Calpain modulates capacitation and acrosome reaction through cleavage of the spectrin cytoskeleton

Yadira Bastián, Ana L Roa-Espitia¹, Adela Mújica¹ and Enrique O Hernández-González¹

Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1 and ¹Departamento de Biología Celular, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Avenue IPN 2508, San Pedro Zacatenco, México DF 07360, Mexico

Correspondence should be addressed to E O Hernández-González; Email: eoton@cell.cinvestav.mx

Y Bastián and A L Roa-Espitia contributed equally to this work

Abstract

Research on fertilization in mammalian species has revealed that Ca^{2+} is an important player in biochemical and physiological events enabling the sperm to penetrate the oocyte. Ca^{2+} is a signal transducer that particularly mediates capacitation and acrosome reaction (AR). Before becoming fertilization competent, sperm must experience several molecular, biochemical, and physiological changes where Ca^{2+} plays a pivotal role. Calpain-1 and calpain-2 are Ca^{2+} -dependent proteases widely studied in mammalian sperm; they have been involved in capacitation and AR but little is known about their mechanism. In this work, we establish the association of calpastatin with calpain-1 and the changes undergone by this complex during capacitation in guinea pig sperm. We found that calpain-1 is relocated and translocated from cytoplasm to plasma membrane (PM) during capacitation, where it could cleave spectrin, one of the proteins of the PM-associated cytoskeleton, and facilitates AR. The aforementioned results were dependent on the calpastatin phosphorylation and the presence of extracellular Ca^{2+} . Our findings underline the contribution of the sperm cytoskeleton in the regulation of both capacitation and AR. In addition, our findings also reveal one of the mechanisms by which calpain and calcium exert its function in sperm.

Reproduction (2010) **140** 673–684

Introduction

Freshly ejaculated mammalian sperm are unable to fertilize mature oocytes; in order to become fertilization competent, they must go through a process called capacitation. Capacitation occurs *in vivo* in the female reproductive tract, but can be mimicked *in vitro* by incubation in specially defined media. Capacitation involves reorganization of the plasma membrane (PM), an increase in protein tyrosine phosphorylation, and hyperpolarization of the PM potential (E_m). During capacitation, there is an increase in the intracellular concentration of Ca^{2+} , cAMP, and pH (Salicioni *et al.* 2007, Abou-haila & Tulsiani 2009). Capacitation is also associated with the appearance of hyperactivated motility (Salicioni *et al.* 2007, Suarez 2008). Once capacitation is completed, sperm are able to undergo the acrosome reaction (AR), an exocytotic process induced by ZP3, a component of the zona pellucida. AR allows the sperm to penetrate the zona pellucida and to fuse with the egg's PM. Both capacitation and AR absolutely require Ca^{2+} influx to activate several indispensable signal pathways (Darszon *et al.* 2005, Publicover *et al.* 2007).

Calpains belong to a family of non-lysosomal Ca^{2+} -dependent cysteine proteases widely expressed in a variety of tissues and cells (Molinari & Carafoli 1997, Goll *et al.* 2003). The most ubiquitous and very well-characterized isoforms are calpain-1 and calpain-2. Because the activation of their 80 kDa large catalytic subunit differs in Ca^{2+} concentration requirements, calpain-1, which is activated at the micromolar range, is also known as μ -calpain, whereas calpain-2, which is activated at the millimolar range, is also called m-calpain (Croall & DeMartino 1991, Goll *et al.* 2003). Calpain is involved in cytoskeleton remodeling, cell adhesion and motility, cell cycle regulation, as well as in cell differentiation and apoptosis (Croall & DeMartino 1991, Lebart & Benyamin 2006). The activity of calpain is tightly regulated by several mechanisms, including its endogenous inhibitor calpastatin, calcium levels, autoproteolytic cleavage, and phosphorylation (Molinari & Carafoli 1997, Goll *et al.* 2003, Franco & Huttenlocher 2005). Calpain plays an important role in the turnover of different molecules related to cell adhesion and motility by the cleavage of many adhesion and cytoskeletal proteins, such as spectrin (Franco & Huttenlocher 2005, Lebart & Benyamin 2006).

In mammalian sperm, calpain-1 and calpain-2 are located within the acrosomal region (Schollmeyer 1986, Ben-Aharon *et al.* 2005) between the PM and the outer acrosomal membrane (Yudin *et al.* 2000). Interestingly, it has been shown that sperm capacitation in the presence of calpain inhibitors results in a reduction of AR (Rojas *et al.* 1999, Aoyama *et al.* 2001, Ben-Aharon *et al.* 2005); however, the role of calpain during capacitation or AR remains unclear.

