

Calpain inhibition prevents flotillin re-ordering and Src family activation during capacitation

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Abstract Prior to fertilization, mammalian sperm undergo several molecular, biochemical and physiological changes in a process termed capacitation. However, the mechanisms explaining the involvement of cytoskeletal remodeling and membrane re-ordering in each process prior to fertilization remain poorly understood. We found that the migration of both flotillin microdomains and Src family kinases towards the apical ridge of guinea pig sperm occurs under capacitating conditions. This re-ordering is associated with spectrin cleavage by calpain. Moreover, Src, Fyn, Lyn and Hck interact with flotillin-1; this interaction increases in a capacitation-dependent manner and the increased autophosphorylation of these kinases is linked to flotillin-1 association. The aforementioned results are prevented by the inhibition of calpain by calpeptin. Thus, spectrin cytoskeleton cleavage during capacitation seems to precede the reorganization of flotillin microdomains and Src family kinases towards the apical ridge of the sperm head in order to initiate the signaling cascade required for proper capacitation and further acrosome reaction. The significance of the Src family kinase reorganization for capacitation is demonstrated by the inhibition of calpain during capacitation also preventing the Src-family-kinase-dependent phosphorylation of FAK at Tyr576/577. Our work further highlights the scaffolding properties of flotillin microdomains

and reveals the importance of their large-scale segregation during capacitation.

Keywords Flotillin microdomain aggregation · Spectrin and sperm membrane domains · Src family kinases · Acrosome reaction · Role of spectrin in capacitation

Introduction

Freshly ejaculated mammalian spermatozoa are unable to fertilize mature oocytes; in order to become competent for fertilization, these cells require a period of time within the female reproductive tract (in vivo) or incubation in a defined medium (in vitro; Austin 1952; Chang 1951). During this time, several biochemical and physiological changes, collectively called capacitation, occur in the spermatozoa (Gangwar and Atreya 2015; Stival et al. 2016). In mammalian sperm, capacitation is defined as a complex process that includes membrane modifications, enzyme activity modulation, cytoskeleton reorganization, membrane hyperpolarization and protein phosphorylation (Aitken and Nixon 2013; Gangwar and Atreya 2015). Furthermore, capacitation is accompanied by an increment in flagellar bend amplitude called sperm hyperactivation (Olson et al. 2011; Suarez 2008). Once capacitation has occurred, sperm are able to undergo a physiologically induced acrosome reaction (AR) in order to become fertilization-competent.

Accumulating evidence has revealed that sperm membranes contain lipid-ordered microdomains termed “lipid rafts” that undergo changes in their spatial distribution under capacitating conditions (Asano et al. 2013; Bou Khalil et al. 2006; Selvaraj et al. 2006) following an apparent increase in the fluidity of lipid microdomains (Hinkovska-Galcheva and Srivastava 1993). These lipid rafts are reorganized towards the apical ridge of the sperm head following capacitation, giving

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rise to a clustering of raft markers that may favor specific signaling and protein interactions (Nixon and Aitken 2009; Shadan et al. 2004; van Gestel et al. 2005). Nonetheless, the underlying mechanisms and importance of this phenomenon are still poorly understood.

Flotillins and caveolins are commonly used as membrane lipid raft markers and are expressed in mammalian sperm (Cross 2004; Miranda et al. 2009; Travis et al. 2001; van Gestel et al. 2005). The flotillin family is composed of two homologous members (flotillin-1 and flotillin-2), which are evolutionarily conserved across distant phyla (Rivera-Milla et al. 2006). They preferentially associate with each other in the form of oligomeric complexes and are capable of forming their own type of non-caveolar membrane microdomains by the formation of homo- and hetero-tetramers (Solis et al. 2007) with scaffolding properties favoring specific protein interactions and cell signaling (Lang et al. 1998; Stuermer et al. 2001). Flotillins are situated at the cytoplasmic face of the plasma membrane and at membranes of intracellular compartments (Lang et al. 1998) through myristoylation and palmitoylation modifications (Morrow et al. 2002; Neumann-Giesen et al. 2004). Several publications have suggested that the scaffolding properties of the flotillin microdomains regulate cell adhesion and signaling events; however, their precise molecular function remains unknown (Stuermer and Plattner 2005). The selective enrichment of proteins within flotillin microdomains includes glycosylphosphatidylinositol (GPI)-anchored proteins (Stuermer et al. 2001), cytoskeleton-associated proteins (Langhorst et al. 2007) and doubly acylated proteins, such as the Src family kinases (SFKs; Langhorst et al. 2006). Diverse evidence indicates that flotillins interact with signaling proteins such as Src, Fyn and Lyn in various cell types (Liu et al. 2005; Nebl et al. 2002; Neumann-Giesen et al. 2007). Since both SFKs and flotillins are present in the guinea pig sperm cell, an interaction between these proteins seems possible.

To date, the Src family of kinases in mammalian cells includes at least eight members, Src (the original member), together with Blk, Fgr, Fyn, Hck, Lck, Lyn and Yes; they all have a common molecular structure, conserved Src-homology 2 (SH2) and Src-homology 3 (SH3) peptide domains and similar molecular weights in the 52–62 kDa range. Nonetheless, each kinase possesses a unique sequence at their N-terminal region and undergoes different acyl modifications from the others (Brown and Cooper 1996; Okada 2012). In somatic cells, SFKs play key roles in various pathways, such as cell differentiation, proliferation, survival, adhesion, morphology and motility (Thomas and Brugge 1997).

The presence of SFKs in the mammalian sperm cell has been determined; however, depending on the species, their individual localization and abundance are highly uneven, suggesting specific functions for each kinase. In order to elucidate the role of SFKs in mammalian sperm, the entire family has

been inhibited by a small-molecule, namely indolinone (SU6656), during capacitation (Goupil et al. 2011; Mitchell et al. 2008), resulting in the discovery that the inhibition decreases tyrosine phosphorylation in human (Mitchell et al. 2008) and murine (Baker et al. 2006) sperm, inhibits the motility of murine sperm (Baker et al. 2006) and blocks the acrosome reaction in human (Tapia et al. 2011), bovine (Etkovitz et al. 2009) and guinea pig (Baltierrez-Hoyos et al. 2012) sperm but also causes a twofold increase in the acrosome reaction in porcine sperm (Bragado et al. 2012). All of the above suggest that perhaps SFKs mediate diverse pathways in mammalian sperm in a species-specific manner.

Various polarized cell types are able to organize and maintain specialized membrane domains in order to coordinate processes within the underlying cortical cytoplasm and cytoskeleton. In order to do so, membrane domains are enriched with selective lipids and proteins stabilized by spectrin, actin, or dystrophin cytoskeletons (Le Rumeur et al. 2010; Machnicka et al. 2014). Spectrin forms a submembranous cytoskeleton comprising α - and β -subunits assembled in antiparallel filamentous heterodimers. The spectrin cytoskeleton is linked to the plasma membrane through ankyrin (Bennett and Healy 2009); however, novel evidence strongly suggests that spectrin associates with membrane domains by means of flotillins in red blood cells and neutrophils (Head et al. 2014; Ludwig et al. 2010; Salzer and Prohaska 2001). The relationship between the spectrin cytoskeleton and flotillins is still poorly understood. On the contrary, the interaction of flotillin-actin has been broadly studied. The immobility and lateral mobility of flotillins depend on the stabilization or disruption of the actin cytoskeleton, respectively (Langhorst et al. 2007).

In the mammalian sperm cell, depending on species, the actin and spectrin cytoskeletons are organized under the plasma membrane in both the head and flagellar regions; however, despite their undoubtedly important role, little is known about the mechanism of cytoskeleton dynamics (Bastian et al. 2010; Camatini et al. 1991; Hernandez-Gonzalez et al. 2000; Xiao and Yang 2007). Actin polymerization is a key process occurring during capacitation, whereas a fast F-actin breakdown is essential to achieve the acrosome reaction (Brenner et al. 2003). Moreover, actin dynamics in the head and flagellum are involved in Izumo1 relocation and sperm motility, respectively (Azamar et al. 2007; Finkelstein et al. 2013; Sosnik et al. 2009).

Calpains are proteases responsible for the degradation of several cytoskeletal elements (Briz and Baudry 2016) and have been associated with motility and the acrosome reaction (Aoyama et al. 2001; Ozaki et al. 2001; Ben-Aharon et al. 2005), because during capacitation, calpain inhibition blocks motility and the acrosome reaction. We have shown that, by inhibiting spectrin cytoskeleton cleavage via calpain, the normal course of the acrosome reaction is blocked in guinea pig

sperm (Bastian et al. 2010). Interestingly, flotillin microdomains have the property of spatially organizing the plasma membrane and allowing specifically located signaling (i.e., active SFKs) by interacting with the cell cortical cytoskeleton, specifically with actin, spectrin and myosin 2a (Langhorst et al. 2007; Ludwig et al. 2010). Significant aggregation of flotillin-1 in the apical acrosomal region has been reported as being a capacitation marker in boar sperm (van Gestel et al. 2005); however, the mechanisms regulating such an aggregation and its involvement in capacitation and the acrosome reaction have not been fully elucidated. Consequently, the purpose of this work has been to study the role of the interaction of flotillin microdomains with SFKs and the relocation of the microdomains with respect to the remodeling of the actin and spectrin cytoskeletons during capacitation of guinea pig sperm.

