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PKC ACTIVITY IN BOAR SPERM

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SUMMARY

Male germ cells undergo different processes within the female reproductive tract to successfully fertilize the oocyte. These processes are triggered by different extracellular stimuli leading to activation of protein phosphorylation. Protein kinase C (PKC) is a key regulatory enzyme in signal transduction mechanisms involved in many cellular processes. Studies in boar sperm demonstrated a role for PKC in the intracellular signaling involved in motility and cellular volume regulation. Experiments using phorbol 12-myristate 13-acetate (PMA) showed increases in the Serine/Threonine phosphorylation of substrates downstream of PKC in boar sperm. In order to gain knowledge about those cellular processes regulated by PKC, we evaluate the effects of PMA on boar sperm motility, lipid organization of plasma membrane, integrity of acrosome membrane and sperm agglutination. Also, we investigate the crosstalk between PKA and PKC intracellular pathways in spermatozoa from this species. The results presented here reveal a participation of PKC in sperm motility regulation and membrane fluidity changes, which is probably associated to acrosome reaction and to agglutination. Also, we show the existence of a hierarchy in the kinases pathway. Previous works on boar sperm suggest a pathway in which PKA is positioned upstream to PKC and this new results support such model.

INTRODUCTION

A competent status of mammalian spermatozoa is required for fertilization. Therefore these male germ cells undergo different processes within the female reproductive tract (motility, capacitation, hyperactivation and acrosome reaction) to successfully fertilize the oocyte. These processes involve changes that are triggered by different extracellular

stimuli such as bicarbonate and Ca^{2+}, which cause intracellular activation of soluble-adenylyl cyclase and cAMP/PKA, leading to stimulation of protein tyrosine phosphorylation (Signorelli *et al.* 2012). Thus, a post-translational modification of pre-existing proteins such as phosphorylation is mainly responsible for the acquisition of spermatozoa functional competence.

Protein kinase C (PKC) is a key regulatory enzyme in signal transduction mechanisms involved in many cellular processes (Nishizuka 1988; Steinberg 2015). Its Ser/Thr kinase activity is triggered by a variety of extracellular stimuli (Nishizuka 1988) and is dependent on calcium, phospholipids, and diacylglycerol. The PKC isozymes family comprises at least 11 different kinases which can be divided into three major groups, namely, classical (cPKCα, βI, βII, and γ), novel (nPKCγ, ε, η, θ, and μ), and atypical PKC (aPKCξ and i) (Breitkreutz *et al.* 2007). The presence of PKC in the mammalian sperm has been previously demonstrated for human (Rotem *et al.* 1990a), bull and ram (Breitbart *et al.* 1992). In addition, PKCδ isoform has been detected in boar spermatids in the seminiferous tubules (Shin *et al.* 1998), suggesting a role in spermatid development. In general, PKC activity has been related to the regulation of sperm motility in different species (Rotem *et al.* 1990b, a) and in the signal transduction pathway underlying acrosomal exocytosis (Lee *et al.* 1987; Rotem *et al.* 1992; O'Toole *et al.* 1996; Liu & Baker 1997).

Effects of the well-known PKC activator, phorbol 12-myristate 13-acetate (PMA) in spermatozoa include stimulation of acrosome reaction and, at high concentrations (15–20 μM), a significant decrease in motility and velocity of human sperm (Liu & Baker 1997). In boar sperm, PKC activity has been studied using different PKC inhibitors, such as Ro-32-0432, assigning a role for PKC in the intracellular signaling involved in motility (Bragado *et al.* 2010); and staurosporine, bismaleimide I and bismaleimide X,

suggesting PKC involvement in the control of sperm cell's volume (Petrunkina *et al.* 2007). Further, PMA effects studied in spermatozoa from this species showed that PMA effectively increases the Ser/Thr phosphorylation of substrates downstream of PKC (Teijeiro & Marini 2012) and one of these substrates was identified as AMPK, which is phosphorylated at threonine 172 (Hurtado de Llera *et al.* 2014). In order to gain knowledge about the sperm cellular processes regulated by protein phosphorylation induced by PKC activity, in this work we evaluate the effects of PMA on boar sperm parameters indicative of spermatozoa's functional state such as the lipid organization of plasma membrane, integrity of the acrosome membrane, sperm agglutination and motility. Moreover, we investigate the crosstalk between PKA and PKC intracellular pathways in spermatozoa from this species.

MATERIALS AND METHODS

Chemicals and Sources

H89 was from Calbiochem (Cat. 19-141. La Jolla, CA, USA). The phosphodiesterase-resistant cell permeable cAMP analogue, 8Br-cAMP (Cat. B5386), phorbol 12-myristate 13-acetate (PMA)(Cat. P1585) and DMSO (D2650) were from Sigma–Aldrich (St Louis, MO, USA). Ethidium homodimer-1 (Cat. E1169) and propidium iodide (Cat. P3566) from Molecular Probes (Leiden, The Netherlands). Tris/Glycine/SDS buffer (10 times concentrated) and Tris/Glycine buffer (10 times concentrated) were from Bio-Rad (Richmond, CA, USA). Hyperfilm ECL, nitrocellulose membranes and anti-rabbit IgG-horseradish peroxidase were from Amersham GE Healthcare (Buckinghamshire, UK). Enhanced chemiluminescence detection reagents were from Pierce (Rockford, IL, USA).

Sperm Incubation Media

Tyrode's complete medium (TCM) was used as spermatozoa's capacitating medium (Aparicio *et al.* 2005) and consisted of 96 mM NaCl, 4.7 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 20 mM HEPES (pH 7.45) and 3 mg/ml BSA. TCM was equilibrated with 95% O_2 and 5% CO_2. A variant of the TCM medium was made by omitting sperm stimuli such as $CaCl_2$, $NaHCO_3$ and BSA and was termed Tyrode's basal medium (TBM). All Tyrode's media were prepared on the day of use and maintained at pH 7.45 at 38°C with an osmolarity of 290–310 mOsm/kg.

