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ORIGINAL RESEARCH

The activation of the chymotrypsin-like activity of the proteasome is regulated by soluble adenyl cyclase/cAMP/protein kinase A pathway and required for human sperm capacitation

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ABSTRACT: One of the first events of mammalian sperm capacitation is the activation of the soluble adenyl cyclase/cAMP/protein kinase A (SACY/cAMP/PKA) pathway. Here, we evaluated whether the increase in PKA activity at the onset of human sperm capacitation is responsible for the activation of the sperm proteasome and whether this activation is required for capacitation progress. Viable human sperm were incubated with inhibitors of the SACY/cAMP/PKA pathway. The chymotrypsin-like activity of the sperm proteasome was evaluated using a fluorogenic substrate. Sperm capacitation status was evaluated using the chlortetracycline assay and tyrosine phosphorylation. To determine whether proteasomal subunits were phosphorylated by PKA, the proteasome was immunoprecipitated and tested on a western blot using an antibody against phosphorylated PKA substrates. Immunofluorescence microscopy analysis and co-immunoprecipitation (IPP) were used to investigate an association between the catalytic subunit alpha of PKA (PKA-Cα) and the proteasome. The chymotrypsin-like activity of the sperm proteasome significantly increased after 5 min of capacitation ($P < 0.001$) and remained high for the remaining incubation time. Treatment with H89, KT5720 or KH7 significantly decreased the chymotrypsin-like activity of the proteasome ($P < 0.001$). IPP experiments indicated that PKA inhibition significantly modified phosphorylation of proteasome subunits. In addition, PKA-Cα colocalized with the proteasome in the equatorial segment and in the connecting piece, and co-immunoprecipitated with the proteasome. This is the first demonstration of sperm proteasome activity being directly regulated by SACY/PKA-Cα. This novel discovery extends our current knowledge of sperm physiology and may be used to manage sperm capacitation during assisted reproductive technology procedures.

Key words: sperm / capacitation / proteasome / protein kinase A / SACY/cAMP/PKA / human

Introduction

Mammalian spermatozoa are unable to fertilize an oocyte immediately after ejaculation. They require a period of priming in the female reproductive tract to acquire fertilizing capacity. During this time, the spermatozoa undergo a series of physiological and functional changes known as capacitation. Sperm capacitation was first reported over six decades ago by Austin (1952) and Chang (1951). *In vitro*, this phenomenon can be mimicked by incubating the spermatozoa in an appropriated culture medium (Harrison, 1996). It appears that certain components of the capacitation medium, such as bicarbonate, serum albumin and calcium play important regulatory roles in promoting sperm capacitation. Bicarbonate activates the spermatozoa soluble

adenyl cyclase (SACY), which results in increased levels of sperm cAMP (Visconti *et al.*, 1995b). Many studies have shown that cAMP plays an important role as a second messenger in the initiation of signalling cascade of sperm capacitation, acting on a cyclic AMP (cAMP)-dependent protein kinase (PKA) (Visconti *et al.*, 1995a; Leclerc *et al.*, 1996; Galantino-Homer *et al.*, 1997; Wennemuth 2003). The PKA is a ubiquitous tetrameric serine/threonine (Ser/Thr) kinase holoenzyme composed of two regulatory (R) and two catalytic subunits (C). Its activity depends on cAMP. The binding of four cAMP molecules to the regulatory subunits (two molecules per subunit) allows tetramer dissociation and activation of the catalytic subunit (Nolan *et al.*, 2004). Once free, the catalytic subunits are active to phosphorylate a wide variety of substrates in the Arg-X-X-Ser/Thr motif of their substrates (Shabb, 2001; Bruce *et al.*, 2002). Indirectly, this increase in cAMP/PKA regulates the phosphorylation of proteins in Tyr residues (P-Tyr) later during capacitation. In human spermatozoa, P-Tyr rises significantly only after 2.5 h of incubation (Leclerc *et al.*, 1996).

Mice that lack the unique sperm PKA catalytic subunit Cα2 are infertile despite normal mating behaviour, and do not show the increase in tyrosine phosphorylation after sperm incubation in capacitating medium (Nolan *et al.*, 2004), suggesting the involvement of PKA in this process. PKA is one of the most prevalent Ser/Thr kinases present in the flagellum of mammalian spermatozoa (Vijayaraghavan *et al.*, 1997). However, little is known about the phosphorylation of proteins at Ser/Thr residues in mammalian spermatozoa.

Both the mammalian and non-mammalian models show the involvement of the ubiquitin-proteasome system (UPS) in the regulation of multiple steps in the cascade of fertilization events (reviewed in Kerns *et al.*, 2016). The UPS degrades most long-and short-lived normal and defective/misfolded intracellular proteins (Goldberg, 2003). In the UPS, substrates are typically marked for degradation by covalent linkages to multiple ubiquitin molecules. Ubiquitin is a monomeric protein of 76 amino acid residues and a molecular weight of 8.5 kDa (Schlesinger *et al.*, 1975; Bebington *et al.*, 2001), and is the essential element of UPS (Ciechanover, 2005). Ubiquitination is one of the most common post-translational protein modifications. Three conserved enzymes, E1 (UBA1), E2 (UBE2) and E3 (UBE3), carry out protein ubiquitination in a sequential manner, first activating (E1), then transporting (E2) and ligating (E3) ubiquitin molecules to their substrates. Their activity culminates with the tandem-binding of multiple ubiquitin molecules to the lysine residues of proteins flagged for elimination/recycling (Hershko and Ciechanover, 1998). Once marked by polyubiquitin chains, proteins are rapidly degraded by the 26S proteasome, typically composed of a 20S core capped with a 19S regulatory particle.

The 20S core is formed by two pairs of homologous rings, each containing seven subunits. The two outer rings contain α-type subunits (PSMA1–7), the function of which is to operate a 'gate' through which proteins enter the catalytic sites (Jung *et al.*, 2009). The β-subunits (PSMB1–7) form the two inner rings. Three of the β-subunits, β1 (PSMB6), β2 (PSMB7) and β5 (PSMB5), are catalytically active and possess the caspase-like (or peptidylglutamyl-peptide), trypsin-like and chymotrypsin-like activities, respectively (Adams, 2004; Jung and Grune, 2012). Given its proteolytic action, the proteasome degrades proteins synthesized with errors or altered post-translationally (Oberdorf *et al.*, 2001; Jung *et al.*, 2009; Bedford *et al.*, 2010; Xie, 2010). The activity of the proteasome can be regulated at several levels. However, its most common post-translational modification is phosphorylation (Konstantinova *et al.*, 2008). Studies in mouse cardiac myocytes indicate that PKA co-immunoprecipitates with the proteasome and that this association increases the chymotrypsin-like and Peptidyl-Glutamyl Peptide-Hydrolyzing (PGPH) activities of the proteasome (Zong *et al.*, 2006). In addition, at least six different proteasomal subunits are phosphorylated on Tyr residues to influence its intracellular localization (Tanaka *et al.*, 1990; Benedict *et al.*, 1995).

In spermatozoa from mouse, boar and rat, phosphoproteome studies have identified several proteins of the UPS undergoing phosphorylation during capacitation. These proteins include the ubiquitin activating enzyme UBE1 (Baker *et al.*, 2010) and multiple proteasome subunits (Arcelay *et al.*, 2008; Baker *et al.*, 2010). In addition, there are differences in the two-dimensional electrophoretic migration pattern of several 19S and 20S proteasome subunits, between capacitated versus non-capacitated sperm from boars (Choi *et al.*, 2008; Sutovsky, 2011; Zigo *et al.*, 2018). This suggests that during capacitation, changes occur in the structure of the sperm proteasome.

Various reports indicate that the activation of PKA is one of the first intracellular events of capacitation since spermatozoa incubated in a capacitating medium show a rapid ($\leq$1 min) increase in PKA activity (Moseley *et al.*, 2005; Martínez-León *et al.*, 2015). Ser/Thr protein phosphorylation rapidly ensued.

To date, there are no studies evaluating the role and the regulation of the proteasome at the onset of sperm capacitation. Thus, the aim of the present work was to characterize the role of the sperm proteasome during the early events of human sperm capacitation and to determine whether the PKA pathway regulates the enzymatic activity of this multicatalytic protease. In the context of this work, capacitation refers to this process *in vitro*.

