PKA and CaMKII mediate PI3K activation in bovine sperm by inhibition of the PKC/PP1 cascade

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Abstract

To enable fertilization, spermatozoa must undergo several biochemical processes in the female reproductive tract, collectively called capacitation. These processes involve protein kinase A (PKA)-dependent protein tyrosine phosphorylation including phosphatidylinositol-3-kinase (PI3K). It is not known how PKA, a serine/threonine (S/T) kinase, mediates tyrosine phosphorylation of proteins. We recently showed that inhibition of S/T phosphatase 1 (PP1) causes a significant increase in phospho-PI3K. In this study, we propose a mechanism by which PKA and PP1 mediate an increase in PI3K tyrosine phosphorylation and implicate calmodulin-dependent kinase II (CaMKII) in this process. Inhibition of sperm PP1 or PKC, stimulated CaMKII phosphorylation/activation, and inhibition of PKC enhanced PP1 phosphorylation/inactivation. Inhibition of CaMKII, using KN-93, caused significant reduction in phospho-PP1, indicating its activation. Moreover, KN-93 prevented the dephosphorylation/inactivation of PKC. We therefore suggest that CaMKII inhibits PKC, leading to PP1 inhibition and the reciprocal auto-activation of CaMKII. Thus, CaMKII can regulate its own activation by inhibiting the PKC/PP1 cascade. Inhibition of Src family kinases (SFK) caused significant inhibition of CaMKII and PP1 phosphorylation, suggesting that SFK activity results in PP1 inhibition and CaMKII activation. Activation of sperm PKA by 8Br-cAMP revealed an increase in phospho-CaMKII, which was inhibited by PKA inhibitor. Tyrosine phosphorylation of PI3K was stimulated by 8Br-cAMP and by PKC or PP1 inhibition and was abrogated by CaMKII inhibition. Furthermore, phosphorylation/activation of the tyrosine kinase Pyk2 was enhanced by PP1 inhibition, and this activation is blocked by CaMKII inhibition. Thus, PKA activates Src, which inhibits PP1, leading to CaMKII and Pyk2 activation, resulting in PI3K tyrosine phosphorylation/activation.

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Introduction

To acquire the ability to fertilize the egg, mammalian spermatozoa must undergo a series of biochemical processes in the female reproductive tract, collectively called capacitation. These processes include cholesterol efflux from the sperm plasma membrane, increased influx of bicarbonate, and Ca^{2+} , which activate soluble adenylyl cyclase (sAC) to produce cAMP, leading to protein kinase A (PKA) activation, protein tyrosine phosphorylation, actin polymerization, and the development of hyperactivated motility (rev. in Breitbart (2003)). The mechanism by which PKA, a serine/threonine (S/T) kinase, mediates protein tyrosine phosphorylation is not fully understood. In murine sperm, PKA co-immunoprecipitates with the tyrosine kinase Src, resulting in Src phosphorylation (Baker *et al.* 2006). We recently reported that PKA can activate Src in bovine sperm (Etkovitz *et al.* 2009).

We have previously shown that PKA activation leads to PKC inhibition (Cohen *et al.* 2004) and that PKA mediates phosphatidylinositol-3-kinase (PI3K) phosphorylation on Tyr-458 and actin polymerization in bovine sperm

(Etkovitz *et al.* 2007). PKA can directly phosphorylate the PI3K (p85) regulatory subunit on Ser-83, leading to its activation (Cosentino *et al.* 2007, De Gregorio *et al.* 2007). PI3K α belongs to PI3K class 1A enzymes, which are primarily responsible for the production of $\text{PIP}_{3(3,4,5)}$ in response to growth factors (Cantley 2002). PI3K is implicated in many biological processes, including cell survival, growth, movement and adhesion, protein synthesis, and cytoskeletal rearrangement. A role for PI3K has been suggested in sperm functions during capacitation and in the acrosome reaction (Fisher *et al.* 1998, Etkovitz *et al.* 2007, Jungnickel *et al.* 2007). The PI3K catalytic and regulatory subunits are present in sperm (Jungnickel *et al.* 2007), and the PI3K catalytic subunit inhibitor wortmannin (10 nM) inhibits PI3K and PIP_3 production in bovine sperm (Etkovitz *et al.* 2007). In other cell types, it has been shown that PKC α can inhibit PI3K activity directly (Sipeki *et al.* 2006) or indirectly (Guan *et al.* 2007). In bovine sperm, activation of PKC leads to PI3K inhibition (Etkovitz *et al.* 2007).