The surface of mammalian sperm displays diverse membrane domains with distinct biochemical and functional characteristics; these domains are very important for capacitation, AR, and motility (Forsman & Pinto da Silva 1989). Diverse cytoskeletal proteins have been found to be associated with these membrane domains, for instance F-actin (Castellani-Ceresa *et al.* 1992, Moreno-Fierros *et al.* 1992, Kann *et al.* 1993, Spungin *et al.* 1995, Yagi & Paranko 1995), spectrin (Virtanen *et al.* 1984, Camatini *et al.* 1991, Hernandez-Gonzalez *et al.* 2000), as well as dystrophin and utrophin (Hernandez-Gonzalez *et al.* 2001, 2005). Interestingly, because F-actin and spectrin form a network associated with the plasma and outer acrosomal membranes (Hernandez-Gonzalez *et al.* 2000) that may act as a physical barrier preventing membrane fusion (Spungin *et al.* 1995, Hernandez-Gonzalez *et al.* 2000), remodeling of the cortical actin cytoskeleton could facilitate capacitation and AR (Brener *et al.* 2003, Cabello-Agueros *et al.* 2003). Several lines of evidence support this hypothesis as follows: 1) F-actin polymerization/depolymerization processes have been observed during capacitation and AR (Spungin *et al.* 1995, Hernandez-Gonzalez *et al.* 2000), 2) phalloidin-induced F-actin stabilization blocks membrane fusion and AR (Spungin *et al.* 1995, Hernandez-Gonzalez *et al.* 2000), and 3) F-actin severing proteins like gelsolin and scinderin have been found within the acrosomal region of mammalian sperm (Pelletier *et al.* 1999, Cabello-Agueros *et al.* 2003). Moreover, spectrin actively participates in the assembly of specialized membrane domains in addition to their conventional maintenance role as scaffolding protein for ion channels and transporters as well as for cell adhesion molecules (Bennett & Healy 2008). Spectrin is associated with the cytoplasmic surface of the sperm PM in the acrosomal region and in the flagella (Camatini *et al.* 1991, Hernandez-Gonzalez *et al.* 2000), although its function in mammalian sperm is unknown.

Since the exact function of calpain in sperm physiology remains unclear, the present study was conducted to 1) evaluate the presence of calpain-1 and calpain-2 as well as its natural regulator calpastatin in guinea pig sperm, 2) distinguish if calpain is activated during capacitation, and 3) to determine the participation of calpain in cytoskeleton remodeling during capacitation.

Results

Calpain-1 and calpastatin are expressed in guinea pig spermatozoa

Consistent with the molecular weight reported for mouse, human, and macaque sperm (Rojas *et al.* 1999, Yudin *et al.* 2000, Ben-Aharon *et al.* 2005), the anti-calpain-1 antibody detected a 80 kDa protein, whereas the anti-calpastatin antibody detected a band of 70 kDa (Fig. 1A and B). The bands we detected were also present in erythrocytes and Jurkat cells, which were used here as positive controls (Sasaki *et al.* 1983, Murakami *et al.* 1988, Porn-Ares *et al.* 1998). Using the same antibodies, we next determined the localization of calpain-1 and calpastatin by indirect immunofluorescence; both calpain-1 and calpastatin were detected in the whole acrosomal region and in the middle piece of non-capacitated sperm (Fig. 1C). Because calpain-2 has been reported in pig, human, macaque, and mouse sperm (Schollmeyer 1986, Rojas *et al.* 1999, Yudin *et al.* 2000, Ben-Aharon *et al.* 2005), we then sought for its presence

Figure 1 Calpain-1 and calpastatin are expressed in guinea pig sperm. Calpain-1 (A) and calpastatin (B) were detected by western blot analysis using 100 µg of whole sperm (S) extracts. Extracts from human erythrocytes (E) and Jurkat cells (J) were used as positive controls. (C) Formaldehyde-fixed sperm were subjected to immunofluorescence to detect the expression of calpain-1 and calpastatin. Sperm preparations subjected to immunofluorescence without addition of primary antibodies were used as controls. In addition, phase contrast micrographs are shown. Images are representative of at least three independent experiments.

in guinea pig sperm using two different specific anti-calpain-2 antibodies; however, we were unable to detect this protein using western blot or indirect immunofluorescence (Supplementary Figure 1, see section on supplementary data given at the end of this article). To confirm that anti-calpain-2 antibodies used here recognize calpain-2 from guinea pig, they were assayed in whole extracts of heart and cerebrum. Both antibodies recognized calpain-2 of guinea pig (Supplementary Figure 2, see section on supplementary data given at the end of this article). These results suggest the possibility that calpain-2 is absent in guinea pig sperm, however, is important to perform more studies to confirm this hypothesis.

Calpain-1 is translocated to the PM during capacitation in a calcium-dependent manner

The activation of calpain during normal and pathologic processes requires increases in intracellular Ca^{2+} and this is probably also necessary for its translocation to the PM, where several calpain target proteins are located (Hood *et al.* 2006). Because Ca^{2+} increase is one of the central intracellular events during capacitation (Coronel & Lardy 1987, Adeoya-Osiguwa & Fraser 1996), we decided to determine the intracellular localization of calpain-1 during capacitation. After 30 min of capacitation, we always observed a relocalization of calpain in both the apical acrosome and the postacrosomal regions