Methods

Ethics statement

The research presented in this manuscript was approved by the Ethics Committee on Scientific Research of the University of Antofagasta, and the Institutional Review Board of The University of Missouri. The institutional review board approved the use of all human semen samples described in this study and followed the current guidelines for human semen studies (Björndahl *et al.*, 2016). All donors, anonymous to researchers, signed a consent form for the use of their spermatozoa for research purpose.

Chemicals and reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK); 7-chlorotetracycline (CTC) hydrochloride; (E)-2-(1H-Benzo [d]imidazol-2-ylthio)-N′-(5-bromo-2-hydroxybenzylidene) propane hydrazide (KH7); bovine serum albumin (BSA; A7030); Ponceau Red; HEPES; EDTA; Hoechst 33258 (H258); 1,4-diazabicyclo [2.2.2.] octane (DABCO); dimethyl sulfoxide (DMSO); Na_3VO_4; NaF; phenylmethylsulfonyl fluoride; aprotinin; and leupeptin. The following compounds were purchased from Enzo Life Sciences (Farmingdale, NY, USA): (9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl] pyrrolo[3,4-i] [1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT5720); N- [2-(p bromocinnamylamino) ethyl]-5-isoquinoline-

sulfonamide 2HCl (H89); N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC); Sp-Cyclic 3′,5′-hydrogen phosphorothioate adenosine hydrate (Sp-cAMPS); 3-isobutyl-1-methylxanthine (IBMX); N-Acetyl-N-methyl-L-isoleucyl-L-isoleucyl-N-[(1S)-3-methyl-1-[[(2R)-2-methyloxiranyl]carbonyl]butyl]-L-threoninamide (epoxomicin). Dako Fluorescent Mounting Medium was purchased from Dako North America.

The following antibodies were used: anti-phospho PKA substrates (RRXS*/T*) rabbit monoclonal antibody (mAb #9624, Cell Signaling Technology, Inc., USA); anti-α-4 proteasome subunit antibody and agarose-immobilized anti-α-4 proteasome subunit antibody (BML-PW8120 and BML-PW005, respectively, Enzo Life Sciences; Farmingdale, NY, USA); anti-PKA catalytic subunit antibody (#4782, Cell Signaling Technology, Danvers, MA, USA); anti β-tubulin antibody (E7, Developmental Studies Hybridoma Bank, Iowa, USA); and R-phycoerythrin-labeled mAb, clone pY20 (#558008 BD Phosflow^{TM}). The following secondary antibodies were used: biotinylated goat anti-rabbit and goat anti-mouse (AP132B and AP124B, respectively, Chemicon International, Temecula, CA, USA), Alexa 594-conjugated chicken anti-rabbit (Thermo Fisher Scientific, Waltham, MA USA) and Alexa 488-conjugated goat anti-mouse (A-21442 and A-10680, respectively, Thermo Fisher Scientific). The chemiluminescence detection system and Immobilon P transfer membranes were purchased from Millipore Corporation (Bedford, MA, USA). The DC protein assay kit was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

None of the inhibitors or solvents used in this study affected negatively sperm viability or motility (data not shown). The deionized water used in these experiments was purified to 18-megohms with an EASYpure UV/UF ion-exchange system (Barnstead/Thermolyne, Dubuque, IA, USA). All other chemicals were of analytical grade and obtained from standard sources.

Culture media

The basic medium used for all the experiments was a modified Tyrode's medium (117 mM NaCl, 19 mM sodium lactate, 2.5 mM glucose, 8.6 mM KCl, 2.4 mM $CaCl_2$, 0.25 mM sodium pyruvate, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 70 µg/ml of penicillin and streptomycin and phenol red). This medium was designated non-capacitating medium (NCM). The capacitating medium (CM) was similar to NCM, except that it was supplemented with 2.6% BSA (w/v) and 25 mM $NaHCO_3$ (Signorelli *et al*., 2013). The pH of all media was adjusted to 7.4–7.45 before use.

Semen collection

Semen samples were provided by healthy donors aged between 20 and 30 years; all donors provided more than one sample. Freshly ejaculated spermatozoa were obtained by masturbation after 2–3 days of sexual abstinence. Samples obtained from the same donor were requested with at least a 2-week interval. Semen was collected in a sterile vessel for which each lot was tested for sperm toxicity. All semen samples used were normospermic according to World Health Organization criteria (WHO, 2010). Semen samples were allowed to liquefy for 30–60 min at 37°C. All semen samples were processed by the same person using compatible equipment, and analyses of volume, pH, sperm concentration and percentages of motile and viable spermatozoa were performed. The mean values for semen parameters are summarized in Supplementary Table SI.

Sperm selection

Motile spermatozoa were obtained using a dual Percoll gradient (40/80%), as described previously (Morales *et al*., 2003). Briefly, aliquots of semen were layered over the upper layer of the Percoll gradient and centrifuged at 300 g for 20 min. The pellet was then resuspended in 10 ml of NCM and centrifuged again at 300 g for 10 min. Finally, the sperm pellet was resuspended in the appropriate medium at the required concentration.

Approximately 10×10^6 spermatozoa/ml were incubated for different times 0 (NCM), 1, 5, 10 15, 30 and 60 min (CM) at 37°C and 5% CO_2 in air, in the presence or absence of inhibitors. None of the treatments had any detrimental effect on sperm motility or viability.

CTC assay

The sperm capacitation status was assessed using the CTC fluorescence assay method, as described previously (Lee *et al*., 1987; Kong *et al*., 2009). CTC is a fluorescent antibiotic whose distribution in the spermatozoa changes during the transition from non-capacitated to capacitated and then to acrosome-reacted state, thereby allowing to differentiation various steps of the sperm capacitation process (Fraser *et al*., 1993; Varner *et al*., 1993; Neild *et al*., 2005). Briefly, the CTC solution was prepared on the day of use and contained 750 µM CTC in a buffer of 130 mM NaCl, 5 mM cysteine, and 20 mM Tris-HCl, pH adjusted to 7.8. This solution was kept wrapped in foil at 4°C until use. A 10 µl aliquot of a sperm suspension supplemented with 10 µg/ml supravital stain Hoechst 33258 (H258) was incubated at 37°C for 10 min; 10 µl of CTC stock solution was then rapidly added, followed within 30 sec by 2 µl of 2% (v/v) glutaraldehyde in 1 M Tris buffer (pH 7.8). A 20 µl aliquot of this suspension was placed on a slide: once it was dried, a drop of DABCO mounting medium was carefully mixed in, to retard fading of the fluorescence, and a cover slip was placed on top. Cells were assessed for their viability using H258, as described previously (Cross *et al*., 1986). In each sample, 200 live cells were assessed for CTC staining patterns, and in all cases, the proportion of dead cells was very low.

Three distinct patterns of CTC fluorescence were classified: the F pattern, with uniform fluorescence over the entire sperm head, was characteristic of non-capacitated, acrosome intact cells; the B pattern, with a fluorescence-free band in the post-acrosomal region, was characteristic of capacitated, acrosome-intact cells; and the AR pattern, with weak or absent fluorescence over the sperm head, was characteristic of capacitated, acrosome-reacted cells (Lee *et al*., 1987).

Flow cytometric analysis

To corroborate CTC, sperm capacitation status was assessed using the pattern of tyrosine phosphorylation. Aliquots of motile sperm suspensions (2×10^6 sperm/ml) were fixed and permeabilized with BD Cytofix/Cytoperm kit (San Jose, CA, USA) for 20 min at 4°C. Permeabilization of spermatozoa is a critical step in the staining, as the tyrosine-phosphorylated sperm proteins are mainly intracellular. Then, the spermatozoa were centrifuged at 14000 g for 2 min and washed with BD Perm/Wash^{TM} buffer (San Jose, CA, USA). The pellet was resuspended in 100 µl of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 4.3 mM Na_2PO_4, pH 7.4) sup-

plemented with 2% (w/v) BSA and incubated with 10 µl of antibody. Tyrosine phosphoproteins were detected using the mAb clone pY20. The optimal working concentration of the anti-phosphotyrosine mAb and the time of coincubation with spermatozoa (2 h) were chosen by titration tests in preliminary experiments. Stained samples were analysed using flow cytometry (FACSJazz Cell Sorter, BD Biosciences, San Jose, CA, USA).

