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Complete List of Authors:	Krapf, Dario; Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET/UNR), Facultad de Ciencias Bioquímicas, O'Brien, Emma; Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET/UNR), Facultad de Ciencias Bioquímicas, Maidagan, María; Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET/UNR), Facultad de Ciencias Bioquímicas, Morales, Enrique; Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET/UNR), Facultad de Ciencias Bioquímicas, Visconti, Pablo; University of Massacusetts, Veterinary and Animal Sciences Arranz, Silvia; Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET/UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas
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Calcineurin regulates progressive motility activation of *Rhinella* (*Bufo*) *arenarum* sperm through dephosphorylation of PKC substrates.

Dario Krapf[1], Emma O´Brien[1], María P. Maidagán[1], Enrique O. Morales[1], Pablo E. Visconti[2] and Silvia E. Arranz[1,*]

[1] Instituto de Biología Celular y Molecular de Rosario (CONICET-UNR) y Facultad de Ciencias Biológicas y Farmacéuticas, UNR, 2000 Rosario, Santa Fe, Argentina. [2] Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003, USA

[*] *Correspondence address:* Silvia E. Arranz, Instituto de Biología Celular y Molecular de Rosario, Ocampo y Esmeralda, 2000 Rosario, Argentina. Tel: +54 (341) 423-7070 ext 649; Fax: +54 (341) 423-7070 ext 607; e-mail: arranz@ibr-conicet.gov.ar

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ABSTRACT

Animals with external fertilization, as amphibians, store their sperm in a quiescent state in the testis. When spermatozoa are released into natural fertilization media, the hypotonic shock triggers activation of sperm motility. *Rhinella (Bufo) arenarum* sperm are immotile in artificial seminal plasma (ASP, resembling testicular plasma tonicity) but acquire *in situ* flagellar beating upon dilution. However, if components from the egg shelly coat are added to this medium, motility shifts to a progressive pattern. Recently, we have shown that the signal transduction pathway required for *in situ* motility activation involves a rise in intracellular cAMP through a transmembrane adenylyl cyclase and activation of PKA, mostly in the midpiece and in the sperm head. In this report, we demonstrate that activation of calcineurin (aka PP2B and PPP3) is required for the shift from in situ to progressive sperm motility. The effect of calcineurin is manifested by dephosphorylation of PKC substrates, and can be promoted by intracellular calcium rise by Ca^{2+} ionophore. Both phosphorylated PKC substrates and calcineurin localized to the flagella, indicating a clear differentiation between compartmentalization of PKA and calcineurin pathways. Moreover, no crosstalk is observed between these signaling events, even though both pathways are required for progressive motility acquisition as discussed.

INTRODUCTION

Over 60 years ago, independent reports demonstrated that ejaculated mammalian spermatozoa require a period of residence in the female reproductive tract before being capable of fertilization (Chang, 1951;Austin, 1952). The changes that take place during this period endow the spermatozoa with a pattern of sperm motility known as hyperactivation and with the ability to undergo the acrosome reaction. This process is known as "capacitation", and correlates at the molecular level with the initiation of complex signal transduction pathways resulting in tyrosine phosphorylation, a hallmark of sperm capacitation (Visconti et al, 2011). Recently, we have shown that capacitation-like changes occur also in *Rhinella arenarum* sperm (aka *Bufo arenarum*). In this species, a short exposure of sperm to factors released from the egg jelly coat during spawning to the surrounding media (named egg water, EW) triggers tyrosine phosphorylation of sperm proteins, resembling capacitation of mammalian sperm (Krapf et al, 2007;Visconti et al, 1995). More importantly, after exposure to egg water, these sperm gained fertilizing capacity (Krapf et al.2007).

Sperm motility is, beyond doubt, crucial for successful fertilization. Initiation of sperm motility is under strict regulation in different species and this regulation is dependent on the environment in which fertilization occurs. Several factors have been reported to regulate sperm motility; for example, high bicarbonate and calcium are essential in mammals to regulate this process (Visconti et al.2011). In external fertilizers such as amphibians, sperm spawning in low osmolarity waters results in the initiation of sperm motility (Inoda and Morisawa, 1987;Hardy and Dent, 1986). At the molecular level, regulation of sperm motility has been linked to cAMP signaling pathways in several animal species, including mammals (Okamura et al, 1985), amphibians (O'Brien et al, 2011) and invertebrates like sea urchins (Beltran et al, 1996). Whereas the soluble adenylyl cyclase, sAC, (aka Adcy10) is involved in mammalian (Hess et al, 2005) and sea urchin (Nomura et al, 2005) sperm motility, a

transmembrane adenylyl cyclase (tmAC) governs cAMP rise involved in flagellar beating of in *Rhinella* sperm (O'Brien et al.2011). Transmembrane ACs are regulated by heterotrimeric G-proteins and stimulated by forskolin (Hanoune and Defer, 2001), and recently demonstrated to regulate acrosome reaction in mouse sperm (Wertheimer et al, 2013). Interestingly, when *Rhinella* sperm in isotonic media (normally immotile) are exposed to forskolin, motility is triggered, together with the phosphorylation of PKA substrates (O'Brien et al.2011).

In boar sperm, cAMP permeable analogues promote hyperactivation through stimulation of PKA and PKC signaling cascades (Harayama and Miyake, 2006). Noteworthy, only a narrow body of work relate to the role of PKC in sperm acquisition of fertilizing capacity. The present work advances on the molecular pathways that regulate motility initiation in Amphibian sperm. Our results indicate that in addition to increased PKA activity, a decrease of the phosphorylated status of PKC substrates is also linked to motility regulation. This decrease is promoted by the activation of the phosphatase calcineurin, rather than by a decrease of PKC activity itself. Interestingly, activation of calcineurin does not affect the phosphorylation status of PKA substrates due to a different compartmentalization: while phosphorylation of PKA substrates is observed in the sperm head (O'Brien et al.2011), both PKC substrates and calcineurin are observed in the tail. Altogether, these data suggest that dephosphorylation of PKC substrates through activation of calcineurin is required for acquisition of sperm fertilizing capacity.