The PKC α isoform belongs to the classical PKC subfamily that comprises S/T kinases and can regulate

cell proliferation, differentiation, and apoptosis by affecting ion channels, receptors, and enzymes (rev. by Hofmann (2004)). In spermatozoa, PKC was found to participate in sperm capacitation, acrosome reaction, and motility (Breitbart *et al.* 1992, Breitbart & Naor 1999, Cohen *et al.* 2004, Almog *et al.* 2008).

A direct role for PKC α in the activation of the S/T phosphatase PP2 resulting in PI3K inhibition through removal of PI3K phosphate was shown in epithelial cells (Guan *et al.* 2007). Both the S/T phosphatases PP1 and PP2 are present in sperm and are found to participate in sperm capacitation, acrosome reaction, and motility (Furuya *et al.* 1993, Ashizawa *et al.* 2006, Chakrabarti *et al.* 2007). In mammals, four PP1c isoforms were identified: PP1 α , PP1 β , PP1 γ 1, and PP1 γ 2, in which PP1 γ 2 is testis/sperm specific and important for sperm motility and morphological processes during spermatogenesis (Chakrabarti *et al.* 2007).

Very little is known about the role and mechanisms of action of PP1 in spermatozoa. In a recent study, we proposed a signaling pathway that involves the regulation of PI3K activation by PKC α , PKA, and PP1 γ 2 in bovine sperm capacitation (Rotman *et al.* 2010). The Ca²⁺/calmodulin-dependent kinase II (CaMKII) is a widely distributed S/T kinase that mediates flagellar and ciliary motility (Smith 2002, Mwimbi *et al.* 2003, Nomura *et al.* 2004). Several studies have implicated CaMKII in sperm capacitation (Ackermann *et al.* 2009). It was also shown that CaMKII mediates the development of hyper-activated motility, a known parameter of sperm capacitation (Ignatz & Suarez 2005) and regulates protein tyrosine phosphorylation (González-Fernández *et al.* 2013), two known parameters of sperm capacitation.

In this study, we propose CaMKII as a key enzyme in the regulation of PI3K activation, which activates Pyk2 to phosphorylate PI3K on Tyr-458. We suggest that CaMKII can undergo self-regulation by inhibiting PKC leading to inhibition of PP1, resulting in CaMK activation. Another pathway to activate CaMK involves PKA-dependent activation of Src, which in turn inhibits PP1 leading to CaMKII activation.

Materials and methods

Materials and antibodies

Calyculin A, bisindolylmaleimide I (GF109203X (GF)), KN-93, and SU6656 were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal anti-PKC α , rabbit polyclonal anti-phospho-PKC α (Thr-497), and polyclonal anti- β -actin (C4)-HRP were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-PP1 α (Thr-320), rabbit polyclonal anti-phospho-PI3K (Tyr-458), and rabbit polyclonal anti-phospho-Pyk2 (Tyr-402) were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal anti-phospho-CaMKII α (Thr-286) was purchased from Abcam

(Cambridge, UK). Mouse monoclonal anti-CaMKII α was purchased from Millipore (Billerica, MA, USA). Rabbit-anti-PP1 γ 2 was kindly provided by Prof. S Vijayaraghavan (Department of Biological Sciences, Kent State University, Kent, OH, USA). All other chemicals were purchased from Sigma-Aldrich Israel Ltd., unless otherwise stated.

Sperm preparation

Ejaculated bull spermatozoa were obtained using artificial vagina, and the 'swim-up' technique was applied to obtain motile sperm. Bovine sperm was supplied by the SION Artificial Insemination Center (Hafetz-Haim, Israel). Sperm cells were washed three times by centrifugation (780 *g* for 10 min at 25 °C) in NKM buffer (110 mM NaCl, 5 mM KCl, and 20 mM 3-*N*-morpholino propane sulfonic acid (MOPS) (pH 7.4)) and the sperm were allowed to swim up after the last wash. The washed cells were counted and maintained at 39 °C until use. Only sperm samples that contained at least 80% motile sperm were used in the experiments.