Preparation of sperm extracts

After capacitation, aliquots of spermatozoa were washed twice in cold PBS and centrifuged at 9000 g for 30 sec. To measure proteasome enzymatic activity, the pellet was resuspended in homogenization buffer (50 mM Hepes, 10% (v/v) glycerol, 200 µM TLCK, pH 7.4), sonicated (Virsonic, Gardiner, NY, USA) with seven 60-W bursts of 30 sec each, and then centrifuged for 30 sec at 5000 g in a Beckman microfuge to remove nuclear and flagellar material (Morales *et al*., 1994). The supernatant was used as the enzyme stock preparation.

For the immunoprecipitation (IPP) analysis, the sperm pellet was resuspended in 200 µl IPP buffer (20 mM Tris HCl pH 7.5, 137 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS) (w/v), 0.5% $C_{24}H_{39}O_4Na$ (sodium deoxycholate, w/v), 1% Triton X-100 v/v). The buffer was supplemented with protease and phosphatase inhibitors. According to previous work from our laboratory, none of the protease inhibitors used have a significant effect on the chymotrypsin-like activity of the sperm proteasome (Pizarro *et al*., 2004). The protein concentration in each sperm extract preparation was measured using the RC DC protein assay. All procedures were performed at 4°C.

IPP

Control sperm aliquots were obtained at the beginning (0 min) and after 15 min of incubation, and sperm aliquots treated with 50 µM H89 were obtained after 15 min. To immunoprecipitate the proteasome these aliquots were then treated with an anti-PSMA7 proteasomal subunit antibody, as described (Diaz *et al*., 2007; Kong *et al*., 2009). Briefly, 400 µl of the sample containing 400 µg of protein were incubated with 7 µl of the anti-proteasome antibody, immobilized on agarose (2 mg/ml). The reaction mixture was incubated on an orbital shaker overnight at 4°C. Antigen-antibody-A/G-agarose protein complexes were concentrated to the bottom of the tube by centrifugation at 14250 g for 30 sec at 4°C. The supernatants were discarded, and the pellets were washed twice with IPP buffer and once with PBS. The washed pellets were mixed with SDS sample buffer and heated in a boiling water bath for 5 min, and the supernatant was subjected to SDS-PAGE.

SDS-PAGE and immunoblotting

SDS-PAGE was performed using 12% gels according to the method of Laemmli (1970). Briefly, sperm extract aliquots were boiled for 5 min with sample buffer (500 µM Tris-HCl, 10% SDS (w/v), 30% glycerol v/v), 0.5% β-mercaptoethanol (v/v) and 0.5% bromophenol blue, pH 6.8 (w/v)) and then immediately stored on ice. Isolated proteins were separated on SDS-PAGE (60 mA, 120 min). Afterwards, proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 250 mA for 60 min at 4°C. Transfer was monitored by Ponceau red stain. The membranes were blocked in TRIS-buffer saline with 0.1% Tween 20 (T-TBS, v/v) and 5% milk (w/v). Then, they were washed six times in T-TBS and incubated with primary antibodies at 4°C overnight. After that, membranes were washed six additional times and incubated with the appropriate biotinylated secondary antibody for 1 h at room temperature. Next, the membranes were washed with T-TBS for the last time and blots were visualized by chemiluminescence (Amersham Corp., Sydney, Australia) according to the manufacturer's instructions. Finally, the signal was imaged using an *In Vivo* F Pro molecular imaging system (Bruker Corporation, Billerica, MA, USA).

Stripping the PVDF membranes

To confirm an equal protein load, blots were stripped and re-probed with an antibody against β-tubulin and PSMA7 proteasome subunit (IPP control). For this procedure, 15 ml of stripping buffer, consisting of 2% (w/v) SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, was added to the membrane for 1 h with constant shaking at 60°C. The membrane was then washed six times for 10 min in TBS, blocked and probed with the primary antibody, as described above.

Measurement of proteasomal enzyme activity

To determine the influence of the SACY/cAMP/PKA pathway on the enzymatic activity of the sperm proteasome, motile spermatozoa were incubated with 50 µM H89, 100 nM KT5720, 25 µM KH7, 100 µM IBMX or 100 µM Sp-cAMPS for 0, 1, 15, 30 or 60 min at 37°C, 5% CO_2. Control aliquots were treated with the inhibitor solvent/vehicle solutions at equivalent volumes. H89, KH7 and IBMX were dissolved in 0.1% (v/v) DMSO. Sp-cAMPS was dissolved in water. KT5720 was dissolved in 0.01% (v/v) methanol. The chymotrypsin-like activity of the sperm proteasome was assayed using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-7-amido-4-trifluoromethylcoumarin (Suc-LLVY-AMC) (Morales *et al*., 2003). Briefly, 50 µl aliquots were incubated in a final volume of 995 µl of homogenization buffer for 15 min at 37°C, 5% CO_2, before adding 10 µM substrate. The assay was run at 37°C, and the fluorescence was monitored with excitation at 380 nm and emission at 460 in a Shimadzu 5301 spectrofluorometer (Kyoto, Japan).

Indirect immunofluorescence

Spermatozoa were fixed in 4% (v/v) paraformaldehyde for 15 min and washed twice by centrifugation with 0.1 M glycine in PBS. Then, the cells were permeabilized for 10 min with 0.1% Triton X-100 diluted in PBS and washed twice with PBS at 14000 g for 2 min. The final pellet was resuspended in PBS supplemented with 1% BSA (BSA-PBS, w/v) for 30 min. The samples were incubated overnight with rabbit polyclonal anti-PKA catalytic subunit antibody (dilution 1:50) and, after three washes with PBS, incubated overnight with mouse monoclonal anti-proteasome PSMA7 subunit antibody (dilution1:50). Samples were washed three times with PBS and incubated with chicken anti-rabbit conjugated to Alexa 594 antibody diluted 1:200 in BSA-PBS for 2 h at room temperature. Samples were washed three times with PBS and incubated with goat anti-mouse conjugated to Alexa 488 antibody diluted 1:500 in BSA-PBS for 2 h at room temperature. Finally, the samples were washed three times for 5 min with PBS and the coverslips were mounted with Dako Fluorescent Mounting Medium

and examined under a confocal microscope (Leica TCS SP8 SMD, Mannheim, Germany). A minimum of 400 cells were analysed in each sample. Controls using a secondary antibody alone were performed to assure specificity.

Statistical analyses

Data were assessed by the Kolmogorov–Smirnov test and were normally distributed. They were analysed by a one-way analysis of variance with Tukey's *post hoc* test. Densitometry analysis was performed using the image 2.0j software and normalized with regard to the internal control tubulin.

Quantitative results are presented as mean values+SEM. In all cases, results with $P < 0.05$ were considered as values with significant differences.

Results

The chymotrypsin-like activity of the sperm proteasome increases during capacitation

The chymotrypsin-like activity of the sperm proteasome started to rise after 5 min of capacitation (Fig. 1, $P < 0.01$), plateaued at 10 min and remained high for the remaining incubation time (Fig. 1, $P < 0.001$). Furthermore, spermatozoa incubated during different times in NCM medium did not show an increase in proteasome chymotrypsin-like activity (Supplementary Fig. S1).

Simultaneously, we evaluated changes in the sperm content of the proteasome during capacitation by western blotting (WB). The results indicate that there were no changes in the density of PSMA7 subunit band (Fig. 1), suggesting that the increase in proteasome activity was not due to changes in the sperm content of proteasomes.

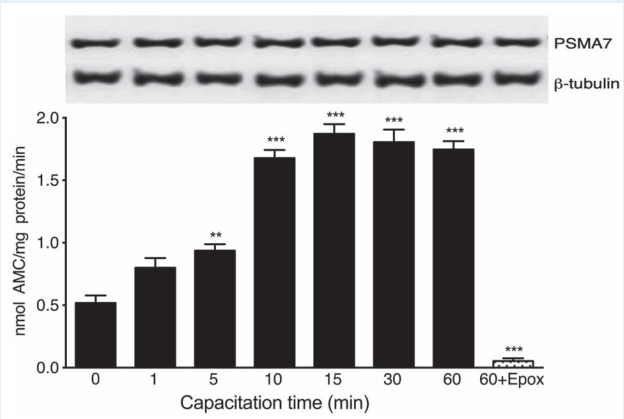

Figure 1 The chymotrypsin-like activity of the sperm proteasome increases at the onset of human sperm capacitation. Spermatozoa were incubated under capacitating conditions for the indicated time periods. The chymotrypsin-like activity of the sperm proteasome was evaluated using the fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC). An additional sperm aliquot was incubated under capacitating conditions for 60 min in the presence of 10 μM epoxomicin (60 + Epox). Data are expressed as mean ± SEM of five experiments. ** $P < 0.01$; *** $P < 0.001$ compared to time 0. In the insert, the upper panel represents the abundance of the PSMA7 subunit of the sperm proteasome at each capacitation time; the lower panel corresponds to the load control evaluated with an anti ß-tubulin antibody.

