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Zn^{2+}-stimulation of sperm capacitation and of the acrosome reaction is mediated by EGFR activation

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ABSTRACT

Extracellular zinc regulates cell proliferation via the MAP1 kinase pathway in several cell types, and has been shown to act as a signaling molecule. The testis contains a relatively high concentration of Zn^{2+}, required in both the early and late stages of spermatogenesis. Despite the clinical significance of this ion, its role in mature sperm cells is poorly understood. In this study, we characterized the role of Zn^{2+} in sperm capacitation and in the acrosome reaction. Western blot analysis revealed the presence of ZnR of the GPR39 type in sperm cells. We previously demonstrated the presence of active epidermal growth factor receptor (EGFR) in sperm, its possible transactivation by direct activation of G-protein coupled receptor (GPCR), and its involvement in sperm capacitation and in the acrosome reaction (AR). We show here that Zn^{2+} activates the EGFR during sperm capacitation, which is mediated by activation of trans-membrane adenylyl cyclase (tmAC), protein kinase A (PKA), and the tyrosine kinase, Src. Moreover, the addition of Zn^{2+} to capacitated sperm caused further stimulation of EGFR and phosphatydil-inositol-3-kinase (PI3K) phosphorylation, leading to the AR. The stimulation of the AR by Zn^{2+} also occurred in the absence of Ca^{2+} in the incubation medium, and required the tmAC, indicating that Zn^{2+} activates a GPCR. The AR stimulated by Zn^{2+} is mediated by GPR39 receptor, PKA, Src and the EGFR, as well as the EGFR down-stream effectors PI3K, phospholipase C (PLC) and protein kinase C (PKC). These data support a role for extracellular zinc, acting through the ZnR, in regulating multiple signaling pathways in sperm capacitation and the acrosome reaction.

Introduction

To acquire the capacity to fertilize the egg, mammalian spermatozoa must undergo a series of biochemical transformations in the female reproductive tract, collectively called capacitat ion. The capacitated spermatozoon can bind to the egg zona-pellucida, which induces the sperm to undergo the acrosome reaction (AR), resulting in penetration and fertilization of the egg. However, a recent publication argued against this paradigm, suggesting that most mouse sperm that fertilize have undergone the AR before reaching the egg (Jin et al., 2011). It is known that AR can be induced in-vitro by various constituents of the female reproductive tract including progesterone (Baldi et al., 1995; Roldan et al., 1994), prostaglandins (Joyce et al., 1987), natriuretic peptide (Rotem et al., 1998; Zamir et al., 1995), epidermal growth factor (EGF) (Etkovitz et al., 2009; Lax et al., 1994), ouabain (Daniel et al., 2010), and other ligands. We also showed that angiotensin II (ATII), which is present in the female reproductive tract (Culler et al., 1986), can induce the AR via activation of AT II-receptor type 1, leading to transactivation of the EGFR (Etkovitz et al., 2009; Gur et al., 1998).

One of the first steps in sperm capacitation is the elevation of cAMP induced by the entry of bicarbonate and Ca^{2+}, which together activate the soluble-adenylyl-cyclase (sAC) (Burton and McKnight, 2007; Demarco et al., 2003), leading to PKA-dependent protein tyrosine phosphorylation (Visconti et al., 1995). The mechanism by which the Ser/Thr kinase, PKA, induces protein tyrosine phosphorylation is not fully understood. It has been suggested elsewhere that inhibition of Ser/Thr phosphatase by Src, enhanced protein tyrosine phosphorylation during sperm capacitation (Battistone et al., 2013; Krapf et al., 2010). In a recent study, we suggested that sperm PKA activates Src which inhibits PP1, leading to CaMKII activation, which in turn phosphorylates Pyk2, subsequently phosphorylating PI3K on tyrosine (Rotfeld et al., 2014). We also showed that sperm PKA/Src activates the EGFR leading to PI3K tyrosine phosphorylation (Etkovitz et al., 2009). In the present study, we probed the mechanisms by which Zn^{2+} affects sperm function. Zn^{2+} required for normal physiological function and development (MacDonald, 2000; Vallee and Falchuk, 1993). The testes contain a high concentration of Zn^{2+} (Bedwal

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and Bahuguna, 1994), which is required in both the earlier and late stages of spermatogenesis (Sorensen et al., 1998; Yamaguchi et al., 2009). It has been suggested that extracellular Zn^{2+} activates a signal transduction pathway by several mechanisms including the activation of ZnR, a member of the GPCR family (Hershfinkel et al., 2001). Moreover, in epithelial cells, Zn^{2+} induces the transactivation of EGFR mediated by Src (Wu et al., 2005).We show here that low concentrations of Zn^{2+} activate PKA, Src, EGFR and PI3K during sperm capacitation. Moreover, Zn^{2+} stimulates EGFR and PI3K in capacitated cells, leading to the AR. Finally, Zn^{2+} can replace Ca^{2+} in the AR pathway.

Materials and methods

Materials and antibodies

Tyrphostin1478, A23187 and protease inhibitor cocktail III were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal anti-phospho-PI3K (Tyr458), Rabbit polyclonal anti-phospho-Src (Tyr416), Rabbit polyclonal anti-phospho-PKA substrate, Rabbit polyclonal anti EGFR, and anti-phospho EGFR (Y845 and Y1068) were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal β-actin (C4)-HRP conjugated was purchased from Santa Cruz Biotechnology (California, USA). Goat anti-mouse IgG-HRP conjugated and goat anti-rabbit IgG-HRP conjugated were purchased from Bio-Rad (Richmond, CA, USA). All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd, Rehovot, Israel), unless otherwise stated.

Sperm preparation

Bovine sperm was supplied by the SION Artificial Insemination Centre (Hafetz-Haim, Israel). Ejaculated bovine spermatozoa were obtained by 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 thrice 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 propanesulfonic 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 room temperature until use. Only sperm preparations that contained at least 80% motile sperm were used in the experiments; motility was not significantly reduced at the end of the incubations.

Sperm capacitation

In vitro capacitation of bovine sperm was induced as described previously (Parrish et al., 1988). Briefly, sperm pellets were resuspended to a final concentration of 10^8 cells/ml in mTALP (Modified Tyrode solution) medium (100 mM NaCl, 3.1 mM KCl, 1.5 mM $MgCl_2$, 0.92 mM KH_2PO_4, 25 mM $NaHCO_3$, 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 μg/ml heparin, and 2 mM $CaCl_2$). The cells were incubated in this capacitation medium for 4 h at 39 °C in 0.5% CO_2 atmosphere. 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 following the addition of A23187 (10 μM) and epidermal growth factor (EGF, 1 ng/ml).

