

Effects of estrogenic compounds on human spermatozoa: evidence for interaction with a nongenomic receptor for estrogen on human sperm membrane

Michaela Luconi, Lorella Bonaccorsi, Gianni Forti, Elisabetta Baldi *

Dipartimento di Fisiopatologia Clinica, Unita' di Andrologia, Universita' di Firenze, viale Pieraccini, 6, I-50139 Florence, Italy

Abstract

Estrogens play an important role in the development and regulation of the male reproductive system. We have earlier shown that a nongenomic receptor for estradiol present on sperm plasma membrane mediates the effects exerted by this hormone on sperm intracellular calcium concentrations ($[Ca^{2+}]_i$), as well as on the biological response to progesterone (P). In particular, 17β -estradiol ($17\beta E_2$) shows an inhibitory effect on P-mediated calcium influx and acrosome reaction (AR). In the present study, the effects of different anti-estrogens and xenoestrogens on $[Ca^{2+}]_i$ and AR stimulated by P have been investigated in human spermatozoa in order to better define the pharmacological characteristics of the sperm membrane estrogen receptor. The anti-estrogens tamoxifen (Tx) and ICI 164384 (ICI) induce only a slight increase of $[Ca^{2+}]_i$, which, however, as in the case of $17\beta E_2$, results in a reduction of P-stimulated calcium influx. Moreover, both the compounds reduce the calcium response to $17\beta E_2$ without affecting $17\beta E_2$ -inhibition of calcium response to P. Concerning AR, Tx alone does not alter either spontaneous or P-stimulated AR but partially revert the inhibitory effect of $17\beta E_2$. These results indicate that the two estrogens act as pharmacological agonists of the membrane estrogen receptors of human spermatozoa. On the other hand, the xenoestrogens bisphenol A (BPA) and octylphenol polyethoxilate (OP) do not exert any direct effect on calcium fluxes and AR in human spermatozoa either in basal conditions or in response to P challenge. Moreover, although these environmental estrogens have been suggested to mimic estrogen effects in the other cell types, probably acting through genomic receptors, in human spermatozoa they do not interfere with $17\beta E_2$ binding to its membrane receptor and with the short-term effects exerted by this steroid. In conclusion, our data indicate that the membrane receptor for estradiol in human spermatozoa shows both biochemical and pharmacological differences respect to the genomic receptor. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Estrogen; Human spermatozoa; Acrosome reaction; Intracellular calcium

1. Introduction

Although estrogens have been considered mainly female reproductive hormones, increasing evidence indicates an important role of these steroids in also regulating male reproductive functions. Estrogens have been detected in the testes and male serum and their receptor isoforms have been identified at different levels of the male gonad (Couse and Korach, 1999). Male mice homozygous for the estrogen recep-

tor α ($ER\alpha$) gene knock-out ($ERKO\alpha$) are infertile and present severe disruption of seminiferous tubules and impaired spermatogenesis. Similar condition also seems to occur in humans as reported in a male patient homozygous for a mutation in the $ER\alpha$ gene (Smith et al., 1994). Earlier studies have demonstrated a detrimental effect on the offspring male reproductive system in laboratory animals following exposure to estrogens and estrogen-like compounds during pregnancy (McLachlan et al., 1975).

Recently, growing interest has been focused on the wide family of environmental estrogens (xenoestrogens), which includes pesticides and industrial by-products with little or no structural homology to estradiol that can bind with estrogen receptors acting

* Corresponding author. Tel.: +39-055-4271366; fax: +39-055-4271371.

E-mail address: e.baldi@dfc.unifi.it (E. Baldi).

either as agonist or antagonists. Among such chemicals with estrogenic activity are bisphenol A (BPA), a monomer of polycarbonate plastics present in food cans and dental resins, and the unrelated compound octylphenol polyethoxilate (OP), present in detergents, paints and pesticides. Although less potent than estrogens, BPA and OP have been described to exert estrogenic activity in different tissues (Steinmetz et al., 1997; Ben-Jonathan and Steinmetz, 1998), through binding to the nuclear genomic receptors. However, little is known on possible interactions of these compounds with the nongenomic estrogen receptors present on plasma membrane, which have recently been described to mediate rapid nongenomic effects in reproductive and non-reproductive tissues (Wehling 1997; Revelli et al., 1998).