The increase in proteasome activity is required for sperm capacitation

We evaluated the capacitation status of human spermatozoa using the CTC and tyrosine phosphorylation assays by flow cytometry and WB. Non-capacitated spermatozoa (0) exhibited a basal level of cells with the B pattern (11 ± 1.1%) (Fig. 2A). When the spermatozoa were incubated under capacitating conditions for 60 min (60), a significant increase in pattern B was observed (34.4 ± 2.0%). In contrast, spermatozoa capacitated for 60 min in the presence of 10 μM epoxomicin (60 + Epox) did not exhibit an increase in capacitation B pattern ($P < 0.001$ versus capacitated control) (Fig. 2A). In regard to the dynamics of global sperm tyrosine phosphorylation (pY) during capacitation, the flow cytometry results show that the percentage of pY increased during capacitation in a time-dependent manner, from 10% after 60 min to 44% pY-positive spermatozoa at 300 min (Fig. 2B). However, spermatozoa capacitated in the presence of 10 μM epoxomicin exhibited a significant decrease in the percentage of pY during capacitation (24%). The mean ± SEM percentage of pY for six experiments is given in the Supplementary materials (Supplementary Fig. S2). In addition, we investigated the capacitation-induced changes in tyrosine phosphorylation of proteins by WB (Leclerc *et al.*, 1996; Lefièvre *et al.*, 2000; Kirkman-Brown *et al.*, 2002; Moseley *et al.*, 2005). Similar to what was observed by flow cytometry, protein tyrosine phosphorylation levels were low in spermatozoa incubated in the presence of a proteasome inhibitor (Supplementary Fig. S3). These results strongly suggest that the sperm proteasome has a role in early capacitation events.

The increase in sperm proteasome activity during capacitation is mediated by the SACY/cAMP/PKA pathway

It is known that proteasomal subunits undergo post-translational modifications, the most common being phosphorylation (Konstantinova *et al.*, 2008). Considering that activation of PKA is one of the first intracellular events of capacitation (Visconti, 2009), the next step was to evaluate if PKA was involved in the regulation of proteasomal activity. The results show that the treatment with 50 μM H89 (Fig. 3A) significantly blocked the increase in chymotrypsin-like activity of the sperm proteasome at all incubation time points ($P < 0.001$). Spermatozoa treated with a different PKA inhibitor, KT520, also failed to increase their proteasomal activity during capacitation (Fig. 3B, $P < 0.001$). These results suggest that PKA regulates the enzymatic activity of the sperm proteasome during capacitation.

Additional experiments were designed to determine if the upstream elements of PKA pathway are involved in increasing the chymotrypsin-like activity of the human sperm proteasome. The activation of PKA requires the second messenger cAMP (Nolan *et al.*, 2004) and the intracellular cAMP concentration is regulated by two opposite enzyme systems: SACY, which generates cAMP and phosphodiesterases (PDE), which degrade cAMP (Leclerc *et al.*, 1996). To test directly whether SACY modulates proteasome activity, spermatozoa were incubated with KH7, a specific small molecule inhibitor of SACY (Hess *et al.*,

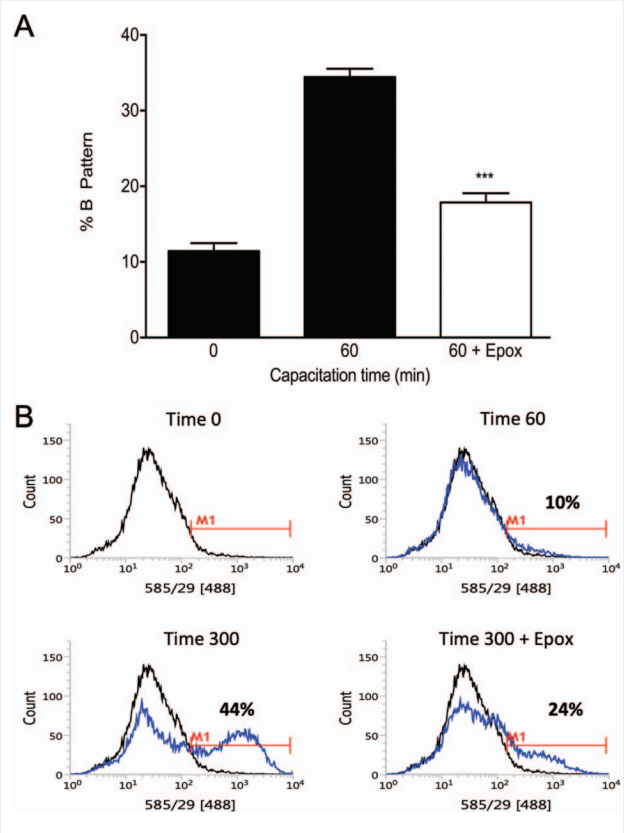

Figure 2 Effect of proteasome inhibition on human sperm capacitation. (**A**) Evaluation of sperm capacitation by the CTC assay. Spermatozoa were incubated for 0 (0) or 60 (60) min with 0.1% v/v DMSO or for 60 min with 10 μM epoxomicin (60 + Epox). Data are expressed as mean ± SEM, N = 7. ***$P < 0.001$ compared to 60 min without inhibitor. (**B**) Evaluation of sperm capacitation by tyrosine phosphorylation, assayed by flow cytometry. A representative histogram out of six replicates with consistent results is shown. Black lines represent the fluorescence distribution pattern of non-capacitated sperm suspensions (Time 0) and the blues lines represent the fluorescence distribution pattern of spermatozoa capacitated for 60 min (Time 60), 300 min (Time 300) and 300 min in the presence of 10 μM epoxomicin (Time 300 + Epox).

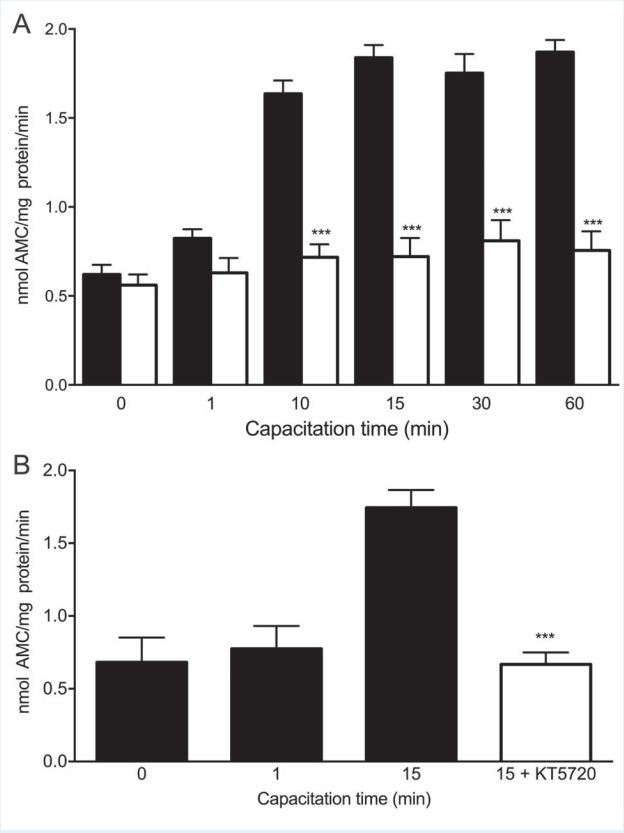

Figure 3 Effect of protein kinase A (PKA) inhibitors on the chymotrypsin-like activity of the sperm proteasome at the onset of human sperm capacitation. (**A**) Sperm aliquots were incubated under capacitating conditions for 0, 1, 10, 15, 30 or 60 min with 0.1% v/v DMSO (black bars) or with 50 μM H89 (white bars). Then, the chymotrypsin-like activity was evaluated using the fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC). ***$P < 0.001$ versus corresponding control, $N = 8$. (**B**) Other sperm aliquots were incubated under capacitating conditions for 0, 1 or 15 min with 0.1% v/v DMSO (black bars) or for 15 min with 100 nM KT520 (white bar). Chymotrypsin-like activity was evaluated using Suc-Leu-Leu-Val-Tyr-AMC. ***$P < 0.001$ versus corresponding control. $N = 5$. Data are expressed as mean ± SEM.

