CDC42 drives RHOA activity and actin polymerization during capacitation

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Abstract

Mammalian sperm cells acquire fertilizing capacity as a result of a process termed capacitation. Actin polymerization is important for capacitation; inhibiting actin polymerization prevents the adhesion and fusion of the sperm with the ovule. The main function of RHO proteins CDC42 and RHOA is to direct actin polymerization. Although these two RHO proteins are present in mammalian sperm, little is known about their role in capacitation, the acrosome reaction, and the way in which they direct actin polymerization. The purpose of this study was to determine the participation of CDC42 and RHOA in capacitation and the acrosome reaction and their relationship with actin polymerization using guinea pig sperm. Our results show that the inhibition of CDC42 and RHOA alters the kinetics of actin polymerization, capacitation, and the acrosome reaction in different ways. Our results also show that the initiation of actin polymerization and RHOA activation depend on the activation of CDC42 and that RHOA starts its activity and effect on actin polymerization when CDC42 reaches its maximum activity. Given that the inhibition of ROCK1 failed to prevent the acrosomal reaction, the participation of RHOA in capacitation and the acrosomal reaction is independent of its kinase 1 (ROCK1). In general, our results indicate that CDC42 and RHOA have different roles in capacitation and acrosomal reaction processes and that CDC42 plays a preeminent role.

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Introduction

Capacitation consists of a series of well-synchronized events experienced by mammalian sperm after spending time in the oviduct. These events allow sperm cells to acquire fertilizing ability. A capacitated sperm cell can be recognized by the presence of two conditions: hypermotility and acrosome reaction (AR) induction. These two events allow sperm to pass through the envelopes of the ovule and adhere and fuse with the plasma membrane of the ovule (Gervasi & Visconti 2016). These events occur in response to changes in the environment to which sperm are exposed, whether the oviductal environment or a specific culture medium, involving the activation of different signaling pathways that lead to the intracellular increase of Ca^{2+} , Cl^- , and HCO_3^- ; plasma membrane hyperpolarization; lipid membrane remodeling; cAMP-related signaling pathway activation; an increase in protein phosphorylation in Tyr residues (Visconti *et al.* 1995a,b, Stival *et al.* 2016); and the remodeling of actin and spectrin cytoskeletons (Breitbart *et al.* 2005, Bastian *et al.* 2010, Roa-Espitia *et al.* 2016). It is important to note that these changes have been reported mainly in human, mouse, bovine,

pig and guinea pig sperm (Breitbart *et al.* 2005, Bastian *et al.* 2010, Gervasi & Visconti 2016, Stival *et al.* 2016).

The polymerization of actin that occurs in the course of capacitation seems to be a generalized process in mammalian sperm, as has been reported for different species, from human beings to bovines, pigs, mice, buffaloes, and guinea pigs (Moreno-Fierros *et al.* 1992, Brener *et al.* 2003, Bernabo *et al.* 2011, Naresh & Atreja 2015, Angeles-Floriano *et al.* 2016, Roa-Espitia *et al.* 2016). In its polymeric status (F-actin), actin has been mainly found in the apical regions of the acrosomal, equatorial, and postacrosomal head, as well as in the flagellum of mature sperm cells (Brener *et al.* 2003, Naresh & Atreja 2015, Angeles-Floriano *et al.* 2016, Roa-Espitia *et al.* 2016). The specific location of the actin cytoskeleton and the inhibition of actin polymerization during capacitation show that F-actin has a role in processes such as motility (Azamar *et al.* 2007, Itach *et al.* 2012), the AR (Spungin *et al.* 1995, Hernandez-Gonzalez *et al.* 2000, Brener *et al.* 2003, Cabello-Agueros *et al.* 2003), and the rearrangement of proteins required by sperm for fertilization, such as Izumo (Sosnik *et al.* 2009).

The way in which actin polymerization is regulated during capacitation has been associated with different mechanisms. Evidence suggests that at least three different signaling pathways could be related to actin polymerization during capacitation: (1) the pathway associated with PKA/PLC/ PKC/PLD/Cofilin, this signaling pathway has been primarily related to human sperm (Breitbart *et al.* 2005, Megnagi *et al.* 2015); (2) the pathway associated with RHOA or RAC1/ROCK-1/ LIMK-1/Cofilin has been related described for bovine and mouse sperm (Fiedler *et al.* 2008, Romarowski *et al.* 2015); and (3) for mouse and guinea pig sperm the pathway associated with CDC42/WASP/ARP2/3 was reported (Delgado-Buenrostro *et al.* 2005, 2016, Angeles-Florianio *et al.* 2016). Blocking CDC42 or RHOA activity is known to inhibit actin polymerization and the normal course of the AR (Brener *et al.* 2003, Baltierrez-Hoyos *et al.* 2012, Angeles-Florianio *et al.* 2016). Data show that RHOA and CDC42 could be the main RHO proteins directing actin polymerization during capacitation. Both of these small GTPases present a conserved localization in human, mouse, guinea pig, bovine, pig, ram, rat sperm; they are located in the acrosomal region and the flagellum (Ducummon & Berger 2006, Baltierrez-Hoyos *et al.* 2012, Salvolini *et al.* 2013, Angeles-Florianio *et al.* 2016, Delgado-Buenrostro *et al.* 2016). However, the dynamics of these proteins during capacitation and how they relate to each other to achieve actin polymerization during capacitation are still unknown.