We have recently identified a novel nongenomic estrogen receptor on human sperm membrane, which, upon stimulation with estradiol, interferes in vitro with the biological response exerted by progesterone (P) both on intracellular calcium transient and acrosome reaction (Luconi et al., 1999).

Spermatozoa may thus represent a suitable model to study the possible effects of xenoestrogens on nongenomic estrogen receptors as well as on functions of male gametes. In the present study we have investigated the effects of BPA and OP on P-induced calcium response and AR in capacitated human spermatozoa. In addition, in order to better elucidate the pharmacological characteristics of the sperm membrane estrogen receptor, we have evaluated the effects of the ER antagonists tamoxifen and ICI 164384 as potential antagonists of natural $17\beta E_2$.

2. Materials and methods

2.1. Chemicals

Percoll was obtained from Pharmacia LKB Uppsala, Sweden). Human serum albumin (HSA)-free Human Tubal Fluid (HTF) was from Irvine (Santa Ana, CA, US). Progesterone (P), 17β -estradiol ($17\beta E_2$), tamoxifen (Tx), bisphenol A (BPA), octylphenol polyethoxilate (OP), cortisol, fluorescein isothiocyanate (FITC)-labeled *Arachis hypogea* (peanut) lectin and all the other chemicals were from Sigma (St. Louis, MI, US). Digitonin and Fura-2/AM were obtained from Calbiochem (La Jolla, CA, US). ICI 164384 was provided by Zeneca Spa (Basiglio, Milan, Italy).

2.2. Preparation of spermatozoa

Human semen was collected, according to the WHO recommended procedure (WHO) by masturbation from normozoospermic men undergoing semen analysis for couple infertility. Samples with a linear progressive

motility of less than 50% and with leukocytes and/or immature germ cell concentration greater than $10^6/\text{ml}$ were not included in the study. Semen samples were processed according to World Health Organization, 1999. Briefly, spermatozoa were separated by swim up collection in HTF medium containing 1% HSA, washed and finally resuspended in the same medium at the indicated concentration and treated as indicated in each experiment.

2.3. Measurement of intracellular calcium concentration

Spermatozoa prepared as described above, were loaded with 2 μM Fura-2/AM for 45 min at 37°C , washed, resuspended in FM medium (125 mM NaCl, 10 mM KCl, 2.5 mM CaCl_2 , 0.25 mM MgCl_2 , 19 mM Na-lactate, 2.5 mM Na-pyruvate, 2 mM HEPES, 0.3% bovine serum albumin (BSA), pH 7.5) and $[\text{Ca}^{2+}]_i$ before and after stimulation with the different agonists was measured as described earlier using the spectrofluorimetric method (Baldi et al., 1991), the only difference being, that in the present experiments we used a Perkin-Elmer LS50B instrument equipped with a fast rotary filter shuttle for alternate 340 and 380 nm excitation. Fluorescence measurements were converted to $[\text{Ca}^{2+}]_i$ by determining the maximal fluorescence (F_{max}) with 0.01% digitonin followed by minimal fluorescence (F_{min}) with 10 mM EGTA, pH 10. $[\text{Ca}^{2+}]_i$ was calculated according to Grynkiewicz (Grynkiewicz et al., 1985) using the ratio 340/380 and assuming a dissociation constant of Fura-2 for calcium of 224 nM.

