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Stimulation of Tyrosine Phosphorylation by Progesterone and Its 11-OH Derivatives: Dissection of a Ca^{2+}-Dependent and a Ca^{2+}-Independent Mechanism

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Progesterone has previously been shown to exert non-genomic effects on human spermatozoa by opening plasma membrane ion channels and by stimulating protein tyrosine phosphorylation. Here we examined how these two activities are influenced by 11-hydroxyl substitution of the steroid molecule either in the α- or in the β-configuration. Both the 11α-OH and the 11β-OH derivatives of progesterone were more effective than progesterone in stimulating tyrosine phosphorylation, although 11α-OH-progesterone was a markedly weaker Ca^{2+}-influx inducing agonist than the other two steroids. In Ca^{2+}-containing medium, the agonist activity of the 11α-OH derivative was weaker than that of the 11β-OH derivative, and it was completely abolished by genistein, whereas that of progesterone and its 11β-OH derivative was inhibited only partly by this drug. In contrast, when applied in Ca^{2+}-free medium, the 11α-OH derivative was the strongest of the three agonists tested, and the effects of all the three steroids were completely abolished by genistein. These data show that the structural motifs of steroid molecules that are responsible for the stimulation of tyrosine phosphorylation are different from those mediating the steroid action on Ca^{2+} influx through plasma membrane channels. The synthesis of selective agonists of both activities may lead to the development of new pharmacological agents to be used in the treament of steroid-dependent pathologies. © 1999 Academic Press

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In addition to the "classical" genomic effects on cells, various steroids also exert rapid non-genomic effects without entering the cell, acting at receptors localized at the plasma membrane. Such effects have been detected in a variety of cell types, such as spermatozoa, oocytes, granulosa cells, pituitary cells, neurons, hepatocytes, myocytes, leucocytes, osteoblasts and others (1, 2). In sperm cells, the known steroid non-genomic effects are exerted by progesterone (P4) and structurally related progestins; they involve the opening of a plasma membrane calcium (3–6), sodium (7) and chloride (8–12) channels, and the stimulation of protein tyrosine phosphorylation (13–16). Recent studies have suggested that the final biological effect resulting from non-genomic steroid action on cells may be due to a superimposition of partial effects, possibly involving different types of steroid receptor; such a dual steroid action may involve different kinds of ion channels (7, 17) or an ion channel and an independent receptor coupled to a protein tyrosine kinase (PTK) activation system (18, 19). Moreover, nongenomic steroid effects are suspected to be implicated in different pathological conditions including carcinogenesis (2). In this context, steroid effects on tyrosine phosphorylation are of particular interest (2, 20).

With regard to the multiplicity of cell signaling pathways that can be simultaneously stimulated by non-genomic action of a steroid at the cell surface, the question arises whether all these different responses are equally dependent on the same structural motifs of the ligand or whether different motifs are preferentially involved in different types of response. To address this question, this study examines the respective activities of P4 and of its two 11-OH derivatives as agonists of two different responses in human spermatozoa, Ca^{2+} influx and PTK activation.

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MATERIALS AND METHODS

Sperm source and preparation. Semen samples were obtained from 16 healthy donors. Each sample was divided into two aliquots that were used for the examination of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) and for the analysis of protein tyrosine phosphorylation, respectively. Ejaculated spermatozoa were washed from seminal plasma and incubated for in-vitro capacitation as described (21). After the incubation (6 h), all samples to be used in further experiments showed >95% motile spermatozoa.

Steroids. All steroids used in this study were purchased from Sigma (St. Louis, Missouri, USA). These were P4 and two C-11 hydroxyderivatives of P4, 11α-OH-P4 and 11β-OH-P4, previously reported to possess different calcium-influx-inducing activities (22). Norgestrel, a steroid known to be a potent agonist of the P4 nuclear receptor but devoid of non-genomic activity in human spermatozoa (22), was used as a negative control in some experiments. Aliquots of capacitated sperm suspensions were treated with different steroids that were added at a standard concentration of 3 μM. This concentration was chosen because, in the case of P4, it gives repeatable response patterns in human spermatozoa, both as to the induction of Ca^{2+} influx (4, 23) and as to the stimulation of tyrosine phosphorylation (13). All steroids were first dissolved in dimethylsulfoxide (DMSO; cell culture grade, Sigma) and then in culture medium (23, 24). DMSO alone was used as solvent control.