Materials and methods

Antibodies and reagents

Rabbit polyclonal antibodies against flotillin-1 (H104), Src (N16), Fyn (FYN3), Lyn (44), Hck (N30) FAK (A-17), p-FAK (Tyr576/577; sc-21831) and Rac-1 (C-11) and mouse anti-flotillin-2 monoclonal antibody (B6) were purchased from Santa Cruz Biotechnology (Dallas, Tex., USA). Rabbit polyclonal antibody against Src family kinases phosphorylated at Tyr⁴¹⁶ (PK1109), polyvinylidene fluoride (PVDF) blotting membranes, calpeptin (03340051) and Immobilon horseradish peroxidase (HRP) substrate (WBKLS0500) were purchased from Merck Millipore (Billerica, MA, USA). The anti-utrophin 71 antibody (Up71) was kindly donated by Dr. Dominique Mornet from INSERM U592 (Paris, France). HRP-conjugated anti-rabbit, HRP-conjugated anti-mouse, HRP-conjugated anti-goat, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa., USA). The Clean-Blot Detection IP Reagent AP (21230) was bought from Thermo Fisher Scientific (Rockford, Ill., USA). Latrunculin A (L5163), anti-chicken spectrin antibody (S1390), Triton X-100, sodium bicarbonate, calcium chloride dihydrate, sodium chloride, sodium pyruvate, sodium azide, polyvinyl alcohol (PVA), glycerol, formaldehyde, glutaraldehyde, bovine serum albumin (BSA), DL-lactic acid 85 %, iodoacetamide, benzamidine, aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), TRIS base (Trizma), soybean trypsin inhibitor, sodium orthovanadate, rhodamine phalloidin, sodium fluoride and chlortetracycline (c-4881) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Complete mini protease inhibitor cocktail was purchased from Hoffmann La-Roche (Basel,

Switzerland). Acrylamide, N,N'-methylenebisacrylamide and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories (Hercules, Calif., USA). Amersham ECL (RPN2106), Hyperfilm ECL (28906836) and Hypercassette autoradiography cassette (RPN11649) were purchased from GE Healthcare Bio Sciences (Pittsburgh, Pa., USA). Fixer and replenisher (1901875), developer and replenisher (1900943) and medical X-ray blue/MXB film (1668144) were bought from Carestream Health (Rochester, N.Y., USA).

Animals

Male Dunkin-Hartley guinea pigs (*Cavia porcellus*) were bred and maintained until they reached 800–900 g. They were housed in a vivarium at Cinvestav-IPN on a 12 h light / 12 h darkness schedule with access to food and water *ad libitum*. All animal handling procedures and experimental designs were approved by the Internal Committee for the Care and Use of Laboratory Animals, Cinvestav-IPN (CICUAL No. 321-02), following American Veterinary Medical Association guidelines. All efforts were made to minimize the potential for animal pain, stress, or distress.

Sperm capacitation

Capacitation was performed as described by Rogers and Yanagimachi (1975). Sperm were obtained from the *ductus deferens* of the guinea pigs and then washed in 154 mM NaCl solution. The sperm cells (35×10^6 cell/ml) were capacitated by incubation at 37 °C in minimal culture medium (MCM-PL) containing: 105.9 mM sodium chloride, 1.71 mM calcium chloride, 25.07 mM sodium bicarbonate, 0.25 mM sodium pyruvate, 20 mM DL-lactic acid 85 %, pH 7.8. Capacitation was conducted for the desired time.

Calpeptin and latrunculin A treatments

In order to avoid spectrin cytoskeleton degradation, sperm cells were treated with calpeptin, a specific calpain inhibitor. Sperm were obtained and washed in a 154 mM NaCl solution supplemented with calpeptin 10 μM. The sperm cells (35×10^6 cell/ml) were capacitated by incubation at 37 °C in MCM-PL supplemented with 10 μM calpeptin. When cells attained 60 min of capacitation, the MCM-PL was supplemented with another dose of calpeptin to reach a final concentration of 20 μM and to preserve the inhibition of calpain until 90 min of capacitation. Additionally, a set of protease inhibitors was supplemented with 10 μM calpeptin and stored at –70 °C. Since the actin cytoskeleton is involved in flotillin microdomain re-ordering and since actin is polymerized during capacitation, sperm were capacitated in MCM-PL supplemented with 50 μM latrunculin A in order to prevent actin polymerization.

Immunofluorescence assays

Ductus deferens sperm were fixed with 3 % (v/v) formaldehyde and 0.2 % (v/v) glutaraldehyde in phosphate-buffered saline (PBS). After 1 h, the sperm were collected by centrifugation. The pelleted sperm (600 g for 3 min) were incubated in 50 mM NH₄Cl for 10 min and rinsed twice with PBS and finally with bi-distilled water. Microscope slides were prepared by using this suspension, air-dried at room temperature overnight and stored at 4 °C. Sperm cells were permeabilized in acetone for 7 min at –20 °C and washed with PBS. Flotillin-1 (1:50), flotillin-2 (1:50), Src (1:25), Fyn (1:50), Lyn (1:50) and Hck (1:25) antibodies were diluted in PBS with 1 % BSA (blocking solution) and incubated on the slides overnight at room temperature. The slides were incubated for 2 h at 37 °C with the appropriate TRITC- or FITC-conjugated secondary antibody diluted in blocking solution. The samples were mounted with glass-coverslips by using Gelvatol, sealed adequately and stored at –20 °C until observed. The stained cells were imaged under a confocal laser scanning microscope (Leica TCS SP8) and analyzed by using the imaging software LAS AF Lite (Ver. 2.6.3).

Western blotting

Ductus deferens sperm were obtained, washed in 154 mM NaCl solution and capacitated when needed. They 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 benzamide, 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 H₂O]). The samples were then incubated for 20 min on ice and centrifuged (14,000 g) for 20 min at 4 °C. The supernatants were collected and their protein concentration was determined (Bradford 1976). For the reduction of protein disulfide bonds, supernatant aliquots were boiled for 5 min in 3× Laemmli buffer (720 mM TRIS-base, 6 % SDS (w/v), 30 % glycerol, 0.03 % bromophenol blue (w/v), pH 10). They were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–12 % polyacrylamide non-gradient gels and subsequently transferred to PVDF membranes and blocked with 5 % skimmed milk in 1× PBS/1 % Triton X-100 (pH 7.5). When probed for spectrin, proteins were initially run on 6 % polyacrylamide gels and transferred to nitrocellulose membranes as performed by Bastian et al. (2010). For other proteins, 10 % polyacrylamide gels were used. The membranes were incubated overnight at 4 °C with the primary antibody. The dilutions for the primary antibodies were as