CDC42 and RHOA are ubiquitously expressed, and although both RHO proteins participate in actin polymerization, these RHO proteins are known to have different dynamics in different cell lines; as a result, they give rise to the formation of different cell structures. The activation of CDC42 results in the formation of filopodia, and the activation of RHOA is associated with the formation of stress fibers (Jaffe & Hall 2005, Hall *et al.* 2011). Mammalian sperm expresses both CDC42 and RHOA; however, these cells do not form cell projections such as filopodia or lamellipodia. Therefore, RHO proteins expressed in sperm must have functions specifically related to sperm physiology. Due to the limited amount of cytoplasm contained in the sperm cell, stress fibers have been observed as short filaments associated with the external plasma and acrosomal membranes (Fouquet *et al.* 1990, Peterson *et al.* 1990, Hernandez-Gonzalez *et al.* 2000).

The purpose of the present study was to determine the role of CDC42 and RHOA in capacitation and the AR. We also focused on the participation of CDC42 and RHOA in actin polymerization dynamics during capacitation, on how specific inhibitors for these RHO proteins affect these sperm processes, and on whether there the activities of CDC42 and RHOA are interrelated. Finally, we studied the participation of RHO kinases (ROCKs) in the capacitation, AR and actin polymerization.

Materials and methods

Antibodies and reagents

Most of the reagents used in this study were purchased from Sigma-Aldrich Co. Protease inhibitor (Complete™ cocktail tablets) were ordered from Roche Diagnostics and Molecular Biochemicals. Nitrocellulose membranes, acrylamide, N,N'-methylene-bisacrylamide, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories. Anti-RHOA (Ab6882) was purchased from Abcam. Anti-CDC42 (sc-57), anti-ROCK1 (sc-5560), and anti-ROCK2 (sc-5561), were acquired from Santa Cruz Biotechnology Inc. C3 RHO inhibitor (CT04) was purchased from Cytoskeleton Inc (Denver, CO). Secondary antibodies labeled with horseradish peroxidase (HRP) or TRITC were obtained from Jackson ImmunoResearch Laboratories Inc. Enhanced chemiluminescence (ECL) reagent was purchased from Amersham or Millipore. Secramine A was kindly donated by Kirchhausen Lab (Harvard Medical School) and the Hammond Lab (University of Louisville), synthesized by Bo Xu and GB Hammond of the University of Louisville.

Animals

Male Dunkin–Hartley guinea pigs (*Cavia porcellus*) weighing 600–800 g were obtained from the Cinvestav-IPN vivarium. All animal experiments and handling procedures were approved by the Internal Ethics committee for Laboratory Animal Care and Use of CINVESTAV-IPN (CICUAL No. 0122-14, April 2017) and conducted following the American Veterinary Medical Association guidelines. Efforts were made to minimize the potential for animal pain, stress, or distress.

Sperm capacitation

Guinea pig sperm were collected from the animals' vas deferens per published procedure (Mujica *et al.* 1991). Sperm were resuspended in 154 mM NaCl and a 25 mM HEPES buffer at pH 7.2. For the non-capacitation condition, sperm were incubated in a solution containing 154 mM NaCl and a 25-mM-HEPES buffer at pH 7.2. For the capacitation condition, sperm were incubated in Tyrode's medium (116.7 mM NaCl, 2.8 mM KCl, 11.9 mM NaHCO₃, 0.3 mM NaH₂PO₄, 0.49 mM MgCl₂, 0.25 mM sodium pyruvate, and 20 mM sodium lactate) at 37°C and pH 7.6. Sperm concentration was maintained at 35 × 10⁶ sperm/mL in all experiments. The process of capacitation is asynchronous; therefore, to define the kinetics of capacitation and acrosomal reaction at defined times (0, 15, 30, 60, and 90 min) of incubation, small aliquots of the sperm suspension were placed on slides and the percentage of motile sperm and the acrosomal reaction were estimated. Those samples that showed low levels of motility (less than 70%) were discarded. Under these conditions, between the 60 to 70 min of capacitation, the sperm initiate the acrosomal reaction.

Sperm cells were also capacitated in the presence of different drugs: Secramine A (SecA, 5 µM), C3 toxin (1 µg/mL), latrunculin A (5 µM), and Y-27632 (0–400 nM), which were added since the beginning of capacitation. Any change is indicated in the text.

Acrosome reaction assay

Spontaneous acrosome reaction

Sperm were capacitated in Tyrode's medium, incubating them for 90 min. Immediately, the sperm were prepared to evaluate the spontaneous AR (sAR) using the CTC test.

Induced acrosome reaction

Sperm were capacitated in Tyrode's medium for 50 min; afterward, AR was induced (iAR) adding progesterone (10 μ M), and the sperm cells were incubated another 20 min. The effects of the different inhibitors on the progesterone-induced AR (iAR) were determined capacitating sperm during 50 min in the presence of any of the following inhibitors: C3 (1 μ g/mL) or SecA (5 μ M) or Y-27632 (100 nM). Next, progesterone (10 μ M) was aggregated, and the sperm were incubated for another 20 min. Through the CTC assay was assessed AR. $iAR = \% \text{ de AR (70min)} - \% \text{ de AR (50 min)}$.

Sperm viability assay

To know whether the drugs used in this work affect sperm viability, this was determined using the methodology described by Brito *et al.* (2003). Sperm were capacitated in Tyrode's medium for 90 min in the presence of some of the following inhibitors: C3 (1 μ g/mL), Sacramine A (5 μ M) and Y-2763 (100 nM). Propidium iodide (PI) solution (1 μ g/mL) was added to sperm samples to a 1:1 ratio and incubated at room temperature for 30 min. Immediately, sperm were washed, and under an epifluorescence microscope, the number of stained sperm was counted (500 cells X sample, $n=3$). As a control, sperm capacitated in the absence of any of the inhibitors were also stained with PI. When the samples had a viability less than 80%, they were discarded. Data are shown in Fig. 1G.

