0021-972X/98/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 83, No. 3
Printed in U.S.A.

Identification and Characterization of Functional Nongenomic Progesterone Receptors on Human Sperm Membrane*

MICHAELA LUCONI, LORELLA BONACCORSI, MARIO MAGGI, PAOLA PECCHIOLI, CSILLA KRAUSZ, GIANNI FORTI, AND ELISABETTA BALDI

Dipartimento di Fisiopatologia Clinica, Unità di Andrologia, Università di Firenze, I-50139 Firenze, Italy

ABSTRACT

The presence of functional nongenomic progesterone (P) receptors in human spermatozoa has been investigated by equilibrium binding studies in intact spermatozoa, ligand blot and Western blot analysis of sperm lysates, as well as determination of the effects of the steroid on sperm intracellular Ca^{2+} concentrations. Binding experiments were performed using progesterone-11α-glucuronide-[^{125}I]iodotyramine as tracer. Computer analysis of competition curves using different steroids as competitors indicated the presence of two distinct binding sites for P. The high affinity site (K_d in the nanomolar range) appears to be specific for P, whereas the low affinity one (K_d in the micromolar range) binds with equal affinity 11β-hydroxyprogesterone (11βOHP) and 17α-hydroxyprogesterone (17αOHP). A significant correlation exists among affinity constants (as determined by binding studies) and EC_{50} values for the effects of P, 11βOHP, and 17αOHP on intracellular Ca^{2+} in fura-2-loaded spermatozoa, strongly indicating the involvement of P-binding sites in the biological effect of the steroid. In particular, dose-response curves for P were biphasic, with an EC_{50} in the nanomolar range and another in the micromolar range. Conversely, curves for 11βOHP and 17αOHP were monophasic, with an EC_{50} just in the micromolar range. Ligand blot analysis of sperm total lysates performed with peroxidase-conjugated P revealed the presence of two binding proteins of 54 and 57 kDa that were specific for P. Indeed, peroxidase-conjugated P binding was blocked by the simultaneous presence of the unconjugated steroid. Using αc262 antibody, which is directed against the P-binding domain of genomic receptor, we detected two proteins of similar molecular mass (54 and 57 kDa), whereas using antibodies directed against the DNA-binding and N-terminal domains of the genomic P receptors, the two proteins were not detected. In addition, p54 and p57 appear to be mostly localized in sperm membranes and virtually absent in the cytoplasm. The involvement of these proteins in the biological effects of P is indicated by the strong inhibitory effect of αc262 on P-induced acrosome reaction of capacitated human spermatozoa. (*J Clin Endocrinol Metab* **83:** 877–885, 1998)

ACROSOME reaction (AR) is an essential event of the process of fertilization. In mammals, physiological AR occurs after interaction with the zona pellucida, the glycoprotein coat overlying the egg plasma membrane (1), and is mediated by specific sperm receptor (2). During the process of fertilization, AR can also be induced by progesterone (P), which is present in high concentrations in the cumulus matrix that surrounds the oocyte and in the follicular fluid (3, 4). In particular, in human spermatozoa, P induces a rapid influx of calcium, calcium-dependent phosphoinositide hydrolysis, an increase in tyrosine phosphorylation of sperm proteins, and chloride efflux, ultimately leading to stimulation of AR (for review, see Ref. 5). In addition, *in vitro* studies on mouse spermatozoa suggest that P exerts a priming effect on zona pellucida 3-induced AR (6). The effects of P are believed to be exerted by a nongenomic pathway (5), as also recently described for other steroids in different cell types (for review, see Ref. 7). A steroid effect is defined as nongenomic when it occurs between a few seconds and 1–2 min after its application and does not involve transcriptional processes. The possible involvement of a γ-aminobutyric $acid_A$ ($GABA_A$) receptor/Cl^- channel complex in the effects of P has been suggested (for review, see Ref. 8), leading to the hypothesis of the existence of at least two different receptors for P, one mediating the influx of calcium and the other mediating the efflux of chloride (9). However, although data obtained in mice (10) suggest that the $GABA_A$ receptor/Cl^- channel complex can account for both P-induced chloride and calcium fluxes, results obtained in human spermatozoa seem to exclude the involvement of $GABA_A$ receptor/Cl^- channel complex involvement in the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) caused by P (11–13), with the exception of a recent report (14).

Several findings suggest that the putative receptor(s) for P in spermatozoa is located on the membrane surface. BSA-conjugated P, which is unable to cross the plasma membrane, produces the same effects as P (15, 16). Moreover, immunohistochemical studies using fluorescein isothiocyanate (FITC)-labeled BSA-conjugated P (17, 18), peroxidase-conjugated P (13), and an antibody directed against the C-terminal domain of the genomic P receptor (19) localize P-binding sites on human sperm heads. Further, sperm P-binding sites are sensitive to treatment with trypsin and aggregate after binding with the agonist (20), suggesting similarities with other surface receptors. Despite all of these studies showing P binding to the surface of ejaculated spermatozoa, the mo-

Received August 8, 1997. Revision received December 1, 1997. Accepted December 8, 1997.

Address all correspondence and requests for reprints to: Elisabetta Baldi, Ph.D., Dipartimento di Fisiopatologia Clinica, Unità di Andrologia, Università di Firenze, Viale Pieraccini 6, I-50139 Florence, Italy. E-mail: e.baldi@mednuc2.dfc.unifi.it.

* This work was supported by grants from the Ministero della Ricerca Scientifica e Tecnologica and Regione Toscana.

lecular, pharmacological, and kinetic characteristics of this putative receptor(s) have not been clearly elucidated. Possible candidates as sperm P receptors include a 94- to 97-kDa protein that is tyrosine phosphorylated upon stimulation with the steroid (21–24) and $GABA_A$ receptor-like/Cl channels with apparent masses of 50 and 75 kDa (reviewed in Ref. 8). Using an antibody directed against the C-terminal binding domain of the P genomic receptor, Sauber *et al.* (19) recently identified a sperm protein with an apparent mass of 50–52 kDa as a possible nongenomic sperm P receptor.

In the present study, we used different approaches to identify and characterize nongenomic P receptors on human sperm surface. By using progesterone-11α-glucuronide-[^{125}I]iodotyramine as the labeled ligand, we identified two distinct P-binding sites on human sperm plasma membrane. By ligand and Western blot analysis we initiated the molecular characterization of P-binding proteins. We identified two proteins of 54 and 57 kDa molecular mass that specifically bind P. In addition, we reexamined the possible involvement of GABA receptors in the effect of P by evaluating the ability of this agonist to displace P binding as well as its effects on sperm $[Ca^{2+}]_i$.

Materials and Methods

Chemicals

Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). Human serum albumin-free human tubal fluid (HTF) was purchased from Irvine (Santa Ana, CA). Progesterone-11α-glucuronide-[^{125}I]iodotyramine (2666 Ci/mmol) and enhanced chemiluminescence Western blotting detection reagent (ECL) were obtained from Amersham (Aylesbury, UK). Mifepristone (RU486) was provided by Roussel-UCLAF (Romainville, France). Steroids, peroxidase-conjugated P (P-POD), antimouse IgG-POD, antirabbit IgG-POD, GABA, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents for SDS-PAGE were purchased from Bio-Rad Laboratories (Hercules, CA). Monoclonal αc262 antibody was obtained from StressGen (Victoria, Canada). Polyclonal anti-DNA-binding domain P receptor antibody was purchased from Dako (Milan, Italy). Monoclonal αPR anti-P receptor was obtained from Biogenex (San Ramon, CA). Ionomycin and fura-2/AM were obtained from Calbiochem (La Jolla, CA).