Assessment of sperm acrosome reaction

Washed cells (10^8 cells/ml) were capacitated for 4 h at 39 °C in mTALP medium. Inducers were then added for another 20 min of incubation. The percentage of acrosome-reacted sperm was determined microscopically on air-dried sperm smears using FITC-conjugated Pisum sativum agglutinin (PSA). An aliquot of spermatozoa (10^6 cells) was smeared on a glass slide and allowed to air-dry. The sperm were then permeabilized by methanol for 15 min at room temperature, washed thrice at 5-min intervals with TBS, air dried, and then incubated with FITC-PSA (50 μg/ml in TBS) for 30 min, washed twice with H_2O at 5-min intervals, and mounted with FluoroGuard Antifade (Bio-Rad Lab). For each experiment, at least 200 cells per slide on duplicate slides were evaluated (total of 400 cells per 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 resuspended in TBS and centrifuged again in order 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 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 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 BCA method (Smith et al., 1985). Sample buffer × 5 was added to the supernatant, and 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 thrice with TBST and incubated for 1 h at room temperature with specific horseradish peroxidase (HRP)-linked secondary antibodies (BioRad Lab., Richmond, CA), diluted 1:5000 in TBST and 1% BSA. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

Immunocytochemistry

Sperm cells were spread on glass slides, air-dried, fixed in methanol for 10 min, washed thrice at 5 min intervals with TBS and dipped for blocking in TBS containing 2% BSA for 120 min. The cells then incubated for 18 h at 4 °C with rabbit polyclonal anti-GPCR (GPR39) antibody diluted 1: 50 in TBS containing 2% BSA. Next, the slides were washed thrice at 5 min intervals with TBS. The bound antibody was detected using Alexa-fluor-488 goat anti-rabbit IgG (Invitrogen molecular probes, 1:200 dilution), incubated for 1 h at 37 °C, and followed by washing thrice at 5 min intervals with TBS. Next, the slides were stained with DAPI for 5 min, thrice at 5 min intervals with TBS. Finally, the slides were mounted in FluoroGuard Antifade. Nonspecific staining was determined by incubating the sperm without primary antibody. Images were captured on an Olympus AX70 microscope at 400 × magnification.

Results and discussion

The effect of extracellular Zn^{2+} on the intracellular signaling pathways in sperm was first described by Clapper et al. (1985).

They showed that Zn^{2+} interacts with the specific ZnR, belonging to the GPCR family. This ZnR induces the release of intracellular Ca^{2+} mediated by a Gαq-protein coupled receptor and activates the MAPK and PI3K pathways (Azriel-Tamir et al., 2004; Besser et al., 2009; Chorin et al., 2011; Sharir et al., 2010). Moreover, it was shown that Zn^{2+} induces transactivation of the EGFR mediated by Src (Wu et al., 2005). We showed previously that physiological activation of bovine sperm lysophosphatydic-acid receptor (LPA-R) or Angiotensin 1-receptor (AT1-R), two receptors belonging to the GPCR family, leads to transactivation of the EGFR mediated by PKA and Src (Etkovitz et al., 2009). We further showed that EGFR is partially activated during sperm capacitation, and the addition of EGF at the end of the capacitation process enhanced further EFGR phosphorylation/activation, leading to Ca^{2+} influx and triggering the acrosome reaction (AR) (Etkovitz et al., 2009). Thus, we hypothesized that Zn^{2+} might affect sperm capacitation and the AR by activation of ZnR, leading to EGFR transactivation. Indeed we found by western blot analysis that bovine, human and mouse sperm cells contain a 51 kDa band identified as GPR39 type ZnR (Fig. 1A arrow). This band disappeared by performing a peptide control experiment using GPR39-antibody that was preincubated with the relevant peptide (Fig. 1B) indicating the specificity of the anti-GPR39-antibody. Cytochemical analysis revealed that the GPR39 is localized to the sperm acrosomal region (Fig. 1B), suggesting its possible involvement in the acrosome reaction as will be seen later. Low concentrations of Zn^{2+} (50 μM) stimulated tyrosine phosphorylation of several proteins during sperm capacitation (Fig. 2A), including Src, EGFR (Fig. 2B and C), and PI3K (Fig. 2D). As shown in Fig. 2B, Zn^{2+} stimulated EGFR phosphorylation on tyr-845, which is the Src-phosphorylating site, as well as Tyr-1068, an autophosphorylation site important for PI3K activation (Cao et al., 2005). These data further support the specificity of the stimulatory effect of Zn^{2+} on Src and PI3K phosphorylation. The levels of the EGFR were constant under all treatments (Fig. 2C); thus, the increase in staining shown with the anti-phospho-antibodies indicates changes in phosphorylation levels. As tyrosine phosphorylation of proteins is part of the capacitation process (Visconti et al., 1995), we suggest that Zn^{2+} stimulates sperm capacitation.

It is known that protein tyrosine phosphorylation depends indirectly on PKA activity (Visconti et al., 1995). Zn^{2+} stimulated the phosphorylation of several PKA-substrates (Fig. 3A), and the PKA inhibitor H89 inhibited Zn^{2+}-dependent tyrosine phosphorylation of EGFR and PI3K (Fig. 3B), indicating that Zn^{2+} activates PKA as well as PKA-dependent tyrosine phosphorylation. We suggested elsewhere that Src mediates PKA-dependent EGFR phosphorylation on Tyr-845, a known site of Src activity (Etkovitz et al., 2009). The activation of PKA activity by Zn^{2+}, as well as Src, EGFR and PI3K phosphorylation/activation, clearly indicate that Zn^{2+} stimulates the cascade PKA-Src-EGFR-PI3K, known to be involved in the capacitation process (Ickowicz et al., 2012). It was shown elsewhere that Zn^{2+} involved in intracellular pH regulation in sea urchin sperm (Clapper et al., 1985). A recent study in sea urchin shows that submicromolar concentrations of free Zn^{2+} activates change in membrane potential, an increase in PHi, $[Ca^{2+}]_i$ and cAMP and activates cGMP-gated K^+ channel (Beltran et al., 2014). It is well accepted that sperm sAC is activated by bicarbonate (Garty and Salomon, 1987; Okamura et al., 1985), and it has been suggested that an increase in pHi could activate sAC by increasing $[HCO_3^-]i$ (Parrish et al., 1989), thus elevating [cAMP]i and activating PKA leads to indirect protein tyrosine phosphorylation and capacitation (Galantino-Homer et al., 2004). This finding further supports the stimulatory effect of Zn^{2+} on sperm capacitation.