Collection, washing and incubation of semen

Semen from Duroc boars (2–4 years old) was used. Animals were housed at a commercial insemination station (Tecnogenext, S.L, Mérida, Spain) and maintained according to Regional Government and European regulations. All boars were housed in individual pens in an environmentally controlled building (15–25°C) and received the same diet. Artificial insemination using preserved liquid semen from boars demonstrated their fertility. Fresh ejaculates were collected by the gloved hand technique, diluted in BTS extender (Minitüb, Tiefenbach, Germany) and stored at 17°C before use. In order to minimize individual boar variations, semen from up to 3 animals was pooled using ejaculates from a minimum of 19 boars in different combinations. Only ejaculates containing at least 80% of morphologically normal spermatozoa and 70% of motile sperm were used. Semen pools were centrifuged at 2000 ***g*** for 4 minutes, washed with PBS and placed in TBM or TCM medium. Samples of 0.5 ml containing 120×10^6 spermatozoa/ml were incubated at 38.5°C in a CO_2 incubator for different times. In order to minimize possible experimental variations, every condition/treatment studied was performed with the same semen pool. When necessary, a control with the

final concentration of the solvent (DMSO 0.1%) was included. In one set of experiments, samples of sperm diluted in BTS were incubated in the presence of 10 µM PMA as follows: 1) 17°C for 24, 48 and 96 h; 2) 17ºC for 24 h + 38,5ºC for 1 h; 3) 17º C for 48 h + 38,5ºC for 1 h, 4) 17ºC for 96 h + 38,5ºC for 1 h; 5) 38, 5 °C for 1 h. In other set of experiments, boar sperm samples were placed in TBM or TCM and treated as follows: 1) incubated for 1 h in the presence of 1 or 10 µM PMA, 2) incubated 1h in the presence of 1 mM of the cAMP analogue 8Br-cAMP, 3) incubated 1h in the presence of PKA inhibitor 50 µM H89, 4) pre- incubated 1h in the presence of 50 µM H89 and then incubated one more hour in the presence of 10 µM PMA and 5) pre-incubated 1h in the presence of 50 µM H89 and then incubated 1 h in the presence of 1 mM of 8Br-cAMP.

Assessment of sperm motility

Sperm motility was assessed using a CASA system (ISAS®, Proiser R+D, Paterna, Valencia, Spain) with a microscope equipped with a 10X negative-phase contrast objective and a stage heated at 38.5°C as described previously (Hurtado de Llera *et al.* 2012). In brief, 2 µl of sperm sample were placed in a pre-warmed counting chamber (Leja®, Luzernestraat, The Netherlands), sperm motility analysis was based on the examination of 25 consecutive digitalized images obtained from a single field and at least 400 spermatozoa per sample were analysed. Images were taken with a time lapse of 1s and objects incorrectly identified as spermatozoa were eliminated from the analysis. The evaluated motility parameters were: VCL (curvilinear velocity, in µm/s), VSL (straight-line velocity in µm/s), VAP (average path velocity, in µm/s), LIN (linearity coefficient in %), STR (straightness coefficient in %), ALH (amplitude of lateral head displacement in µm), WOB (wobble coefficient in %), BCF (beat cross frequency in Hz). Those spermatozoa with VAP<10µm/s were considered immotile,

while spermatozoa with a velocity ≥10μm/s were considered motile; spermatozoa motility was considered progressive (MP) when STR>80%. Spermatozoa with a VAP velocity >80μm/s were considered as rapid.

Flow cytometry analyses

Flow cytometry analyses were performed using a Coulter EPIC XL flow cytometer (Beckman Coulter Ltd.). The fluorophores were excited by a 200mV argon ion laser operating at 488nm. A total of 10,000 gated events (bases on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample with sample running rates of approximately 500 events/sec. Fluorescence data were collected in the logarithmic mode. The fluorescence values of peanut agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC), YoPro-1 and SYBR-14 were collected in the FL1 sensor using a 525nm band pass (BP) filter. Propidium iodide fluorescence was collected in the FL3 sensor using a 620nm BP filter, and M-540 fluorescence was collected in the FL2 sensor using a 575 nm BP filter. Flow cytometry data were analyzed using a FacStation computer and EXPO™ 32 ADC software (Beckman Coulter, Inc.).

Assessment of sperm viability

As described previously (Aparicio *et al.* 2007), fluorescent staining using the LIVE/DEAD Sperm Viability Kit (Leiden, The Netherlands) was used to assess porcine spermatozoa viability. Briefly, 5 μL of SYBR-14 (2 μmol/l) and 10 μL of PI (5 μmol/l) were added to 500 μL of diluted semen samples (30 x 10^6 cells/ml) in isotonic buffered diluent (Coulter Isoton II Diluent, Beckman Coulter, Inc., Brea, CA, USA) and incubated 20 min at 38°C. After incubation, cells were analyzed by flow cytometry, and the results were expressed as the average percentage of SYBR-14-positive and PI-negative spermatozoa ± SEM.

Assessment of spermatozoa outer acrosome membrane integrity

The outer acrosome membrane status of spermatozoa was assessed after staining the sperm with PNA-FITC (Sigma, Saint Louis, Missouri, USA), as a marker of outer acrosome membrane integrity, and PI (Waterhouse *et al.* 2004). Aliquots of 100 µl of each semen sample (30×10^6 cells/ml) were incubated at room temperature in the dark for 5 min with 1 µg/ml of PNA-FITC and 6 µmol/l of PI. Just before analysis, 400 µl of isotonic buffered diluent were added to each sample and remixed before flow cytometry analysis. After incubation, cells were analyzed and the results were expressed as the average of the percentage of PNA-positive and propidium iodide-negative spermatozoa ± SEM.

Evaluation of the degree of plasma membrane lipid organization of spermatozoa

As described previously (Martin-Hidalgo *et al.* 2011), fluorescent staining using the membrane probes merocyanine M540, as a lipid fluidity marker, and YoPro-1, as a marker of changes in plasma membrane permeability was performed to assess changes in the lipid architecture of spermatozoa plasma membrane. Briefly, aliquots of 100 µl of each semen sample (35 x 10^6 cells/ml) were diluted in 400 µl of isotonic buffer containing 75 nmol/l of YoPro-1, mixed and incubated at 38.5ºC for 15min. Then, M540 was added to each sample to a final concentration of 2 µmol/l, incubated for 2 min and remixed before flow cytometry analysis. The spermatozoa were categorized by labelling as follows: YoPro-1^+ shows non-viable spermatozoa, YoPro-1^-/M540$^+$ shows viable cells with plasma membrane scrambling. The M540$^+$ staining in spermatozoa can be divided in either low or high, representing low and high plasma membrane scrambling, respectively. Results referred to membrane scrambling are expressed as the average of the percentage of YoPro-1^-/M540$^+$ spermatozoa ± SEM.

Assessment of sperm head-to-head agglutination.

We considered that spermatozoa are agglutinated when these male cells meet the following requirements detected by CASA system: head-to-head aggregation; maintain vigorous flagellum beating; maintain motility, especially progressive motility of small aggregates of sperm (Supplementary video). In order to quantify sperm agglutination, we proceeded to measure the area of the particles formed by agglutinated sperm. Since the observation by CASA system seemed to indicate that agglutinated sperm form bigger particles upon increasing PMA concentration and considering that particles are also observed in TCM medium, we measured the area of the agglutinated sperm particles by ImageJ, a public domain Java image processing program created by the National Institutes of Health (Schneider *et al.* 2008). This software allowed measuring the maximal and minimal area of the particles in micrographs using arbitrary units and then comparing these data statistically.