Assay to detect and quantify F-actin

F-actin was quantified using the method described by Roa-Espitia *et al.* (2016). Thus, non-capacitated and capacitated sperm were fixed (1.5% formaldehyde and 0.1% glutaraldehyde, in PBS). After 1 h, the sperm cells were collected by centrifugation (600 g for 3 min) and the cellular pellet was incubated in 50 mM NH_4Cl (10 min) and rinsed three times with PBS. Microscope slides were prepared. Sperm cells were permeabilized in PBS with Triton (0.1%) for 20 min at room temperature and then washed with PBS.

Sperm were incubated for 1 h at 37°C with phalloidin-TRITC or phalloidin-FITC (1:25) and washed five times in PBS. Samples were mounted with glass-coverslips in Gelvatol. All fluorescence images were obtained using a confocal laser scanning microscope (Leica TCS SP8), and 500 sperm cells were analyzed using the NIS-Elements 3.1 imaging software.

Chlortetracycline assay

The procedure used in this essay was first described by Ward and Storey (1984), and it has been adapted to guinea pig

sperm (Maldonado-Garcia *et al.* 2017). At specific times of capacitation, 45 μ L of sperm suspension was mixed with 45 μ L chlortetracycline (CTC) solution (750 mM CTC in 130 mM NaCl, 5 mM cysteine, 20 mM TRIS, pH 7.8) and incubated for 20 s in a water bath at 37°C. Immediately after incubation, the CTC-sperm suspension was fixed with glutaraldehyde (0.1%, final concentration) in 0.5 mM TRIS (pH 7.4) at room temperature. A 10 μ L sample of the CTC-sperm suspension was smeared onto a glass slide and covered with a coverslip, adequately sealed, and stored for 48 h at -20°C to clear strong background fluorescence. The CTC solution was kept in a light-shielded container at 4°C at all times. All fluorescence images were obtained using an epifluorescence microscope (Olympus BX5) and registered using the Nis-Element 3.1 software. Five hundred sperm cells per sample were classified as expressing one of three CTC staining patterns: F pattern, characterized by faint fluorescence in the acrosome region, typical of non-capacitated acrosome-intact cells; B pattern, characterized by bright fluorescence in the acrosomal region and a band along the equatorial segment, typical of capacitated acrosome-intact cells; and AR pattern, characterized by fluorescence in the post-acrosomal region and the equatorial segment, typical of physiologically capacitated acrosome-reacted cells. CTC excitation at 330–380 nm, emission at 420 nm.

CDC42 activation assay

To detect CDC42-GTP specifically, we used the CDC42 G-LISA Activation Assay Biochem Kit (BK127. Cytoskeleton Inc., Denver, CO, USA) per the manufacturer's instructions and as reported by Baltierrez-Hoyos *et al.* (2012). Thus, 50 μ L of duplicate samples of sperm lysates from non-capacitated and capacitated sperm (0, 5, 10, 20, 30, 60, and 90 min) at a concentration of 0.3 mg protein/mL were added to each well. Plain lysis buffer and a standard curve of constitutively active CDC42 protein were added to duplicate wells as a blank and positive control, respectively. After binding, anti-CDC42 primary antibody was added to each well followed by secondary antibody labeled HRP, which was developed by adding HRP reagent. Each well was read at OD 490 nm on a 96-well plate spectrophotometer. Control sperm cells were capacitated in the presence of SecA (5 μ M). Purified GTP-bound CDC42, supplied by the manufacturer, was used to establish a standard curve.

RHOA activity assay

RHOA-GTP was evaluated by pull-down assay using the RHOA Pull-down Activation Assay Biochem Kit (Cytoskeleton). Proteins (300 μ g) from non-capacitated sperm, capacitated sperm, or sperm capacitated in the presence of inhibitors (1 μ g/mL C3 or 5 μ M SecA) were incubated with 50 μ g agarose-conjugated Rhotekin-RBD at 4°C for 1 h. Proteins not bound to rhotekin-RBD were recovered by centrifugation at 5000 g at 4°C for 3 min, after three washes. The RHOA-GTP associated with Rhotekin-RBD was released by boiling in Laemmli sample buffer and separated by SDS-PAGE, transferred to nitrocellulose membranes, and then subjected to immunoblot analysis.

Figure 1 Actin polymerization antagonists alter capacitation and acrosomal reaction differently. (A) CTC staining patterns. F, non-capacitated sperm; B, capacitated sperm; AR, sperm that have experienced acrosomal reaction, corresponding to the spontaneous acrosomal reaction. (B) Pattern B was assessed in sperm capacitated in the absence or presence of C3 toxin (1 μM), Secamine (5 μM). AR pattern of CTC staining. (C) The AR pattern was assessed in sperm capacitated in the absence or presence of C3 toxin (1 μM), Secamine (5 μM), or Y-27632 (100 nM). AR pattern of CTC staining. Mean $\pm$ S.E.M., $n=3$ independent experiments. Five hundred cells per experiment were counted. (D) Whole extracts obtained from sperm capacitated under different conditions were used to determine the PYP levels. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. PYP was analyzed using an anti-p-Tyr antibody. SecA, Secamine A (5 μM); Y-27, Y-27632 (100 nM). C3 (1 μg/mL). The image is a representative of three independent experiments. (E) Densitometric analysis of PYP. Means $\pm$ S.E.M., $n=3$ independent experiments. (F) Acrosome reaction progesterone-induced. In sperm capacitated in the presence or absence of C3, SecA, or Y-27632, the AR was induced by progesterone and compared with sAR. Means $\pm$ S.E.M., $n=3$ independent experiments. (G) Sperm viability assessment. Sperm were capacitated in the presence of any of the following inhibitors: SecA (5 μM), C3 (1 μg/mL) or Y-27632 (100 nM). After 90 min of incubation in Tyrode's medium, sperm viability was assessed using propidium iodide (see Materials and methods). Means $\pm$ S.E.M., $n=3$ independent experiments.