In our recent study, we suggested a mechanism by which PKA mediates the tyrosine phosphorylation of PI3K in sperm capacitation (Rotfeld et al., 2014). In this cascade, PKA activates Src, which in turn mediates the inhibition of the serine/threonine phosphatase, PP1. PP1 dephosphorylates CaMKII; thus, the inhibition of PP1 by Src enhances the phosphorylation/activation state of CaMKII, leading to phosphorylation of the tyrosine kinase Pyk2, which in turn- phosphorylates PI3K on tyr-467 (Rotfeld et al., 2014). Activation of the EGFR leads to PI3K-tyrosine phosphorylation (Rev. by Schlessinger (2000). As this occurs in bovine sperm as well, we suggest that Zn^{2+} can activate the PKA-Src-EGFR-PI3K and/or the PKA-Src-PP1-CaMKII-Pyk2-PI3K cascades in sperm capacitation.

We showed in several studies that activation of EGFR and PI3K in capacitated sperm leads to the AR (Etkovitz et al., 2007; Jaldety et al., 2012; Lax et al., 1994). Other authors also suggested a role for PI3K in sperm capacitation and the AR (Fisher et al., 1998; Jungnickel et al., 2007). Here, we demonstrate that the addition of Zn^{2+} to capacitated bovine sperm, enhanced EGFR and PI3K tyrosine phosphorylation/activation (Fig. 4A). Moreover, Zn^{2+} induced occurrence of the AR, when added to capacitated sperm, and this induction was inhibited by tyrphostin 1478 (AG) or wortmannin (10 nM) (Fig. 4B), known inhibitors of EGFR or PI3K, respectively. Thus, Zn^{2+} can induce the AR mediated by EGFR and PI3K activation.

Zn^{2+} binds and activates the ZnR, a known GPCR (Hershfinkel et al., 2007). Activation of GPCR leads to activation of tmAC, which catalyzes cAMP production from ATP. In sperm, the most frequent AC is the soluble-AC (sAC), activated by bicarbonate (Chen et al., 2000) and Ca^{2+} (Jaiswal and Conti, 2003; Litvin et al., 2003). Bicarbonate is a necessary component in the capacitation medium (Esposito et al., 2004; Hess et al., 2005), indicating the importance of sAC for the capacitation process. Although the role of tmAC in mammalian sperm function is still controversial, we showed elsewhere that specific activation of bovine sperm GPCRs leads to PKA activation (Etkovitz et al., 2009), indicating the activation of tmAC in mammalian sperm capacitation. tmACs are regulated by heterotrimeric G-proteins and stimulated by forskolin (Hanoune and Defer, 2001). Indeed, forskolin was found to stimulate bovine (Vijayaraghavan et al., 1985) and human (Liu et al., 2003) sperm motility. Moreover, truncation of the AC3 gene, encoding a ubiquitous eukaryotic tmAC, disrupts mouse sperm motility (Livera et al., 2005). Also, tmAC mediates the AR in mouse sperm (Wertheimer et al., 2013) and both sAC and tmAC mediate the AR in sea urchin sperm (Beltran et al., 2007).

Here, we wished to determine whether Zn^{2+} mediates the AR via the activation of sAC and/or tmAC. As shown in Fig. 5B, inhibition of sAC by 2-OH-estradiol (2-OH) caused about 60% reduction, whereas inhibition of tmAC by ddAdo caused 100% inhibition of the Zn^{2+}-induced AR. Similar results were obtained by these inhibitors when AR was induced by Angiotensin II (AngII) which activates the GPCR AT1-R (Fig. 5B). Thus, the similarity between the effects of Zn^{2+} and Ang II on AR and the complete inhibition of Zn^{2+}-induced AR by inhibition of the tmAC support the involvement of the ZnR in sperm AR. It was shown that ddAdo specifically targets the p-site of the tmAC and does not affect sAC (Bitterman et al., 2013; Corcoran and Stetler-Stevenson, 1995; Wuttke et al., 2001). Thus, our data indicate that Zn^{2+} activates the tmAC, probably via activation of ZnR. When EGFR was activated directly by adding EGF, or when the Ca^{2+}-ionophore A23187 was used to induce the AR, the two AC inhibitors did not affect the acrosome reaction (Fig. 5B) indicating the specificity of the AC inhibitors, since bypassing the AC step using the downstream effectors EGF or Ca^{2+}-ionophore, eliminated the effect on AR mediated by these AC inhibitors.

The stimulation of EGFR phosphorylation by Zn^{2+} was also inhibited by the two AC inhibitors (Fig. 5A).The inhibition of p-EGFR by ddAdo indicates that Zn^{2+} mediates tmAC activation via ZnR, known to activate the tmAC. Regarding the effect of sAC

