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Bovine sperm acrosome reaction induced by G protein-coupled receptor agonists is mediated by epidermal growth factor receptor transactivation ☆

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ABSTRACT

We have previously demonstrated the presence of active epidermal growth factor receptor (EGFR) and its involvement in sperm capacitation and the acrosome reaction; however, the mechanism of EGFR activation was not clear. We show here that the sperm EGFR can be transactivated by angiotensin II or by lysophosphatydic acid, two ligands which activate specific G-protein-coupled receptors (GPCR), or by directly activating protein kinase A using 8Br-cAMP. This transactivation occurs in noncapacitated sperm and is mediated by PKA, SRC and a metalloproteinase. We also show that the EGFR is activated in sperm incubated under *in vitro* capacitation conditions, without any added ligand, but not in bicarbonate-deficient medium or when PKA is blocked. Despite the fact that EGFR is activated in capacitated sperm, this state is not sufficient to induce the acrosome reaction. We conclude that the EGFR is stimulated during capacitation via PKA activation, while further activation of the EGFR in capacitated sperm is required in order to induce the acrosome reaction. The acrosome reaction can be induced by GPCR via the transactivation of the EGFR by a signaling pathway involving PKA, SRC and metalloproteinase and the EGFR down-stream effectors PI3K, PLC and PKC.

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Introduction

Ejaculated mammalian spermatozoa must reside in the female genital tract for some time before gaining the ability to fertilize the egg. During this time, spermatozoa undergo a series of physiological changes called capacitation (reviewed in (Visconti et al., 2002). This step is necessary to enable the sperm–egg interaction.

After binding to the egg zona pellucida, the spermatozoon undergoes an exocytotic process called the acrosome reaction (AR) (reviewed in (Breitbart, 2003). This event is required for fertilization, because it enables passage of the spermatozoon through the zona pellucida and its subsequent fusion with the egg oolema. Therefore, elucidation of the mechanisms regulating the acrosome reaction is important for understanding the process of mammalian fertilization. A variety of agonists derived from the zona pellucida or constituents of the female reproductive tract trigger the AR *via* receptor-mediated mechanisms (Wassarman, 1987). Although zona pellucida-derived glycoproteins are thought to be the physiological inducers of the AR (Arnoult et al., 1996; Wassarman, 1987), the reaction 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), atrial natriuretic peptide (Rotem et al., 1998; Zamir et al., 1993) epidermal growth factor (EGF) (Lax et al., 1994) and other ligands. These agonists may have a direct and/or synergistic effect on the zona pellucida (Roldan et al., 1994). We recently showed that Angiotensin II (AngII), which is present in the female genital tract (Culler et al., 1986; Heimler et al., 1995; Husain et al., 1987; Palumbo et al., 1989) can induce the AR in bovine sperm (Gur et al., 1998). Moreover, we showed that the AGTR1 (Angiotensin II receptor type 1), which is a high-affinity receptor for AngII, is synthesized and expressed on the bovine sperm head during sperm capacitation (Gur et al., 1998). In human and rat sperm, the AGTR1 is localized in the sperm tail and low concentrations of AngII enhance the motility of these sperm (Vinson et al., 1995). The AGTR1 belongs to the family of G-protein-coupled-receptors (GPCRs), which are known to activate conventional-adenylyl cyclase (CAC) to produce cAMP (Fraser et al., 2005). AngII is also found in seminal plasma at concentrations higher than in blood plasma (O'Mahony et al., 2000) and it was shown to accelerate capacitation (Mededovic and Fraser, 2005) and induce hyperactivated-motility in sperm (Ball et al., 2003). The lysophosphatydic-acid receptor (LPAR) also belongs to the GPCR family, and we showed elsewhere that LPA activates PKC and induces the AR and actin polymerization in bovine sperm (Cohen et al., 2004; Garbi et al., 2000). LPA is present in the seminal plasma (Hama et al., 2002; Tanaka et al., 2004) and in the follicular fluid (Tokumura et al., 1999) indicating a potential role for LPA in fertilization.

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Protein kinase A (PKA)-dependent protein tyrosine phosphorylation occurs during sperm capacitation (Visconti et al., 1995b); however, its mechanism of action is not clear. It was shown that cAMP levels are enhanced during capacitation (Aitken et al., 1998; White and Aitken, 1989) and this affects protein tyrosine phosphorylation (Aitken et al., 1995; Baker et al., 2004; Rivlin et al., 2004; Visconti et al., 1995a). The sperm PKA catalytic subunit $C\alpha_2$ (Cs) is the only PKA catalytic subunit present in the mature sperm (San Agustin et al., 1998). It was shown that PKACs is required for bicarbonate to speed the flagellar beat and facilitate $Ca^{(2+)}$ entry channels, and in addition, is needed for the protein tyrosine phosphorylation during mouse sperm maturation (Baldi et al., 1995; Nolan et al., 2004). Confocal microscopy of ovine and murine sperm revealed that PKACs was detected predominantly in the tail, and less in the head (San Agustin et al., 1998). Immunofluorescent labeling of human sperm with a PKACs specific antibody indicated that Cs was localized in the midpiece region of the spermatozoon (Reinton et al., 2000). However, recently it was shown that PKAC is localized to the head and tail of human sperm (Mitchell et al., 2008). Moreover, in sea urchin it was shown that PKA is active in the head as well as in the flagellum (Su et al., 2005). The regulatory subunits of PKA: PKA1A, PKA1B, PKA2A, PKA2B are also present in bovine sperm in which PKA1A and PKA1B were localized to the acrosome region (Vijayaraghavan et al., 1997). This activity in the head is in agreement with the involvement of this kinase in the AR (Lefievre et al., 2002; Su et al., 2005).

In spermatozoa, about 95% of the adenylyl cyclase consists of soluble-adenylyl cyclase (SAC) known to be activated by bicarbonate and calcium ions (Chen et al., 2000). It was shown that the SAC is localized to the tail and head of sea urchin sperm and was found to participate in the AR (Beltran et al., 2007). Thus, do sperm cells need GPCRs for cAMP production? It was shown that sperm contain the Gs-protein, which activates the conventional-adenylyl cyclase (or transmembrane-adenylyl cyclase) (Fraser et al., 2005). Moreover, it was found that the conventional-adenylyl cyclase (CAC) is localized to the sperm tail and head and was shown to participate in the AR (Baxendale and Fraser, 2003; Beltran et al., 2007). Furthermore, AngII, the ligand of the AGTR1, can induce cAMP production in sperm (Fraser et al., 2005) indicating its involvement in conventional-adenylyl cyclase activation. Because AngII and LPA have been implicated in sperm functions, we further investigated the mechanism of action of these ligands in the AR.

We previously showed that bovine sperm contains epidermal-growth-factor-receptor (EGFR) involved in the AR and in actin polymerization during sperm capacitation (Brener et al., 2003; Lax et al., 1994). Recently it was shown that boar sperm also contain the EGFR involved in the motility of sperm. This EGFR were localized at higher extent to the acrosome region then to the post-acrosome and the flagellum (Oliva-Hernandez and Perez-Gutierrez, 2008). In other cell types, GPCR signaling is mediated by receptor tyrosine kinases (RTK) such as EGFR, in a process called transactivation (Jorissen et al., 2003; Prenzel et al., 1999; Shah and Catt, 2003; Wetzker and Bohmer, 2003). The majority of RTK transactivation by GPCRs, in many cell types, is mediated by metalloproteinase-dependent shedding, or by release of growth factor-like substances such as heparin binding–EGF (HB-EGF), known as triple-membrane-passing signals (Prenzel et al., 2000). In this mechanism, the GPCR activates a Zn^{2+}-dependent metalloproteinase to cleave pro-heparin-binding EGF, releasing an EGF-like ligand, which binds to the EGFR and activates it. In some cases, SRC mediates the GPCR-EGFR transactivation process (Nair and Sealfon, 2003) by phosphorylating EGFR-Y845, known to be the SRC target (Liu et al., 2004; Prenzel et al., 2001; Tice et al., 1999). This phosphorylation (Y845) in the kinase domain is implicated in stabilizing the activation loop and maintaining the active state of the receptor (Cooper and Howell, 1993; Hubbard et al., 1994). EGFR-Y845 phosphorylation can lead to the activation of the EGFR by autophosphorylation that leads to the activation of various cascades. These cascades include the MAPK cascade (Rojas et al., 1996) and PI3K-Akt-PDPK2 cascade (Cao et al., 2005) which are activated by phosphorylation of EGFR-Y1068.

In human sperm SRC was found in the flagellum and head and was localized to membrane fraction (Lawson et al., 2008). SRC is also involved in protein tyrosine phosphorylation and motility during sperm capacitation (Baker et al., 2006; Mitchell et al., 2008). It was shown that SRC forms a complex with PKA which can phosphorylate and activate SRC (Baker et al., 2006; Lawson et al., 2008). Recently, SRC was localized to the post-acrosomal region of the head, neck and midpiece of human sperm (Lawson et al., 2008). SRC was found to be activated during human sperm capacitation and appears to be involved in regulating sperm capacitation, calcium fluxes, tyrosine phosphorylation and the AR (Varano et al., 2008).

We show here that the AR induced by AngII or LPA is mediated by the transactivation of the EGFR *via* a mechanism involving SRC and a metalloprotease. Moreover, we suggest that PKA mediates this transactivation, and it is localized upstream to SRC.

Materials and methods

Materials

AG1478, bisindolylmaleimide I (GF109203X (GF)), protease inhibitor cocktail, and GM6001 were purchased from Calbiochem (San Diego, CA). Antibodies against EGFR and specific polyclonal antibodies against phospho (Y845 or Y1068)EGFR were purchased from Cell Signaling (Beverly, MA). Goat Anti-Rabbit IgG (H+L)-Alexa Fluor 568, Goat Anti-mouse IgG (H+L)-Alexa Fluor 568 and Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Antibodies against SRC were purchased from Santa-Cruz (Santa Cruz, CA). Antibody against PKACs was a kind gift from G.B. Witman and J.T. San Agustin. Goat anti mouse IgG-HRP conjugated and Goat anti rabbit IgG-HRP conjugated were purchased from Bio-Rad (Richmond, CA). All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd. Rehovot, Israel) unless otherwise stated.