2.4. Acrosome reaction assay

Acrosome reacted spermatozoa were evaluated using the fluorescent probe fluorescein isothiocyanate (FITC)-labeled *A. hypogea* (peanut) Lectin according to Aitken (Aitken et al., 1993) as earlier described (Krausz et al., 1996). Briefly, after 2 h capacitation spermatozoa ($10^6/\text{ml}$) were pre-incubated for 10 min with E_2 and other compounds at different concentrations and then stimulated with P 10 μM , or appropriate control solvent (DMSO) for 2 h at 37°C . After staining with fluorescent lectin, fluorescence was observed under a fluorescent microscope (Leitz, Type 307-148.002, Wetzlar, Germany) and acrosome reaction was evaluated on a total of 10Q spermatozoa per slide. According to Aitken (Aitken et al., 1993), only curly tailed spermatozoa were considered viable and thus scored.

2.5. Analysis of experimental results

Data are expressed as mean $\pm$ standard error (S.E.M.). Statistical analysis was made with Student's *t*-test and one-way analysis of variance (ANOVA).

3. Results

3.1. Effects of Tamoxifen and ICI 164384 on intracellular calcium concentrations in human spermatozoa

Since we had earlier reported (Luconi et al., 1999) that $17\beta E_2$ strongly inhibits the $[Ca^{2+}]_i$ increase in response to a subsequent administration of P, we investigated the possible interference of the two known estrogen antagonists Tx and ICI 164384 (ICI) on calcium response to progesterone and on estrogen inhibitory effect. While the maximum effect of $17\beta E_2$ was obtained with a concentration of 10 μM (Luconi et al., 1999), Tx and ICI were used at 1 μM since the two compounds have been demonstrated to be effective on nongenomic estrogen receptors at this concentration (Watters et al., 1997). The administration of Tx to Fura-2 loaded capacitated human spermatozoa had only a little effect per se on $[Ca^{2+}]_i$ while it significantly reduced the increase of $[Ca^{2+}]_i$ stimulated by P (10 μM)

both in the peak and plateau components of calcium wave (Fig. 1A). Similar results were obtained with the pure estrogen antagonist ICI, although it showed a slightly less efficacy in inhibiting both the phases of P response (Fig. 1A). The average inhibitory effect of the two compounds on plateau and peak phases of P response in several experiments are shown in Fig. 2 (A for plateau and B for peak). Besides the direct effects of the two anti-estrogens on calcium response to P, both the compounds also interfere with calcium waves evoked by a subsequent administration of $17\beta E_2$ (10 μM) (Fig. 1B Fig. 3). In fact, a first administration of Tx or ICI reduces the increase of $[Ca^{2+}]_i$ stimulated by a subsequent addition of $17\beta E_2$ (Fig. 1B Fig. 3), without significantly affecting calcium response to a further challenge with P (Fig. 1B Fig. 2A and B). It thus appears that the two anti-estrogens have similar effects as $17\beta E_2$ instead of inhibiting its effect.

To further investigate whether the inhibitory effects on calcium response to P exerted by both $17\beta E_2$ and its antagonists could be mediated by interaction with the

