Protein kinase A and protein kinase C α /PPP1CC2 play opposing roles in the regulation of phosphatidylinositol 3-kinase activation in bovine sperm

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Abstract

In order to acquire fertilization competence, spermatozoa have to undergo biochemical changes in the female reproductive tract, known as capacitation. Signaling pathways that take place during the capacitation process are much investigated issue. However, the role and regulation of phosphatidylinositol 3-kinase (PI3K) in this process are still not clear. Previously, we reported that short-time activation of protein kinase A (PRKA, PKA) leads to PI3K activation and protein kinase C α (PRKCA, PKC α) inhibition. In the present study, we found that during the capacitation PI3K phosphorylation/activation increases. PI3K activation was PRKA dependent, and down-regulated by PRKCA. PRKCA is found to be highly active at the beginning of the capacitation, conditions in which PI3K is not active. Moreover, inhibition of PRKCA causes significant activation of PI3K. Similar activation of PI3K is seen when the phosphatase PPP1 is blocked suggesting that PPP1 regulates PI3K activity. We found that during the capacitation PRKCA and PPP1CC2 (PP1 γ 2) form a complex, and the two enzymes were degraded during the capacitation, suggesting that this degradation enables the activation of PI3K. This degradation is mediated by PRKA, indicating that in addition to the direct activation of PI3K by PRKA, this kinase can enhance PI3K phosphorylation indirectly by enhancing the degradation and inactivation of PRKCA and PPP1CC2.

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Introduction

For the acquisition of the eminent and vivacious ability of fertilizing an egg, mammalian spermatozoa have to undergo a series of biochemical transformations in the female reproductive tract, collectively called capacitation. There is no clear recognizable marker for the occurrence of capacitation, although several intracellular changes are known to occur, including increase in cholesterol efflux, membrane fluidity, intracellular Ca²⁺ and cAMP concentrations, protein tyrosine phosphorylation, actin polymerization, and changes in swimming patterns and chemostatic motility (reviewed in Breitbart (2003)). The elevation in cAMP concentration derived from entry of bicarbonate ions which together with calcium ions activate the soluble adenylyl cyclase (sAC; reviewed in Burton & McKnight (2007)), leading to protein kinase (PRKA, PKA) activation. The PRKA pathway is known to regulate the elevation of protein tyrosine phosphorylation during sperm capacitation (Visconti *et al.* 1995); however, the mechanism by which serine/threonine (S/T) kinase induces tyrosine phosphorylation is not fully understood.

In murine sperm, PRKA co-immunoprecipitates with the tyrosine kinase SRC, and this interaction leads to SRC phosphorylation (Baker *et al.* 2006). Activation of the PRKACA2 (PKA-C α 2) catalytic subunit is also involved in increasing flagellar beat frequency during the capacitation (Carlson *et al.* 2007). Inhibitors of PRKA blocked sperm capacitation and the associated increase in protein tyrosine phosphorylation (Leclerc *et al.* 1996).

We have previously shown that PRKA activation leads to protein kinase C α (PRKCA, PKC α) inhibition (Cohen *et al.* 2004), and that PRKA activation during the capacitation leads to phosphatidylinositol 3-kinase (PI3K) activation, leading to actin polymerization (Etkovitz *et al.* 2007). Recently, it has been shown that PRKA can directly phosphorylate PI3K (p85) regulatory subunit on Ser83, leading to its activation (Cosentino *et al.* 2007, De Gregorio *et al.* 2007) and further phosphorylation on tyrosine 467 (Etkovitz *et al.* 2007). PI3K α belongs to PI3K class IA enzymes which are primarily responsive for the production of PIP_{3(3,4,5)} in response to growth factors (Cantley 2002). PI3K is implicated in many biological processes, including cell survival, cell growth,

cell movement and adhesion, protein synthesis, and cytoskeletal rearrangements. A role for PI3K has been suggested in sperm functions during sperm capacitation and the acrosome reaction (Fisher *et al.* 1998, Etkovitz *et al.* 2007, Jungnickel *et al.* 2007). It was also shown that the PI3K catalytic and regulatory subunits are present in sperm (Jungnickel *et al.* 2007), and the PI3K catalytic subunit inhibitor wortmannin (WT) inhibited PI3K and PIP₃ production in bovine sperm (Etkovitz *et al.* 2007). In other cell types, it has been shown that PRKCA can inhibit PI3K activity directly (Sipeki *et al.* 2006) or indirectly (Guan *et al.* 2007).

The PRKCA isoform belongs to the classical PRKC subfamily which comprises S/T kinases that have been implicated in the regulation of a variety of cellular functions such as proliferation, differentiation and apoptosis, and regulation of ion channels, receptors, and enzymes (reviewed in Hofmann (2004)). PRKC roles in spermatozoa of many species have been widely investigated at our laboratory and by others, and PRKC was found to participate in many sperm functions, i.e. capacitation, acrosome reaction, and sperm motility (Breitbart *et al.* 1992, Breitbart & Naor 1999, Cohen *et al.* 2004, Almog *et al.* 2008).

A direct role for PRKCA in the activation of the S/T phosphatase PP2A that resulted in PI3K inhibition through removal of PI3K phosphorylation was recently shown in epithelial cells (Guan *et al.* 2007). The S/T phosphatases PPP1 and PPP2 are present in sperm cells, and were found to participate in the capacitation and acrosome reaction processes, and sperm motility (Furuya *et al.* 1993, Ashizawa *et al.* 2006, Chakrabarti *et al.* 2007). In the mammalian phylum, four PPP1C isoforms were identified: PPP1CA (PP1c α), PPP1CB (PP1c β), PPP1CC1 (PP1c γ 1), and PPP1CC2 (PP1c γ 2), of which the latter two are alternative splice variants of the *PPP1CC* gene. Current studies revealed PPP1CC2 as a testis-specific isoform that has been found to be required for flagellar and structural wholeness. In addition, it is also required for morphogenic processes during the spermiogenesis (Chakrabarti *et al.* 2007).

Very little is known about the role and mechanism of action of PPP1 in spermatozoa. The interesting connection between PRKCA and phosphatases in the regulation of PI3K, together with the fact that these proteins are involved in sperm functions, although never been investigated during the capacitation process, led us to investigate their possible relationships during the capacitation, with respect to PRKA activity. The changes in phosphorylation and the total level of these proteins were detected during the capacitation process.

In the present study, we propose a signaling pathway that involves the regulation of PI3K activation mediated by PRKCA, PRKA, and PPP1CC2 during bovine sperm capacitation.