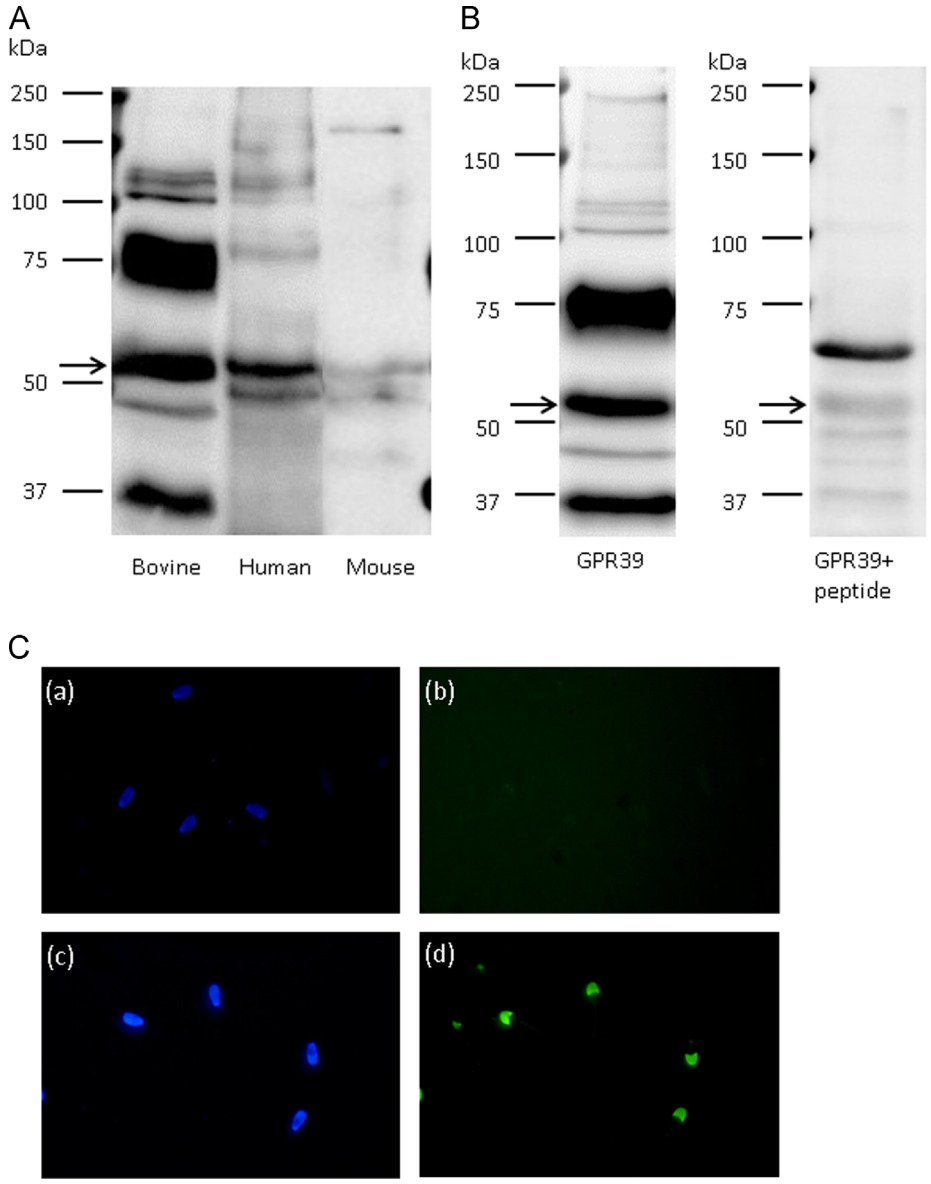

Fig. 1. Detection of ZnR GPR39 in sperm. (A) Bovine, human and mouse spermatozoa were incubated in capacitation medium for 4 h, 3 h and 1.5 h, respectively. Proteins were extracted and analyzed by western blot using anti-GPCR GPR39 antibody. The results shown are representative of three independent experiments. (B) To determine the specificity of the 51 kDa band, we used an immunizing peptide blocking experiment. Bovine spermatozoa were incubated in capacitation medium for 4 h. Proteins were extracted and analyzed by western blot using anti- GPR39 antibody or anti- GPR39 antibody that undergone pre-absorption with GPR39 peptide. (C) Bovine sperm was incubated for 4 h under capacitation conditions. At the end of the incubation, samples were taken out, and the cells were stained with DAPI (a,c) and with anti-GPCR (GPR39) antibody (d) followed by Alexa-fluor-488 antibody, photographed under a fluorescence microscope to determine fluorescence intensity in the sperm. To determine the specificity of secondary antibody, cells were stained without primary antibody.

inhibition on p-EGFR, it is known that sAC is activated in the capacitation process by bicarbonate and Ca^{2+}, leading to cAMP/PKA activation and EGFR tyrosine phosphorylation (Etkovitz et al., 2009). Thus, inhibiting the sAC is expected to result in p-EGFR inhibition (Fig. 5A).

To define more specifically the role of the ZnR, we tested whether Zn^{2+} could replace Ca^{2+} in the AR process. To test this possibility, we washed capacitated sperm to eliminate Ca^{2+}, and Zn^{2+} was added to medium containing the Ca^{2+}-chelator, EGTA. Indeed, 50 μM Zn^{2+} induced the AR in the absence of Ca^{2+} in the incubation medium, and the Ca^{2+}-ionophore A23187 further stimulated this effect. It was shown that A23187 can facilitate Zn^{2+} transport into cells (Erdahl et al., 1996; Wang et al., 1998), explaining the enhanced effect of A23187 on Zn^{2+}-induced AR in absence of extracellular Ca^{2+} (Fig. 6). Moreover, this effect of Zn^{2+} was completely blocked by La^{3+} (Fig. 6) an inhibitor of Ca^{2+} transport mechanisms (Hu et al., 2009). These data demonstrate that Zn^{2+} enters the cell and can affect intracellular processes. The fact that A23187 could not induce the AR in absence of added Ca^{2+} (Fig. 5C) indicates that the medium is depleted of Ca^{2+} further support our conclusion that Ca^{2+} can be replaced by Zn^{2+} in the AR mechanism. The stimulation of the AR by Zn^{2+} in the absence of Ca^{2+} is completely inhibited by ddAdo, with no inhibition by 2-OH (Fig. 5C) indicating that tmAC but not sAC, mediates the Zn^{2+}-induced AR by activating a ZnR. However, when both Ca^{2+} and Zn^{2+} are present we see inhibition of AR by the two AC inhibitors (Fig. 5B) suggesting that under these conditions sAC as well as tmAC are both active in the AR mechanism. The inhibition of AngII-induced AR is also inhibited by the two inhibitors (Fig. 5B), suggesting both sAC and tmAC mediate the AR under these