Preparation of spermatozoa

Human semen was collected, according to the WHO recommended procedure (25), 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 (11). Briefly, spermatozoa were separated on 40% and 80% Percoll gradients, combined, washed in HTF medium containing 0.3% fatty acid-free BSA, and finally resuspended in the same medium at the indicated concentration. Spermatozoa were capacitated for 2 h or otherwise indicated in 0.3% BSA-containing HTF.

Equilibrium binding studies

Binding studies were performed on human intact spermatozoa. After 2 h of capacitation, spermatozoa were washed in phosphate-buffered saline (PBS) and resuspended in 0.3% BSA-PBS. One hundred-microliter aliquots of spermatozoa (8×10^7/mL) were incubated for 1 h at 4 C with 77 pmol/L progesterone-11α-glucuronide-[^{125}I]iodotyramine in 0.3% BSA-PBS in the presence of increasing concentrations (1E-11 to 1E-3 mol/L) of unlabeled compounds: P, 17α-hydroxyprogesterone (17αOHP), 11β-hydroxyprogesterone (11βOHP), RU486, GABA, dihydrotestosterone (DHT), and Norgestrel (Sigma). After incubation, 1 mL iced Tris (50 mmol/L) was added to each tube. Samples were centrifuged at 3000 rpm for 10 min at 4 C, and radioactivity associated with the obtained pellets was measured in a γ-counter at 70% efficiency. In some experiments, pellets were resuspended in 1 mL glycine acid buffer (pH 2), incubated for 10 min on ice, and then centrifuged, and the obtained pellets and supernatants were counted in a γ-counter to determine the loss of radioactivity after the acid wash.

Measurement of $[Ca^{2+}]_i$

Spermatozoa, prepared as described above, were loaded with 2 μmol/L fura-2/AM for 45 min at 37 C, washed, and 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 sodium lactate, 2.5 mmol/L sodium pyruvate, 2 mmol/L HEPES, and 0.3% BSA, pH 7.5), and $[Ca^{2+}]_i$ before and after stimulation with the different agonists was measured as described previously using the spectrofluorometric method (11). Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (F_{max}) with ionomycin (8 μmol/L, final concentration) followed by minimal fluorescence (F_{min}) with 10 mmol/L ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 810. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz (26), assuming a dissociation constant of fura-2 for calcium of 224 nmol/L.

Preparation of uterine lysate

Human uterine samples in the proliferative phase of the menstrual cycle obtained at surgery were minced with sharp scissors, suspended in lysis buffer (20 mmol/L Tris, pH 7.4; 150 mmol/L NaCl; 0.25% Nonidet P-40; 1 mmol/L Na_3VO_4; and 1 mmol/L phenylmethylsulfonylfluoride), and homogenized (Teflon-glass). The homogenates were centrifuged at 1500 rpm for 10 min at 4 C, and supernatants corresponding to total lysates were subjected to protein measurement.

Sperm membrane preparation

Spermatozoa stored in liquid nitrogen were lysed in lysis buffer for 1 h on ice. Then, the samples were subjected to two subsequent cycles of homogenizing (Teflon-glass) and sonicating (three times, 15-s 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. The supernatants (cytosolic fractions) were dried in a speed vacuum and resuspended in lysis buffer. Proteins were measured in cell lysates using a Bio-Rad kit (Bio-Rad, Hercules, CA).

SDS-PAGE

After 5-h capacitation in 0.3% BSA-containing HTF, sperm samples were processed for SDS-PAGE as previously described (22, 27). Briefly, they were centrifuged at $400 \times g$ at 4 C for 10 min, washed twice in PBS, and resuspended in 10 μL lysis buffer. After measurement of proteins, the sperm extracts, containing approximately 30–50 μg proteins, were diluted in equal volume of 2-fold concentrated Laemmli sample buffer, vortexed, incubated at 95 C for 5 min, and then loaded onto 10% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Sigma Chemical Co.).

Ligand blot analysis

The nitrocellulose was incubated for 30 min in 3% Nonidet P-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 finally overnight in 0.3% BSA/0.1% Tween-20/PBS containing P-POD (0.5 μmol/L) in the presence or absence of a high concentration of free P (10 μmol/L). After several washes in 0.1% Tween-20/PBS, reacted proteins were simultaneously revealed by an ECL system (Amersham). Particular attention was paid in these experiments to treat the samples (with or without free P) simultaneously and for exactly the same incubation times.

Western blot analysis

Transferred nitrocellulose was blocked for 3 h at room temperature in TTBS (0.1 Tween-20, 20 mmol/L Tris, and 150 mmol/L NaCl) con-

taining 5% BSA, then washed repeatedly in TTBS and incubated for 2 h in 2% BSA-TTBS containing αc262 (1:400). After washing, nitrocellulose were incubated with goat antimouse IgG-POD (1:5000 in 2% BSA-TTBS). In other experiments, different primary antibodies were used (αPR and αDNAbd-PR, 1:100 dilution). After several washes in TTBS, reacted proteins were revealed by the ECL system (Amersham).

AR assay

Acrosome-reacted spermatozoa were evaluated using the fluorescent probe FITC-labeled *Arachis hypogea* (peanut) lectin according to the method of Aitken (28) as previously described (29). Briefly, after 2-h capacitation, spermatozoa (10^6/mL) were preincubated for 30 min with αc262 antibody or αPR (1:100) and then stimulated with P (10 μmol/L), A23187 (10 μmol/L), or the appropriate control solvent (dimethylsulfoxide) for 2 h at 37 C. After staining with fluorescent lectin, fluorescence was observed under a fluorescent microscope (type 307–148.002, Leitz, Wetzlar, Germany), and the AR was evaluated on a total of 100 spermatozoa/slide. Using the method of Aitken (28), only curly-tailed spermatozoa were considered viable and thus scored.

Analysis of experimental results

The binding data were evaluated quantitatively with nonlinear least squares curve fitting using the computer program Ligand (30). The program provides objective measures of goodness of fit in terms of both magnitude and randomness of residuals. The computer program Allfit (31) was used for the analysis of sigmoidal dose-response curves obtained in binding and calcium studies. AR data were analyzed by Student's *t* test and one-way ANOVA after data transformation in arcsins of the root square. Each data point represents the mean ± SEM.

Results

Competitive equilibrium binding studies in human spermatozoa

To identify and pharmacologically characterize P-binding sites on human spermatozoa, we performed binding studies in pooled capacitated human spermatozoa using progesterone-11α-glucuronide-[^{125}I]iodotyramine as tracer (Fig. 1, *inset*). Preliminary time-course experiments indicated that progesterone-11α-glucuronide-[^{125}I]iodotyramine binding was time dependent and reached apparent equilibrium within 60 min at 4 C (Fig. 1). Accordingly, all subsequent binding experiments were conducted using these experimental conditions. The steric compliance and the hydrophilic properties of the substitution group should prevent 11α-glucuronide-[^{125}I]iodotyramine from crossing the plasmalemma, as indicated by the dramatic decrease in radioactivity (73.96 ± 0.74%; n = 10) after acid washing of samples. To exclude the possibility that binding of progesterone-11α-glucuronide-[^{125}I]iodotyramine to BSA could be responsible for the observed binding, these experiments were also conducted in the absence of BSA with results similar to those obtained in its presence (results not shown).