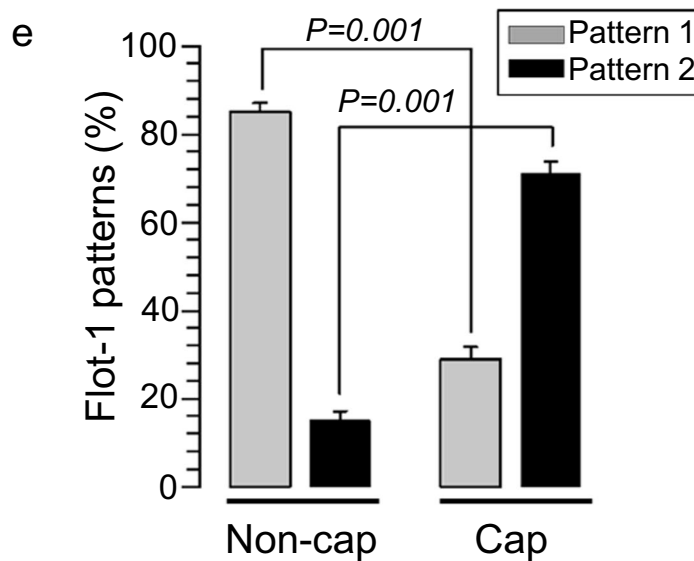
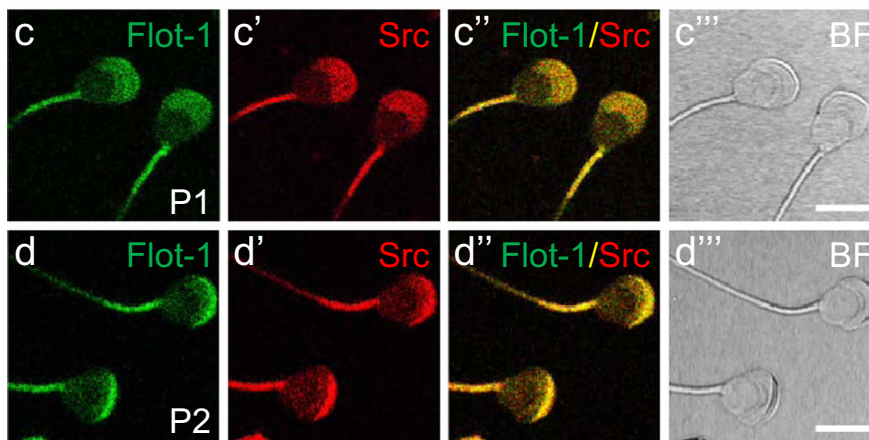
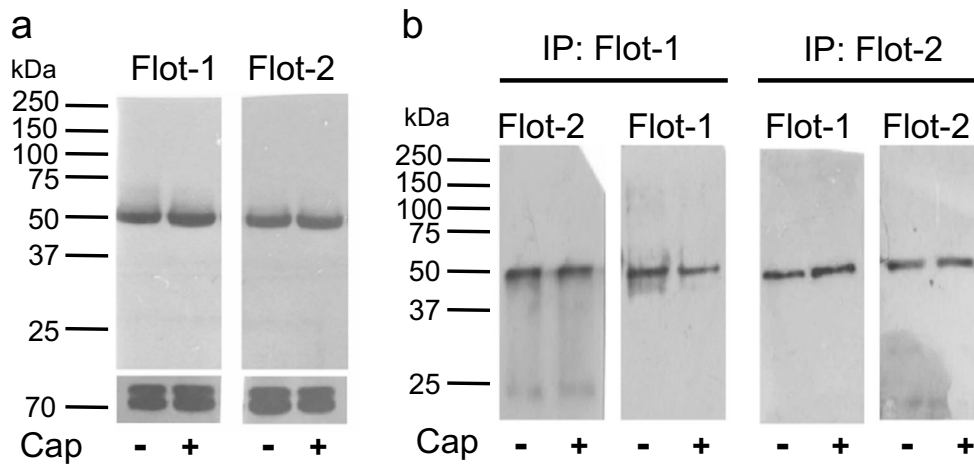
follows: flotillin-1 (1:500), flotillin-2 (1:500), Src (1:500), Fyn (1:500), Lyn (1:500), Hck (1:250), FAK (1:500), p-FAK (1:500) and Rac-1 (1:250). Membranes were next incubated with their respective HRP secondary antibody: HRP-conjugated anti-rabbit (1:5000), HRP-conjugated anti-mouse (1:1250), or HRP-conjugated anti-goat (1:2000). The PVDF membranes were bathed with Amersham ECL and exposed to a MXB film inside an autoradiography cassette. Gel loading was normalized to Up71 (1:500). When the antibody for Src family kinases phosphorylated at Tyr⁴¹⁶ (anti-p-Tyr⁴¹⁶, 1:1000) was used, membranes were blocked for 2 h with 5 % BSA in 1× PBS/1 % Triton X-100 (pH 7.5) and incubated overnight at 4 °C with 3 % BSA in 1× PBS/1 % Triton X-100 (pH 7.5), followed by the Clean-Blot Detection IP Reagent HRP-conjugate (1:2000; this reagent is optimized for post-immunoprecipitation) in order to minimize heavy and light chain IgG bands. Finally, the PVDF membranes were bathed with Immobilon HRP substrate and exposed to a Hyperfilm ECL inside an autoradiography cassette to optimize resolution and reduce exposure time.

Co-immunoprecipitation assay

Co-immunoprecipitation experiments were performed by using the Crosslink Immunoprecipitation Kit (26147) purchased from Thermo Fisher Scientific. This method involves the capture of 20 µg IP antibody on Protein A/G beaded agarose resin and covalently immobilizing it on the support by crosslinking with 2.5 mM disuccinimidyl suberate (DSS). The antibody resin is then incubated at 4 °C for 12 h with 500 µg pre-cleared guinea pig sperm protein extracts, allowing the antibody-antigen complex to form. Proteins bound to the respective antibodies were eluted, recovered by low speed centrifugation (3000 g) and stored at –20 °C. The entire procedure was performed in a microcentrifuge spin cup. Only the antigen is eluted in the procedure, enabling it to be identified and further analyzed with minimal interference from antibody fragments. For protein disulfide reduction, supernatant aliquots were boiled for 5 min in 3× Laemmli buffer (pH 10) containing 2-mercaptoethanol and the proteins were then separated by SDS-PAGE. Next, proteins were transferred to PVDF membranes for immunodetection. A secondary antibody (Clean-Blot Detection Reagent AP) specific for IP assays was used to eliminate detection-interference from both heavy and light chain IgG fragments.

Phalloidin staining assays

Epididymal sperm cells were fixed on slides as described above. The cells on the slides were permeabilized in acetone for 7 min at –20 °C and washed with PBS. In order to detect F-actin staining patterns, cells were incubated for 1 h at 37 °C with TRITC-phalloidin (1:20) and washed 5 times in PBS.



The samples were mounted on glass-coverslips by using Gelvatol, sealed adequately, and stored at $-20\text{ }^{\circ}\text{C}$. The stained cells were imaged under an optic epifluorescence microscope

(Olympus BX50) and analyzed by using the imaging software NIS-Elements AR (Ver. 3.1). All washes were carried out 5 times during a period of 5 min with PBS.

Fig. 1 Flotillin-1 (*Flot-1*) and flotillin-2 (*Flot-2*) remain associated after capacitation and re-order in a capacitation-dependent manner. **a** Whole sperm extracts from non-capacitated (–) and 90-min (+) capacitated (*Cap*) guinea pig sperm were subjected to immunoblotting in order to determine the presence of flotillins and possible changes that these proteins could undergo during capacitation. The protein utrophin of 71 kDa (Up71) was used to ensure equal protein loading (*bottom*). Western blot images are representative of three independent experiments. **b** Co-immunoprecipitation (*IP*) assays were carried out with total sperm extracts from non-capacitated and 90-min capacitated sperm by using anti-flotillin-1 antibody. The precipitated proteins were subjected to SDS–PAGE and Western blot analysis and were probed for flotillin-1 as a positive control and with anti-flotillin-2 antibody to determine its association. The reciprocal strategy was performed with anti-flotillin-2 antibody. Western blot images are representative of three independent experiments. **c–d** Flotillin-1 and flotillin-2 were immunolabelled in non-capacitated (**c–c'**) and capacitated (**d–d'**) sperm (90 min) with intact acrosome. Co-localization was analyzed by confocal microscopy. *P1* represents a dispersed pattern in the acrosome, and *P2* represents an apical clustered pattern (*BF* bright field images). Images are representative of at least three independent experiments. Bars 10 μm . **e** Quantification of patterns *P1* and *P2* showed a significant difference in both patterns under capacitating conditions, pattern *P1* decreased (to 15 ± 3.60) and pattern *P2* increased (to 71 ± 4.58). Graph represents means \pm SEM, $n = 3$ independent experiments, 300 cells per experiment were counted

Chlortetracycline fluorescence assay

This procedure, which was previously described by Ward and Storey (1984), was modified for guinea pig sperm as follows. At the time of the assay, 45 μl non-capacitated or capacitated sperm was mixed with 45 μl 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 0.75 μl glutaraldehyde (12.5 %) in 0.5 mM TRIS (pH 7.4) to give a final concentration of 0.1 % glutaraldehyde, at room temperature. A 10- μl 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 by using a fluorescence microscope (Olympus BX5). In each sample, 100 spermatozoa were classified as expressing one of three CTC staining patterns: “F”, with faint fluorescence in the acrosome region, as is characteristic of non-capacitated acrosome-intact cells; “B”, with bright fluorescence in the acrosomal region and with a band along the equatorial segment, as is characteristic of capacitated acrosome-intact cells; “AR”, with fluorescence in the post-acrosomal region, as is characteristic of physiologically capacitated acrosome-reacted cells. If the sperm samples did not match the statistic criteria, the samples were not used for further experiments. The presence or absence of the acrosomal cap on each cell was verified under phase-contrast illumination.

Statistical analysis

Statistical analyses were performed by using the *t*-test with SigmaPlot version 12.0. Statistical significance is indicated in the figure legends.

Results

Flotillins are re-ordered during capacitation

For the first time, flotillin-1 and flotillin-2 were detected by Western blot analyses in sperm extracts from guinea pigs at their respective Mr of ~50 kDa (Fig. 1a). Since flotillin-1 and -2 have almost 44 % of amino acid sequence identity (Babuke and Tikkanen 2007) and to avoid a false positive band on flotillins detection, we used antibodies raised against the non-conserved region of flotillins of human origin, from the amino acid sequences 324–427 and 151–240, for the detection of flotillin-1 and flotillin-2, respectively. Immunofluorescence analyses revealed that flotillin-1 (Fig. 1c) and flotillin-2 (Fig. 1c') were located in the acrosomal region and mid-piece of non-capacitated sperm (Fig. 1c–c''); however, under capacitating conditions, both proteins relocated to the apical ridge of the sperm head, whereas their presence in the mid-piece remained without change (Fig. 1d–d'). The entire sperm population with intact acrosomes exhibited two types of flotillin-1 immunolabelling patterns in the sperm head: a dispersed pattern in the acrosome, which is a characteristic of non-capacitated cells (*P1*) and an apical ridge re-ordered pattern, which is a characteristic of capacitated cells (*P2*). The re-ordering of flotillin-1 (*P2*) showed more than a four-fold increment when the sperm samples underwent 90 min of capacitation (Fig. 1e). As a capacitation control, we determined chlortetracycline (CTC) fluorescence patterns to evaluate the physiological states of the guinea pig sperm samples. After 90 min of capacitation, sperm displayed approximately 70 % of pattern B (Fig. S1), which correlated with the number of sperm that exhibited the flotillin re-ordering pattern after capacitation (Fig. 1e). All of the above results suggest that flotillin re-ordering is associated with capacitation in guinea pig sperm and that the association of flotillin-1 with flotillin-2 remains intact under capacitation conditions.