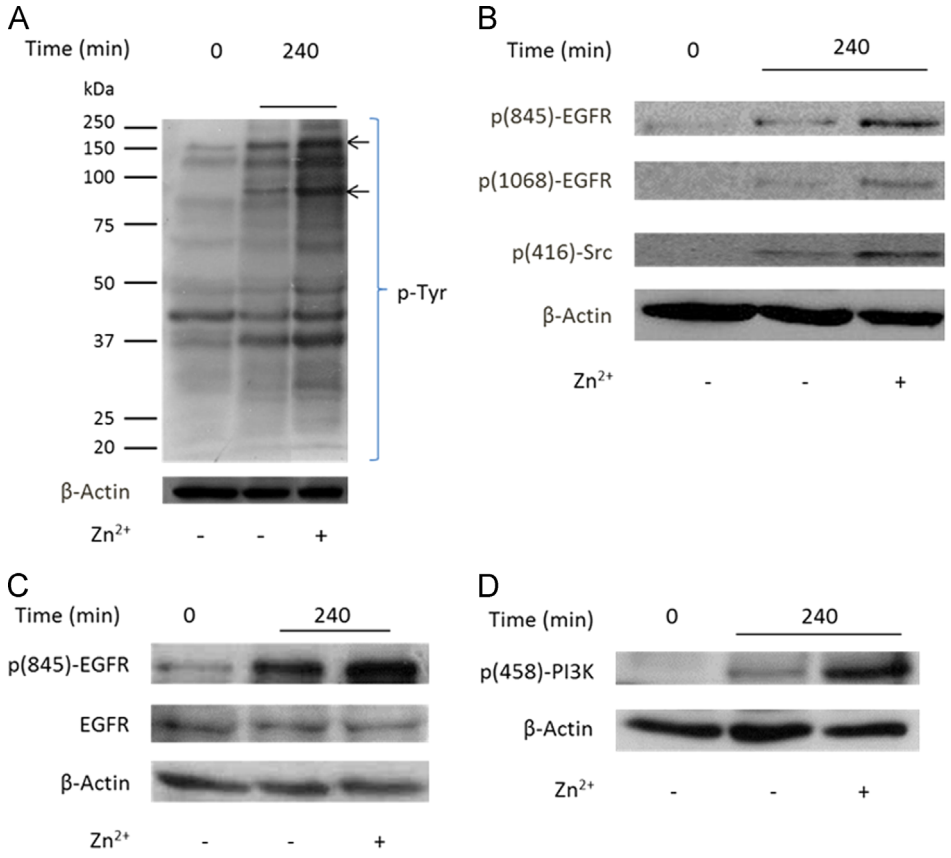

Fig. 2. Effect of Zn^{2+} on protein tyrosine phosphorylation during sperm capacitation: Bovine spermatozoa were incubated in capacitation medium with or without $ZnCl_2$ (50 μM) for 4 h. Proteins were extracted and analyzed by western blot using: (A) anti-phospho-Tyrosine antibody, (B) anti-phospho-EGFR (Tyr845 and 1068) and anti-phospho-Src (Tyr416) antibodies, (C) anti-EGFR and anti-phospho-EGFR (Tyr845) antibodies, or (D) anti-phospho-PI3K (Tyr458) and anti-β-actin (loading control) antibodies. The results shown are representative of three independent experiments.

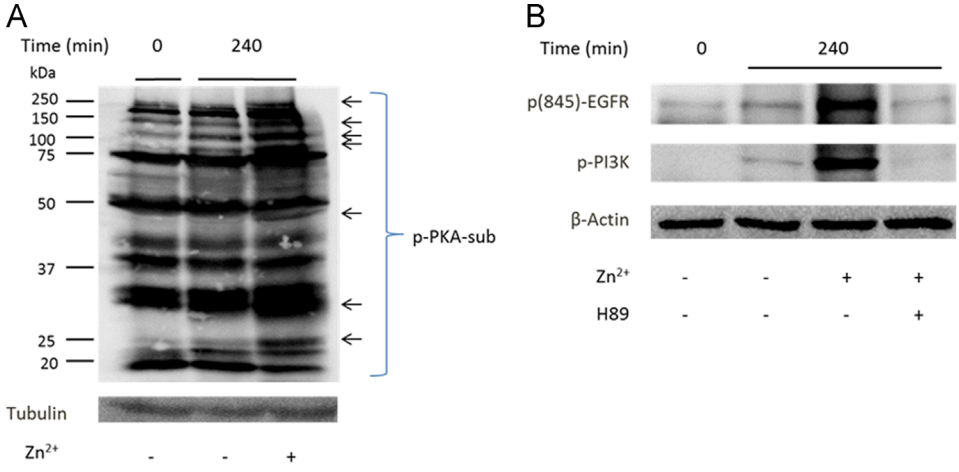

Fig. 3. Zn^{2+} activates PKA-dependent protein tyrosine phosphorylation: (A) Bovine spermatozoa were incubated in capacitation medium with or without $ZnCl_2$ (50 μM) for 4 h. Proteins were extracted and analyzed by western blot using anti-phospho-PKA substrate and anti-tubulin antibodies. (B) Bovine spermatozoa were incubated in capacitation medium with or without H-89 (50 μM) and $ZnCl_2$ (50 μM). H-89 was added at the beginning of capacitation for 10 min, followed by addition of $ZnCl_2$ for the remainder of the 4 h capacitation. Proteins were extracted and analyzed by western blot using anti-phospho-EGFR (Tyr845), anti-phospho-PI3K (Tyr485), and anti-β-actin antibodies. The results shown are representative of three independent experiments.

conditions. There is evidence of direct interaction between Zn^{2+} and GPR39, inducing IP_3-mediated Ca^{2+} release from intracellular stores (Holst et al., 2007; Yasuda et al., 2007). Indeed, we found that AR induced by Zn^{2+} is completely blocked by the phospholipase C(PLC) inhibitor, U73122 and there is no effect by the inactive analog U73343 (Fig. 7C) indicating the specificity of the inhibitor. Thus, intracellular Zn^{2+} might replace Ca^{2+} in the mechanism leading to AR, or may activate PLCβ, known to be activated by ZnR in other cell types (Hershfinkel et al., 2001), or PLCγ, known to be activated by EGFR. PLC can catalyze the hydrolysis of PIP_2 to produce IP_3 which can activate IP_3R of the outer acrosomal membrane to release acrosomal Ca^{2+}, leading to store-operated-Ca^{2+}-channel (SOCC) activation and the occurrence of the AR (Meizel and Turner, 1993; Spungin and Breitbart,