Evaluation of $[Ca^{2+}]i$. Spermatozoa were loaded with indo-1 by incubation with 5 μM indo-1/AM (Sigma) and analyzed under continuous stirring in a Hitachi F-2000 fluorescence spectrometer. During the recording period, additions of different steroids (3 μM) were made. After calibration (23), $[Ca^{2+}]i$ was expressed in nM. For comparison of the agonist activity of different steroids, the Ca^{2+} response after the addition of individual steroids was expressed as percent increase over the value measured for solvent control. Details of all these methods, including the composition of incubation media, sperm loading with indo-1, the conditions of $[Ca^{2+}]i$ measurement, and calibration of $[Ca^{2+}]i$ curves, have been described in detail elsewhere (23, 24).

Evaluation of protein tyrosine phosphorylation by Western blotting. Before dividing into aliquots to be treated with individual steroids, sperm suspensions were carefully homogenized to distribute spermatozoa as equally as possible among the aliquots. The estimated number of spermatozoa per aliquot was comprised between 5×10^6 and 10×10^6 in individual experiments. Each aliquot was incubated with a steroid at 37°C for 25 min followed by centrifugation at $10,000 \times g$ for 5 min. Triton-soluble sperm proteins were extracted as described (25), and the samples were subsequently supplemented with SDS-sample buffer (26), boiled for 5 min and subjected to electrophoresis in 10% polyacrylamide slab gels (26). Some aliquots were incubated with the PTK inhibitor genistein or its inactive analog daidzein (both purchased from Sigma and added at a concentration of 100 μg/ml) for 30 min preceding the steroid addition and during the steroid treatment. This concentration of genistein has been shown previously to produce a significant inhibition of P4-induced tyrosine phosphorylation in human spermatozoa without producing any apparent toxic effects (13).

Western blots were prepared essentially as described (27) but for the use of polyvinylidendifluoride membranes instead of nitrocellulose. After blocking with bovine serum albumin, blots were incubated with RC-20 anti-phosphotyrosine monoclonal antibody conjugated with horseradish peroxidase (Transduction Laboratories, Lexington, Kentucky, USA). The immunoreactivity was detected using an enhanced-cheminiluminiscence system ECL (Amersham, Buckinghamshire, UK) and quantified with the use of the Bio-Image Whole Band programme (Millipore, Bedford, Massachusetts, USA).

Evaluation of tyrosine phosphorylation by metabolic labeling and autoradiography. Suspensions of capacitated spermatozoa ($\sim 150 \times 10^6$/ml) were incubated for 5 h with $NaH_2\ ^{32}PO_4$ (ICN, Costa Mesa, California, USA) at a radioactivity concentration of 50 μCi/ml. This incubation was carried out at 37°C in B2 medium (Bio-Mérieux, Marcy l'Etoile, France) equilibrated with 5% CO_2 in air (pH 7.4). To assess the eventual superimposition of the respective steroid effects on the calcium influx and on protein tyrosine phosphorylation, metabolically labeled spermatozoa were further treated both in Ca^{2+}-free and Ca^{2+}-containing media. In experiments evaluating the response of spermatozoa to steroids in Ca^{2+}-free medium, spermatozoa were first loaded with controlled levels of calcium by incubation for 5 min at 37°C in B2 medium containing 10 μM ionophore A23187 (Sigma). This medium contained 1.7 mM Ca^{2+}. After washing twice in PBS containing 0.02% EDTA (PBS/EDTA; Sigma), the cells were resuspended in PBS/EDTA with 1 mM sodium orthovanadate (Sigma), in which subsequent steroid additions were made. Steroid solutions were prepared as above but for the use of PBS/EDTA as the final incubation medium. In experiments evaluating the response of spermatozoa to steroids in Ca^{2+}-containing medium, spermatozoa were exposed to steroids while in PBS supplemented with 1.7 mM $CaCl_2$ and 1 mM sodium orthovanadate, and no ionophore pretreatment was used.