Western blotting

Spermatozoa were treated with 8Br-cAMP or PMA in TBM for 1h and then centrifuged 30s at 7000 **g**, washed with phosphate buffered saline (PBS) supplemented with 0.2 mmol/l Na3VO4 and lysed in a buffer consisting of 50 mmol/l Tris/HCl, pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 1% deoxycholate, 1 mmol/l EGTA, 0.4 mmol/l EDTA, protease inhibitors cocktail (Complete, EDTA-free, Cat. P8340, Sigma–Aldrich, St Louis, MO, USA), 0.2 mmol/l Na3VO4, and 1 mmol/l PMSF by sonication for 5s at 4°C. After 20 min at 4°C samples were centrifuged at 10,000 **g** (15 min, 4°C) and the supernatant (lysate) was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blotting was performed as previously described (Teijeiro & Marini 2012) using anti-phospho-PKA Substrate (RRXS*/T*)(Cat # 9624)

and anti-Phospho-PKC substrate (Cat # 2261) polyclonal antibodies (Cell Signaling Technology Beverly, Massachusetts). Mouse monoclonal anti-α tubulin antibody was supplied by Santa Cruz Biotechnology, Inc (CA) (Cat sc-8035).

Statistical analysis

The mean and standard error were calculated for descriptive statistics. The effect of treatments on spermatozoa variables was assessed by analysis of variance (ANOVA). When F-test results were significant in ANOVA, individual means were further tested by Tukey's multiple range test (Motulsky, 1995). To analyze the percentage of motile and rapid spermatozoa we used the Pearson Chi-square test. All analyses were performed using SPSS v11.0 for MacOs X software (SPSS Inc. Chicago, IL). The level of significance was set at $p<0.05$.

RESULTS

PMA-induced PKC activity does not affect boar sperm viability.

We initially assayed possible secondary effects of the PKC activator PMA that could lead to sperm death. Boar spermatozoa were incubated for 1 hour at 38.5°C with different concentrations of PMA, ranging from 1 to 10 µM and cell viability was evaluated by flow cytometry using SYBR-14 and PI as probes. Although nanomolar concentrations of PMA have been shown to exert effects in various human blood cell lines as HL-60 (Aihara *et al.* 1991) and K562 (Murray *et al.* 1993), previous experiments using boar sperm demonstrated that at these concentrations only a slight increase in serine and threonine phosphorylation is noticed (Teijeiro & Marini 2012). Furthermore, micromolar concentrations of PMA showed significant changes in experiments using human (Liu & Baker 1997) or boar sperm (Teijeiro & Marini 2012; Hurtado de Llera *et al.* 2014). Therefore, micromolar concentrations of PMA were used in this study. As seen in Figure 1A, no significant reduction on sperm viability was

observed after incubation with 10 μM PMA, the highest concentration used, neither in unstimulating (TBM) nor stimulating (TCM) medium.

PKC activity induced by PMA regulates the lipid organization of sperm plasma membrane. Changes in fluidity of sperm plasma membrane due to lipid disorganization are associated to important sperm processes as capacitation, agglutination and/or acrosome reaction, which are triggered by different sperm stimuli. Therefore, we first investigated whether PKC activation is involved in the lipid disorganization of boar sperm plasma membrane that occurs under different extracellular spermatozoa conditions. Boar spermatozoa were incubated (1 h) at physiological temperature (38.5°C) with PMA (10 μM) under unstimulating (TBM) or HCO_3^- and Ca^{2+}-stimulating medium (TCM). Lipid organization of plasma membrane was assessed by flow cytometry using merocyanine 540 as probe. As expected, due to calcium and bicarbonate's presence, basal membrane disorganization in TCM medium is higher than that on TBM (Fig. 1B). Moreover, as seen in Figure 1B, PKC activity induced by PMA significantly increased the lipid disorganization of the sperm plasma membrane independently of the media of incubation, as it occurs in approximately the same magnitude (2-fold) either in TBM or TCM.

PMA-induced PKC activity deteriorates acrosome membrane integrity in boar sperm. Although investigated previously (Teijeiro & Marini 2012), the effect of PMA treatment on acrosome membrane integrity in boar spermatozoa was further evaluated in this work using flow cytometry and PNA-FITC as probe, a more sensitive technique that allows screening a bigger amount of sperm cells. Since TCM is a capacitating medium and may produce spontaneous acrosome reaction, mainly to the presence of calcium, an expected increase in acrosome disorganization in TCM respect to TBM medium was observed (Fig. 1C). Moreover, PKC activity induced by 10 μM PMA

during 1 h incubation at 38.5°C significantly increased the percentage of PNA^+ live spermatozoa regardless of the incubation medium (Fig. 1C). This is indicative of alteration on acrosome membranes.

The increase in PKC activity induced by PMA leads to head-to-head boar sperm agglutination.

Observation of boar spermatozoa after PMA treatment in the microscope fields captured by the CASA system indicated head-to-head agglutination (Figure 2). Surprisingly, those agglutinated spermatozoa formed bigger particles at greater PMA concentration, showing progressive motility with an intense flagellar beating (see video in supplementary material). Therefore, we decided to investigate this sperm agglutinating effect of PMA and to study whether sperm agglutination is modified by different environmental conditions as temperature (physiological, 38.5°C or semen preservation, 17°C), incubation media (unstimulating, TBM or stimulating, TCM), PMA concentration (0.3-10µM) and time (5-60 min at 38.5°C or 24-96 h at 17°C). As seen in Figure 2, the sperm agglutinating effect induced by PKC activation at boar physiological temperature occurred either in TBM (Fig. 2A) or TCM medium (Fig. 2B), although it was clearly higher and faster in the HCO_3^- and Ca^{2+}-containing medium. Despite the incubation media, the sperm agglutinating effect was dependent on the time of incubation and PMA concentration. Thus, spermatozoa incubated with PMA in TBM showed visible agglutination at 30 min with 0.3 µM, which was higher at 1 and 10 µM of PMA. Moreover, sperm incubated in TCM medium (Fig. 2B) showed visible PMA-induced head-to-head agglutination as fast as 5 min, which increased presenting the highest agglutination at 60 min, again showing a clear PMA concentration effect.