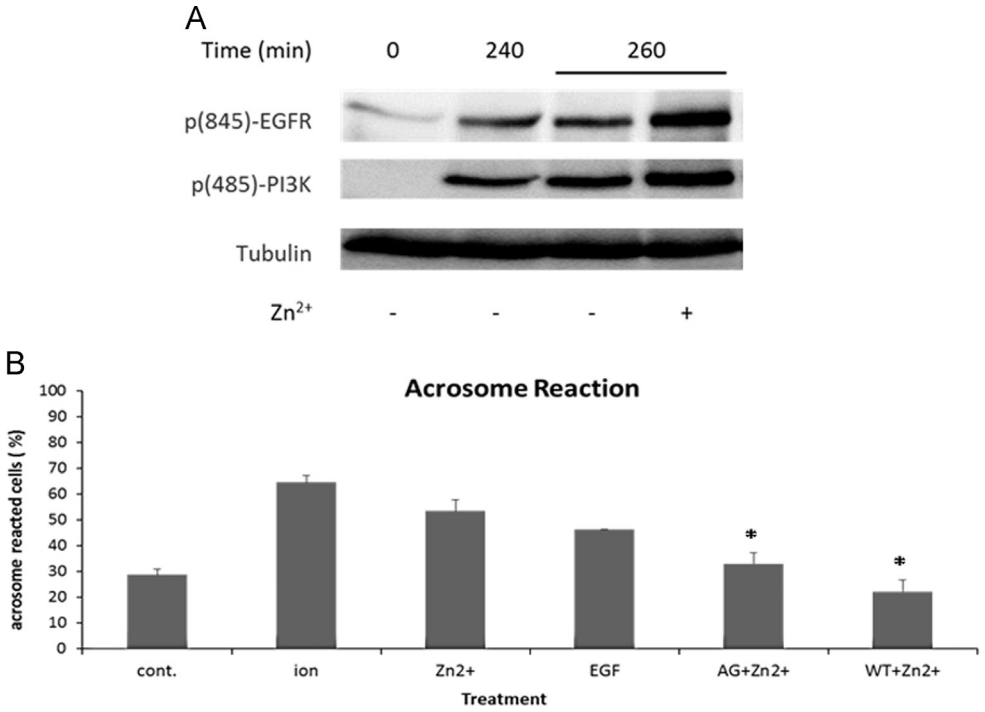

Fig. 4. Effect of Zn^{2+} on protein tyrosine phosphorylation and acrosome reaction in capacitated sperm cells: (A) Bovine spermatozoa were incubated in capacitation medium for 4 h. $ZnCl_2$ (50 μM) was added for an additional 20 min. Proteins were extracted and analyzed by western blot using anti-phospho-EGFR (Tyr845), anti-phospho-PI3K (Tyr485) and anti-tubulin antibodies. (B) Bovine spermatozoa were incubated in capacitation medium for 4 h. Tyrphostin1478 (AG) (5 μM) or Wortmannin (WT) (10 nM) were added for the last 10 min, and then $ZnCl_2$ (50 μM), EGF (1 ng/ml) or calcium ionophore A23187 (ion) (10 μM) were added for an additional 20 min. Sperm samples were smeared on slides to determine acrosome reaction, as described in "Experimental Procedures". ∗Significant difference versus Zn^{2+} only. $p < 0.05$.

1996; Walensky and Snyder, 1995). We showed elsewhere that bovine sperm exhibit SOCC activity (Dragileva et al., 1999), and here we show that activation of SOCC using thapsigargin, which inhibits the Ca^{2+}-ATPase of the outer acrosomal membrane, led to strong stimulation of the AR (Fig. 7A). However, it is not clear if Zn^{2+} can be transported by SOCC. It was shown in salivary cells that Zn^{2+} (20 μM) is a competitive inhibitor of Ca^{2+} influx via SOCC but does not permeate through the SOCC (Gore et al., 2004). Nevertheless, the effect of Zn^{2+} on the AR in the presence of thapsigargin, was significantly higher (55% stimulation) than its effect without thapsigargin (Fig. 7A), suggesting that SOCC can serve as a Zn^{2+} transporter. It is also possible that Zn^{2+} triggers Ca^{2+} release from the acrosome leading to stimulation of the AR, or can activate CatSper in the plasma membrane due to the elevation of PHi. The effect of Ca^{2+} on the AR in the presence of thapsigargin was 43% higher than the effect of Zn^{2+} (Fig. 7A). Similarly, Ca^{2+}-ionophore induced a higher rate of AR in the presence of Ca^{2+} compared to Zn^{2+} (Fig. 6). Thus, it seems that Ca^{2+} is more efficient than Zn^{2+} in its influx rate via the SOCC or the ionophore and/or the induction of the AR. However when the Ca^{2+} transporters are inactive or in absence of the Ca^{2+}-ionophore, Zn^{2+} can trigger the AR whereas Ca^{2+} cannot (Fig. 5C).

The major route for Zn^{2+} entry into many cells are the voltage operated Ca^{2+} channels (Atar et al., 1995; Sensi et al., 1997; Snider et al., 2000). Alternatively, Zn^{2+} transporters (members of the ZIP-protein family) are expressed in various tissues including liver, ovarian, and testicular cells (He et al., 2009). The fact that Zn^{2+} can induce the AR, while Ca^{2+} is not effective unless its transport mechanism is activated, suggest that Zn^{2+} must activate the AR by a different mechanism than Ca^{2+}, probably via activation of a GPCR such as the ZnR. This idea is supported by showing complete inhibition of Zn^{2+}-induced AR by pre-treatment of sperm with anti-GPR39 antibodies, but no effect of peptide-preabsorbed GPR39 antibodies (Fig. 7B).These data clearly indicate that Zn^{2+}-induced AR is mediated by GPR39. Using antibodies to inhibit GPCR was recently described in other cell types (Hagemann et al., 2014). Activation of PLC which catalyzes the hydrolysis of PIP_2 to produce Diacylglycerol (DAG), an activator of PKC, suggests that PLC/PKC system might mediate the Zn^{2+}-induced AR. Indeed, we found that the PLC inhibitor U73122 (but not the inactive analog U73343) and the PKC inhibitor GF, completely inhibited the Zn^{2+}-induced AR (Fig. 7C). It is well accepted that activation of GPCRs lead to PLCβ/PKC activation, thus the involvement of PLC/PKC in the mechanism in which Zn^{2+} induces the AR support the activation of GPR39 by Zn^{2+}. Moreover, the inhibition of Zn^{2+}-induced AR by La^{3+} suggests that although extracellular Zn^{2+} can activate ZnR/PLCβ in the absence of extracellular Ca^{2+}, the AR cannot occur under these conditions unless Zn^{2+} enters the cells. The complete inhibition of Zn^{2+}- induced AR in Ca^{2+}-deficient medium by AG1478 (Fig. 7C) demonstrates that Zn^{2+}-induced AR is mediated by the EGFR. Moreover, this finding indicates that the GPCR by itself cannot induce the AR unless transactivation of the EFGR occurs. This conclusion is further supported by our previous study in which we show complete inhibition by an EGFR inhibitor when the AR is induced by GPCR activation (Etkovitz et al., 2009).

In conclusion, Zn^{2+} stimulates sperm capacitation and the AR mediated by activation of ZnR leading to PKA/Src dependent transactivation of the EGFR. Zn^{2+} can activate sAC by increasing intracellular pH and bicarbonate and enhance tmAC activity by activating ZnR. The fact that Zn^{2+}-induced AR is mediated by tmAC, and the inhibition of this effect by anti-GPR39 antibody suggests that Zn^{2+} activates a GPCR, probably the GPR39, in sperm.