Figure 2 shows a typical Scatchard plot for progesterone-11α-glucuronide-[^{125}I]iodotyramine binding to human spermatozoa. The curvilinear relationship indicates heterogeneity of P binding in human spermatozoa. To further characterize P-binding sites, we performed 10 experiments of competition curves for progesterone-11α-glucuronide-[^{125}I]iodotyramine binding using increasing concentrations of P, 17αOHP, and 11βOHP as competitors for the radioactive tracer. Competition experiments were conducted in sperm derived from different subjects or in pooled sperm from different donors (n = 10). Simultaneous mathematical analysis of experimental results strongly indicated the presence of at least two distinct classes of sites. The introduction of a second independent class of sites dramatically improved the fitting of experimental results ($P < 0.0001$). A typical family of competition curves is shown in Fig. 3. Affinity constants and concentrations of receptors are reported in Table 1. We found a high affinity, low capacity binding site that is apparently selective for P and a low affinity, high capacity site that binds with virtually equal affinity all three progestins. Note that GABA, DHT, and Norgestrel did not displace binding even at micromolar concentrations. To exclude the possibility that displacement of radioactive tracer by P might be due to induction of the AR and, thus, loss of acrosome in a high percentage of cells, we evaluated the AR in spermatozoa treated with P (1 nmol/L, 0.1 μmol/L, and

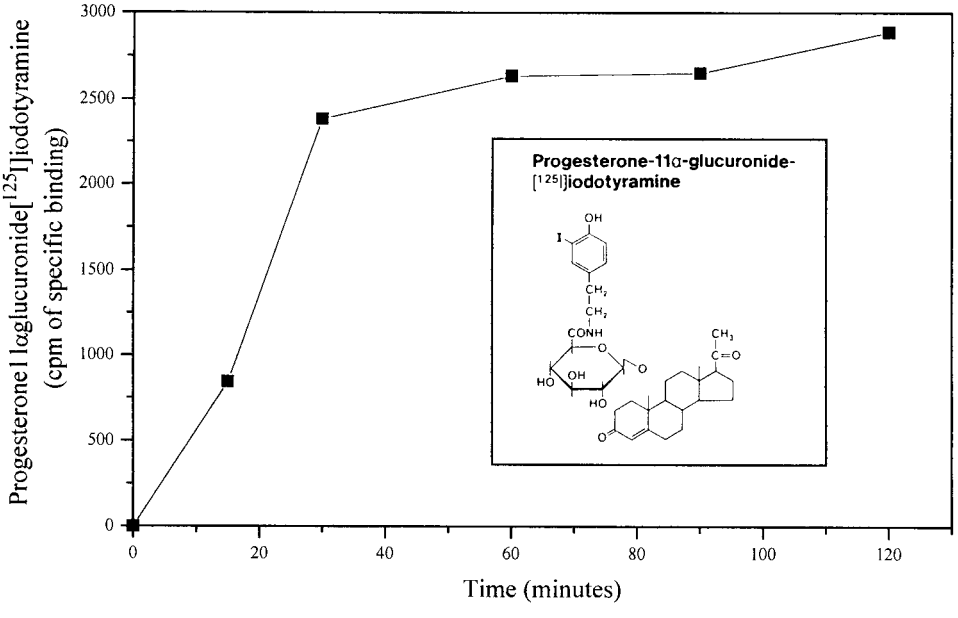

FIG. 1. Time course of progesterone-11α-glucuronide-[^{125}I]iodotyramine binding to human capacitated spermatozoa. Intact spermatozoa (8×10^6) were incubated at 4 C in the presence of 77 pmol/L progesterone-11α-glucuronide-[^{125}I]iodotyramine for the indicated times. Specific binding was determined by subtracting nonspecific (obtained with 10 μmol/L P) from total binding. In the *inset*, the molecular structure of progesterone-11α-glucuronide-[^{125}I]iodotyramine is shown.

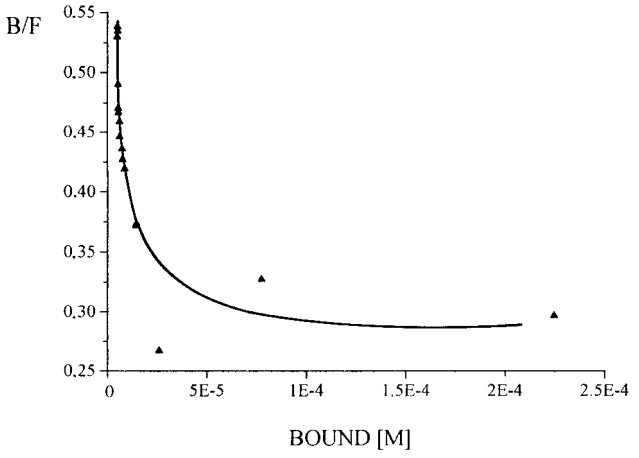

FIG. 2. Typical Scatchard plot analysis of progesterone-11α-glucuronide-[^{125}I]iodotyramine binding to human spermatozoa. The curvilinear plot was derived from the predicted relationship for the two-site model for P binding, as in Table 1. *Ordinate*, bound to free ratio (B/F); *abscissa*, concentration of bound ligand.

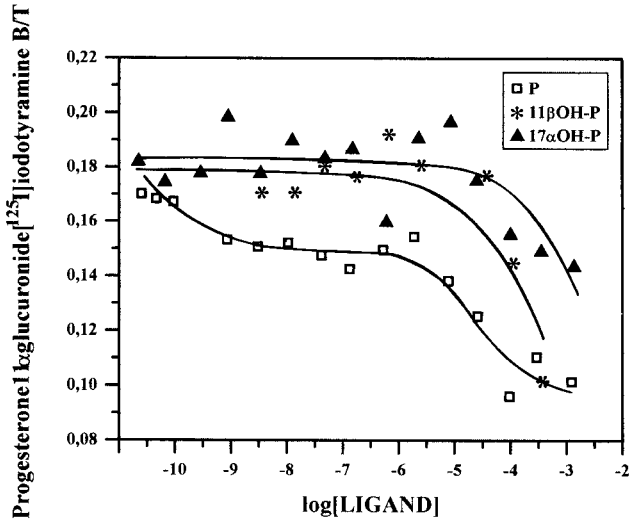

FIG. 3. Representative binding experiment showing heterologous displacement curves of progesterone-11α-glucuronide-[^{125}I]iodotyramine using P, 11βOHP, and 17αOHP as competitors. *Ordinate*, bound to total ratio (B/T); *abscissa*, concentration of ligands (molar). Smooth curves show the predicted relationships for the two-site model shown in Table 1.

10 μmol/L) for 60 min on ice (same experimental conditions as those used for binding experiments). We found that in these conditions, P was unable to stimulate the AR (mean ± SEM acrosome-reacted sperm, 15.5 ± 2.0 in controls, 13.2 ± 2.7 with 1 nmol/L P, 12.5 ± 3.5 with 0.1 μmol/L P, and 17.5 ± 4.4 with 10 μmol/L P; n = 2). Similar results were obtained by Tesarik and Mendoza (32) using FITC-BSA-conjugated P.

