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Estradiol inhibits the effects of extracellular ATP in human sperm by a non genomic mechanism of action

Marco Rossato[1], Marco Ferigo[2], Carlo Galeazzi[2] & Carlo Foresta[2]
[1]*Department of Medical and Surgical Sciences, Clinica Medica 3, University of Padova, Italy;* [2]*Department of Histology, Microbiology and Medical Biotechnologies, Center of Male Gamete Cryopreservation, University of Padova, Padova, Italy*

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Abstract

Steroid hormones, beside their classical genomic mechanism of action, exert rapid, non genomic effects in different cell types. These effects are mediated by still poorly characterized plasma membrane receptors that appear to be distinct from the classic intracellular receptors. In the present study we evaluated the non genomic effects of estradiol ($17\beta E_2$) in human sperm and its effects on sperm stimulation by extracellular ATP, a potent activator of sperm acrosome reaction. In human sperm $17\beta E_2$ induced a rapid increase of intracellular calcium (Ca^{2+}) concentrations dependent on an influx of Ca^{2+} from the extracellular medium. The monitoring of the plasma membrane potential variations induced by $17\beta E_2$ showed that this steroid induces a rapid plasma membrane hyperpolarization that was dependent on the presence of Ca^{2+} in the extracellular medium since it was absent in Ca^{2+} free-medium. When sperm were pre-incubated in the presence of the K^+ channel inhibitor tetra-ethylammonium, the $17\beta E_2$ induced plasma membrane hyperpolarization was blunted suggesting the involvement of K^+ channels in the hyperpolarizing effects of $17\beta E_2$. Extracellular ATP induced a rapid plasma membrane depolarization followed by acrosome reaction. Sperm pre-incubation with $17\beta E_2$ inhibited the effects of extracellular ATP on sperm plasma membrane potential variations and acrosome reaction. The effects of $17\beta E_2$ were specific since its inactive steroisomer $17\alpha E_2$ was inactive. Furthermore the effects of $17\beta E_2$ were not inhibited by tamoxifen, an antagonist of the classic $17\beta E_2$ intracellular receptor.

Introduction

Several recent studies have reported that steroid hormones exert important effects in human sperm through a non genomic mechanism of action by interacting with specific binding sites/receptors present on sperm plasma membrane [1–4]. In particular progesterone has been shown to induce an influx of Ca^{2+} and Na^+ within sperm cytoplasm determining a rapid rise in $[Ca^{2+}]_i$ and plasma membrane depolarization [5–7]. These effects were followed by acrosome reaction, a biological event that is fundamental for sperm–oocyte interaction [8]. More recently estradiol ($17\beta E_2$), another steroid present at high concentration in follicular fluid from ovulatory follicles together with progesterone [9], has been shown to possess specific binding sites on human sperm plasma membrane [10] and it has been demonstrated that $17\beta E_2$ induces a rise in sperm $[Ca^{2+}]_i$ and tyrosine phosphorylation of sperm proteins resulting in the inhibition of progesterone stimulated $[Ca^{2+}]_i$ increase and acrosome reaction [10]. Although the physiological trigger of sperm acrosome reaction in human sperm has not been clearly discovered, there is a general agreement in considering the oocyte ZP3 as the natural activator of sperm acrosomal exocytosis. Beside ZP3, a number of different agents have been shown to stimulate acrosome reaction in human sperm activating different signaling pathways [11 and references therein, 12]. In previous studies we demonstrated that extracellular ATP was a rapid and potent inducer of human sperm acrosome reaction through the interaction with a specific P2X receptor expressed on sperm plasma membrane which activation activates an influx of Na^+ from the external medium and a plasma membrane depolarization leading to acrosome reaction and fertilizing ability acquisition [13, 14]. It has been shown that $17\beta E_2$ negatively modulates a wide range of biological effects in a number of different cell types through a non genomic mechanism of action and recently Darszon's group demonstrated that this steroid inhibits Ca^{2+} channels activity in mouse spermatogenetic cells [15–17]. In the present study we evaluated the effects of $17\beta E_2$ on extracellular ATP stimulated effects in human sperm.

