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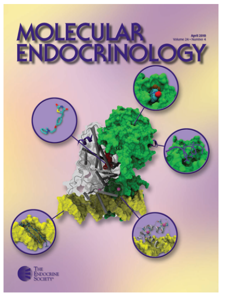

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Identification and Characterization of a Novel Functional Estrogen Receptor on Human Sperm Membrane That Interferes with Progesterone Effects

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ABSTRACT

The presence of a novel functional estrogen receptor on the human sperm surface has been demonstrated by using different experimental approaches. Ligand blot analysis of sperm lysates, using peroxidase-conjugated estradiol as probe, identified a specific estradiol-binding protein of approximately 29-kDa apparent molecular mass. The same protein band was also revealed by using αH222 antibody, which is directed against the steroid binding domain of the genomic estrogen receptor. The biological effects of estrogen receptor were investigated by analyzing calcium fluxes, tyrosine phosphorylation, and acrosome reaction (AR) in response to 17β-estradiol (17βE_2) and by measuring the steroid influence on calcium and AR in responses to progesterone (P), a well-known physiological stimulus for human spermatozoa. Our results demonstrate that 17βE_2 induces a rapid and sustained increase of intracellular calcium concentrations ($[Ca^{2+}]_i$). This effect is totally dependent on the presence of extracellular calcium, because it is completely abolished in a calcium-depleted medium. The dose-response curve for calcium increase to 17βE_2 is biphasic with a first component in the nanomolar range (effective concentration 50 = 0.60 ± 0.12 nmol/L) and a second component in the micromolar range (EC_{50} = 3.80 ± 0.26 μmol/L). 17βE_2 stimulates tyrosine phosphorylation of several sperm proteins, including the 29-kDa protein band, and determines a reduction of calcium response to P, finally resulting in inhibition of P-stimulated sperm AR. Conversely, no direct effect of 17βE_2 is observed on AR. 17βE_2 effects on calcium are clearly mediated by a membrane receptor, because they are reproduced by the membrane-impermeable conjugate of the hormone BSA-E_2 and reduced by sperm preincubation with αH222 antibody. Taken together, our results clearly show the presence of a functional surface estrogen receptor, of 29 kDa, on human spermatozoa. This receptor may play a role in the modulation of nongenomic action of P in these cells during the process of fertilization. (*J Clin Endocrinol Metab* **84:** 1670–1678, 1999)

SEVERAL steroid hormones have been demonstrated to exert rapid effects on cells by interacting with specific receptors present on surface (1). In particular, estrogen has been described as affecting intracellular calcium concentrations ($[Ca^{2+}]_i$), cAMP levels, mitogen-activated protein kinase activity (2), phospholipase C (3) and A_2 (4), and protein kinase C (5) in different tissues and cell lines. Both progesterone (P) and estrogen are present at high levels in follicular fluid (6–8). In particular, average estradiol concentrations in follicular fluid from mature oocyte are in the micromolar range (6–8). Rapid effects of both estradiol and P have been extensively demonstrated in human oocytes, as well as their role in oocyte activation and development (for review, see Ref. 9). Although nongenomic effects of P on human spermatozoa have been well elucidated, showing that P stimulates a cascade of signaling pathways leading to induction of acrosome reaction (AR) (10) and functional P surface receptor have been recently characterized (11), little is known about the estrogen effects in these cells (for review, see Ref. 9). The influence exerted by the steroid depletion in estrogen receptor knock-out mice has been investigated recently on maturation of spermatozoa (12), showing reduced motility and absence of fertilizing potential. Moreover, several competitive binding (13, 14) and immunofluorescence (15) studies suggest the presence of specific binding sites for 17β-estradiol (17βE_2) on human sperm surface. However, although Cheng *et al.* (16) could not detect specific binding sites for 17βE_2 in the sperm cytosolic and nuclear fractions, the effects exerted by this steroid on sperm motility and fertilization potential seem to be inhibited by the classical genomic receptor antagonist tamoxifen (17). Thus, at present, the nature of these receptors remains unclear.

Interestingly, some interactions between P and estrogen at membrane level have been suggested both in spermatozoa and brain tissues. P competes with [^{3}H]17βE_2 binding to intact human spermatozoa (18), and estradiol can displace iodide P binding to a protein of 29 kDa identified on mouse brain membrane lysates (19, 20).

In the present study, we report identification and partial characterization of a novel receptor for estrogen on human sperm membrane, using functional and biochemical approaches similar to those applied by our group to characterize the nongenomic receptor for P on human sperm surface (11). We investigated the biological effects of 17βE_2 on intracellular calcium levels in fura-2-loaded spermatozoa and on AR. In addition, we examined the possible interference exerted by this steroid on calcium and AR in response to P. Finally, by ligand and Western blot analysis of sperm

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lysates, we initiated the molecular characterization of estrogen receptor and investigated the modulation of tyrosine phosphorylation pattern of this protein in response to administration of the steroid.

Materials and Methods

Chemicals

Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). Human serum albumin-free human tubal fluid (HTF) was from Irvine (Santa Ana, CA). All free steroids, peroxidase-conjugated estradiol (E_2-POD), 6-(O-carboxymethyl)oxime-estradiol conjugated with BSA (BSA-E_2), secondary conjugated antibodies, fluorescein isothiocyanate-labeled Arachis hypogea (peanut) lectin, and all the other chemicals were from Sigma Chemical Co. (St. Louis, MO). Reagents for SDS-PAGE and for protein measurement were from Bio-Rad Laboratories, Inc. (Hercules, CA). Monoclonal αH222 antibody was a kind gift of Prof. Geoffrey Greene (The Ben May Institute for Cancer Research, University of Chicago, Chicago, IL). Peroxidase-conjugated monoclonal (PY20-HRP) antiphosphotyrosine antibodies were from ICN (Costa Mesa, CA). Digitonin and Fura-2/AM were obtained from Calbiochem (La Jolla, CA). The BM enhanced-chemiluminescence system was from Boehringer (Mannheim, Germany).

Preparation of spermatozoa

Human semen was collected, according to the World Health Organization (WHO)-recommended procedure (21) 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/mL were not included in the study. Semen samples were processed as previously described (22). Briefly, spermatozoa were separated on 40 and 80% Percoll gradients, combined, washed in HTF medium containing 0.3% fatty acid free-BSA, and resuspended in the same medium at the indicated concentration. Alternatively, sperms were separated by swim-up collection, according to the WHO-recommended procedure (21). Spermatozoa were capacitated for 2 h or otherwise indicated in 0.3% BSA-containing HTF and treated as indicated in each experiment.

Preparation of sperm membranes

Sperm membranes were prepared as previously described (11). Briefly, spermatozoa were lysed in lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.25% Nonidet P40, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethanesulfonyl flouride] for 1 h on ice. Then the samples were subjected to two subsequent cycles of homogenizing (teflon-glass) and sonicating 3 × 15-sec 8 burst. The homogenates were centrifuged at 1,500 rpm for 10 min at 4 C, and supernatants were ultracentrifuged at 48,000 rpm for 45 min at 4 C. The resulting pellets (cellular membranes) were resuspended in lysis buffer and homogenized.

Preparation of uterine and myometrial cell lysates

Human uterine samples in the proliferative phase of the menstrual cycles, obtained at surgery, were processed as previously described (23).

Myometrial cells, obtained as previously described (23), were resuspended in lysis buffer (see above). After protein measurement (Biorad kit, Bio-Rad Laboratories, Inc.), aliquots of cell extracts were applied onto SDS-polyacrylamide gels.