Sperm preparation

Ejaculated bull 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 Center (Hafetz-Haim, Israel). Sperm cells were washed three times by centrifugation (7803×*g* for 10 min at 25 °C) in NKM buffer that contained 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, and the 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 containing: 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 with 5% CO_2. The capacitation state of the sperm was confirmed after the 4-h incubation in mTALP by examining the ability of the sperm to undergo the acrosome reaction. In all experiments the control cells were treated with appropriate vehicle (Me_2SO or water).

Assessment of sperm acrosome reaction

Washed cells (10^8 cells/ml) were capacitated for 4 h at 39 °C in mTALP medium (Parrish et al., 1988). The inhibitors indicated were added after 4 h of incubation for 10 min in order to inhibit only the AR in capacitated sperm. 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 (10^8 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 three times 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 150 cells per slide on duplicate slides were evaluated (total of 300 cells for one experiment). Cells with green staining over the acrosomal cap were considered acrosome intact; those with equatorial green staining or no staining were considered acrosome reacted.

Determination of intracellular calcium

The intracellular concentration of free Ca^{2+} was assessed using the fluorescent calcium indicator, Fura-2. Washed cells (1×10^8/ml) were incubated in mTALP for 3.5 h, then 4 μM Fura-2/AM was added for a further 30 min. The loaded cells were then washed three times to remove extracellular Fura-2. The cells were used immediately for fluorescence measurements using a Shimadzu (Columbia, MD) RF-5000 spectrofluorophotometer, with an excitation wavelength of 340 nm and emission of 510 nm. During fluorescence measurements, sperm suspensions were maintained at 37 °C with stirring.

Immunoblot analysis

Sperm were washed by centrifugation for 5 min at 10,000×*g* at 4 °C and then the supernatant was discarded and TBS was added to the pellet. Sperm lysates were then prepared by the addition of lysis buffer containing: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 6% SDS, protease inhibitor cocktail 1:100 (Calbiochem), 50 μM NaF, 50 μM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM Na_3VO_4, to the pellet, and the lysate vortexed vigorously for 20 min at room temperature. Lysates were then centrifuged for 5 min at 10,000×*g* at 4 °C, the supernatant was removed, and the protein concentration was determined by the Bradford method (Bradford, 1976). Sample buffer ×2 was added to the supernatant and boiled for 5 min. The extracts were separated on 10% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked with 1% BSA in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. Kinases were immunodetected using anti-EGFR, anti-SRC, anti-PKACs and phospho-specific antibodies diluted 1:3000. 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 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

Phospho-specific anti-EGFR, anti-EGFR, anti-SRC and anti-PKACs antibodies were used at 1:50 dilution on permeabilized sperm smears, to determine intracellular localization of kinases and levels of phosphorylated EGFR residues, as described previously (Etkovitz et al., 2007). Nonspecific staining was determined by incubating the sperm without primary antibody in the presence of Goat Anti-Rabbit IgG (H+L)-Alexa Fluor 568 or Goat Anti-mouse IgG (H+L)-Alexa Fluor 568 diluted 1:200, and no staining was detected.

Microscopy

All images were captured on an Olympus AX70 microscope at a magnification of ×400. This microscope was equipped with an Olympus DP50 digital camera and with "Viewfinder Lite" software (version 1 from Pixera Corporation (Los Gatos, California, USA)). All fluorescence determinations were done under non-saturated conditions. Each experiment and staining were performed on the same day, and sperm were photographed within 24 h to reduce fading. All cell preparations from a single experiment were photographed during the same session and at the same exposure time.

Statistical analysis

Data are expressed as mean ± SD of at least three experiments for all determinations. Statistical significance was calculated by ANOVA with Bonferroni's *post hoc* comparison test using SPSS software (Chicago, IL).

Results

Transactivation of EGFR by AngII, LPA or cAMP

Activation of GPCRs can induce the transactivation of the EGFR in several cell types (Prenzel et al., 1999). Bovine sperm express the EGFR, and its activation by EGF induces the AR (Lax et al., 1994). We also showed that activation of the EGFR can promote sperm capacitation (Brener et al., 2003; Spungin et al., 1995a). In this study, we tested whether activation of AGTR1 by AngII or of the LPAR by LPA will transactivate the EGFR *via* a PKA-dependent mechanism.

EGFR antibodies recognize a single band at 170 kDa (Fig. 1) which is in agreement with our previous findings (Lax et al., 1994).

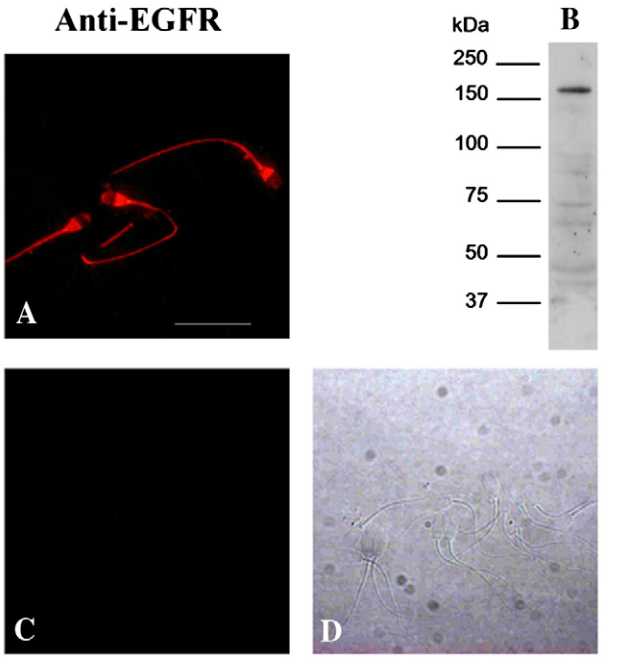

Fig. 1. Expression and localization of EGFR in bovine sperm. Bovine sperm were stained with antibodies directed against the EGFR (a) or only with second antibody (c and d) as described in Materials and methods or lysed and separated by SDS-PAGE, then blotted with anti-EGFR (b). The data represent one experiment, typical of three repetitions performed with sperm from three different bulls. Scale bar represents 20 μm.

EGFR was localized to the sperm head and midpiece but not to the principal region of the tail (Fig. 1). Using two antibodies directed against two different specific tyrosines of the EGFR, Y845 and Y1068, we found by indirect immunocytochemistry a significant increase in tyrosine phosphorylation induced by LPA, AngII, 8Br-cAMP or EGF (Fig. 2A). These antibodies recognize a single band at 170 kDa when incubated with 8Br-cAMP for 5 min as shown in Fig. 2B. Furthermore, western blot analysis indicated a significant increase in the EGFR-Y845 phosphorylation by AngII, LPA, 8Br-cAMP or EGF (Fig. 4). It is known that Y845 is a specific EGFR-tyrosine phosphorylated by SRC that stabilizes the receptor in its active form (Cooper and Howell, 1993; Hubbard et al., 1994; Prenzel et al., 2001; Stover et al., 1995; Tice et al., 1999); thus our data suggest that SRC is activated under these conditions. Since SRC is known to be activated by PKA in sperm (Baker et al., 2006; Lawson et al., 2008; Varano et al., 2008), and the EGFR is localized to the sperm head, and we assumed that these three proteins mediate the AR, we used specific antibodies directed against these kinases in order to confirm their localization in bovine sperm. PKACs antibodies recognize a single band at around 40 kDa (Fig. 3Ab) which is in agreement with previous findings (San Agustin and Witman, 2001). SRC antibodies recognize a single band at 60 kDa (Fig. 3Bb) which is also in agreement with previous findings (Baker et al., 2006; Lawson et al., 2008). We show by indirect immunocytochemistry that PKACs is localized to the acrosome region and tail (Fig. 3Aa) while SRC is localized to the midpiece, post acrosome and the acrosome regions (Fig. 3Ba).

The increased phosphorylation of Y845 on EGFR induced by the addition of AngII, LPA, 8Br-cAMP or EGF to starved sperm was inhibited by tyrphostin 1478 (AG), a specific inhibitor of the EGFR (Figs. 4A, C). Furthermore, the increase in EGFR phosphorylation induced by AngII, LPA or 8Br-cAMP was inhibited by H89, a specific inhibitor of PKA (Fig. 4A), however, no inhibition by H89 was detected in the presence of EGF (Fig. 4C), indicating the specificity of this inhibitor to EGFR-upstream effectors. Thus, the increase in phospho-EGFR by 8Br-cAMP and the inhibition of EGFR phosphorylation on Y845 by H89 indicates that EGFR activation is mediated by PKA. It is known that H89 at relatively high concentration can inhibit PKC as well; however we show elsewhere that 50 μM H89 induces PKC-dependent actin polymerization in bovine sperm and PKCα is activated when PKA activity is omitted (Cohen et al., 2004). Moreover, we showed here that H89 does not inhibit the acrosome reaction induced by EGF (Fig. 7) whereas inhibition of PKC by GF does inhibit this activity (Fig. 8) indicating that PKC mediates the acrosome reaction induced by EGF and H89 does not affect this activity. All together, these findings show clearly that the inhibition of EGFR phosphorylation by H89 occurs due to PKA inhibition and not by PKC inhibition.