(Fig. 2A). Then, we confirmed the participation of Ca^{2+} in the localization changes of calpain-1 by incubating the cells for 90 min in minimal culture medium containing lactate and pyruvate (MCM-PL) capacitating medium without Ca^{2+} (see Materials and Methods). In the absence of calcium, the distribution of calpain-1 did not show any change, and its localization was identical to that of non-capacitated sperm (Fig. 2A). The proportion of sperm immunostained in the apical acrosome and postacrosomal regions was clearly different between sperm capacitated in complete MCM-PL and those capacitated in MCM-PL without Ca^{2+} (Fig. 2B). Next, we evaluated whether or not calpain-1 is translocated from the cytoplasm to the sperm membranes during capacitation; western blot of purified PM proteins showed that calpain-1 was not detected in the PM of non-capacitated sperm; however, during capacitation, calpain-1 was associated with the PM, increasing the amount associated throughout capacitation (Fig. 2C). Moreover, the translocation of calpain-1 to PM was Ca^{2+} dependent as shown by the absence of calpain-1 associated with the PM isolated from sperm capacitated in the absence of Ca^{2+} (Fig. 2C). To confirm that calpain-1 was translocated from cytosol to the PM, the presence of calpain-1 in cytosolic proteins was assayed by western blot. Using both non-capacitated and capacitated sperm in the absence of extracellular Ca^{2+} , the amount of cytosolic calpain-1 detected was similar to that detected in proteins from whole sperm

Figure 2 Calpain-1 is translocated to plasma membrane during capacitation in a calcium-dependent manner. (A) Immunolocalization of calpain-1 at indicated times during capacitation in the presence or absence of extracellular calcium; time 0 represents non-capacitated sperm and is used as reference. AR, acrosome-reacted sperm. Lower images are phase contrast micrographs of the same fields shown above. (B) Quantification of the proportion of sperm showing calpain-1 immunostaining in both the apical acrosome and postacrosomal regions after capacitation (60 min) in the presence or absence of calcium (mean $\pm$ S.E.M., $n=3$, 250 cells per experiment). PAR, postacrosomal region; AAR, apical acrosome region. (C) Western blot comparison of the presence of calpain-1 in plasma membranes proteins (PMP) and cytosolic proteins (CSP) of non-capacitated sperm versus capacitated sperm in the presence or absence of extracellular Ca^{2+} . Time 0 (min) represents non-capacitated sperm. Lower panel corresponds to calpain-1 detection in whole sperm extract (WSE). Western blot shown is representative of at least three independent experiments.

extract, while the amount of cytosolic calpain-1 in capacitated sperm underwent a decrease (Fig. 2C), which could be correlated with the increase of calpain-1 in PM (Fig. 2C). Total amount of calpain-1 did not change in non-capacitated and capacitated sperm before PM was isolated (Fig. 2C).

Co-immunoprecipitation of calpain and calpastatin

It is generally well accepted that calpastatin down-regulates the activity of calpain through a direct interaction that inhibits its proteolytic activity (Melloni *et al.* 2006). With this in mind, we performed co-immunoprecipitation assays to evaluate the association of calpain to calpastatin during capacitation. When calpain-1 was immunoprecipitated from whole sperm extracts, calpastatin was co-immunoprecipitated (Fig. 3A). Western blot analysis of immunoprecipitates showed that calpain-1 is interacting with calpastatin in non-capacitated sperm; moreover, such calpain–calpastatin association was found to clearly decrease with the time of capacitation (Fig. 3A and B). However, when sperm were capacitated in the absence of Ca^{2+} , the interaction of calpain with calpastatin was similar to the non-capacitated sperm used as control (data not shown).

Figure 3 Calpain-1 is associated with calpastatin. Whole sperm extracts obtained from non-capacitated and capacitated sperm at indicated times were immunoprecipitated with anti-calpain-1 (A) or anti-phosphoserine (p-Ser) (C) antibodies. Immunoprecipitates were then analyzed by western blot (WB) to detect calpastatin. (B) Densitometric analysis of calpastatin co-immunoprecipitated. Blot images represent at least three independent experiments.

Because the reduction of the physical association between calpain and calpastatin is known to be mediated by serine phosphorylation of calpastatin by protein kinase C (PKC; Aversa *et al.* 1999, Melloni *et al.* 2006), we decided to evaluate the calpastatin phosphorylation levels during capacitation. As can be seen in Fig. 3C, initial calpastatin phosphorylation in non-capacitated sperm was increased during capacitation and was inversely proportional to the association levels with calpain.

Effect of calpain inhibitors on the AR

Our results clearly show that calpain is active throughout the course of capacitation. In order to determine whether calpain participates on AR, we explored the effect of two calpain inhibitors; sperm were capacitated for 90 min in the presence of calpeptin (0–10 μM) or *N*-acetyl-Leu-Leu-Nle-CHO (ALLN; 0–500 μM), then afterwards, AR was evaluated. Both calpeptin and ALLN significantly reduced the AR in a dose-dependent manner, with a maximal inhibitory effect at 5 and 250 μM respectively (Fig. 4A).

We have reported that the cytoskeleton associated with the PM is modified during capacitation (Hernandez-Gonzalez *et al.* 2000). To explore the involvement of calpain in the alteration of this cytoskeleton, membrane-associated cytoskeleton from non-capacitated and capacitated sperm treated or not with calpeptin or ALLN was obtained, and then analyzed by electron microscopy. The examination of membrane cytoskeleton from non-capacitated sperm showed typical cross-linked filaments forming a dense cytoskeletal network (Fig. 4B), whereas in samples from capacitated sperm, the membrane-associated cytoskeleton exhibited empty spaces, where the cytoskeleton was not present (Fig. 4B). As expected, when sperm were capacitated in the presence of calpeptin, their cytoskeleton exposed a similar structure to samples from non-capacitated sperm (Fig. 4B). Similar results were obtained using ALLN (data not shown). Therefore, our results indicate that calpain is involved in capacitation and that its activity affects the cytoskeleton.