Effects of different steroids and other agonists on $[Ca^{2+}]_i$ in human spermatozoa

To investigate the biological function of the two binding sites identified with equilibrium binding studies, we evaluated the effects of increasing concentrations of P, 17αOHP, and 11βOHP on $[Ca^{2+}]_i$ of fura-2-loaded spermatozoa. Figure 4 reports the percent increase in $[Ca^{2+}]_i$ in response to increasing concentrations of P, 17αOHP, and 11βOHP, as generated by the computer program Allfit after simultaneous analysis of the different curves. The dose-response curve for P was clearly biphasic, with a first component in the nanomolar range (EC_{50} = 55.6 ± 15.4 nmol/L; n = 12) and a second component in the micromolar range (EC_{50} = 40.1 ± 20.1 μmol/L; n = 12). On the contrary, dose-response curves for 17αOHP and 11βOHP were monophasic, with EC_{50} values, respectively, of 53.4 ± 41.4 μmol/L (n = 7) and 5.8 ± 4.4 μmol/L (n = 8). A significant correlation was observed between pK and logEC_{50} (r = 0.946; P = 0.05). Interestingly, at low concentrations (10–50 nmol/L), the intracellular calcium increase in response to P was significantly higher ($P < 0.05$) than that of the other steroids, whereas at micromolar concentrations, the three progestins induced similar responses, as previously reported (11, 33). The addition of different concentrations of solvent control (0.01–1% dimethylsulfox-

TABLE 1. Concentration (B_{max} = sites/cell) and affinity constant (K_d) of the two predicted binding sites (R_1 and R_2) for the indicated ligands

	R_1	R_2
B_{max}	274 ± 87	39 ± 12 × 10^6
Agonists	K_d (nmol/L)	K_d (μmol/L)
Progesterone	0.58 ± 0.42	26 ± 4
11βOH-P	ND	81 ± 40
17αOH-P	ND	273 ± 70
RU486	ND	7.6 ± 2.8
GABA	ND	>10,000
DHT	ND	>10,000
Norgestrel	ND	>10,000

Values ± SEM were derived from computer modeling using the program Ligand (30) of 10 families of competitive curves for progesterone-11α-glucuronide-[^{125}I]iodotyramine. ND, Not detectable.

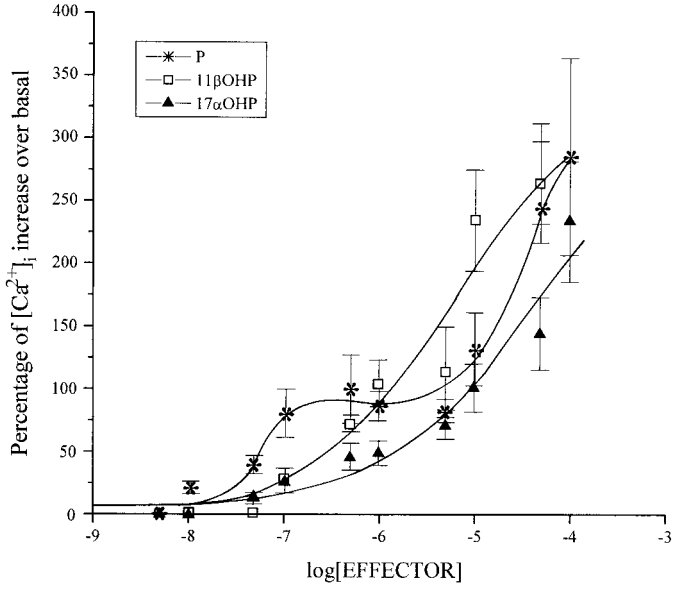

FIG. 4. Dose-response curves of the effects of different steroids on intracellular calcium levels in fura-2-loaded human spermatozoa. The percentage of the $[Ca^{2+}]_i$ increase is reported on the *ordinate*. Each *point* represents the mean ± SEM of at least four different experiments. The curves were generated by the program Allfit after simultaneous analysis of the different dose-response curves.

ide) did not modify sperm $[Ca^{2+}]_i$ (not shown). In Table 2, the percent stimulation of $[Ca^{2+}]_i$ *vs.* basal in response to several agonists at two different concentrations is reported. At the concentration of 50 nmol/L, only P induced a significant response, whereas at 50 μmol/L P, 11βOHP and 17αOHP stimulated a similar influx of calcium. The other tested molecules did not stimulate an appreciable increase in sperm $[Ca^{2+}]_i$ (Table 2).

In somatic cells, agonists whose biological effects are mediated by membrane receptors are subjected to a phenomenon termed desensitization or down-regulation (34). We tested the possibility that this also occurs in human spermatozoa stimulated with progestins by measuring the effect of two repeated administrations of steroid on $[Ca^{2+}]_i$. As shown in Fig. 5, a first dose of 50 nmol/L P induced an increase in $[Ca^{2+}]_i$ that could not be reproduced by a subsequent equimolar administration of the same agonist (Fig. 5a), indicating the occurrence of homologous desensitization (34). No response and no modification of the response to subsequent administration of P were observed with 50 nmol/L 11βOHP or 17αOHP (not shown). At the concentration of 50 μmol/L, homologous and heterologous (34) desensitization occurred with all three steroids. At this concentration, a first administration of P (Fig. 5b) or 11βOHP (Fig. 5c) induced similar intracellular calcium transients (see also Table 2), which was not followed by a response to a subsequent equimolar dose of P (compared with the response to P in the same subject; Fig. 5d). Similar results were obtained with 17αOHP (not shown).

As stated above, controversial results have been obtained concerning the possible involvement of $GABA_A$ receptors in calcium transients stimulated by P (10–14). We have reexamined this issue by studying the direct effects of GABA on basal and P-stimulated $[Ca^{2+}]_i$ as well as on AR. As shown in Fig. 6, GABA did not stimulate an increase in $[Ca^{2+}]_i$ at either low (50 nmol/L; Fig. 6a) or high (50 μmol/L; Fig. 6c) concentrations. Moreover, GABA did not affect calcium transient in response to a subsequent equimolar administration of P at both low (Fig. 6, a *vs.* b) and high (Fig. 6, c *vs.* d) concentrations compared to the respective control values. In addition, in our hands, GABA at concentrations ranging from 50 nmol/L to 50 μmol/L did not induce acrosome reaction of capacitated human spermatozoa or modify the response to P (percent AR; mean ± SEM of three experiments; control, 8 ± 1.1; 10 μmol/L P, 14 ± 1.1; 50 nmol/L GABA, 8 ± 1.0; 500 nmol/L GABA, 8 ± 1.0; 5 μmol/L GABA, 7.5 ± 0.5; 50 μmol/L GABA, 8.6 ± 0.3; 50 nmol/L GABA plus 10 μmol/L P, 16 ± 4; 50 μmol/L GABA plus 10 μmol/L P, 14.6 ± 2.7).

TABLE 2. Effects of the indicated ligands on intracellular calcium levels in fura-2-loaded human spermatozoa

	50 nmol/L (% stimulation)	50 μmol/L (% stimulation)
Progesterone	41.2 ± 7.4 (11)	252.3 ± 33.1 (6)
11βOH-P	2.2 ± 1.4 (4)	271.1 ± 50.2 (7)
17αOH-P	14.6.0 ± 5.6 (6)	151.0 ± 28.6 (4)
DHT	0 (2)	11.6 ± 1.6 (3)
Testosterone	0 (2)	0 (2)
GABA	0 (3)	0 (3)

Percentages of $[Ca^{2+}]_i$ increase *vs.* basal for two doses (50 nmol/L and 50 μmol/L) of the ligands are reported. Data represent the mean ± SEM of the number of experiments indicated in *parentheses*.

