Regulation of the sperm EGF receptor by ouabain leads to initiation of the acrosome reaction

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The sperm acrosome reaction occurs after the binding of the capacitated sperm to the egg zona pellucida. This study describes a novel mode of regulation of the sperm epidermal growth factor receptor (EGFR) under physiological conditions and its relevance to the acrosome reaction. Ouabain, a known Na/K ATPase blocker is present in the blood and in the female reproductive tract. We show here that physiological concentrations (nM) of ouabain enhance phosphorylation of EGFR on tyr-845, stimulate Ca²⁺ influx and induce the acrosome reaction in sperm. These effects could be seen only in the presence of very low concentrations of EGF (0.1 ng/ml or 0.016 nM) added together with nano-molar ouabain. Phosphorylation, Ca²⁺ influx, and the acrosome reaction are inhibited by an EGFR blocker, suggesting that trans-activation of the EGFR is involved. Moreover, our data revealed that protein kinase A and the family of tyrosine kinase, SRC, shown before to be involved in EGFR activation in sperm, mediate the acrosome reaction induced by ouabain. Ouabain alone (without EGF) at relatively high concentration (10 µM) could enhance EGFR phosphorylation, Ca²⁺ influx and acrosome reaction, and these processes were inhibited by EGFR blockers. Moreover, we show here that PKA and SRC family are involved in the activation of EGFR by 10 µM ouabain, further demonstrating that ouabain induces the acrosome reaction by a mechanism mediated by the trans-activation of EGFR. In conclusion, this study describes an interesting regulatory path of EGFR by physiological concentrations of ouabain and EGF found in the female reproductive tract. Neither of these compounds can activate the EGFR alone at such low physiological levels; however, when both are present, the interaction of ouabain with the Na/K ATPase leads to the priming of the EGFR, which undergoes its full activation by EGF.

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Introduction

Mammalian spermatozoa must reside in the female genital tract for several hours before gaining the ability to fertilize the egg. Sperm cells undergo a series of physiological changes called capacitation, which render the spermatozoa capable of interacting with and fertilizing the egg.

After binding to the egg zona pellucida, the spermatozoon undergoes an exocytotic process called the acrosome reaction (AR) (reviewed by Breitbart, 2003). This event is required for fertilization, as 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 AK (Arnout 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), and other ligands.

Recently it was also shown that boar sperm contain EGFR involved in sperm motility (Oliva-Hernandez and Perez-Gutierrez, 2008). In other cell types, EGFR can be activated by GPCR or through a process called trans-activation (Jorissen et al., 2003; Jung et al., 2006; Prenzel et al., 1999; Shah and Catt, 2003; Wetzker and Bohmer, 2003). We recently...
showed that EGFR in bovine sperm can be activated by agonists of two GPCRs, AT1-R and LPA-R (Etkovitz et al., 2009), indicating that EGFR trans-activation occurs in sperm, as well.

The so-called EGFR signal trans-activation can also be induced by treating cells with ouabain, a specific inhibitor of Na+/K+–ATPase (Haas et al., 2000). In the past few years, it has become apparent that mammals naturally produce the cardiac glycoside, ouabain, which has hormone-like activity and is thought to be synthesized in the adrenal glands. Individuals with a low-renin form of hypertension have abnormally high blood levels of ouabain, as do patients with congestive heart failure (Armon et al., 2000; Schoner, 2001). The Na+/K+–ATPase is a heteromeric, integral membrane Na+ K+ exchange protein (Blanco and Mercer, 1998; Lingrel and Kuntzweiler, 1994; Sweadner, 1989). The enzyme consists of two subunits, the α subunit contains the catalytic function and the cation, ouabain and ATP-binding sites (Lingrel and Kuntzweiler, 1994), whereas the β subunit is necessary for localization to the plasma membrane (Beguin et al., 1998; Geering, 1991; Hasler et al., 1998; McDonough et al., 1990) and stabilization of the K+–occluded intermediate form of the protein (Eakle et al., 1994; Lutsenko and Kaplan, 1993). Different isoforms of the α and β subunits exhibit unique tissue expression profiles (Aristarkhova and Sweadner, 1997; Blanco and Mercer, 1998; Malik et al., 1996; Orlowski and Lingrel, 1988; Shamraj and Lingrel, 1994) and specific functions (James et al., 1999). The α4 isoform has been identified only in the testes of several species (James et al., 1999; Shamraj and Lingrel, 1994; Underhill et al., 1999; Woo et al., 2000) and in bovine sperm (Newton et al., 2009). This isoform shows high affinity to ouabain, Na+ and K+ and its activity is inhibited by low concentrations of ouabain (10 µM) (Blanco et al., 1999; Woo et al., 2000), which also inhibits sperm motility. In recent studies, it was shown that 100 µM ouabain induces bovine sperm capacitation without any effect on sperm motility (Newton et al., 2009; Thundathil et al., 2006).

