFR during capacitation. Human sperm was incubated under capacitation conditions either untreated or with SU5565 (50 μM). At the indicated times, samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-gelsolin (Tyr-483)/anti-P-EGFR (Tyr-845)/anti-gelsolin and anti-tubulin antibodies. The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments with different donors.

2007). Additionally, we show in a recent work that the actin severing protein, gelsolin, is inhibited during capacitation in order to allow actin polymerization to occur (Finkelstein et al., 2010). Based on this finding, and on the ability of PIP_2 to bind and inhibit gelsolin activation (Finkelstein et al., 2010; Guttman et al., 2002), we hypothesized that gelsolin is involved in regulation of sperm motility and the development of HA motility in sperm capacitation. We therefore proposed the following pathway: increase in PIP_2 and F-actin in the sperm head during capacitation leads to the translocation of gelsolin from the tail to the head, allowing the increase of F-actin in the tail, resulting in the development of HA motility. To support our hypothesis we asked if gelsolin is indeed involved in controlling cell motility. To this end, we found that activation of gelsolin by PBP10 or WT caused 40–50% inhibition of sperm total motility and complete inhibition of HA motility (Fig. 1). The inhibitory effect of PBP10 and WT on total motility is almost completely reversed by adding exogenous PIP_2 which binds and inhibits free gelsolin (Fig. 1), indicating that the inhibition of sperm motility by PBP10 and WT is due to the release of gelsolin from its binding to PIP_2. Also, inactivation of gelsolin by increasing endogenous PIP_2 levels using spermine or PA, showed significant increase in motility (Fig. 1A and B). Thus, we conclude that gelsolin regulates sperm motility and the development of HA. The relationships between actin polymerization and sperm motility were recently described by us in mouse sperm (Itach et al., 2012). Here, we

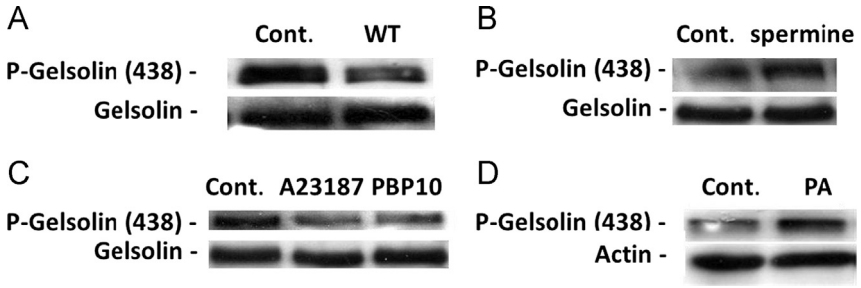

Fig. 8. Phosphorylation of gelsolin under various conditions: (A) Effect of wortmannin on gelsolin phosphorylation: human sperm was incubated under capacitation conditions in HAM F-10 for 30 min. The cells were incubated with wortmannin (10 μM). After 30 min samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-gelsolin (Tyr-483). The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments with different donors. (B) Effect of spermine on gelsolin phosphorylation: human sperm was incubated under capacitation conditions in HAM F-10 for 30 min. The cells were incubated with spermine (10 μM). After 30 min samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-gelsolin (Tyr-483). The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments with different donors. (C) Effect of A23187 and PBP10 on gelsolin phosphorylation: human sperm was incubated under capacitation conditions in HAM F-10 for 180 min. PBP10 (1 μM) or A23187 (10 μM) was added at 180 min for 30 min. At 210 min samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-gelsolin (Tyr-483). The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments with different donors. (D) Effect of PA on gelsolin phosphorylation: human sperm was incubated under capacitation conditions in HAM F-10. PA (3 mg/ml) was added for 3 min. Samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-gelsolin (Tyr-483). The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments with different donors.

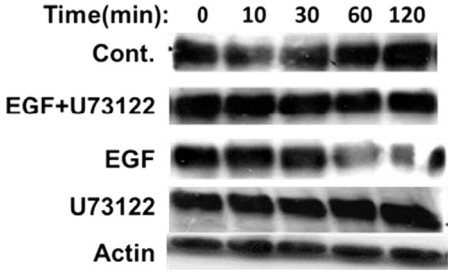

Fig. 9. PLC and EGF regulate gelsolin phosphorylation during capacitation. Human sperm was incubated under capacitation conditions in HAM F-10. EFG (1 ng/ml) and U73122 (1 μM) were added from the beginning. At different time, samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-gelsolin (Tyr-483). The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments using different donors.

showed the relationships between the cellular levels of PIP_2, actin polymerization and sperm motility. We showed that spermine enhances PIP_2 levels and F-actin in the sperm whereas WT inhibits these effects (Figs. 2B and 3B). Moreover, we observed an increase of PIP_2 and F-actin in the head during sperm capacitation (Fig. 4B). Interestingly, PIP_2 in the head is increased up to 60 min of incubation, and later on a decrease is observed, although F-actin in the head remains high (Fig. 4B upper pictures), indicating that gelsolin is inactive. We showed that the increase of PIP_2 in the head is essential for gelsolin translocation to the head (Fig. 6A) leading to its phosphorylation/inactivation (Fig. 8B). Thus, the late decrease in PIP_2 in the head does not affect F-actin, since gelsolin is inactive due to its phosphorylation.