Measurement of intracellular calcium concentration

Spermatozoa, prepared as described above, were loaded with 2 μmol/L Fura-2/AM for 45 min at 37 C, washed, resuspended in FM medium (125 mmol/L NaCl, 10 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 0.25 mmol/L $MgCl_2$, 19 mmol/L Na-lactate, 2.5 mmol/L Na-pyruvate, 2 mmol/L HEPES, 0.3% BSA, pH 7.5), and $[Ca^{2+}]_i$, before and after stimulation with the different agonists, was measured (as described previously) using a spectrofluorimetric method (22), except that, in the present experiments, we used a Perkin-Elmer Corp. (Foster City, CA) LS50B instrument equipped with a fast rotary filter shuttle for alternate 340- and 380-nm excitation. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence with 0.01% digitonin, followed by minimal fluorescence with 10 mmol/L EGTA, pH 10. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz (24) using the ratio 340/380 and assuming a dissociation constant (K_d), of Fura-2 for calcium, of 224 nmol/L.

SDS-PAGE

After the different incubations, as indicated, samples were processed for SDS-PAGE as previously described (25). Briefly, sperm samples containing 10^7 cells/mL were added with 1 mmol/L Na_3VO_4, centrifuged at 400 × g at 4 C for 10 min, washed in PBS, and resuspended in 20 μL lysis buffer. After protein measurement (Biorad kit, Bio-Rad Laboratories, Inc.), the sperm extracts, containing the same protein amount, were diluted in an equal volume of reducing 2× loading buffer (1× = 62.5 mmol/L Tris (pH 6.8), 10% glycerol, 20% SDS, 2.5% pyronin, and 100 mmol/L dithiotheitrol), incubated at 95 C for 5 min, and loaded onto 10% polyacrylamide-bisacrylamide midi- and minigels. After SDS-PAGE, proteins were transferred to nitrocellulose membranes.

Ligand blot analysis

Nitrocelluloses filters with transferred proteins were treated for ligand blot analysis of sperm proteins, as previously described (11), with slight modification. Briefly, the membranes were incubated for 30 min in 3% NP-40/PBS, then for 2 h in 0.3% BSA/0.1% Tween-20/PBS for 10 min in 0.1% Tween-20/PBS, and overnight in 0.3% BSA/0.1% Tween-20/PBS containing peroxidase-conjugated estradiol (E_2-POD, 0.5 μmol/L). After several washes in 0.1% Tween-20/PBS, reacted proteins were revealed by a BM chemiluminescence system.

Western blot analysis

Nitrocelluloses filters with transferred proteins were blocked overnight at 4 C in TTBS (0.1% Tween-20, 20 mmol/L Tris, 150 mmol/L NaCl) containing 5% BSA, then washed repeatedly in TTBS, and incubated for 2 h in 2% BSA-TTBS containing PY20-HRP antibody (1:1000). After several washes in TTBS, reacted proteins were revealed by a BM chemiluminescence system. In some experiments, blots were washed for 30 min at 50 C in stripping buffer (10 mmol/L Tris (pH 6.8), 1% SDS, 5 mmol/L β-mercaptoethanol), to remove bound antiphosphotyrosine antibodies, then immunostaining was performed by 3-h incubation with αH222 antibody (1:400 in 2% BSA-TTBS), followed by 1-h incubation with antirat IgG-POD (1:4800 in 2% BSA-TTBS). Finally, the bands were visualized by the BM system. The immunospecificity of PY20 was determined by preadsorbing the antibody with 40 mmol/L o-phospho-DL-tyrosine for 1 h at room temperature.

AR assay

Acrosome-reacted spermatozoa were evaluated using the fluorescent probe fluorescein isothiocyanate-labeled Arachis hypogea (peanut) lectin, according to Aitken *et al.* (26), as previously described (27). Briefly, after 2-h capacitation, spermatozoa (10^6/mL) were preincubated for 10 min with 17βE_2 at different concentrations and then stimulated with P (10 μmol/L), or appropriate control solvent (dimethyl sulfoxide) 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 AR was evaluated on a total of 100 spermatozoa/slide. According to Aitken *et al.* (26), only curly-tailed spermatozoa were considered viable and thus scored.

Analysis of experimental results

The computer program ALLFIT (28) was used for the analysis of sigmoidal dose-response curves obtained in calcium studies. Data are expressed as mean ± SEM. Statistical analysis was made with Student's *t* test and one-way ANOVA.

Results

Effects of estradiol on intracellular calcium concentrations in human spermatozoa

Addition of 17βE$_2$ to fura-loaded spermatozoa induced a rapid and sustained rise of $[Ca^{2+}]_i$ in a dose-dependent manner. Figure 1 reports the typical calcium waves in response to increasing concentrations of 17βE$_2$ (0.1 nmol/L–100 μmol/L). The dose-response curve for the calcium effect of 17βE$_2$, as generated by the simultaneous computer analysis with the program ALLFIT (28), is biphasic (Fig. 2), showing a first component with an effective concentration 50 of 0.60 $\pm$ 0.12 nmol/L and a second component with an EC$_{50}$ of 3.80 $\pm$ 0.26 μmol/L. Also, because P stimulates a rapid calcium influx in human spermatozoa with a similar biphasic dose-response curve (11), and interactions between P and estrogen have been reported (19, 20), we tested the hypothesis of an eventual interference between the effects of the two steroids. Interestingly, the shapes of P- and 17βE$_2$-induced calcium waves were different: P induced first a rapid peak, followed by a long sustained plateau, whereas 17βE$_2$ induced a slow sustained response (Fig. 1). The typical $[Ca^{2+}]_i$ transient in response to P (10 μmol/L) was reduced in a dose-dependent manner by a previous administration of 17βE$_2$, both in the peak and plateau components (Fig. 1, also see Table 1). Table 1 reports the percentage peak and plateau $[Ca^{2+}]_i$ increases in response to P (10 μmol/L) alone or after previous administration of increasing concentrations of 17βE$_2$. Inhibition of the plateau phase was statistically significant for all the tested doses of estradiol, whereas peak inhibition was statistically significant only for high concentrations (Table 1). The effect of 17βE$_2$ was specific for P-response, because the steroid did not affect $[Ca^{2+}]_i$ increase obtained after stimulation with the endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin (10 μmol/L, Fig. 3), previously shown to increase calcium levels (29, 30) and AR (31) in human spermatozoa. Effects of 17βE$_2$, both on calcium levels and on calcium response to P, were not

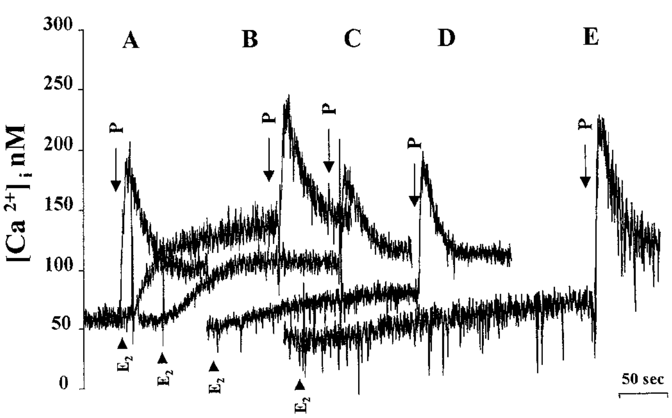

FIG. 1. Effects of increasing concentrations of 17βE$_2$ on basal and P-stimulated $[Ca^{2+}]_i$ in fura-2-loaded spermatozoa. Representative calcium waves, in response to P (10 μmol/L) in control conditions (A) and after a previous challenge with 100 μmol/L (B), 10 μmol/L (C), 10 nmol/L (D), and 0.1 nmol/L (E) 17βE$_2$ are shown. Note that 17βE$_2$ stimulates a dose-dependent increase of $[Ca^{2+}]_i$. All tracings were obtained in the same subject.