Sperm capacitation

In vitro capacitation of bovine sperm was induced as described previously (Parrish *et al.* 1999). Briefly, sperm pellets were re-suspended to a final concentration of 10⁸ cells/ml in mTALP (modified Tyrode solution) medium (100 mM NaCl, 3.1 mM KCl, 1.5 mM MgCl₂, 0.92 mM KH₂PO₄, 25 mM NaHCO₃, 20 mM HEPES (pH 7.4), 0.1 mM sodium pyruvate, 21.6 mM sodium lactate, 10 IU/ml penicillin, 1 mg/ml BSA, 20 μ g/ml heparin, and 2 mM CaCl₂). The cells were incubated in this capacitation medium for 4 h at 39 °C. The capacitation state of the sperm was routinely confirmed after the 4-h incubation in mTALP by examining the ability of the sperm to undergo the acrosome reaction induced by the addition of the calcium ionophore A23187 (10 μ M).

Assessment of sperm acrosome reaction

Washed cells (10⁸ cells/ml) were capacitated for 4 h at 39 °C in mTALP medium. Acrosome reaction (AR) inducer was then added for another 20 min of incubation. The percentage of acrosome-reacted sperm was determined microscopically using FITC-conjugated *Pisum sativum* agglutinin (PSA). An aliquot of spermatozoa (10⁶ cells) was smeared on a glass slide and allowed to air-dry. The sperm were then permeabilized with methanol for 15 min at room temperature, washed three times at 5-min intervals with TBS, air dried, and then incubated with FITC-PSA (50 μ g/ml in TBS) for 30 min, washed twice with H₂O at 5-min intervals, and mounted with FluoroGuard Antifade (Bio-Rad Laboratories). For each experiment, at least 200 cells/slide on duplicate

slides were evaluated (total of 400 cells for one experiment). Cells with green staining over the acrosomal cap were considered acrosome intact; those with equatorial green staining or no staining were considered acrosome reacted.

Immunoblot analysis

Sperm were washed by centrifugation for 5 min at 10 000 *g* at 4 °C and then the supernatant was discarded and the pellet was re-suspended in TBS and centrifuged again to remove remaining traces of BSA. Sperm lysates were prepared by the addition of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 6% SDS, protease inhibitor cocktail 1:100, 50 μ M NaF, 50 μ M Na-pyrophosphate, 0.2 mM Na₃VO₄, and freshly added 1 mM phenylmethylsulfonyl fluoride), to the pellet, and the lysate vortexed vigorously for 15 min at room temperature. Lysates were then centrifuged for 5 min at 10 000 *g* at 4 °C, the supernatant was transferred, and the protein concentration was determined by the bicinchoninic acid (BCA) method (Smith *et al.* 1985). Sample buffer 5 $\times$ was added to the supernatant, and the sample was boiled for 5 min. The extracts were separated on 7 or 10% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. Blots were routinely stained with Ponceau solution to confirm equal loading and even transfer. The blots were blocked with 1% BSA in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. The membranes were incubated overnight at 4 °C with the primary antibodies diluted in 1% BSA in TBST. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with specific HRP-linked secondary antibodies (Bio-Rad Laboratories), diluted 1:5000 in TBST and 1% BSA. The membranes were washed three times with TBST and visualized by ECL (Amersham).

Results

We previously showed an increase in tyrosine phosphorylation of PI3K during capacitation of bovine sperm (Etkovitz *et al.* 2007). We also showed that the level of this phosphorylation is significantly enhanced by treating the sperm cells with calyculin A, a S/T phosphatase inhibitor with high specificity toward PP1 (Rotman *et al.* 2010). In this study, we explain the mechanism by which the inhibition of the S/T phosphatase PP1 enhances the tyrosine phosphorylation level of PI3K.

CaMKII phosphorylation is affected by PP1, PKC, and Src

Sperm incubated under capacitation conditions in the presence of the PP1 inhibitor, calyculin A, showed

Figure 1 PP1 inactivation enhances CaMKII and PI3K phosphorylation. Bovine spermatozoa were incubated in capacitation medium with or without calyculin A (100 nM) for 2 h. Proteins were extracted and analyzed by western blot using anti-phospho-PI3K, anti-phospho-CaMKII α , and anti-actin (loading control) antibodies. The results shown are representative of three independent experiments.