Correspondence to: Dr Marco Rossato, Department of Medical and Surgical Sciences, Clinica Medica 3, University of Padova, Via Ospedale 105, 35128 Padova, Italy. Tel: +39-049-8218747; Fax: +39-049-8213332; E-mail: marco.rossato@unipd.it

Materials and methods

Materials

Tamoxifen, $17\beta E_2$, $17\alpha E_2$, pisum sativum-FITC, ATP, propidium iodide have been purchased by Sigma chemical Co (Milan, Italy). Fura-2/AM and bis-oxonol were obtained by Molecular Probes (Eugene, OR). All other chemicals were of analytical grade.

Semen collection

We evaluated five normozoospermic healthy men referring to our center because of semen donation. Semen culture was negative and antisperm antibodies were absent in all subjects. Nobody had a history of previous cryptorchidism, testicular torsion and genital tract infections.

Experimental protocol

Semen samples were collected after 3 days of sexual abstinence in sterile containers. After fluidification at room temperature for 30 min, standard seminal parameters were examined according to the WHO laboratory manual [18]. All semen samples showed normal standard parameters. To evaluate the effects of $17\beta E_2$ on human sperm, isolated motile sperm semen samples were divided in two equivalent aliquots and then washed with BWW medium. After washing, each sperm sample was suspended in BWW at a concentration of 10×10^6/ml and incubated in the presence and absence of $17\beta E_2$ at different concentrations (0.01, 0.1, 1.0 and 10.0 μM) for 120 min. After incubation sperm viability, motility and acrosome reaction were evaluated. In separate experiments we evaluated the effects of $17\beta E_2$ (1.0 μM) on sperm acrosome reaction induced by extracellular ATP (2.5 mM). When evaluating the effects of $17\beta E_2$, some experiments were preceeded by sperm preincubation with the inactive stereoisomer $17\alpha E_2$ (1.0 μM) and with the $17\beta E_2$ receptor antagonist tamoxifen (1.0 μM) for 15 min before $17\beta E_2$ addition.

Evaluation of sperm motility and viability

Sperm motility was assessed by means of light microscopy examining an aliquot of each sperm sample before and after incubation in the presence and absence of $17\beta E_2$ at different concentrations and extracellular ATP (2.5 mM). Sperm motility was expressed as percentage of total motile sperm. Sperm viability was evaluated by means of red-eosin exclusion test and expressed as percent of viable sperm. Both $17\beta E_2$ (up to the concentration of 10.0 μM) and ATP (2.5 mM) did not significantly modify sperm motility and viability.

$[Ca^{2+}]_i$ measurement in human sperm

$[Ca^{2+}]_i$ was measured utilizing the fluorescent probe fura-2/AM as previously described [2]: sperm isolated as above were incubated for 30 min at 37 °C in the presence of fura-2/AM (2 μM). After loading, sperm were washed by centrifugation at $800 \times g$ for 10 min, resuspended in BWW medium and maintained at room temperature until used. $[Ca^{2+}]_i$ was measured in a LS50B Perkin Elmer fluorometer equipped with a thermostatted and magnetically-stirred cuvette holder and utilizing 1.0 ml sperm aliquots. The excitation wavelenght was alternated between 350 and 380 nm and emission fluorescence was continuously monitored at 505 nm.