After the addition of individual steroids, the incubation was continued for an additional 30 min. After two cycles of centrifugation ($500 \times g$, 15 min) and resuspension in PBS (or in PBS/EDTA for experiments in Ca^{2+}-free medium), each sperm suspension was pelleted again by centrifugation, and Triton-soluble proteins were extracted as described (13). After clearing, each sperm extract was divided into two aliquots. One aliquot was used, after precipitation with trichloracetic acid, for measurement of total radioactivity incorporated to sperm phosphoproteins. This measurement was performed with the use of a Beckman LS 6000TA β-counter. After the addition of 35 μl sample buffer (26) to the other aliquot and incubation for 5 min at 100°C, the sample was processed by electrophoresis in 10% polyacrylamide slab gels (26). Dried gels were covered with Hyperfilm MP high performance autoradiography film (Amersham) and left for 10 days at room temperature in an Amersham Hypercassette. Autoradiographs were subjected to image analysis using Adobe Photoshop 4.0 software. After exposure, the portion of each gel containing the 94-kDa protein band was cut out and used for radioactivity determination as described (13).

Statistical analysis. Statistical analysis was performed by analysis of variance (ANOVA) using the StatView II statistical package (Abacus Concepts, Berkeley, California, USA). Means were compared by paired or unpaired Student's *t*-test.

RESULTS

When added to suspensions of living indo-1-loaded spermatozoa, P4 and 11β-OH-P4 showed a similar activity as agonists inducing calcium influx into spermatozoa, whereas 11α-OH-P4 was a significantly weaker agonist (Table 1). The effect of norgestrel was not significantly different from that of solvent control (Table 1).

In Western blots probed with the RC-20 anti-phosphotyrosine antibody, a number of bands, corresponding to proteins whose apparent molecular weight was comprised between 30 kDa and 115 kDa, were distinguished (data not shown). However, only three regions with a strong reaction with the antibody, corresponding to proteins of 75-85 kDa, 90-100 kDa and 105-115 kDa, were consistently present in the blots

TABLE 1

Comparison of Different P4-Related Steroids as Agonists Inducing Ca^{2+} Influx into Spermatozoa

Component added	Maximum $[Ca^{2+}]i$ increase (nM)[a]
Solvent control	7 ± 2[b]
P4	709 ± 74[c]
11α-OH-P4	381 ± 40[d]
11β-OH-P4	698 ± 71[c]
Norgestrel	9 ± 2[b]

[a] Calculated as the difference between the highest $[Ca^{2+}]i$ value achieved after each respective addition and the baseline value before the addition. Values are mean ± SEM (n = 6).

[b,c,d] Values with different superscripts are significantly different from each other ($P < 0.05$).

and were subjected to quantitative evaluation. Of these three regions, only proteins in the 90-100-kDa region showed a significant increase in the level of tyrosine phosphorylation in response to P4 as compared to solvent control (Table 2). Interestingly, the relative increase in tyrosine phosphorylation of proteins within this region was not lower for 11α-OH-P4 as compared to P4 and 11β-OH-P4, although 11α-OH-P4 was a significantly weaker agonist as to the induction of Ca^{2+} influx (Table 2). The values for norgestrel were similar to those for solvent control (Table 2). Genistein (100 μg/ml), but not its inactive homologue daidzein, decreased the basal tyrosine phosphorylation level in all of the three blot regions evaluated and inhibited the steroid-induced increase in tyrosine phosphorylation of proteins in the 90–100-kDa region (data not shown).

Measuring of ^{32}P radioactivity incorporated to total Triton-soluble sperm protein extract after sperm exposure to steroids in Ca^{2+}-containing medium showed

TABLE 2

Relative Efficiency of Different P4-Related Steroids as Agonists Stimulating Ca^{2+} Influx and PTK Activity in Spermatozoa

		Relative increase in tyrosine phosphorylation[a]		
Component added	Ca^{2+} response[a] (%)	75-85 kDa (%)	90-100 kDa (%)	105-115 kDa (%)
P4	567 ± 59[b]	2 ± 1[b]	24 ± 4[b]	4 ± 3[b]
11α-OH-P4	304 ± 32[c]	3 ± 2[b]	26 ± 5[b]	2 ± 2[b]
11β-OH-P4	558 ± 56[b]	2 ± 1[b]	25 ± 4[b]	1 ± 1[b]
Norgestrel	7 ± 2[d]	2 ± 1[b]	1 ± 1[c]	1 ± 1[b]

[a] Calculated as percent increase over the value (in nM for Ca^{2+} and in arbitrary units for optical density of protein regions in western blots probed with anti-phosphotyrosine antibody) measured for solvent control (mean ± SEM).