The concentration of Zn^{2+} in the seminal plasma is very high (~3 mM) (Abdul-Rasheed, 2009), and it has been suggested that 0.25 mM Zn^{2+} and higher concentration inhibit sperm motility and the AR in-vitro (Andrews et al., 1994; Aonuma et al., 1981). Thus, the high concentrations of Zn^{2+} in the seminal plasma are

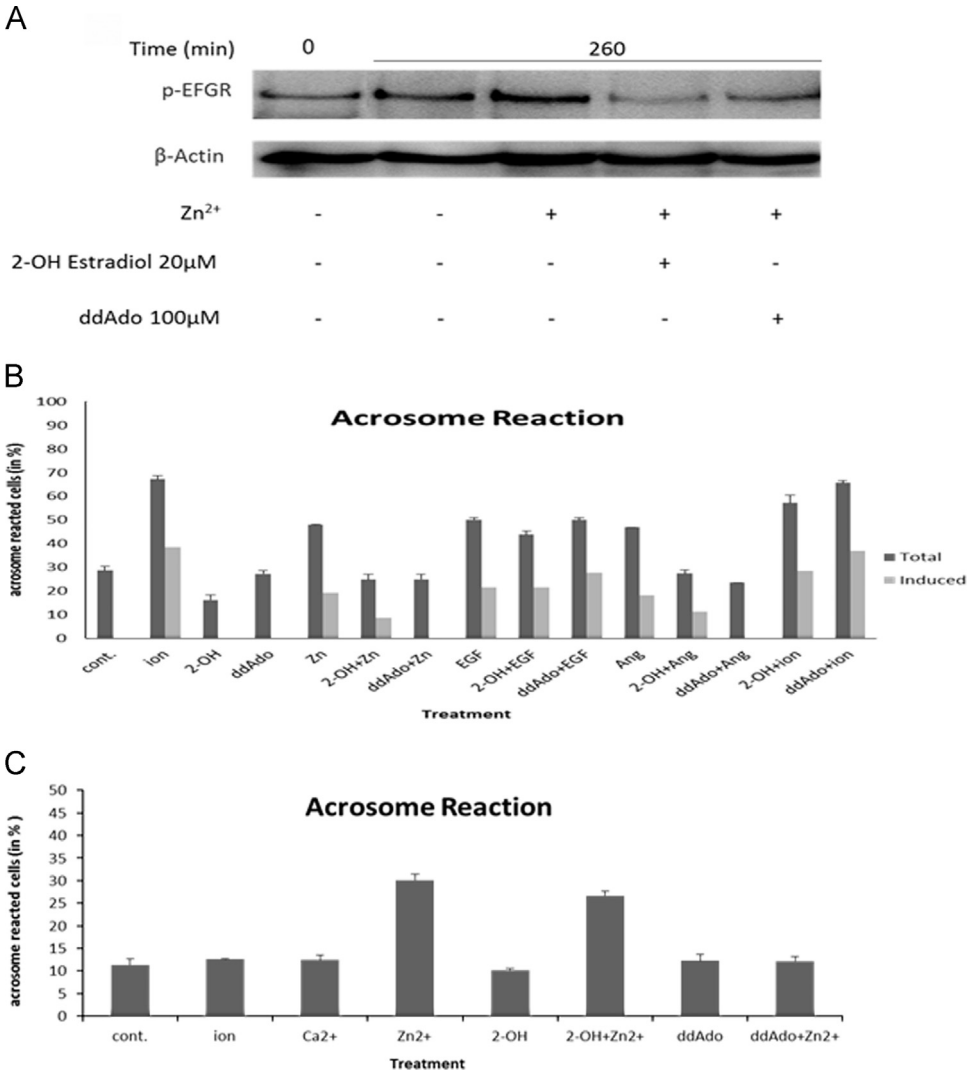

Fig. 5. Zn^{2+}-induced EGFR phosphorylation and AR are mediated by AC: (A) Bovine spermatozoa were incubated in capacitation medium for 4 h. 2-OH Estradiol (2-OH) (20 μM) or ddAdo (100 μM) was added for the last 10 min, and then $ZnCl_2$ (50 μM) was added for an additional 20 min. Proteins were extracted and analyzed by western blot using anti-phospho-EGFR (Tyr845), anti-phospho-PI3K (Tyr485), and anti-β-actin antibodies. (B) Bovine spermatozoa were incubated in capacitation medium for 4 h. 2-OH Estradiol (20 μM) or ddAdo (100 μM) was added for the last 10 min, and then $ZnCl_2$ (50 μM), calcium ionophore A23187 (ion) (10 μM), EGF (1 ng/ml), or AngiotensinII (Ang) (10 nM) were added as indicated for an additional 20 min. Sperm samples were smeared on slides to determine the acrosome reaction, as described in "Experimental Procedures". Induced acrosome reaction was calculated after subtracting the corresponding controls.(C) Bovine spermatozoa were incubated in capacitation medium for 4 h. The samples were washed in Ca^{2+}-free medium and EGTA (1 mM) was added to remove extracellular Ca^{2+}. 2-OH Estradiol (2-OH) (20 μM) or ddAdo (100 μM) was added for 10 min, and then $ZnCl_2$ (50 μM), $CaCl_2$ (2 mM) or calcium ionophore A23187 (ion) (10 μM) was added for an additional 20 min. Sperm samples were smeared onto slides to determine acrosome reaction, as described in "Experimental Procedures".

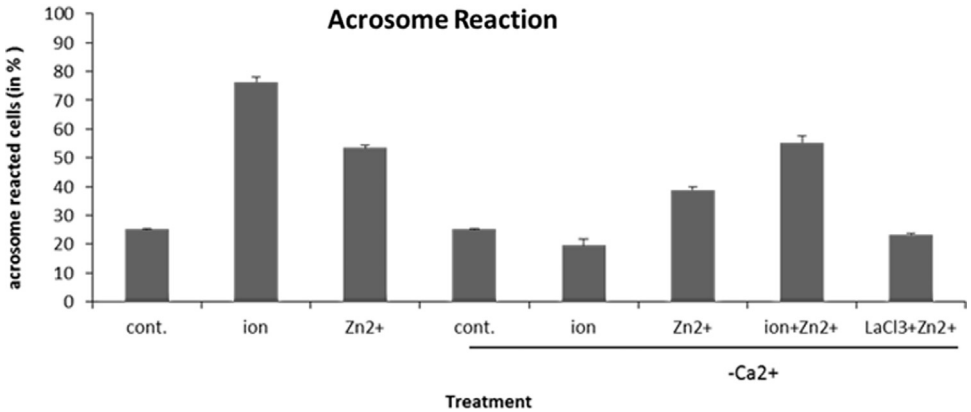

Fig. 6. Induction of AR by Zn^{2+} in Ca^{2+}-free medium: Bovine spermatozoa were incubated in capacitation medium for 4 h. An aliquot of each sample was washed in Ca^{2+}-free medium, and EGTA (1 mM) was added to remove extracellular Ca^{2+}. $LaCl_3$ (250 μM) was added for the 10 min, and then $ZnCl_2$ (50 μM) or the calcium ionophore A23187 (ion) (10 μM) was added for an additional 20 min. Sperm samples were smeared on slides to determine acrosome reaction, as described in "Experimental Procedures".