Evaluation of sperm plasma membrane potential changes

Sperm plasma membrane changes were monitored utilizing the potential sensitive fluorescent dye bis-oxonol as previously described [13]. Briefly, 1.5×10^6 sperm were placed in a cuvette thermostatted at 37 °C containing the bis-oxonol solution (200 nM) in saline. After stabilization of the fluorescent signal, additions of test substances were made. Excitation and emission wavelenghts were 540 and 580 nm, respectively. In some experiments evaluating the role of the $17\beta E_2$ receptor antagonist tamoxifen and of the K^+ channel-blocker tetraethylammonium (TEA) on $17\beta E_2$ induced plasma membrane potential variations, sperm suspensions were pre-incubated with each specific blocker for 15 min before $17\beta E_2$ addition.

Acrosomal status evaluation

In preliminary experiments we tested the effects of different $17\beta E_2$ concentrations on $[Ca^{2+}]_i$ variations in human sperm and found that at 1.0 μM the steroid exerted its maximal effects and then we utilized this concentration for the experiments evaluating sperm acrosome induction. Sperm acrosome reaction was evaluated after 120 min incubation in the presence and absence of $17\beta E_2$ (1.0 μM). In separate experiments we evaluated the effects of $17\beta E_2$ (1.0 μM) on sperm acrosome reaction induced by extracellular ATP (2.5 mM). When evaluating the effects of $17\beta E_2$, some experiments were preceeded by sperm preincubation with the estrogen receptor antagonist tamoxifen for 15 min before $17\beta E_2$ addition. The acrosomal status was evaluated utilizing FITC-conjugated pisum sativum agglutinin (PSA-FITC) and a flow cytometric analysis. In brief, after incubation in the different experimental conditions described above, sperm aliquots were incubated with PSA-FITC (1.0 μg/ml) for 30 min at 37 °C. Propidium iodide (PI, 10 μg/ml) was added to each sperm suspension after 20 min from PSA-FITC addition and incubated for 10 min. After incubation each aliquot was washed with prewarmed PBS (37 °C) for 10 min at 600 *g*. After washing, each sperm pellet was suspended in PBS and the flow cytometric analysis was performed by means of flow cytometry (FACSCAN, Becton & Dickinson, Milan, Italy). Non viable sperm (PI positive) were detected using fluorescence detector 3 (FL3, detecting photons with a wavelength > 670 nm) while acrosome reacted sperm (PSA-FITC-positive and PI negative) were detected using fluorescence detector 1 (FL1, detecting photons with a wavelength in the range 515–545 nm). Non sperm events

were gated out of the fluorimetric analysis as determined from the forward-scatter and side-scatter analysis. Ten thousand gated-events were recorded for each analysis.

Statistical analysis

Data are expressed as mean ± S.D. and analysed using the Student's *t*-test and the analysis of variance (ANOVA, StatView, Abacus Concepts, Cary, NC, USA). A P value < 0.05 was chosen as the limit for statistical significance.

Results

Effects of 17βE$_2$ on sperm [Ca^{2+}]$_i$ and plasma membrane potential

Stimulation of fura-2 loaded human sperm with 17βE$_2$ induced a rapid and dose dependent Ca^{2+} rise that was sustained and completely dependent on the presence of Ca^{2+} in the external medium since it was abrogated by Ca^{2+} chelation with EGTA (Figure 1). The effects of 17βE$_2$ on sperm plasma membrane potential variations have not been explored yet. Then we analyzed the modifications of sperm plasma membrane potential induced by 17βE$_2$ utilizing the potential sensitive fluorescent dye bis-oxonol as previously described [13]. As shown in Figure 2 (trace a) 17βE$_2$ induced a rapid plasma membrane hyperpolarization that was dependent on K^+ efflux from the sperm cytoplasm since when sperm were pre-incubated in the presence of TEA, a well known K^+ channel blocker, the plasma membrane hyperpolarization induced by 17βE$_2$ was inhibited (Figure 2, trace b). Given the effects of 17βE$_2$ on sperm $[Ca^{2+}]_i$ we analyzed the role of Ca^{2+} ions in 17βE$_2$ induced plasma membrane hyperpolarization. When incubated in Ca^{2+}-free medium, 17βE$_2$ addition did not induced any plasma membrane potential variation (Figure 2, trace c) but when Ca^{2+} was added back to the sperm suspension a prompt plasma membrane hyperpolarization occurred demonstrating that the hyperpolarizing effects of 17βE$_2$ were dependent on $[Ca^{2+}]_i$ rise induced by this steroid. The effects of 17βE$_2$ were specific since its inactive isomer 17αE$_2$ did not induce any increase in $[Ca^{2+}]_i$ (not shown) nor any modification of sperm plasma membrane potential at the concentration of 1.0 μM without modifing the effects of 17βE$_2$ (Figure 2, trace d). Furthermore the rapid plasma membrane hyperpolarizing effects of 17βE$_2$ were not inhibited by sperm pre-incubation with tamoxifen, a well known antagonist of the classic receptor for 17βE$_2$ (Figure 2, trace e).