Results

PI3K phosphorylation and PRKCA dephosphorylation/degradation during sperm capacitation

The phosphorylation state of Tyr467 on PI3K regulatory subunit p85 and of Ser657 and Thr497 on PRKCA, which indicate their activation, and the total level of these proteins were analyzed by western blot analysis using specific antibodies. Whole blot presentation shows the specificity of each of the antibodies used here (Fig. 1C and D). Bovine sperm proteins were extracted at the beginning and after 4 or 7 h of incubation in the capacitation medium. As shown in Fig. 1A and D, there was an elevation in PI3K phosphorylation during the incubation in the capacitation medium but not in non-capacitation conditions, while its total level was not changed (Fig. 1C). On the contrary, PRKCA underwent degradation and dephosphorylation ($27.05\% \pm 10.74$, and $37.76\% \pm 8.18$ respectively, as was determined from six different experiments) during the incubation in the capacitation medium but not under non-capacitative conditions (Fig. 1A, B, and D). The high phosphorylation rate of Thr497 as well as of Ser657 at time zero points to the possibility that PRKCA is highly active at the beginning of the capacitation and is down-regulated towards its end. This indication was further confirmed by using the fractionation method that revealed a prominent level of PRKCA in the membrane fraction at the beginning of the capacitation and its reduction during 4 h of incubation in the capacitation medium (Fig. 1E). It is well known that the translocation of PRKC to the membrane indicates its activation (Johnson *et al.* 1996). The main amount of membrane-bound PRKCA was phosphorylated at the beginning of the capacitation and underwent prominent dephosphorylation during this process, further indicating loss of activity (Fig. 1E). The presence of phosphorylated PRKCA in the cytosol fraction is not surprising since PRKCA is known to be present in closed-phosphorylated conformation in this fraction (Hansra *et al.* 1999). After 4 h of incubation, a small amount of phosphorylated PRKCA was present in the cytosol, and it almost disappeared from the membrane fraction, further supporting our notion regarding PRKCA desensitization during the capacitation process. Altogether, these data suggest that dephosphorylation and degradation of PRKCA during the incubation time are accompanied by phosphorylation/activation of PI3K, and these processes are capacitation dependent.

Activation of PI3K by cAMP/PRKA in sperm capacitation

The fact that PI3K phosphorylation was increased during the capacitation led us to explore which is the positive regulator of PI3K phosphorylation during this process. In our previous work in bovine sperm, we showed that PRKC is activated when PRKA activity is blocked, and

Figure 1 PRKCA is inhibited and PI3K is activated during sperm capacitation. Bovine sperm was incubated for 4 or 7 h in the mTALP capacitation medium (A–E) or NKM medium (non-capacitation medium) (A and B). (A) Capsulated or non-capsulated sperm proteins were extracted at the indicated times and western blotted as described in ‘Materials and Methods’. p-(Tyr467)-PI3K, p-(Ser657)-PRKCA, and PRKCA were detected using specific antibodies. (B) Capsulated or non-capsulated sperm proteins were extracted at the indicated times and western blotted. p-(Thr497)-PRKCA and PRKCA were detected using specific antibody. (C) PI3K was detected in sperm extracts at the indicated times using specific antibody. (A–C) β -Actin antibody was used to equalize the loading quantities. (D) p-(Tyr467)-PI3K, PRKCA, p-(Thr497)-PRKCA, or p-(Ser657)-PRKCA were detected at the indicated times, using specific antibodies on western blotted sperm extracts. Whole blots are presented. (E) Cells were fractionated into cytosol (Cyto) and membrane (Mem) fractions using Subcellular ProteoExtract kit, Calbiochem, according to the manufacturer’s instructions, and western blotted as described in ‘Materials and Methods’. PRKCA and p-(Thr497)-PRKCA were detected using specific antibodies. The results represent at least three independent experiments.

that activation of PRKA reduces PRKC activity (Cohen *et al.* 2004). In addition, our recent study revealed that PI3K is activated by PRKA and inhibited by PRKC in bovine sperm after short time of incubation (Etkovitz *et al.* 2007). Together, these data led us to investigate the requirement for PRKA activity in contrary to PRKCA activity, for the capacitation-dependent PI3K phosphorylation. First, PRKA activity in bovine sperm during the capacitation was assessed using an antibody that recognizes PRKA-phosphorylated substrates. Cells were incubated for 4 h in the capacitation medium or in bicarbonate-deficient medium in which the soluble adenylyl cyclase is not activated, cAMP is not produced, and PRKA is not activated (reviewed in Burton & McKnight (2007)), or in the presence of the PRKA inhibitor – H89 or with 8Br-cAMP, which activates PRKA. As shown in Fig. 2A, PRKA activity was increased during 4 h of incubation in the capacitation medium. This activity was inhibited when H89 was present in the medium and enhanced when 8Br-cAMP was added. Moreover, H89 abrogated the 8Br-cAMP-induced PRKA activation. Incubation in bicarbonate-deficient medium for 4 h did not allow the elevation in PRKA-phosphorylated substrates, but upon addition of 8Br-cAMP to this medium, PRKA was activated. H89 also prevented the 8Br-cAMP-dependent activation of PRKA in the

bicarbonate-deficient medium. In addition, PRKC inhibition by GF or Ro did not influence the phosphorylation level of PRKA-phospho-substrates (data not shown), further indicating the specificity of H89 for PRKA inhibition.

In order to investigate the possible effect of PRKA activity on PI3K activation, cells were incubated in the capacitation medium in the presence of the PRKA activator 8Br-cAMP, and time-dependent phosphorylation/activation of PI3K was examined. As shown in Fig. 2B, the addition of 8Br-cAMP to the capacitation medium led to remarkable and earlier elevation in PI3K phosphorylation (at 30 min compared with 2 h in the control). In addition, PI3K phosphorylation did not elevate when PRKA activation was prevented in bicarbonate-deficient medium (Fig. 2C). Treatment with the PRKA inhibitor H89 resulted in absence or very little PI3K phosphorylation after 4 h of incubation in the capacitation medium (mTALP; see Figs 3E and 4D). The addition of 8Br-cAMP also restored PI3K phosphorylation when added to bicarbonate-deficient medium (Fig. 2C). The addition of H89 together with 8Br-cAMP to mTALP medium or to bicarbonate-deficient medium prevented PI3K phosphorylation indicating the specificity of H89 (Fig. 2C). Together, these data point to the significant role of PRKA activity for PI3K activation.

Figure 2 Activation of PI3K by cAMP/PRKA in sperm capacitation. Bovine sperm was incubated for 4 h in the capacitation medium (mTALP) (A–C) or in the bicarbonate-deficient medium (mTALP W/O HCO_3^-) (A and C) in the presence or absence of (50 μM) H89 (A and C), (0.1 mM) 8Br-cAMP (cAMP) (A–C), and (162 nM) PMA (C). Sperm proteins were extracted at the indicated times and western blotted as described in ‘Materials and Methods’. PRKA-phospho-substrates (A) and p-PI3K (B and C) were detected using specific antibodies, and β -actin antibody was used to equalize the loading quantities. The results represent at least three independent experiments.