significant enhancement of PI3K phosphorylation on tyrosine 458 and CaMKII on threonine 286 (Fig. 1). As inhibition of PP1 also enhanced CaMKII phosphorylation (Fig. 1) and it was previously shown that CaMKII can activate the tyrosine kinase Pyk2 (Della Rocca *et al.* 1997, Soltoff 1998, Zwick *et al.* 1999, Heidinger *et al.* 2002, Fan *et al.* 2005, Montiel *et al.* 2007), which can tyrosine phosphorylate PI3K (Dikic *et al.* 1996, Schlaepfer & Hunter 1996, Avraham *et al.* 2000, Rocic *et al.* 2001), we also tested whether CaMKII can mediate PI3K tyrosine phosphorylation in sperm capacitation. CaMKII was already phosphorylated at the beginning of the capacitation process, with some decrease in the phosphorylation after 2 h of incubation (Fig. 2). The calmodulin inhibitor W-7 caused significant reduction in p-CaMKII (Fig. 2). It is known that CaMKII undergoes self-phosphorylation, and inhibition of this activity by KN-93 causes significant reduction in p-CaMK (as shown later), indicating that KN-93 can be used as CaMKII inhibitor in sperm cells. KN-93 is a membrane-permeable CaMKII inhibitor that prevents Ca²⁺-calmodulin binding to CaMKII and thereby inhibits its activation (Hudmon & Schulman 2002, Hunter & Schulman 2005). No change in percent of motile sperm was observed in KN-93-treated sperm.

It is known that PP1 dephosphorylates/inactivates CaMKII in other cell types (Hwang *et al.* 1996, Lisman & Zhabotinsky 2001). It was shown elsewhere (Srinivasan & Begum 1994) that activation of PKC leads to PP1 activation. We suggested that PKC α mediates the activation of PP1 in bovine sperm (Rotman *et al.* 2010). Thus, we assumed that inhibition of PKC would affect CaMKII activity via inhibition of PP1. To detect the phosphorylation state of PP1, we used an antibody against p-(Thr-320)-PP1 α able to recognize phosphorylated PP1 γ 2. It has been shown elsewhere (Huang & Vijayaraghavan 2004) that an antibody against p-PP1 α can recognize phosphorylated PP1 γ 2. This is due to the fact that testis- and sperm-specific PP1 γ 2 has the sequence of TRPTPPR, which differs by only one amino acid from the corresponding sequence in PP1 α but is the

Figure 2 Phosphorylation of CaMKII in sperm capacitation. Bovine spermatozoa were incubated in mTALP (capacitation medium) with or without the CaM inhibitor W-7 (50 μ M) for 2 h. Proteins were extracted at the indicated times and analyzed by western blot using anti-phospho-CaMKII α , anti-CaMKII α , and anti-actin (loading control) antibodies. The results shown are representative of three independent experiments.

same as that of PP1 γ 1. Sperm treatment with GF, a PKC inhibitor, caused an increase in p-PP1 and p-CaMKII, indicating PP1 inactivation and CaMKII activation (Fig. 3A). Thus, as was found for direct inhibition of PP1 by calyculin A (Fig. 1), indirect inhibition of PP1 by inhibiting PKC activates CaMKII. It was suggested elsewhere that the Src family kinases (SFK) are able to phosphorylate PP1 α resulting in PP1 inactivation (Srinivasan & Begum 1994, Villa-Moruzzi & Puntoni 1996, Mallozzi *et al.* 2005, Mao *et al.* 2005). We assumed that sperm PP1 might also be inhibited by SFK, and indeed, we showed that treatment of sperm with SU6656, a known SFK inhibitor, caused a decrease in CaMKII and PP1 phosphorylation levels (Fig. 3B). These data suggest that dephosphorylation/activation of PP1 by inhibiting SFK causes inactivation of CaMKII. Moreover, we found that the CaMKII inhibitor KN-93 caused a dephosphorylation/activation of p-PP1 (Fig. 3C) and prevented the dephosphorylation/inactivation of PKC α (Fig. 3C), suggesting that CaMK inhibits PP1 activation through inactivation of PKC α .