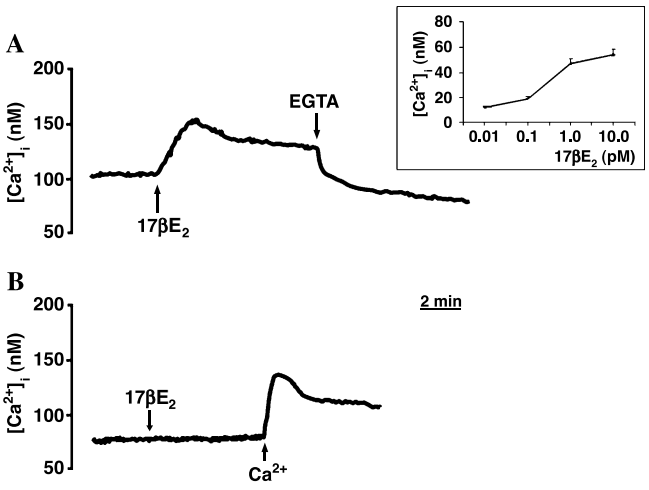

Figure 1. Effects of 17βE$_2$ on $[Ca^{2+}]_i$ in human sperm. Isolated motile human sperm suspensions (4.0–5.0 × 10^6 cells) were loaded with fura-2/AM as described in the Material and Methods section. (A) Sperm were suspended in Ca^{2+} containing medium. (B) Sperm were suspended in Ca^{2+}-free medium. Where indicated 17βE$_2$ (1.0 μM), EGTA (2.0 mM) and Ca^{2+} (2.0 mM) were added. Traces are representative of a typical experiment of three. *Inset*: dose response of $[Ca^{2+}]_i$ increase induced by increasing concentrations of 17βE$_2$ (0.01, 0.1, 1.0 and 10.0 μM) in human sperm. Results represent mean ± S.D. of three separate experiments.