The effects of PRKC agonist and antagonists on PI3K and PRKCA phosphorylation and degradation

In order to assess the influence of the PRKCA isoform on PI3K during the capacitation time, cells were incubated in the presence of two PRKC inhibitors, GF which is a general PRKC inhibitor and Ro which specifically inhibits the PRKCA isoform. The treatment with GF or Ro resulted in rapid and massive elevation in PI3K phosphorylation (Fig. 3A and B respectively), indicating the negative regulation of PRKCA or other isoform of PRKC on PI3K activity. The higher PI3K phosphorylation resulting from GF treatment as shown in Fig. 3A compared to relative low enhancement caused by Ro as shown in Fig. 3B is due to normal differences between sperm cells taken from different bulls. Therefore, when these agents were applied to sperm cells from the same bull, the elevation of PI3K phosphorylation in the presence of GF or Ro was similar suggesting that PRKCA is the negative regulator of PI3K (Fig. 3D). GF and Ro also decelerated the rate of PRKCA degradation (Fig. 3C). It is

established that in order to undergo degradation PRKCA should be active (Hansra *et al.* 1999).

In order to investigate the importance of PRKA as a PI3K activator as opposed to PRKCA as PI3K inhibitor, cells were long-time (4 h) treated with phorbol 12-myristate 13-acetate (PMA) in bicarbonate-deficient medium. As shown in Fig. 2C, PMA-induced PI3K phosphorylation did not occur when cells were incubated in bicarbonate-deficient medium. Moreover, PRKA inhibition by H89 in the capacitation medium that contained PMA prevented the PMA-induced PI3K phosphorylation (data not shown). In addition, in order to show that PI3K phosphorylation is PRKA dependent and is not a result of PRKCA inhibition, cells were incubated in the presence of GF or Ro and H89. The inhibition of PRKA by H89 abrogated the elevation in PI3K phosphorylation in the presence of GF or Ro (Fig. 3D). Together, these data indicate that PRKCA inhibition by itself could not activate PI3K unless PRKA is active.

The activation of PI3K was further investigated using immunocytochemistry method which measured the level of PI3K direct product, $\text{PIP}_{3(3,4,5)}$. The $\text{PIP}_{3(3,4,5)}$ level in sperm cells was examined after 4 h of incubation in the capacitation medium in the presence of PRKC inhibitor GF or H89. WT, inhibitor of the PI3K catalytic subunit, was used as positive control. As shown in Fig. 3E, treatment with 10 nM WT reduced the $\text{PIP}_{3(3,4,5)}$ production, while GF that has been shown to elevate PI3K phosphorylation on Tyr467 (Fig. 3A) led to remarkable $\text{PIP}_{3(3,4,5)}$ production (Fig. 3E). WT abolished the GF-induced $\text{PIP}_{3(3,4,5)}$ production, since it inhibits the catalytic activity of PI3K. Treatment with H89 also abolished $\text{PIP}_{3(3,4,5)}$ production, indicating the prominence of PRKA activation for PI3K phosphorylation/activity (Fig. 3E). This result is in correlation with the absence of PI3K phosphorylation on Tyr467 that was observed when H89 was present in the incubation medium (Fig. 3D), further strengthening the correlation between PI3K activation and its phosphorylation on Tyr467.

The role of PRKCA in regulating PI3K activation was further confirmed by using PMA, known to function as classical and novel PRKC isoforms activator; however, chronic activation leads to PRKC down-regulation (Hansra *et al.* 1999). The effect of PMA on PRKCA and PI3K during the capacitation process was assessed by western blot analysis. PMA treatment accelerated the desensitization (degradation and dephosphorylation) of PRKCA that could be seen after 1 h of incubation in the capacitation medium comparing with the control in which PRKCA degradation and dephosphorylation could be seen only after 4–7 h of incubation (Fig. 5A). Short-time (30 min) treatment with calcium ionophore (A23187) did not cause PRKCA degradation (Fig. 5C) confirming that the PMA-induced-PRKCA reduction is due to the degradation and not because of the acrosome reaction enhancement. The PMA-dependent PRKCA

Figure 3 PRKC/PRKCA down-regulation enhances PI3K phosphorylation/activation. Bovine sperm was incubated for 4 h in mTALP in the presence or absence of PRKC inhibitors (0.1 nM GF3200109X (GF) (A, C, and D) or (15 nM) Ro 32-0432 (Ro) (B–D) or with PRKA inhibitor (50 μM) H89 (D). Sperm proteins were extracted at the indicated times and western blotted as described in ‘Materials and Methods’. p-PI3K (A, B, and D) and PRKCA (C) were detected using specific antibodies, and β-actin or α-tubulin antibodies were used to equalize the loading quantities. (E) For detection of PI3K activity, sperm cells were incubated in mTALP with or without wortmannin (WT; 10 nM), GF (0.1 nM) or H89 (50 μM) for 4 h. Sperm cells were stained with anti-PIP_{3(3,4,5)}, and the images were captured as described in ‘Materials and Methods’. The results represent at least five independent experiments.

down-regulation led to early and remarkable elevation in PI3K phosphorylation compared with the control (Fig. 5A). However, short-time (10 min) PMA treatment resulted in PRKCA translocation from the cytosol to the membrane fraction indicating its activation (Cohen *et al.* 2004; Fig. 5B) and prevented PI3K phosphorylation (Etkovitz *et al.* 2007). Since PRKCA is highly activated at the beginning of the capacitation (highly phosphorylated and appears in the membrane fraction (Fig. 1)), we do not see elevation in PRKCA phosphorylation upon short-time (10 min) PMA treatment, but translocation to the membrane fraction is visible (Fig. 5B), indicating that it is more active. These data further support our assumption regarding the regulation of PI3K activation by PRKCA. It is worth noting that PRKA inhibition by H89 abolished the PMA-induced PRKCA degradation (see Fig. 6B), indicating that PRKA mediates this process.

S/T phosphatases inhibit PI3K phosphorylation downstream to PRKCA

To explore the signaling pathway underlying PRKCA-mediated inhibition of PI3K activation, we examined the involvement of protein S/T phosphatases in this pathway using two S/T phosphatases inhibitors: calyculin A and

okadaic acid (OA). This strategy was chosen based on a recent publication about the ability of PRKC agonists to enhance PP2A activity in IEC-18 cells (Guan *et al.* 2007). In addition, new evidences are gathered regarding the possibility that protein kinases can function as a targeting/modulating subunits in phosphatases complexes (Cohen 2002). This approach will allow us to determine whether PI3K inhibition by PRKCA is direct or indirect.