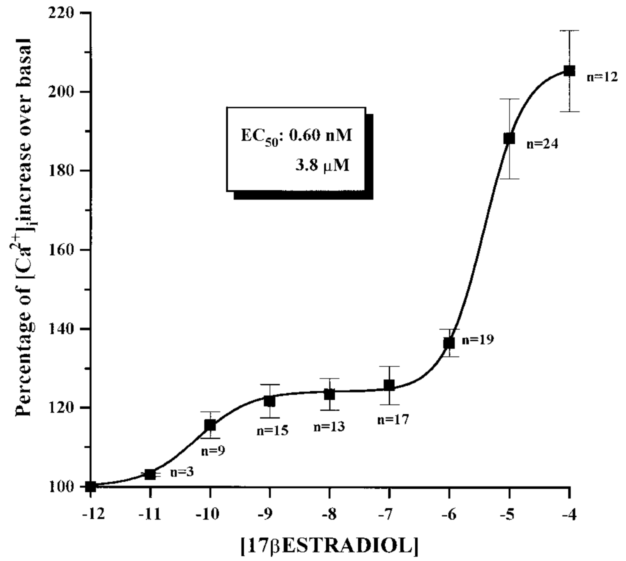

FIG. 2. Dose-response curve of the effect of 17βE$_2$ on $[Ca^{2+}]_i$ in fura-2-loaded human spermatozoa. The percentage of the $[Ca^{2+}]_i$ increase is reported on the *ordinate* and concentration of 17βE$_2$ on the *abscissa*. Each point represents the mean $\pm$ SEM of percentage stimulation for the number of the indicated experiments. The curve is generated by the computer program ALLFIT (28) after simultaneous analysis of the different dose-response curves. EC$_{50}$ values of 17βE$_2$ for the two components of the curve are reported in the *inset*.

antagonized by the cytosolic estrogen receptor antagonist tamoxifen (not shown), suggesting that the classical estrogen receptors are not involved. The effect of 17βE$_2$ seemed to be specific, because comparable concentrations of 17α-estradiol (17αE$_2$), even at 10 μmol/L concentration, neither stimulated $[Ca^{2+}]_i$ rise nor interfered with P-induced response (Fig. 4). To further demonstrate that the effect of 17βE$_2$ on $[Ca^{2+}]_i$ was mediated by a receptor present on sperm membrane, we used the membrane-impermeable estradiol conjugate BSA-E$_2$. This compound induced an $[Ca^{2+}]_i$ increase similar to that of 17βE$_2$ (Fig. 5), whereas the addition of BSA alone, the macromolecular component of the conjugate, was ineffective (not shown). BSA-E$_2$ was also able to mimic the inhibitory effect exerted by the free steroid on P-induced calcium waves (Fig. 5). The biological effects of BSA-E$_2$ were observed until 0.1 μmol/L concentration was achieved, which elicited an increase of basal $[Ca^{2+}]_i$ of about 1.16-fold (data not shown). Taken together, all these data demonstrate that 17βE$_2$ acts through interaction with a surface receptor. The increase in $[Ca^{2+}]_i$ after addition of 17βE$_2$ was totally dependent on the presence of extracellular calcium, because the response was absent when spermatozoa was stimulated in calcium-depleted medium in the presence of 2 mmol/L EGTA, and it was restored by subsequent replacement of external calcium to normal levels (Fig. 6B). Similarly, the calcium wave induced by BSA-E$_2$ (1 μmol/L) was blunted in the absence of extracellular calcium and was restored when $[Ca^{2+}]_e$ was replaced (Fig. 6C).

TABLE 1. Effects of different doses of $17\beta E_2$ on percentage peak and plateau $[Ca^{2+}]_i$ increases in response to progesterone (10 μM) in fura-2-loaded human spermatozoa

$17\beta E_2$ (M)	PEAK % $[Ca^{2+}]_i$ increase	Stat	PLATEAU % $[Ca^{2+}]_i$ increase	Stat
0	193.95 ± 17.75 (n = 42)		103.23 ± 6.70 (n = 41)	
1 mM	143.39 ± 22.16 (n = 16) 26%	n.s.	49.52 ± 6.06 (n = 15) 52%	$P < 0.001$
100 mM	123.67 ± 19.10 (n = 17) 36%	$P < 0.02$	35.92 ± 4.10 (n = 16) 65%	$P < 0.001$
10 mM	79.88 ± 7.91 (n = 26) 59%	$P < 0.001$	14.60 ± 1.54 (n = 24) 86%	$P < 0.001$

Data represents the mean ± SEM percentage stimulation for the number of experiments indicated in *parenthesis*. Statistical significance (stat) of each point is calculated *vs.* respectively peak and plateau values in response to P in the absence of $17\beta E_2$ (0). Percentage of inhibition for each $17\beta E_2$ concentration is also reported. n.s., Not significant.

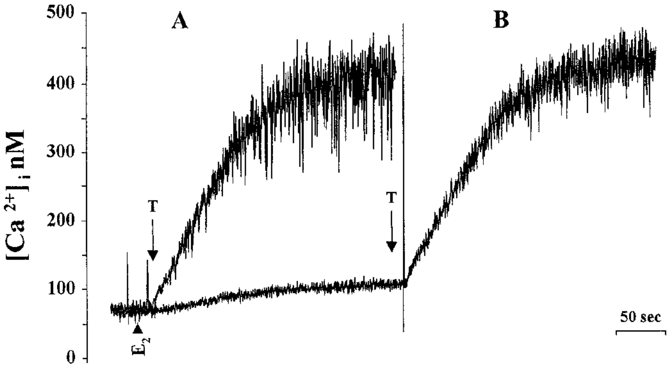

FIG. 3. Effects of $17\beta E_2$ on calcium response to thapsigargin. Sperm $[Ca^{2+}]_i$ increases, in response to thapsigargin (10 μmol/L) under control conditions (A) or after a previous challenge with 10 μmol/L $17\beta E_2$ (B), are shown. Results are representative of two similar experiments.

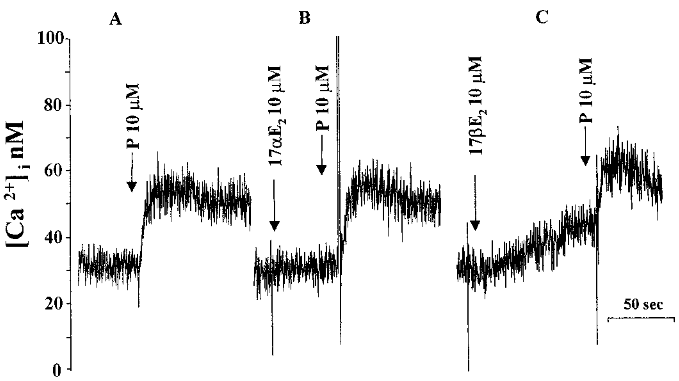

FIG. 4. Effects of $17\alpha E_2$ on basal sperm $[Ca^{2+}]_i$ and on calcium response to P. Intracellular calcium waves, in response to P (10 μmol/L) alone (A) and after a previous challenge with 10 μmol/L $17\alpha E_2$ (B) or $17\beta E_2$ (C), are shown. Note that $17\alpha E_2$ does not affect either basal or P-stimulated $[Ca^{2+}]_i$. Results are representative of three similar experiments.