To analyse the sperm agglutinating effect of PMA during boar sperm conservation at 17°C, seminal doses prepared in BTS extender were incubated for several days (24-96

h) with 10μM PMA. In the absence of PMA, no visible sperm agglutination was observed up to 96 h of semen preservation (Fig 3). However, PMA clearly induced sperm agglutination, which was appreciable at 24h and increased towards 96h of semen preservation. To mimic the variation of temperature inherent to artificial insemination techniques in this species, semen preservation at 17°C during different times was followed by 1h treatment at the physiological temperature of 38.5°C. Under these artificial insemination-mimicking conditions, PMA treatment showed sperm agglutination at the same degree than at 17ºC (Fig. 3).

In order to quantify the effect of PMA on sperm agglutination, micrographs were analysed using ImageJ software. As shown in table 1, the area of the particles, which augmented with the number of agglutinated sperm, increased with PMA concentration and with time. Also, the effect was greater in TCM than in TBM medium. In figure 4 an example of the imaging processing is shown.

Effect of the increase in PKC activity induced by PMA on boar sperm motility.

Analysis of motility parameters evaluated with CASA system after treatment of boar spermatozoa with 1 and 10 μM PMA for 60 minutes was done. As shown in Table 2 there was a significant effect of PMA, with an increase in the percentage of static sperm and a decrease in the percentage of rapid spermatozoa (VAP>80μm/s) and straight-linear velocity (VSL) at 10 μM PMA, either in TBM or TCM medium. Also, a decrease in curvilinear velocity (VCL) and in the percentage of average velocity (VAP) in sperm treated with 10 μM PMA was observed, but only in TCM medium. Linearity index (LIN=VSL/VCL), straightness index (STR=VSL/VAP), oscillation index (WOB=VAP/VCL), amplitude of lateral head displacement (ALH), beating frequency (BCF), and progressive motility (spermatozoa showing more than 80% of STR) were not affected by PMA treatment either in TBM or TCM medium.

Effect of cAMP/PKA pathway in the lipid disorganization of sperm plasma membrane produced by PMA-induced PKC activity.

To further investigate the intracellular pathway by which PKC leads to a modification of lipid organization on boar sperm plasma membranes, we evaluated membrane fluidity by flow cytometry after sperm incubation with PMA in the presence of the PKA inhibitor H89. As a positive control of the experiment we included a sample of boar sperm in TBM treated with a non-hydrolysable cAMP analogue, 8Br-cAMP.As expected, this cAMP analogue potently increased the lipid disorganization of the membrane, and the effect was partly blocked in the presence of the PKA inhibitor H89, confirming the action of the inhibitor. As shown in figure 1B and in figure 5, a significant increase in lipid disorganization of the plasma membrane was detected in sperm samples treated with PMA either in TBM or TCM media. Interestingly, this inhibitor showed divergent effects depending on the conditions in which sperm were incubated. Under non-capacitating conditions in TBM, it slightly but significantly ($p<0.05$) increased plasma membrane lipid reorganization regardless of the presence of PMA, whereas under capacitating conditions H89 had no effect on its own, but prevented the stimulating effect of PMA (Fig. 5, TCM+H89+PMA).

Effect of PMA-induced PKC and 8Br-cAMP-induced PKA activities on protein phosphorylation.

We next evaluated the changes in the pattern of sperm phosphorylated proteins induced by PKA or PKC stimulated activity using 8Br-cAMP (1 mmol) and PMA (10 μM), respectively, in TBM medium. As seen in figure 6, stimulation of boar spermatozoa with 8Br-cAMP lead to a potent increase in the phosphorylation of sperm proteins recognized with anti-PKA substrate antibodies (Fig. 6A. Lane 2) and also to a clear increase in the phosphorylation of sperm proteins recognized with anti-PKC substrate

antibodies (Fig. 6B. Lane 2). As expected, stimulation of boar spermatozoa with PMA lead to a clear increase in the phosphorylation of sperm proteins recognized with anti-PKC substrate antibodies (Fig. 6B. Lane 3), however, PMA stimulation did not induce any change on the phosphorylation pattern of sperm proteins recognized with anti-PKA substrate antibodies.

DISCUSSION

Protein kinase C comprises a family of serine- and threonine-specific protein kinases and is considered as the major phorbol ester receptor (Parker *et al.* 1986). These enzymes, in general, can be activated by DAG, one of the products of phosphatidylinositol turnover. Permeable synthetic DAG (OAG) and phorbol 12-myristate 13-acetate (PMA) are known to activate PKC (Nishizuka 1984). Immunohistochemical analyses of human spermatozoa using both light (Rotem *et al.* 1990b, a) and electron microscopy (Kalina *et al.* 1995) indicate the presence of isoforms of PKC in both the head and tail. While PKCδ is detected in boar and bull spermatids (Shin *et al.* 1998), the presence in mouse spermatozoa is inferred by using PMA (Lee *et al.* 1987). Although nanomolar amounts of PMA are used for PKC induction in experiments using human blood cell lines (Aihara *et al.* 1991) experiments with sperm from different animals require micromolar amounts of the inducer (O'Toole *et al.* 1996; Liu & Baker 1997).

In previous works, we demonstrated that PKC activation by PMA on boar sperm produces protein phosphorylation/dephosphorylation in the specific motif recognized by PKC, increases phosphorylation at tyrosine residues (Teijeiro & Marini 2012), and produces specific phosphorylation of boar sperm AMPK at threonine 172 (Hurtado de Llera *et al.* 2014), with a maximum of phosphorylation at 10 μM PMA. It is interesting

to note that although 0.3 μM PMA did not cause the phosphorylation/dephosphorylation of all the proteins that change the phosphorylation state at 1 μM PMA (Teijeiro & Marini 2012), it caused sperm agglutination in a medium lacking calcium and bicarbonate (Fig. 2). Since PKC is involved in lipid's signaling pathways, we thought that one obvious target of PKC activation would be the plasma membrane. Thus, in this work we analyzed the effect of activation of PKC through PMA treatment on sperm plasma membrane fluidity. The fluidity of the sperm plasma membrane, evidenced by using merocyanine and flow cytometry, is increased by PMA treatment (Fig. 1B) suggesting that PKC is involved in this process; also, such plasma membrane fluidity is enhanced in TCM compared with TBM, in coincidence with previous reports supporting membrane disorganization by capacitating agents such us calcium and bicarbonate (Flesch *et al.* 2001; Gadella & Van Gestel 2004). As expected, membrane acrosome integrity is also affected by those components (Purohit *et al.* 1999; Visconti *et al.* 1999; Breitbart 2002; Ickowicz *et al.* 2012), but enhanced in each medium by PMA treatment. As an interconnection between PKA and PKC in boar sperm signalling pathway has been suggested (Bragado *et al.* 2010), we also studied the effect of H89, a PKA inhibitor, on plasma membrane fluidity. Noteworthy, the obtained results suggest the presence of a PKA-dependent and calcium-independent mechanism/pathway involved in the control of the fluidity of boar sperm plasma membrane (see fig. 5). In line with these findings, H89 was able to block the effect of the PKC activator PMA in a calcium dependent manner (Fig. 5. TCM+H89+PMA). However, this effect was not observed in calcium depleted media. This result suggests that PKA lies upstream of PKC in the boar sperm intracellular pathway, supporting a previous work by us (Bragado *et al.* 2010), and that it is also involved in lipid organization of the plasma membrane. Interestingly, PKC-stimulated membrane fluidity is higher when calcium is