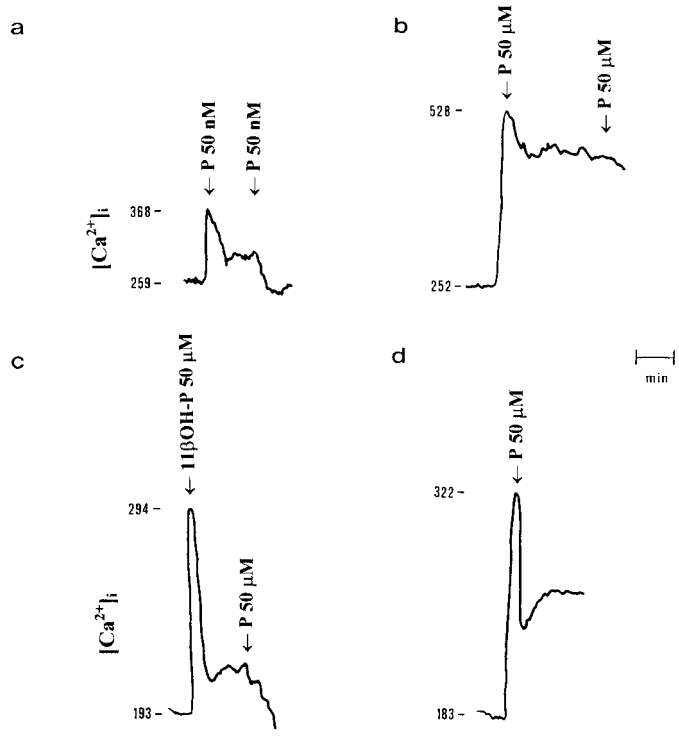

FIG. 5. Calcium waves in response to two repeated administrations of progestins in fura-2-loaded spermatozoa. The $[Ca^{2+}]_i$ transient induced by 50 nmol/L (a) and 50 μmol/L (b) P and the absence of response after a second administration of an equimolar dose of the same steroid are shown. c, 11βOHP (50 μmol/L) induces an $[Ca^{2+}]_i$ transient comparable to that of P in the same subject (d) and completely blocks the calcium response to a subsequent equimolar dose of P.

Identification of P-binding proteins on human spermatozoa

To identify the molecular mass of P-binding proteins in capacitated human spermatozoa, we performed ligand blot experiments using P-POD (0.5 μmol/L) as probe. This molecule has been shown to be effective in inducing AR in human spermatozoa (13) and thus is an interesting tool to perform this type of experiment. As shown in Fig. 7A, P-POD identifies two protein bands on total sperm lysate (*right panel*) of 54 and 57 kDa, which totally disappear when P-POD is added in the presence of a high concentration (10 μmol/L) of free P (*left panel*), suggesting that these proteins bind P in a specific manner. These results have been consistently reproduced in six different sperm samples.

We also performed Western blot experiments using the monoclonal antibody αc262, which is directed against the C-terminal tail of genomic P receptor (35). Recent data demonstrated that this antibody is able to counteract the effect of P in human spermatozoa and stains some protein bands in sperm lysates (19). As shown in Fig. 7B (*left panel*), Western blot immunostaining of sperm lysates with αc262 identified two protein bands of similar molecular mass (57 and 54 kDa) as those revealed by ligand blot with P-POD (Fig. 7A) and an additional band at 66 kDa. In uterine lysates, used as a control

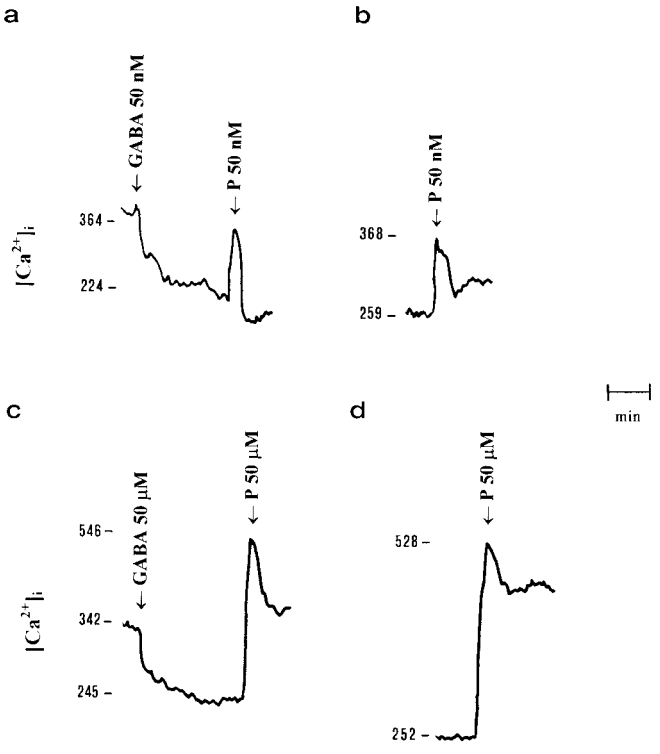

FIG. 6. Effects of GABA on sperm $[Ca^{2+}]_i$ and on the calcium response to P. Both low (50 nmol/L; a) and high (50 μmol/L; c) concentrations of GABA induce a slight decrease in basal $[Ca^{2+}]_i$ without affecting the calcium response to a subsequent equimolar dose of P (a, 50 nmol/L; c, 50 μmol/L) compared to the relative control value in the same subjects (b, 50 nmol/L; d, 50 μmol/L).

for P genomic receptor, the antibody detected several bands. Among these, two bands of about 100–110 and 60 kDa, which correspond to known isoforms of genomic P receptor (35, 36), are not present in spermatozoa, confirming the absence of genomic P receptors in these cells (37).

The same sperm and uterine total lysates as those in Fig. 7B (*left panel*) were stained with antibodies directed against different regions of the genomic P receptor, respectively the N-terminal (αPR) and the DNA-binding domains of the genomic receptor. Interestingly, αPR detected only the 66-kDa band (Fig. 7B, *right panel*), whereas no protein bands were revealed by the antibody directed against the DNA-binding domain (data not shown), indicating that the putative 57- and 54-kDa P-binding proteins do not show homology with the classical nuclear receptor family at the N-terminal and DNA-binding sequences. To exclude the possibility that the 66-kDa protein band detected with α-c262 and αPR is due to the residual presence of BSA, we performed Western blot analysis of BSA standard with the two antibodies. In this case, no protein bands were detected (not shown), indicating that the 66-kDa band revealed in sperm lysates is indeed a sperm protein.

The possible sperm surface localization of 54- and 57-kDa P-binding proteins was demonstrated by Western blot analysis with αc262 of purified sperm membrane and cytosol shown in Fig. 7C. The monoclonal antibody detected the same three bands of 66, 57, and 54 kDa as in total lysate (Fig. 7B, *left panel*), whereas in the cytosolic fraction, only the 66-kDa band was observed.

In Table 3, we summarize the results of Western and ligand blot experiments on sperm P-binding proteins (six experiments for ligand and four experiments for Western blot). We believe that only the 54- and 57-kDa proteins are specific P-binding proteins, as only these two proteins were detected by both αc262 and P-POD and disappeared when P-POD was used in the presence of a high concentration of free P (Table 3). Moreover, our results confirm that genomic P receptors are not present in spermatozoa (37), as no protein bands are detected by the antibody directed against the DNA-binding domain of genomic receptor.

We also tried to perform immunoprecipitation of P receptors in sperm lysates with αc262 antibody, but the heavy chain of the antibody (~60 kDa) used to immunoprecipitate masks the proteins of interest as they comigrate at the same molecular mass (data not shown).