Western blot and densitometry assays

Non-capacitated sperm and sperm capacitated at different times were centrifuged (600 g , for 3 min) and suspended in RIPA buffer (25 mM TRIS HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (5 mg/mL soybean trypsin inhibitor, 100 mg/mL benzamidine, 30 mg/mL pepstatin, 30 mg/mL leupeptin, 30 mg/mL aprotinin, 1 mM PMSF diluted in dimethylsulfoxide, 20 mg/mL iodoacetamide, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 2.5% complete mini protease inhibitor cocktail, 1 tablet diluted in 1 mL H_2O). Samples were then incubated for 20 min in ice and centrifuged (5000 g) for 30 min at 4°C. Supernatants were collected, and protein concentration was determined using the Bradford assay. Samples were then boiled for 5 min in sample Laemmli buffer pH 7 and separated by SDS-PAGE to be subsequently transferred to nitrocellulose membranes and blocked with 5% skim milk in PBS added with 1% Triton (pH 7.5). The membranes were incubated overnight at 4°C with the primary antibodies: anti-CDC42 (1:1000), anti-RHOA (1:500), anti-ROCK1 (1:1000), and anti-ROCK2 (1:1000).

Next, membranes were incubated with their respective secondary antibody: HRP-conjugated anti-rabbit (1:10,000), HRP-conjugated anti-mouse (1:5000), or HRP-conjugated anti-goat (1:5000). The nitrocellulose membranes were bathed with ECL, and chemiluminescence was registered with the Odyssey Fc Imaging System. Images were analyzed using the ImageJ software.

Immunocytochemistry assay

Non-capacitated and capacitated sperm were fixed as indicated previously. Sperm cells were permeabilized in PBS with 0.1% Triton for 20 min at room temperature and washed with PBS. ROCK1 (1:50) and ROCK2 (1:50) antibodies were diluted in blocking solution (PBS with 1% BSA) and incubated on slides overnight at room temperature. The slides were washed three times with PBS and then incubated for 2 h at 37°C with the appropriate TRITC-conjugated secondary antibody diluted in blocking solution. Samples were mounted using glass coverslips and Gelvatol, adequately sealed, and stored at -20°C until the observations. The sperm cells were

imaged under a confocal laser scanning microscope (Leica TCS SP8) and analyzed using LAS AF Lite (Ver. 2.6.3).

Statistical analysis

All data are presented as means $\pm$ s.e. Statistical significance was analyzed using *t*-test or ANOVA for comparisons between two groups and multiple comparisons, respectively. SigmaPlot version 11.0 was used for the analysis, and $P < 0.05$ was considered statistically significant.

Results

CDC42 and RHOA antagonists alter capacitation and the acrosome reaction differently

To define how CDC42 and RHOA antagonists affect capacitation and spontaneous acrosome reaction (sAR), these two physiological states of sperm were quantified by CTC assay and their kinetics were determined from 0 to 90 min of capacitation. Sperm capacitated in the absence of any of the antagonists showed a constant increase in pattern B until 30 min of incubation; after this time, pattern B decreased (Fig. 1B). This decrease coincided with the increase in the AR pattern, which reached its maximum at 90 min of capacitation (Fig. 1C). These events are similar to those observed in mouse sperm (Angeles-Floriano *et al.* 2016).

When sperm were capacitated in the presence of SecA, (5 μ M), pattern B increased at 30 min incubation (75.00 ± 2.64 s.e.), similar to the control sample (75.67 ± 0.88 s.e. Fig. 1B). However, unlike the control, the percentage of pattern B declined only marginally; after 90 min of capacitation in the presence of SecA, pattern B levels maintained values that were not significantly different (65.67 ± 4.84 s.e.) with respect to the values at 30 min of capacitation (Fig. 1B). This result explains why the sAR does not increase in sperm incubated in the presence of SecA (Fig. 1C). Taken together, these results indicate that CDC42 inhibition has no effect on the normal course of capacitation, although it does block the AR, similar to what happens in mouse sperm (Angeles-Floriano *et al.* 2016).

When the sperm cells were capacitated in the presence of C3 (1 μ M), pattern B experienced a slight increase that stopped at 15 min, remaining unchanged until 90 min of incubation (Fig. 1B). The sAR remained unchanged, maintaining similar values from the beginning of capacitation (25.00 ± 2.47 s.e.) until 90 min (19.67 ± 2.40 s.e. Fig. 1C). These data confirm that the inhibition of RHOA prevents sperm from experiencing capacitation and, as a consequence, the sAR (Brener *et al.* 2003).

The AR pattern assessed in the previous results corresponds to sperm that experienced sAR. To know if the different inhibitors used in this work have the same effect on AR induced (iAR) by progesterone, sperm were capacitated in the presence of C3 (1 μ g/mL) or SecA

(5 μ M) for 50 min. Then, AR was induced by adding progesterone (10 μ M), and the sperm were incubated for another 20 min. The results show that iAR was significantly higher ($P = 0.001$) than sAR (Fig. 1F). While in sperm capacitated in the presence of C3 or SecA, the iAR was significantly lower compared with the controls sAR and iAR in sperm capacitated in the absence of the inhibitors (Fig. 1F).

It is important to note that the inhibitors used in the present work (C3, SecA, and Y-2763) at the concentrations used did not alter the viability of the sperm concerning sperm non-capacitated and capacitated sperm in the absence of the inhibitors (Fig. 1G). Only the data of non-capacitated and capacitated at 90 min are shown because there were no significant changes in sperm viability at times tested (0, 15, 30, 60, 75, and 90 min).