As shown in Fig. 4A, calyculin-A induced early PI3K phosphorylation during the incubation in the capacitation medium. This effect on PI3K phosphorylation was remarkably faster and higher than OA (Fig. 4B), and occurred at a very short time (30 min) compared with OA (180 min; Fig. 4A and B respectively; for control, see Fig. 5). It is known that calyculin-A inhibits PPP1 at lower IC₅₀ than PP2A, while OA inhibits PP2A and PPP1 at higher and similar concentrations. Therefore, we carried out a concentration curve. Sperm cells were treated with varied calyculin-A (1–100 nM) or OA (10–1000 nM) concentrations during the capacitation, and the phosphorylation level of PI3K was examined. As shown in Fig. 4C, 1 nM calyculin-A caused the same levels of PI3K phosphorylation that can be seen with 10 nM of OA, and the effect of 10 nM calyculin-A was

Figure 4 Serine/threonine phosphatase inhibitors induce time-dependent PI3K phosphorylation. Bovine sperm was incubated for 4 h in mTALP in the presence of calyculin-A (Caly; 100 nM) (A) or okadaic acid (OA; 1 μ M) (B). (C) Bovine sperm was incubated for 4 h in mTALP in the presence or absence of calyculin-A (Caly) or okadaic acid (OA) at different concentrations as indicated. (D) Bovine sperm was incubated for 4 h in the capacitation medium (mTALP) or in the bicarbonate-deficient medium (mTALP W/O HCO₃⁻) in the presence or absence of (50 μ M) H89 or (100 nM) calyculin-A (Caly). Sperm proteins were extracted at the indicated times and western blotted as described in 'Materials and Methods'. p-P13K (A–D), p-(Ser657)-PRKCA (A and B), and PRKCA (A and B) were detected using specific antibodies, and β -actin (A, B, and D) or tubulin (C) antibodies were used to equalize the loading quantities. The results represent at least three independent experiments.

similar to that of 100 nM OA. In addition, the treatment with 10 nM of calyculin-A caused high PI3K phosphorylation that was only little elevated by higher calyculin-A concentrations, while the effect of OA on PI3K phosphorylation was markedly elevated with every concentration elevation. Together, these observations point to the phosphatase PPP1 crucial role in regulating PI3K phosphorylation, since calyculin-A inhibits the activity of protein phosphatases PPP1 and PP2A, unlike OA which reduces PP2A activity, but has little effect on PPP1 (Ishihara *et al.* 1989). It was noted that the phosphatase inhibitors did not affect the level and phosphorylation rate of PRKCA (Fig. 4A and B), despite significant and early elevation in PI3K phosphorylation (mainly in Fig. 4A), indicating that PPP1 is localized downstream to PRKCA and upstream to PI3K in this system.

Regarding the role of the phosphatases versus PRKA in activating PI3K, it is shown in Fig. 4D that addition of the PRKA inhibitor H89 to cells incubated in the capacitation medium markedly diminished PI3K phosphorylation level induced by calyculin-A treatment. In bicarbonate-deficient medium, when PRKA is not activated, calyculin-A treatment caused very small increase in PI3K phosphorylation compared with its effect in bicarbonate-containing medium (TALP; Fig. 4D), further supporting the necessity for PRKA

activation in order to activate PI3K. It was noted that calyculin-A treatment caused very slight elevation in PI3K phosphorylation in bicarbonate-deficient medium, and this elevation was not inhibited by H89. This result may point to a basal phosphatases activity within the cells that operate constantly to remove the phosphate from PI3K. The demonstration that PRKCA or PPP1 inhibition is not sufficient for PI3K phosphorylation supports the biological significance of PRKA activity for PI3K activation.

PRKCA and PPP1CC2 operate as a complex that inhibits PI3K phosphorylation

Based on the prominence of PPP1 in regulating PI3K phosphorylation, we examined the involvement of the sperm-specific PPP1CC2 isoform which is known to be the main S/T phosphatase in sperm cells, and it is expressed exclusively in testis and spermatozoa (Chakrabarti *et al.* 2007). First, we characterized PPP1CC2 in bovine sperm during the capacitation time. The presence of PPP1CC2 was assessed by western blot analysis (Fig. 7A). The specificity of the PPP1CC2 antibody is demonstrated in Fig. 7B. As shown in Fig. 7A, PPP1CC2 underwent degradation during the incubation which was capacitation dependent, as was found for PRKCA (Fig. 1). Therefore, we assumed that PRKCA

Figure 5 PMA-induced PRKCA degradation enhances PI3K phosphorylation/activation. (A) Bovine sperm was incubated for 7 h in mTALP in the presence or absence of PRKC activator PMA (162 nM). Sperm proteins were extracted at the indicated times and western blotted as described in 'Materials and Methods'. p-PI3K, p-(Ser657)-PRKCA, and PRKCA were detected using specific antibodies, and β -actin antibody was used to equalize the loading quantities. The result represents at least four independent experiments. (B) Bovine sperm was incubated for 10 min in mTALP in the presence or absence of PMA (162 nM). At the indicated times, cells were fractionated into cytosol (Cyto) and membrane (Mem) fractions using Subcellular ProteoExtract kit, Calbiochem, according to the manufacturer's instructions, and western blotted as described in 'Materials and Methods'. PRKCA was detected using specific antibody. The result represents two independent experiments. (C) Bovine sperm was incubated for 30 min in mTALP in the presence or absence of 10 μ M calcium ionophore A23187 (AR inducer). Sperm proteins were extracted at the indicated times and western blotted as described in 'Materials and Methods'. PRKCA was detected using specific antibody, and tubulin antibody was used to equalize the loading quantities. The result represents three independent experiments.

might be in interaction with PPP1CC2. Short-time (60 min) treatment with calcium ionophore (A23187) did not cause PPP1CC2 degradation (Fig. 7E) confirming that PPP1CC2 reduction is indeed due to degradation and does not indicate enzyme loss due to the acrosome reaction.

The interaction between PRKCA and PPP1CC2 was investigated by immunoprecipitation assay. PRKCA was immunoprecipitated (IP) from sperm cells extracts and immunoblotted with PPP1CC2-specific antibody and vice versa as shown in Fig. 7C. Our results point to the existence of PRKCA–PPP1CC2 complex in bovine sperm cells incubated under capacitation conditions as indicated by the detection of PPP1CC2 in the IP of PRKCA (Fig. 7C left) and by the detection of PRKCA in the IP of PPP1CC2 (Fig. 7C right). For negative control, an anti-GFP antibody was used in the same IP conditions. As shown in Fig. 7D, PPP1CC2 was immunoprecipitated only in the IP of PRKCA and not in the IP of GFP. As additional control, the IP was repeated using different lysis buffer that contained 0.1% Triton, and the findings were identical (data not shown).