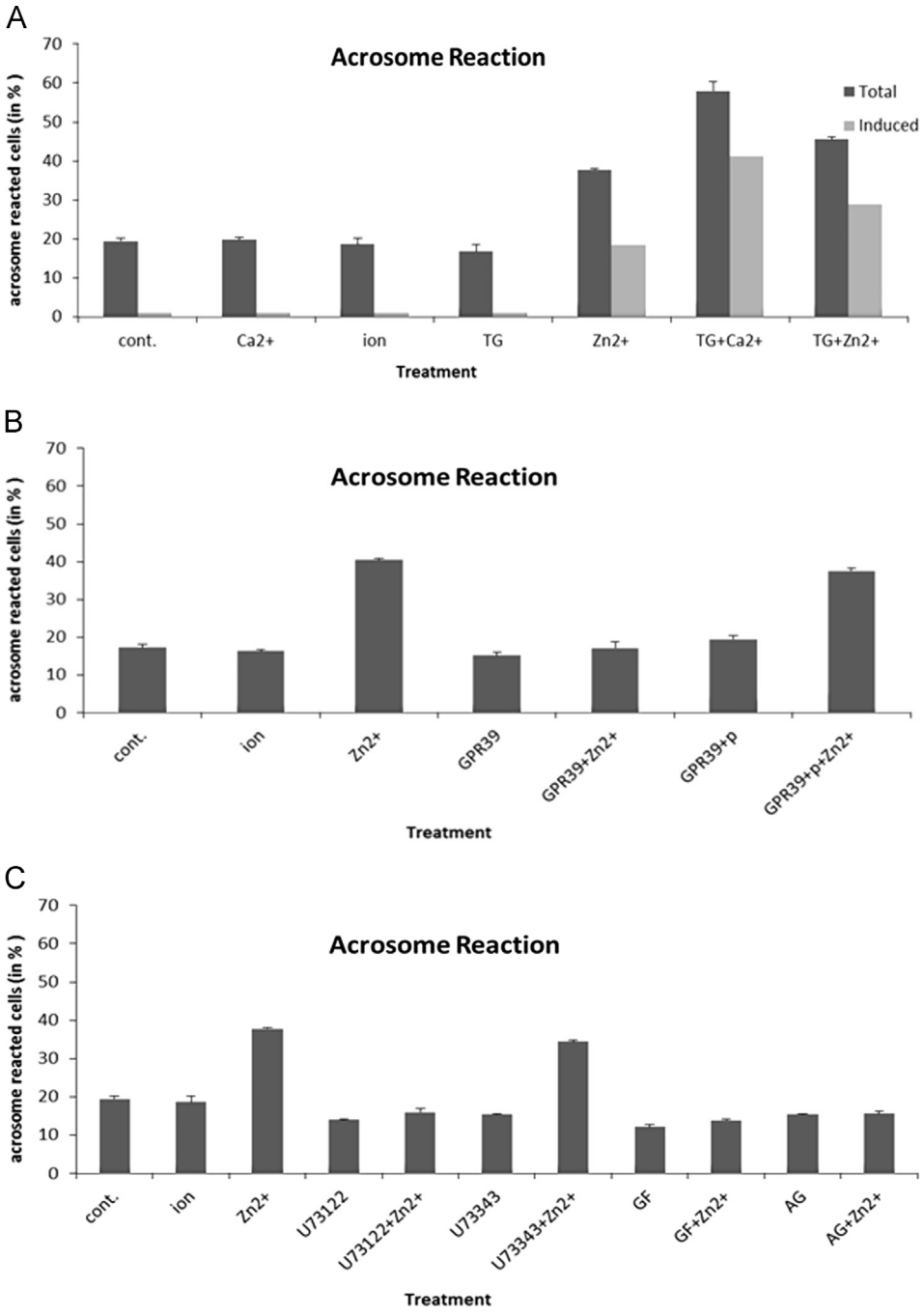

Fig. 7. Involvement of SOCC, PLC, PKC and EGFR in Zn^{2+}-induced AR in Ca^{2+}-free medium: (A) Bovine spermatozoa were incubated in capacitation medium for 4 h. The samples were washed in Ca^{2+}-free medium, and EGTA (1 mM) was added to remove extracellular Ca^{2+}. Thapsigargin (TG) (10 μM) was added for 10 min, and then $ZnCl_2$ (50 μM), $CaCl_2$ (2 mM) or calcium ionophore A23187 (ion) (10 μM) was added for an additional 20 min. Sperm samples were smeared onto slides to determine acrosome reaction, as described in "Experimental Procedures". The induced acrosome reaction was calculated after subtracting the corresponding controls. (B) Bovine spermatozoa were incubated in capacitation medium for 4 h. The samples were washed in Ca^{2+}-free medium, and EGTA (1 mM) was added to remove extracellular Ca^{2+}. GPR39 antibody (1 μg/ml) (PGR39) or GPR39 antibody that undergone pre-absorption with GPR39 peptide (1 μg/ml) (GPR39+P) were added for 10 min, and then $ZnCl_2$ (50 μM) or calcium ionophore A23187 (ion) (10 μM) was added for an additional 20 min. Sperm samples were smeared onto slides to determine acrosome reaction, as described in "Experimental Procedures". The induced acrosome reaction was calculated after subtracting the corresponding controls. (C) Bovine spermatozoa were incubated in capacitation medium for 4 h. The samples were washed in Ca^{2+}-free medium, and EGTA (1 mM) was added to remove extracellular Ca^{2+}. U73122 (1 μM), U73343 (1 μM), GF (0.1 nM), or Tyrphostin1478 (AG) (5 μM) were added for 10 min, and then $ZnCl_2$ (50 μM), calcium ionophore A23187 (ion) (10 μM), or $CaCl_2$ (2 mM) was added for an additional 20 min. Sperm samples were smeared onto slides to determine acrosome reaction, as described in "Experimental Procedures".

inhibitory for sperm functions. However, after penetration into the female reproductive tract, the sperm is exposed to 1.0–1.5 μM Zn^{2+} (Menezo et al., 2011) which is about 2000 times lower compared to its concentration in the seminal plasma. Thus, under physiological conditions in the female reproductive tract Zn^{2+} might enhance sperm capacitation and fertilization ability.

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