PKA mediates CaMKII phosphorylation

In our previous study, we showed that activation of sperm PKA causes significant increase in tyrosine phosphorylation of PI3K (Etkovitz *et al.* 2007) and further suggested that PKA can activate Src, leading to EGFR phosphorylation on Tyr-845, the site known to be phosphorylated by Src (Etkovitz *et al.* 2009). It has been proposed that Src is activated by a cAMP-mediated pathway via inhibition of Csk, the tyrosine kinase responsible for inactivation of c-Src in other cell types (Baker *et al.* 2006). Here, we showed that inhibition of Src by SU6656 caused a significant reduction in phospho-CaMKII (Fig. 3B). Thus, we assumed that PKA might mediate CaMKII phosphorylation. The data in Fig. 4A show that H-89, a known PKA inhibitor, caused significant reduction in p-CaMKII, and addition of the permeable 8Br-cAMP, which activates PKA, enhanced p-CaMKII (Fig. 4B). Addition of 8Br-cAMP enhanced PI3K phosphorylation, which was inhibited by the

CaMKII inhibitor KN-93 (Fig. 4C). Thus, a possible mechanism by which PKA mediates CaMKII phosphorylation is as follows: PKA activates Src which in turn blocks PP1 leading to an increase in p-CaMKII.

Figure 3 The dual relationship between PKC/PP1 and CaMKII. (A) Bovine spermatozoa were incubated in NKM (starvation medium) for 1.5 h with or without GF109203X (0.1 nM) and then transferred to mTALP (capacitation medium) for 15 or 30 min. Proteins were extracted and analyzed by western blot using anti-PP1 γ 2, anti-phospho-PP1 α , anti-phospho-CaMKII, and anti-actin (loading control) antibodies. (B) Bovine spermatozoa were incubated in NKM (starvation medium) for 1.5 h with or without SU6656 (50 μ M) and then transferred to mTALP (capacitation medium) for 2 h. Proteins were extracted and analyzed by western blot using anti-PP1 γ 2, anti-phospho-PP1 α , anti-phospho-CaMKII, and anti-tubulin (loading control) antibodies. (C) Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) for 4 h. Proteins were extracted at the indicated times and analyzed by western blot using anti-phospho-PP1 α , anti-PP1 γ 2, and anti-tubulin (loading control) antibodies. (D) Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) for 2 h. Proteins were extracted and analyzed by western blot using anti-phospho-PKC α , anti-PKC α , and anti-tubulin (loading control) antibodies. The results shown are representative of three independent experiments.

Figure 4 The effect of PKA activation on CaMKII. (A) Bovine spermatozoa were incubated in capacitation medium with or without H-89 (50 μ M) for 2 h. Proteins were extracted and analyzed by western blot using anti-phospho-CaMKII and anti-tubulin (loading control) antibodies. (B) Bovine spermatozoa were incubated in capacitation medium with or without 8Br-cAMP (1 mM) for 2 h. Proteins were extracted and analyzed by western blot using anti-phospho-CaMKII and anti-actin (loading control) antibodies. (C) Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) or 8Br-cAMP (1 mM) for 2 h. Proteins were extracted and analyzed by western blot using anti-phospho-PI3K and anti-actin (loading control) antibodies. The results shown are representative of three independent experiments.

Tyrosine phosphorylation of PI3K is affected by CaMKII, PKC, and PP1

Sperm incubated under capacitation conditions showed an increase in tyrosine phosphorylation/activation of PI3K after 2–4 h of incubation (Fig. 5A). This phosphorylation was abrogated in cells treated with KN-93 (Fig. 5A), indicating the involvement of CaMKII in PI3K activation. Inhibition of PKC using GF or long incubation with 12-o-tetradecanoyl phorbol-13-acetate (PMA) revealed a significant increase in p-PI3K, which was completely blocked by KN-93 (Fig. 5B). Moreover, inhibition of PP1 by calyculin A also enhanced p-PI3K, and the increased phosphorylation was completely blocked by KN-93 (Fig. 5C). These results suggest that CaMK might inhibit PKC, leading to inhibition of PP1, and resulting in an increase of p-PI3K. Indeed, as shown in Fig. 3D, KN-93 prevented the dephosphorylation/inactivation of PKC during sperm capacitation, indicating that CaMKII induced inhibition of PKC activity.

CaMKII activates the tyrosine kinase Pyk2

In other cell types, CaMKII can activate Pyk2 (Della Rocca *et al.* 1997, Soltoff 1998, Zwick *et al.* 1999, Heidinger *et al.* 2002, Fan *et al.* 2005, Montiel *et al.* 2007), which can activate PI3K (Dikic *et al.* 1996, Schlaepfer & Hunter 1996, Avraham *et al.* 2000, Rocic *et al.* 2001). Thus, we expected Pyk2 to be activated by CaMKII during sperm capacitation, leading to PI3K activation. Indeed, we found that calyculin A enhanced Pyk2 phosphorylation on tyrosine 402, and this phosphorylation was blocked by KN-93 (Fig. 6). These data indicate that CaMKII activates Pyk2. Moreover, the enhanced phosphorylation by calyculin A of CaMKII on threonine and of Pyk2 on tyrosine indicates that PP1 indirectly affects the Pyk2 phosphorylation state and