[b,c,d] Values with different superscripts in each column are significantly different from each other ($P < 0.05$).

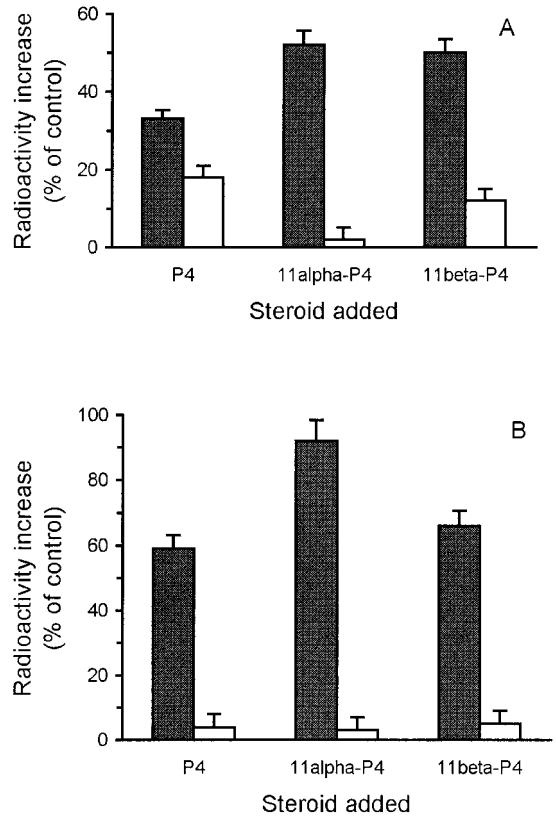

FIG. 1. Effects of P4, 11α-OH-P4 (11alpha-P4) and 11β-OH-P4 (11beta-P4) on the incorporation of ^{32}P to Triton-soluble sperm proteins in the absence (full bars) and in the presence (empty bars) of genistein (100 μg/ml). Panels A and B show the effects of the steroids added in Ca^{2+}-containing and Ca^{2+}-free medium, respectively.

that the increase in radioactivity occurring in response to each of the 11-OH-derivatives of P4 was significantly higher ($P < 0.05$) as compared to unsubstituted P4, whereas no difference was found between the two 11-OH-derivatives (Fig. 1A). In contrast, when spermatozoa were exposed to steroids in Ca^{2+}-free medium, 11α-OH-P4 was a significantly stronger ($P < 0.05$) agonist than the other two steroids (Fig. 1B). Moreover, the effects of P4 and 11β-OH-P4 on ^{32}P incorporation to total sperm proteins were inhibited by genistein only partly, whereas the inhibition by genistein of the 11α-OH-P4 effect was complete (Fig. 1A). This contrasted with the effects of the three steroids produced in Ca^{2+}-free medium, which were equally and completely inhibited by genistein (Fig. 1B).

Autoradiographic analysis of protein extracts of spermatozoa metabolically labeled with ^{32}P and exposed to individual steroids confirmed the preferential incorporation of ^{32}P to a protein band of 94 kDa, although labeling intensity of a protein band of ~67 kDa was also increased after sperm exposure to the steroids (Fig. 2). Quantitative evaluation of ^{32}P incorporation to the 94-kDa protein band in response to the three steroids tested showed the same relationships as for ^{32}P

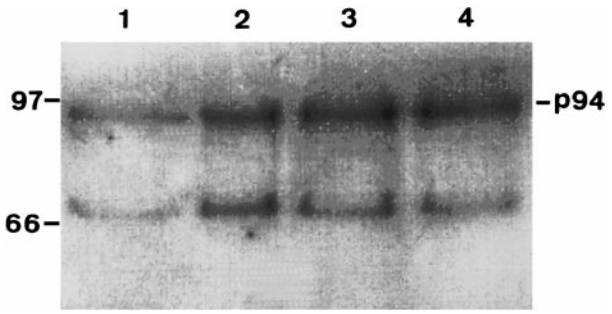

FIG. 2. Representative autoradiograph of Triton-soluble sperm proteins after sperm incubation with $^{32}PO_4$ and exposure, in Ca^{2+}-containing medium, to different steroids: lane, solvent control; lane 2, P4; lane 3, 11β-OH-P4; lane 4, 11α-OH-P4. The positions of molecular weight markers and of the main tyrosine-phosphorylated protein (p94) are indicated to the left and to the right, respectively.

incorporation to total Triton-soluble protein fraction (data not shown).