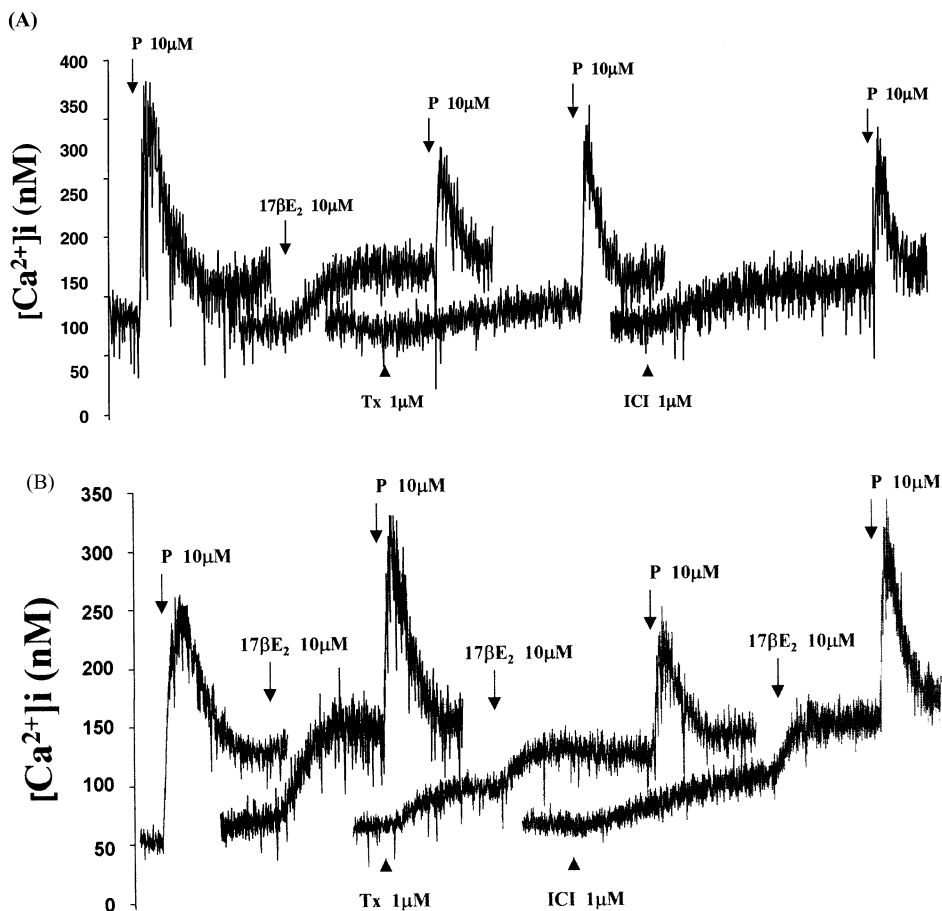


Fig. 1. Panel A, effects of Tx and ICI on basal and P-stimulated $[Ca^{2+}]_i$ in fura-2-loaded spermatozoa. For comparison, effects of P and $17\beta E_2$ alone in sperm samples from the same subject are shown. Panel B, effects of Tx and ICI on the $[Ca^{2+}]_i$ increase stimulated by $17\beta E_2$ and on the inhibition of $17\beta E_2$ exerted on calcium response to P. For comparison, effects of P and $17\beta E_2$ alone in sperm samples from the same subject are shown.