Ouabain induces signaling through plasma membrane receptors, resulting in intracellular Ca2+ oscillations and transcriptional activation (Aizman et al., 2001). By interacting with the Na+/K+–ATPase, ouabain trans-activates the EGFR and releases reactive oxygen species from the mitochondria (Xie and Askari, 2002). It was recently suggested that Na+/K+/ATPase acts as a signaling molecule in bovine sperm capacitation (Newton et al., 2009; Thundathil et al., 2006).

In other cell types, it was reported that low concentrations of ouabain can trans-activate EGFR via the activation of SRC (Jung et al., 2006; Mohammadi et al., 2001; Xie and Askari, 2002) which phosphorylates EGFR on Y845, known to be the SRC target (Liu et al., 2004; Preznel 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). Y845 phosphorylation can also lead to the activation of the EGFR by autophosphorylation, and to the activation of various downstream effectors, including the MAPK (Rojas et al., 1996) and PI3K-Akt-PDK2 (Cao et al., 2005; Jung et al., 2006; Mohammadi et al., 2001; Xie, 2003).

We recently showed that bovine sperm contains the tyrosine kinase, SRC, which is localized to the midpiece, post-acrosome and the acrosome regions (Etkovitz et al., 2009). This kinase was also shown to participate in the trans-activation of EGFR leading to AR induction (Etkovitz et al., 2009). In human sperm, SRC is activated during capacitation and appears to be involved in regulating sperm capacitation, calcium fluxes, tyrosine phosphorylation and the acrosome reaction (Varano et al., 2008). SRC is also involved in protein tyrosine phosphorylation and motility during sperm capacitation (Baker et al., 2006; Mitchell et al., 2008). SRC forms a complex with Protein kinase A (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).

PKA-dependent protein tyrosine phosphorylation is known to occur during sperm capacitation (Visconti et al., 1995). In our recent study, we showed that PKA is localized to the head of bovine sperm and is involved in the trans-activation of EGFR (Etkovitz et al., 2009), in agreement with the involvement of PKA in the AR (Lefievre et al., 2002; Su et al., 2005).

A role for ouabain in bovine sperm capacitation was suggested previously (Thundathil et al., 2006); however, the physiological relevance of this process in the female reproductive organ remains to be elucidated. It is known that ouabain is present in the blood plasma at very low concentration (50 pM to 80 nM) (Goto et al., 1992; Gottlieb et al., 1992; Schoner et al., 2003; Vakkuri et al., 2000; Valente et al., 2009). We showed here that ouabain at nano-molar concentration is present in bovine semen and in vaginal fluids of the cow, and these concentrations of ouabain together with a very low concentration of EGF, the physiological ligand for EGFR, can induce the acrosome reaction mediated by EGFR activation.

Materials and methods

Materials

AG1478, bisindolylmaleimide I (GF109203X (GF)), protease inhibitor cocktail, and PPI were purchased from Calbiochem (San Diego, CA, USA). Antibodies against phospho (845)-EGFR were purchased from Abcam (Cambridge, MA, USA). Antibodies against phospho–PKA substrate (RRXS/T) (100G7) were purchased from Cell Signaling (Beverly, MA, USA). Goat anti-mouse IgG–HRP conjugated and goat anti-rabbit IgG–HRP conjugated were purchased from Bio-Rad (Richmond, CA, USA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd. Rehovot, Israel) unless otherwise stated.