Methods

Reagents. H-89, 2-dibutyryladenosine cAMP (db-cAMP), β-glycerophosphate, sodium vanadate, p-Nitrophenyl phosphate (pNPP), Gö6983, thymeleatoxin and cocktail of protease inhibitors were obtained from Sigma-Aldrich (St. Louis, MO). Forskolin (FK) was purchased from Calbiochem (San Diego, CA). Cyclosporin A and okadaic acid were from LC Laboratories (Woburn, MA). Anti-phospho-PKA substrate antibodies (#9621) (anti-pPKAs)

and anti-phospho-PKC substrate antibodies (#2261) were obtained from Cell Signaling (Danvers, MA), anti-Actin (I-19) from Santa Cruz Biotechnology (SCB; Santa Cruz, CA). Anti-calcineurin (PP2B) (#610259) were obtained from BD Biosciences (Lexington, KY). Secondary rabbit IgG-HRP labeled antibody was from Amersham (Pittsburg, PA) and secondary mouse IgG-HRP labeled antibody from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were of reagent grade. Stock solutions of chemicals were prepared in distilled water or dimethyl sulfoxide (DMSO) according to manufacturer's instructions. The concentration of DMSO in the incubation media was kept constant between treatments and never exceeded 1% (v/v), a condition that did not affect sperm motility.

Animals and preparation of gametes. *Rhinella arenarum* sexually mature specimens (150 g) were collected in the neighborhood of Rosario, Argentina, and maintained in a dark moist chamber between at 16 °C until used. Experiments were performed according to the guide for care and use of laboratory animals of Facultad de Ciencias Biológicas y Farmacéuticas, Universidad Nacional de Rosario. Approval has been obtained from the Ethics and Animal Care and Use Committee from same institution. Sperm suspensions were obtained as described elsewhere (Krapf et al, 2006). Briefly, Sperm suspensions to be used in quantitative sperm binding assays were prepared by mincing testes in ice cold ASP (see below). Debris were removed by centrifugation at 130*g* for 10 min at 4°C. Mature sperm were pelleted by centrifugation at 650*g* for 10 min at at 4°C. Spermatozoa were suspended in ice cold ASP to a final concentration of $1–1.4\times10^8$ cells/ml and used within 3 h. All incubations were performed at 20°C unless otherwise stated. Live–dead staining (2% of eosin in ASP) was performed after every treatment in order to analyze possible sperm toxicity (O'Brien et al.2011). Oocytes were recovered from female toads that were hormonally stimulated with a homologous hypophysis extract. After 12 hrs, strings of oocytes were removed from ovisacs (Barisone et al, 2007).

Composition of media used. Egg water (EW) was obtained as described (Diaz Fontdevila et al, 1991). Briefly, strings of oocytes were removed from ovisacs and incubated for 10 min in distilled water. The resultant solution, named EW, had a final protein concentration of 70 µg/ml and pH 8.2. Artificial Seminal Plasma (ASP, in mM: 105 NaCl, 40 KCl, 1.4 $CaCl_2$, 10 Tris, pH 7.2) medium was the isotonic medium used to keep cells completely immotile (O'Brien et al.2011). Ten percent of ASP corresponds to a 1/10 dilution of ASP. Changing the pH of the control isotonic media from 7.4 to 8.2 did not significantly alter motility parameters (O'Brien et al.2011). Calcium concentrations of media containing EGTA was calculated using Ca-EGTA Calculator v1.2 (http://maxchelator.stanford.edu/CaEGTA-NIST.htm)

Immunoblotting. Sperm suspensions were diluted in the appropriate medium depending on the experiment. Sperm protein extracts were performed in Triton X-100 lysis buffer (1% Triton X-100, 5 mM EDTA, 1% cocktail of ROCHE protease inhibitors, 1 mM sodium vanadate, 100 nM okadaic acid, 30 mM β-glycerophosphate, 5 mM pNPP, 150 mM NaCl, 10 mM Tris pH 7.6). The supernatants were mixed with sample buffer containing 50 mM DTT, incubated 10 min at 70 °C and subjected to 10% SDS–PAGE (Laemmli, 1970). Each lane was loaded the equivalent to with 7×10^6 cells. Proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, UK) at 250 mA (constant) for 2 h at 4 °C. Membranes were blocked with 5% BSA in TBS + 0.1% Tween 20 (T-TBS) for 1 hr at RT. Immunoblotting was performed using a dilution 1/1,000 anti-phospho-PKC substrate (anti-pPKCs) antibodies, 1/1,000 anti-phospho-PKA substrate (anti-pPKAs) antibodies, 1/1000 dilution of anti- calcineurin or 1/10,000 anti-actin antibodies. Secondary antibodies used were 1/10,000 dilution of a secondary rabbit IgG-HRP labeled antibody (Amersham) and 1/10,000 of a secondary mouse IgG-HRP labeled antibody (Jackson Labs). All antibodies wre diluted in T-TBS. Membranes were developed with the enhanced chemiluminescence detection kit

(Thermo Scientific SuperSignal West Femto Substrate). All membranes were stripped (Krapf et al, 2012) and western blots for actin detection were performed as loading controls.

Fractionation of Heads and tails. Separation of sperm heads and tails was performed by sonicating a sperm suspension (500 µl containing $1x10^8$ cells) at 80W during 30 sec in ASP. This suspension was layered over 4 ml of Percoll, consisting of a 63% Percoll lower phase and a 45% Percoll upper phase in ASP, and centrifuged at 650 g for 20 min at RT. The interphase between ASP and 45% Percoll phase contained mostly broken flagella, while sperm heads were found at the bottom of the tube. Both fractions were collected and diluted to a final volume of 1 ml of ASP and further centrifuged for 2 min at 13,000 g. The pellet was treated with lysis buffer for SDS/PAGE.

Indirect Immunofluorescence. Immunolocalization in fixed cells was performed as described (Martinez and Cabada, 1996). Briefly, alter sperm incubation according to the experiment performed, 10 µl of sperm suspensión $5x10^6$ sp/ml) were deposited over 10 µl of 3% formaldhehide in ASP on polylysine coated coverslips, and incubated for 20 min at RT. Cells were then washed with TBS and permeabilized with 0.2% Triton X-100 in ASP. Blocking was performed with 3% BSA in TBS for 1 hr at RT. Immunodetection was performed using a 1/100 dilution of anti-pPKCs antibodies. Sperm nuclei were stained with Hoechst (33258). Samples were analyzed with a Nikon Eclipse TE-2000-E2 confocal microscope (Natick, MA).