In other cell types, transactivation of the EGFR can be mediated by SRC (Liu et al., 2004; Nair and Sealfon, 2003; Wetzker and Bohmer, 2003). Therefore, we looked for the involvement of SRC family in the transactivation of sperm EGFR induced by AngII, LPA or 8Br-cAMP. The phosphorylation of the EGFR on Y845 which is the SRC target, was inhibited by the SRC family inhibitors PP2 or

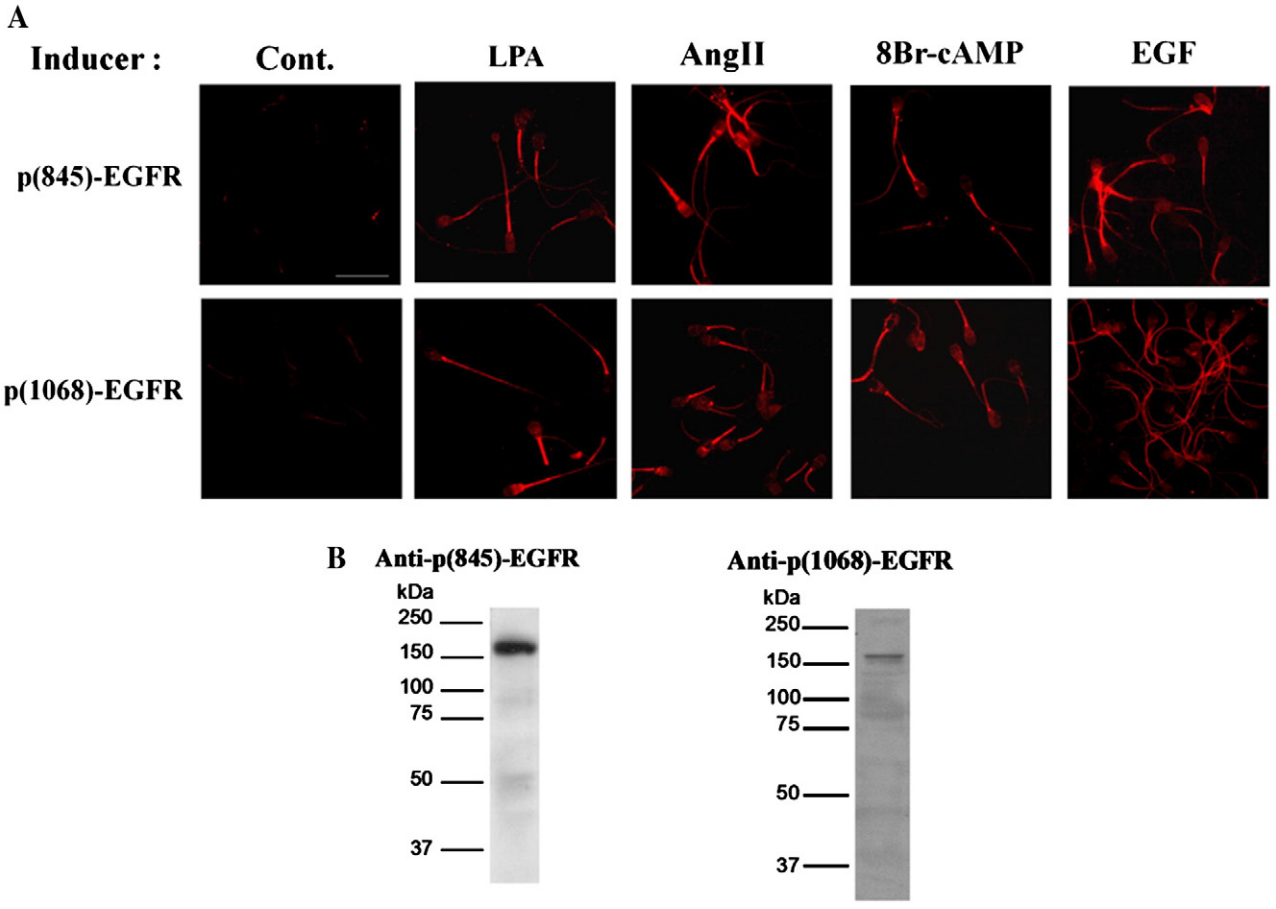

Fig. 2. LPA, AngII and 8Br-cAMP increase EGFR Y845 and Y1068 phosphorylation. Bovine sperm were starved for 2 h in NKM buffer before incubation in mTALP. The inducers 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP or 1 ng/ml EGF were added to the cells for 5 min and sperm cells were stained with antibodies directed against p(845) or p(1068) of the EGFR (A) as described in Materials and methods or lysed and separated by SDS-PAGE (8Br-cAMP treatment), then blotted with antibodies directed against p(845) or p(1068) (B). The data represent one experiment, typical of three repetitions performed with sperm from three different bulls. Scale bar represents 20 μm.

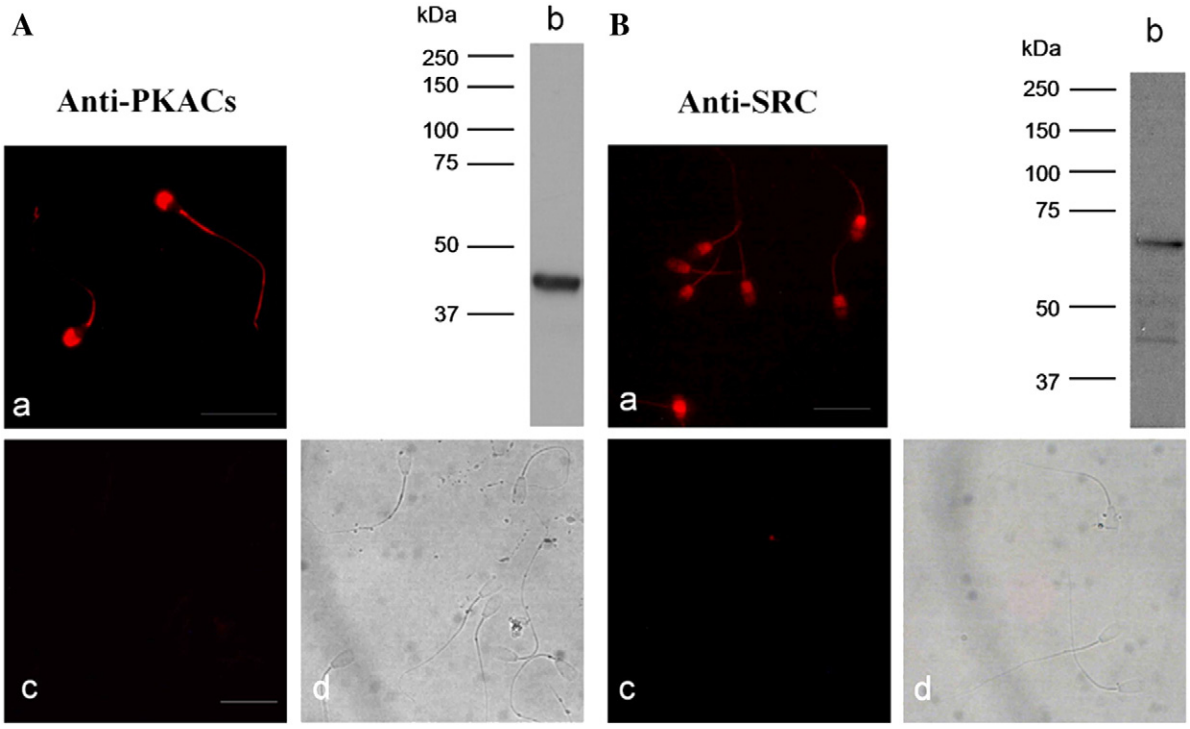

Fig. 3. Expression and localization of PKA and SRC in bovine sperm. Bovine sperm were stained with antibodies directed against PKACs (Aa) or SRC (Ba) or only with second antibody (Ac and Ad for PKACs Bc and Bd for SRC) as described in Materials and methods or lysed and separated by SDS-PAGE, then blotted with anti-PKACs (Ab) or anti-SRC (Bb). The data represent one experiment, typical of three repetitions performed with sperm from three different bulls. Scale bar represents 20 μm.

PP1 (Figs. 4A, B), but not by its inactive analogue PP3 (not shown) or when the phosphorylation of the EGFR was induced by EGF (Fig. 4C). The phosphorylation of the EGFR on Y845 and the inhibition of this phosphorylation by PP2 and PP1 but not by PP3, clearly indicate that SRC family mediates EGFR transactivation.

To further support the EGFR-transactivation by GPCR or 8Br-cAMP, we tested the involvement of metalloproteases (MMP), known intermediates in such transactivation Prenzel (Jorissen et al., 2003; Prenzel et al., 1999; Shah and Catt, 2003; Wetzker and Bohmer, 2003). Galardin (GM6001) is a specific inhibitor of MMP activity (Wahl and

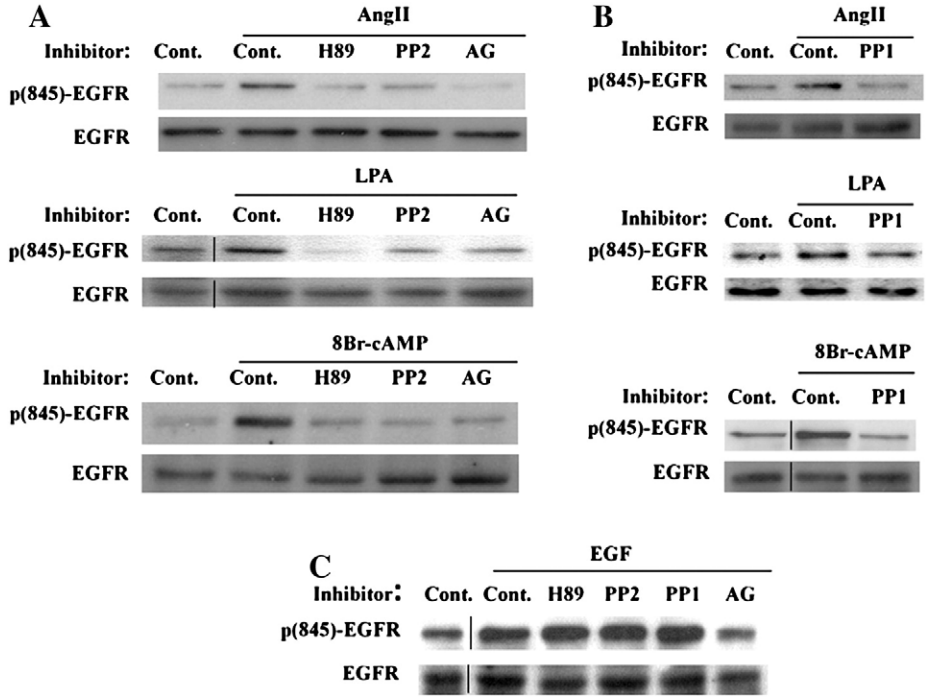

Fig. 4. LPA, AngII and 8Br-cAMP increase EGFR Y845 phosphorylation by a PKA, SRC and EGFR-dependent mechanism. Bovine sperm were starved for 2 h in NKM before incubation in mTALP with or without 50 μM H89, 5 μM PP2 or 5 μM AG1478 (AG) (A and C) or 5 μM PP1 (B and C) for 15 min. Then, the inducers 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP (A and B) or 1 ng/ml EGF (C) were added to the cells for 5 min. The sperm were then lysed and separated by SDS-PAGE, then blotted with anti p(845)EGFR and anti-EGFR. The data represent one experiment, typical of five repetitions performed with sperm from five different bulls.