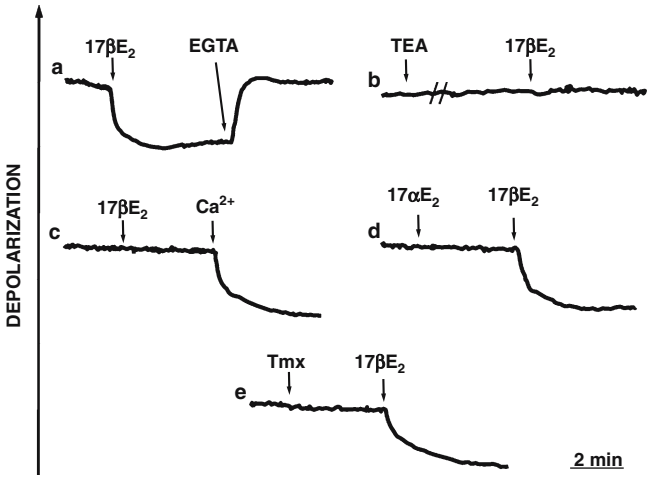

Figure 2. Effects of 17βE$_2$ on sperm plasma membrane potential. Isolated motile human sperm (1.5 × 10^6 cells) were suspended in the presence of 200 nM bis-oxonol, as described in the Materials and methods section, in control medium (*trace a*), tetraethylammonium (TEA, 20 mM) containing medium and preincubated for 15 min before 17βE$_2$ addition (*trace b*), Ca^{2+}-free medium (*trace c*), Ca^{2+} containing medium (*traces d* and *e*). Where indicated, 17βE$_2$ (1.0 μM), EGTA (2.0 mM), tetraethylammonium (TEA, 20 mM), calcium (Ca^{2+}, 2.0 mM), 17αE$_2$ (1.0 μM) were added. Traces represent the result of a single from three similar experiments.

Effects of 17βE$_2$ on plasma membrane potential variations induced by extracellular ATP

We have previously demonstrated that extracellular ATP activates human sperm inducing a rapid plasma membrane depolarization depending on an influx of Na^+ from the extracellular medium. In Figure 3 (trace a) it is shown that extracellular ATP induces a rapid plasma membrane depolarization confirming previous data [13]. The depolarizing effects of ATP were not maximal since gramicidin D, a polypeptide antibiotic that forms pores in the cell membrane allowing Na^+ to enter the cell thus inducing plasma membrane depolarization [19], was able to further depolarize plasma membrane potential (Figure 3, trace a). The addition of 17βE$_2$ (1.0 μM) to sperm suspension just

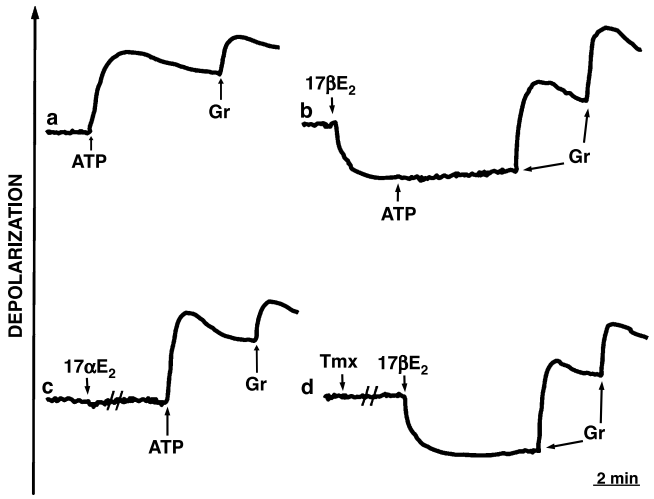

Figure 3. Effects of $17\beta E_2$ on ATP induced sperm plasma membrane potential variations. Isolated motile human sperm (1.5×10^6 cells) were suspended in the presence of 200 nM bis-oxonol as described in the Material and Methods section in calcium containing medium before addition of the different agonists. Where indicated ATP (2.5 mM), gramicidin D (Gr, 1.0 μM), $17\beta E_2$ (1.0 μM), $17\alpha E_2$ (1.0 μM) and tamoxifen (1.0 μM) were added. Traces represent the result of a single from three similar experiments.