Next, we carried out co-immunoprecipitation assays with flotillin-1 obtained from whole cell extracts of non-capacitated and capacitated sperm cells, resulting in the co-immunoprecipitation of flotillin-2 (Fig. 1b). The association of flotillin-1 with flotillin-2 remained constant regardless of capacitation. Since the immunofluorescence patterns of both flotillins were strongly co-localized in the majority of the sperm population and since immunoprecipitation assays revealed that both flotillins remained associated upon

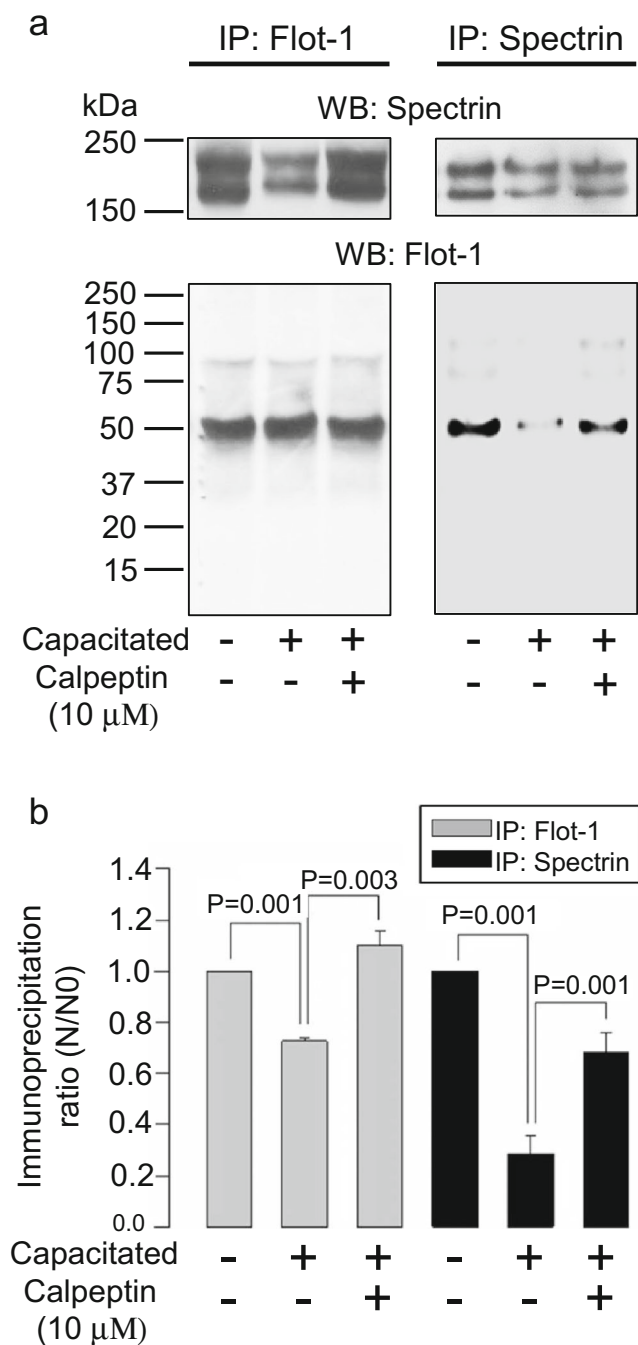


Fig. 2 Spectrin-flotillin association is reduced during capacitation. **a** *Left* Co-immunoprecipitation assays with flotillin-1 (*Flot-1*) were carried out on total sperm extracts (500 μg) from non-capacitated 90-min capacitated, and 90-min capacitated cells in the presence of calpeptin (10 μM). The precipitated proteins were subjected to SDS-PAGE and Western blot (WB) analysis with flotillin-1 as a positive control and probed with anti-spectrin antibody to determine its association. *Right* The reciprocal strategy was performed with anti-spectrin antibody. Western blot images are representative of three independent experiments. **b** Densitometric analysis of co-immunoprecipitated proteins. The results are expressed as the ratio *N/N0*, where *N* is the total amount of co-immunoprecipitated spectrin or flotillin-1 and *N0* is the total amount of immunoprecipitated flotillin-1 or spectrin. Means ± SEM, *n* = 3 independent experiments

capacitation, we chose to use flotillin-1 for pattern quantification and further experiments.

The above results indicated that flotillin microdomains underwent a re-ordering triggered by capacitation in the guinea pig sperm. Subsequently, we performed co-immunoprecipitation assays to determine whether spectrin and flotillin-1 were associated in the guinea pig sperm, as proposed in other cell types (Head et al. 2014; Ludwig et al. 2010; Salzer and Prohaska 2001). The Western blot analysis of co-immunoprecipitated proteins revealed that spectrin co-immunoprecipitated together with flotillin-1 in non-capacitated sperm, whereas in capacitated sperm, a significantly lower amount of spectrin co-immunoprecipitated with flotillin-1 (Fig. 2a, b). To define spectrin cytoskeleton participation, spectrin degradation was inhibited by using calpeptin, a specific inhibitor of calpain. We found that the amount of spectrin that co-immunoprecipitated with flotillin-1 on capacitated sperm in the presence of calpeptin was similar to that found in non-capacitated guinea pig sperm (Fig. 2a, b). Additionally, reciprocal assays were carried out and similar results were obtained; flotillin-1 co-immunoprecipitated together with spectrin at a lower amount in capacitated sperm than in non-capacitated sperm. We also found that the amount of flotillin-1 that co-immunoprecipitated with spectrin on capacitated sperm in the presence of calpeptin was similar to that found in non-capacitated guinea pig sperm (Fig. 2a, b).

Flotillin-1 associates and re-orders with SFKs

Previous studies strongly suggest the presence of SFKs in guinea pig sperm (Baltierrez-Hoyos et al. 2012). We therefore first sought to identify, for the first time, the presence and localization of four selected Src family kinases. Through Western blot analyses, we found that four SFKs (Src, Fyn, Lyn, Hck) were present in their respective ~58- to 60-kDa band region and their quantity remained constant in both non-capacitated sperm and sperm capacitated for 90 min (Fig. 3a; for a negative control, see Fig. S2). Then, by immunofluorescence confocal microscopy analysis, we showed that flotillin-1 and the four Src family members (Src, Fyn, Lyn, Hck) had identical immunolabeling patterns on the acrosome and mid-piece of non-capacitated guinea pig sperm (Fig. 4a”, c”, e”, g”). Under capacitating conditions, these four kinases migrated towards the sperm head apical ridge and strongly co-localized with flotillin-1; co-localization in the mid-piece remained intact during capacitation of guinea pig sperm (Fig. 4b”, d”, f”, h”). To identify whether the association of flotillin-SFKs became stronger or weaker, we performed co-immunoprecipitation assays with non-capacitated and capacitated sperm extracts. Immunoprecipitation of flotillin-1 from whole cell extracts of non-capacitated sperm resulted in the co-immunoprecipitation of Src, Fyn, Lyn and Hck (Fig. 3b, c). When a similar assay was performed on whole cell extracts of