Effects of αc262 and αPR antibodies on AR

As P is a well known stimulus for acrosome reaction (5), we investigated whether αc262 antibody affected this effect. Human capacitated spermatozoa were stimulated with P (10 μmol/L) in the presence or absence of monoclonal antibody αc262 or αPR antibody (1:100 dilution). As shown in Table 4, αc262 antibody did not alter the spontaneous AR, whereas it totally blunted the effect of P. No effects of the antibody were observed on A23187-induced AR (data not shown). These results strongly indicate that the membrane p54 and p57 proteins revealed by αc262 are involved in transducing P biological effects in human spermatozoa. Conversely, the αPR antibody, which did not detect the 57- and 54-kDa protein bands, but only the 66-kDa one, in Western blot analysis of sperm lysates, did not inhibit the P-stimulated AR (Table 4), suggesting that the 66-kDa band is not involved in mediating P activity.

Discussion

The present study demonstrates the presence of distinct membrane surface receptors for P in capacitated human spermatozoa. This result is supported by data obtained using three different experimental approaches: binding on intact spermatozoa, ligand blot, and Western blot analysis in sperm lysates. The biological functions of these receptors in human spermatozoa were established by measuring intracellular calcium fluxes in response to different concentrations of P and other agonists.

Computerized analysis of binding displacement curves with the program Ligand strongly indicates the presence of two distinct P-binding sites on human spermatozoa. The high affinity, low capacity site is displaced, among the different molecules used in our experiments, only by P, whereas the low affinity, high capacity site is displaced by P, 11βOHP, 17αOHP, and RU486. Both binding sites appear to be relevant for P-induced calcium fluxes in human spermatozoa. Indeed, we found that the dose-response curve of P-induced $[Ca^{2+}]_i$ increase was characterized by two distinct components, with EC_{50} values, respectively, in the nanomolar and in the micromolar ranges, whereas dose-response curves for

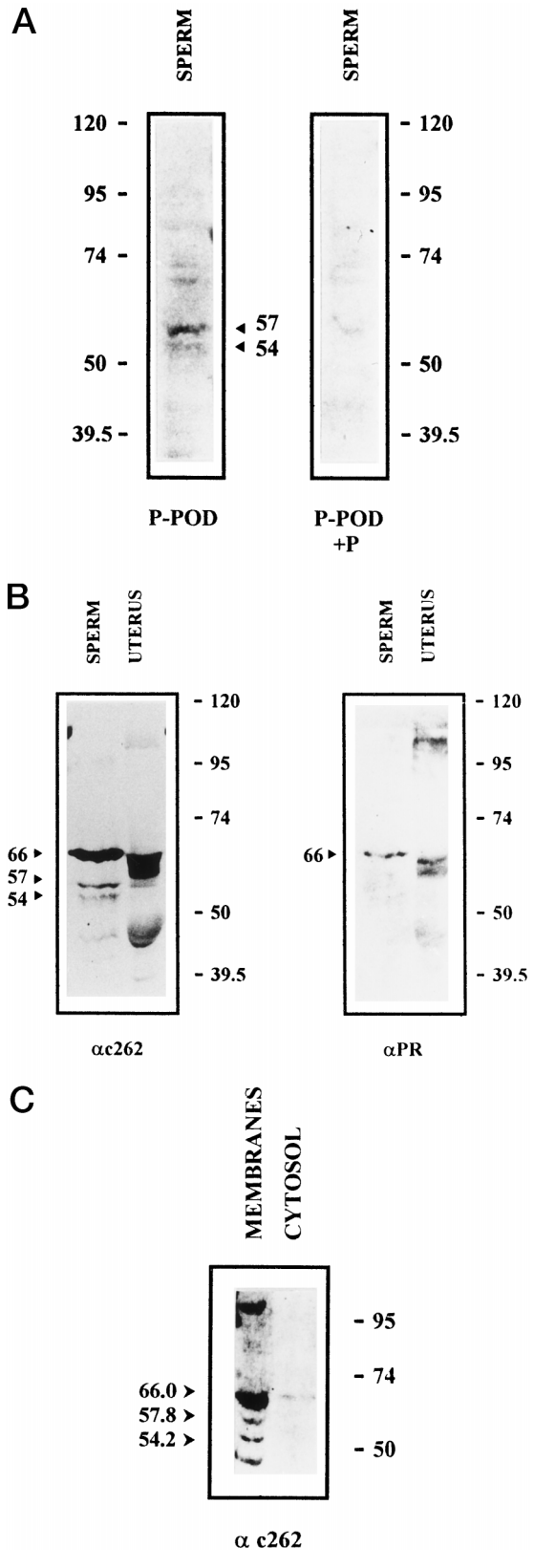

FIG. 7. A, Ligand blot analysis of whole human sperm lysates using P-POD to reveal P-binding proteins. Sperm proteins (50 μg/lane) separated by reducing 10% SDS-PAGE were blotted with P-POD (0.5 μmol/L) in the absence (*left panel*) or presence (*right panel*) of an excess of free P (10 μmol/L). On sperm lysates, P-POD reveals two protein bands (*arrowheads*) of 57 and 54 kDa (*left panel*) that disappear in the presence of free P (*right panel*). Similar results were obtained in four different experiments. Molecular mass markers are indicated on both sides of the figure. B, Western blot analysis of whole human sperm and uterine lysates with antibodies directed against different domains of the genomic P receptor. Sperm and uterine proteins (50 μg/lane) separated by reducing 10% SDS-PAGE were revealed by αc262 (1:400 dilution; *left panel*) and αPR (1:100 dilution; *right panel*). On sperm lysates, αc262 detects three protein bands of 66, 57, and 54 kDa (*left panel*), whereas αPR reveals the 66-kDa band only (*right panel*). On uterine lysates, both antibodies reveal a similar pattern of proteins. Similar results were obtained in four different experiments. Molecular mass markers are indicated to the *right* of both panels. C, Western blot analysis of human sperm membrane and cytosolic lysates with αc262. Total, membrane, and cytosolic proteins (30 μg), obtained as described in *Materials and Methods*, were separated by reducing 10% SDS-PAGE and revealed by αc262 (1:400 dilution). In particulate membranes, three proteins of 66, 57, and 54 kDa apparent molecular mass were detected, whereas in the cytosolic fraction only the 66-kDa band was detected. Molecular mass markers are indicated to the *right* of figure.

11βOHP and 17αOHP were characterized by a single component with an EC_{50} in the micromolar range. Most relevant, EC_{50} values for $[Ca^{2+}]_i$ dose-response curves and binding affinity constants for the three progestins were correlated, indicating that the two surface receptors identified with equilibrium binding studies are involved in P-induced calcium influx. As P is present at micromolar levels in the cumulus oophorus surrounding the oocyte (3), it can virtually bind both the high and low affinity receptors present on spermatozoa; hence, it can be hypothesized that both sites are physiologically involved in the action of the steroid in human spermatozoa. Moreover, the ability of other molecules sharing a progestin structure, such as 17αOHP and 11βOHP, to displace the low affinity site suggests that 17αOHP, which is also present in high concentrations in follicular fluid and cumulus matrix (3), might be physiologically relevant during the process of fertilization. The low affinity site is also displaced by RU486, a potent antagonist of the genomic P receptor. This finding is consistent with its reported inhibition of P biological effects in human spermatozoa when used at micromolar doses (38). In a previous report, Neulen *et al.* (39), using [^{3}H]P as ligand, found a single site for P with a K_d of about 10 nmol/L in human seminal plasma and spermatozoa. However, a comparison of these results with the present data is difficult, because these researchers used as tracer [^{3}H]P, which can easily cross the plasma membrane, and performed most of the experiments in seminal plasma (39).