Carpenter, 1988). Since the activation of the EGFR by MMP is not mediated by SRC/Y845 phosphorylation, we determined the EGFR-Y1068 phosphorylation. This phosphorylation induced by AngII, LPA or 8Br-cAMP was inhibited by GM6001 (Fig. 5A) however, no inhibition of Y1068 phosphorylation was detected in the presence of EGF (Fig. 5B), indicating the specificity of this inhibitor for EGFR-upstream effectors. These data suggest that the triple-membrane-passing signals mechanism is involved in the transactivation of the EGFR in bovine sperm.

Activation of EGFR during sperm capacitation

We previously showed that direct activation of the EGFR by its ligand (EGF) can promote sperm capacitation (Brener et al., 2003; Spungin et al., 1995b). We showed above that starved cells EGFR can be activated by EGF, by activating GPCR or by adding 8Br-cAMP. However, to our surprise, we found that the EGFR is gradually phosphorylated on Y845 during the 4-h incubation under capacitation conditions even without adding any EGFR ligand (Fig. 6A), but not in the absence of bicarbonate (Fig. 6B). It was shown elsewhere that the presence of bicarbonate in sperm capacitation medium is required for the activation of soluble adenylyl cyclase (Chen et al., 2000) resulting in cAMP/PKA activation and protein tyrosine phosphorylation (Visconti et al., 1995b). The EGFR-tyrosine phosphorylation in sperm incubated in bicarbonate-deficient medium could be recovered by adding 8Br-cAMP to the medium (Fig. 6B) supporting the possible involvement of PKA in EGFR transactivation during sperm capacitation. Sperm incubation under capacitation conditions induced EGFR-tyrosine phosphorylation that was increased following the addition of 8Br-cAMP to the medium and was inhibited by the PKA inhibitor, H89 (Figs. 6A, C). Furthermore, inhibition of PKA, SRC family, metalloproteinase or EGFR during incubation under capacitation conditions inhibited EGFR-Y1068 phosphorylation (Fig. 6D), indicating that sperm EGFR is activated during capacitation in the absence of any known EGFR ligand, probably *via* the transactivation mechanism mediated by PKA, SRC and metalloproteinase.

The acrosome reaction induced by AngII or LPA is mediated by PKA

In an early study from our laboratory we showed that EGFR activation by EGF induces the AR (Lax et al., 1994). In light of our results, we examined the involvement of EGFR transactivation during the AR. The addition of LPA, AngII, 8Br-cAMP or EGF to capacitated bovine sperm resulted in a significant enhancement of the percentage of acrosome-reacted cells (Fig. 7). AR induced by AngII, LPA or 8Br-cAMP was 82%, 88% or 91% inhibited, respectively, by the PKA inhibitor H-89, whereas the AR induced by EGF was not affected (Fig. 7).

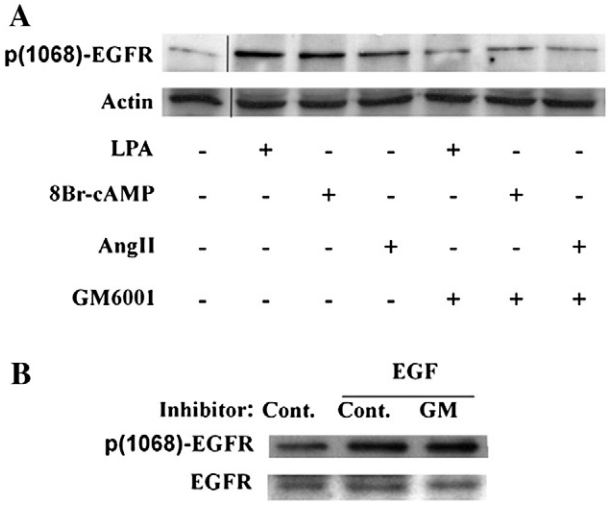

Fig. 5. LPA, AngII and 8Br-cAMP increase EGFR Y1068 phosphorylation *via* a mechanism involving metalloproteinase. Bovine sperm were serum starved for 2 h in NKM before incubation in mTALP with or without 20 μM GM6001 (GM) for 15 min. Then, the inducers 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP (A) or 1 ng/ml EGF (B) were added to the cells for 5 min. The sperm were lysed and separated by SDS-PAGE, then stained with anti p(1068)EGFR, anti EGFR or anti actin. The results shown represent one experiment, typical of five repetitions performed with sperm from five different bulls.

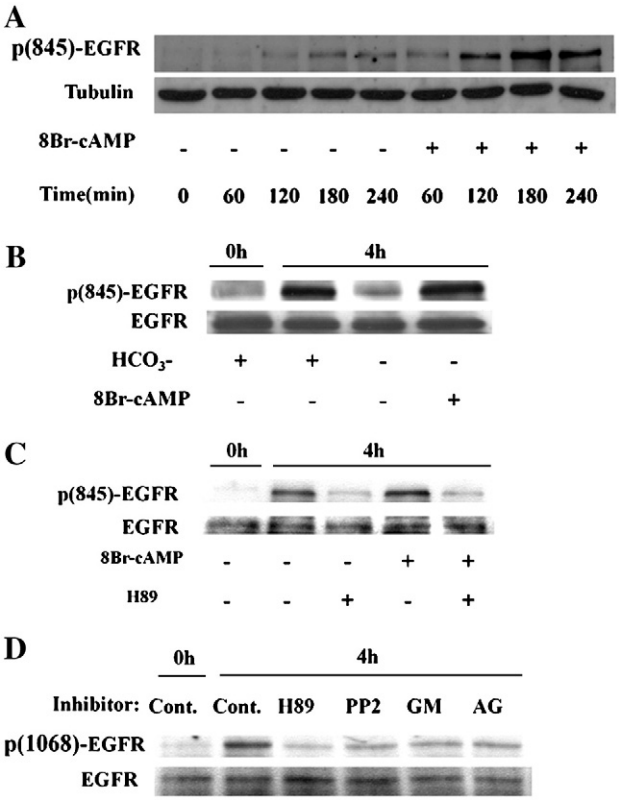

Fig. 6. Transactivation of EGFR during capacitation is mediated by PKA. (A) Bovine sperm were incubated in mTALP with or without 1 mM 8Br-cAMP for 4 h and sperm were lysed at the indicated times. (B) Bovine sperm were incubated in mTALP with or without bicarbonate and 1 mM 8Br-cAMP for 4 h. (C) Bovine sperm were incubated in mTALP with or without 50 μM H89 for 10 min; then, 1 mM 8Br-cAMP was added, as indicated, for 4 h. (D) Bovine sperm were incubated in mTALP with 50 μM H89, 5 μM PP2, 5 μM AG1478 (AG) or 20 μM GM6001 (GM) for 4 h. Sperm were lysed at the indicated times and proteins separated by SDS-PAGE, then stained with anti p(845)EGFR, anti-EGFR or anti-tubulin. The data represent one experiment, typical of three repetitions performed with sperm from three different bulls.

The AR induced by AngII, LPA or cAMP is mediated by EGFR, SRC family and metalloprotease

To further show that EGFR is the mediator of AngII, LPA or 8Br-cAMP induced AR, we examined the effect of EGFR inhibitor on this process. Fig. 7 shows that the AR induced by the addition of AngII, LPA, 8Br-cAMP or EGF to capacitated sperm is 83%, 92%, 80% or 92% inhibited, respectively, by the EGFR specific inhibitor, AG 1478 (Fig. 7). These data suggest that the AR induced by these agents is mediated by the EGFR. To further support this suggestion, we looked for the involvement of SRC family in the AR. We showed that the AR induced by AngII, LPA or 8Br-cAMP is significantly inhibited by PP1 or PP2 (96%, 82%, 80% by PP1 and 84%, 75% or 76% by PP2) two SRC-family-specific inhibitors (Fig. 7). Incubation of sperm with PP3, which is an inactive analogue of PP2, revealed a very small and insignificant inhibition of the AR (Fig. 7), indicating the specificity of PP1 and PP2 for SRC family inhibition. Moreover, the acrosome reaction induced by EGF was only slightly and