Spectrin is cleaved by calpain-1 during capacitation

Calpain is an important regulator of the cytoskeleton; it cleaves different cytoskeletal proteins such as the non-erythroid spectrin (α and β subunits), utrophin (Up71), and filamin-1. To determine whether the effect of calpain in the PM-associated cytoskeleton (shown in Fig. 4B) was due to cleavage of cytoskeletal proteins, we first determined the presence of these proteins in the same region where calpain-1 is located. As shown in Fig. 5A, spectrin, Up71, and filamin-1 were located in the acrosomal region; in addition, spectrin was also

Figure 4 Calpain-1 inhibits the acrosome reaction and alters the plasma membrane-associated cytoskeleton. (A) Sperm capacitation was performed in the presence of calpeptin or ALLN at the indicated concentrations to inhibit calpain. The graphs represent the quantification of the proportion of sperm that showed acrosome reaction (mean $\pm$ S.E.M., $n=5$, 500 cells per experiment). (B) Inhibition of calpain-1 during capacitation prevents the alteration of the plasma membrane-associated cytoskeleton. Triton-resistant cytoskeletons obtained from plasma membranes of non-capacitated or capacitated sperm in the absence or presence of calpeptin were stained with uranyl acetate and electron microscopy micrographs taken. Images show the substructure of the plasma membrane-associated cytoskeleton. The micrographs were taken directly from negative film.

located in the equatorial segment as well as in the principal piece of the flagella, whereas Up71 was found along the flagella and filamin-1 in both the equatorial segment and middle piece (Fig. 5A). We next looked for the presence of calpain-mediated breakdown products of these cytoskeletal proteins in membranes from non-capacitated and capacitated sperm. Western blot analysis of 30 μ g of membrane proteins from non-capacitated sperm showed spectrin as two bands corresponding to its α (240 kDa) and β (220 kDa) chains, whereas Up71 and filamin-1 were detected as bands of 72 and 280 kDa respectively (Fig. 5B). However, when membrane proteins from capacitated sperm were analyzed, breakdown products were not detected (Fig. 5B), very likely because of the low amount of protein used. Nonetheless, when 150 μ g of membrane protein was used, spectrin breakdown products (SBPs) were detected in low amounts in non-capacitated sperm, whereas SBPs were greatly found in capacitated sperm. In line with previous reports, we detected two major SBPs of $\sim$ 150 kDa (SBP-1) and 85 kDa (SBP-2; Fig. 6A). As expected, capacitation in the presence of calpeptin (10 μ M) inhibited the production of SBPs (Fig. 6A and B). Densitometric analysis of the SBPs showed that $\sim$ 50% of spectrin was cleaved during capacitation (Fig. 6B). On the other hand, using the same membrane samples and same conditions, we were unable to detect any breakdown products for Up71 or filamin-1 (Fig. 6C and D).

Effect of calpain on the acrosome cytoskeleton

To further explore the changes in spectrin during capacitation, we examined its localization pattern during capacitation as well as the effect of calpeptin treatment on its localization. Spectrin was always found in the principal piece of non-capacitated and capacitated sperm (Figs 5A and 7A); however, when the staining pattern of spectrin in the acrosomal region was analyzed, two differences were clearly observed: non-capacitated sperm displayed a solid uniform fluorescence pattern in the whole acrosome and throughout the equatorial segment (pattern 1 (P1)), whereas in capacitated sperm, the acrosomal staining pattern of spectrin was observed like spots with unstained spaces and the equatorial segment was not stained (pattern 2 (P2)). In all cases, acrosomes were intact as they can be observed in phase contrast micrographs (Fig. 7B). These results thus confirm the alteration undergoing by spectrin cytoskeleton during capacitation. To further determine the involvement of calpain in these changes, sperm capacitated in the presence of calpeptin (10 μ M) were immunostained for spectrin; we found that their acrosomal spectrin-staining pattern was similar to that of non-capacitated sperm (Fig. 7B). The quantification of the proportion of sperm showing P1 and P2 in non-capacitated versus capacitated sperm exhibited a significant difference between them, difference that was inhibited by calpeptin (Fig. 7C). The changes observed in capacitated sperm were also Ca^{2+}

Figure 5 Localization of calpain targets in the guinea pig sperm. The expression of spectrin, Up71, and filamin-1 was determined in guinea pig sperm by immunofluorescence of formaldehyde-fixed sperm (A) as well as by western blot (B) using 30 µg of whole sperm extracts obtained from non-capacitated (1) and capacitated sperm (2). Lower images in panel (A) are phase contrast micrographs of the same fields shown above them. All proteins in panel (B) were found in the Mr reported for them: 240/220 kDa for α/β -spectrin, 70 kDa for Up71, and 280 kDa for filamin-1. Images shown are representative of at least three independent experiments.

dependent; sperm capacitated in the absence of Ca^{2+} showed a spectrin-staining pattern similar to that of non-capacitated sperm, with the sole exception that spectrin was not found in the equatorial segment (Fig. 7B).