Motility analysis. Sperm suspensions (1×10^8 cells/ml) were diluted 25 times in the desired media and the suspension placed on a standard count 20 micron (Spectrum Technologies, Heldsburg, CA) chamber slides to assess sperm motility. Trajectories were recorded using an Olympus BH-2 microscope connected to a Nikon DS-Fi1 camera (Natick, MA) at 200× magnification. The percentage of motile sperm was assessed from the video recordings. Movies were converted to image sequences (virtualdub.org) and analyzed with ImageJ software (http://rsb.info.nih.gov/ij) as described previously (O'Brien et al.2011).Motility index

were classified as 1) immotile, 2) *in situ* (sperm flagella beating without midpiece movement) and 3) progressive movement. Sperm were counted as "motile" when they either exhibited progressive movement or flagellar beatings. At least three independent experiments analyzing more than 100 sperm each time were performed for each condition tested.

Statistical analysis. Data were analyzed with paired Student's t test for comparing mean values, and with Mann–Whitney test to compare medians. Analysis of variance (ANOVA) was used for comparing multiple groups. Models were further tested according to Nagarsenker (1984), and Shapiro and Wilk (1965). Statistical significances are indicated in the text.

RESULTS

Motility activation triggered by hypotonic media correlates with a decrease in the phosphorylated state of PKC substrates.

When activated, most PKC isozymes are able to phosphorylate substrates containing Ser or Thr residues, with Arg or Lys at the -3, -2, and +2 positions, and a hydrophobic amino acid at +1 position. Thus, the role of these PKC isoforms in the regulation of Amphibian sperm motility, if any, could be analyzed using antibodies that specifically detect protein substrates bearing a phosphorylated Ser residue at the above mentioned sequence (named anti-pPKCs antibodies). To investigate whether PKC is activated during motility initiation, *Rhinella* sperm were incubated in ASP (artificial seminal plasma, an isotonic medium that keeps sperm immotile (O'Brien et al.2011)) or in the hypotonic 10% ASP in order to promote flagellar motility. After 5 min, protein extracts were analyzed by western blot, using anti-pPKCs antibodies (see Materials). Contrary to what originally expected, a decrease in the phosphorylated status of sperm proteins that correlate to motility initiation was detected (Fig. 1A). Proteins extracted from quiescent sperm (in ASP) displayed a subset of phosphorylated

proteins in the range from 60 to 110 kDa. The phosphorylated state of PKC substrates was significantly lowered upon activation of flagellar motility (see Figs 1A and B). Since sperm exposure to EW (hypotonic solution containing factors released from the egg jelly) not only activates flagellar motility but also prepares the sperm for a physiologically induced acrosome reaction (Krapf et al.2007;Krapf et al, 2009), the effect of EW on the phosphorylated state of PKC substrates was also analyzed. As seen in figure 1A, EW also promotes dephosphorylation of PKC substrates. As previously demonstrated, EW also promotes progressive movement (Fig. 1B) thus lowering *in situ* motility as shown in Fig. 1C (Krapf et al.2007). Noteworthy, hypotonic shock triggers both increase of PKA activity (O'Brien et al.2011) and decrease of PKC related phosphorylation cascades involved in sperm motility, without triggering tyrosine phosphorylation which have been shown to have a role in the preparation for the agonist-induced acrosome reaction (Krapf et al.2009;Krapf et al.2007). Considering these data, 10% ASP is an excellent media for studying motility associated signaling events independently of the acrosome reaction.

Although anti-pPKCs antibodies have been previously used in sperm from other species (Alnagar et al, 2010;White et al, 2007;Harayama et al.2006), we performed experiments aiming to validate this antibody in our system. In order to ascertain that the observed phosphoproteins responded to PKC activity, sperm suspensions were supplemented with thymelaea toxin (TXA), a diterpene which activates many PKC isozymes (Kazanietz et al, 1993). These incubations were performed both in ASP and in 10% ASP. As shown (Fig. 1D), the phosphorylated status of PKC substrates was augmented when both ASP and 10% ASP sperm suspensions were supplemented with 100 nM TXA. To further ascertain the specificity of these antibodies, sperm suspensions were incubated in the presence of the pan PKC inhibitor Gö6983. Upon incubation of 60 min, PKC inhibition was observed with an IC_{50} of ~10 μM Gö 6983 (Fig. 1E). Sperm membrane integrity was assessed by eosin staining,

indicating no detrimental effects of this inhibitor (not shown). To further ascertain sperm viability, sperm were first exposed to 30 μM Gö 6983 during 60 min, washed, and further exposed to 100 nM TXA during 5 min. Phosphorylation increase of PKC substrates was clearly observed (Fig 1F, right panel). As a control, when sperm that were first exposed to 30 μM of the PKC inhibitor Gö 6983 for 60 min were then exposed to 100 nM TXA without washing, the PKC agonist was in this case not able to promote phosphorylation of PKC targets (Fig 1F, left panel). Altogether, these data demonstrate that this antibody (anti-pPKCs) is a valid tool to analyze the phosphorylation status of PKC substrates. More importantly, the phosphorylated status of PKC targets lowers upon motility activation.

Presence of phosphatases in Rhinella sperm

Worth noticing, during experiments with the PKC inhibitor Gö 6983, it was observed that long incubation periods were required to observe a decrease of the phosphorylated stated of PKC substrates. Since the phosphorylated state of a protein responds to a balance between activity of kinases and phosphatases, we hypothesized that the phosphorylation/dephosphorylation turn-over of PKC substrates was almost negligible in quiescent sperm, and that during hypotonic shock, a ser/thr phosphatase would be activated and responsible for the observed protein dephosphorylation. In support of this hypothesis, the ser/thr PP1γ1 (a splice variant of the Ppp1cc gene) has been previously shown to be present in Amphibian sperm (Chakrabarti et al, 2007). In addition, the presence of both PP2A and calcineurin (PP2B) has been confirmed in this work by western blot analysis (Fig 2A and B). Therefore, we aimed to explore the possible involvement of these phosphatases in the dephosphorylation of PKC substrates.