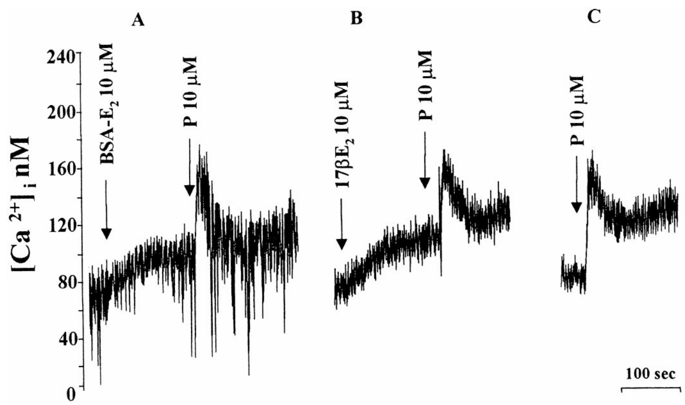

FIG. 5. Effects of the impermeable conjugate compound BSA-E_2 on basal $[Ca^{2+}]_i$ and on response to P. BSA-E_2 (A) (10 μmol/L) stimulates an increase of $[Ca^{2+}]_i$ and reduces calcium response to P (10 μmol/L), similar to $17\beta E_2$ (B). Control response to P (10 μmol/L) in the same subject is shown in C. Results are representative of four similar experiments.

Effects of estradiol on AR

Because a $[Ca^{2+}]_i$ rise, induced by P, leads to an increase in AR of human spermatozoa, we next investigated whether $17\beta E_2$ effects on calcium were also involved in regulation of AR. As shown in Fig. 7, 2-h incubation of capacitated spermatozoa with increasing concentrations of $17\beta E_2$ induced only a slight stimulation of AR at the highest dose used (10 μmol/L). Interestingly, all the three doses of $17\beta E_2$ blunted AR in response to P (Fig. 7).

Identification of estradiol receptor by ligand and Western blot analysis of human sperm lysates

To characterize $17\beta E_2$-binding proteins in human spermatozoa, we performed, on total sperm lysates, ligand blot experiments using E_2-POD as probe, and Western analysis with the monoclonal antibody αH222, directed against the steroid-binding domain of the genomic receptor (32). E_2-POD has been shown to bind to a membrane estrogen receptor in pancreatic islet cells (33), indicating that such molecule is a good tool to investigate this type of receptor. αH222 antibody has been shown to recognize a membrane estrogen receptor in rat pituitary tumor cells (34). Moreover, the approach of using an antibody produced against the steroid binding sequence of the genomic receptor was applied by our (11) and other groups (35, 36) to identify the putative membrane receptors for P in human spermatozoa. In addition, preincubation of sperm samples with αH222 antibody (1:20, Fig. 8B), but not with normal rat serum (1:20, Fig. 8C), reduced $17\beta E_2$ stimulation of calcium influx (Fig. 8A), suggesting that the sperm membrane receptor for estradiol is recognized by this antibody. A single band, of approximately 29-kDa molecular mass, is revealed both by E_2-POD (0.5 μmol/L, Fig. 9A) and αH222 antibody (1:400, Fig. 9B). An estrogen-binding protein of similar molecular mass has been described also in other cell types (19, 20, 37). The same pro-

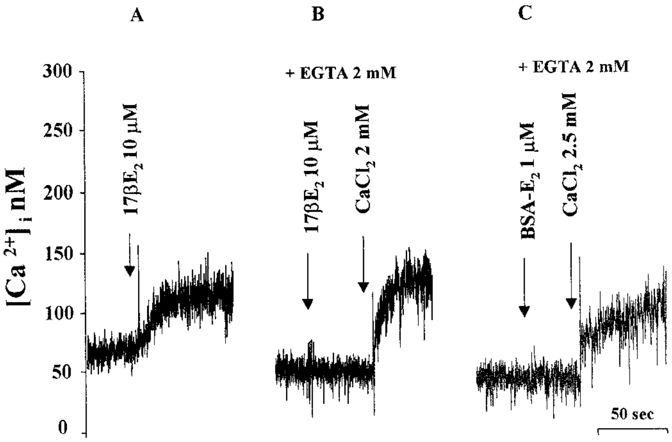

FIG. 6. Effect of EGTA on $17\beta E_2$- and BSA-E_2-induced $[Ca^{2+}]_i$ increases. Calcium responses to $17\beta E_2$ (10 μmol/L) in calcium-complete medium (A) and in the absence of extracellular calcium (Ca^{2+}-free medium + 2 mmol/L EGTA) (B) are shown. Similarly, the calcium influx induced by BSA-E_2 (1 μmol/L) is absent in calcium-depleted medium (C). The response to the two agonists is restored by subsequent addition of 2.5 mmol/L $CaCl_2$. All tracings were obtained in the same subject. Results are representative of three similar experiments.

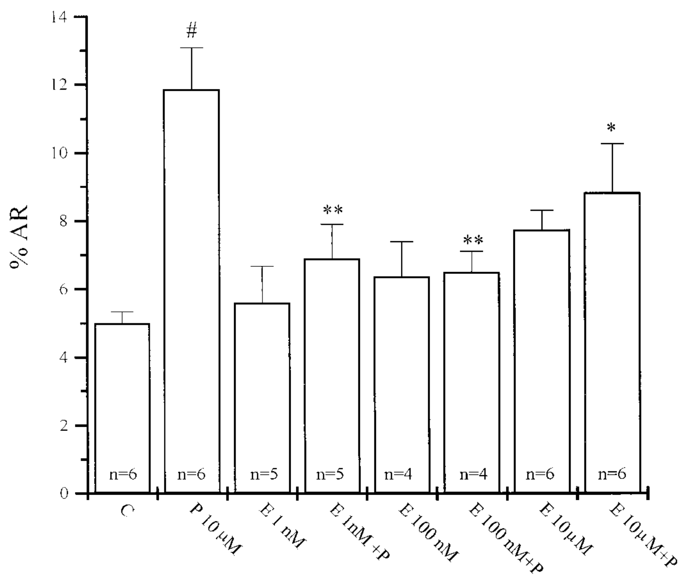

FIG. 7. Effect of $17\beta E_2$ on basal and P-stimulated AR in human spermatozoa. Capacitated spermatozoa were treated for 2 h with P (10 μmol/L) or with increasing concentrations of $17\beta E_2$ (1 nmol/L, 100 nmol/L, 10 μmol/L), both in the presence or absence of P (10 μmol/L) and AR, evaluated as described in *Materials and Methods*. C, Untreated control sample. Values represent the mean ± SEM percentage of AR for the indicated number of experiments. *, $P < 0.05$ *vs.* P; **, $P < 0.005$ *vs.* P; #, $P < 0.001$ *vs.* C.

tein band of 29 kDa was detected on purified sperm membranes stained with αH222 antibody (Fig. 9D). Longer exposures of the αH222-stained blots revealed the presence of two additional bands, of about 42–45 kDa and 54–58 kDa (Fig. 9E). A protein band, at the expected 54–58 kDa molecular mass range, probably corresponding to one of the known isoforms of the genomic estrogen receptor (38, 39), was detected by αH222 both on myometrial cell and total