In order to confirm the results obtained employing the CTC technique, the levels of phosphorylation in Tyr of the sperm proteins (PYP) were evaluated by WB. The results show a significant increase in PYP in capacitated sperm ($P = 0.001$) compared to non-capacitated sperm (Fig. 2D and E). When sperm were capacitated in the presence of SecA, PYP levels were similar to that exhibited by sperm capacitated in the absence of SecA (Fig. 2D and E). On the other hand, sperm capacitated in the presence of C3 show significantly lower levels of PYP compared to sperm capacitated in the absence of C3 (Fig. 2D and E).

RHO protein antagonists have various effects on the polymerization of actin

Different studies have shown that drugs that inhibit RHO proteins such as C3 toxin (RHOA) and SecA (CDC42)

Figure 2 RHO protein antagonists alter actin polymerization differently. (A) Fluorescence patterns of sperm treated with different RHO protein inhibitors and stained with phalloidin-TRITC. (B) Fluorescence levels of phalloidin-TRITC were quantified using the NIS-Elements 3.1 software to define the time course of F-actin during capacitation in the presence or absence of C3 toxin (C3) or Secamine A (SecA) (Mean $\pm$ s.e.m., $n = 3$ independent experiments). * $P = 0.044$, ** $P = 0.044$.

prevent the polymerization of actin during capacitation (Moreno-Fierros *et al.* 1992, Brener *et al.* 2003, Angeles-Floriano *et al.* 2016, Roa-Espitia *et al.* 2016). However, little is known about the temporary effect of these drugs on the remodeling of the actin cytoskeleton. To study the temporary effect of C3 and SecA, the present study assessed the amount of F-actin present in the sperm at different times of incubation in a capacitation medium in the absence or presence of these actin polymerization antagonists. As can be appreciated in Fig. 1A, at 90 min of capacitation, both C3 (1 µg/mL) and SecA (5 µM) inhibited actin polymerization. To determine whether these effects were similar at any time of capacitation, the amount of F-actin was assessed at different times of capacitation (0, 15, 30, 60, and 90 min). Figure 2B exhibits the different effects of these drugs. Control: sperm cells capacitated in the absence of these drugs presented increasing amounts of F-actin levels during capacitation until approximately 30 min of incubation and a less pronounced increase until 90 min of incubation. Secramine A: Although F-actin levels increased between the beginning of capacitation and after 30 min of capacitation, the increase was not significantly different from the different capacitation times (Fig. 2B). C3: In the case of sperm capacitated in the presence of C3, the increase in F-actin levels was similar to that observed in the control between 0 to 15 min; after that the levels decayed rapidly to levels comparable to those shown by SecA (Fig. 2B).

Effects of CDC42 inhibition on actin polymerization and the acrosome reaction

Our previous results suggest that CDC42 could play an important role in actin polymerization during the early stages of capacitation. Four different assays were performed to test the previous hypotheses; in these essays, CDC42 was inhibited at different times of capacitation by adding SecA (5 µM). F-actin was evaluated at different times (0, 15, 30, 60, and 90 min) in all four essays. Assay 1: Sperm capacitated in the absence of SecA. Results showed that F-actin increased steadily until 30 min of capacitation and continued increasing less intensely until 90 min (Fig. 3A). Assay 2: SecA added at the beginning of capacitation (0 min). Although SecA allowed a small increase in F-actin, especially at 90 min of incubation, the increase was not significant with respect to the different times tested (Fig. 3A). Assay 3: SecA added 15 min after the beginning of capacitation. Results showed an increase in the concentration of F-actin until 30 min of capacitation, and after this time, the amount of F-actin maintained its level without experiencing changes (Fig. 3A). Assay 4: SecA added 30 min after the beginning of capacitation. In this case, during capacitation, the actin was polymerized as in Assay 1 (Fig. 3A). Interestingly,

Figure 3 CDC42 inhibition at different times of capacitation has different effects on the polymerization of F-actin, but not on the AR. (A) Sperm were capacitated in the absence or presence of Secramine A (5 µM) following four different tests: Assay 1: Sperm capacitated in the absence of Secramine A. Assay 2: Sperm capacitated in the presence of Secramine A. Assay 3: Secramine A added at 15 min of capacitation. Assay 4: Secramine added at 30 min of capacitation. The fluorescence levels of phalloidin-TRITC were quantified using the NIS-Elements 3.1 software to define the time course of F-actin during capacitation in the presence or absence of Secramine. * $P=0.002$. (B) The acrosomal reaction was assessed at 90 min of capacitation in the different assays. * $P=0.05$ (mean $\pm$ S.E.M., $n=3$ independent experiments).

the sAR was significantly inhibited with respect to the control in all assays using SecA (Fig. 3B).

RHOA does not increase its activity when CDC42 is inhibited during capacitation

The results presented thus far suggest that CDC42 has a major effect on RHOA at the beginning of actin polymerization during capacitation. To test this hypothesis, we assessed the activation of CDC42 and RHOA chronologically during capacitation. Figure 4A shows that the amount of CDC42-GTP increases significantly ($P=0.01$) at 10 min of capacitation with respect to non-capacitated sperm (time 0 min of incubation). The maximum amount of CDC42-GTP was detected between 10 and 20 min of capacitation, decreasing significantly ($P=0.022$) at 30 min, but maintaining significantly higher levels with respect to time 0 min ($P<0.05$, Fig. 4A). In the case of RHOA-GTP, the immunoblot analysis showed an increase with respect to capacitation time, an increase that was inhibited by the C3 toxin (Fig. 4B). The densitometric analysis of immunoblots showed that the level of RHOA-GTP increased significantly ($P=0.001$) at 20 min of capacitation compared to non-capacitated sperm (Fig. 4A). The maximum level of RHOA-GTP was reached until 60 min of capacitation. These data indicate that the CDC42 and RHOA proteins are activated sequentially: CDC42 is activated early during capacitation, and that is not until CDC42 reaches its maximum activity that RHOA activity increases significantly.