Loss of phosphorylation of PKC substrates as a consequence of calcineurin activity

In order to address the possible role of ser/thr phosphatases in the dephosphorylation of PKC substrates that correlates to motility activation, sperm were exposed to ser/thr phosphatase

inhibitors during hypotonic shocks. Considering the presence of both PP1γ1 and PP2A, we analyzed the effect of the inhibitor okadaic acid (Krapf et al, 2010). Only a slight inhibition could only be detected when 1μM okadaic acid was used (data not shown). Considering that Okadaic acid inhibits both PP1 and PP2A at the nanomolar range, but calcineurin at micromolar concentrations, we decided to assay the effect of the calcineurin inhibitor Cyclosporin A (CsA). This inhibitor forms a complex with the ubiquously expressed cyclophilin A, which in turn competitively binds to and inhibits calcineurin by limiting the access of protein substrates to the active site of CsA. As shown in figure 3A, CsA effectively prevented dephosphorylation of PKC substrates, at a concentration of 10 nM. Moreover, CsA inhibited the acquisition of progressive motility upon incubation in EW. These sperm, in turn, showed an increase of *in situ* motility (Fig. 3C). As expected, *in situ* motility of sperm in 10% ASP was not affected (Fig 3C). Moreover, the presence of CsA in EW affected the acquisition of fertilizing capacity, as shown by the dramatic drop in *in vitro* fertilization rates (Fig 3D).

Dephoshorylation of PKC substrates by calcineurin requires extracellular Ca^{2+}

The above presented results indicate that calcineurin has a direct role on the steady-state phosphorylation levels of PKC substrates and participates in sperm motility regulation. Since calcineurin is a Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase, the absence of Ca^{2+} would jeopardize its activity. To further substantiate the role of calcineurin in sperm physiology, *Rhinella* sperm were incubated in either EW or 10% ASP containing different concentrations of EGTA. Considering the concentration of Ca^{2+} in 10% ASP (0.14 mM (O'Brien et al.2011)) and EW (0.28 mM (O'Brien et al.2011)), the presence of 2, 5 or 10 mM EGTA leaves 4, 1.6 and 0.8 pM and 65, 25 and 12 pM of free Ca^{2+} in 10% ASP and EW respectively. These values were calculated as described in Methods using the Ca-EGTA Calculator v1.2 program. Dephosphorylation of PKC substrates was attenuated in the presence of 5 mM EGTA compared to control sample, whereas at 10 mM EGTA

dephosphorylation did not occur (Fig. 4A). At similar concentrations, EGTA blocked progressive motility; the EGTA effect was observed both in 10% ASP and EW (Fig. 4B). This result indicates that either depletion of extracellular Ca^{2+} or intracellular Ca^{2+} dialysis driven by EGTA affects dephosphorylation. Moreover, sperm exposure to 10 μM Ca^{2+} ionophore A23187 in ASP media during 5 min, which freely diffuses calcium across sperm membranes, induces a significant dephosphorylation of PKC substrates (Fig. 4C). Altogether, these results are consistent with the hypothesis that dephosphorylation of PKC substrates associated with sperm motility occurs upon activation of calcineurin mediated by an intracellular calcium increase..

Subcelular localizacion of PKC substrates in Bufo sperm

The above results suggest that dephosphorylation of PKC substrates plays a role in the shift from *in situ* to progressive motility of *Rhinella* sperm. In order to examine the subcellular localization of PKC substrates, sperm were incubated in either ASP or 10% ASP and immunostained using anti-pPKCs antibodies. In sperm incubated in ASP, the fluorescence signal mainly localized to the flagellum (arrows in Fig. 5A panels 1 and 2). This signal was absent when sperm were incubated in hypotonic conditions (10% ASP) (Fig. 5A panel 3 and 4), indicating that dephosphorylation of PKC substrates occurs in the tail region. Noteworthy, as previously shown, sperm incubation in 10% ASP also triggers phosphorylation of PKA substrates. In contraposition, this phosphorylation of ser/thr residues was mostly observed in the head (O'Brien et al.2011). This opposite behavior led us to hypothesize that the tight regulation between phosphorylation of PKA substrates and dephosphorylation of PKC substrates was orchestrated by the specific compartmentalization of calcineurin to the flagella. Despite our efforts to immunolocalize calcineurin in *Rhinella* sperm with different unmasking protocols, no signal was obtained. Thus, the subcellular localization was analyzed using highly pure (>95%) fractionation of isolated sperm heads and tails by sonication and

purification in percoll gradients (Fig. 5B). Western-blot analysis revealed that calcineurin was present in the tail preparations (Fig. 5C), coincident with the major site of ser/thr dephosphorylation of PKC substrates (see Fig 5A).

Crosstalk between PKA and PKC/calcineurin signaling events.

Previous results have shown that motility activation of *Rhinella* sperm depends on PKA activation. Both the transmembrane adenylyl cyclase activator forskolin and cAMP permeable analogues, promoted an increase on the phosphorylation state of PKA substrates along with motility activation even in isotonic ASP media (O'Brien et al.2011). In order to analyze a possible link between activation of PKA and dephosphorylation of PKC substrates, sperm were incubated in different conditions that either stimulate or block these pathways. First, ASP was supplemented with forskolin, in order to promote PKA activation (Fig 6) (O'Brien et al.2011). In this case, no dephosphorylation of PKC substrates was promoted, indicating that PKA does not activate calcineurin. Second, the presence of CsA in 10% ASP, which prevented dephosphorylation of PKC substrates through calcineurin blockade, did not impair phosphorylation of PKA substrates promoted by hypotonic shock. In addition, the presence of the PKA specific inhibitor H-89 in 10% ASP did not prevent dephosphorylation of PKC substrates. Moreover, the activation of calcineurin through Ca^{2+} ionophore A23187 (Fig. 4C), did not promote PKA activation (not shown). Altogether, these data point towards independent regulation of PKC/ calcineurin and PKA signaling pathways.

DISCUSSION

Mature epidydimal sperm of mammalian species remain in a quiescent state until ejaculation (Vadnais et al, 2013). The exact mechanism of sperm quiescence is not clearly understood. Regardless of the lack of epididymis in external fertilizers, sperm of these species also remain in a quiescent state in the testis. In Amphibians, sperm become motile upon exposure to

freshwater. In both mammalian and amphibians, the molecular pathways leading to sperm motility are still not completely understood.