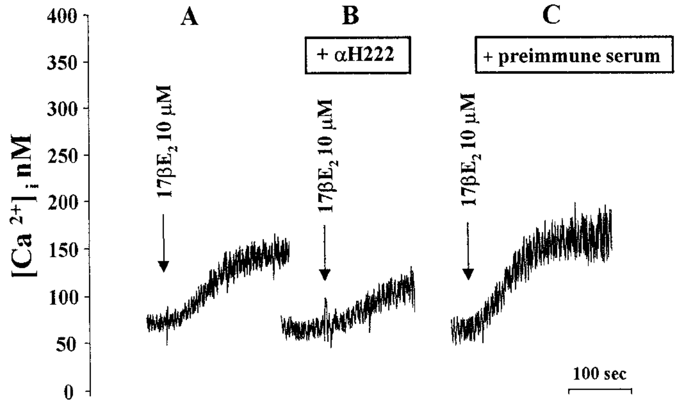

FIG. 8. Effect of αH222 antibody on calcium response to $17\beta E_2$ in fura-2-loaded human spermatozoa. The $[Ca^{2+}]_i$ increase, induced by $17\beta E_2$ (10 μmol/L) (A), was partially reverted after 10-min incubation of samples with αH222 antibody (1:20) (B). C, Calcium transient, stimulated by $17\beta E_2$ (10 μmol/L), after preincubation with normal rat serum in the same subject. Results are representative of three similar experiments.

uterus lysates, used as control for genomic estrogen receptors (Fig. 10). Interestingly, myometrial cells also show the presence of a 29-kDa protein band (Fig. 10).

Because phosphorylation of estrogen genomic receptor has been described as one of the mechanisms of receptor transactivation (40, 41), we investigated whether p29 was phosphorylated in tyrosine in human spermatozoa. Reprobing the blots shown in Fig. 9, A and B, with PY20 antibody revealed that this protein was phosphorylated on tyrosine residues (Fig. 9C). Moreover, a rapid (10 min) stimulation of capacitated spermatozoa with $17\beta E_2$ induced an increase of phosphorylation in tyrosine residues of several protein bands, including the p29 kDa one (Fig. 11A). A comigration between this tyrosine phosphorylated band (Fig. 11A) and the putative estrogen receptor was observed when the blot was washed thoroughly and reprobed with αH222 antibody (Fig. 11B).

Discussion

Our paper demonstrates the presence of a functional estrogen receptor on human sperm surface. This receptor apparently is involved in the activation of two different signal transduction pathways, namely an increase of $[Ca^{2+}]_i$ and of tyrosine phosphorylation of proteins, resulting in inhibition of P-stimulated calcium influx and AR. Ligand and Western analyses of sperm lysates, using E_2-POD and αH222 antibody as probes, reveal the presence of a protein band with an apparent molecular mass of 29 kDa. Location of this receptor on sperm surface is demonstrated both by the ability of the impermeable conjugate E_2-BSA to induce similar calcium waves, as $17\beta E_2$, as well as by the detection of the 29-kDa protein band in purified sperm membranes by Western analysis. The possible involvement of such a protein in the biological effects of $17\beta E_2$ is suggested by the inhibition of $17\beta E_2$-stimulated $[Ca^{2+}]_i$ increase by αH222 antibody, which probably competes with the steroid for the binding domain of the membrane receptor. Similarly, Morey *et al.* (42) showed that αH222 antibody reverted the biological effects of estrogen in vascular smooth muscle cells. A protein with a mo-

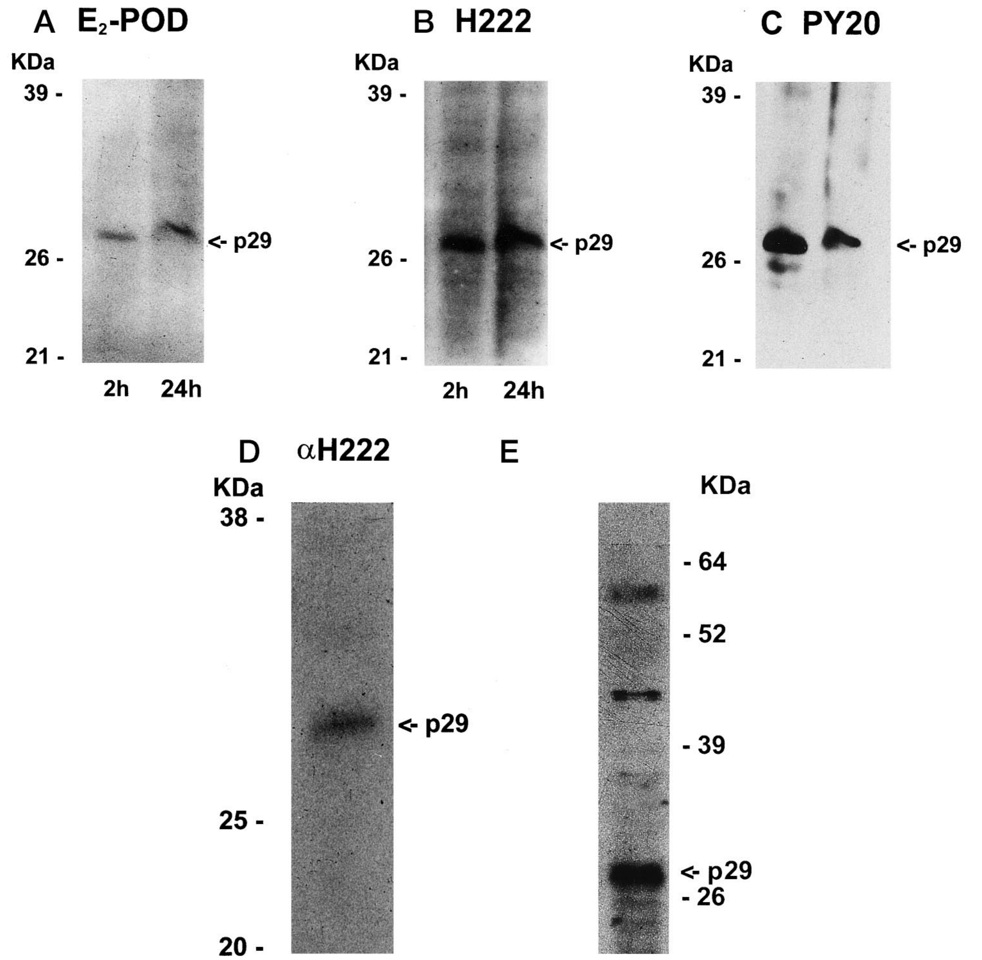

FIG. 9. Identification of sperm $17\beta E_2$-binding proteins by ligand and Western analysis. Total extracts (30 μg/lane) or purified membranes (50 μg/lane), from 2- and 24-h capacitated spermatozoa, were separated on reducing 10% SDS-PAGE. A, Ligand blot analysis of the sperm lysates using peroxidase-conjugated estradiol (E_2-POD, 0.5 μmol/L) reveals a single binding protein of 29-kDa molecular mass. After stripping, the same blot as in A was first probed for tyrosine-phosphorylated proteins with peroxidase-conjugated PY20 antibody (C) and then for estrogen receptor with αH222 antibody (1:400) (B), followed by the BM detection system. An exact alignment of the blots indicates that the E_2-POD-binding protein of 29 kDa (A) coincides with the band revealed both by αH222 (B) and PY20 (C) antibodies. D, Western blot analysis with αH222 (1:400) on purified sperm membranes reveals the same 29-kDa protein band as in A and B. E, Higher BM exposure of a αH222 antibody-stained blot of total sperm lysates showing other protein bands besides the 29-kDa one. Molecular weight markers are indicated to the *left* or the *right* of each blot. Results are representative of two similar experiments.