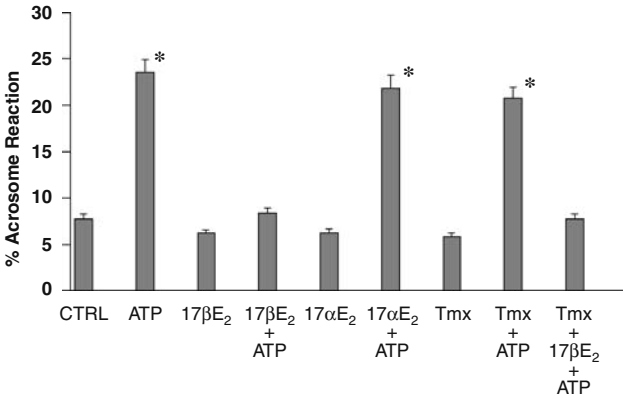

Figure 5. Effects of $17\beta E_2$ on ATP induced acrosome reaction in human sperm. Isolated motile human sperm were incubated for 120 min in control medium (CTRL) and in the presence of ATP (2.5 mM) with and without the presence of $17\beta E_2$ (1.0 μM), $17\alpha E_2$ or tamoxifen (Tmx, 1.0 μM). Data are expressed as mean ± S.D. of results from three separate experiments. $^*P < 0.01$ vs control and samples treated with $17\beta E_2$.

before stimulation with extracellular ATP inhibited the ATP induced plasma membrane depolarization (Figure 3, trace b) wih an IC_{50} of about 0.1 μM (Figure 4). The inhibitory effects of $17\beta E_2$ were so rapid that it is not possible to ascribe them to the classical genomic action of the steroid but to a non genomic rapid effect via the activation of putative receptors located on the sperm plasma membrane as previously suggested [20–22]. The effects of $17\beta E_2$ in reducing the effects of extracellular ATP in human sperm were specific since its inactive isomer $17\alpha E_2$ was not effective (Figure 3, trace c). Pre-incubation of sperm in the presence of the classic $17\beta E_2$ genomic receptor inhibitor tamoxifen did not modify the effects of $17\beta E_2$ on sperm plasma membrane thus confirming that the action of this steroid in human sperm are not due to the interaction with its classical cytoplasmic receptor (Figure 3, trace d).

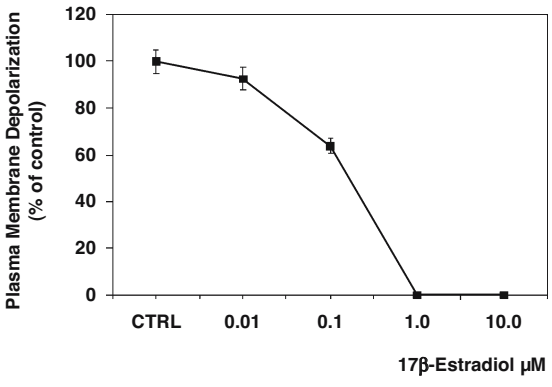

Figure 4. Dose dependence of the inhibition by $17\beta E_2$ of the ATP induced plasma membrane depolarization in human sperm. Isolated sperm were suspended in the presence of 200 nM bis-oxonol as described in the Materials and methods section. Sperm suspensions were stimulated with $17\beta E_2$ at different doses (0.01, 0.1, 1.0 and 10.0 μM) for 2 min before addition of ATP (2.5 mM). Plasma membrane depolarization is expressed as percentage of the plasma membrane depolarization induced by ATP determined in the absence of $17\beta E_2$ (100%). Results are mean ± S.D. of three separate experiments.

Effects of $17\beta E_2$ on basal and extracellular ATP stimulated acrosome reaction

Previous results from other Authors have clearly shown that $17\beta E_2$ did not influence acrosomal loss [10, 23]. Furthermore we demonstrated that extracellular ATP rapidly induced acrosomal exocytosis in mammalian and human sperm [5, 13, 24–26]. The results of the present study confirm those previous observations and demonstrate that $17\beta E_2$ inhibits sperm acrosome reaction induced by extracellular ATP while the inactive steroisomer $17\alpha E_2$ was without effect (Figure 5). The non genomic feature of $17\beta E_2$ action on human sperm was further confirmed by the lack of any effect of the classic $17\beta E_2$ genomic receptor antagonist tamoxifen on the inhibitory action of $17\beta E_2$ on ATP-induced acrosome reaction (Figure 5).