To determine whether the activity of RHOA depends on the activity of CDC42, the levels of RHOA-GTP

Figure 4 CDC42 and RHOA are activated at different times of capacitation. (A) Whole extracts obtained from sperm capacitated at different times were used to determine the activity of CDC42 by the quantification of CDC42-GTP (see Materials and methods for details). RHOA-GTP was isolated from whole extracts obtained from sperm capacitated at different times (see Materials and Methods for details). Sperm capacitated in the presence of C3 (1 μ M) were used as control. (B) The WB of RHOA was analyzed by densitometry. The results are expressed as the ratio N/N0, where N is the total amount of RHOA-GTP and N0 is the total amount of RHOA. Both CDC42 and RHOA normalized concerning non-capacitated (0 min). Means $\pm$ S.E.M., $n=3$ independent experiments. Images are representative of three independent experiments.

from capacitated sperm were assessed using the assays described in a previous section (Effects of CDC42 inhibition on actin polymerization and the acrosome reaction). Assay 1: Sperm capacitated in the absence of SecA. The amount of RHOA-GTP isolated from sperm increased significantly ($P=0.05$) during capacitation with respect to non-capacitated sperm (0 min, Fig. 5A and B). Assay 2: SecA added from the beginning of capacitation (0 min). The amount of RHOA-GTP recovered in this assay was similar to that obtained from non-capacitated sperm in the absence of SecA (Fig. 5A and B). Assay 3: The amount of RHOA-GTP recovered from sperm capacitated in the presence of SecA, added 15 min after the beginning of capacitation, was similar to that of non-capacitated sperm in the absence of SecA. Although an increase in the amount of isolated RHOA-GTP was observed, such increase was not significantly larger than that of sperm capacitated for 30 or 60 min in the absence of SecA (Fig. 5A and B). Assay 4: The amount of RHOA-GTP isolated from sperm capacitated in the presence of SecA added 30 min after the beginning

Figure 5 RHOA was not activated when sperm were capacitated in the presence of Secramine A. (A) Whole extracts obtained from sperm capacitated in the absence or presence of SecA (5 μ M) following four different tests: Assay 1: Sperm capacitated in the absence of SecA. Assay 2: Sperm capacitated in the presence of SecA. Assay 3: SecA added at 15 min of capacitation. Assay 4: SecA added at 30 min of capacitation. These extracts were used to isolate RHOA-CDC42 by pull-down. WB images correspond to three independent experiments. (B) RHOA and RHOA-GTP protein bands were analyzed by densitometry, and the results are expressed as an N/N0 ratio, where N is the total amount of RHOA-GTP and N0 is the total amount of RHOA and normalized with respect to non-capacitated sperm (0 min). Means $\pm$ S.E.M., $n=3$ independent experiments. * $P=0.007$, ** $P=0.04$.

of capacitation was similar to that of sperm in Assay 1, both at 30 and 60 min of incubation (Fig. 5A and B).

ROCK1 inhibition affects actin polymerization but not the acrosome reaction

Considering that the main effectors of RHOA-GTP are RHOA 1 and 2 kinases (ROCK1 and 2), we analyzed the role of ROCKs on capacitation, the AR, and actin polymerization. First, we determined the presence of ROCK1 and 2 in the sperm. Using a specific ROCK1 antibody and Western blotting, we detected only a protein band of approximately 160 kDa, both for sperm and the MCF7 cell line, which was used as a control (Fig. 6A). In the case of ROCK2, it was only detected in the MCF7 cell line and not in sperm (Fig. 6A). Additionally, ROCK1 was located in the acrosomal region and the sperm flagellum. ROCK2 was not found in guinea pig sperm (Fig. 6B). These data suggest that, as with other mammalian sperm, guinea pig sperm express only ROCK1 (Ducummon & Berger 2006, Romarowski et al. 2015).

Figure 6 ROCK1 is expressed in guinea pig sperm and does not participate in the acrosomal reaction. (A) Immunodetection of ROCK1 and ROCK2 in guinea pig sperm in complete protein extracts of sperm cells (SC) and MCF7 cells (MC). ROCK1 and ROCK2 proteins were detected using specific antibodies. The images represent independent experiments ($n=3$). (B) Immunolocalization of ROCK1 and ROCK2 in guinea pig sperm. Top panels: immunolocalization of ROCK1 and ROCK2. Lower panels: brightfield images. The images represent three independent experiments. (C) Effects of ROCK inhibitor (Y-27632) on the AR. Sperm were capacitated in the presence or absence of different concentrations of Y-27632, and its effects on the AR were assessed by CTC assay. Means $\pm$ S.E.M., $n=3$ independent experiments. (D) Effects of Y-27632 inhibitor on actin polymerization. Sperm were capacitated in the presence or absence of Y-27632, F-actin was stained with phalloidin-FITC, and fluorescence was assessed using the NIS-Elements 3.1 software. Means $\pm$ S.E.M., $n=3$ independent experiments. * $P=0.007$. (E) Sperm were capacitated (60 min) in the absence or presence of the ROCKs inhibitor, Y-27632 (100 nM), and then stained with phalloidin-FITC to reveal F-actin. The images represent three independent experiments.