lecular mass of about 29 kDa, identified by photoaffinity labeling with progesterone-11α-hemisuccinate-(2-[^{125}I)iodohistamine), and specifically displaced by incubation with estradiol, has been detected in mouse brain membranes (19, 20) and has been suggested as the putative membrane binding site for estrogen (20). Moreover, Monje and Boland (37), using monoclonal antibodies against different domains of the intracellular estrogen receptor, identified on uterine membranes a 28- to 32-kDa protein, besides the expected 65-kDa band representing one of the genomic receptors. Such molecular mass (29 kDa) is quite different from the known classical α and β estrogen receptors (38, 39). Higher exposures of films in our Western blot analysis of sperm lysates reveal that αH222 antibody faintly detects two additional sperm protein bands, the higher of which shows a molecular weight similar to one of the classical genomic receptors. Although we have used all the necessary precautions to minimize eventual protein cleavage, the possibility that the 29-kDa protein band is a proteolytic fragment of the full-length estrogen receptor cannot be excluded. Also, it possible that a specific regulatory protein cleavage is involved in synthesis of the functional estrogen receptor in spermatozoa. The fact that other bands are seen with αH222 antibody suggests this possibility. On the other hand, a 66-kDa estrogen receptor that comigrates with a similar protein in the endometrial tissue has been detected, with a different antibody in human spermatozoa, by Durkee *et al.* (15). However, these authors could not discriminate whether this form represented the genomic receptor or not. Interestingly, the same authors detected, by RT-PCR analysis of sperm RNA, two different amplified nucleotidic bands (15), suggesting the presence of different messenger RNAs for estrogen receptors in human spermatozoa, as also described in other cell types (43, 44). So far, the question of whether classical genomic estrogen receptors are present in human spermatozoa still remains open. On the other hand, it is unlikely that the genomic estrogen receptor, if present, could be functional, because mature spermatozoa are transcriptionally silent. Moreover, our experiments clearly show that the 29-kDa protein band is the only one detected by both ligand and Western blot analyses, strongly indicating that this protein represents the membrane receptor for estrogen in human spermatozoa.

The rapid increase of $[Ca^{2+}]_i$ and phosphorylation induced by $17\beta E_2$ in human spermatozoa confirms the findings in other cell types for nongenomic/rapid actions of estrogens (37, 45–49). As in the case of P (50), both $17\beta E_2$- and BSA-E_2-stimulated $[Ca^{2+}]_i$ increases in spermatozoa are strictly dependent on the presence of extracellular calcium. Because this steroid is present in the follicular fluid (6–8) and in the male genital tract (12) at concentrations similar to those inducing the biological effects observed *in vitro* in human spermatozoa, it is conceivable that these effects may be physiologically relevant. The increase of calcium and tyrosine phosphorylation of proteins stimulated by 17βE_2 in human spermatozoa is not followed by induction of AR; rather, these effects interfere with those exerted by P. Indeed, a previous addition of 17βE_2 inhibits, in a dose-dependent manner, the subsequent calcium and AR responses to P. In particular, the plateau phase of P calcium response is significantly reduced after a first priming with very low concentrations of 17βE_2. Because the plateau phase of P-induced $[Ca^{2+}]_i$ increase has been associated with induction of AR (50), it is conceivable that inhibition of P-stimulated AR by 17βE_2 is attributable to inhibition of the plateau phase. Stimulation of tyrosine phosphorylation of its own receptor may be involved in the modulation of receptor binding activity. Indeed, modulation of the phosphorylation state of the estrogen receptor by the steroid itself or other substances has been associated with transactivation of the classical genomic estrogen receptor (40, 41). In particular, tyrosine phosphorylation occurs in the ligand binding domain of the genomic receptor (52).

The precise mechanism involved in 17βE_2 inhibition of calcium and AR response to P in human spermatozoa is still unclear. Other groups reported rapid inhibitory effects of 17βE_2 on vascular smooth muscle contraction (53–56) and on neuron hyperpolarization (57). In particular, the rapid inhibitions of coronary artery contraction [either basal (56) or induced by PG $F_{2\alpha}$, extracellular potassium (54), and endothelin (55)] seem to be mediated by reduction of cellular calcium influx via blockage of L-type Ca^{2+} channels (53). Lagrange *et al.* (57) reported 17βE_2 reduction of μ-opioids' ability to hyperpolarize guinea pig hypotalamic neurons via G protein-coupled receptors. However, in all these cases, the inhibitory effects of 17βE_2 are never associated with an increase of calcium influx induced by the steroid itself, as in our experiments. It is possible that the partial stimulation by 17βE_2 of the same signal transduction pathways of P interferes with the biological response to the latter, leading to inhibition of AR. However, the possibility that 17βE_2 and P compete for the same receptors cannot be excluded.

Interestingly, the sperm calcium curve, in response to 17βE_2, shows a biphasic behavior, with two components (one in the nanomolar and the other in the micromolar range), similar to the calcium curve obtained for P (11). Although this result may suggest the presence of two different binding sites for estradiol, we have constantly observed the presence of a single 29-kDa protein band in ligand blot experiments with E_2-POD. On the other hand, binding of ^3H-17βE_2 to intact human spermatozoa revealed the presence of a single binding site, with a an apparent K_d of 0.6 nmol/L (13), consistent with the first component of our curve. Similarly, the effect of 17βE_2 on P-induced AR and plateau phase of calcium increases was observed at nanomolar concentrations.

Inhibition of rapid responses to 17βE_2 by tamoxifen is controversial (45, 49, 58). Indeed, whereas Lantin-Hermoso *et al.* (58) described a complete inhibition by tamoxifen on estradiol acute stimulation of nitric oxide synthase activity in artery endothelium, Watters *et al.* (49) found no effect of tamoxifen on rapid membrane effects of estrogen in neuroblastoma cells. Moreover, Morley *et al.* (45) showed that tamoxifen could not affect the rapid estrogen-triggered $[Ca^{2+}]_i$ increase in chicken granulosa cells. In our hands, this

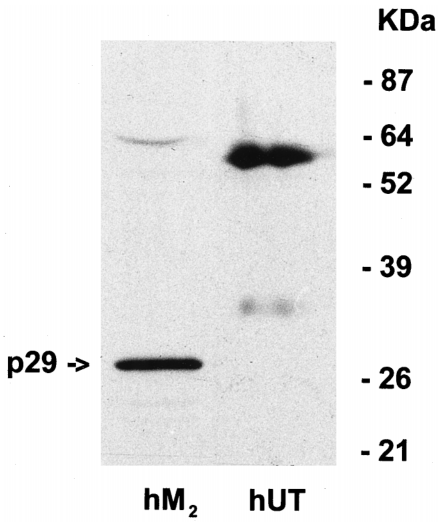

FIG. 10. Western blot analysis of total lysates from human myometrial cells and human uterus with αH222 antibody. Human uterus (hUT) and myometrial cell (hM_2) lysates were obtained as described in *Materials and Methods*, and proteins extracts were separated on 10% reducing SDS-PAGE. Western blot analysis with αH222 antibody (1:400) reveals two protein bands in the molecular mass range of 20–64 kDa. In particular, a 54- to 58-kDa protein band is detected by αH222, both on myometrial and uterus lysates. A 29-kDa protein band is observed in myometrial cells. Molecular weight markers are indicated to the *right* of the blot.