2005). When spermatozoa were incubated for 15 min with 25 μM KH7, the increase in proteasome activity was prevented (Fig. 4A; $P < 0.001$). In contrast, when spermatozoa were incubated for 15 min with the broadly specific PDE inhibitor IBMX, the chymotrypsin-like activity of the sperm proteasome was significantly enhanced (Fig. 4B; $P < 0.01$). These results suggest that the increase in chymotrypsin-like activity during early capacitation is mediated by an increase in sperm intracellular cAMP levels.

The importance of PKA in the modulation of proteasome activity during capacitation was corroborated using the PKA activator Sp-cAMPS. When spermatozoa were incubated for 15 min in the presence of Sp-cAMPS, the chymotrypsin-like activity of the sperm proteasome was significantly increased (Fig. 4C; $P < 0.001$). Finally, we carry out experiments designed to recover PKA activity in conditions that the SACY/cAMP/PKA pathway was inhibited (Fig. 4D). When spermatozoa were incubated in the presence of SP-cAMPS in NCM for 15 min, proteasome activity increased ($P < 0.001$). Likewise, when the spermatozoa were incubated in CM in the presence of SP-cAMPS and KH7, we observed significant increases in proteasome activity ($P < 0.001$).

Proteasome subunits are phosphorylated by PKA during human sperm capacitation

The next experiment was designed to determine whether proteasomal subunits are phosphorylated by PKA at the onset of capacitation. Spermatozoa were incubated under capacitating conditions for 0 and 15 min with 0.1% DMSO or for 15 min with 50 μM H89. To immunoprecipitate the proteasome, the sperm extracts were treated with an anti-PSMA7 proteasomal subunit antibody, as described in Materials and Methods. The precipitated proteins were tested on a western blot using an antibody against phosphorylated PKA substrates. Spermatozoa incubated for 15 min exhibited an increase in the band

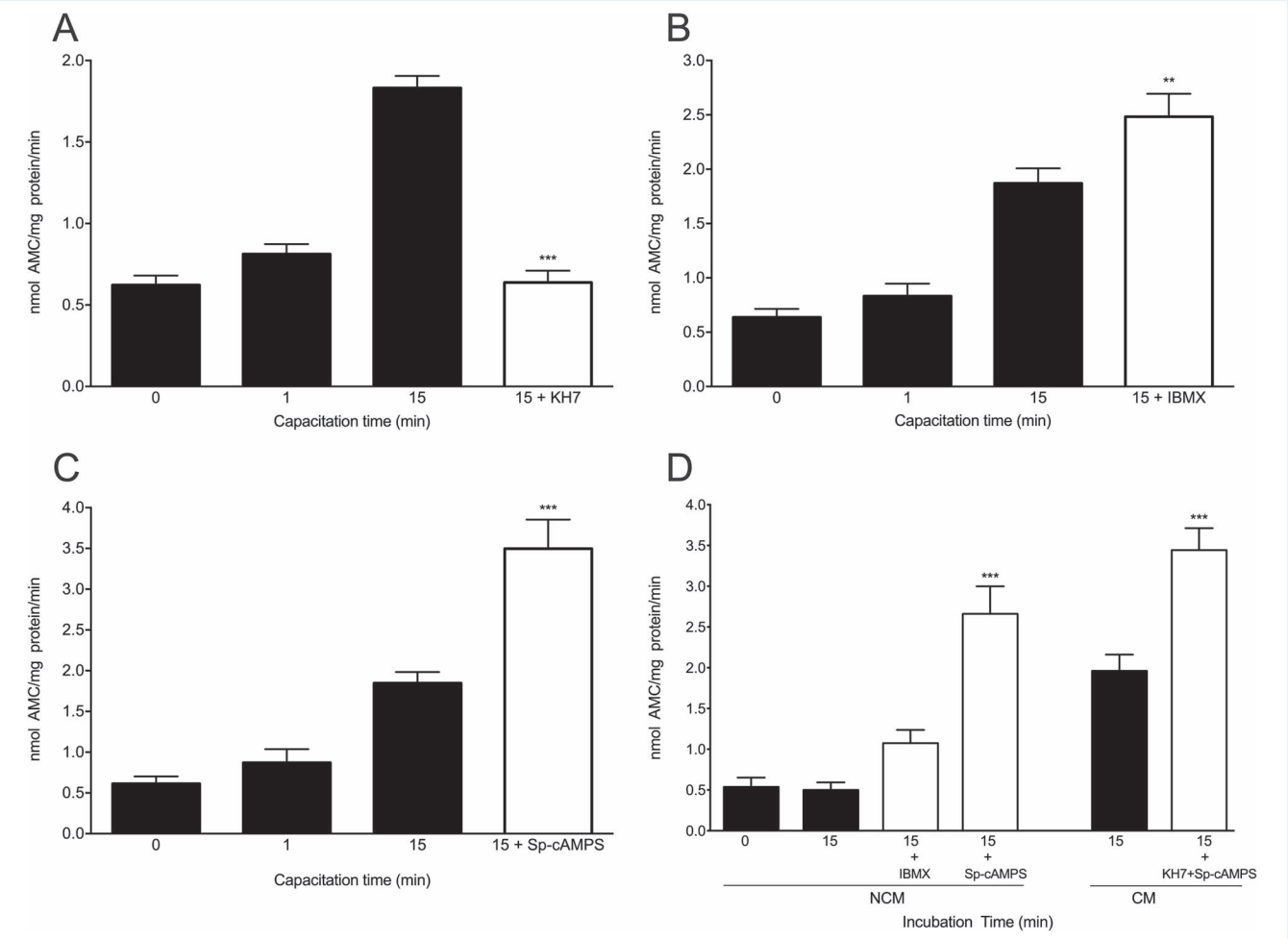

Figure 4 Effect of interference with the soluble adenyl cyclase/cAMP/protein kinase A (SACY/cAMP/PKA) pathway on the chymotrypsin-like activity of the sperm proteasome at the onset of human sperm capacitation. Spermatozoa were incubated under capacitating conditions for 0, 1 or 15 min with 0.1% v/v DMSO (black bars) or with (**A**) 25 μM KH7; (**B**, **D**) 100 μM IBMX; or (**C**, D) 100 μM Sp-cAMPS (white bars). (D) Other sperm aliquots were incubated simultaneously with 25 μM KH7 and 100 μM Sp-cAMPS for 15 min. The chymotrypsin-like activity of the sperm proteasome was evaluated using the fluorogenic substrate N N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC). Data are expressed as mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control.

density of proteasomal subunits phosphorylated by PKA (Fig. 5A; $P < 0.01$). However, spermatozoa treated with H89 did not increase their content of PKA phosphorylated proteasome subunits ($P < 0.01$). There were no differences between non-capacitated spermatozoa (0 min) and those capacitated for 15 min in the presence of H89. These changes were not due to an artefact or unequal protein loading, as demonstrated by the α4 proteasome subunit control (Fig. 5, bottom). As a negative control, lysis buffer and the antibody attached to the agarose used for IPP were incubated. As shown in Fig. 5, there was no artefactual cross-reaction.

Catalytic alpha subunit of PKA colocalizes with the proteasome complexes during sperm capacitation

Finally, we investigated if there is an association between the catalytic subunit alpha of the PKA (PKA-Cα) and the proteasome during human sperm capacitation. Immunofluorescence revealed that PKA-Cα was present in all spermatozoa analysed. The labelling of PKA-Cα was homogeneously localized in the principal piece, strong in the midpiece and absent from the sperm tail end piece (Fig. 6B). However, differences in the localization of PKA-Cα were observed in the sperm head. The average percentage of spermatozoa that showed PKA-Cα labelling in the head is shown in Supplementary Table SII. More than half of the spermatozoa presented labelling in the connecting piece of the tail and about a quarter of them presented labelling in the head equatorial segment. In addition, 20% presented labelling in the connecting piece of tail and in the equatorial segment (Fig. 6B). The antibody PKA-Cα specificity was demonstrated by western blot analysis (Supplementary Fig. S4). A single band is observed of an approximate molecular weight of 42 kDa.