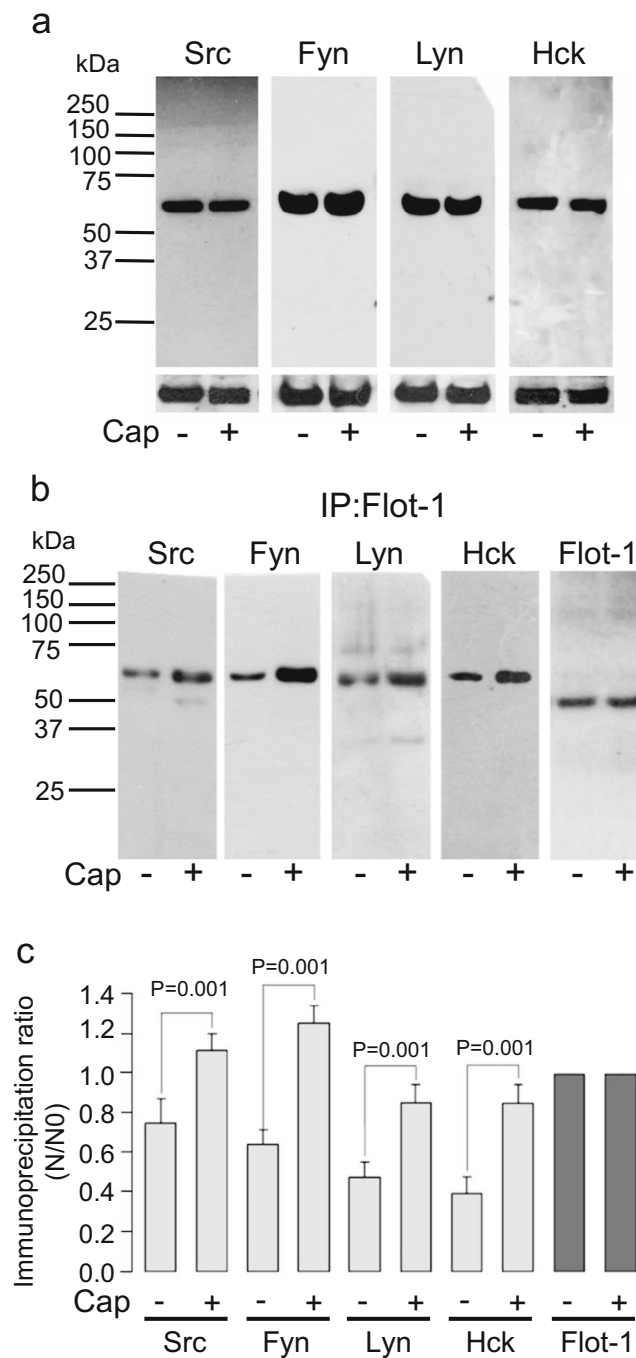
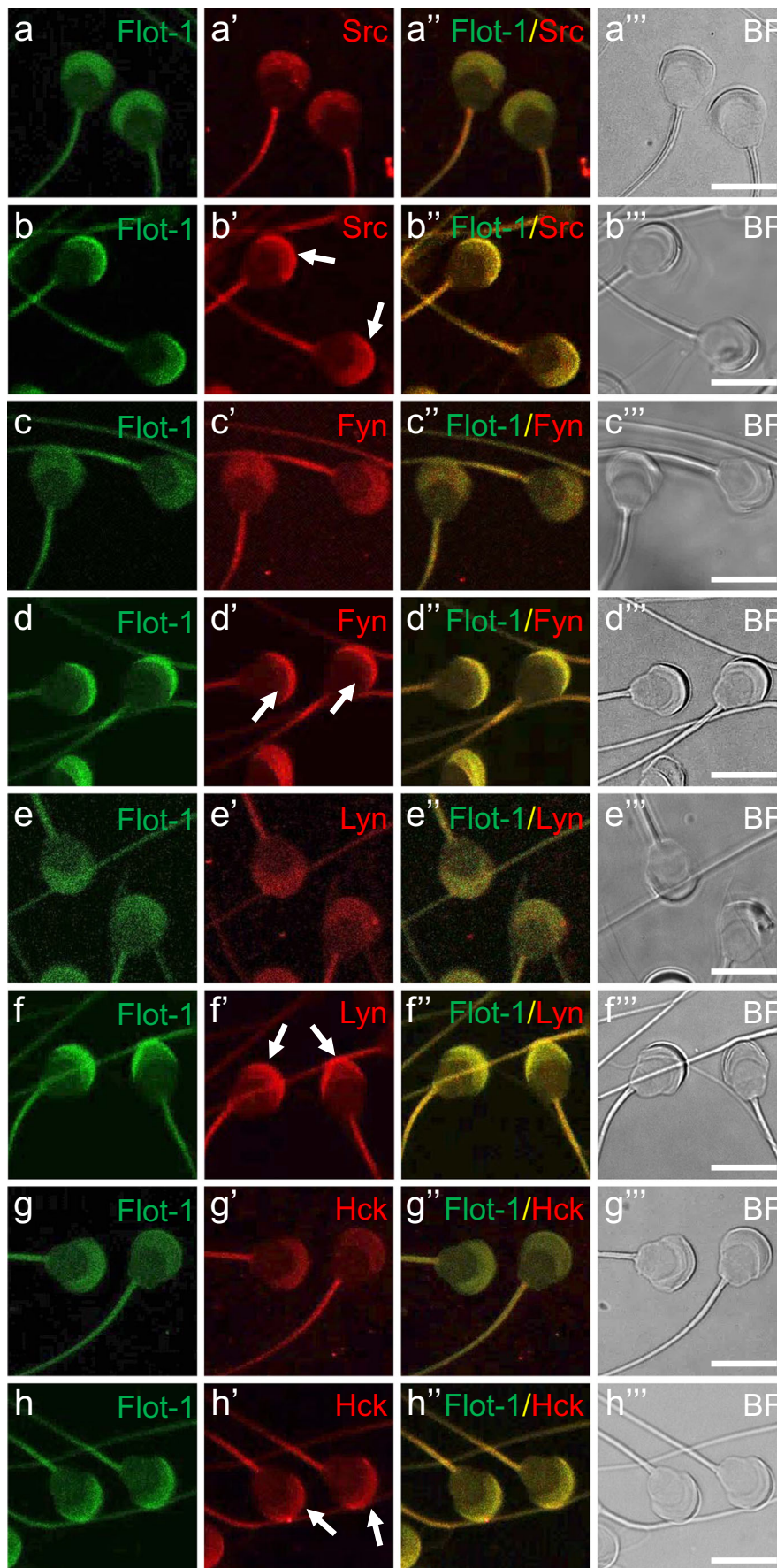


Fig. 3 Src family kinases (SFKs) increase their association with flotillin-1 during capacitation in guinea pig sperm. **a** Whole guinea pig sperm extracts (300 μ g) from non-capacitated (-) and 90-min (+) capacitated (Cap) cells were probed for Src (N16), Fyn (FYN3), Lyn (44) and Hck (N30) antibodies to determine their presence in sperm and the changes that these proteins can undergo during capacitation. Following SFK detection, the membranes were stripped and re-probed with anti-Up71 to ensure equal protein loading (bottom). Western blot images are representative of three independent experiments. **b** Co-immunoprecipitation assays with flotillin-1 were performed with total sperm extracts (500 μ g)

from non-capacitated and capacitated (90 min) sperm. The precipitated proteins were subjected to SDS-PAGE and Western blot analysis probed for flotillin-1 (*Flot-1*) as a positive control and probed with each SFK antibody to determine its association. Western blot images are representative of three independent experiments. **c** Densitometric analysis of co-immunoprecipitated proteins. Results are expressed as the ratio N/N_0 , where N is the total amount of protein co-immunoprecipitated together with flotillin-1 (SFKs and flotillin-1) and N_0 is the total amount of immunoprecipitated flotillin-1. Means \pm SEM, $n = 3$ independent experiments

capacitated guinea pig sperm, the amount of co-immunoprecipitated protein significantly increased for Src,

Fyn, Lyn and Hck (Fig. 3b), as evidenced by densitometric analysis (Fig. 3c).



◀ **Fig. 4** SFKs co-localize and re-order with flotillin-1 during capacitation. Non-capacitated (a–a’’, c–c’’, e–e’’, g–g’’) and 90-min capacitated sperm (b–b’’, d–d’’, f–f’’, h–h’’) were doubly stained for flotillin-1 (*Flot-1*) and for the following SFKs: Src (a–b’’), Fyn (c–d’’), Lyn (e–f’’) and Hck (g–h’’). Their co-localization was analyzed by confocal microscopy. The *first column* shows the marker with respect to the flotillin-1 antibody visualized with an FITC-conjugated secondary antibody (*green*), whereas the *second column* shows the marker with respect to the corresponding SFK antibody coupled to a TRITC-conjugated secondary antibody (*red*). The *third column* shows the merge between flotillin-1 and the respective SFK, whereas the *fourth column* shows the respective bright field (*BF*) micrographs. *Arrows* indicate the re-ordering towards the apical ridge of SFKs in the capacitated sperm. Images are representative of five independent experiments. *Bars* 10 μm

Cytoskeleton remodeling precedes flotillin re-ordering

To shed light into the functional significance of the dynamics of the cortical cytoskeleton on flotillins mobility, our next step was to determine whether the re-ordering of flotillin-SFKs depended on cytoskeleton remodeling. First of all, spectrin cleavage was inhibited during capacitation: sperm were capacitated in the presence of calpeptin and after 90 min of incubation, flotillin-1 and SFKs were immunolocalized. By inhibiting spectrin cytoskeleton degradation during capacitation, flotillin-1 and SFKs were not re-ordered, since the staining patterns were similar to those of non-capacitated sperm (Fig. 5a–e). The actin cytoskeleton is known also to regulate the mobility and stabilization of flotillin domains (Langhorst et al. 2007). Therefore, we carried out assays to determine whether the actin cytoskeleton participated in flotillin re-ordering. When the capacitation-associated actin polymerization was inhibited by inducing capacitation in the presence of latrunculin A in the capacitation medium, the immunolocalization of flotillin-1 and SFKs showed that, although no actin polymerization occurred (Fig. 5f–f’), the re-ordering of these proteins remained unaltered (Fig. 5g–k). These results demonstrate, for the first time, that flotillin microdomain mobility is spectrin-cleavage dependent in guinea pig sperm.

SFK autophosphorylation is related to spectrin remodeling

Once we had defined that SFKs associate with and relocate with flotillin-1 during capacitation and that spectrin degradation precedes this microdomain aggregation, our next step was to determine whether the re-ordering of flotillin microdomains impacted on SFK Tyr416 autophosphorylation. Therefore, we immunoprecipitated Src, Fyn, Lyn and Hck (Fig. 6a–d) from whole sperm protein extracts by using different capacitation times in the presence or absence of calpeptin and probed them with a specific antibody that recognizes the Tyr416

phosphorylation of any SFK member (anti-SFK-pTyr416). Western blot analysis showed that p-Tyr416 on Src, Fyn, Lyn and Hck was almost absent in non-capacitated sperm (time: 0 min) and also during the first 15 min of capacitation. However, after 30 min of capacitation, Tyr416 phosphorylation increased in a coordinated and significant manner, reaching the highest levels of activation at 90 min of capacitation (Fig. 6a–d). The increment in p-Tyr416 previously described for Src, Fyn, Lyn and Hck was statistically lower when the sperm were capacitated in the presence of calpeptin (Fig. 6a’–d’). These results suggest that both spectrin degradation and SFK relocalization are prerequisites for SFK autophosphorylation in sperm and allow physiological capacitation and further acrosome reaction.