We have recently shown that a solution that resembles testicular ionic composition and osmolarity (292 mOsm/kg), named Artificial Seminal Plasma (ASP), keeps amphibian sperm in a quiescent state (O'Brien et al.2011). Sperm dilution into hypoosmotic media activates phosphorylation events by PKA that correlate to motility activation, similarly to what is observed upon mammalian sperm capacitation (Krapf et al.2010); moreover, amphibian sperm motility is abrogated in the presence of the PKA inhibitor H-89 (O'Brien et al.2011). In *Rhinella* sperm, we have shown that PKA activity was preceded by activation of a transmembrane adenylyl cyclase triggered by hypoosmotic shock (O'Brien et al.2011). Interestingly, it has been recently shown that a transmembrane adenylyl cyclase also is also present in mouse sperm, and participates in the acrosome reaction without activation of PKA (Wertheimer et al.2013) Worth noticing, in boar sperm capacitation, cAMP leads to activation of both PKA and PKC (Harayama et al.2006). On the other hand, during capacitation of bovine sperm, it has been proposed that PKA inactivates phospholipase C, preventing PKC activation . In the present study, we aimed to study the possible participation of PKC in *Rhinella* sperm motility activation. Amphibian sperm are an attractive model which allows to discriminate between signaling events leading to acrosomal responsiveness (Krapf et al.2009) from those related to motility (O'Brien et al.2011). In this manuscript, we have shown for the first time the high steady state level of phosphorylation of PKC substrates in quiescent sperm. These levels are suddenly decreased during activation of flagellar motility that follows hypotonic shock. In *Rhinella* sperm, hypotonic shock promoted PKA activation (Fig 6) and down-regulated phosphorylation of PKC substrates. However, down-regulation of PKC substrate phosphorylation was not due to a decrease of PKC activity *per se*, but to an up-regulation of the ser/thr phosphatase calcineurin. Pharmacological inhibition of calcineurin

through cyclosporine A impaired dephosphorylation of PKC substrates triggered by hypotonic shock. Moreover, the blockade of dephosphorylation of PKC substrates was correlated to an impairment of progressive movement, without affecting total motility (in situ + progressive), demonstrating the role of calcineurin on progressive motility. In addition, CsA decreased fertilizing capacity of sperm in IVF experiments, with an IC_{50} at the low nanomolar range.

Calcineurin is a PPP family ser/thr phosphatase, activated by Ca^{2+}-calmodulin. The presence of EGTA in the incubation media prevented both dephosphorylation of PKC substrates as well as sperm progressive motility. Moreover, when sperm incubated in isotonic ASP were exposed to Ca^{2+} ionophore A23187, dephosphorylation of PKC substrates was promoted. These results further substantiate the role of Ca^{2+}- calcineurin in this pathway. Worth noticing, when dephosphorylation was promoted by Ca^{2+} ionophore in isotonic ASP media, sperm motility was not triggered. These data suggest that calcineurin activation alone does not support sperm motility, but instead, supports a shift from *in situ* to progressive motility in a hypotonic environment. We have recently demonstrated that activation of PKA induces *in situ* motility in *Rhinella* sperm (O'Brien et al.2011). In this regard, it could be hypothesized that activation of both signaling pathways would support progressive motility. This was not the case when PKA was activated by permeable cAMP analogues (dibutyryl cAMP) and calcium ionophore (A23187) triggered calcineurin activity, even though both activation of PKA and dephosphorylation of PKC substrates were observed (not shown). Faliure to develop progressive motility could arise fromexcess flooding of calcium to the intracellular sperm milieu, not compatible with physiological calcium requirements for sperm motility. In mouse sperm, ionophore treatement has been shown to promote capacitation, provided that ionophore is washed away after a short exposure (Tateno et al, 2013). The role of this calcium requirement is still unclear.

In summary, our data suggest that phosphorylation of PKA substrates, and dephosphorylation of PKC substrates by calcineurin are independent processes according to the following evidence: 1) direct activation of PKA by cAMP permeable analogues does not promote calcineurin activity; 2) PKA inhibition by H89 does not prevent calcineurin activity; 3) calcineurin blockade does not impair PKA activity in hypotonic media; and 4) phosphorylation of PKA substrates takes place in the mid-piece and head regions, while both calcineurin and phosphorylation of PKC substrates localize to the flagella, evidencing a different compartmentalization of these pathways. Altogether, these data indicate that both pathways are acting in parallel and are both required for progressive sperm motility. How these pathways complement to each other warrants further investigation.

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FIGURE LEGENDS

Figure 1. Activation of *Rhinella* sperm motility correlates to dephosphorylation of PKC susbtrates. (*A*) Sperm suspensions were incubated in ASP, 10% ASP or EW during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control. **(*B* and *C*)** Sperm motility analysis after 5 min incubations in isotonic solution (ASP) or hypotonic solutions (10% ASP or EW). Motility patterns were identified as progressive movement (B) (rapid forward movement) and *in situ* (C) (indicates only flagellar beating). Data represent mean±SD; n=10; $^{**}p<0.001$ compared to ASP). **(*D*)** Sperm suspensions were incubated in 10% ASP or ASP during 5 min in the presence of different concentrations of thymeleatoxin (TXA). Each lane contains sperm proteins detected as described in panel A. **(*E*)** Sperm suspensions were incubated in ASP during 60 min in the presence of different concentrations of Gö6983 (Gö). **(*F*)** Sperm suspensions were incubated in ASP during 60 min in the presence or absence of 30μM Gö6983 (Gö), and further exposed or not to 100 nM TXA (left panel). Alternatively, sperm were washed after the fisrt 60 min incubation under the presence (or not in controls) of Gö6983.

Figure 2. Presence of phosphatases in *Rhinella* sperm. (*A*) 1% Tritón X-100 soluble proteins of 7×10^6 *Rhinella* sperm or 1% SDS soluble mouse sperm proteins (control, $1x10^6$ cells) were analyzed by 10% SDS/PAGE and Western blot using anti-PP2A antibodies. Western blot for actin was performed as loading control. Negative control was performed over same membrane, without incubation with anti-PP2A antibodies. **(*B*)** 1% Tritón X-100 soluble proteins of 7×10^6 *Rhinella* sperm or 1% SDS soluble rat brain proteins (20μg, control) were analyzed by 10% SDS/PAGE and Western blot using anti-calcineurin antibodies. Western blot for actin was performed as loading control. Negative control was performed over same membrane, without incubation with anti-calcineurin antibodies. Asterisk indicates a non specific band.