Figure 5 PP1 inactivation enhances PI3K phosphorylation through activation of CaMKII. (A) Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) for 4 h. Proteins were extracted at the indicated times and analyzed by western blot using anti-phospho-PI3K and anti-actin (loading control) antibodies. (B) Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) for 10 min and then PMA (100 ng/ml) or GF109203X (0.1 nM) was added for additional 2 h of incubation. Proteins were extracted and analyzed by western blot using anti-phospho-PI3K and anti-actin (loading control) antibodies. (C) Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) for 10 min and then calyculin A (100 nM) was added for additional 2 h of incubation. Proteins were extracted and analyzed by western blot using anti-phospho-PI3K and anti-actin (loading control) antibodies. The results shown are representative of three independent experiments.

Figure 6 CaMKII activates PyK2 during capacitation. Bovine spermatozoa were incubated in capacitation medium with or without KN-93 (50 μ M) for 10 min and then calyculin A (100 nM) was added for additional 2 h of incubation. Proteins were extracted and analyzed by western blot using anti-phospho-PyK2, anti-phospho-CaMKII, and anti-actin (loading control) antibodies. The results shown are representative of three independent experiments.

further suggests that PP1 dephosphorylates/inactivates CaMKII, leading to dephosphorylation/inactivation of PyK2.

CaMKII prevents the spontaneous acrosome reaction

It was shown elsewhere that CaMKII recruits synaptic vesicles to the active zone of the presynaptic nerve terminal (Greengard *et al.* 1993, Leal-Ortiz *et al.* 2008). This pathway resembles the situation in the acrosome reaction, in which outer acrosomal membrane-specific sites interact with the overlying plasma membrane. Moreover, it was shown that inhibition of CaMKII using the permeable peptide AIPII or the calmodulin inhibitor W-7, but not KN-93, causes a significant increase in the occurrence of spontaneous acrosomal exocytosis in mouse sperm (Ackermann *et al.* 2009). We found that incubation of bovine sperm under capacitation conditions in the presence of KN-93 or W-7 caused a significant increase (72 and 45% respectively) in the spontaneous acrosome reaction rate compared with the control cells (24%) (Fig. 7). Moreover, it was shown that KN-93 inhibits hyper-activated motility in bovine sperm (Ignatz & Suarez 2005). Thus, it seems that sperm sensitivity to KN-93 differs among species. As the spontaneous acrosome reaction is very high (72%) in KN-93-treated sperm, the ionophore-induced acrosome reaction is only 6% compared with the 48% found in the control cells. Surprisingly, the ionophore-induced acrosome reaction was inhibited by W-7 (Fig. 7), suggesting that calmodulin might be a positive regulator of the induced acrosome reaction. In conclusion, these data suggest that active CaMKII protects sperm from undergoing spontaneous acrosome reaction, a process known to negatively affect the fertilization rate (Wiser *et al.* 2013).

Discussion

In this study, we describe possible mechanisms by which PP1 regulates tyrosine phosphorylation of PI3K. We showed that inhibition of PP1 enhanced PI3K as well as

CaMKII phosphorylation (Fig. 1). In our previous study in bovine sperm, we suggested that PP1 can be activated by PKC (Rotman *et al.* 2010). Here, inhibition of PKC caused significant increase in p-CaMKII and p-PP1 (Fig. 3A), indicating the activation of CaMKII and inhibition of PP1. Moreover, we show that KN-93 inhibits dephosphorylation/inactivation of PKC α (Fig. 3D), suggesting that CaMKII inhibits PKC α activity by enhancing its dephosphorylation. Altogether, our data suggest that CaMKII can inhibit PKC leading to inactivation of PP1 and in return an increase in CaMKII phosphorylation/activation occurs. Thus, CaMKII can regulate its own activity by the inhibition of PP1 α and preventing self-dephosphorylation. This conclusion is supported by reports demonstrating that PP1 can dephosphorylate/inactivate CaMKII (Hwang *et al.* 1996, Lisman & Zhabotinsky 2001). Another factor that can affect PP1 is Src (Villa-Moruzzi & Puntoni 1996, Huang & Vijayaraghavan 2004), whose inhibition revealed complete reduction/inactivation of p-CaMKII, and reduction/activation of p-PP1 α (Fig. 3B). Thus, Src mediates PP1 α inhibition leading to CaMKII activation. Altogether, we suggest that PP1 is activated by PKC leading to CaMKII inhibition, and inhibition of PP1 by Src leads to CaMKII activation. The possible involvement of Src in PP2A inhibition in mouse sperm has been suggested elsewhere (Krapf *et al.* 2010). Furthermore, we show in bovine sperm that Src mediates PKA-dependent EGFR phosphorylation on Tyr-845, a known Src-specific phosphorylation site (Etkovitz *et al.* 2009). We also show that Src mediates Tyr-438 phosphorylation/inactivation of the actin-severing protein, gelsolin, leading to an increase in actin polymerization and hyper-activated motility during human sperm capacitation (Finkelstein *et al.* 2013). In our previous study,