Finally, in order to determine whether spectrin and F-actin localize to the same regions, sperm non-capacitated and capacitated in the presence or absence of calpeptin were immunostained for spectrin, while F-actin was stained using TRITC-labeled phalloidin. As we reported previously (Moreno-Fierros *et al.* 1992) that F-actin was located in the acrosomal region and along the flagella in both non-capacitated and capacitated sperm, and although the fluorescence intensity was not evaluated, an increase in fluorescence levels was observed in capacitated sperm when compared to non-capacitated (Fig. 7B), a phenomenon already reported in other mammalian sperm (Brener *et al.* 2003).

F-actin also presented a clear co-localization with spectrin in the principal piece and did not undergo any changes during capacitation (Fig. 7A). In the acrosome region, spectrin and F-actin showed a low level of co-localization in non-capacitated and capacitated sperm that was not modified by the treatment with calpeptin or by the absence of Ca^{2+} (Fig. 7B).

Discussion

Calpain is a cysteine protease family, where the two major and more ubiquitous members are calpain-1 and calpain-2, which are involved in processes where the cleaving of cytoskeletal proteins is a key event. The activity of calpain is tightly regulated by cellular factors such as intracellular calcium concentration and calpastatin and very likely, by translocation to the PM. In the present study, we show for the first time evidence of the activation and regulation of calpain-1 in mammalian sperm. We also present evidence of the role of calpain in remodeling the sperm spectrin cytoskeleton, a process that can be of vital importance to achieve capacitation and AR.

The activation of calpain during both normal and pathologic processes requires an increase in $[\text{Ca}^{2+}]_i$, as well as their translocation to the PM, where several calpain targets are located (Hood *et al.* 2006). Our data provide cellular and molecular evidence suggesting that calpain-1 has to undergo different changes during capacitation to become active. Previous studies and our present work demonstrate that calpain-1 and calpain-2 are located in the acrosome and in the middle piece of flagella of non-capacitated mammalian sperm (Schollmeyer 1986, Rojas *et al.* 1999, Yudin *et al.* 2000, Aoyama *et al.* 2001, Ozaki *et al.* 2001, Ben-Aharon *et al.* 2005). In addition, during capacitation, calpain-1 is translocated to PM and redistributed into different sperm regions; apical acrosome and postacrosomal regions (see Fig. 2A and C). We can consider the non-capacitated sperm as a non-stimulated state in which $[\text{Ca}^{2+}]_i$ is low, and although it is not known how, when sperm are incubated in a medium that allows capacitation, the $[\text{Ca}^{2+}]_i$ increase is triggered (Coronel & Lardy 1987, Adeoya-Osiguwa & Fraser 1996). This increase in $[\text{Ca}^{2+}]_i$ could be linked with the translocation of calpain-1 to PM, since calpain-1 was not detected in PM from non-capacitated sperm, and both the redistribution and translocation of calpain-1 do not happen when sperm were capacitated in the absence of Ca^{2+} .

The presence of calpastatin, the natural inhibitor of calpain, has been demonstrated in at least two sperm species: human (Rojas *et al.* 1999) and macaque (Yudin *et al.* 2000). Our results show that calpain-1 is already associated with calpastatin in non-capacitated sperm and that the interaction decreases during capacitation. Very likely, calpain-1 is kept in the sperm cytoplasm associated with calpastatin, and during capacitation, this

Figure 6 Calpain-1 cleaves spectrin. Whole sperm extracts (150 µg) from non-capacitated and capacitated cells in the presence or absence of calpeptin were subjected to western blot to look for the presence of breakdown products of spectrin (A), Up71, (C), and filamin-1 (D). Breakdown products were detected only when spectrin was analyzed (A). (B) Densitometric analysis of spectrin (Spct) and spectrin breakdown products (SBP-1 and SBP-2) in non-capacitated and capacitated sperm in the absence or presence of calpeptin. The graph represents the mean $\pm$ s.e.m. of at least three independent experiments. Western blot images are representative of three independent experiments.

interaction is reduced allowing calpain-1 to move towards the PM. One possible mechanism to explain this reduction in interaction could involve an increase in serine phosphorylation of calpastatin, which takes place before the translocation of calpain-1 to the PM. Furthermore, it has been reported that calpastatin phosphorylation in serine residues by PKC reduces both its capacity to interact with the inactive form of calpain and its inhibitory efficiency on the active form of calpain. Moreover, calpastatin phosphorylation also increases the concentration of Ca^{2+} required to induce the formation of the calpain–calpastatin complex (Averna *et al.* 1999, Melloni *et al.* 2006).