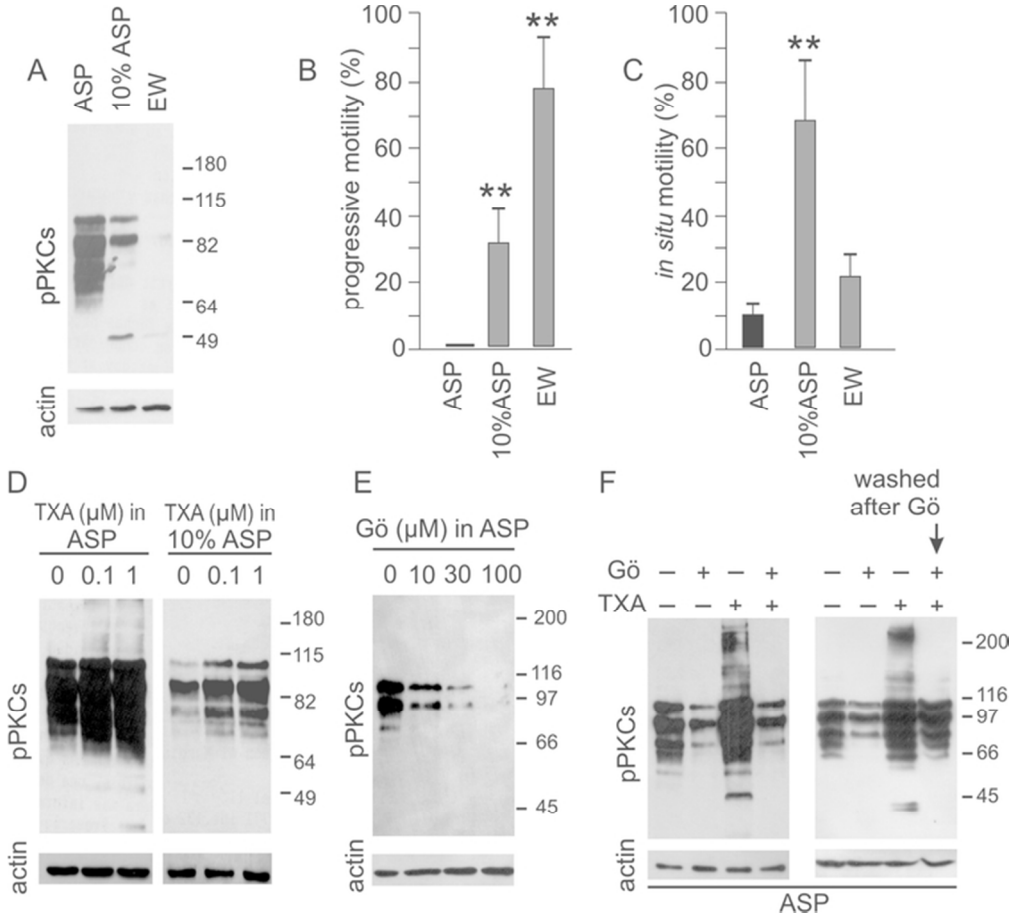
Figure 3. Inhibition of calcineurin affects both progressive sperm motility and fertilizing capacity of *Rhinella* sperm. (*A*) Sperm suspensions were incubated in either ASP or 10% ASP supplemented with different concentrations of Cyclosporin A (in nM), during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control. **(*B* and *C*)** Sperm motility analysis after 5 min incubations in 10% ASP or EW, supplemented with different concentrations of Cyclosporin A. Motility patterns were identified as progressive movement (B) (rapid forward movement) and *in situ* (C) (only flagellar beating). Data represent mean±SEM; n=10; $^{*}p<0.05$ and $^{**}p<0.001$ compared to untreated sample). **(*D*)** Sperm suspensions were incubated in ASP supplemented with different concentrations of Cyclosporin A (in nM), during 5 min, and further used for IVF of intact jelly eggs in CsA free media.

Figure 4. Calcium is involved in both dephosphorylation of PKC substrates and progressive sperm motility. (*A*) Sperm suspensions were incubated in either ASP or 10% ASP supplemented with different concentrations of EGTA (in mM), during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control. **(*B*)** Sperm motility analysis after 5 min incubations in 10% ASP or EW, supplemented with different concentrations of EGTA. Motility patterns

were identified as progressive movement (rapid forward movement) and *in situ* (only flagellar beating). Data represent mean±SD; n=10; **p*<0.05 and ***p*<0.001 compared to untreated sample). **(*C*)** Sperm suspensions were incubated in ASP or ASP supplemented with 10 μM Ca^{2+} ionophore A23187 during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control.

Figure 5. Phosphorylated PKC substrates locate to the flagella, where calcineurin is observed. (*A*) *Rhinella* sperm were incubated in ASP (panels 1 and 2) or EW (panels 3 and 4), fixed, permeabilized and stained with anti-pPKCs antibodies. Arrows indicate the flagellum of the toad sperm. Barr represents 10 μm. **(*B*)** Heads and flagella were obtained by sonication and fractionated by percoll gradient centrifugation. Preparation purity is shown in the micrographs. Barr represents 10 μm. **(*C*)** Heads and tails fractions were used for detection of calcineurin. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 heads or flagella analyzed by 10% SDS/PAGE and Western blot using a calcineurin antibody. Actin is only detected in the head fraction.

Figure 6. Simultaneous analyses of PKA and PKC phosphorylation status of sperm proteins. Sperm suspensions were incubated in either ASP or 10% ASP supplemented with 50 μM forskolin (FK), 10 nM CyclosporinA (CsA) or 30 μM H89 during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 sperm analyzed by 10% SDS/PAGE and Western blot using **(*A*)** anti phospho-PKA substrates antibody (anti-pPKAs) or **(*B*)** anti phospho-PKC substrates antibody (anti-pPKCs). Western blot for actin was performed as loading control