Discussion

The results of the present study confirm that $17\beta E_2$ rapidly increases $[Ca^{2+}]_i$ in human sperm as previously demonstrated [10] and extend our knowledge on the rapid non genomic effects of this steroid in human sperm showing for the first time that it induces a rapid plasma membrane hyperpolarization that is dependent on the influx of Ca^{2+} from the external medium that activates an efflux of K^+ ions from the sperm cytoplasm. The existence of Ca^{2+}-activated K^+ channels in human sperm has been previously reported [27] and the plasma membrane hyperpolarizing effects of the different agonists reported so far have been shown to inhibit important sperm functions by modifing sperm responsiveness to putative physiological inducers

of the acrosome reaction [28 and references therein]. The other main result of the present study is the demonstration that $17\beta E_2$ inhibits sperm plasma membrane depolarization and acrosome reaction induced by extracellular ATP, a putative physiological activator of human sperm acting through a still poorly characterized P2X receptor subtype [5, 13]. $17\beta E_2$ itself did not induce any increase of sperm acrosome reaction despite the fact that it enhances $[Ca^{2+}]_i$ but probably other mechanisms, that are not activated by $17\beta E_2$, are involved in the induction of acrosomal exocytosis other than $[Ca^{2+}]_i$ increase.

Purinergic receptors are classified in two families, P2X and P2Y. The P2Y family encompasses a number of different receptors activated by ATP but also by other nucleotides and are linked to inositol phosphate and diacylglycerol formation leading to Ca^{2+} leakage from intracellular stores and protein kinase C activation [29, 30]. These purinergic receptors have not been identified in human sperm yet [13] although their presence in mammalian male reproductive tract has been reported [24, 31]. The other P2 purinergic receptor family is the P2X constituted by different multimeric ligand-gated ion channels activated by ATP and allowing the passage of small ions as well as Ca^{2+} and Na^+ inducing increases in $[Ca^{2+}]_i$ and plasma membrane depolarization. This subgroup comprises seven different subtypes (named P2X1 to P2X7) and are activated by ATP [32]. In human sperm extracellular ATP does not induce any modification of $[Ca^{2+}]_i$ thus probably excluding the presence of P2Y receptor subtype in these cells. On the contrary this nucleotide induced a rapid plasma membrane depolarization due to the activation of a Na^+-permeable channel as previously demonstrated [5, 13].

The effects of $17\beta E_2$ on human sperm were rapid, stereospecific and not inhibited by the classic nuclear estrogen receptor antagonist tamoxifen. All these characteristics, together with the knowledge that mature sperm do not retain protein synthesis activity [33], confirm that the effects of $17\beta E_2$ in human sperm are non genomic. The rapid, non genomic hyperpolarizing effects of $17\beta E_2$ have been previously described in neurons from pre-optic area where this steroid inhibits their excitability by inducing a plasma membrane hyperpolarization due to the opening of K^+ channel thus reducing their firing rate [34, 35]. $17\beta E_2$ has been shown to induce plasma membrane hyperpolarization also in hypothalamic GnRH neurons in guinea pig [36]. From this point of view the similarity of the spermatozoon with "a neuron with the tail" as suggested recently by Stanley Meizel is further underlined considering the similar effects of $17\beta E_2$ on cell plasma membrane [37]. Previous studies have identified sperm plasma membrane for progesterone and in the present study we show rapid effects of $17\beta E_2$ in human sperm thus hypothizing the presence of binding sites also for this steroid on human sperm surface as previously suggested [10]. The membrane receptors for $17\beta E_2$ mediating rapid non genomic effects in human sperm appear to be molecularly and pharmacologically different from the classical intracellular $17\beta E_2$ receptors. On the other hand it has been demonstrated that in ERKO mice $17\beta E_2$ actions appear to be genetically and pharmacologically different from those of the classic $17\beta E_2$ receptor thus supporting the hypothesis that $17\beta E_2$ possess also non genomic actions although some discordant data exist [38, 39].