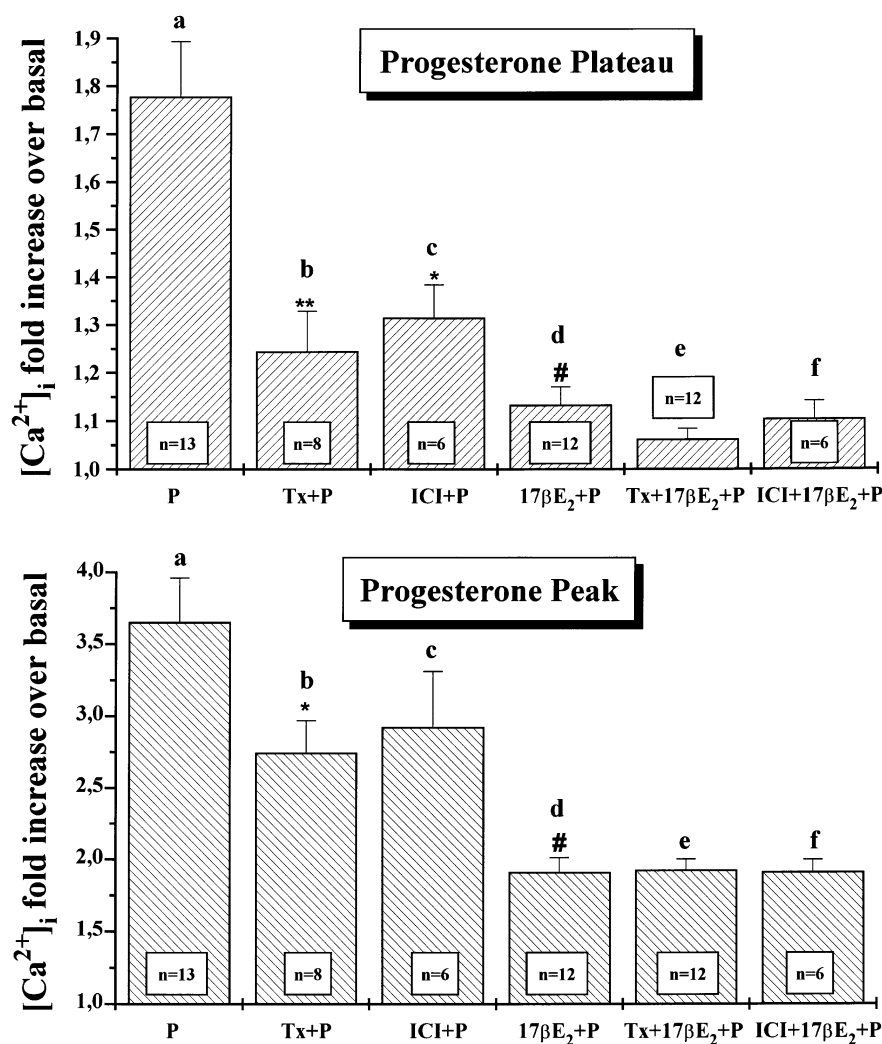


Fig. 2. Effects of Tx (1 μ M) and ICI (1 μ M) on plateau (panel A) and peak (panel B) phases of calcium responses to P (10 μ M) (columns b and c) or on the response to 17 β E₂ (10 μ M) followed by P (10 μ M) (columns e and f). The effects of P alone and of 17 β E₂ on P response are shown, respectively, in columns a and d. * P < 0.05 vs. a; ** P < 0.05 vs. a; #, P < 0.0001 vs. a.

same membrane receptor(s) we studied possible interference between Tx, ICI and 17 β E₂ on their respective calcium responses. As shown in Fig. 3, pre-treatment with Tx, and to a lesser extent with ICI, significantly reduced the [Ca²⁺]_i increase induced by 17 β E₂. Similarly, 17 β E₂ inhibited both Tx (Fig. 3) and ICI (not shown) evoked calcium waves. The higher effectiveness of Tx versus ICI could be argued from Tx ability to reduce to about 50% the ICI subsequent calcium response while ICI does not affect calcium response to a subsequent administration of Tx (Fig. 3).

3.2. Effects of Tamoxifen on AR in human spermatozoa

Since the plateau phase of calcium response to P has been demonstrated to be associated with induction of AR (Thomas et al., 1989), we next evaluated whether the interference exerted by Tx on P response at calcium

level could also affect the downstream process of AR. As shown in Fig. 4, 17 β E₂ (1 and 10 μ M) totally inhibits P-stimulated AR confirming earlier results (Luconi et al., 1999). However, despite the effect on P-stimulated calcium influx (see above), Tx did not affect AR stimulated by the steroid (Fig. 4). In addition, in the presence of 1 μ M Tx the inhibitory effect exerted by 17 β E₂ on P-stimulated AR is much less evident (Fig. 4). Indeed, in the presence of Tx and 17 β E₂ in the incubation medium, the increase of AR in response to P is partially restored (Fig. 4). Moreover, the small increase of AR induced by 10 μ M 17 β E₂ is not inhibited by the concomitant presence of Tx (Fig. 4).

3.3. Effects of xenoestrogens on intracellular calcium concentrations in human spermatozoa

Although xenoestrogens have been reported to be able to induce rapid effects in different cell types by