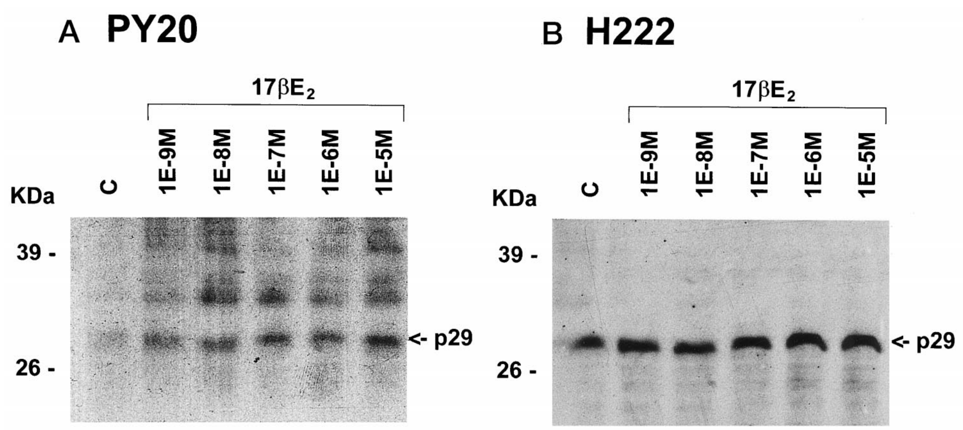

FIG. 11. Effect of 17βE_2 on tyrosine phosphorylation of p29 kDa protein band in human spermatozoa. Capacitated spermatozoa were stimulated for 10 min with increasing concentrations of 17βE_2 (1 nmol/L–10 μmol/L). Protein extracts, separated on 10% reducing SDS-PAGE, were first probed with PY20-HRP antibody (A), washed, and reprobed with αH222 antibody (1:400, B). The phosphorylated p29 protein band in A exactly aligns with the 29-kDa protein detected in B. C, unstimulated control. Molecular weight markers are indicated to the *left* of each blot. Results are representative of two similar experiments.

cytosolic estrogen receptor antagonist was ineffective in counteracting estradiol action on intracellular calcium, further suggesting that the estrogen receptor in spermatozoa differs from the genomic one.

In conclusion, our results demonstrate the presence of a biologically active sperm receptor for estrogen in human spermatozoa, suggesting a novel role for estradiol, in the process of fertilization, as a possible physiological modulator of P action on spermatozoa. Because levels of estradiol in the follicular fluid are similar to those inducing the observed nongenomic effects, the strict cross-talk between sperm membrane receptors for $17\beta E_2$ and P may be important for an appropriate timing of capacitation and AR in the female genital tract. Further studies are required to elucidate whether environmental chemicals with estrogen action might have similar effects on human sperm and to evaluate whether the absence of sperm response to P in several cases of idiopathic male infertility (27, 59) may be attributable to alteration in the interactions between these two steroids.