Figure 7 CaMKII enhances the spontaneous acrosome reaction. Bovine spermatozoa were incubated in capacitation medium for 4 h with or without KN-93 (50 μ M) or W-7 (50 μ M). Calcium ionophore (A23187, 10 μ M) was added for an additional 20 min. Sperm samples were smeared on slides to determine acrosome reaction, as described in the 'materials and methods' section. These data represent the mean $\pm$ s.d. of duplicates from at least three experiments. *Significant difference from the corresponding control, $P < 0.05$.

Figure 8 Schematic presentation of the mechanisms underlying CaMKII activation during bovine sperm capacitation. During capacitation, PKA is activated by cAMP generated by the bicarbonate-activated soluble adenylyl cyclase (sAC). PKA phosphorylates/activates SRC, which in turn inhibits PP1, leading to CaMKII activation. CaMKII activates PyK2, which phosphorylates PI3K.

we suggested that Src can be activated by PKA in bovine sperm (Etkovitz *et al.* 2009). This conclusion was further supported by others in human sperm (Mitchell *et al.* 2008). Here, we show that inhibition of PKA caused significant inhibition of CaMKII phosphorylation and activating PKA by adding 8Br-cAMP to the cells stimulated CaMKII phosphorylation (Fig. 4A and B). Furthermore, the enhanced effect of 8Br-cAMP on PI3K phosphorylation was inhibited by KN-93 (Fig. 4C). These data suggest that PKA mediates CaMKII activation through the following mechanism: PKA activates Src that inhibit PP1 α leading to CaMKII activation as suggested earlier. In a recent study, we suggested that PKA can mediate PKC α dephosphorylation/inactivation; however, PP1 is not responsible for direct PKC dephosphorylation, as inhibition of PP1 did not influence the PKC phosphorylation level (Rotman *et al.* 2010). In this study, we suggest a mechanism for this effect, in which PKA activates Src, which mediates PP1 α inhibition, leading to CaMKII activation and PKC inhibition. It was shown elsewhere that PKA can directly phosphorylate/activate CaMK (Ferrero *et al.* 2007); however, in this study, we showed that CaMKII phosphorylation is completely inhibited by inhibition of Src (Fig. 3B); thus, we conclude that Src mediates CaMKII phosphorylation in bovine sperm.

Next, we tried to understand the relationships between CaMKII and PI3K. We show that PI3K tyrosine phosphorylation during sperm capacitation is inhibited by KN-93 (Fig. 5A), indicating that CaMK mediates this phosphorylation. Inhibition of PKC by GF or its down-regulation by long incubation with PMA or inhibition of PP1 caused a significant increase in p-PI3K, which was inhibited by KN-93 (Fig. 5B and C), further supporting the phosphorylation of PI3K by CaMK. This finding is also supported by the fact that inhibition of PKC or PP1 caused CaMKII activation, as well (Figs 1 and 3A). In order to explain these relationships, we tested the involvement of Pyk2, and we found that inhibition of PP1 enhanced Pyk2 phosphorylation, which is inhibited by KN-93 (Fig. 6), indicating that CaMKII activates Pyk2, a known activator of PI3K. Other studies showed that

CaMKII can activate the tyrosine kinase Pyk2 (Della Rocca *et al.* 1997, Soltoff 1998, Zwick *et al.* 1999, Heidinger *et al.* 2002, Fan *et al.* 2005, Montiel *et al.* 2007), which can phosphorylate PI3K on tyrosine (Dikic *et al.* 1996, Schlaepfer & Hunter 1996, Avraham *et al.* 2000, Rocic *et al.* 2001).