Ligand blot analysis of sperm lysates using P-POD identified two protein bands of about 57 and 54 kDa that were displaced in the presence of unconjugated P. Two proteins with similar molecular masses were also identified by Western blot analysis of sperm proteins with an antibody directed against the C-terminal domain of the P genomic receptor (α-c262). At present, we do not have any evidence that these two proteins are related to the two receptors evidentiated with equilibrium binding studies. On the other hand, the inhibitory effect of the α-c262 antibody of P-induced AR (Ref. 19 and the present study) and the increase in $[Ca^{2+}]_i$ (19) strongly suggests that the proteins detected with this antibody are involved in the biological effects of P. P-POD has been used as a probe in ligand blot analysis of

TABLE 3. Protein bands detected by Western blot and ligand blot analysis on human sperm lysates

kDa + SEM	P-POD	P + P-POD	αc262 total lysate	αc262 membrane	αc262 cytosol	αPR	αDNA bd-PR
54.2 ± 0.4	+	−	+	+	−	−	−
57.8 ± 0.3	+	−	+	+	−	−	−
66.0 ± 0.05	−	−	+	+	+	+	−

In Western analysis, antibodies directed against different domains of progesterone genomic receptor, respectively against C-terminal progesterone-binding sequence (αc262), N-terminal sequence (αPR), and DNA-binding sequence (αDNAbd-PR), were used. In ligand blot analysis, P-POD in the presence or absence of free progesterone (P) was used as probe. The mol wt of protein bands are the mean ± SEM of at least four experiments for each tool used.

TABLE 4. Effect of αc262 antibody on basal (C) and progesterone-stimulated (P) acrosome reaction in human spermatozoa

% Acrosome Reaction					
C (n = 6)	P (n = 6)	αc262 (n = 4)	P + αc262 (n = 4)	αPR (n = 2)	P + αPR (n = 2)
5.16 ± 1.04	12.5 ± 0.76[a]	4.75 ± 2.06	4.50 ± 1.29	7.00 ± 1.41	13.00 ± 0.00[b]

C, Control; P, progesterone.
[a] $P < 0.001$ *vs.* C.
[b] $P < 0.05$ *vs.* αPR.
Capacitated spermatozoa were treated for 2 h with progesterone (10 μmol/L) in the presence or absence of αc262 antibody (against C-terminal progesterone-binding sequence) or αPR (against DNA-binding sequence; both at the final dilution of 1:100) and then processed as described in *Materials and Methods*. Values are the mean ± SEM percentage of acrosome reaction in four different subjects.

sperm proteins by Benoff *et al.* (18), who identified two proteins of 70 and 58 kDa present on sperm crude membranes. It must be also mentioned that in Western blot analysis of sperm proteins with α-c262 antibody, Sauber *et al.* (19) detected two major proteins with molecular masses of 50–52 and 46–48 kDa and additional bands at lower molecular masses. The discrepancies in apparent molecular mass of the two P-binding proteins identified in these studies may be due to covalent modifications (glycosylation, phosphorylation) of the proteins that could be altered by the different methods of protein preparation. Among proteins capable of binding P, cortisol-binding globulin (40) and albumin (41) show molecular masses similar to those of p54 and p57. Using pure BSA as a positive control, we excluded αc262 cross-reaction with albumin, confirming data reported by Benoff *et al.* (18). In addition, Sauber *et al.* (19) did not detect any immunoreactivity with antibody against cortisol-binding globulin in washed spermatozoa. In our study we detected an additional protein band at 66 kDa, which is also present in Western blot performed with an antibody directed against a different domain of the P genomic receptor (αPR). However, the lack of effect of this antibody on P-stimulated AR suggests that this band is not specific and is not involved in the biological effect of the steroid. Using proteins lysates from partially purified sperm membranes, we demonstrated that the two binding proteins of 57 and 54 kDa are selectively localized in the membranes and absent in the cytosol, in agreement with immunofluorescence studies demonstrating surface localization of the staining with α-c262 (19). Recently, high affinity binding sites for P have been characterized and purified in porcine liver membranes (42). Interestingly, binding studies using [^{3}H]P as ligand identified two binding sites for P with apparent K_d values of 11 and 286 nmol/L, while purification of P-binding proteins revealed the presence of two proteins of 56 and 28 kDa (42). From these studies it appears that nongenomic P receptors show profound structural and functional differences compared to the genomic receptor family. In keeping with this conclusion, spermatozoa from transgenic mice lacking the nuclear genomic P receptor show normal surface P receptors (43), suggesting the presence of two different genes encoding for genomic and nongenomic receptors for the steroid.

As stated above, the possible involvement of GABA receptors in the mechanism of action of P in mammalian sperm is still a matter of discussion, as both positive (9, 10, 14) and negative (11–13) results have been reported. Binding sites for GABA have been shown in spermatozoa (9, 44). However, competitive displacement of [^{3}H]GABA tracer by nipecotic acid, a blocker of GABA transport protein, suggests the presence of GABA transport proteins more than true GABA receptors (44). [^{3}H]GABA transport has been demonstrated in human spermatozoa (45). By immunofluorescence studies, a $GABA_A$ receptor/Cl^- channel complex has been localized on the surface of the sperm head at the equatorial segment (9). It has been proposed that the effects of P may be mediated by such a channel on the surface of human sperm (8). However, despite different results obtained in mice (10), GABA has been reported to only weakly stimulate the AR in human spermatozoa (9) and, in contrast to the effects of P, to be devoid of stimulatory effects on sperm-oocyte fusion (13). At least in the mouse, the effect of GABA appears to be mediated by the influx of calcium (10). We were unable to demonstrate any effect of GABA on AR in either the absence or presence of P. Moreover, our data confirm that GABA does not stimulate an influx of calcium in human spermatozoa or modify P-induced calcium influx, as reported previously (11–13). In addition, competitive binding studies demonstrate no interaction of GABA with either the high or the low affinity P-binding sites. Taken together, our results seem to exclude the involvement of GABA receptors in calcium signaling stimulated by P in human spermatozoa. In a recent report, Meizel *et al.* (14) demonstrated, by image analysis of single sperm cells loaded with fura-2, that the effect of P could be partially reverted by picrotoxin, suggesting the involvement of $GABA_a$ receptors. However, direct effects of GABA on calcium influx were not investigated in this study (14), and using an identical experimental approach, Aitken *et al.* (13) did not demonstrate any effect of GABA on sperm calcium.

As the P-induced AR clearly requires chloride efflux and activation of $GABA_A$ receptor/Cl^- channel complex (8), it is possible that P receptors and $GABA_A$ receptor/Cl^- channel complex are distinct and cooperate to induce the AR.

In conclusion, our results demonstrated the presence of two functional membrane P receptors in human spermatozoa. The p54 and p57 P-binding molecules detected in our study may represent novel nongenomic P receptors on human sperm membrane that can mediate the steroid effects. Isolation, sequencing, and further functional characterization of these two protein candidates of nongenomic P receptors are presently under investigation in our laboratory. As we have recently shown that a correlation exists between sperm responsiveness to P and sperm fertilizing ability (29, 46), identification and characterization of nongenomic P receptor(s) may be clinically relevant not only in future research in male infertility, but also in the development of new contraceptive tools.

Acknowledgments

We thank Prof. Mario Serio for critical reading of manuscript. We also thank Dr. Guido Fantoni (Department of Anatomy, University of Florence, Florence, Italy) for helping us with the binding experiments. We are grateful to Dr. E. Paglierani for providing the antibody against genomic P receptor.