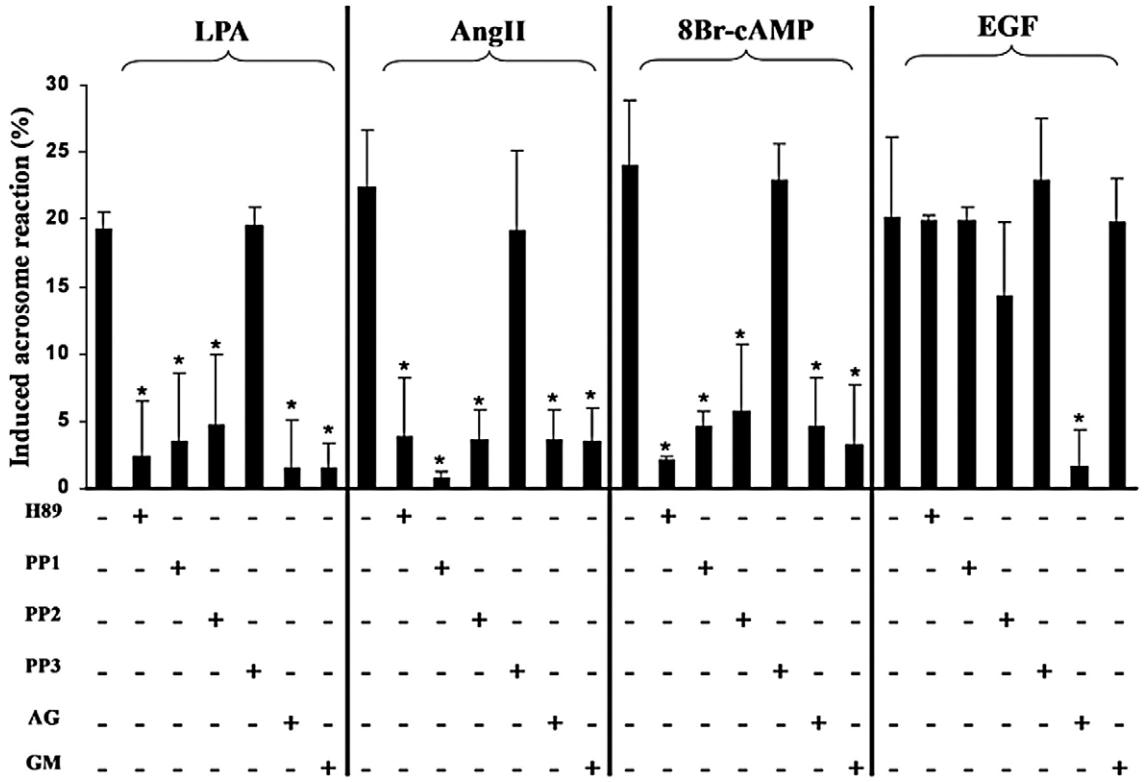

Fig. 7. The induction of the acrosome reaction by LPA, ANGII and 8Br-cAMP is mediated by the EGFR. Bovine sperm were incubated in mTALP for 4 h. At the end of this incubation 50 μM H89, 5 μM PP1, 5 μM PP2, 5 μM PP3, 0.5 μM AG1478 (AG), or 20 μM GM6001 (GM) were added as indicated for 10 min. After this incubation, 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP, or 1 ng/ml EGF were added to cells for additional 20 min of incubation. Acrosome-reacted cells were identified by PSA staining as described in the Materials and methods. The percentage of acrosome-reacted cells at the end of the 4 h incubation (22%) was subtracted to obtain the induced percentage. The data represent the mean ± SD of duplicates from at least five experiments. *Significant difference from the corresponding control, $P<0.05$.

insignificantly inhibited by PP2 but not by PP1 (Fig. 7). This finding further supports the specificity of PP1 and PP2 for SRC family inhibition.

Next, we tested the involvement of the triple-membrane-passing signals mechanism. AR induced by AngII, LPA or 8Br-cAMP were almost completely inhibited by GM6001 (Fig. 7); however, AR induced

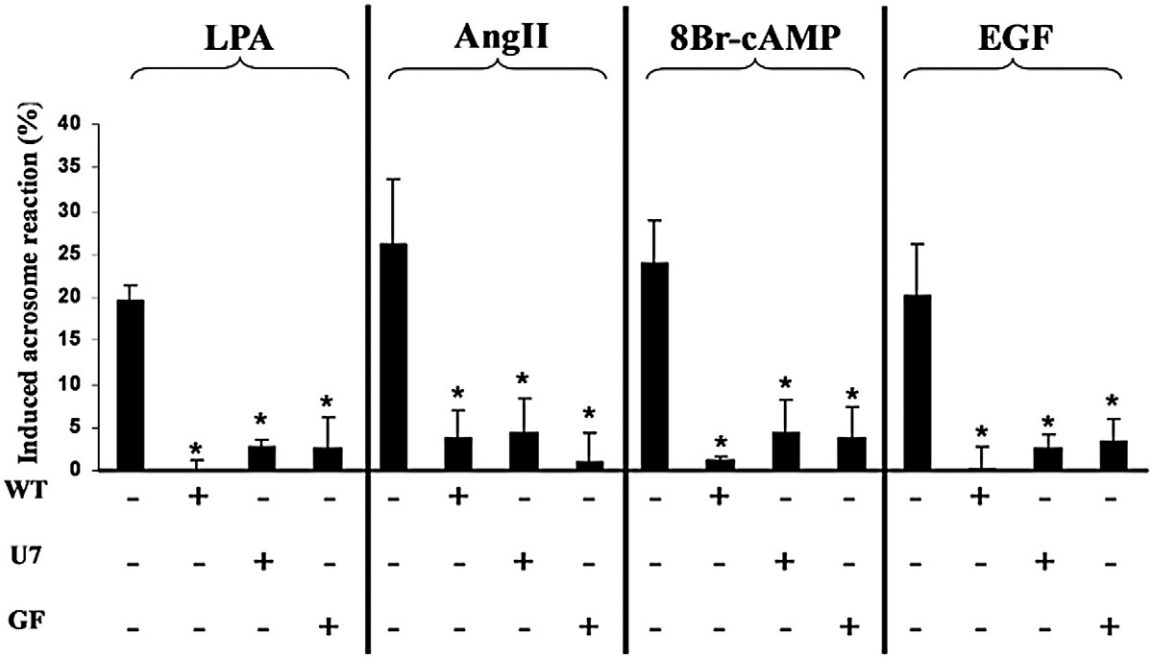

Fig. 8. LPA ANGII and 8Br-cAMP induced AR is inhibited by downstream effectors of EGFR. Bovine sperm were incubated in mTALP for 4 h. At the end of this incubation, 10 nM Wortmannin (WT), 1 μM U73122 (U7) and 0.1 nM bisindolylmaleimide I (GF) were added, as indicated, for 10 min. After this incubation, 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP, or 1 ng/ml EGF were added to the cells for an additional 20 min of incubation. The percentage of acrosome-reacted cells at the end of 4 h incubation (22%) was subtracted to obtain the induced percentage. The data represent the mean ± SD of duplicates from at least five experiments. *Significant difference from the corresponding control, $P<0.01$.

by direct activation of the EGFR by EGF was not affected by GM6001, confirming the specificity of this inhibitor (Fig. 7). These data suggest that the AR induced by GPCR agonists or PKA activation is mediated by a triple-membrane-passing signals mechanism.

The acrosome reaction induced by EGFR transactivation is mediated by PI3K, PLC and PKC

The data so far suggest that the AR induced by AngII, LPA or 8-Br-cAMP is mediated by the transactivation of the EGFR. We therefore looked for the involvement of EGFR-downstream effectors including PI3K, PLC and PKC in the AR induced by GPCR or cAMP. It can be seen in Fig. 8 that 10 nM wortmannin (WT), U73122 or bisindolylmaleimide I (GF), specific inhibitors of PI3K, PLC or PKC, respectively, caused almost complete inhibition of AR induced by AngII, LPA or 8Br-cAMP. These results suggest that these three enzymes mediate the AR induced by GPCR agonists or PKA activators downstream to the transactivation of the EGFR.

EGFR mediates intracellular Ca^{2+} increase induced by AngII, LPA or cAMP

The AR depends on the elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) (reviewed in (Breitbart, 2003). We showed in Fig. 7 that AngII, LPA, 8Br-cAMP or EGF induced the AR. In Fig. 9 we showed that these four compounds also caused a rapid increase in $[Ca^{2+}]_i$, and this effect was completely blocked when EGFR was inhibited by AG1478 (Fig. 9 inserts). These data further support the notion that transactivation of the EGFR by GPCRs agonists or cAMP is required for intracellular Ca^{2+} elevation.

Determination of EGFR transactivation by AngII, LPA or cAMP during the acrosome reaction

The results in the previous sections suggested that AngII, LPA or cAMP can activate the EGFR under capacitation conditions resulting in intracellular Ca^{2+} elevation leading to the occurrence of the acrosome reaction. We also showed that EGFR is already phosphorylated to some extent on Y845 by the end of the capacitation. If so, why do these cells do not undergo spontaneous acrosome reaction? In order to clarify this point, we examined whether the EGFR is further tyrosine phosphorylated during the AR. Fig. 10A shows a time-dependent increase in EGFR phosphorylation on Y845, in capacitated sperm during the AR, indicating that the EGFR is further activated under conditions that induce the acrosome reaction. These results suggest that the phosphorylation seen after 4 h of incubation in capacitation medium is not sufficient to drive the sperm to undergo the acrosome reaction. Moreover, the enhanced phosphorylation of