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References

1. **Wehling M.** 1997 Specific nongenomic actions of steroid hormones. Annu Rev Physiol. 59:365–393.
2. **Nemere I, Farach-Carson MC.** 1998 Membrane receptors for steroid hormones: a case for specific cell surface binding sites for vitamin D metabolites and estrogens. Biochem Biophys Res Commun. 248:443–449.
3. **Le Mellay V, Grosse B, Lieberherr M.** 1997 Phospholipase C β and membrane action of calcitriol and estradiol. J Biol Chem. 272:11902–11907.
4. **Schwartz Z, Gates PA, Nasatzky E, Sylvia VL, Mendez J, Dean DD, Boyan BD.** 1996 Effect of 17β-estradiol on chondrocyte membrane fluidity and phospholipid metabolism is membrane-specific, sex-specific, and cell maturation-dependent. Biochim Biophys Acta. 1282:1–10.
5. **Sylvia VL, Hughes T, Dean DD, Boyan BD, Schwartz Z.** 1998 17β-estradiol regulation of protein kinase C activity in chondrocytes is sex dependent and involves nongenomic mechanisms. J Cell Physiol. 176:435–444.
6. **Frederick JL, Francis MM, Macaso TM, Lobo RA, Sauer MV, Paulson RJ.** 1991 Preovulatory follicular fluid steroid levels in stimulated and unstimulated cycles triggered with human chorionic gonadotropin. Fertil Steril. 55:44–47.
7. **Lobo RA, diZerega GS, Marrs RP.** 1985 Follicular fluid steroid levels in dysmature and mature follicles from spontaneous and hyperstimulated cycles in normal and anovulatory women. J Clin Endocrinol Metab. 60:81–87.
8. **Andersen CY.** 1993 Characteristics of human follicular fluid associated with successful conception after *in vitro* fertilization. J Clin Endocrinol Metab. 77:1227–1234.
9. **Revelli A, Massobrio M, Tesarik J.** 1998 Nongenomic actions of steroid hormones in reproductive tissue. Endocr Rev. 19:3–17.
10. **Baldi E, Luconi M, Bonaccorsi L, Forti G.** 1998 Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. Front Biosci. 3:d1051–1059.
11. **Luconi M, Bonaccorsi L, Maggi M, et al.** 1998 Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. J Clin Endocrinol Metab. 83:877–885.
12. **Eddy EM, Washburn TF, Bunch DO, et al.** 1996 Target disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology. 137:4796–4805.
13. **Hernandez-Perez O, Ballesteros LM, Rosado A.** 1979 Binding of 17-β-estradiol to the outer surface and nucleus of human spermatozoa. Arch Androl. 3:23–29.
14. **Cheng CY, Boettcher B, Rose RJ. Kay DJ, Tinneberg HR.** 1981 The binding of sex steroids to human spermatozoa. An autoradiographic study. Int J Androl. 4:1–17.
15. **Durkee TJ, Mueller M, Zinaman M.** 1998 Identification of estrogen receptor protein and messenger ribonucleic acid in human spermatozoa. Am J Obstet Gynecol. 178:1288–1297.
16. **Cheng CY, Boettcher B, Rose RJ.** 1981 Lack of cytosol and nuclear estrogen receptors in human spermatozoa. Biochem Biophys Res Commun. 1200:840–846.
17. **Idaomar M, Guerin JF, Lornage J, Monchamont P, Czyba JC.** 1987 Effects of estradiol and its antagonist-tamoxifen-on motility and metabolism of human spermatozoa. Adv Contracept. 3:337–341.
18. **Hyne RV, Boettcher B.** 1977 The selective binding of steroids to human spermatozoa. Contraception. 15:163–174.
19. **Bukusoglu C, Krieger NR.** 1994 Photoaffinity labeling with progesterone-11α-hemisuccinate-(2-[^{125}I)iodohistamine) identifies four protein bands in mouse brain membranes. J Neurochem. 63:1434–1438.
20. **Bukusoglu C, Krieger NR.** 1996 Estrogen-specific target site identified by progesterone-11α-hemisuccinate-(2-[^{125}I)iodohistamine) in mouse brain membranes. J Steroid Biochem Mol Biol. 58:89–94.
21. **World Health Organization.** 1992 WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions. 3rd ed. Cambridge, UK: Cambridge University Press;
22. **Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, Forti G.** 1991 Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl. 12:323–330.
23. **Maggi M, Fantoni G, Baldi E, et al.** 1994 Antagonist for the human oxytocin receptor: an *in vitro* study. J Reprod Fertil. 101:345–352.
24. **Grynkiewicz G, Poenie M, Tsien RY.** 1985 A generation of a Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem. 260:3440–3450.
25. **Luconi M, Krausz C, Forti G, Baldi E.** 1996 Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. Biol Reprod. 55:207–216.
26. **Aitken RJ, Buckingham DW, Fang HG.** 1993 Analysis of response of human spermatozoa to A23187 employing a novel technique for assessing acrosome reaction. J Androl. 14:132–141.
27. **Krausz CS, Bonaccorsi L, Maggio P, et al.** 1996 Two functional assays of sperm responsiveness to progesterone and their predictive values in *in vitro* fertilization. Hum Reprod. 11:1661–1667.
28. **De Lean A, Munson PJ, Rodbard D.** 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol. 235:E97–E102.
29. **Blackmore PF.** 1992 Thapsigargin elevates and potentiates the ability of progesterone to increase intracellular free calcium in human sperm: possible role of perinuclear calcium. Cell Calcium. 14:53–60.
30. **Bonaccorsi L, Luconi M, Forti G, Baldi E.** 1995 Tyrosine kinase inhibition reduces the plateau phase of the calcium increase in response to progesterone in human sperm. FEBS Lett. 364:83–86.
31. **Meizel S, Turner KO.** 1993 Initiation of the human sperm acrosome reaction by thapsigargin. J Exp Zool. 267:350–355.
32. **Greene GL, Sobel NB, King WJ, Jensen EV.** 1984 Immunochemical studies of estrogen receptors. J Steroid Biochem Mol Biol. 20:51–56.
33. **Nadal A, Rovira JM, Laribi O, et al.** 1998 Rapid insulinotropic effect of 17β-estradiol via a plasma membrane receptor. FASEB J. 12:1341–1348.
34. **Pappas TC, Gametchu B, Watson CS.** 1995 Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J. 9:404–410.
35. **Sauber K, Edwards DP, Meizel S.** 1996 Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction. Biol Reprod. 54:993–1001.
36. **Cheng FP, Gadella BM. Voorhout WF, et al.** 1998 Progesterone-induced acrosome reaction in stallion spermatozoa is mediated by a plasma membrane progesterone receptor. Biol Reprod. 59:733–742.
37. **Monje P, Boland R.** 1999 Characterization of membrane estrogen binding proteins from rabbit uterus. Mol Cell Endocrinol. 147:75–84.
38. **Ikeda M, Ogata F, Curtis SW, et al.** 1993 Characterization of the DNA-binding domain of the mouse uterine estrogen receptor using site-specific polyclonal antibodies. J Biol Chem. 268:10296–10302.
39. **Ogawa S, Inoue S, Watanabe T, et al.** 1998 The complete primary structure of human estrogen receptor β (hERβ) and its heterodimerization with ER α *in vivo* and *in vitro*. Biochem Biophys Res Commun. 243:122–126.
40. **Ram PT, Kiefer T, Silverma M, Song Y, Brown GM, Hill SM.** 1998 Estrogen receptor transactivation in MCF-7 breast cancer cells by melatonin and growth factors. Mol Cell Endocrinol. 141:53–64.
41. **Weigel NL.** 1996 Steroid hormone receptors and their regulation by phosphorylation. Biochem J. 319:657–667.
42. **Morey AK, Pedram A, Razandi M, et al.** 1997 Estrogen and progesterone inhibit vascular smooth muscle proliferation. Endocrinology. 138:3330–3339.
43. **Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH.** 1998 Estrogen receptor-β mRNA variants in human and murine tissues. Mol Cell Endocrinol. 138:199–203.
44. **Griffin C, Flouriot G, Sonntag-Buck V, Nestor P, Gannon F.** 1998 Identification of novel chicken estrogen receptor-alpha messenger ribonucleic acid isoforms generated by alternative splicing and promoter usage. Endocrinology. 139:4614–4625.
45. **Morley P, Whitfield JF, Vanderhyden BC, Tsang BK, Schwartz JL.** 1992 A

new, nongenomic estrogen action: the rapid release of intracellular calcium. Endocrinology. 131:1305–1312.
46. **Fiorelli G, Gori F, Frediani U, et al.** 1996 Membrane binding sites and nongenomic effects of estrogen in cultured human preosteoclastic cells. J Steroid Biochem Mol Biol. 59:233–240.
47. **Sanchez-Bueno A, Sancho MJ, Cobbold PH.** 1991 Progesterone and oestradiol increase cytosolic Ca^{2+} in single rat hepatocytes. Biochem J. 280:273–276.
48. **Benten WPM, Lieberherr M, Giese G, Wunderlich F.** 1998 Estradiol binding to cell surface raises cytosolic free calcium in T cells. FEBS Lett. 422:349–353.
49. **Watters JJ, Campbell JS, Cunningham MJ, Krebs EG, Dorsa DM.** 1997 Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology. 138:4030–4033.
50. **Blackmore PF, Neulen J, Lattanzio F, Beebe S.** 1991 Cell surface-binding site for progesterone mediate calcium uptake in human sperm. J Biol Chem. 266:18655–18659.
51. **Tesarik J, Mendoza C.** 1996 Single cell analysis of tyrosine kinase dependent and independent Ca^{2+} fluxes in progesterone induced acrosome reaction. Mol Hum Reprod. 2:225–232.
52. **Castoria G, Migliaccio A, Green S, DiDomenico M, Chambon P, Auricchio F.** 1993 Properties of a purified estradiol-dependent calf uterus tyrosine kinase. Biochemistry. 32:1740–1750.
53. **Kitakazawa T, Hamada E, Kitakazawa K, Gaznabi AK.** 1997 Non-genomic mechanism of 17β-oestradiol-induced inhibition of contraction in mammalian vascular smooth muscle. J Physiol. 499:497–511.
54. **Jiang C, Sarrel PM, Poole-Wilson PA, Collins P.** 1991 Endothelium-independent relaxation of rabbit coronary artery by 17β-oestradiol *in vitro*. Br J Pharmacol. 104:1033–1037.
55. **Jiang C, Sarrel PM, Poole-Wilson PA, Collins P.** 1992 Acute effect of 17β-estradiol on rabbit coronary artery contractile response to endothelin-1. Am J Physiol. 263:H271–H275.
56. **Ruhelmann DO, Steinert JR, Valverde MA, Jacob R, Mann GE.** 1998 Environmental estrogenic pollutants induce acute vascular relaxation by inhibiting L-type Ca^{2+} channels in smooth muscle cells. FASEB J. 12:613–619.
57. **Lagrange AH, Ronnekleiv OK, Kelly MJ.** 1997 Modulation of G protein-coupled receptors by an estrogen receptor that activates protein kinase A. Mol Pharmacol. 51:605–612.
58. **Lantin-Hermoso RL, Rosenfeld CR, Yuhanna IS, German Z, Chen Z, Shaul P.** 1997 Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. Am J Physiol. 273:L119–L126.
59. **Benoff S, Barcia M, Hurley IR, et al.** 1996 Classification of male factor infertility relevant to IVF insemination strategies using mannose ligands, acrosome status and anti-cytoskeletal antibodies. Hum Reprod. 11:1905–1918.