Using a Y-27632 antagonist, specific for both ROCKs, we determined the role of ROCK1 in the sAR and the polymerization of actin. Sperm were capacitated in the presence of different concentrations of the Y-27632 inhibitor (0–400 nM). After 90 min of incubation in the capacitation-supportive medium, the sAR was assessed by CTC assay. Results showed that the sAR was unaffected by any concentration of Y-27632 (Fig. 6C). We also determined the kinetics of the capacitation and sAR processes from 0 to 90 min of incubation. Figure 1B shows that in sperm capacitated in the presence of Y-27632 (100 nM) the pattern B increased until 30 min of capacitation. After 30 min, the pattern B suffered a non-significant decrease that coincides with an accelerated increase in the pattern AR (Fig. 1C), that at 60 min of capacitation is significantly greater ($P=0.001$) than exhibited by the control, capacitated sperm in the absence of any of the inhibitors. The levels of the pattern AR in the presence of Y-27632 did not exceed those reached by the control (Fig. 1C). In order to know whether the iAR is affected by Y-27632, sperm were

capacitated in the presence of this ROCKs inhibitor (100 nM), and the AR was induced by progesterone (10 μ M). In essence, iAR in the presence of Y-27632 shows similar values to iAR in the absence of the inhibitor (Fig. 1F). These results suggest that the development of sperm capacitation is not affected even when ROCK-1 is inhibited. This suggestion was confirmed by analyzing PYP levels. PYP levels were similar for capacitated sperm in the presence or absence of the ROCK1 inhibitor (Fig. 2D and E).

By contrast, when sperm were capacitated in the presence of Y-27632 (100 nM), the polymerization of actin was significantly modified. In the first 15 min of capacitation, the amount of F-actin increased to the same extent as the control, sperm capacitated in the absence of Y-27632 (Fig. 6D). After this time, the amount of F-actin remained unchanged, and its levels remained similar until 90 min of capacitation, significantly lower than those determined for the control at the same incubation times (30–90 min, Fig. 6D). Interestingly, the amount of F-actin between 60 to 90 min of capacitation

is significantly higher ($P \leq 0.01$) than those shown when sperm were capacitated in the presence of C3 and SecA (Figs 2B and 6D). The location of F-actin in sperm capacitated in the presence of Y-27632 (100 nM) shows that the majority of the polymerized actin is found in the apical region of the acrosome, while the rest of the head and the flagellum show a lesser fluorescence (Fig. 6E). These results suggest that ROCK1 participates in actin polymerization of the flagella and head, but not in the apical region of the acrosome.

Discussion

RHO GTPase is well known for its role in the regulation of the dynamics of the actin cytoskeleton, and although there are approximately 22 different RHO proteins (Narumiya & Thumkeo 2018), some of the most studied have been RHOA and CDC42. Several studies have reported the expression of different RHO proteins such as RHOA, RHOB, RHOC, and CDC42 in mammalian sperm (Delgado-Buenrostro *et al.* 2005, Ducummon & Berger 2006, Romarowski *et al.* 2015). Although we are aware that the subcellular locations of RHOA and CDC42 are similar, the acrosomal region and the flagellum (Ducummon & Berger 2006, Baltierrez-Hoyos *et al.* 2012, Delgado-Buenrostro *et al.* 2016), little is known about the involvement of these RHO proteins in physiological processes such as capacitation, the AR, and actin cytoskeleton dynamics. The expression of other RHO proteins, such as RHOB and RHOC, has also been reported in guinea pig and mouse sperm; however, the location of RHOB is limited to the perinuclear region (Delgado-Buenrostro *et al.* 2005), while the subcellular location of RHOC is unknown (Romarowski *et al.* 2015). The present paper provides the first evidence of the relationship between RHOA and CDC42 in the dynamics of the actin cytoskeleton and how they participate in capacitation and the AR.

Our results showed that only SecA inhibits actin polymerization since the first minutes of capacitation, while that F-actin presents a transient increase (15 min of capacitation) in the treatment with C3 (Fig. 1B). Although the sperm were incubated with C3 1 h before their placement in the capacitation medium, C3 may require these 15 min to reach an intracellular concentration that allows for the total inhibition of RHOA and, consequently, of actin polymerization. C3 is known not to inhibit CDC42 (Aktories *et al.* 1995, Just *et al.* 1995), which suggests that the transient increase could be due to the activity of CDC42. However, these two possibilities fail to explain why actin polymerization is depleted after 15 min of capacitation in the presence of C3 even though CDC42 continues to be active after 15 min of capacitation (Fig. 4). Our research suggests that, at the beginning of capacitation (0–30 min), CDC42 is required to initiate actin polymerization, perhaps forming nucleation sites through WASP/ARP2/3

(Jaffe & Hall 2005) proteins present in guinea pig sperm (Delgado-Buenrostro *et al.* 2005), sites required by RHOA to regulate the formation of actin filaments (Jaffe & Hall 2005). This hypothesis is supported by the following facts: (1) only the inhibition of CDC42 prevents the total polymerization of actin from the beginning of capacitation; (2) the increase in actin polymerization is prevented when CDC42 activity is inhibited before 30 min of capacitation, but not after 30 min of capacitation; and (3) RHO protein activation tests indicate that maximum CDC42 activity is reached after 15 min of capacitation, and after this time, the activity decreases to a baseline level, higher than shown in the non-capacitated state. In addition, RHOA activity reaches significant levels at 20 min of capacitation, increasing its activity constantly during capacitation (Fig. 4). These facts indicate that CDC42 directs actin polymerization during the first 20 min of capacitation. They also suggest that RHOA takes over in actin polymerization after 20 min of capacitation and that CDC42 activity is possibly not required for actin polymerization but necessary for the activation of other signaling pathways, such as those related with the AR (Baltierrez-Hoyos *et al.* 2012). This hypothesis is supported by an important finding, the inhibition of CDC42 by SecA, that prevents normal activation of RHOA until before 30 min of capacitation (Fig. 5). These results suggest that the activity of RHOA during the first minutes of capacitation depends on the activity of CDC42 and that such dependence is lost after 30 min of capacitation since, after this time, the activity of RHOA was not prevented by the inhibition of CDC42 (Fig. 5) and actin polymerization occurred normally (Fig. 3A). It should be noted that SecA is a very specific CDC42 inhibitor, which does not alter the activity of other RHO proteins (Pelish *et al.* 2006); therefore, the inhibition of RHOA activity by CDC42 is not caused by SecA. On the other hand, it has been reported that an insufficient expression of CDC42 prevents the sperm to develop actin polymerization normally, even if RHOA levels are normal (Angeles-Floriano *et al.* 2016). Taken together, these suggest a cross-talk between CDC42 and RHOA, a cross-talk that plays an important role not only in actin polymerization, but also in capacitation and acrosomal reaction.