In line with previous reports, our data show that AR is inhibited by calpain antagonists (Rojas *et al.* 1999, Aoyama *et al.* 2001, Ben-Aharon *et al.* 2005). One of the major roles of calpain in non-pathological cells is a limited cleavage of different cytoskeletal proteins in a Ca^{2+} -dependent manner. We have previously reported that the structure of the PM-associated cytoskeleton is disturbed during capacitation (Cabello-Aguero *et al.* 2003), and now we add another piece of the puzzle by showing that calpain is involved in such alteration through the cleavage of spectrin. We found a clear correlation between the disruption of the spectrin

cytoskeleton and AR since calpain inhibitors not only interfered with AR, but they also inhibited spectrin cleavage. Moreover, $\sim 50\%$ of spectrin remained intact after capacitation, and considering that the distribution pattern of spectrin showed only alteration in the acrosome region in capacitated sperm, our results suggest that the cleavage of spectrin is a compartmentalized process. Finally, our data indicate that the activity of calpain is specific; it does not alter the actin cytoskeleton or other cytoskeletal proteins such as utrophin and filamin-1, although as in other cells, calpain might have other non-cytoskeletal substrates associated with PM such as Ca^{2+} channels or receptors (Sandoval *et al.* 2006, Croall & Ersfeld 2007). On the other hand, although our data suggest that calpain-1 could be one of the calpains involved in spectrin cleavage and AR, we do not discard the possibility that other calpain may be implicated in such processes, since the calpain inhibitors are not specific for calpain-1, and calpain-11 has been reported in mammalian sperm (Ben-Aharon *et al.* 2006).

Different and important membrane domains are established in both the acrosome and flagella of mammalian sperm, which display distinct biochemical and physiological functions; however, little is known

Figure 7 The staining pattern of spectrin changes in the acrosome during capacitation. The co-localization of spectrin (red) and F-actin (blue) was determined in formaldehyde-fixed non-capacitated sperm (A) and in capacitated sperm in the presence of calpeptin or in the absence of calcium (B). Confocal microscopy analysis showed two major staining patterns for spectrin that were denominated P1 (in non-capacitated sperm) and P2 (in capacitated sperm). Quantification of the proportion of sperm showing P1 and P2 is presented in (C). The graph represents means $\pm$ S.E.M., $n=3$, 25 cells per experiment. Cap, capacitated; PhCM, phase contrast micrographs. Images shown are representative of at least three independent experiments.

about the role of the cytoskeleton in the establishment of these membrane domains. Our findings showing the localization of spectrin indicate that it could constitute and stabilize such domains, and also suggest that considering the cytoskeletal proteins associated with PM, flagella is formed by two different membrane subdomains: the middle piece, whose membrane cytoskeleton is mainly conformed by the short dystrophin Dp71/F-actin (Hernandez-Gonzalez *et al.* 2001), and the principal piece, whose membrane cytoskeleton is mainly conformed by spectrin/F-actin. In particular, these cytoskeletons do not undergo changes during capacitation or AR. In the same way as the principal piece, the cytoskeleton associated with PM in the acrosomal region is mainly composed of spectrin/F-actin, but with the difference that it is remodeled during capacitation. During capacitation and AR, proteins associated with PM as well as with membrane lipids compartmentalize in

the acrosome region; they are redistributed inside the acrosome or distribute towards the equatorial segment and postacrosomal region as well (Rochwerger & Cuasnicu 1992, Da Ros *et al.* 2004, Selvaraj *et al.* 2007, Tsai *et al.* 2007, Pasten-Hidalgo *et al.* 2008). These molecules in some way are maintained as compartmentalized in the acrosome before capacitation. We propose that the spectrin network works as a barrier that keeps these molecules compartmentalized. During capacitation, spectrin is severed and in consequence, its network disrupted, just then, the molecules can redistribute. Even though lipid membrane composition has been regarded as the principal mechanism to maintain and stabilize the sperm membrane domains (Boerke *et al.* 2008), we also consider that the spectrin cytoskeleton plays an important role to maintain these membrane domains, specially when taking into account that spectrin contains phospholipid-binding sites, through which it interact with the

PM (Hryniewicz-Jankowska *et al.* 2004, An *et al.* 2005, Bok *et al.* 2007); such interaction is facilitated by cholesterol (Diakowski *et al.* 2006).

In conclusion, based on our findings, we propose a model in which calpain-1 is activated during capacitation, and although its effects are visualized until AR, the activity of calpain can be of great importance for capacitation. We also propose that one of the main roles of calpain-1 during capacitation is to cleave spectrin, and as a consequence, the specific disruption of the spectrin cytoskeleton. It is important to point out that the presence of several different cytoskeletal proteins associated with PM in the mammalian sperm is already known; however, their participation in sperm physiology is unclear. In this way, our study opens the possibility to understand the role of the spectrin cytoskeleton, the signal pathways that regulate the sperm cytoskeleton, as well as its importance for capacitation and AR.