Next, we evaluated the subcellular localization of the proteasomal subunit PSMA7. Immunofluorescence revealed that the said subunit and by extrapolation proteasomes were not present in the flagella of all spermatozoa evaluated. In the sperm head, differences in the localization of the proteasomes were observed (Supplementary Table SIII).

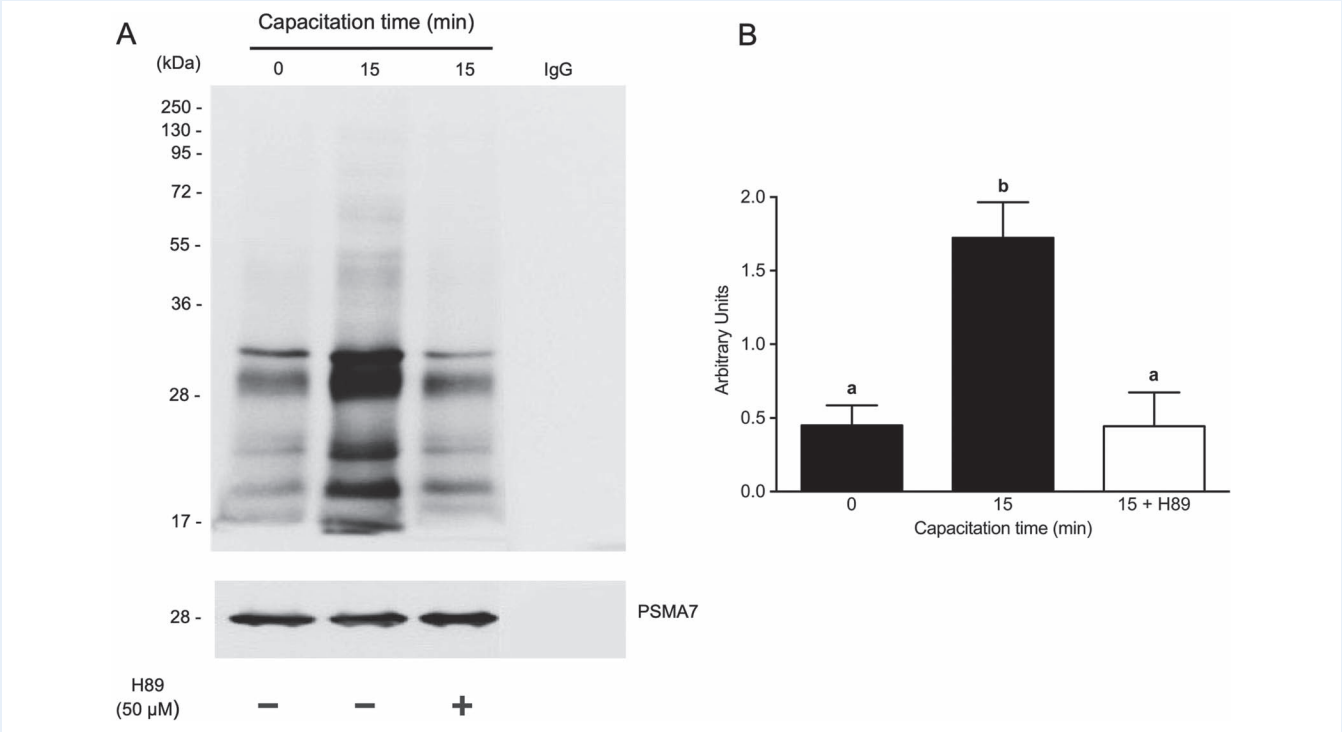

Figure 5 Protein kinase A (PKA)-dependent phosphorylation of proteasomal subunits at the onset of human sperm capacitation. (**B**) Spermatozoa were incubated under capacitating conditions for 0 or 15 min with 0.1% v/v DMSO or for 15 min with 50 μM H89. Then, the proteasome was immunoprecipitated with an anti-PSMA7 proteasome subunit antibody. The phosphorylation of proteasomal subunits by PKA was evaluated by western blots using an antibody against phosphorylated PKA substrates. (**B**) For the densitometric analysis, the PSMA7 subunit was used as a control. The bars represent the mean ± SEM of total signal from phosphorylated proteosome components. $N = 4$. Different letters indicate statistically significant differences ($P < 0.01$) between the groups.

Labelling was observed in the connecting-piece/implantation fossa, equatorial segment, acrosome and post acrosomal sheath. Finally, immunofluorescence revealed that PKA-Cα colocalized with the proteasome in the connecting piece and equatorial segment. However, only 21% of the spermatozoa evaluated after 15 min of capacitation showed evidence of colocalization between PKA-Cα and proteasome. As a negative control, trials with secondary antibody only were carried out (Supplementary Fig. S5).

Because PKA-Cα and the proteasome partially overlap in human spermatozoa, the next experiment was designed to examine possible molecular interaction between PKA-Cα and the proteasome. A co-IPP experiment revealed that PKA-Cα was successfully co-precipitated with the proteasome (Fig. 7A). The co-precipitation of PKA-Cα with the proteasome was significantly higher in spermatozoa capacitated for 15 min ($P < 0.01$) versus non-capacitated spermatozoa (time 0). It can be seen in the total cell extract that the content of PKA-Cα is constant during the time points evaluated. To rule out non-specific IPP, the blots were stripped and reprobed with an antibody against β-actin (Supplementary Fig. S6). The β-actin was detected only in the extracts and not in the immunoprecipites.

Discussion

In the present study, we report that the enzymatic activity of the sperm proteasome increases as early as 5 min after the onset of capacitation. This increase seems to be required for capacitation to occur since proteasome inhibition with the specific inhibitor epoxomicin significantly blocked capacitation. When we evaluated capacitation using the CTC assay, the percentage of spermatozoa with the capacitated B pattern significantly increased after 60 min of in vitro capacitation. These results are consistent with published work (Signorelli *et al.*, 2013; Martínez-León *et al.*, 2015). In contrast, spermatozoa capacitated in the presence of epoxomicin did not exhibit this increase, a pattern similar to that described by Kong *et al.* (2009). Protein tyrosine phosphorylation has been associated with capacitation and the acquisition of mouse sperm ability to undergo acrosomal exocytosis (Visconti *et al.*, 1995a, 1999a). Thus, we also evaluated sperm capacitation by measuring the level of protein tyrosine phosphorylation and detected a significant increase in the global phosphotyrosine content. These results, obtained by flow cytometry, were similar to those reported by Barbonetti *et al.* (2008). We also found that sperm incubation with epoxomicin significantly blocked this increase in tyrosine phosphorylation during capacitation. The observation that proteasome inhibition prevents sperm capacitation strongly suggests that the sperm proteasome has a role early in the capacitation process. Our preliminary evidence indicates that epoxomicin does not have a direct effect on PKA activity (Zapata-Carmona *et al.*, unpublished results).