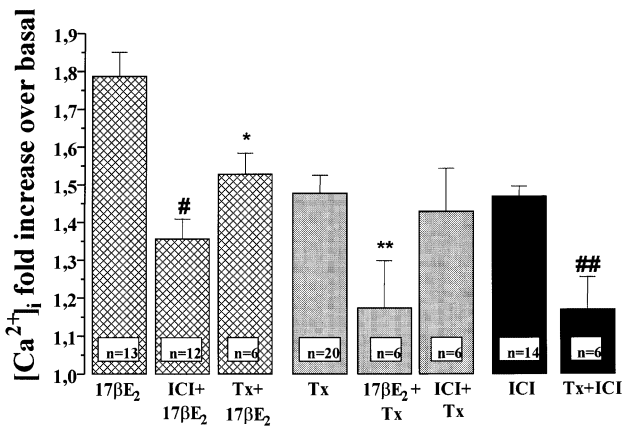


Fig. 3. Inhibitory effects of an earlier challenge with 17βE₂ (10 μM), Tx (1 μM) and ICI (1 μM) on calcium response to a subsequent challenge with each indicated stimulus, in fura-2-loaded spermatozoa. Values represent the mean ± S.E.M. [Ca²⁺]_i fold increase in response to the stimuli over basal for the indicated number of experiments. The effects of 17βE₂, Tx and ICI alone are shown for comparison. **P* < 0.01 vs. E₂; ***P* < 0.05 vs. Tx; #, *P* < 0.0001 vs. E₂; ##, *P* < 0.0005 vs. ICI.

possibly mimicking estrogen binding to its receptors (Steinmetz et al. 1997; Loomis and Thomas, 2000), little is known on the interaction of these molecules with nongenomic receptors. We evaluated the effects of BPA on intracellular calcium levels both in basal conditions and on calcium response to 17βE₂ and P in Fura-2 loaded spermatozoa. Although BPA slightly stimulates a modest influx of calcium, it does not affect the calcium response to a subsequent addition of 17βE₂ (1 μM) or P (10 μM) both in the peak and plateau phases (Fig. 5), suggesting that in human spermatozoa BPA does not interact with membrane receptors of either

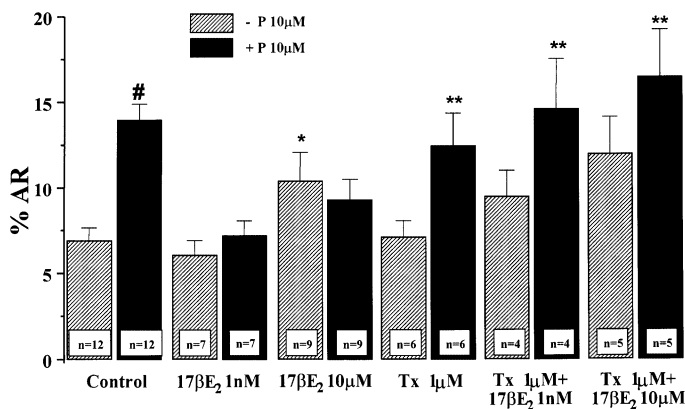


Fig. 4. Effects of Tx (1 μM) on 17βE₂ mediated inhibition of P-induced AR. Capacitated spermatozoa were treated for 2 h with the indicated stimuli. Acrosome reaction was evaluated as described in Section 2. Values represent the mean ± S.E.M. percentage of acrosome reaction for the indicated number of experiments. *, *P* < 0.01 vs. Tx; **, *P* < 0.01 vs. Tx + E₂ 1 nM; ***, *P* < 0.01 vs. Tx + E₂ 10 μM; # *P* < 0.0001 vs. Control.

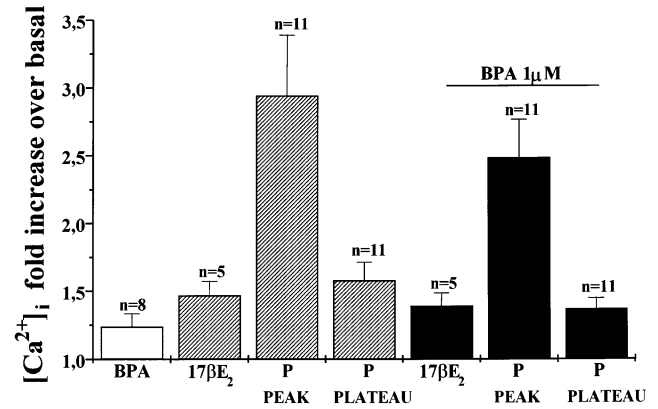


Fig. 5. Effects of an earlier challenge with 1 μM BPA on plateau and peak phases of calcium waves in response to 1 μM 17βE₂ and to 10 μM P in fura-2-loaded spermatozoa. Values represent the mean ± S.E.M. [Ca²⁺]_i fold increase in response to the stimuli over basal for the indicated number of experiments.