PRKA regulates PRKCA/PPP1CC2 degradation

The above findings revealed that PI3K phosphorylation was negatively regulated by PRKCA and PPP1 during sperm capacitation. Moreover, PRKCA and PPP1CC2 were degraded in capacitation-dependent manner (Figs 1A and B, and 7A respectively); therefore, we looked for the possible connection between PRKA activation and PRKCA/PPP1CC2 degradation.

As shown in Fig. 6A, the addition of 8Br-cAMP to cells that were incubated in the capacitation medium resulted in marked degradation (after 4 h) or almost complete degradation (after 7 h) of PRKCA and PPP1CC2 in comparison with the control, and also enhanced PRKCA dephosphorylation. In bicarbonate-deficient medium when PRKA activation is largely prevented, the degradation and the dephosphorylation of PRKCA, and the degradation of PPP1CC2 were markedly inhibited (in 4 or 7 h; Fig. 6A); however, upon the addition of 8Br-cAMP, the degradation rates of PRKCA and PPP1CC2 were elevated and were similar to the control (Fig. 6A). Inhibition of PRKA by H89 also slowed down the degradation rate of the two proteins (Fig. 6B). Notably, long-time treatment with PMA (4 h) enhanced not only PRKCA degradation but also PPP1CC2 degradation (Fig. 6B). This result is very interesting because no direct effect of PMA on protein phosphatases is known (Srinivasan & Begum 1994, Sim & Scott 1999). H89 treatment inhibited the PMA-induced degradation of PRKCA and PPP1CC2 (Fig. 6B), indicating that the PMA-induced degradation of these two proteins is mediated by PRKA.

Discussion

In the present study, we suggest a dual role for PRKA in the regulation of PI3K activity during bovine sperm capacitation. First, PRKA mediates PI3K activation, and secondly, PRKA mediates PRKCA and PPP1CC2 degradation which is significant for PI3K activation (Fig. 8).

Figure 6 PRKA mediates the degradation PRKCA and PPP1CC2.

(A) Bovine sperm was incubated for 7 h in the capacitation medium (mTALP) or in the bicarbonate-deficient medium (mTALP W/O HCO₃⁻) in the presence or absence of (0.1 mM) 8Br-cAMP (cAMP). (B) Bovine sperm was incubated for 4 h in the capacitation medium in the presence or absence of (50 μ M) H89 or (162 nM) PMA. Sperm proteins were extracted at the indicated times and western blotted as described in 'Materials and Methods'. p-Ser657-PRKCA (A), PRKCA and PPP1CC2 (A and B) were detected using specific antibodies, and α -tubulin antibody was used to equalize the loading quantities. The results represent at least three independent experiments.

These conclusions are supported by the following data: 1) inhibition of PRKA during sperm capacitation revealed no phosphorylation of PI3K and no elevation of PIP_{3(3,4,5)} and significant inhibition of PRKCA/PPP1CC2 degradation (Figs 2C, 3D and E, and 6B). 2) Prevention of PRKA activation during sperm capacitation revealed no activation of PI3K and relatively small degradation of PRKCA/PPP1CC2. These activities could be restored by adding 8-Br-cAMP to the incubation medium (Figs 2C and 6A), and the restored activities were inhibited by the PRKA inhibitor H89 (Fig. 2C). 3) PMA treatment could not enhance PI3K activation or PRKCA/PPP1CC2 degradation when PRKA was inhibited (Figs 2C and 6B). 4) GF or Ro treatment could not enhance PI3K activation when PRKA was inhibited. However GF and Ro treatment inhibited PRKCA/PPP1CC2 degradation (Fig. 3). 5) The enhanced effect of calyculin-A on PI3K activation was significantly abrogated when PRKA activity was inhibited (Fig. 4D).

The data revealed that PRKCA was active at the beginning of the capacitation process as indicated by its high level and phosphorylation on Ser657 and Thr497, by its high membrane localization (Fig. 1), and by its high phosphorylation level at the membrane fraction at this time (Fig. 1). The presence of phosphorylated PRKCA in the cytosol fraction can be explained by the presence of closed-phosphorylated conformation of the enzyme in this fraction as was shown by others (Hansra *et al.* 1999). Nevertheless, the main amount of phosphorylated PRKCA was present in the membrane-bound fraction. With the ongoing of the capacitation process, PRKCA appeared to undergo prominent degradation and dephosphorylation, while PI3K phosphorylation level on

Tyr467 was increased with no change in its total amount (Fig. 1), indicating its activation. These processes did not occur when cells were incubated under non-capacitation conditions (Fig. 1), indicating that they might be part of the capacitation process. The degradation of PRKCA cannot be explained by acrosome loss since at the end of the capacitation there is only ~10–15% of spontaneous acrosome loss, while the amount of PRKCA was twofold lower. Moreover, when PMA was present during the capacitation process, PRKCA underwent prominent degradation (Fig. 5A), while there is no significant loss of acrosome (data not shown). In addition, short-time treatment at the beginning of the capacitation with the AR inducer calcium ionophore did not affect the level of PRKCA or PPP1CC2 (Figs 5C and 7E), suggesting that the loss of acrosome is not the cause of PRKCA or PPP1CC2 protein loss.

PRKA is known to mediate capacitation (Visconti *et al.* 1995). It is also established that PRKA can directly phosphorylate PI3K regulatory subunit on Ser83 (Cosentino *et al.* 2007, De Gregorio *et al.* 2007). Our results identified PRKA as an important 'positive' regulator of PI3K activation in sperm since PI3K phosphorylation was correlated with PRKA activation or inhibition (Fig. 2). Moreover, we showed here that PIP_{3(3,4,5)} levels were higher at the end of the capacitation, and this elevation was inhibited by H89 (Fig. 3E).