Previous studies have shown that RHOA or CDC42 inhibition prevents either capacitation or AR (Brenner *et al.* 2003, Baltierrez-Hoyos *et al.* 2012, Angeles-Floriano *et al.* 2016). The present study analyzed these events using a technique (CTC) that allowed us to define the physiological states that sperm undergo during their incubation in a medium that supports capacitation (Ward & Storey 1984, Shi & Roldan 1995, Mattioli *et al.* 1996, Cordero-Martinez *et al.* 2018). Recently, we reported that a low CDC42 expression prevents actin polymerization; however, capacitation is achieved, but AR is interrupted (Angeles-Floriano *et al.* 2016). The data in the present study are consistent with those reported

by Angeles-Floriano *et al.*, they show that the majority of sperm remains capacitated without experiencing the AR (Fig. 2). It should be noted that SecA inhibited both, the sAR and iAR, in all of our essays (Fig. 3B), suggesting that the participation of CDC42 in the AR goes beyond actin polymerization. We propose that the sperm cells fail to experience the AR because CDC42 is required to form and activate the VAMP2-Syntaxin 1A complex, which is required in exocytosis processes (Alberts *et al.* 2006, Nevins & Thurmond 2006, Bretou *et al.* 2014), as the AR. Similarly RHOA is required for SNAP-25 activation, another SNARE involved in the process of exocytosis (Horvath *et al.* 2017). It has been proposed that the inhibition of actin polymerization by C3, a toxin that blocks the activity of RHO proteins (RHOA, RHOB, and RHOC), results in the inhibition of the AR (Brener *et al.* 2003, Breitbart *et al.* 2005). Our results suggest that RHOA inhibition prevents capacitation, and as a consequence, the sperm no experience AR (Fig. 2). Together, our results confirm that CDC42 could be more involved in AR than in capacitation, while RHOA, unlike CDC42, would be principally related to capacitation.

The signaling pathway that leads to actin polymerization involves RHO proteins and ROCK1 or ROCK2 (Fiedler *et al.* 2008, Amano *et al.* 2010, Romarowski *et al.* 2015); therefore, the inhibition of ROCKs during capacitation should inhibit actin polymerization and, consequently, capacitation and the AR (Brener *et al.* 2003, Breitbart *et al.* 2005). Our results show that the inhibition of ROCK1 does not interrupt the actin polymerization in the acrosome apical region and the course of the AR, but it is interrupted in the flagellum (Fig. 6D). Similar results have been reported for avian sperm (Ashizawa *et al.* 2006). The analysis of actin polymerization kinetics during capacitation in the presence of Y-27632 indicates that, when RHOA is inhibited, F-actin increases until 15 min incubation and that their level is preserved until 90 min of capacitation (Fig. 6). This increase occurs in the acrosome apical region apical and may be sufficient for the development of the sAR and iAR. The signaling pathway that links RHOA with capacitation and the AR could involve an effector different from ROCK1, perhaps a member or members of the diaphanous-related formin-1 (mDIA) family (Sakamoto *et al.* 2012) that would maintain adequate levels of F-actin for capacitation and the AR to happen. Another possibility is that other RHO protein, not inhibited by Y-27632, would be related to the actin polymerization between the external plasma and acrosomal membranes, an event required to avoid early AR, and that this sperm process occurs in the usual way (Spungin *et al.* 1995, Hernandez-Gonzalez *et al.* 2000, Breitbart *et al.* 2005). We have recently reported that RAC1 could be related to the formation of this cytoskeleton (Ramirez-Ramirez *et al.* 2020).

It is important to note that inhibition of ROCK1 during capacitation accelerates AR presentation, possibly as a

result of sperm being rapidly subjected to capacitation process. A similar effect has been reported when FAK is inhibited: it inhibits actin polymerization, allows early PYP and consequently the early presentation of AR (Roa-Espitia *et al.* 2016). Therefore, we hypothesize a FAK/RHOA/ROCK1/Actin polymerization relationship with PYP regulation and therefore with capacitation. Further study is required to prove this hypothesis.

In summary, we have provided evidence indicating that CDC42 and RHOA proteins have specific and essential functions in sperm physiology. CDC42 seems to have an important role in the initiation of actin polymerization during capacitation. Although its inhibition does not impact capacitation, it does affect AR. On the other hand, RHOA would seem to have a predominant role in capacitation, which consequently impacts AR. This conclusion is supported by the fact that ROCK1 would appear to have no participation in the AR. We also identified that RHOA activity is dependent on CDC42 activity and that they act in tandem during capacitation; CDC42 initiates the polymerization of actin and after RHOA takes over. However, both RHO proteins are essential for the normal remodeling of the actin cytoskeleton to take place during capacitation. Finally, we suggest that the different RHO proteins (CDC42, RAC1, and RHOA) present in mammalian sperm regulate actin polymerization in specific regions of sperm and thus participate in the diverse physiological processes that sperm experience to acquire fertilizing ability.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T M R carried out the experiment and analyzed the data. R B H helped to carry out the experiment. A L R E helped supervise the project, standardized the CTC assay and administered the project. E O H G conceived of the presented idea, planned the experiments, analyzed the data and wrote the manuscript.

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