17βE₂ or P. Similar results were obtained with OP (not shown).

3.4. Effects of xenoestrogens on acrosome reaction in human spermatozoa

To further confirm the absence of direct effects of xenoestrogens on human spermatozoa, we next evaluated the ability of BPA and OP on P-stimulated AR. While 2 h incubation of spermatozoa with 17βE₂ (1 μM) blunts P-induced AR, neither BPA (1 μM) or OP (not shown) could affect P stimulation Fig. 6). Moreover, both the xenoestrogenic compounds do not alter the spontaneous AR (Fig. 6).

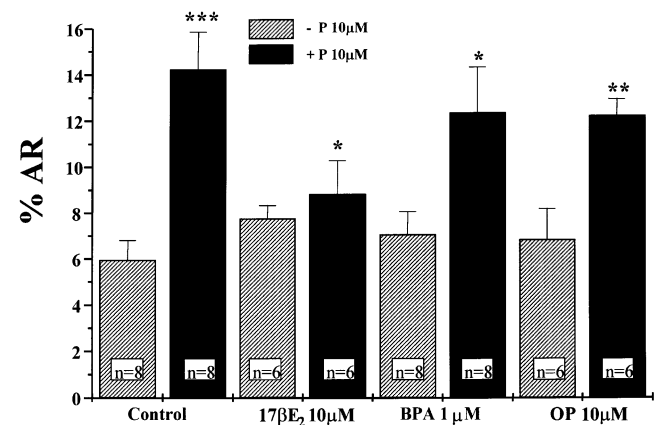


Fig. 6. Effect of BPA and OP on basal and P-stimulated acrosome reaction in human spermatozoa. Capacitated spermatozoa were treated for 2 h with P (10 μM) or with 17βE₂, BPA, OP (1 μM) both in the presence or absence of P (10 μM) and acrosome reaction evaluated as described in Section 2. Values represent the mean ± S.E.M. percentage of acrosome reaction for the indicated number of experiments. For comparison, effect of 17βE₂ on basal and P-stimulated AR is also shown. **P* < 0.05 vs. P; ***P* < 0.005 vs. BPA; ****P* < 0.005 vs. OP; #, *P* < 0.0001 vs. control.

4. Discussion

4.1. Effects of antiestrogens on human sperm functions

The present paper demonstrates that two different estrogen antagonists, Tx and ICI 164384 have rapid effect on human spermatozoa probably by interacting with nongenomic estrogen receptor on sperm membrane. Essentially, the two anti-estrogen show similar effects as $17\beta E_2$ (Luconi et al., 1999) determining a rapid increase of sperm $[Ca^{2+}]_i$ and inhibition of the subsequent calcium influx stimulated by P. However, despite the observed effects on $[Ca^{2+}]_i$, the two anti-estrogens did not result, differently from $17\beta E_2$, in inhibition of AR stimulated by the steroid, actually partially reverting the inhibitory effect of $17\beta E_2$. This result might indicate that the inhibition of the plateau and peak components of P-calcium response obtained with the two anti-estrogens (Fig. 2), which is lower than that obtained with $17\beta E_2$, may be not sufficient to determine inhibition of AR. However, when the two antiestrogens are given together with $17\beta E_2$, the inhibition of both the calcium components of P response are similar to those obtained with $17\beta E_2$. Yet, no inhibition but rather partial restoration of AR in response to P is obtained in this case. It is possible that other signal transduction pathways, stimulated by P, such as activation of tyrosine kinases (Luconi et al., 1999) or stimulation of chloride efflux (Meizel, 1997), are disrupted by estradiol, but not by the two anti-estrogens. We have, for instance, demonstrated that $17\beta E_2$ stimulates tyrosine phosphorylation of sperm proteins (Luconi et al., 1999) possibly interfering with tyrosine kinase activation by P. Further studies about effects of TX and ICI on the other signal transduction pathways activated in human spermatozoa are needed to clarify this point. Taken together our data suggest that Tx and ICI may act as partial agonists of the nongenomic sperm estrogen receptor and that, by occupying the receptor, they partially counteract the action of a subsequent addition of estradiol, restoring AR in response to P. Between the two anti-estrogens, Tx appears to be more potent than the ICI compound. These data are in agreement with the recent data about pharmacological action of antiestrogens. Indeed, although initially considered an anti-estrogen such as ICI, Tx has been recently reclassified as a selective estrogen receptor modulator (SERM), since its action depends on the tissue analyzed. In fact, while in some tissues it can act as estrogen antagonist, in others, such as cardiovascular system and bone, it shows partial or complete agonist activity (Wijayarathne et al., 1999). Such a different activity of ICI and Tx has been described in different tissues both in vivo and in vitro. Moreover, although on nuclear estrogen receptor, Tx and ICI have been