Determination of ouabain levels

The levels of ouabain in the semen and vaginal fluid were determined by quantitative ELISA based on anti-ouabain antibodies, as described previously (Lichtstein et al., 1998).

Sperm preparation

Ejaculated bull spermatozoa were obtained by using artificial vagina, and the ‘swim up’ technique was applied to obtain motile sperm. Bovine sperm were 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 propane sulfonic acid (MOPS) (pH 7.4) and the sperm were allowed to swim up after the last wash. The washed cells were counted and maintained at room temperature until use. Only sperm preparations that contained at least 80% motile sperm were used in the experiments, and the motility was confirmed to not be 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 108 cells/ml in mTALP (Modified Tyrode solution) medium containing 100 mM NaCl, 3.1 mM KCl, 1.5 mM MgCl2, 0.92 mM KH2PO4, 25 mM NaHCO3, 20 mM Hepes (pH 7.4), 0.1 mM sodium pyruvate, 21.6 mM sodium lactate, 10 IU/ml penicillin, 1 mg/ml BSA, 20 µg/ml heparin, and 2 mM CaCl2. The cells were incubated in this capacitation medium for 4 h at 39 °C with 5% CO2. 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 the appropriate vehicle (Me2SO or water).
Assessment of sperm acrosome reaction

Washed cells (10⁶ cells/ml) were capacitated for 4 h at 39 °C in mTALP medium (Parrish et al., 1988). The indicated inhibitors 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 an additional 20 min of incubation. The percentage of acrosome-reacted sperm was determined microscopically on air-dried smear using FITC-conjugated Pisum sativum agglutinin (PSA). An aliquot of spermatozoa (10⁶ cells) was smeared on a glass slide and allowed to air-dry. The sperm were then permeabilized by methanol at 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 H₂O at 5 min intervals, and mounted with FluoroGuard Antifade (Bio-Rad Lab., Richmond, CA). 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 Ca²⁺ was assessed using the fluorescent calcium indicator, Fura-2. Washed cells (1 × 10⁸/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 spectrofluorometer, 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 Na₃VO₄, to the pellet, and the lysate was 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-phospho-tyrosine-(845)-EGFR, phospho-PKA subunit α-4, phospho-PKA subunit α-1, and anti-tubulin [loading control] (Sigma), diluted 1:10000. 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 (Bio-Rad Lab., Richmond, CA), diluted 1:10000 in TBST and 1% BSA. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK). Densitometric quantification was done using the metamorph “ImageJ” (National Institutes of Health).

Statistical analysis

Statistical significance was calculated by ANOVA with Bonferroni’s post-hoc comparison test using SPSS software (Chicago, IL, USA).

Results

Ouabain is present in semen and vaginal fluid

Sperm capacitation and the acrosome reaction occur in the female reproductive tract; therefore it was first essential to determine whether ouabain as a possible physiological regulator of this process is present in the female reproductive tract. Ouabain present in semen might also affect capacitation/AR after its interaction with the sperm. The concentrations of ouabain in cow vaginal fluids and bull semen were determined by ELISA. Ouabain was present in cow vaginal fluid at concentration of 17.86 ± 12.6 nM (n = 4) and in bovine semen at 11.4 ± 3.65 nM (n = 5). We were next interested in determining whether these physiological concentrations of ouabain are able to affect the acrosome reaction and by what mechanisms.