present in the medium, showing that the canonical calcium pathway involved in membrane fluidity is also present and functional (Fig. 5). In addition to calcium, BSA and bicarbonate in the capacitating medium are well-known to affect membrane fluidity either directly or indirectly through activation of sperm adenylyl cyclase and increase in intracellular cAMP concentration in species such as mouse and human (Wang *et al. 2007;* Battistone *et al.* 2013). Phorbol esters have been reported as capable of initiating acrosomal exocytosis in capacitated human sperm (De Jonge *et al.* 1991; Rotem *et al.* 1992) and in zona-induced mouse sperm (Lee *et al.* 1987); as well as of producing early events leading to acrosomal exocytosis in bull (Lax *et al.* 1997). These previous data seem to indicate that there is a plasma membrane preparation for acrosomal reaction that could be initiated by PKC activation, and that an increase in membrane fluidity may be an early event necessary for this process. More recently, it was found that MARCKS protein is phosphorylated by PKC during human acrosomal exocytosis (Rodriguez Pena *et al.* 2013) supporting the role of PKC in acrosomal exocytosis preparation. Also, PKC activity has been involved in early events leading to acrosomal exocytosis in other mammals, and a more recent work demonstrated that PMA enhances ZP-induced AR in human sperm (Liu *et al.* 2013). Despite our previous results (Teijeiro & Marini 2012), which showed no effect of PMA on boar sperm acrosome reaction, here we demonstrate a slight but significant effect of PMA on acrosome reaction, detected by a more sensitive and exhaustive technique, flow cytometry. This increase in AR may be related to an increase in membrane fluidity and might be the product of further manipulation and transit through the cytometer instead of true acrosome reaction.

Previous studies on boar spermatozoa motility performed using PKC inhibitors indicate that PKC activity is related to the motility process (Bragado *et al.* 2010). In addition, other study demonstrates that PMA stimulation contributes to the activation through

phosphorylation of boar sperm AMPK, a cell energy sensor kinase involved in sperm motility (Hurtado de Llera *et al.* 2014). In this work we find that the effect of PKC activation increases the percentage of static sperm and reduces the percentage of rapid spermatozoa, VAP>80 µm/s. Also, parameters such as VCL and VAP are reduced. This is in line with the results previously reported by us, where PMA induces phosphorylation of threonine 172 of AMPK (Hurtado de Llera *et al.* 2015), indicating that phosphorylation of AMPK at threonine 172 is related to reduction of sperm motility. Remarkably, reduction in motile sperm parameters, VCL and VAP, upon PMA treatment is observed by Hurtado de Llera and coworkers (2014), and the same effect is observed in this study. Taking into account that phosphorylation of AMPK at threonine 172 contributes to lipids disorganization (Hurtado de Llera *et al.* 2014) and affects sperm motility parameters, and that phosphorylation at this residue is enhanced when spermatozoa are incubated with 10 µM PMA, it is possible to link phospho-Thr^{172}-AMPK to PKC activity, to achieve the sperm cellular functions described.

The most notorious cytological effect of PKC activation with PMA is head-to-head agglutination with permanent flagellar motility, which provides clusters of sperm in motion (see video in supplementary material). Previous works have shown that sperm head-to-head agglutination occurs between spermatozoa with intact plasma acrosome membranes (Yang *et al.* 2012; Leemans *et al.* 2016). Moreover, Harayama and coworkers (2003) showed that boar sperm agglutination is promoted by bicarbonate and PKA activation. In addition, they concluded that cytoplasmic free Ca^{2+} is involved in sperm head-to-head agglutination and that agglutination is not the resultant of acrosome reaction (Harayama *et al.* 2003). Sperm head-to-head agglutination has been proposed to be an early stage of the capacitation process *in vitro* (Harayama *et al.* 2000), which physically prevents sperm from binding to the oviduct epithelium (Leemans *et al.*

2016). It is to note that flagellar activity is maintained, suggesting that the fluidity of the flagellar plasma membrane is not affected. Thus, we interpret this boar spermatozoa head-to-head agglutination as a product of an increase in membrane fluidity specifically in the head of sperm as results of stimulation by a PKC-mediated mechanism. This conclusion is based on the following: 1) head-to-head agglutination is PMA dose and time-dependent 2) increases in sperm membrane fluidity and head-to-head agglutination are clearly higher and faster in a HCO_3^- and Ca^{2+}-containing medium and 3) PKC stimulation by PMA causes a clear and significant increase in both sperm membrane fluidity and head-to-head agglutination in a non-capacitating medium without calcium and bicarbonate (TBM) (Fig. 1B and 2);.

The last point suggests the existence of a calcium-independent pathway involved in membrane fluidity, the participation of a PKC isoform such as δ, ε, η or θ, which contain C2 domains that do not recognize calcium, can be suspected.

As sperm agglutination is a common event during manipulation of sperm and is a problem during artificial insemination procedures; we decided to investigate the possible effect of PKC activation on boar sperm in extender solution. We found that activation of PKC on boar sperm elicits head-to-head agglutination in extender either at 17°C or 38.5 °C. This result is important as it provides a possible explanation to this phenomenon, which is observed during semen handling. A calcium-independent agglutination pathway has been inferred by previous experiments, given that the BTS extender, which is also used in this work, contains calcium depleting EDTA and bicarbonate (Pursel & Johnson 1975), and our results are in accordance with that preliminary conclusion. However, evaluation of PMA stimulation for 96 h may have caused down-regulation of PKCs and the results may be due to DAG- dependent PKCs inhibition. Alternatively, the treatment may have unmasked DAG-independent PKCs,

which were now amplified and may have induced the effect. More experiments are necessary to clarify this point

Finally, when boar sperm are incubated with 8Br-cAMP in TBM, an expected effect on protein phosphorylated at motifs recognized by PKA is observed, however no effect is seen on PMA treated sperm (Fig. 6A). Surprisingly, when anti-phospho-PKC substrate antibodies are used, a notorious effect on sperm treated with 8Br-cAMP is observed. This suggests that there is a hierarchy in the kinases pathway and reinforces the hypothesis of previous works on boar sperm that suggest a pathway in which PKA is positioned upstream of PKC (Bragado *et al.* 2010).