In our previous publication, we showed PKA-dependent activation of PI3K and inhibition by PKC in bovine sperm capacitation (Etkovitz *et al.* 2007). However, the activation of PI3K by PKA is not due to its possible inhibitory effect on PKC, as inhibition of PKA abrogated the enhanced effect of PKC inhibition on p-PI3K (Rotman *et al.* 2010). Thus, PKC inhibition by itself cannot activate PI3K unless PKA is active. It is known that PKA can phosphorylate p85, the regulatory subunit of PI3K on Ser-83, which is important for enzyme activation (Cosentino *et al.* 2007). Here, we further suggest a mechanism by which PKA mediates Tyr-458 phosphorylation of PI3K. We propose the following cascade: PKA activates Src, which inhibits PP1, leading to CaMK activation. This leads to Pyk2 activation and tyrosine phosphorylation of PI3K. Incubation of the sperm in non-capacitation medium (NKM medium) revealed relatively rapid dephosphorylation of CaMKII (Fig. 2). Under these conditions, PKA cannot be activated as there is no bicarbonate in the medium. Bicarbonate activates sAC that comprises 95% of sperm AC; thus, in the absence of bicarbonate, cAMP is not practically produced and no PKA activation occurs. According to our model, when PKA is inactive, PP1 is active, resulting in CaMKII dephosphorylation.

It is not clear what function CaMKII fulfills in sperm physiology besides its regulation of PI3K, which participates in actin polymerization during capacitation (Etkovitz *et al.* 2007) and in the acrosome reaction (Etkovitz *et al.* 2009, Breitbart *et al.* 2010). Here, we showed that inhibition of CaMK caused high increase in spontaneous acrosome reaction (Fig. 7). This may suggest that active CaMKII protects the sperm cell from prematurely undergoing the acrosome reaction. In a recent study, it was suggested that CaMKII interacts with the protein, MUPP1, and prevents spontaneous

acrosomal exocytosis in mouse sperm (Ackermann *et al.* 2009). MUPP1 is present in the acrosomal region of sperm of various mammals, including bovine sperm (Heydecke *et al.* 2006), and mediates the recruitment of molecules that control the initial tethering of acrosome-specific sites to the plasma membrane (Ackermann *et al.* 2009). It is possible that CaMKII catalyzes the phosphorylation of proteins responsible for keeping the acrosomal vesicles in an intermediate pre-assembled fusion state (Wang 2008), thereby preventing spontaneous acrosome reaction. Although a recent study shows that fertilizing mouse sperm undergoes the acrosome reaction within the cumulus (Jin *et al.* 2011), it is still accepted that the physiological acrosome reaction occurs after the binding of the capacitated sperm to the egg zona pellucida (Ward & Kopf 1993). Thus, sperm that undergo a spontaneous acrosome-like reaction before reaching the egg region cannot fertilize the egg. We recently showed that human sperm with a relatively high spontaneous acrosome reaction rate exhibit poor IVF, while low spontaneous acrosome reaction is associated with high fertilization potential (Wiser *et al.* 2013).

Figure 8 summarizes the pathway suggested by our findings. CaMKII is known to undergo self-phosphorylation and we suggest that this phosphorylation is also maintained by the ability of CaMKII to inhibit PKC/PP1. Therefore, inhibition of PP1 by calyculin A activates CaMKII and Pyk2, leading to PI3K activation. Moreover, PKA activates Src, which in turn mediates PP1 inhibition leading to CaMKII activation. Our data support our previous conclusion that the ratio between PKA and PKC activity during capacitation regulates PI3K activity (Etkovitz *et al.* 2007); activation of PKA enhances PI3K activity whereas activation of PKC reduces PI3K activity (Etkovitz *et al.* 2007). This study sheds light on the mechanism of this regulation, which is now better understood. We suggest that PKA activates PI3K via Src-dependent PP1 inhibition, and PKC inactivates PI3K via activation of PP1. Thus, PP1 plays a key role in this process, as its activity is enhanced by PKC and inhibited via PKA/Src, or CaMKII, which inhibits PKC leading to PP1 inhibition. PP1 activation causes dephosphorylation/inactivation of CaMKII, while PP1 inhibition maintains CaMKII in a high phosphorylation/activation state, leading to the activation of the tyrosine kinase Pyk2, which phosphorylates PI3K on tyrosine 458.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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