Effect of ouabain on the acrosome reaction

It was recently shown that ouabain (0.1 mM) can replace heparin in capacitation medium and can induce bovine sperm capacitation (Thundathil et al., 2006). We therefore tested whether even lower concentrations of ouabain could induce capacitation. Here we showed that 10 µM ouabain induces the acrosome reaction when added to capacitated bovine sperm (Fig. 1). Only 5.5% AR was seen when ouabain was added in the presence of the Ca²⁺ chelator EGTA, indicating that ouabain induces a true Ca²⁺-dependent AR. Ouabain at 10 µM can inhibit the testes specific α-4 subunit of the Na⁺/K⁺-ATPase known to be present in sperm including bovine sperm (Newton et al., 2009; Sanchez et al., 2006). In order to clarify whether the effect of ouabain on the AR is due to the inhibition of the Na⁺-pump, we blocked pump activity by incubating the cells in low K⁺ (1 mM) medium. Under these conditions no effect on AR was observed (not shown) indicating that inhibition of Na⁺ pump activity does not induce the occurrence of the AR. Moreover, in the presence of 10 mM ouabain, which inhibits the α-1 subunit of the Na⁺/K⁺-ATPase, the stimulatory effect observed in the presence of 10 µM ouabain was almost completely abrogated (Fig. 1). These results suggest that the induction of the AR by micromolar ouabain is not due to the inhibition of the sodium pump activity.

It was shown in other cell types that ouabain at relatively low concentrations (0.1 mM) can trans-activate the EGFR (Jung et al., 2006; Mohammadi et al., 2001; Xie, 2003). We showed elsewhere that the AR in bovine sperm can be induced by EGFR activation (Etkovitz et al., 2009; Lax et al., 1994). Moreover, we recently showed that the EGFR can be trans-activated in capacitated bovine sperm by activating...
GPR, resulting in the AR (Etkovitz et al., 2009). We therefore wished to determine whether ouabain and EGF might have a synergistic effect in triggering the AR. Significant induction of the AR by 1.0 ng/ml EGF or by 10 µM ouabain was observed, but no effect was seen following the addition of 0.1 ng/ml EGF or a physiological concentration of ouabain (1 nM or 10 nM) (Fig. 1). However, when both 0.1 ng/ml EGF and 1.0 nM ouabain were present, a significant synergistic increase in the rate of AR was observed (Fig. 1). Moreover, the stimulatory effect of 10 µM ouabain or 1 nM ouabain plus 0.1 ng/ml EGF on AR was inhibited by 84% and 96% respectively, by tyrphostin 1478, a specific inhibitor of EGFR (Fig. 2A). Furthermore, when SRC family, a known activators of EGFR, was inhibited by PP1, the AR induced by 10 µM ouabain or by 1 nM ouabain plus 0.1 ng/ml EGF was 74% and 84% inhibited, respectively (Fig. 2A). Other SRC family inhibitors, herbimycin-A or PP2, but not the inactive analogue PP3, also caused significant inhibition of the AR induced by 10 µM ouabain (not shown). We showed that SRC family in sperm can be activated by PKA (Etkovitz et al., 2009). Here, we show that AR induced by ouabain is completely blocked by inhibition of PKA by H89 (Fig. 2A), suggesting that ouabain leads to PKA/SRC family activation. Inhibition of the EGFR downstream enzyme P3K by 10 nM Wortmannin (WT), revealed 79% and 91% inhibition of the AR induced by 10 µM ouabain or 1 nM ouabain plus 0.1 ng/ml EGF, respectively (Fig. 2B). Inhibition of PLC or PKC which are also downstream effectors to EGFR also caused significant inhibition of the AR (Fig. 2B).

![Fig. 2.](image)

**Fig. 2.** The induction of the acrosome reaction by ouabain or ouabain plus EGF is mediated by PKA, SRC and EGFR signaling. Bovine sperm were incubated in mTALP for 4 h. At the end of this incubation 50 µM H89, 10 µM PP1 or 10 µM AG1478 (A), or 10 nM Wortmannin (WT), 1 µM U73122 or 0.1 nM GF (B) were added for 15 min. After incubation with inhibitors, 10 µM ouabain or 1 nM ouabain plus 0.1 ng/ml EGF were added to cells for an additional 30 min incubation. Acrosome-reacted cells were identified by PSA staining as described in the Materials and methods. The percentage of acrosome-reacted cells in the end of the 4 h incubation (22% for A and 18% for B) was subtracted to obtain the induced percentage. The data represent the mean ± SD of duplicates from at least four experiments. * Indicates significant difference from the corresponding control, P<0.05.

these data suggest that ouabain induces the AR via trans-activation of the EGFR by a mechanism mediated by PKA/SRC family activation. In addition, the dependence of ouabain-induced AR on extracellular Ca2+ indicates that ouabain activates the AR via a mechanism that involves Ca2+ influx into the cell (Fig. 6).