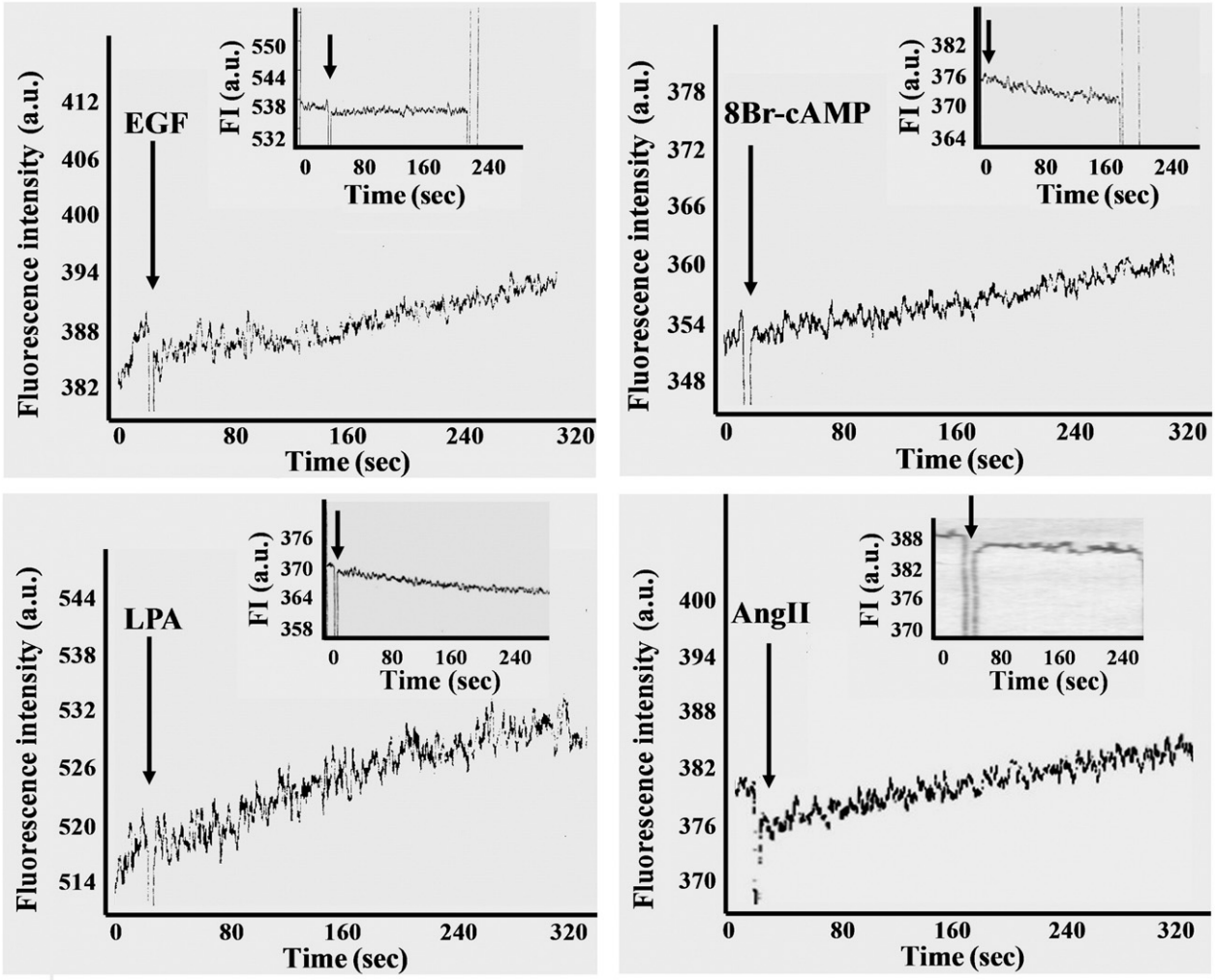

Fig. 9. AngII, LPA, 8Br-cAMP and EGF increase $[Ca^{2+}]i$ by an EGFR-dependent mechanism. (A) Bovine sperm were incubated in mTALP for 4 h and loaded with Fura 2AM for the final 30 min. At the end of this incubation, cells were washed and the fluorescence intensity was measured with or without the addition of 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP and 1 ng/ml EGF. The data represent one experiment, typical of three repetitions performed with sperm from three different bulls. (Inserts) Sperm loaded with Fura 2AM were washed as in A and incubated with 5 μM AG1478 for 30 min. Then, 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP, or 1 ng/ml EGF were added (arrow) and the fluorescence intensity was measured.

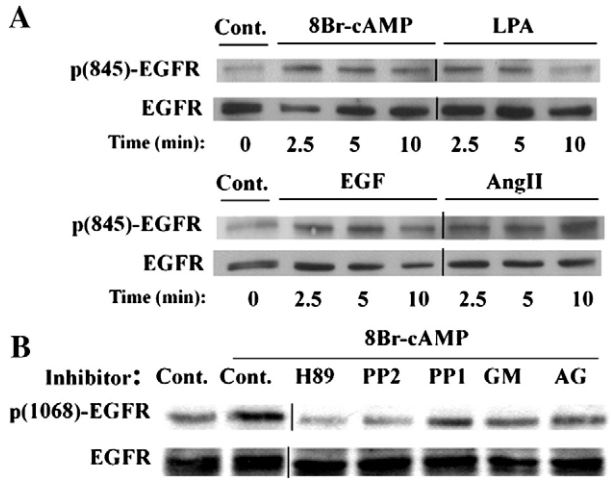

Fig. 10. LPA AngII and 8Br-cAMP increase EGFR phosphorylation in capacitated sperm. (A) Bovine sperm were incubated in mTALP for 4 h. Then, the inducers 1 μM LPA, 10 nM AngII, 1 mM 8Br-cAMP, or 1 ng/ml EGF were added to cells for 10 min. The sperm were lysed at the indicated times and separated by SDS-PAGE, then stained with anti p(845) EGFR and anti-EGFR. (B) Bovine sperm were incubated in mTALP for 4 h. At the end of this incubation, the inhibitors 50 μM H89, 5 μM PP1, 5 μM PP2, 5 μM AG1478 or 20 μM GM6001 were added for 15 min. Then, 1 mM 8Br-cAMP was added to the cells for 5 min. The sperm were lysed at the indicated times, separated by SDS-PAGE, and then stained with anti p(1068)EGFR and anti-EGFR. The data represent one experiment, typical of four repetitions performed with sperm from three different males.

the EGFR on Y1068 by 8-Br-cAMP was inhibited by H89, PP1, PP2, GM6001 or AG1478 (Fig. 10B), further supporting our suggestion.

Discussion

We showed here that sperm EGFR can be transactivated by the agonists of AGTR1 or LPAR. We suggest that this activation is mediated by PKA, SRC and metalloproteinase. A direct proof for this transactivation was provided by following EGFR activation/phosphorylation on two tyrosine residues known to be involved in the receptor activation. Phosphorylation was demonstrated using specific antibodies in two independent assays, western blot analysis and indirect immunocytochemistry. In order to demonstrate this increase in EGFR phosphorylation, the sperm cells were starved for 2 h and then incubated in capacitation medium for a short time (5 min) in the presence of AngII, LPA, 8Br-cAMP or EGF, and EGFR phosphorylation was determined. The enhanced effect of GPCR agonists or 8Br-cAMP on the phosphorylation of EGFR-Y845 (Figs. 2, 4) (known to be the SRC target (Prenzel et al., 2001; Stover et al., 1995; Tice et al., 1999), and the inhibition of this phosphorylation following inhibition of PKA, SRC family or EGFR, clearly indicate that EGFR-transactivation is mediated by PKA and SRC family (Fig. 4). Furthermore, the enhanced effect of GPCR agonists or 8Br-cAMP on EGFR-Y1068 phosphorylation, known to be autophosphorylated when EGFR is activated, was inhibited by blocking metalloproteinase's (Fig. 5). These results suggest that EGFR-transactivation is mediated by PKA, SRC family and metalloproteinase's (Figs. 4, 5). The localization of EGFR, PKA and SRC in the sperm head and midpiece support the possible interactions among these three proteins (Figs 1, 3). It is known that EGF receptor phosphorylation on Y845 leads to autophosphorylation of additional tyrosines (Stover et al., 1995; Tice et al., 1999), and indeed we showed here that in addition to Y854, Y1068 is phosphorylated as well (Figs. 2, 4, 5), a fact that further supports the activation of the EGFR by GPCR agonists and 8Br-cAMP. It was shown before that AngII and other GPCR agonists stimulate cAMP production in both capacitated and uncapacitated sperm (Mededovic and Fraser, 2004), a fact which supports our conclusion regarding cAMP/PKA stimulation using GPCR agonists.

To our surprise, we found that the EGFR is gradually activated when sperm are incubated under capacitating conditions for 4 h. This phosphorylation is remarkable, since no EGFR or GPCR ligands were included in the incubation medium. Furthermore, adding 8Br-cAMP to the incubation medium gave an early and enhanced EGFR phosphorylation. However, when bicarbonate was omitted from the medium, EGFR was not activated (Fig. 6B). PKA-dependent protein tyrosine phosphorylation is known to occur during sperm capacitation (Visconti et al., 1995b) and the majority of cAMP for PKA activation is produced by the SAC, which depends on bicarbonate for its

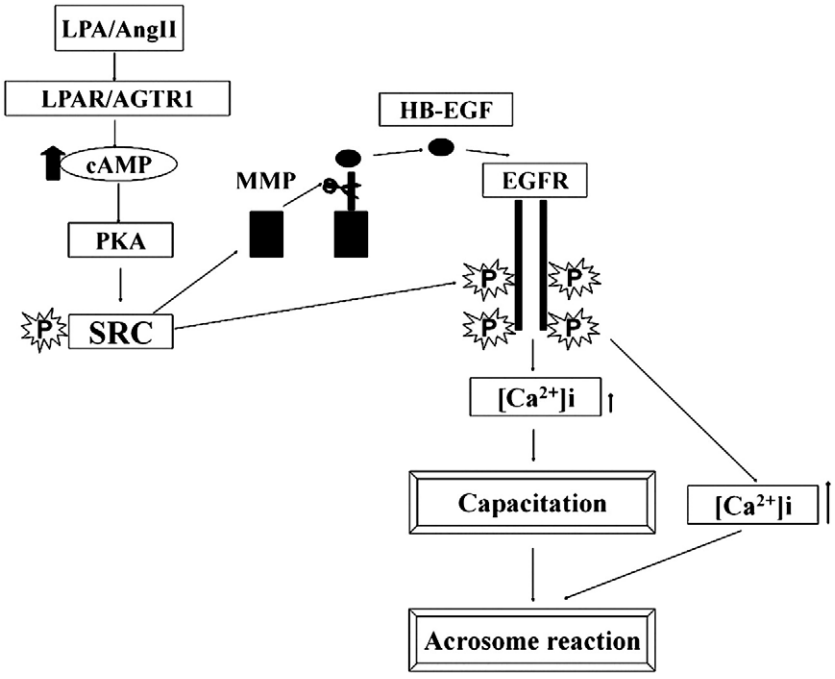

Fig. 11. A model for the transactivation of EGFR by GPCR agonists or PKA activation. Activation of AGTR1 or LPAR by agonists increases cAMP which activates PKA followed by SRC activation. SRC can activate EGFR directly or indirectly *via* MMP. The activation of the EGFR is part of the capacitation process; however during capacitation the EGF-receptors are only partially activated which cause relatively small increase in intracellular Ca^{2+}. Further activation of the EGFR is required in order to induce much higher increase in intracellular Ca^{2+} leading to the acrosome reaction.

activation (Chen et al., 2000). Thus our data suggest that PKA mediates EGFR activation during capacitation. This conclusion is supported by showing that EGFR-tyrosine phosphorylation in sperm incubated in bicarbonate-deficient medium could be restored by adding 8Br-cAMP to the cells (Fig. 6B) and the inhibition of EGFR-tyrosine phosphorylation occurred during capacitation by the PKA inhibitor, H89 (Fig. 6C).