Taken together, the results presented in this work demonstrate pleiotropic effects of PKC on boar sperm affecting motility and membrane fluidity.

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Teijeiro, Juan and Garcia-Marin, Luis performed the research and were involved in the study conception and design, data analysis and interpretation, and in manuscript writing. Marini, Patricia & Bragado Julia analyzed the data and wrote the paper.

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Figure Legends.

Figure 1. A-Effect of PMA-induced PKC activation on boar sperm viability. Five hundred microliters containing 60×10^6 spermatozoa from semen pools were incubated for 1 hour at 38.5°C in TBM and TCM, and in both media with the addition of different concentrations of PMA, ranging from 1 to 10 μM. Viability was assessed by flow cytometry detection of SYBR-14-positive and propidium iodide-negative spermatozoa. Viability is expressed as percentage of viable sperm. For PMA treatment, only the results for the higher concentration used are shown, TMB+PMA and TCM+PMA correspond to 10 μM PMA. T0 corresponds to sperm from pools, before incubation in any media. Each value corresponds to the average of 8 replicates. No significant differences were found ($p<0.05$). **B-Effect of PMA-induced PKC activation on plasma membrane lipids organization.** After treatment with 10 μM PMA sperm were analysed by flow cytometry using merocyanine 540 as probe. As shown in the graphic, an increase in membrane disorganization on sperm treated with PMA was observed in either TBM or TCM medium compared to the control. (*) indicates significant differences. Each value corresponds to the average of 8 replicates. **C- Effect of PMA-induced PKC activation on boar sperm acrosome reaction.** PKC activity was induced in sperm from semen pools by incubation in TBM or TCM media with 10 μM PMA, and acrosome status was assessed by staining with PNA, followed by flow cytometry detection. Incubation in TCM and in TBM devoid of PMA was used as control. (*) indicates slight but significant increase of the percentage of acrosome-reacted spermatozoa in capacitating TCM medium (n=8).

Figure 2. PMA-induced PKC activation causes head-to-head sperm agglutination in TBM and TCM. PKC activity was induced in sperm from semen pools by incubation in TBM (A) or TCM (B) media with 0.3, 1 and 10 μM PMA, at different incubation times, indicated in the figures. Samples (n=8) were observed with negative

phase contrast, at 100x magnification. TBM and TCM without PMA addition were used as controls. Scale bar represents 50 μM

Figure 3. PMA-induced PKC activity causes head-to-head sperm agglutination on BTS extender. Pools of fresh ejaculates were diluted and maintained in BTS extender or BTS with 10 μM PMA, at 17 ºC for up to 96 hours. Also, after incubation, as indicated in the figure, samples were further exposed to 38.5 ºC for 1 hour to mimic the temperature change suffered upon insemination. Samples (n=8) were observed with negative phase contrast at 100x magnification at the times indicated in the figure. Scale bar represents 50 μM

Figure 4. Example of image processing using ImageJ. A tool for particles analysis provided by Image software enables to measure particles formed by head-to-head agglutination. An example is provided, showing the recorded numbers and the delimitated areas generated by the software (C and D), from images captured by CASA of a control (A) and a sperm sample treated with 10 μM PMA in TCM**.** Scale bar represents 50 μM

Figure 5. Effect of PMA-induced PKC activation and PKA inhibition over changes in plasma membrane lipids organization. Sperm pools were incubated in TBM or TCM media, and in the presence of PMA, PKA inhibitor H89 or both. As a control, sperm were also incubated with 8Br-cAMP, and preincubated 30 minutes with H89 prior 8Br-cAMP. Membrane lipid organization was estimated using merocyanine 540 as probe, and flow cytometry technique. a,b,c,d,e,f indicate significant differences, $p<0.05$ n=8

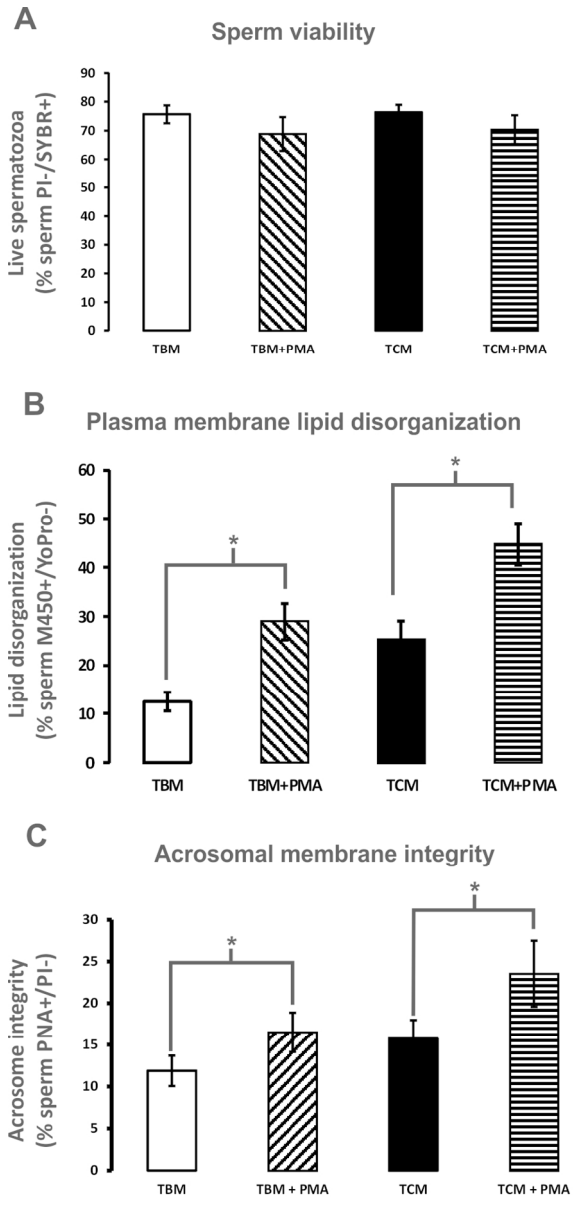
Figure 6. PMA-induced PKC activation and 8Br-cAMP- induced PKA activation on sperm protein phosphorylation pathways. Sperm pools were treated with 8Br-cAMP or PMA in TBM medium for 1 h at 38.5ºC. Specific phosphorylated protein profiles were identified by western blot with **(A)** anti-PKA-substrate and **(B)** anti-PKC-substrate antibodies. Arrows indicate differences on migration of detected bands. Loading control was performed with anti-tubulin antibodies.