**Ouabain activates the EGFR**

The data in the previous section suggest that AR induced by ouabain is mediated by PKA, SRC family and EGFR activation. In order to support this pathway, we followed the effect of ouabain on EGFR phosphorylation/activation of SRC tyrosine-845, a known site for SRC phosphorylation, and an indicator of SRC and EGFR activation (Liu et al., 2004; Prenzel et al., 2001; Tice et al., 1999). Fig. 3 shows significant increase in tyrosine-845 phosphorylation by 1.0 ng/ml EGF or by 10 µM ouabain and significant inhibition of the ouabain effect by tyrphostin 1478. These data demonstrate that ouabain activates sperm EGFR. A smaller increase of Y-845 phosphorylation was induced by 1 nM ouabain or 0.1 ng/ml EGF, however, a significant increase in this phosphorylation was observed when both 1 nM ouabain and 0.1 ng/ml EGF were present (Fig. 3C). These effects were also significantly inhibited by tyrphostin 1478 (Fig. 3C) further indicating that the EGFR is activated under these conditions. It was interesting to observe fast elevation (within 5 min) of p-EGFR levels when sperm were treated with 10 µM ouabain or 1.0 ng/ml EGF (Fig. 3A and B), whereas the increase in p-EGFR induced by 1 nM ouabain or 0.1 ng/ml EGF or both together appeared only after 30 min of incubation (Fig. 3C). Moreover, the fast effect seen with 1.0 ng/ml EGF was significantly decreased after 30 min, suggesting transient activation when only 1.0 ng/ml EGF is added to the cells.

To further investigate the pathway through which ouabain and EGF trigger AR, we tested inhibitors of downstream pathways involved in the AR. The stimulatory effect of ouabain plus EGF on EGFR-Y-845 phosphorylation was significantly reduced by inhibition of PKA by H89 (Fig. 4A) or by inhibition of SRC family by PP1 (Fig. 4B), indicating the involvement of PKA and SRC family in EGFR activation under these conditions. To further test this pathway, we determined the activation of PKA by ouabain by following phosphorylation of PKA substrate phosphorylation using specific antibodies. Long incubation of sperm with 10 µM ouabain caused significant enhancement of PKA substrate phosphorylation which was strongly inhibited by H89 (Fig. 5A). In capacitated sperm, the PKA substrate is already highly phosphorylated; therefore it was difficult to see an increase in this phosphorylation by ouabain treatment under these conditions. Nevertheless, a small increase of PKA substrate phosphorylation by 10 µM ouabain or by 1 nM ouabain plus 0.1 ng/ml EGF (see arrows in Fig. 5B) is still evident; more importantly, this phosphorylation was inhibited by the PKA H89 (Fig. 4B), indicating that PKA is activated under these conditions, as well.

**Ouabain enhances intracellular calcium concentrations**

The AR depends on the elevation of intracellular calcium ([Ca2+]i) ions (reviewed by Breitbart, 2003). In the absence of added Ca2+ ions (reviewed by Breitbart, 2003), capacitated sperm, the PKA substrate is already highly phosphorylated; therefore it was difficult to see an increase in this phosphorylation by ouabain treatment under these conditions. Nevertheless, a small increase of PKA substrate phosphorylation by 10 µM ouabain or by 1 nM ouabain plus 0.1 ng/ml EGF (see arrows in Fig. 5B) is still evident; more importantly, this phosphorylation was inhibited by the PKA H89 (Fig. 4B), indicating that PKA is activated under these conditions, as well.