Activation of *Rhinella* sperm motility correlates to dephosphorylation of PKC susbtrates. (A) Sperm suspensions were incubated in ASP, 10% ASP or EW during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×10^6 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control. (B and C) Sperm motility analysis after 5 min incubations in isotonic solution (ASP) or hypotonic solutions (10% ASP or EW). Motility patterns were identified as progressive movement (B) (rapid forward movement) and in situ (C) (indicates only flagellar beating). Data represent mean±SD; n=10; **p<0.001 compared to ASP). (D) Sperm suspensions were incubated in 10% ASP or ASP during 5 min in the presence of different concentrations of thymeleatoxin (TXA). Each lane contains sperm proteins detected as described in panel A. (E) Sperm suspensions were incubated in ASP during 60 min in the presence of different concentrations of Gö6983 (Gö). (F) Sperm suspensions were incubated in ASP during 60 min in the presence or absence of 30μM Gö6983 (Gö), and further exposed or not to 100 nM TXA (left panel). Alternatively, sperm were washed after the fisrt 60 min incubation under the presence (or not in controls) of Gö6983.
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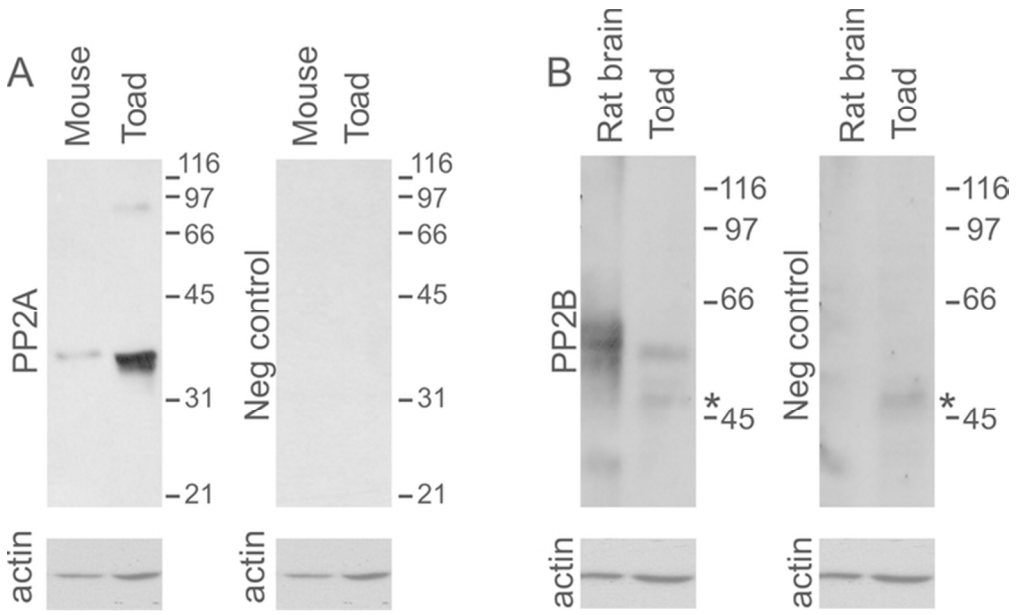

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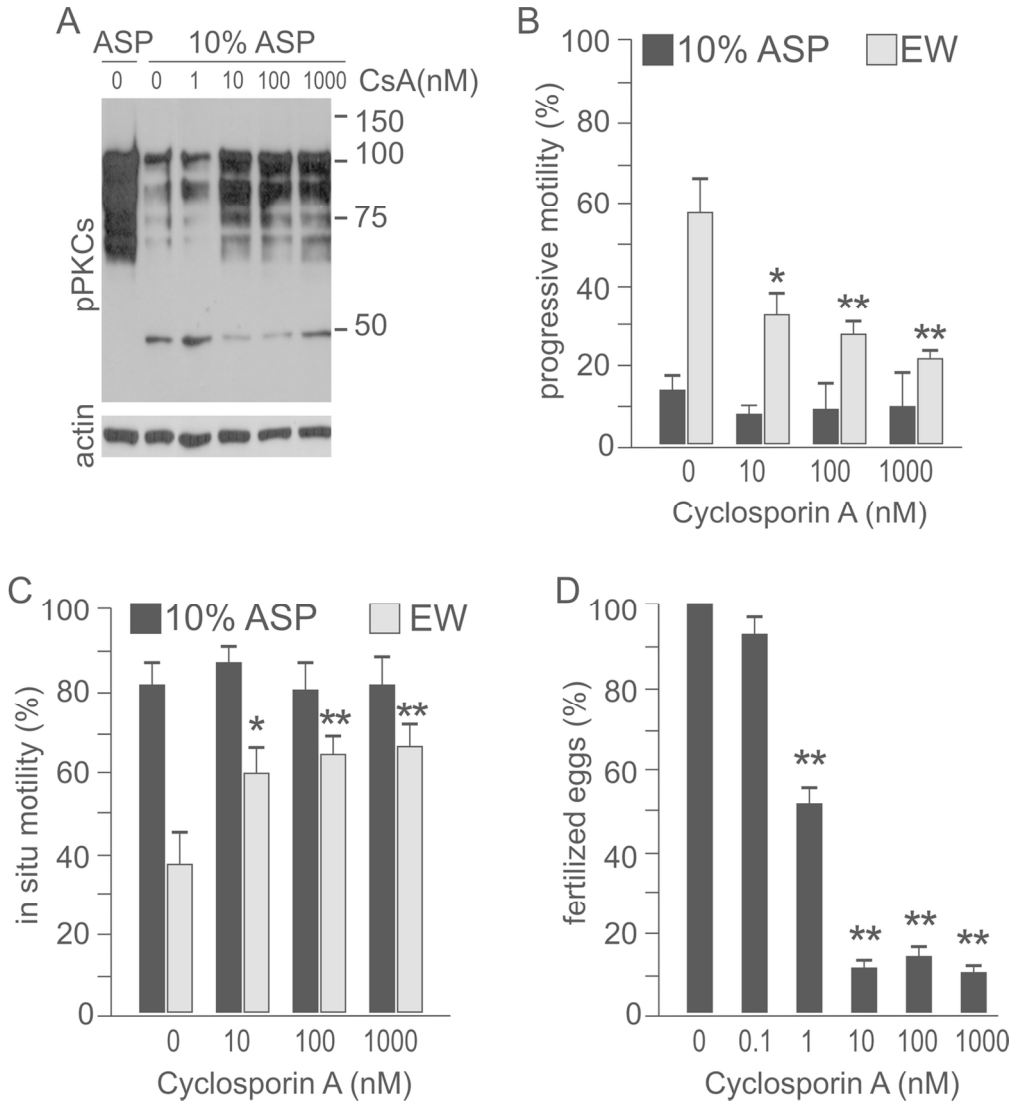