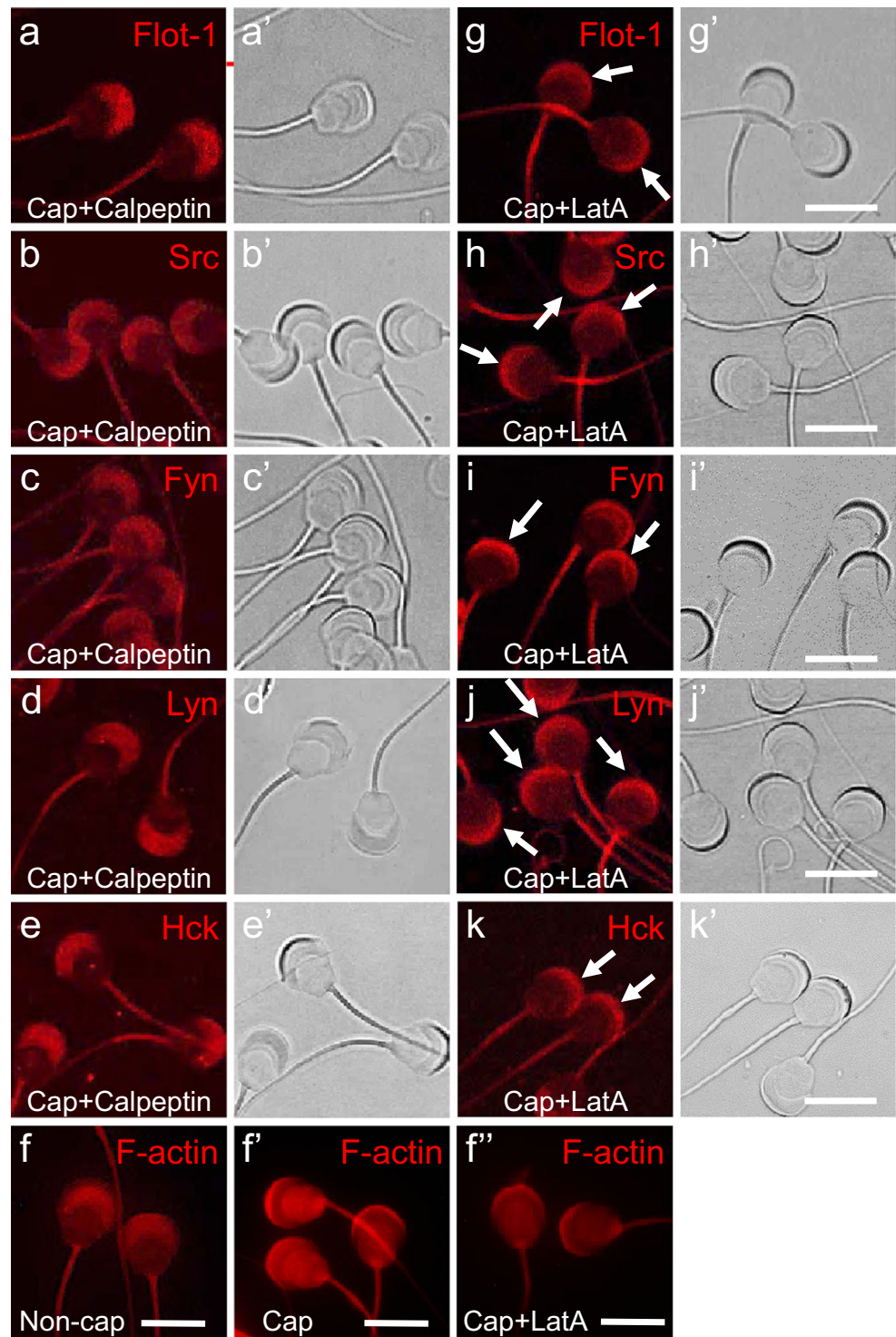
Spectrin remodeling and SFK re-ordering precede FAK Tyr576/Y577 phosphorylation

Finally, to elucidate the importance of SKF relocalization and autophosphorylation in the guinea pig sperm, we analyzed whether FAK Tyr576/577 phosphorylation was affected by the inhibition of SFK re-ordering and activation. Tyr576/577 of FAK is a site specifically phosphorylated by SFK members (Calalb et al. 1995). As we previously showed, the inhibition of spectrin cleavage by calpeptin prevented SKF relocalization and autophosphorylation. Therefore, before and during capacitation, sperm were incubated in the presence of calpeptin. Western blot analysis showed that FAK Tyr576/577 phosphorylation increased in capacitated sperm, an increment that was not reached when the sperm were treated with calpeptin (Fig. 7a, b).

Discussion

The spectrin-actin cytoskeletal meshwork associated with the plasma membrane forms a structural platform that stabilizes membrane microdomains for further focalized signal triggering (Machnicka et al. 2012). This cytoskeletal network, through scaffold proteins such as ankyrin and flotillins, anchors several signaling proteins at the membrane domains, e.g., receptors, ion channels, transporters, kinases and adhesion molecules (Bennett and Healy 2009; Bennett and Lorenzo 2016). The spectrin cytoskeleton is known to be associated with membrane microdomains defined by flotillins (Domingues et al. 2010; Salzer and Prohaska 2001); however, the precise molecular function of spectrin and flotillin in mammalian sperm is still not known. In the present study, we provide initial evidence concerning the role of sperm flotillin microdomains that function as a scaffolding structure for Src family kinases involved in capacitation and the acrosome reaction and whose re-ordering and activation during capacitation are probably driven by spectrin cleavage.

Fig. 5 Calpain inhibition suppresses flotillin-1 and SFK re-ordering in guinea pig sperm. Immunostaining of 90-min capacitated (*Cap*) sperm in presence of calpeptin (+*Calpeptin*; **a–e'**) or latrunculin A (+*Lat-A*; **g–k'**) with intact acrosomes by antibodies against flotillin-1 (*Flot-1*; **a, g**), Src (**b, h**), Fyn (**c, i**), Lyn (**d, j**) and Hck (**e, k**). Analysis by confocal microscopy. The *first* and *third* columns show the marker with respect to the primary antibody visualized with a TRITC-conjugated secondary antibody (*red*), whereas the *second* and *fourth* columns show the respective bright field (*BF*) micrographs. *Arrows* indicate the re-ordering of the selected proteins towards the apical ridge. Images are representative of at least three independent experiments. **f–f''** As a positive control for latrunculin A treatment on actin cytoskeleton remodeling, the sperm were stained with TRITC-phalloidin. *Bars* 10 μ m



A hallmark event that occurs during capacitation is the redistribution of membrane microdomain markers (Gadella and Boerke 2016). Flotillins and caveolins are the principal protein markers in the membrane microdomains and are found dispersed throughout the acrosome region. During capacitation, these proteins undergo a reorganization that results in their increased concentration at the apical ridge of the

acrosome (van Gestel et al. 2005). Our results are in agreement with the finding that, in an entire sperm population with intact acrosomes, two types of flotillin-1 and -2 patterns are found in the sperm head: a dispersed pattern in the acrosome, characteristic of non-capacitation (Fig. 1c–c'') and an apical ridge re-ordered pattern, characteristic of capacitated sperm (Fig. 1d–d''). Therefore, we suggest that the re-ordering of