The induction of the acrosome reaction by ouabain or ouabain plus EGF is mediated by PKA, SRC and EGFR signaling. Bovine sperm were incubated in mTALP for 4 h. At the end of this incubation 50 µM H89, 10 µM PP1 or 10 µM AG1478 (A), or 10 nM Wortmannin (WT), 1 µM U73122 or 0.1 nM GF (B) were added for 15 min. After incubation with inhibitors, 10 µM ouabain or 1 nM ouabain plus 0.1 ng/ml EGF were added to cells for an additional 30 min 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% for A and 18% for B) was subtracted to obtain the induced percentage. The data represent the mean ± SD of duplicates from at least four experiments. * Indicates significant difference from the corresponding control, P<0.05.
Discussion

In mammals, the acrosome reaction is regulated by agonists originating from the egg zona pellucida glycoproteins or by other components of the female reproductive tract (Yanagimachi, 1994). The present study suggests that ouabain could serve as such an agonist. We show here that normal vaginal fluid from cows contains \(~18\) nM ouabain. A recent in vitro study on bovine sperm suggested that ouabain can serve as an important component for the capacitation process (Thundathil et al., 2006). These authors also showed that the β1 subunit of the Na-K-ATPase is localized to the acrosomal region of bovine sperm, suggesting a possible role for this pump in the acrosome reaction. In addition, the α4 subunit of the Na-K-ATPase is localized in the entire sperm head before capacitation, and it concentrates at the post-acrosomal region during capacitation (Newton et al., 2009). We showed that the EGFR is localized in bovine sperm head mainly in the acrosomal region (Etkovitz et al., 2009). Thus, the localization of α4 and EGFR in the acrosomal region supports the possible interaction between these two components. Here, we show that 10 µM but not 1 nM ouabain induces the occurrence of the AR (Fig. 1); however, when 1 nM ouabain plus 0.1 ng/ml EGF were added, a synergistic effect on the induction of the AR was observed. When AR is induced by 10 µM ouabain or 1 ng/ml EGF, it results in a relatively strong increase in EGFR phosphorylation on Y-845 (Fig. 2). Although 1 nM ouabain or 0.1 ng/ml EGF alone result in a small increase in phosphorylated-EGFR (p-EGFR), no increase in acrosome reaction could be seen unless the two agents were added together, conditions that also result in a relatively strong increase in p-EGFR (Figs. 1 and 2). These data suggest that a mild increase in p-EGFR is not enough to initiate the AR. It is possible that this low increase in p-EGFR represents partial activation of the receptor. This idea is further supported in our recent study in which we observed an increase in p-EGFR during bovine sperm capacitation; however, no AR is induced unless the EGFR is further activated by EGF (Etkovitz et al., 2009). Moreover, we showed here that neither 1 nM ouabain nor 0.1 ng/ml EGF alone is sufficient to stimulate Ca²⁺ influx (Fig. 4), suggesting that the partial activation of the EGFR cannot induce Ca²⁺ influx or the resulting AR. However, when both are
present at low concentrations, p-EGFR, Ca\(^{2+}\) influx and the AR are all enhanced (Figs. 2 and 5). Thus, we suggest that under physiological conditions, ouabain concentrations in the nano-molar range can induce the AR only in the presence of very low concentrations of EGF. In fact, EGF concentration in the follicular fluid is 1.4–4.7 nM (8–28.5 ng/ml) which is much higher than the concentration used here (0.1 ng/ml) (Westergaard et al., 1990).

Our data support the suggestion that the AR induced by ouabain is mediated by EGFR trans-activation. Under conditions by which ouabain induces the AR, there is a significant increase in the phosphorylation/activation of the EGFR. Second, the AR induced by ouabain is significantly inhibited by tyrphostin 1478 (Fig. 1), a known specific inhibitor of the EGFR. Moreover, the ouabain-induced AR or p-EGFR is inhibited by PKA or SRC family inhibitors (Figs. 1 and 3).
indicating that PKA and SRC family mediate the ouabain-induced trans-activation of the EGFR, as suggested in other cell types for SRC (Haas et al., 2000). PKA and SRC are present in the head of bovine sperm and are involved in EGFR activation induced by GPCR agonists (Etkovitz et al., 2009). SRC was also found in human (Leclerc and Goupil, 2002) and mouse (Baker et al., 2006) sperm, suggesting its possible involvement in sperm function. We show here that ouabain can lead to PKA activation (Fig. 4), and it was shown elsewhere that relatively high concentration of ouabain (0.1 mM) stimulates PKA-dependent protein tyrosine phosphorylation in bovine sperm. The activation of PKA by ouabain treatment is further supported by showing the involvement of EGFR downstream effectors including PI3K, PLC or PKC in the AR induced by ouabain; significant reduction in the ouabain-induced AR is seen following inhibition of these enzymes (Fig. 1). Finally, ouabain or EGF can induce an increase in intracellular calcium in capacitated sperm which is inhibited by tyrophostin 1478 (Fig. 5). The AR depends on the increase in [Ca^{2+}]_i, which was also shown here. Thus, our data clearly indicate that the AR induced by ouabain is mediated by EGFR trans-activation (Fig. 7).