Materials and Methods

Chemicals

Sodium pyruvate, lactic acid, DL-dithiothreitol, sucrose, Triton X-100, iodoacetamide, benzamidine, aprotinin, leupeptin, pestatin, *p*-aminobenzamidine (pAB), phenylmethyl-sulfonyl fluoride (PMSF), trizma base, ALLN, TRITC-labeled phalloidin, sodium orthovanadate, and sodium fluoride were purchased from Sigma Chemical Co. Protein A-agarose and protease inhibitors Complete cocktail tablets were purchased from Roche Diagnostics and Molecular Biochemicals. Nitrocellulose membrane, acrylamide, *N,N'*-methylene-bis-acrylamide, and SDS were purchased from Bio-Rad Laboratories. Immobilon membrane was purchased from Millipore (Billerica, MA, USA). The antibodies mouse monoclonal anti-calpain-1 (C-266 and C-5736), monoclonal anti-calpastatin (C-270 and C-2), monoclonal anti-calpain-2 (C-268), anti-human spectrin (S-1515), and anti-chicken spectrin (S-1390) were purchased from Sigma Chemical Co.; anti-filamin-1 (H-300, sc-28284) and anti-calpain-2 (C-19, sc-7532) were obtained from Santa Cruz Biotechnology (San Jose, CA, USA); anti-utrophin 71 (Up71) was kindly donated by Dr Dominique Mornet from INSERM U-592, France; rabbit anti-phosphoserine was obtained from Zymed Laboratories Inc. (South San Francisco, CA, USA); HRP-linked goat anti-mouse IgG and TRITC-labeled goat anti-mouse IgG were purchased from Jackson Immuno-research Laboratories Inc. (West Grove, PA, USA), whereas anti-calpeptin was obtained from Calbiochem (San Diego, CA, USA). The ECL reagent was obtained from Amersham.

Animals

All animal handling procedures and experimental design were approved by the Internal Committee for the Care and Use of the Laboratory Animal CINESTAV-IPN (CICUAL 321-02), following the American Veterinary Medical Association guidelines. All efforts were made to minimize the potential for animal pain, stress, or distress.

Capacitation in the presence and absence of ALLN or calpeptin

Capacitation was performed as described elsewhere (Cabello-Agueros *et al.* 2003, Pasten-Hidalgo *et al.* 2008); briefly, *ductus deferens* guinea pig sperm were obtained and washed in 154 mM NaCl solution. Sperm cells (3.5×10^7 cell/ml) were capacitated by incubation at 37 °C in MCM-PL without glucose. Cells were preincubated with different concentrations of the calpain inhibitors ALLN (0–500 µM) or calpeptin (0–500 µM) for 15 min before capacitation in 154 mM NaCl; cells were then centrifuged and capacitated for 60–90 min in MCM-PL in the presence of the same concentrations of ALLN or calpeptin. Immediately after capacitation, samples were fixed using 1.5% formaldehyde (final concentration) in PBS. As control, depending on the experiment, cells were incubated in 154 mM NaCl, MCM-PL, or MCM-PL plus vehicle; in all cases, cells were incubated and fixed in parallel with treated samples (Rogers & Yanagimachi 1975, Sanchez-Gutierrez *et al.* 2002).

Estimation of acrosome-reacted sperm

AR was evaluated by light microscopy based upon the presence of motile sperm without the acrosome (Yanagimachi & Bhattacharyya 1988). Sperm were incubated in MCM-PL for 90 min (although an evaluation of AR was performed after 60 min of incubation), and then sperm aliquots were fixed in 1.5% formaldehyde. Quantification of AR was performed by triplicate for each experiment using a hemocytometer. To normalize the data, sperm were incubated in medium without calcium, and sperm without acrosome were quantified. Reported values represent the percentage of spermatozoa without acrosome after normalization.

Immunofluorescence procedures

Cells were fixed in 1.5% formaldehyde in PBS, permeabilized using acetone at –20 °C for 7 min, and washed three times in PBS and once in distilled water. Water-resuspended cells were used to prepare smears, which were air dried at room temperature and rinsed with PBS. Smears were then incubated with the primary antibody dilutes (anti-calpain 1:100, anti-calpastatin 1:100, anti-filamin-1 1:100, anti-Up71 1:100, or anti-spectrin 1:250) in blocking solution (1% BSA in PBS), under cover glass slides for 12 h at 4 °C in humid conditions. Exhaustive PBS washes were carried out, and then the cells were incubated for 1 h at 37 °C under humid conditions with the appropriate TRITC- or Cy5-labeled secondary antibodies. In all cases, smears were exhaustively washed with PBS, and for observation, they were mounted under cover glass slides using gelvatol.

F-actin detection

The localization of F-actin cytoskeleton was revealed using TRITC-labeled phalloidin (30 µM) for 45 min at room temperature after spectrin immunolabeling was completed. Smears were exhaustively washed with PBS and mounted as mentioned above. Images were acquired using an Olympus

BX50 photomicroscope equipped with phase contrast and epifluorescence or using a Leica TCS SP2 confocal microscope as required.

Electrophoresis and western blot

Cells (350×10^6) were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM PMSF, Complete, 1 mg/ml aprotinin, 10 mM sodium orthovanadate, 25 mM sodium fluoride, and 1% Triton X-100) as previously reported (Pasten-Hidalgo *et al.* 2008). Samples were then centrifuged at 5000 *g* for 5 min at 4 °C, supernatants were collected, and protein concentration was determined (Bradford 1976). Samples were then boiled for 5 min in sample buffer (Laemmli 1970), and proteins were resolved in 7 or 10% SDS-PAGE and transferred onto nitrocellulose membranes (Towbin *et al.* 1979). Membranes were blocked using Tris-buffered saline containing 5% dried fat-free milk and 0.1% Tween-20. Membranes were then incubated overnight at 4 °C with the respective antibody (anti-calpain-1, 1:1000; anti-calpain-2, 1:1000; anti-calpastatin, 1:1000; anti-spectrin, 1:2000; anti-filamin-1, 1:1000; or anti-utrophin, 1:1000). After five 7 min washes, membranes were incubated with the appropriated HRP-labeled secondary antibody (1:10 000). Finally, immuno-reactive proteins were detected by chemiluminescence using an ECL western blot detection kit (Amersham Biosciences).