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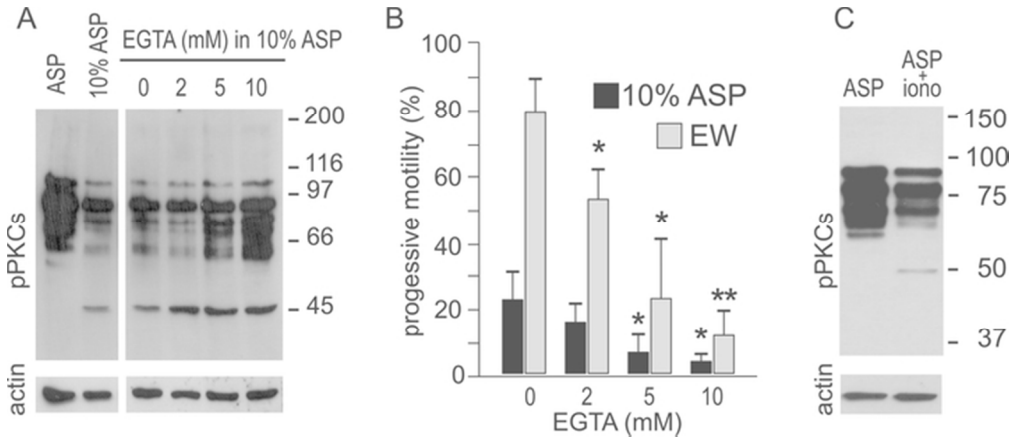
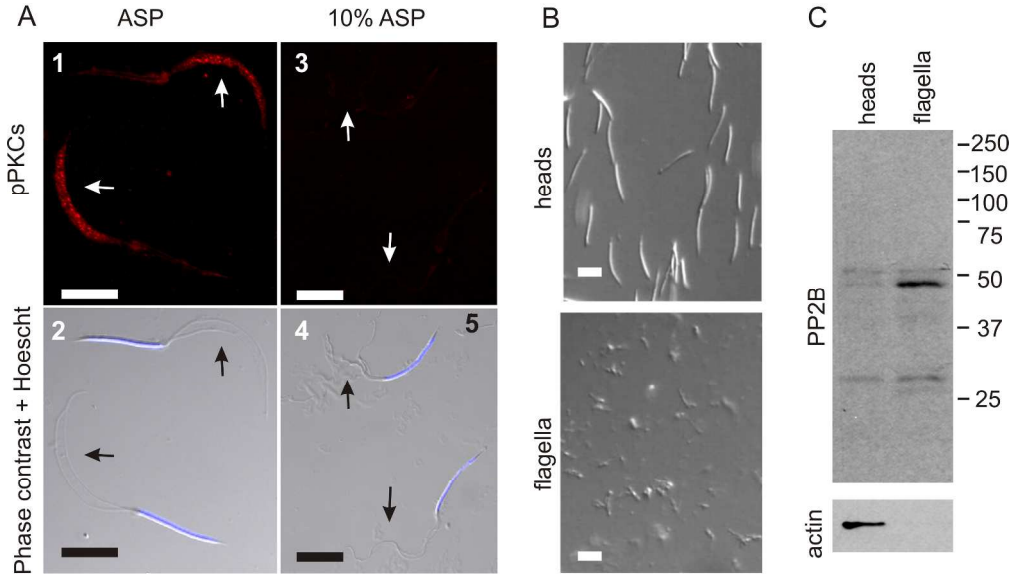
126x138mm (300 x 300 DPI)



Calcium is involved in both dephosphorylation of PKC substrates and progressive sperm motility. (A) Sperm suspensions were incubated in either ASP or 10% ASP supplemented with different concentrations of EGTA (in mM), during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×106 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control. (B) Sperm motility analysis after 5 min incubations in 10% ASP or EW, supplemented with different concentrations of EGTA. Motility patterns were identified as progressive movement (rapid forward movement) and in situ (only flagellar beating). Data represent mean±SD; n=10; *p<0.05 and **p<0.001 compared to untreated sample). (C) Sperm suspensions were incubated in ASP or ASP supplemented with 10 μM Ca2+ ionophore A23187 during 5 min. Each lane contains 1% Tritón X-100 soluble proteins of 7×106 sperm analyzed by 10% SDS/PAGE and Western blot using a phospho-PKC substrate-specific antibody (anti-pPKCs). Western blot for actin was performed as loading control.
60x26mm (300 x 300 DPI)



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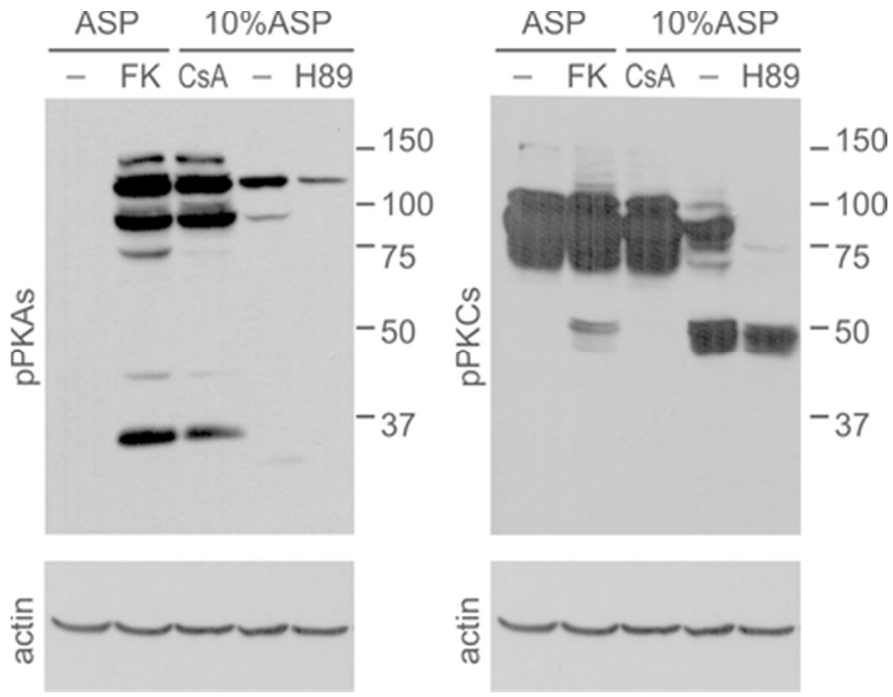

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37x28mm (300 x 300 DPI)