Previously, we showed in bovine sperm that activation of PRKCA leads to inhibition of PI3K activation (Etkovitz *et al.* 2007); however it was not clear which PRKCA isoform mediates this phenomena. In other cell types, it was shown that PRKCA can inhibit PI3K directly or indirectly (Sipeki *et al.* 2006, Guan *et al.* 2007). We showed here that upon PRKCA inhibition (by inhibitors or by long-time PMA treatment), PI3K phosphorylation was enhanced (Figs 3 and 5). PRKCA or PRKCA inhibition (GF and Ro respectively) led to similar enhancement and acceleration of PI3K phosphorylation (Fig. 3D), suggesting that the down-regulation of the PRKCA isoform mediates PI3K phosphorylation/activation. Furthermore, PIP_{3(3,4,5)} levels were up-regulated when GF was present in the incubation medium confirming that PI3K is activated (Fig. 3E). Interestingly, PI3K was highly activated, although PRKCA degradation was decelerated in the presence of GF or Ro, suggesting that PRKCA dephosphorylation, but not degradation, is the critical event that leads to PI3K phosphorylation/activation (Fig. 3C). Two possible mechanisms for PI3K inhibition by PRKCA were proposed: 1) PRKCA directly phosphorylates PI3K catalytic subunit, as was found in other cell type (Sipeki *et al.* 2006), leading to autophosphorylation on Ser608 in the regulatory subunit, resulting in PI3K inhibition (Foukas *et al.* 2004). 2) PRKCA activates protein phosphatases which remove the phosphate from Ser83 in the p85 subunit of PI3K (activation site; Cosentino *et al.* 2007, De Gregorio *et al.* 2007), leading to PI3K inhibition. This possible

mechanism is supported by [Guan *et al.* \(2007\)](#) work that shows a direct activation of the S/T phosphatase PP2A by PRKCA which result in PI3K inhibition by dephosphorylation of PI3K. The S/T protein phosphatases PPP1 and PPP2 are found in sperm cells and participate in the capacitation and acrosome reaction processes ([Furuya *et al.* 1993](#), [Ashizawa *et al.* 2006](#), [Chakrabarti *et al.* 2007](#)). Following this information, we hypothesized that the PRKCA-mediated PI3K inhibition during sperm capacitation occurs through PRKCA-dependent activation of PPP1 and/or PPP2. Sperm treatment with calyculin A or OA enabled us to prefer the phosphatase PPP1 as the PRKCA-downstream-PI3K regulator ([Fig. 4](#)). This result support our assumption regarding the indirect inhibition of PI3K by PRKCA, since, if PRKCA directly phosphorylates PI3K leading to its inhibition, the phosphatases inhibition would result in even greater PI3K inhibition, and this was not the case. An opposite regulation of PI3K by phosphatases was found in 3T3-L1 adipocytes in which overexpression of PP2C α resulted in direct activation of PI3K by dephosphorylating the inhibitory site Ser608 in PI3K regulatory subunit – p85 ([Yoshizaki *et al.* 2004](#)). The fact that PI3K has many phosphorylation sites which are activatory or inhibitory ([Yoshizaki *et al.* 2004](#), [Cosentino *et al.* 2007](#), [De Gregorio *et al.* 2007](#)), together with the established existence of variety of modulators, targeting and regulatory subunits that function as complexes with phosphatases and regulate their specific activity ([Cohen 2002](#)), points to the possibility that different phosphatases have specific sites for dephosphorylation on PI3K. Therefore, we assume that PPP1 dephosphorylates PI3K on 'positive'/stimulatory-phospho-site, leading to PI3K inhibition. This assumption does not contradict the possible existence of other phosphatases (like PP2C), which remove 'inhibitory' phosphorylation resulting in PI3K activation. In accordance with this idea, [Qiu *et al.* \(2007\)](#) showed in MC3T3-E1 cells that calyculin A caused elevation of Ser437 phosphorylation site but not of Thr308 in AKT (by PP2C inhibition), indicating the specificity of the phosphatases activity. The isoform PPP1CC2 was found to be the abundant PPP1-specific isoform in spermatozoa, and it is a testis-specific isoform ([Chakrabarti *et al.* 2007](#)). Here we showed that PRKCA and PPP1CC2 exist as a complex within the cells, that PPP1CC2 is degraded during the capacitation process simultaneously with PRKCA degradation/dephosphorylation and PI3K phosphorylation, and that PPP1CC2 (similar to PRKCA) is hyper-degraded in response to long PMA treatment ([Figs 6B and 7](#)), further confirming the relationship between PRKCA and PPP1CC2 ([Srinivasan & Begum 1994](#), [Sim & Scott 1999](#), [Cohen 2002](#)). In agreement with PPP1CC2 degradation, we found an elevation in protein serine phosphorylation during the capacitation (data not shown). This is in accordance with other works that show that calyculin-A stimulates protein phosphorylation and capacitation in human

Figure 7 PRKCA and PPP1CC2 are in complex at the time of capacitation. (A) Bovine sperm was incubated for 7 h in mTALP. Sperm proteins were extracted at the beginning, after 4 h, and after 7 h of incubation and western blotted as described in 'Materials and Methods'. PPP1CC2 was detected using specific antibody, and β -actin antibody was used to equalize the loading quantities. This result represents at least five independent experiments. (B) PPP1CC2 was detected at the indicated times, using specific antibody on western blotted sperm extracts. Whole blots are presented. (C) Left panel shows that PRKCA was immunoprecipitated from cells at the beginning of the incubation time as described in 'Materials and Methods', and western blotted. The membrane was incubated with anti-PPP1CC2, then was stripped and incubated with anti-PRKCA antibody. Right panel shows that PPP1CC2 was immunoprecipitated from cells at the beginning of the incubation time as described in 'Materials and Methods', and western blotted. The membrane was incubated with anti-PRKCA, then was stripped and incubated with anti-PPP1CC2 antibody. Whole cell lysate (WCL) constitutes a positive control. This result represents three independent experiments. (D) Immunoprecipitation assay was performed, using anti-PRKCA or anti-GFP antibody, in cells at the beginning of the incubation time as described in 'Materials and Methods', and western blotted. The membrane was incubated with anti-PRKCA. Whole cell lysate (WCL) constitutes a positive control. (E) Bovine sperm was incubated for 60 min in mTALP in the presence or absence of 10 μ M calcium ionophore A23187 (AR inducer). Sperm proteins were extracted at the indicated times and western blotted as described in 'Materials and Methods'. PPP1CC2 was detected using specific antibody, and tubulin antibody was used to equalize the loading quantities. The result represents three independent experiments.

sperm ([Furuya *et al.* 1993](#)). It was shown in MAST cells that PMA treatment led to PRKC and PP2A translocation to the plasma membrane suggested that these two proteins may held in a complex on one anchoring protein ([Sim & Scott 1999](#)). It is established that S/T phosphatases operate as complexes consist of 2–3 subunits which include one catalytic subunit attached to one or more regulatory subunits. There is a variety of

regulatory subunits which can direct the phosphatase to different domains, and to diverse substrates, and with the ongoing research, more and more proteins that can form complexes with phosphatases and regulate them are revealed (Cohen 2002). Interestingly, some protein kinases have been found to function as regulatory subunits of specific phosphatase complex (Cohen 2002). Our results point to the possibility that PRKCA function as a regulatory subunit in the PPP1CC2 complex, or that these two proteins are held as a complex by an anchoring protein (Westphal *et al.* 1999).