Co-immunoprecipitation

Protein extracts were incubated with 10 µl of protein A-agarose and 1 µl of antibody under constant agitation for 18 h at 4 °C. Immunoprecipitates were recovered by centrifugation at 5000 *g* and washed three times with 500 µl buffer A (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 20 mM sodium orthovanadate, 20 mM sodium molybdate, 50 mM sodium fluoride, Complete, and 1% Triton X-100, pH 7.5). Samples were next boiled for 5 min after Laemmli sample buffer was added.

PM and cytosolic proteins preparation

Cells (300×10^6) were suspended in 1 ml buffer B (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 µg/ml soybean trypsin inhibitor, 300 µl Complete, 1 µM aprotinin, 1 µM pestatin, 1 µM leupeptin, 10 µM benzamidine, 10 mM sodium orthovanadate, and 50 mM sodium fluoride). Samples were sonicated for 30 s at 4 °C using an Ultrasonic Processor CP130PB-1 (Cole Palmer Co., Vernon Hill, IL, USA) set at amplitude of 40 W, and then were centrifuged at 5000 *g* for 5 min at 4 °C. Supernatants, which contained cytosolic proteins and membranes (plasma and acrosomal membranes), were recovered and centrifuged now at 100 000 *g* for 2 h at 4 °C to separate the cytosolic proteins from membranes. Supernatants, which contained cytosolic proteins, were recovered and their protein concentration was determined. The pellets, which contained the membranes, were washed twice by centrifugation at 100 000 *g* for 2 h at 4 °C with buffer B and then solubilized in buffer B containing 2% SDS. Protein concentration was determined (Markwell *et al.* 1978) and subjected to SDS-PAGE as described above.

Isolation of sperm PMs

PM preparations were obtained as previously described (Hernandez-Gonzalez *et al.* 2000); briefly, cells were resuspended in buffer AH (70 mM KH_2PO_4 , 90 mM sucrose, 2 mM MgSO_4 , 1 mM EDTA, 25 mM 4-morpholineethanesulfonic acid, and 10 mM HgCl_2 , pH 6.2) containing 2 mM pAB, 2 mM benzamidine, 1 µM leupeptin, 1 µM pestatin, and 1 µM aprotinin, and homogenized at 8000 load/min for 30 s. Homogenates were centrifuged at 2000 *g* for 30 min (Tekmar Mark II, IKA Labortechnik, Staufen, Germany), and supernatants were collected and further processed as follows: supernatants were centrifuged at 100 000 *g* for 2 h at 4 °C; pellets containing sperm membranes obtained after centrifugation were washed twice by centrifugation with buffer B at 100 000 *g* for 2 h at 4 °C and then solubilized in buffer B (50 mM Tris-HCl, 1 mM EDTA, pH 7.4 added with 2% of SDS, 10 mM sodium orthovanadate, and 50 mM sodium fluoride); and protein concentration was determined (Markwell *et al.* 1978). Proteins from both fractions were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for western blot analysis.

PM-associated cytoskeleton

PM-associated cytoskeleton was obtained as previously described (Hernandez-Gonzalez *et al.* 2000). Supernatants obtained after homogenization and centrifugation as in isolation of sperm PMs were fixed in 1.5% formaldehyde in PBS for 30 min. A drop of this membrane suspension was plated on collodion-carbon-coated grids and left to adhere for 15 min, and aldehyde groups were blocked by incubation with 50 mM NH_4Cl in PBS for 10 min. Grids were then rinsed with PBS, treated with 0.2% Triton X-100, and post-fixed in Karnovsky's solution for 10 min. After three washes with PBS and twice with double-distilled water, samples were stained for 1 min with 0.2% of uranyl acetate in 70% ethanol, washed three times for 5 min with 70% ethanol, air dried, and examined using a JEOL 2000EX electron microscope.

Statistics analysis

All results are representative of at least three independent experiments and are expressed as average $\pm$ S.E.M. Results comparing two samples were analyzed by paired Student's *t*-test. Significance levels were set at $P < 0.05$.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-09-0545>.

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.

Funding

This work was supported by CONACYT grant 79921 to E O Hernández-González.

Acknowledgements

We thank the staff of both Unidad de Microscopia Electrónica (UME, CINVESTAV-IPN) and Unidad de Microscopia Confocal (Dpto. Biología Celular, CINVESTAV-IPN) for providing electron microscopy and confocal facilities. We also thank Dr Alfredo Mendez for critical review of this manuscript.

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Received 9 December 2009

First decision 20 January 2010

Revised manuscript received 8 July 2010

Accepted 17 August 2010