Proteasomes are present in spermatozoa of numerous species and several studies have shown that human spermatozoa have all com-

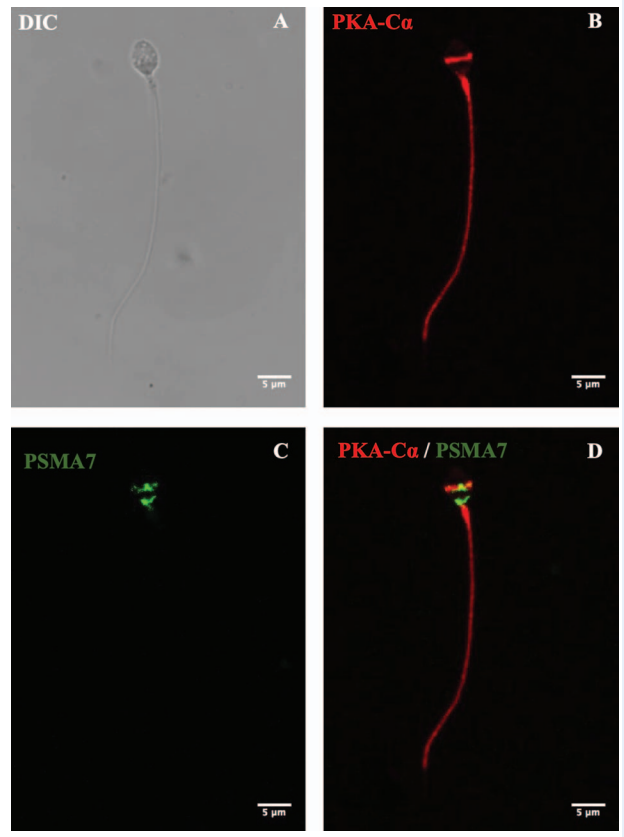

Figure 6 Subcellular localization of the catalytic subunit of protein kinase A (PKA) and its colocalization with the proteasome in human spermatozoa. Spermatozoa were incubated under capacitating conditions for 15 min. The cells were fixed and labelled with a PKA catalytic subunit primary antibody (red; [**B**]) and primary antibody against proteasomal subunit PSMA7 (green; [**C**]). DIC: differential interference contrast. Scale bar is 5 µm in all photos.

ponents/subunits of the 26S proteasome (Tipler *et al.*, 1997; Wojcik *et al.*, 2000; Morales *et al.*, 2003). Additionally, multiple ubiquitin specific proteases (Baker *et al.*, 2008) and ubiquitin conjugating enzymes E2 (Sutovsky *et al.*, 2000; Fischer *et al.*, 2005) and E3-ligases (Rodriguez and Stewart, 2007; Rivkin *et al.*, 2009; Zimmerman *et al.*, 2014) have been identified in mammalian spermatozoa. It is known that reproductive success of mammalian species requires precise orchestration of multiple events, all regulated through the cAMP dependent protein kinase A (PKA) pathway. It has been shown that PKA activity peaks at 1 min after the beginning of capacitation (Moseley *et al.*, 2005; Martínez-León *et al.*, 2015) and it has been referred as an 'earlier' event of capacitation. In addition, PKA inhibitors effectively prevent sperm capacitation *in vitro* (Kong *et al.*, 2009; Battistone *et al.*, 2013).

The present results indicate that the increase in proteasome activity takes place after 5 min of capacitation, giving support to the concept that proteasome activation is also an early event of sperm capacitation. In addition, the stable expression of the proteasome during capacitation suggests that the initial increase is related to a regulatory mechanism and not to the amount of proteasomes present. One possible mechanism involves post-translational modification of the proteasomal subunits. Phosphorylation is a well-studied post-translational modification (reviewed by Guo *et al.*, 2017). Under physiological conditions, the proteasome typically consists of two multi-subunit complexes, a 20S proteasome capped with one or two 19S regulatory particles. Both 20S and 19S multi-subunit complexes undergo phosphorylation to become active (Iwafune *et al.*, 2002; Kikuchi *et al.*, 2010). Here, we present evidence that two validated PKA inhibitors, H89 and KT5720, prevented the increase in proteasome activity at the beginning of capacitation. These results suggested that an increase in proteasome activity is necessary for capacitation to occur and that this increase seems to be mediated by PKA and its downstream elements. As a precedent, proteasome function is regulated by the PKA pathway in other cellular systems. It has been shown that PKA is capable of stimulating the proteolytic activity of the proteasome by phosphorylation of Ser120 of the Rpt6 subunit in NRK cell line. This phosphorylation of Rpt6 by PKA directly stimulates the chymotrypsin and trypsin-like activities of the 20S proteasome (Zhang *et al.*, 2007). Asai *et al.* (2009) showed that the exogenous or endogenous stimulation of PKA speeds up and enhances the assembly of the 26S proteasome and its enzymatic activity in canine heart cells. Metcalfe *et al.* (2012) reported a dibutyryl-cAMP stimulated proteasome activity and reduced prostaglandin-induced proteasome inhibition in cultured cortical neurons. They proposed that targeting cAMP/PKA to boost proteasome activity in a sustainable manner could offer an effective approach to avoid early accumulation of ubiquitinated proteins, possibly preventing/delaying Alzheimer's disease-associated neurodegeneration (Metcalfe *et al.*, 2012).

In this study, proteasome phosphorylation by PKA during sperm capacitation has been confirmed by activating PKA with Sp-cAMPS (direct effect) and IBMX (indirect effect through inhibition of PDE), or by using PKA inhibitors such as H89 and KT5720. Furthermore, we used the SACY inhibitor KH7. The results strongly suggest that SACY/cAMP/PKA pathway stimulates the chymotrypsin-like activity of the sperm proteasome at the onset of capacitation. To our knowledge, this is the first report that suggests that subunits of the sperm proteasome are phosphorylated by PKA during capacitation. The results obtained with the antibody that recognizes the PKA-phosphorylated substrates, showing that the subunits of the core 20S increased their phosphorylation level at the beginning of capacitation, support this conclusion. When the spermatozoa were incubated in the presence of H89, there was a decrease in the degree of PKA substrate phosphorylation. This observation suggests that PKA phosphorylation positively regulates the 20S proteasome. Kong *et al.* (2009) showed that the sperm proteasome was phosphorylated on Thr and Tyr residues in spermatozoa incubated for 18 h (late capacitation events) and that the observed phosphorylation was not confined to the 20S core, but to subunits of the proteasomal 19S regulatory complexes. These phosphorylations may also be related to acrosomal exocytosis or the initial acrosomal remodelling during sperm capacitation. Our results agree with reports in other cell types, where the activation of the proteasome is related to the phosphorylation of the 20S core subunits. Zong *et al.* (2006) reported that PKA phosphorylates in cardiomyocytes the α1 (PSMA6), α2 (PSMA2), α3 (PSMA4), β2 (PSMB7), β3 (PSMB3) and β7 (PSMB4) subunits of the 20S proteasome *in vitro*, increasing its chymotrypsin and PGPH