Another possible mechanism considers the fact that in other cell types inhibition of the Na\(^+-\)K\(^+-\)ATPase can enhance intracellular Ca\(^{2+}\) concentration due to the reduced activity of the Na\(^+\)/Ca\(^{2+}\) exchanger (Aizman et al., 2001). Micromolar concentrations of ouabain can block the sperm specific α4 subunit of the Na\(^+\)/K\(^+\)-ATPase (Blanco et al., 1999; Woo et al., 2000); however, when we used mM ouabain or 1 mM K\(^+\) conditions under which the α1 and α4 subunits are inhibited, no induction of the AR was observed (Fig. 1). Thus, 1 mM ouabain does not inhibit either the α1 or α4 subunits, although ouabain at this concentration may interact with the Na-K-ATPase. Thus, ouabain probably interacts with the Na-K-ATPase without affecting its activity; this interaction leads to EGFR activation resulting in the occurrence of the acrosome reaction. This idea is further supported in other cell types in which low concentrations of ouabain which do not inhibit Na-K-ATPase activity, stimulate signaling cascades (Jung et al., 2006; Mohammadi et al., 2001; Xie, 2003). Moreover, it was shown that ouabain stimulates Ca\(^{2+}\) transients in arterial smooth muscle without raising cytosolic Na\(^+\) concentration (Arnon et al., 2000). However, a recent study in bovine sperm showed that 100 μM ouabain causes membrane depolarization, indicating that the Na\(^+\)-pump is inhibited at this concentration of ouabain, although no increase in intracellular calcium which usually occurs when the Na\(^+\)-pump is blocked was demonstrated (Thundathil et al., 2006).

This study demonstrates a synergistic effect between ouabain and EGF in the induction of the AR, suggesting that under in vivo conditions, when their concentrations are limited, the combination of these factors can cause the activation of the EGFR. It is possible that pre-activation or priming of the EGFR by ouabain precedes the final activation that is induced by the suboptimal levels of EGF. Our unpublished data reveal that the EGFR mediates zona pellucida-induced AR in mouse sperm. Thus, it is possible that priming of the EGFR by ouabain occurs prior to the sperm-egg interaction facilitating subsequent AR, penetration and fertilization.

The AR which allows sperm penetration occurs on the egg zona pellucida. There is still an open question regarding the role of various AR inducers found in the female reproductive tract. It is well established that acrosome-reacted sperm cannot bind to the zona pellucida and no fertilization can occur. It is possible that the occurrence of the AR before reaching the egg might be a way of selection which prevents these cells from fertilizing the egg. Also, it is possible that enzymes like hyaluronidase and acrosin secreted from the acrosome-reacted sperm might help to “open the way”, via the egg envelope cells, to the selected intact sperm to reach the egg.

In summary, ouabain present in the female reproductive tract might be a physiological regulator of the EGFR in a mechanism mediated by PKA/SRC family and may lead to the AR. Although the AR is usually induced by the egg zona pellucida, there are other physiological components of the female reproductive tract such as progesterone that can induce the AR. The role of these components is not clear, they may prime the sperm before or during their interaction with the ZP (Roldan et al., 1994); alternatively, they may cause premature AR in a process of selection, so that the acrosome-reacted cells cannot interact with the ZP and would not fertilize the egg.

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References