Another point to be emphasized is the finding that PRKA activity affects the PRKC–PPP1CC2–PI3K signaling pathway in more than one way. We suggest that PRKA regulates the degradation rate of PRKCA and PPP1CC2 (Fig. 6) which enables PI3K phosphorylation/activation during the capacitation process. An interesting significance has been raised for PRKA activation and for PPP1CC2 degradation during the capacitation towards the acrosome reaction. Variety of works revealed that S/T kinase (specifically PRKA) is a positive regulator of the acrosome reaction, whereas S/T phosphatase (especially PP2A) is a negative regulator of this process (De Jonge *et al.* 1991, Jha & Shivaji 2002). In our previous work, we suggested that PRKA inhibits PLC, preventing PRKC activation (Cohen *et al.* 2004). In that work, we concluded that PRKC activity is capacitation disturbing, therefore it should be kept low during this process, and this become possible by the activation of PRKA. However, towards the end of capacitation, PRKC activity should be enhanced because PRKC is involved in the sperm acrosome reaction (Breitbart *et al.* 1992); therefore, we concluded that PRKA activity should be down-regulated towards the end of the capacitation, allowing PLC activation and PRKC activation. Our current findings deprive the PRKCA isoform from being involved in the proposed mechanism. First, we saw degradation and dephosphorylation of PRKCA towards the end of the capacitation process pointing to its down-regulation at this time (Fig. 1). Secondly, we have found that specific inhibition of PRKCA by Ro did not inhibit the ionophore-induced acrosome reaction (data not shown), pointing to the involvement of another PRKC isoform in this process.

The finding that PRKCA and PPP1CC2 are degraded during the capacitation process (Figs 1 and 7A) argued for the involvement of degradation system/s which regulate the cellular levels of these proteins. The degradation of diverse PRKC isoforms is a well-studied issue (Lee *et al.* 1997, Kang *et al.* 2000), whereas there is not any documentation regarding protein phosphatase degradation. The two major proteolytic pathways in eukaryotic cells are: the ubiquitin/proteasome system and the lysosomal pathway. Since lysosomes do not exist in sperm cells, and proteasomes do exist (Moreno & Alvarado 2006), we suggest that the ubiquitin/proteasome pathway is involved in PRKCA degradation and/or PPP1CC2. Proteasomes and ubiquitin are likely to be

expressed in mammalian gametes and embryos at any given developmental step, and found to be involved in assortment of processes like sperm maturation, elimination of paternal mitochondria, destruction of defective spermatozoa, and penetration of zona pellucida by the acrosome-reacted sperm (reviewed in Sutovsky (2003)). Recently, it was found that the proteasome is involved in human sperm capacitation (Kong *et al.* 2009). Previous studies have implicated the proteasomal activity in PRKCA degradation (Lee *et al.* 1997, Kang *et al.* 2000). Most notably, Lu *et al.* (1998) showed that PRKC ubiquitination is a physiological response, and is not unique as response to phorbol ester treatment. We showed that PRKA activation mediates PRKCA and PPP1CC2 degradation (Fig. 6). Most recently, it was reported that the proteasomal activity is important to human sperm capacitation, and that PRKA activity regulates the chymotrypsin-like activity of the proteasome during capacitation (Kong *et al.* 2009). Another study revealed that PRKA can positively regulate the proteasomal activity by phosphorylation of Rpt6, one of the six AAA-ATPases in the 19S regulatory subunit of the proteasome (Zhang *et al.* 2007). In addition, we found that bovine sperm expresses both Rpt6 and the proteasome catalytic α -C2 subunit (data not shown). Together, these findings support our current results regarding the influence of PRKA activation on PRKCA/PPP1CC2 degradation. We hypothesize that due to the bicarbonate-induced activation of PRKA at the beginning of the capacitation the proteasomal activity is enhanced leading to ubiquitin-tagged proteins degradation via the proteasome; however, further investigations are needed in order to establish this hypothesis.

Our results also implied PRKA as a PRKCA dephosphorylation mediator (Fig. 6A). The way by which PRKA can influence PRKCA dephosphorylation is intriguing. A possible mechanism is that PRKA and a S/T phosphatase may held together in a multimeric complex on one anchoring protein, i.e. AKAP as was found in other cells (Wong & Scott 2004), and upon PRKA activation the phosphatase is also activated, maybe by dissociating from the complex, and can dephosphorylate PRKCA. Apparently, the protein phosphatases PPP1 or PP2A are not responsible for PRKCA dephosphorylation in our system, since their inhibition by calyculin A or OA during the capacitation did not influence PRKCA phosphorylation level (Fig. 4A and B). A possible phosphatase can be the calcium-calmodulin-activated phosphatase calcineurin (protein phosphatase PP2B) which was found to dephosphorylate PRKCA in endothelial cells (Lum *et al.* 2001). In addition, it was found that in neuronal cells, PRKA, PRKC, and PP2B are associated with AKAP79 in one signaling complex (Klauck *et al.* 1996). To date, there is no evidence for AKAP79 presence in spermatozoa; however, at least six other AKAPs have been identified (Fiedler *et al.* 2008),

Figure 8 Diagrammatic representation of the mechanisms underlying PRKA-mediated PI3K activation during bovine sperm capacitation. At the beginning of the capacitation process, PRKA and PPP1CC2 are active and present in high levels and inhibit (directly or indirectly) PI3K phosphorylation. During the capacitation, PRKA is activated by cAMP that is generated by the bicarbonate-activated soluble adenylyl cyclase (sAC). PRKA phosphorylates PI3K leading to its activation. PI3K activation cannot be seen at beginning of the capacitation, because the PRKA-activated PPP1CC2 removes PI3K phosphorylation. With the ongoing capacitation, PRKA mediates PRKA and PPP1CC2 degradation, which can be proteasome dependent. Therefore, PI3K inhibition by PRKA/PPP1CC2 is removed, and PI3K phosphorylation/activation by PRKA can occur.

some of them are known to bind protein phosphatases (Wong & Scott 2004). Using immunoprecipitation assay, we found a complex of AKAP3 and PRKA in bovine sperm cells (data not shown). In addition, we found that the addition of calcium ionophore A23187 for 0.5 h to sperm cells in the capacitation medium caused PRKA dephosphorylation even at the beginning of the capacitation when the degradation is not seen yet (data not shown). This finding implies on a calcium-dependent phosphatase that functions as the PRKA dephosphorylation mediator; however, future work is required in order to gain a better insight into this possible signaling pathway.