In mammals, $17\beta E_2$ influences neuronal activity via non genomic mechanism of action by changing the cellular responsiveness to the activation of different receptor systems to their respective agonist [40]. Indeed this was the case also for the effects of extracellular ATP in human sperm that were specifically inhibited by $17\beta E_2$. The inhibitory action of $17\beta E_2$ on extracellular ATP induced effects are not novel since $17\beta E_2$ has been previously shown to inhibit the response to extracellular ATP in neurons [41], in PC12 [42] and kidney cells [43] and were suggested to be due to interference with ion channels.

Recently it has been suggested that $17\beta E_2$ inhibitory action on ATP stimulated effects were due to P2 purinergic receptor inhibition [42] although a role of plasma membrane potential variations or alterations of plasma membrane physical properties have been suggested [10, 15]. This could be the case also for human sperm since $17\beta E_2$ has been previously shown to attenuate the stimulatory effects of different agonists in human sperm and mammalian spermatogenic cells [10, 15] and in a number of other cellular systems [41–43]. Activation of P2 purinergic receptors by extracellular ATP in human sperm induced Na^+ influx, plasma membrane depolarization and acrosome reaction through the activation of a not yet identified P2X purinergic receptor subtype [5, 13]. To this respect it is of interest that $17\beta E_2$ seems to exert its inhibitory actions just interfering with P2X receptor subtype as demonstrated by Tourmaniaz et al. [43]. However we cannot completely rule out the possibility that $17\beta E_2$ might directly interact with Na^+ channel (as for Ca^{2+} channels) or the P2X receptor activated by extracellular ATP.

The possible interference of $17\beta E_2$ with Na^+ channel coupled to P2X receptor activation by extracellular ATP appears to be a novel finding although further studies are required to corroborate this hypothesis. To this regard it has to be remembered that the activity of membrane spanning proteins, as plasma membrane ion channels, is regulated also by the physical properties of the plasma membrane lipid bilayer via lipid–protein interactions [15, 44–46]. Very recently some Authors have described that plasma membrane Na^+ channel function is regulated by protein (channel)-lipid bilayer hydrophobic interactions [44, 45]. Then it is also possible that $17\beta E_2$, a highly hydrophobic molecule, might modify the ATP-gated Na^+-channel through its interaction with the plasma membrane lipid bilayer. This hypothesis could be further supported by the fact that $17\beta E_2$ concentrations required for fully influencing sperm functions *in vitro* are quite higher than physiological plasma levels of this hormone above all in men. However we have to consider that $17\beta E_2$ concentrations within the testis are about 75 times higher than plasma levels and that seminal plasma levels of $17\beta E_2$ are higher than those of serum [47]. Together with the demonstration that human sperm possess a biologically active aromatase, the enzyme responsible for the aromati-

zation of testosterone to estradiol [48] it is conceivable that sperm are exposed to elevated $17\beta E_2$ concentrations from the site they are produced within the testis and along the whole male reproductive tract. Furthermore we have also to consider $17\beta E_2$ concentrations within the female genital tract secretions where sperm are spawn after ejaculation and that, at the time of ovulation, are close to $17\beta E_2$ concentrations utilized in the present study [10]. Finally the effects of $17\beta E_2$ on sperm functions may be further amplified during ovarian hyperstimulation when $17\beta E_2$ plasma levels are much higher than those found in situations of unstimulated follicular growth as during spontaneous ovulation. Then it appears that during their trip to the oocyte, sperm are costantly exposed to different $17\beta E_2$ concentrations negatively modulating its functions. Thus it could be possible that some forms of infertility might be due to interferences of $17\beta E_2$ with the physiological mechanisms leading to oocyte fertilization. To this regard an increase in $17\beta E_2$ concentrations has previously described in seminal plasma of infertile men [47].

In conclusion the present study demonstrates that $17\beta E_2$ interferes with ATP-dependent activation of human sperm through non genomic mechanisms of action suggesting a possible physiopathological role of this steroid in some forms of infertility.

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