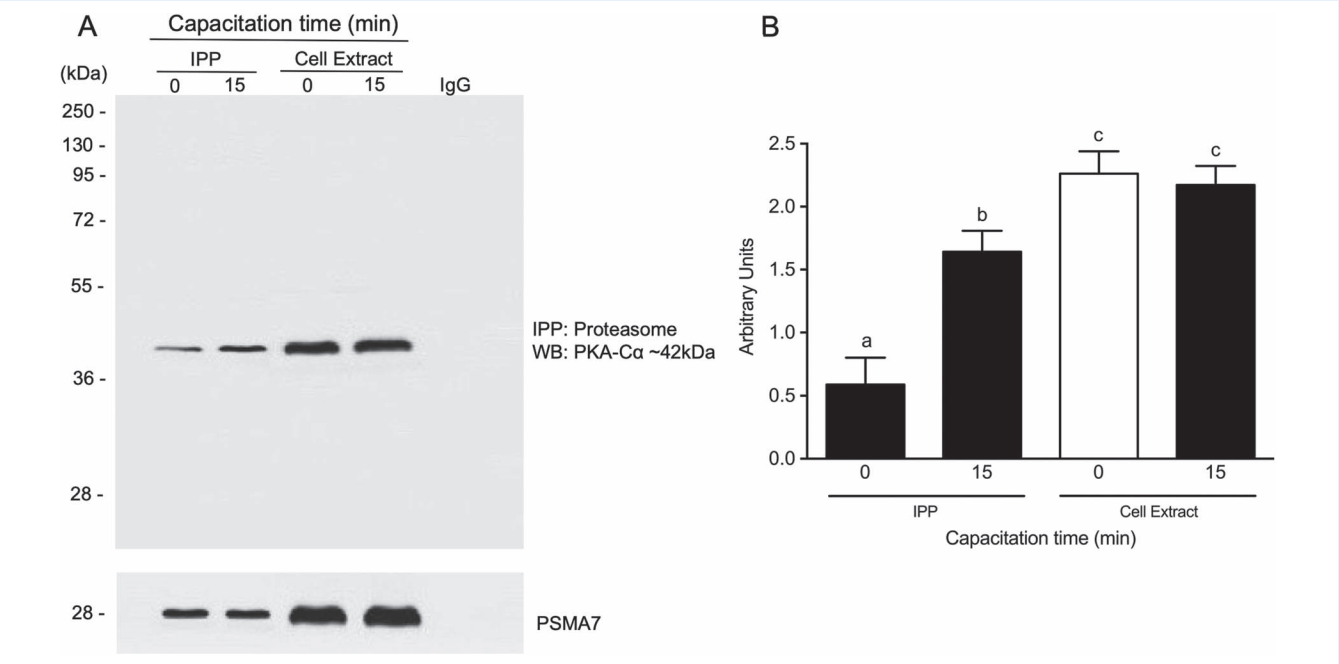

Figure 7 Association of the catalytic protein kinase A (PKA) subunit with proteasome during human sperm capacitation. (**A**) Co-immunoprecipitation (IPP) of the proteasome and the catalytic subunit of PKA. Spermatozoa were incubated for 0 or 15 min. After incubation, the spermatozoa were lysed in lysis buffer and immunoprecipitated overnight with an anti-PSMA7 proteasome subunit antibody. The PKA catalytic subunit was detected by western blotting (WB). Cell extracts correspond to the lysates of individual treatment groups. (**B**) For densitometry, the α4 subunit was used as a control. Bars represent the mean ± SEM of three different experiments. Different letters indicate statistically significant differences ($P < 0.01$) between the groups.

activities. This has also been demonstrated in human kidney cells, where purified PKA phosphorylated some 28–30 kDa subunits *in vitro*, leading to evident up-regulation of peptidase activity (Marambaud *et al.*, 1996).

The increase of cAMP levels and activation of PKA are considered early events of sperm capacitation. Upon activation, PKA induces the phosphorylation of target proteins on Ser/Thr residues. Our results indicate that the proteasome is a substrate for PKA at the beginning of the capacitation. The role of cAMP in the pathway conducive to sperm capacitation has been elucidated over the years by both biochemical and pharmacological approaches; increased cAMP levels promote the release of the PKA-Cα from the regulatory subunits, stimulating the activity of the kinase (Nolan *et al.*, 2004). It has been shown that PKA activation is a necessary element for sperm capacitation; animals lacking the PKA-Cα subunit are infertile, despite of normal mating behaviour (Nolan *et al.*, 2004). In the present work, we also report the localization of PKA-Cα in the subcellular compartments in human spermatozoa during capacitation. The subcellular localization of the PKA-Cα is consistent with published work in human sperm (Neuhaus *et al.*, 2006; Mitchell *et al.*, 2008). We show that PKA-Cα is readily detected in the spermatozoa and that there is a strong labelling in the midpiece part of the flagellum and in the equatorial segment of the sperm head. The subcellular localization of the proteasome that we report here agrees with observations of human spermatozoa reported by others (Wojcik *et al.*, 2000; Biały *et al.*, 2001; Morales *et al.*, 2004). The sperm proteasome is mainly located in the acrosomal and post-acrosomal head regions and in the tail connecting piece. We also show for the first time that PKA-Cα and the proteasome overlap in the same subcellular compartment. In addition, PKA-Cα interacts with the proteasome and this interaction may induce the increase in chymotrypsin-like proteasomal core activity at the beginning of capacitation. These results suggest a strong interaction between PKA-Cα and the proteasome during the early events of sperm capacitation. These findings agree with other cell models. For instance, it has been reported that the 20S proteasomes isolated from murine heart also contain PKA (catalytic) subunit as confirmed by WB and mass spectrometry, and the PKA-mediated *in vitro* phosphorylation enhances peptidase activities of 20S proteasomes isolated from both heart and liver (Zong *et al.*, 2006; Lu *et al.*, 2008).

In conclusion, our results indicate that PKA phosphorylation positively regulates the 20S proteasome during early stages of capacitation. Our current model (Fig. 8) indicates that at the onset of capacitation, the entry of HCO_3^- activates soluble adenyl cyclase (SACY) and increases the intracellular pH. The activation of SACY produces an increase in cAMP levels. The binding of cAMP to the regulatory subunits of PKA allows the dissociation of the tetramer and the activation of the catalytic subunit. Once free, the catalytic subunits remain active to phosphorylate a wide variety of substrates on Ser/Thr residues. Once active, PKA is responsible for phosphorylating multiple subunit of the proteasome, thus increasing its enzymatic activity. Finally, this proteasome

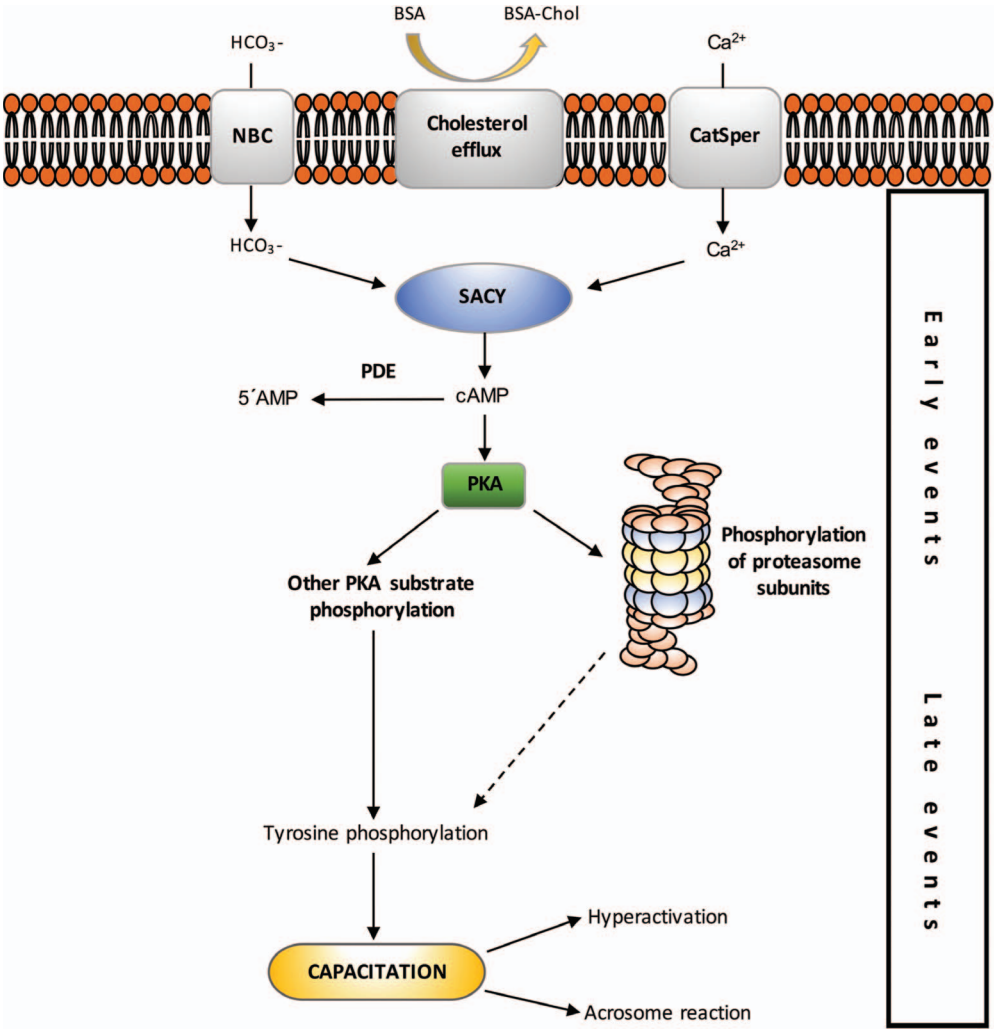

Figure 8 Proposed model of protein kinase A (PKA) and proteasome participation in sperm capacitation-signalling. The arrows represent stimulation. Dashed line indicates that the presence of a signal activating tyrosine phosphorylation by the proteasome has been proposed but the mechanism remains to be proven.

activation by PKA is necessary for regulation in the capacitation process.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

HZ-C performed the experiments, analysed the data, and wrote the manuscript. LB and EZD processed sperm samples, and LB performed the experiments and analysed the data. LZ and MK drafted the manuscript and prepared the figures. ESD designed the experiments. PM conceived and designed the experiments, performed the experiments, analysed the data and wrote the manuscript. PS advised LB in experimental design and co-wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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