In summary, our results show that at the beginning of the capacitation the level of PI3K phosphorylation is low and phospho-PRKA is high. The activation of PRKA in the capacitation (White & Aitken 1989) leads to PI3K phosphorylation/activation and to PRKA dephosphorylation and degradation (Fig. 8). Although PRKA is activated at very early time of the capacitation process (Harrison 2004), PI3K phosphorylation/activation is not distinguishable yet, possibly due to the high activity of PRKA/PPP1CC2 that removes the PRKA-mediated PI3K phosphorylation, causing inhibition of PI3K phosphorylation/activation (Fig. 8). Towards the end of the capacitation, phospho-PRKA undergoes dephosphorylation, and both PRKA and PPP1CC2 are degraded, allowing the PRKA-mediated PI3K phosphorylation/activation to occur (Fig. 8), and the activated PI3K can function in the acrosome reaction (Fisher *et al.* 1998, Jungnickel *et al.* 2007). The degradation of PRKA/PPP1CC2, which along with PI3K phosphorylation

were capacitation- and PRKA-dependent processes, was possibly mediated by the ubiquitin/proteasome pathway, pointing to an additional role for PRKA in regulating this signaling pathway.

Materials and Methods

Materials and antibodies

Bisindolylmaleimide I (GF109203X (GF)), Ro 32-4320, A23187, protease inhibitor cocktail III, calyculin A and OA were purchased from Calbiochem (San Diego, CA, USA). Specific rabbit polyclonal antibody against phospho-PI3K p85 (Tyr458)/p55 (Tyr199) antibody and phospho-PRKA substrate (RRXS/T) (100G7) rabbit mAb were purchased from Cell Signaling (Beverly, MA, USA). Goat polyclonal anti-PI3K p85 α (S-20), rabbit polyclonal anti-PRKA (C-20), goat polyclonal anti-GFP (T-19), and donkey anti-goat IgG-HRP conjugate were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-PRKA (Ser657) (rabbit polyclonal IgG) was purchased from Upstate (Lake Placid, NY, USA). Specific phospho-PRKA (Thr497) rabbit antibody was purchased from Epitomics (Burlingame, CA, USA). Goat anti-mouse IgG-HRP conjugate and goat anti-rabbit IgG-HRP conjugate were purchased from Bio-Rad. Re-Blot plus Strong Solution (10 $\times$) was purchased from Millipore (Temecula, CA 92590, USA). Mouse anti-PIP_{3(3,4,5)} (biotin-conjugated) antibody was purchased from Echelon (Salt Lake City, UT, USA). Texas Red-conjugated streptavidin was obtained from Oncogene (La Jolla, CA, USA). All other chemicals were purchased from Sigma (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 Centre (Hafetz-Haim, Israel). Sperm cells were washed three times by centrifugation (780 *g* for 10 min at 25 $^{\circ}$ C) in NKM buffer (110 mM NaCl, 5 mM KCl, and 20 mM 3-*N*-morpholino propanesulfonic acid (pH 7.4)), and the sperm cells were allowed to swim up after the last wash. The washed cells were counted and maintained at room temperature until use. Only sperm preparations that contained at least 80% motile sperm were used in the experiments, and the motility was not significantly reduced at the end of the incubations.

Sperm capacitation and drug treatments

In vitro capacitation of bovine sperm was induced as described previously (Parrish *et al.* 1999). Briefly, sperm pellets were resuspended 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 CaCl_2). The cells were incubated in this capacitation medium for 4 h at 39 °C with 0.5% CO_2 . PRKCA was inhibited in these cells by treatment with GF (0.1 nM), or Ro32-0432 (15 nM). PMA (100 ng/ml) was used to activate PRKCA in short time (up to 1 h), and then to down-regulate it. Control cells were treated with appropriate vehicle (Me_2SO or water). PP2A and/or PPP1 activity was inhibited using 100 nM calyculin A or 1 μM OA. PRKA activity was inhibited or was not induced using 50 μM H89 or bicarbonate-deficient medium respectively. One mM 8Br-cAMP was used to activate PRKA. Ten nM WT (Sigma–Aldrich Israel Ltd) were used to inhibit PI3K activity. 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 by the addition of A23187.

Immunoblot analysis

Sperm cells were washed by centrifugation for 5 min at 10 000 *g* at 4 °C, and then the supernatant was discarded, and the pellet was resuspended in Tris-buffered saline (TBS) and centrifuged again in order to remove BSA leftover. Sperm lysates were then 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 μM NaF, 50 μM pyrophosphate, 0.2 mM Na_3VO_4 , and freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF)), to the pellet, and the lysate was 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 Bradford method (Bradford 1976) or by the BCA method (Smith *et al.* 1985). Sample buffer $\times 2$ was added to the supernatant and boiled for 5 min. The extracts were separated on 8 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 TBS, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. Kinases and phospho-kinases were immunodetected by specific antibodies (diluted 1:5000 and 1:3000 respectively). 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 Lab), diluted 1:5000 in TBST and 1% BSA. The membranes were washed three times with TBST and visualized by ECL (Amersham). In order to use more than one antibody, the membrane was stripped for 30 min at room temperature in gentle agitation using Re-Blot Plus Strong Solution, then blocked twice with 1% BSA in TBST for 30 min at room temperature and subjected to another antibody. Each figure in this article represents the same membrane/blot that was subjected to different antibodies using stripping buffer. The antibodies were in the order of: phospho-PRKCA, PRKCA, phospho-PI3K, actin or tubulin, and finally PPP1CC2, unless otherwise stated. Distinct bands that were cut from the same membrane and exposure time are separated by a thin black line.

Immunoprecipitation assay

Cells (4×10^8) were suspended in homogenization buffer (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 1 mM benzamidine, 1 mM Na_3VO_4 , 10% (w/v) glycerol, 5 mM NaF, 30 mM NaH_2PO_4 , protease inhibitor cocktail III, and freshly added 1 mM PMSF), and were sonicated in three pulses 10 s each, with 5 s gaps, in 40 intensity/strength in Vibra cell sonicator. The homogenate was gently agitated for 30 min in 4 °C, and cells debris were removed by centrifugation in 10 000 *g*, for 10 min, in 4 °C. The lysate was precleared by addition of 50 μl protein A/G plus-agarose (sc-2003, Santa Cruz Biotechnology) for 2 h in gentle agitation at 4 °C. After the removal of beads (by centrifugation), 3–5 μl of antibody were added to the clarified cell extract and incubated overnight at 4 °C. The next day, 50 μl of protein A/G plus-agarose beads were added and incubated for further 2 h in gentle agitation at 4 °C. Beads were then subjected to three washes with 0.1% Triton in TBS buffer and boiled for 5 min with SDS sample buffer.

Fractionation assay

Fractionation assay was performed using Subcellular ProteoExtract kit, Calbiochem, according to the manufacturer's instructions.

Immunocytochemistry

The anti-PIP_{3(3,4,5)} antibody was used to determine the intracellular levels of PIP_{3(3,4,5)} as described previously (Etkovitz *et al.* 2007).

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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