These data together with the data on motility (Fig. 1) strongly support our hypothesis regarding the relationships between the elevation of PIP_2 and F-actin in the head and gelsolin dependent sperm motility during capacitation. Interestingly, spermin showed no significant change in the total motility of cells which show relatively high motility (Fig. 1A) although it enhanced HA motility (Fig. 1B). These data suggest that the basal levels of PIP_2 in the cells might affect total and HA motility. Moreover, it seems that HA motility is more sensitive to the changes of PIP_2 in the cell. We show elsewhere that reducing F-actin levels, below the basal levels, blocks mouse sperm motility (Itach et al., 2012). As a result of these data we determined the basal levels as well as the levels after capacitation of PIP_2 and F-actin in human sperm with preliminary high or low motility. Sperm with high motility showed higher basal levels of PIP_2 and F-actin, whereas sperm with low motility exhibited the opposite results (Fig. 3A and C). Moreover, the levels of PIP_2, F-actin and HA motility after capacitation were much lower in poorly motile sperm compared to high motility sperm (Fig. 3A and C). This relationship between motility rate, intracellular levels of PIP_2 and F-actin support our idea regarding the regulation of sperm motility by intracellular levels of PIP_2 and F-actin.

The second part of our hypothesis deals with gelsolin translocation to the sperm head during capacitation. Our previous work exhibits that gelsolin translocate from the sperm tail to the head during capacitation (Finkelstein et al., 2010). Accordingly, we show here that this phenomenon is more pronounced in low motility samples, since in high motility sperm, most of gelsolin is already localized to the sperm head before capacitation due to its high basal levels of PIP_2 and F-actin (Fig. 5). The translocation of gelsolin to the head is prevented by PBP10 or Ca^{2+}-ionophore and these compounds caused reverse translocation of gelsolin from the head to the tail, in high motility sperm (Fig. 5). Additionally, wortmannin inhibited and spermin or PA enhanced gelsolin translocation to the head (Fig. 6). All together these data clearly indicate that PIP_2 and/or F-actin levels in the head regulate the translocation of gelsolin to the head. This localization of gelsolin in the sperm head has a significant importance as a key protein responsible for depolymerization of the F-actin barrier prior to the acrosome reaction (Finkelstein et al., 2010).

We previously showed that gelsolin is phosphorylated on tyrosine during capacitation and suggested that in order to enable actin polymerization during capacitation, gelsolin should be inactivated by its binding to PIP_2 and phosphorylated on tyrosine (Finkelstein et al., 2010). Here we demonstrated that gelsolin phosphorylation depends on binding to PIP_2. Previous studies in other cell types showed strong stimulation of phosphorylation on gelsolin (Tyr 438) only in the presence of PIP_2 or LPA (De Corte et al., 1999). In the present study, as expected, gelsolin phosphorylation was increased during sperm capacitation (Fig. 7), maintaining its inactivated state, and allowing actin polymerization to progress. This phosphorylation was inhibited by SU5565 (Fig. 7), a known specific inhibitor of SRC-family-kinase (SFK) (Visconti et al., 2011), indicating the involvement of SFK in tyrosine phosphorylation of gelsolin during sperm capacitation. Interestingly, SU6656 caused partial degradation of gelsolin after 3 h of incubation, suggesting a possible increase in gelsolin sensitivity to degradation

when it undergoes dephosphorylation. Indeed it was shown elsewhere that SU6656 caused activation of caspace 3 (Riffell et al., 2011). Caspase 3 is known to cause cleavage of gelsolin in smooth muscle cells (Geng et al., 1998). This notion provides additional possible role for gelsolin phosphorylation to protect it from degradation by caspases.

Spermin and PA which enhance PIP_2 levels, stimulate gelsolin–tyrosine phosphorylation while wortmannin, which inhibits PIP_2 synthesis, Ca^{2+}-ionophore (A23187), which activates phospholipase C and stimulates PIP_2 hydrolysis, and PBP10, which competes with gelsolin binding to PIP_2, inhibits gelsolin phosphorylation (Fig. 8A–C). Moreover, stimulation of PLC_γ activity by activating the EGFR with EGF reduces the rate of gelsolin phosphorylation and this reduction is inhibited by U73122, a known PLC inhibitor, indicating that PIP_2 hydrolysis by PLC_γ caused the reduction in gelsolin phosphorylation. These data clearly indicate that the phosphorylation of gelsolin on tyrosine-438 depends on its binding to PIP_2. Thus, the two processes, binding to PIP_2 and its tyrosine-phosphorylation, which inhibit gelsolin activation, occur during sperm capacitation. It is possible that binding of gelsolin to PIP_2 stabilizes the tyrosine-phosphorylation state and protects it from dephosphorylation by tyrosine phosphatase. The free phospho-gelsolin which is released after PIP_2 hydrolysis is activated by tyrosine-phosphtase which dephosphorylates the phospho-tyrosine. The active gelsolin severs F-actin and afterwards it can be downregulated by caspases cleavage.

In conclusion, the data presented here support our hypothesis that the increase in PIP_2 and F-actin levels in the sperm head during capacitation enhances gelsolin translocation to the head, its phosphorylation/inactivation—leading to stimulating sperm hyper-activated motility. Moreover, the basal levels of PIP_2 and F-actin are critical for basal sperm motility and for the development of hyper-activated motility in sperm capacitation.

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