described to act at the level of ligand-binding domain, interfering with the receptor interaction with DNA (Brzozowski et al., 1997), very little is known on the antiestrogenic action of these two molecules on the nongenomic estrogen receptors. Differential activity of Tx has been demonstrated also on membrane receptors in different cell types and tissues (Lagrange et al., 1997; Watters et al., 1997). In the human sperm we found, both by ligand blot analysis with estradiol or western blot analysis with an antibody directed against the ligand binding domain of the genomic estrogen receptor, a protein band of about 29 kDa (Luconi et al., 1999), which presumably retains a similar estrogen-binding domain as the genomic receptor. In this light, the finding of a partial agonistic activity of the two anti-estrogens is not surprising.

4.2. Effects of xenoestrogens on human sperm functions

Several studies investigated xenoestrogens activity as endocrine disrupters, especially at the level of male reproductive system. In particular, possible effects of exogenous estrogens exposure during fetal and neonatal life on increasing incidence of disorders in male reproductive tract and the falling of male fertility have been addressed (Sharpe and Skakkebaek, 1993; Sharpe, 1995). So far, only few studies have been designed to investigate the xenoestrogens effects on adult male testis, and on possible effects of these substances mediated by nongenomic receptors. In particular, Loomis and Thomas (Loomis and Thomas, 2000) demonstrated that BPA binds to an estrogen membrane receptor from Atlantic Croacker testis, interfering with the androgen production. Human sperm responsiveness to P and $17\beta E_2$ in terms of calcium waves and AR may be considered a good model to study the steroid effects mediated by binding to an atypical nongenomic membrane receptor. For this reason, we tested on this model eventual rapid effect of xenoestrogens present in the environment. In the present study, we excluded any direct effect of xenoestrogens on human sperm functions, since incubation of spermatozoa with xenoestrogens did not result in any alteration of intracellular calcium levels or AR both in basal conditions and in response to P. Despite this, we have shown no effect of xenoestrogens on mature human spermatozoa. It must be mentioned that a specific binding to testicular membrane estrogen receptors has been described for a variety of xenobiotic estrogens in Atlantic Croaker testes (Loomis and Thomas, 2000). However, in this case, xenoestrogen disrupting activity in adult testes seems to be related to the presence of nongenomic estrogen receptor on Leydig cells, since these compounds specifically inhibit testicular normal production of androgens.

Acknowledgements

We thank Professor Gianna Fiorelli (Dipartimento di Fisiopatologia Clinica, Umta' Endocrinologia, Università di Firenze, Italy) for kindly providing the anti-estrogen ICI 164 384. This paper was supported by a grant of the Italian Public Health Project 'Human Exposure to Xenobiotics with Potential Endocrine Activities: Evaluation of the Risks for Reproduction and Development'.

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