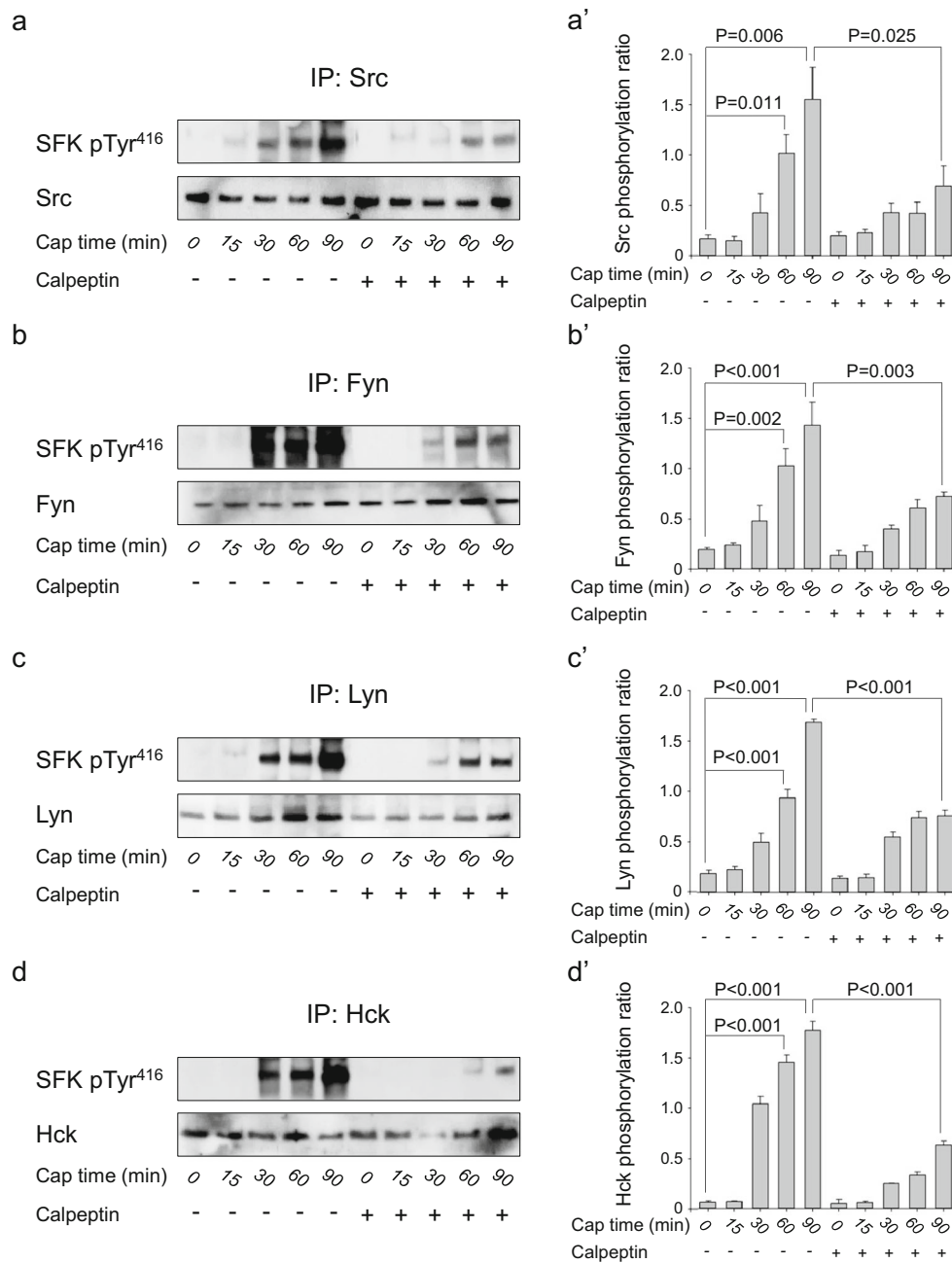


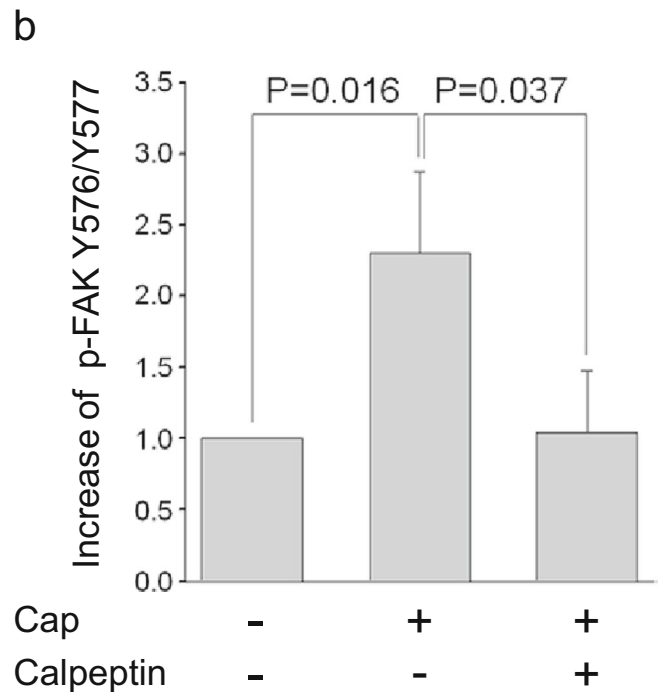
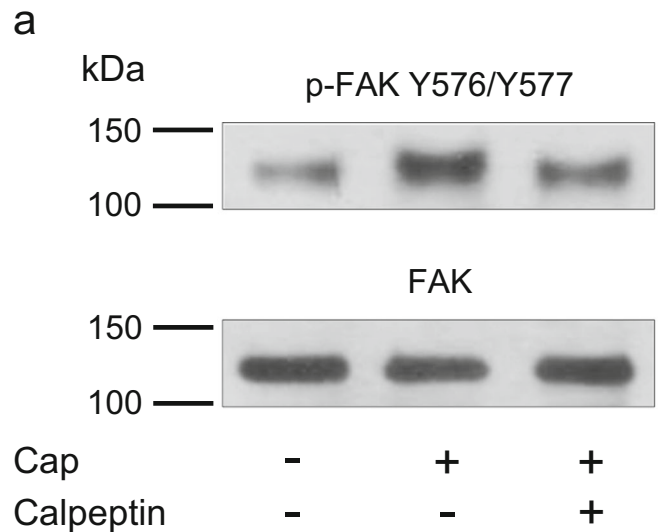
Fig. 6 Calpain inhibition suppresses the SFK Tyr416 autophosphorylation that occurs during the course of capacitation. *Left* Co-immunoprecipitation assays were carried out with total sperm extracts (500 μ g) from non-capacitated (0 min) and capacitated (*Cap*: 15, 30, 60, and 90 min) sperm in the presence or absence of calpeptin. Co-immunoprecipitation assays were performed for the following SFKs: Src (**a**), Fyn (**b**), Lyn (**c**) and Hck (**d**). Immunoprecipitated proteins were

probed with a specific antibody that recognizes any SFK member phosphorylated at Tyr416. Western blot images are representative of three independent experiments. *Right* Graphs showing densitometric analyses for the following SFKs: Src (**a'**), Fyn (**b'**), Lyn (**c'**) and Hck (**d'**). The respective SFK phosphorylation ratio is expressed as the ratio of SFK phosphorylated at Tyr⁴¹⁶/total SFK. Means \pm SEM, $n = 3$ independent experiments

the flotillins is an important capacitation event that can be considered as a marker of this spermiatic process; our proposal is supported by our results in which approximately 70 % of the capacitated sperm exhibit pattern P2 (Fig. 1e), a similar percentage being reported for capacitated porcine (~75 %) and mouse (~90 %) sperm (Boerke et al. 2013; Angeles-Floriano et al. 2016). We also questioned whether the localization of

each flotillin varies, finding that, in sperm, the flotillins strongly co-localize and co-immunoprecipitate, suggesting that both flotillins coexist in the same microdomain, in accordance with flotillin properties (Solis et al. 2007). These results show that flotillin-1 and flotillin-2 share a similar behavior, which is probably the result of both proteins forming hetero-oligomers that are not dissociated during capacitation

Fig. 7 Calpain inhibition prevents capacitation-dependent FAK Tyr576/Y577 phosphorylation. **a** Whole guinea pig sperm extracts (300 µg) from non-capacitated, 60-min capacitated and 60-min capacitated sperm in presence of calpeptin (10 µM) were probed with anti-FAK Tyr576/Y577 antibody. Membranes were stripped and re-probed with anti-FAK antibody to ensure equal protein loading. Western blot images are representative of three independent experiments. **b** Densitometric analysis of FAK Tyr576/577 phosphorylation during capacitation (Cap) in the presence and absence of calpeptin. Means ± SEM, *n* = 3 independent experiments



(Fig. 1b, c). Even though the capacitation of guinea pig sperm in vitro is performed in the absence of BSA or another cholesterol-depletion molecule, guinea pig sperm are fertile (Rogers and Yanagimachi 1975) and the reorganization of flotillin-1 and -2 occurs normally (Fig. 1d–d”, e). Our results are in agreement with the finding that cytoskeleton dynamics are responsible for raft mobilization (Langhorst et al. 2007) and that albumin-mediated sterol depletion preferentially forms non-lipid raft pools on the surface of the sperm (van Gestel et al. 2005). Our results also support the proposal that lipid membrane microdomains are not disrupted during capacitation (Shadan et al. 2004) and that the accumulation of microdomain markers such as caveolin-1 and flotillins in the

apical ridge surface area characterizes the capacitation-dependent reorganization of these microdomains (van Gestel et al. 2005). Depending on the species, the sperm membrane might be reinforced with different sterol compositions, which may be the reason that sperm from other species require BSA or another cholesterol depletion molecule to induce protein relocation and to achieve a capacitated state in vitro.

Despite flotillin reorganization being a capacitation-associated event, the mechanism that regulates the redistribution and its importance in capacitation are still unknown (Boerke et al. 2014; van Gestel et al. 2005). One explanation for the observations mentioned above is that the removal of selected glycosylphosphatidylinositol (GPI)-anchored

proteins from the capacitating sperm is associated with the reorganization of lipid membrane microdomains and with the acrosome reaction (Watanabe and Kondoh 2011). However, the experiments performed are not conclusive in explaining the mechanism of the way that flotillins are re-ordered (Boerke et al. 2014). The results of the present study suggest another possible mechanism related to the spectrin cytoskeleton. Two important pieces of evidence from the literature suggest the participation of spectrin in this mechanism: (1) spectrin and flotillins are found in lipid membrane microdomains of various cell types (Nebl et al. 2002) and (2) the spectrin cytoskeleton binds to the inner face of the plasma membrane and creates a physical restrictive barrier that maintains a specific protein localization by avoiding the free diffusion of cytoplasmic and plasma membrane proteins (Bennett and Lorenzo 2016; Susuki et al. 2016). Thus, our results strongly imply that spectrin and flotillin-1 are associated with each other in non-capacitated sperm, a physiological state in which the meshwork of spectrin keeps its integrity, possibly by inhibiting the diffusion of spectrin-associated proteins such as flotillin-1 and -2. This spectrin meshwork changes during the course of capacitation in such a way that the spectrin barrier is disassembled when it is cleft by calpain (Bastian et al. 2010); as a result, flotillin-1 dissociates from spectrin and reorganizes at the acrosomal ridge (Fig. 1d–d', Fig. 2a, b). Therefore, our proposed mechanism is supported by the evidence that, when spectrin cleavage is avoided by calpain inhibition, it prevents the dissociation of flotillin-1 from spectrin (Fig. 2a, b) and also inhibits flotillin-1 redistribution (Fig. 5a–e).