We suggest that the phosphorylation level of the EGFR that occurs during sperm capacitation (see Fig. 6) is not sufficient for the induction of the AR. In order to trigger this reaction, an inducer must be added to the capacitated sperm (Fig. 7). Moreover, our data reveal that the AR induced by GPCR agonists, by 8Br-cAMP or by EGF, is inhibited by AG1478 (Fig. 7), indicating that the AR induced under these conditions is mediated by EGFR activation. These data suggest that further activation of the EGFR in capacitated sperm is needed in order to induce the AR. This notion is supported by showing that GPCR agonists, 8Br-cAMP or EGF added to capacitated sperm induce a time-dependent further increase in EGFR phosphorylation on Y845 (Fig. 10A). Moreover, inhibition of PKA, SRC family, metalloproteinase or EGFR at the end of capacitation inhibited both the phosphorylation of Y1068 on the EGFR (Fig. 10B) and the AR (Fig. 7), indicating that EGFR is transactivated through the same mechanism as occurs at the beginning of capacitation (see Figs. 2, 4 and 5). We assume that during capacitation the EGF-receptors are only partially activated which cause relatively small increases in intracellular Ca^{2+} (Fig. 9) and these receptors should be further activated at the end of the capacitation in order to induce a much higher increase in intracellular Ca^{2+} leading to the AR. This assumption is further supported by the enhancement of $[Ca^{2+}]_i$ by these compounds and the abrogation of this effect when EGFR was blocked (Fig. 9). Thus, activation of the EGFR causes an increase in $[Ca^{2+}]_i$ which is a crucial step for the AR. These data explain why the AR induced by AngII, LPA, cAMP or EGF is blocked by inhibiting the EGFR activities.

Inhibition of the EGFR down-stream effectors PI3K, PLC and PKC blocked the induction of AR by GPCR agonists or 8Br-cAMP (Fig. 8), further supporting our conclusion regarding EGFR transactivation under these conditions.

It is known that PKA, which is a serine/threonine kinase, mediates protein tyrosine phosphorylation in sperm capacitation (Visconti et al., 1995a); however, it is not clear how this mechanism functions. Several reports in somatic cells suggest that PKA activates SRC (Patschinsky et al., 1986; Schmitt and Stork, 2002), a tyrosine kinase known to phosphorylate the EGF receptor leading to its activation (Baker et al., 2006; Bertelsen et al., 2004; Jorissen et al., 2003; Prenzel et al., 1999; Shah and Catt, 2003; Shah et al., 2006; Stover et al., 1995; Tice et al., 1999; Wetzker and Bohmer, 2003). In murine sperm, SRC co-immunoprecipitates with PKA and this interaction leads to SRC phosphorylation (Baker et al., 2006). Here we showed that inhibition of SRC family by PP2, PP1 or inhibition of the EGFR, blocked the AR induced by AngII, LPA or 8Br-cAMP (Fig. 7), indicating the involvement of SRC family in EGFR activation. Moreover, the AR induced by these compounds was also blocked by metalloproteinase inhibition (Fig. 7), suggesting the additional involvement of metalloproteinase in EGFR activation. Transactivation of EGFR by GPCRs was previously demonstrated in other cell types (Bertelsen et al., 2004; Jorissen et al., 2003; Shah and Catt, 2003; Shah et al., 2006; Wetzker and Bohmer, 2003). In GTI-7 cells it was shown that activation of the GnRH-R, which is a GPCR, transactivates the EGFR mediated by SRC and metalloproteinase (Shah et al., 2006), a finding which supports our data. However, in C9 cells activation of AGTR1 transactivates the EGFR mediated by metalloproteinase but not by SRC (Shah et al., 2006). In HEK 293 cells, it was suggested that AGTR1 or LPAR can activate PI3K by a mechanism in which EGFR-transactivation is not involved (Shah et al., 2006). Thus, different GPCRs function with somewhat different mechanisms. Here we showed for the first time that sperm GPCRs transactivate the EGFR in a process mediated by PKA, SRC family and metalloproteinase in both noncapacitated or in capacitated sperm during the acrosome reaction.

In conclusion, we like to suggest a model which explains our findings (Fig. 11). During *in vitro* sperm capacitation, EGFR is partially activated by cAMP/PKA using cAMP produced by the bicarbonate/SAC system. Further activation of the EGFR in the capacitated sperm is required in order to trigger the AR. This activation can occur by direct activation of the EGFR by EGF, or by transactivation using GPCR agonists or PKA activators. AngII and LPA are found in the seminal plasma (O'Mahony et al., 2000) and AngII was already shown to accelerate capacitation (Mededovic and Fraser, 2005). Under *in vivo* sperm capacitation in the female reproductive tract, sperm are probably exposed to AngII and/or LPA, two ligands found in the female reproductive organs which activate the CAC/cAMP/PKA system. PKA can activate SRC which can activate the EGFR directly or via metalloproteinase activation which in turn hydrolyzes the HB-EGF to yield HB-EGF which activates the EGFR. The activation of EGFR during sperm capacitation is part of the capacitation process. Under *in vivo* conditions, the AR of capacitated sperm is induced by the egg zona-pellucida; however capacitated sperm might undergo a non-specific AR by other inducers present in the female reproductive tract like EGF, AngII or LPA. The reason for this non-specific AR might be as a result of selection priorities, since only intact non-acrosome-reacted sperm can interact with the egg zona-pellucida and fertilize the egg.

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References

Aitken, R.J., Paterson, M., Fisher, H., Buckingham, D.W., Van Duim, M., 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J. Cell Sci. 108, 2017–2025.

Aitken, R.J., Harkiss, D., Knox, W., Paterson, M., Irvine, D.S., 1998. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J. Cell Sci. 111, 645–656.

Arnoult, C., Zeng, Y., Florman, H., 1996. ZP 3-dependent activation of sperm cation channels regulates acrosomal secretion during mammalian fertilization. J. Cell Biol. 134, 637–645.

Baker, S.S., Thomas, M., Thaler, C.D., 2004. Sperm membrane dynamics assessed by changes in lectin fluorescence before and after capacitation. J. Androl. 25, 744–751.

Baker, M.A., Hetherington, L., Aitken, R.J., 2006. Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. J. Cell Sci. 119, 3182–3192.

Baldi, E., Krausz, C., Forti, G., 1995. Nongenomic actions of progesterone on human spermatozoa. Trends in Endocrin. Metab. 6, 198–205.

Ball, B.A., Gravance, C.G., Wessel, M.T., Sabeur, K., 2003. Activity of angiotensin-converting enzyme (ACE) in reproductive tissues of the stallion and effects of angiotensin II on sperm motility. Theriogenology 59, 901–914.

Baxendale, R.W., Fraser, L.R., 2003. Evidence for multiple distinctly localized adenylyl cyclase isoforms in mammalian spermatozoa. Mol. Reprod. Dev. 66, 181–189.

Beltran, C., Vacquier, V.D., Moy, G., Chen, Y., Buck, J., Levin, L.R., Darszon, A., 2007. Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction. Biochem. Biophys. Res. Commun. 358, 1128–1135.

Bertelsen, L.S., Barrett, K.E., Keely, S.J., 2004. G(s) protein-coupled receptor agonists induce transactivation of the epidermal growth factor receptor in T-84 cells—implications for epithelial secretory responses. J. Biol. Chem. 279, 6271–6279.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Breitbart, H., 2003. Signaling pathways in sperm capacitation and acrosome reaction. Cell. Mol. Biol. 49, 321–327.

Brener, E., Rubinstein, S., Cohen, G., Shternall, K., Rivlin, J., Breitbart, H., 2003. Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. Biol. Reprod. 68, 837–845.

Cao, Z., Liu, L., Van Winkle, D.M., 2005. Met5-enkephalin-induced cardioprotection occurs via transactivation of EGFR and activation of PI3K. Am. J. Physiol. Heart Circ. Physiol. 288, H1955–H1964.

Chen, Y., Cann, M.J., Litvin, T.N., Iourgenko, V., Sinclair, M.L., Levin, L.R., Buck, J., 2000. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 289, 625–628.