Table 1. Area measurement of particles formed by agglutinated sperm. This experiment was performed at least 5 times. Mean and standard error of the mean are showed. Statistical differences are shown as a, b, c, d and f when $p <0.0001$ between treatments (columns)

Table 2. Motility analysis performed by CASA. Sperm were incubated 60 minutes in the indicated medium**.** This experiment was performed at least 5 times and results express the mean ± standard error of the mean. Statistical differences are shown as a, b when $p < 0.0001$ between treatments (columns)

Supplementary material: TCM 60. Video of sperm incubated 60 minutes in TCM medium

TCM 60 + 10 PMA. Video of sperm incubated 60 minutes in TCM medium supplemented with 10 μM PMA.



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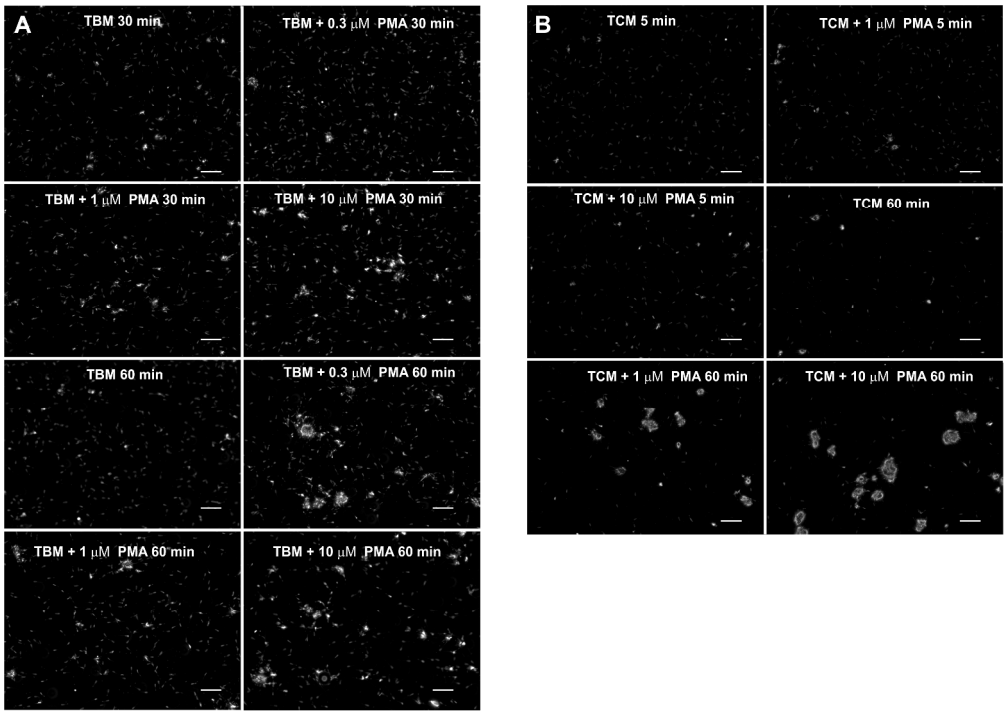

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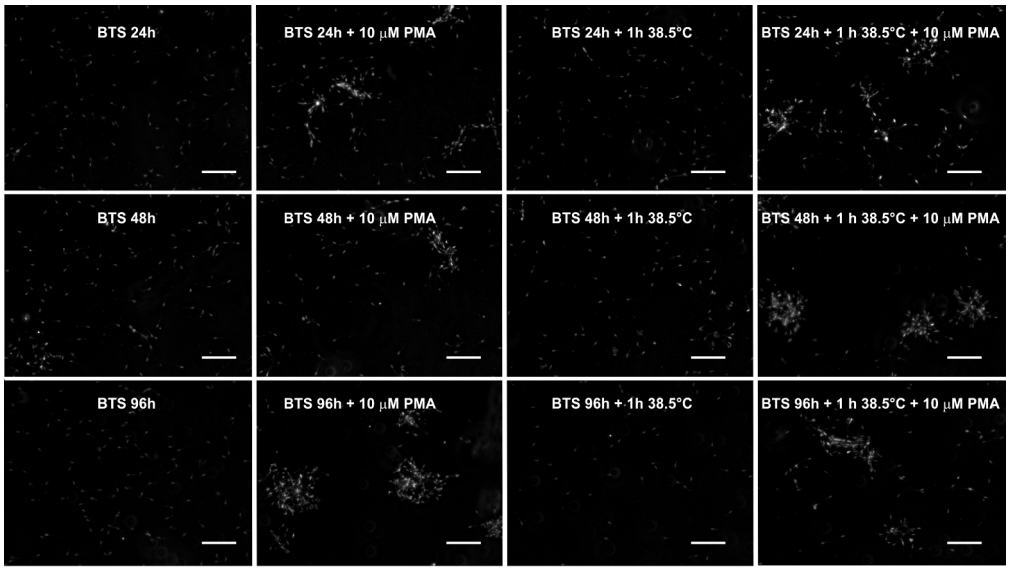

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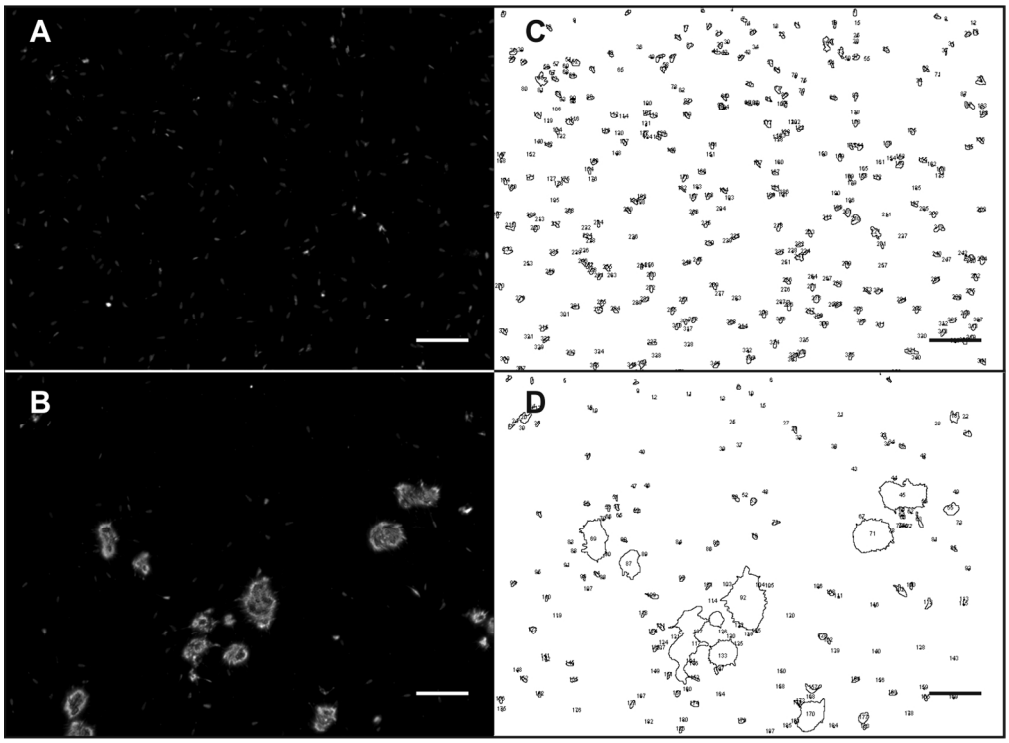