References

1. **Yanagimachi R.** 1994 Mammalian fertilization. In: Knobil E, Neil JD, eds. The physiology of reproduction, 2nd ed. New York: Raven Press; 189–317.
2. **Snell WJ, White JM.** 1996 The molecules of mammalian fertilization. Cell. 85:629–637.
3. **Osman RA, Andria ML, Jones AD, Meizel S.** 1989 Steroid induced exocytosis: the human sperm acrosome reaction. Biochem Biophys Res Commun. 160:828–833.
4. **Thomas P, Meizel S.** 1989 Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon calcium influx. Biochem J. 264:539–546.
5. **Baldi E, Krausz C, Forti G.** 1995 Nongenomic actions of progesterone on human spermatozoa. Trends Endocrinol Metab. 6:198–205.
6. **Roldan ERS, Murase T, Shi Q-X.** 1994 Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science. 266:1578–1581.
7. **Wehling M.** 1994 Nongenomic actions of steroid hormones. Trends Endocrinol Metab. 5:347–353.
8. **Meizel S.** 1997 Amino acid neurotrasmitter receptor/chloride channels of mammalian sperm and the acrosome reaction. Biol Reprod. 56:569–574.
9. **Wistrom CA, Meizel S.** 1993 Involvement of a human sperm putative GABAa receptor/Cl channel complex in the progesterone-initiated acrosome reaction. Dev Biol. 159:679–690.
10. **Shi Q-X, Roldan ERS.** 1995 Evidence that a GABAa-like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Biol Reprod. 52:373–381.
11. **Baldi E, Casano R, Falsetti C, Krausz CS, Maggi M, Forti G.** 1991 Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl. 12:323–330.
12. **Blackmore PF, Im WB, Bleasdale JE.** 1994 The cell surface progesterone receptor which stimulates calcium influx in human sperm is unlike the A ring reduced steroid site on the $GABA_A$ receptor/chloride channel. Mol Cell Endocrinol. 104:237–243.
13. **Aitken RJ, Buckingham DW, Irvine DS.** 1996 The extragenomic action of progesterone on human spermatozoa: evidence for ubiquitous response that is rapidly down-regulated. Endocrinology. 137:3999–4009.
14. **Meizel S, Turner KO, Nuccitelli R.** 1997 Progesterone triggers a wave of increased free calcium during the human sperm acrosome reaction. Dev Biol. 182:67–75.
15. **Blackmore PF, Neulen J, Lattanzio F, Beebe J.** 1996 Cell surface binding sites for progesterone mediate calcium uptake in human sperm. J Biol Chem. 266:18655–18659.
16. **Meizel S, Turner KO.** 1991 Progesterone acts at the plasma membrane of human sperm. Mol Cell Endocrinol. 11:R1–R5.
17. **Tesarik J, Mendoza C, Moos J, Carreras A.** 1992 Selective expression of a progesterone receptor on the human sperm surface. Fertil Steril. 58:748–792.
18. **Benoff S, Rushbrook JI, Hurley IR, et al.** 1995 Co-expression of mannose-ligand and non-nuclear progesterone receptors on motile human sperm identifies an acrosome-reaction inducible subpopulation. Am J Reprod Immunol. 34:100–115.
19. **Sauber K, Edwards DP, Meizel S.** 1996 Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction. Biol Reprod. 54:993–1001.
20. **Tesarik J, Mendoza C.** 1993 Insight into the function of a sperm-surface progesterone receptor: evidence of ligand-induced receptor aggregation and the implication of proteolysis. Exp Cell Res. 205:111–117.
21. **Tesarik J, Moos J, Mendoza C.** 1993 Stimulation of protein tyrosine phosphorilation by a progesterone receptor on the cell surface of human sperm. Endocrinology. 133:328–335.
22. **Luconi M, Bonaccorsi L, Krausz Cs, Gervasi G, Forti G, Baldi E.** 1995 Stimulation of protein tyrosine phosphorilation by platelet activing factor and progesterone in human spermatozoa. Mol Cell Endocrinol. 108:35–42.
23. **Bonaccorsi L, Luconi M, Forti G, Baldi E.** 1995 Tyrosine kinase inhibition reduces the plateu phase of the calcium increase in response to progesterone in human sperm. FEBS Lett. 364:83–86.
24. **Emiliozzi C, Cordonier H, Guerin JF, Ciapa B, Benchaib M, Fenichel P.** 1996 Effects of progesterone on human spermatozoa prepared for in-vitro fertilization. Int J Androl. 19:39–47.
25. **World Health Organization.** 1992 WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions, 3rd ed. Cambridge: Cambridge University Press
26. **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.
27. **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.
28. **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.
29. **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.
30. **Munson PJ, Rodbard D.** 1980 Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem. 107:220–239.
31. **De Lean A, Munson PJ, Rodbard D.** 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassy, radioligand assay, and physiological dose-response curves. Am J Physiol. 235:E97–E102.
32. **Tesarik J, Mendoza C.** 1993 Insights into the function of a sperm-surface progesterone receptor: evidence of ligand-induced receptor aggregation and the implication of proteolysis. Exp Cell Res. 205:111–117.
33. **Blackmore PF, Beebe SJ, Danforth DR, Alexander N.** 1990 Progesterone and 17α-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. J Biol Chem. 265:1367–1380.
34. **Chuang TT, Iacovelli L, Sallese M, De Blasi A.** 1996 G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. Trends Pharmacol Sci. 17:416–421.
35. **Weigel NL, Beck CA, Estes PA, et al.** 1992 Ligands induce conformational changes in the carboxyl-terminus of progesterone receptors which are detected by a site-directed antipeptide monoclonal antibody. Mol Endocrinol. 6:1585–1597.
36. **Wei LL, Miner R.** 1994 Evidence for the existence of a third progesterone receptor protein in human breast cancer cell line T47D. Cancer Res. 54:340–343.
37. **Castilla JA, Gil T, Molina J, et al.** 1995 Undetectable expression of genomic progesterone receptor in human spermatozoa. Hum Reprod. 10:1757–1760.
38. **Yang J, Serres C, Philibert D, Robel P, Baulieu EE, Jouannet P.** 1994 Progesterone and RU486: opposing effects on human sperm. Proc Natl Acad Sci USA. 91:529–533.
39. **Neulen J, Ishikawa K, Blackmore PF, Beebe ST.** 1993 Identification and characterization of progesterone binding proteins in human semen. Endocr J. 397–404.
40. **Hammond GL.** 1995 Potential functions of plasma steroid-binding proteins. Trends Endocrinol Metab. 6:298–304.
41. **Romeu AM, Martino EE, Stoppani, AOM.** 1976 Structural requirements for steroid binding and quenching of albumin fluorescence in bovine plasma albumin. Biochim Biophys Acta. 439:175–193.
42. **Meyer C, Schmid R, Scriba PC, Wehling M.** 1996 Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. Eur J Biochem. 239:726–731.
43. **Tripp BM, Tibbetts T, Lydon JP, O'Malley BW, Lipshultz LI, Lamb DJ.** The presence fo a distinct sperm surface progesterone receptor in transgenic mice lacking the nuclear progesterone receptor. Proc of the 52nd Annual Meet of the Am Soc for Reprod Med. 1996; S78.
44. **Aanesen A, Fried G, Andersson E, Gottlieb C.** 1995 Evidence for γ-aminobutyric acid specific binding sites on human spermatozoa. Mol Hum Reprod. 1:1885–1890.
45. **Aanesen A, Fried G, Andersson E, Gottlieb C.** 1996 Progesterone stimulates GABA uptake in human spermatozoa. Biochem Biophys Res Commun. 226:88–93.
46. **Krausz C, Bonaccorsi L, Luconi M, et al.** 1995 Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with *in vitro* fertilization. Hum Reprod. 10:120–124.