In various cell types, the cortical actin cytoskeleton modulates the organization and lateral mobility of flotillin microdomains (Langhorst et al. 2007). Nonetheless, flotillin-1 redistribution in sperm cells is not perturbed by latrunculin A, a specific drug that inhibits actin polymerization (Spector et al. 1989). In spite of the low levels of F-actin displayed by sperm treated with latrunculin A, flotillin-1 still migrates towards the acrosomal ridge. This suggests that actin cytoskeleton polymerization might not be necessary for flotillin mobilization. The role of actin cytoskeleton polymerization might be to stabilize the re-ordered flotillin microdomains, since the accumulation of F-actin in this region is observed after capacitation (Fig. 5f'). Even though the mechanism proposed in this study can partially explain the way that flotillins are re-ordered in a capacitation-dependent manner, we do not exclude the idea that GPI-anchored protein removal has a function in this process; hence, further experiments are necessary to clarify the manner in which the two mechanisms regulate flotillin redistribution.

The four SFKs studied here were localized in the mid-piece and acrosomal region, which is a similar localization previously reported for Src and Fyn in human, mouse and rat sperm (Goupil et al. 2011; Kierszenbaum et al. 2009; Mitchell et al.

2008). In mouse sperm, Lyn was detected in the acrosomal region and principal end pieces but was absent from the mid-piece, whereas Hck was restricted to the principal piece of the flagellum (Goupil et al. 2011). This suggests that all these different localizations depend on each Src family member in a species-specific manner. In spite of the discrepancies in SFK localization found among sperm from various species, SFKs are key regulators of capacitation and the acrosome reaction, because the inhibition of their activity prevents the normal course of both processes (Krapf et al. 2010; Stival et al. 2015; Varano et al. 2008).

Membrane microdomains participate in focalized signaling events by assembling specific signaling molecules and by providing a structure in which proteins can meet and initiate a downstream signaling process (Foster et al. 2003). Membrane microdomain markers, such as caveolin-1 and flotillins, accumulate at the apical ridge and may function as protein scaffolds that concentrate specific molecules in regions in which they are required. Interestingly, several SFKs have been localized in specialized membrane microdomains (Simons and Toomre 2000; Yasuda et al. 2002). Accordingly, Src and Fyn have been found associated and co-clustered at various regions in the N-terminal sequence of flotillins in adipocyte cells (Liu et al. 2005). Similarly, we show that SFKs co-immunoprecipitate with flotillin-1 and their interaction increases under capacitating conditions (Fig. 3b, c); this might be related to the increment in SFK autophosphorylation (Fig. 6a–d'). Furthermore, during the course of capacitation, Src, Fyn, Lyn and Hck migrate towards the apical ridge of the sperm head. Together, the above evidence suggests that, during capacitation, the SFKs interact strongly with flotillin microdomains. Nevertheless, we failed to demonstrate whether the flotillin-1-SFK interaction occurs in a direct manner or through other proteins present in the flotillin microdomain.

Among all SFK members, the autophosphorylation of Tyr416 is highly conserved and is required to promote the kinase activity of all family members (Roskoski 2015). In other cell types, Fyn associates constantly with flotillin-1 through the SPFH domain (Liu et al. 2005), whereas the interaction of Lyn with flotillin-1 increases after Lyn activation (Kato et al. 2006). On the same note, our data also suggest that the association of SFKs with flotillins in sperm is important for SFK autophosphorylation. Indeed, SFK autophosphorylation might depend on two steps: first, at spectrin cleavage and secondly, on association with flotillin-1. This occurs in such a way that, when the spectrin cleavage is inhibited, SFK autophosphorylation is greatly avoided (Fig. 6a–d'). Based on these results, we suggest that the cytoskeleton meshwork organized by spectrin stabilizes membrane microdomains from the acrosomal region and does not allow a free redistribution of flotillins, with a consequent lack of activation of signaling proteins being required for proper capacitation

and the acrosome reaction, as for the tyrosine kinase Src (for a review, see Bailey 2010).

Flotillins not only are important scaffold proteins but also influence the activation of various signaling proteins, because their mis- and downregulations affect the high activation levels of Rho-GTPases such as Rac, Rho and Cdc42, which modify actin cytoskeleton dynamics (Stuermer 2011). Furthermore, in mammalian sperm, SFKs are not the only proteins whose activity is related to membrane microdomain lipid changes; phospholipase B is activated by sterol removal (Asano et al. 2013). Adding our data concerning SFKs and flotillins to the work from others, we reinforced the proposal that flotillins are scaffold proteins that concentrate signaling molecules in specific sperm regions.

Flotillin-1 and SFKs co-localize in the mid-piece (Fig. 4a", c", e", g") but do not undergo changes in their localization during capacitation (Fig. 4b", d", f", h"). Interestingly, this sperm region lacks spectrin (Bastian et al. 2010) but is rich in actin-F (Fig. 5f–f"); probably, the complex flotillin-1-SFK present in the mid-piece is stabilized by the actin cytoskeleton. These results support the importance of spectrin in structuring sperm domains and suggest that flotillin-1-SFKs influence mitochondrial functions, as proposed by Hebert-Chatelain (2013).

Our data show that the inhibition of spectrin cleavage had two important effects on Src, Fyn, Lyn and Hck autophosphorylation: (1) SFK re-ordering occurs late during capacitation (Fig. 4b", d", f", h") and (2) the increase in the phosphorylation Tyr416 that occurs during the normal course of capacitation is significantly restricted (Fig. 6a–d'). The localization of the various Src family tyrosine kinases in membrane microdomains is known to be crucial for their activation in several cell lines, because they have substantially lower kinase activity outside of the membrane microdomains (Hitosugi et al. 2007; Kato et al. 2006; Young et al. 2003). Additionally, the inhibition of spectrin cleavage prevents the phosphorylation increment of FAK Tyr576/Y577 (Fig. 7a, b), which are SFK-mediated phosphorylation sites when FAK is previously Tyr397 phosphorylated (Schlaepfer et al. 1999). In the guinea pig sperm, FAK contributes to acrosome integrity by activating several signaling pathways and by regulating actin polymerization (Roa-Espitia et al. 2016). FAK Tyr397 autophosphorylation increases in the apical ridge and mid-piece in guinea pig sperm during capacitation (Roa-Espitia et al. 2016), regions in which SFKs reside and relocate. Therefore, we propose that FAK phosphorylation and the autophosphorylation of Src, Fyn, Lyn and Hck in guinea pig sperm depends on spectrin cleavage in order to release flotillin-1, thereby allowing a relocation and a stronger interaction with SFKs and a further focalized signaling process by p-FAK Tyr576/577 and other SFK targets. Our results imply that a fine regulation of SFK autophosphorylation occurs at the right time and space within the cell. With reference to time,

the autophosphorylation of SFKs increases after 30 min of capacitation; before this time, SFK autophosphorylation is extremely low, since capacitation cannot be initiated until the required levels of Ca^{2+} are present to cleave spectrin through an activated calpain. Concerning space, the highest SFK autophosphorylation ratio is reached when these kinases are carried by flotillin-1 to the apical region of the acrosome (Fig. 4a–h", 6a–d'), where possibly target proteins are found, such as p-FAK Tyr397 (Roa-Espitia et al. 2016), since most of the sperm, after 60 min of capacitation, show the pattern 2 staining that is correlated with the highest levels of p-Tyr416 for SFKs (Fig. 6a–d').

In conclusion, our results support the hypothesis that the cytoskeleton structure of spectrin stabilizes various membrane domains where scaffolding proteins reside and interact with diverse signaling molecules, thereby affecting their activity. Therefore, we consider that the spectrin cytoskeleton is one of the molecular structures that inhibits capacitation in guinea pig sperm and that, when it is disassembled, it triggers the apical aggregation of flotillin microdomains and various signaling cascades important for capacitation and the acrosome reaction. The observations generated by this study demonstrate that spectrin not only serves as a structural protein but is also involved in membrane microdomain stabilization prior to capacitation. Our findings show, for the first time, that, once spectrin cytoskeleton cleavage occurs during capacitation, it allows flotillin microdomains to migrate towards the head apical ridge, together with SFKs, to trigger FAK to its maximal catalytic state, like other targets, to initiate a localized intense signaling cascade required for the proper capacitation and further acrosome reaction that are essential for guinea pig sperm to become fertilization-competent.

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Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists that could be perceived as prejudicing the impartiality of this scientific work.

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