Cohen, G., Rubinstein, S., Gur, Y., Breitbart, H., 2004. Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. Dev. Biol. 267, 230–241.
Cooper, J.A., Howell, B., 1993. The when and how of Src regulation. Cell 73, 1051–1054.
Culler, M.D., Tarlatzis, B.C., Fernandez, L.A., De Cherney, A.H., Negro Vilar, A., Naftolin, F., 1986. Angiotensin II-like immunoreactivity in human ovarian follicular fluid. J. Clin. Endocrinol. Metab. 62, 613–615.
Etkovitz, N., Rubinstein, S., Daniel, L., Breitbart, H., 2007. Role of PI3-kinase and PI4-kinase in actin polymerization during bovine sperm capacitation. Biol. Reprod. 77, 263–273.
Fraser, L.R., Adeoya-Osiguwa, S., Baxendale, R.W., Mededovic, S., Osiguwa, O.O., 2005. First messenger regulation of mammalian sperm function via adenylyl cyclase/cAMP. J. Reprod. Dev. 51, 37–46.
Garbi, M., Rubinstein, S., Lax, Y., Breitbart, H., 2000. Activation of protein kinase Ca in the lysophosphatidic acid-induced bovine sperm acrosome reaction and phospholipase D1 regulation. Biol. Reprod. 63, 1271–1277.
Gur, Y., Breitbart, H., Lax, Y., Rubinstein, S., Zamir, N., 1998. Angiotensin II induces acrosomal exocytosis in bovine spermatozoa. Am. J. Physiol. 275, E87–E93.
Hama, K., Bandoh, K., Kakehi, Y., Aoki, J., Arai, H., 2002. Lysophosphatidic acid (LPA) receptors are activated differentially by biological fluids: possible role of LPA-binding proteins in activation of LPA receptors. FEBS Lett. 523, 187–192.
Heimler, I., Rawlins, R.G., Binor, Z., Aiman, J., Raff, H., Hutz, R.J., 1995. Elevated follicular fluid angiotensin II and pregnancy outcome. Fertil. Steril. 63, 528–534.
Hubbard, S.R., Wei, L., Ellis, L., Hendrickson, W.A., 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature 372, 746–754.
Husain, A., Bumpus, F.M., DeSilva, P., Speth, R.C., 1987. Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries. Proc. Natl. Acad. Sci. U. S. A. 84, 2489–2494.
Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W., Burgess, A.W., 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp. Cell Res. 284, 31–53.
Joyce, C.L., Nuzzo, N.A., Wilson, L., Zaneveld, L.J.D., 1987. Evidence for the role of cyclooxygenase (prostaglandin synthetase) and prostaglandins in the sperm acrosome reaction and fertilization. J. Androl. 8, 74–82.
Lawson, C., Goupil, S., Leclerc, P., 2008. Increased activity of the human sperm tyrosine kinase SRC by the cAMP-dependent pathway in the presence of calcium. Biol. Reprod. 79, 657–666.
Lax, Y., Rubinstein, S., Breitbart, H., 1994. Epidermal growth factor induces acrosomal exocytosis in bovine sperm. FEBS Lett. 339, 234–238.
Lefievre, L., Jha, K.N., de Lamirande, E., Visconti, P.E., Gagnon, C., 2002. Activation of protein kinase A during human sperm capacitation and acrosome reaction. J. Androl. 23, 709–716.
Liu, J., Liao, Z., Camden, J., Griffin, K.D., Garrad, R.C., Santiago-Perez, L.I., Gonzalez, F.A., Seye, C.I., Weisman, G.A., Erb, L., 2004. Src homology 3 binding sites in the P2Y2 nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. J. Biol. Chem. 279, 8212–8218.
Mededovic, S., Fraser, L.R., 2004. Angiotensin II stimulates cAMP production and protein tyrosine phosphorylation in mouse spermatozoa. Reproduction 127, 601–612.
Mededovic, S., Fraser, L.R., 2005. Mechanisms of action of angiotensin II on mammalian sperm function. Reproduction 129, 211–218.
Mitchell, L.A., Nixon, B., Baker, M.A., Aitken, R.J., 2008. Investigation of the role of SRC in capacitation-associated tyrosine phosphorylation of human spermatozoa. Mol. Hum. Reprod. 14, 235–243.
Nair, V.D., Sealfon, S.C., 2003. Agonist-specific transactivation of phosphoinositide 3-kinase signaling pathway mediated by the dopamine D2 receptor. J. Biol. Chem. 278, 47053–47061.
Nolan, M.A., Babcock, D.F., Wennemuth, G., Brown, W., Burton, K.A., McKnight, G.S., 2004. Sperm-specific protein kinase A catalytic subunit Calpha2 orchestrates cAMP signaling for male fertility. Proc. Natl. Acad. Sci. U. S. A. 101, 13483–13488.
O'Mahony, O.A., Djahanbahkch, O., Mahmood, T., Puddefoot, J.R., Vinson, G.P., 2000. Angiotensin II in human seminal fluid. Hum. Reprod. 15, 1345–1349.
Oliva-Hernandez, J., Perez-Gutierrez, J.F., 2008. Localization of the epidermal growth factor (EGF) in the epididymis and accessory genital glands of the boar and functional effects on spermatozoa. Theriogenology 70, 1159–1169.
Palumbo, A., Jones, C., Lightman, A., Carcangiu, M.L., De Cherney, A.H., Naftolin, F., 1989. Immunohistochemical localization of renin and angiotensin II in human ovaries. Am. J. Obstet. Gynecol. 160, 8–14.
Parrish, J.J., Susko-Parrish, J., Winer, M.A., First, N.L., 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38, 1171–1180.
Patschinsky, T., Hunter, T., Sefton, B.M., 1986. Phosphorylation of the transforming protein of Rous sarcoma virus: direct demonstration of phosphorylation of serine 17 and identification of an additional site of tyrosine phosphorylation in p60v-src of Prague Rous sarcoma virus. J. Virol. 59, 73–81.
Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., Ullrich, A., 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402, 884–888.
Prenzel, N., Zwick, E., Leserer, M., Ullrich, A., 2000. Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. Breast Cancer Res. 2, 184–190.
Prenzel, N., Fischer, O.M., Streit, S., Hart, S., Ullrich, A., 2001. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr. Relat. Cancer 8, 11–31.
Reinton, N., Orstavik, S., Haugen, T.B., Jahnsen, T., Tasken, K., Skalhegg, B.S., 2000. A novel isoform of human cyclic 3′,5′-adenosine monophosphate-dependent protein kinase, c alpha-s, localizes to sperm midpiece. Biol. Reprod. 63, 607–611.
Rivlin, J., Mendel, J., Rubinstein, S., Etkovitz, N., Breitbart, H., 2004. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. Biol. Reprod. 70, 518–522.
Rojas, M., Yao, S., Lin, Y.Z., 1996. Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. J. Biol. Chem. 271, 27456–27461.
Roldan, E.R.S., Murase, T., Shi, Q.X., 1994. Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science 266, 1578–1581.
Rotem, R., Zamir, N., Keynan, N., Barkan, H., Breitbart, H., Naor, Z., 1998. Atrial natriuretic peptide induces acrosomal exocytosis of human sperm. Am. J. Physiol. 274, E218–E223.
San Agustin, J.T., Witman, G.B., 2001. Differential expression of the C(s) and Calpha1 isoforms of the catalytic subunit of cyclic 3′,5′-adenosine monophosphate-dependent protein kinase testicular cells. Biol. Reprod. 65, 151–164.
San Agustin, J.T., Leszyk, J.D., Nuwaysir, L.M., Witman, G.B., 1998. The catalytic subunit of the cAMP-dependent protein kinase of ovine sperm flagella has a unique amino-terminal sequence. J. Biol. Chem. 273, 24874–24883.
Schmitt, J.M., Stork, P.J., 2002. PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. Mol. Cell. 9, 85–94.
Shah, B.H., Catt, K.J., 2003. A central role of EGF receptor transactivation in angiotensin II-induced cardiac hypertrophy. Trends Pharmacol. Sci. 24, 239–244.
Shah, B.H., Neithardt, A., Chu, D.B., Shah, F.B., Catt, K.J., 2006. Role of EGF receptor transactivation in phosphoinositide 3-kinase-dependent activation of MAP kinase by GPCRs. J. Cell Physiol. 206, 47–57.
Spungin, B., Margalit, I., Breitbart, H., 1995a. A 70kDa protein is transferred from the outer acrosomal to the plasma membrane during capacitation. FEBS Lett. 357, 98–102.
Spungin, B., Margalit, I., Breitbart, H., 1995b. Sperm exocytosis reconstructed in a cell-free system. Evidence for the involvement of phospholipase C and actin filaments in membrane fusion. J. Cell Sci. 108, 2525–2535.
Stover, D.R., Becker, M., Liebetanz, J., Lydon, N.B., 1995. Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. J. Biol. Chem. 270, 15591–15597.
Su, Y.H., Chen, S.H., Zhou, H., Vacquier, V.D., 2005. Tandem mass spectrometry identifies proteins phosphorylated by cyclic AMP-dependent protein kinase when sea urchin sperm undergo the acrosome reaction. Dev. Biol. 285, 116–125.
Tanaka, M., Kishi, Y., Takanezawa, Y., Kakehi, Y., Aoki, J., Arai, H., 2004. Prostatic acid phosphatase degrades lysophosphatidic acid in seminal plasma. FEBS Lett. 571, 197–204.
Tice, D.A., Biscardi, J.S., Nickles, A.L., Parsons, S.J., 1999. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. Proc. Natl. Acad. Sci. U.S.A. 96, 1415–1420.
Tokumura, A., Miyake, M., Nishioka, Y., Yamano, S., Aono, T., Fukuzawa, K., 1999. Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of *in vitro* fertilization patients. Biol. Reprod. 61, 195–199.
Varano, G., Lombardi, A., Cantini, G., Forti, G., Baldi, E., Luconi, M., 2008. Src activation triggers capacitation and acrosome reaction but not motility in human spermatozoa. Hum. Reprod. 23, 2652–2662.
Vijayaraghavan, S., Goueli, S.A., Davey, M.P., Carr, D.W., 1997. Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility. J. Biol. Chem. 272, 4747–4752.
Vinson, G.P., Puddefoot, J.R., Ho, M.M., Barker, S., Mehta, J., Saridogan, E., Djahanbakhch, O., 1995. Type 1 antigiotensin II receptors in rat and human sperm. J. Endocrinol. 144, 369–378.
Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Old-Clarke, P., Kopf, G.S., 1995a. Capacitation in mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121, 1129–1137.
Visconti, P.E., Moore, G.D., Bailey, J.L., Laclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995b. Capacitation in mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121, 1139–1150.
Visconti, P.E., Westbrook, V.A., Chertihin, O., Demarco, I., Sleight, S., Diekman, A.B., 2002. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J. Reprod. Immunol. 53, 133–150.
Wahl, M., Carpenter, G., 1988. Regulation of epidermal growth factor-stimulated formation of inositol phosphates in A-431 cells by calcium and protein kinase C. J. Biol. Chem. 263, 7581–7590.
Wassarman, P.M., 1987. Early events in mammalian fertilization. Annu. Rev. Cell Biol. 3, 109–142.
Wetzker, R., Bohmer, F.D., 2003. Transactivation joins multiple tracks to the ERK/MAPK cascade. Nat. Rev. Mol. Cell Biol. 4, 651–657.
White, D.R., Aitken, R.J., 1989. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Res. 22, 163–177.
Zamir, N., Riven-Kreitman, R., Manor, M., Makler, A., Blumberg, S., Ralt, D., Eisenbach, M., 1993. Atrial natriuretic peptide attracts human spermatozoa *in vitro*. Biochem. Biophys. Res. Commun. 197, 116–122.