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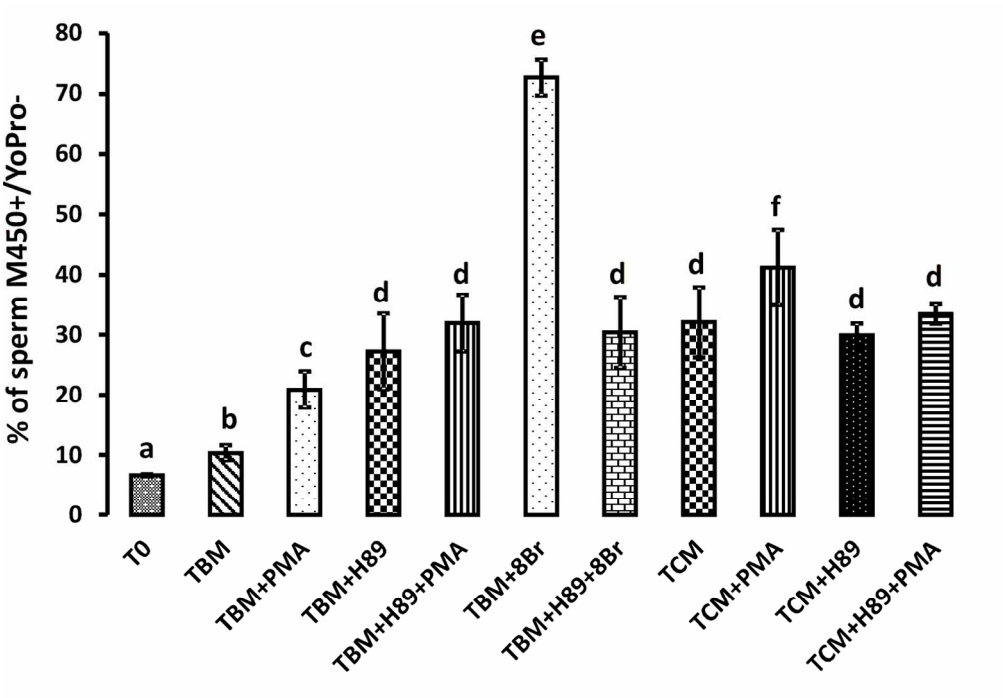
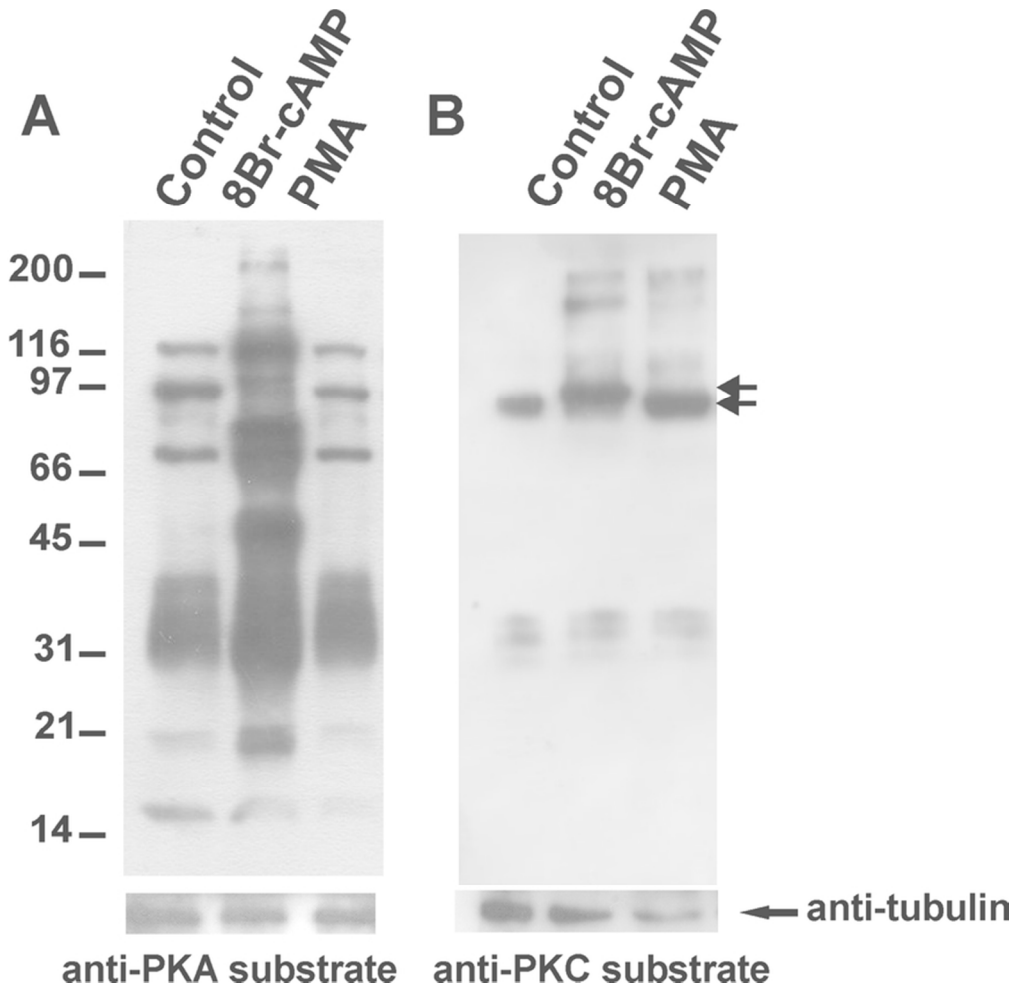

% of sperm M450+/YoPro-
80
70
60
50
40
30
20
10
0
a
b
c
d
d
e
d
d
f
d
d
T0
TBM
TBM+PMA
TBM+H89
TBM+H89+PMA
TBM+8Br
TBM+H89+8Br
TCM
TCM+PMA
TCM+H89
TCM+H89+PMA



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