DISCUSSION

The results presented in this study have shown that the activity of P4 as an agonist operating Ca^{2+} influx into human spermatozoa can be modified by OH-substitution on C-11. In fact, 11α-OH-P4 was a markedly weaker agonist as compared to 11β-OH- P4 or unsubstituted P4. This finding is in agreement with previously published data (22). If the stimulation of tyrosine phosphorylation, another known effect of P4 on human spermatozoa, were mediated by the P4-induced Ca^{2+} influx, it could be expected that the ability of 11α-OH- P4 to stimulate tyrosine phosphorylation would also be lower as compared to P4 and 11β-OH-P4. This study, however, did not show any difference in the PTK-stimulating activity of these three steroids, when added to spermatozoa in Ca^{2+}-containing medium. This is in agreement with our previous findings showing that the induction of Ca^{2+} influx and the stimulation of tyrosine phosphorylation are two ar least partly independent actions exerted by P4 at cell surface receptors (18).

To compare the Ca^{2+}-independent activity of the three steroids as PTK stimulators, the incorporation of ^{32}P to sperm phosphoproteins was determined after sperm exposure to steroids in Ca^{2+}-free medium. The results of this experiment have shown that 11α-OH-P4 is a markedly stronger PTK stimulator as compared to P4 and 11β-OH-P4, both of which displayed a similar activity. This contrasted with the findings obtained when spermatozoa were exposed to steroids in Ca^{2+}-containing medium, where all the three steroids under study stimulated tyrosine phosphorylation to the same extent. This difference can be explained by the existence of two mechanisms mediating the nongenomic effect of steroids on tyrosine phosphorylation – one Ca^{2+}-dependent and the other Ca^{2+}-independent.

The existence of two different mechanisms of steroid-induced PTK activation is also supported by the present experiments using the PTK inhibitor genistein. In agreement with a previous study (13), inhibition of P4-induced tyrosine phosphorylation by genistein is incomplete even with the highest nontoxic concentration of this drug. The same applies to 11β-OH-P4. In contrast, genistein produced a complete inhibitition of the tyrosine phosphorylation stimulated by 11α-OH-P4. Moreover, tyrosine phosphorylation induced by any of these three steroids was inhibited completely by genistein when spermatozoa were treated with the steroids in Ca^{2+}-free medium. Consequently, it appears that different types of PTK, with distinct sensitivities to genistein, are preferentially activated by the steroids in the presence and in the absence of external Ca^{2+}, respectively. The PTK preferentially involved in the former case appears to be stimulated by the steroid-induced Ca^{2+} influx. On the other hand, the PTK involved in the latter mechanism appears to be activated by the steroid ligand either directly or by means of association with a steroid-bound receptor.

The PTK involved in the Ca^{2+}-independent steroid response appears to be identical with Hu9, a sperm-specific receptor PTK whose physiological agonist is ZP3, a sperm-activating glycoprotein from the mammalian egg's zona pellucida (28). This contention is supported by the following arguments. Firstly, phosphoaminoacid analysis of proteins phosphorylated in human spermatozoa in response to P4 has shown that 98.5% of ^{32}P incorporated to the 94-kDa phosphoprotein of the human sperm extract actually corresponds to phosphorylation on tyrosine residues, whereas other proteins incorporating ^{32}P became preferentially phosphorylated on serine and threonine (13). Secondly, except Hu9, no tyrosine-phosphorylated protein with a similar molecular weight, subcellular distribution and solubility in detergents appears to be present in human spermatozoa. In fact, the major sperm substrates for tyrosine phosphorylation, A-kinase anchoring protein and its precursor, have a slightly larger molecular weight, are associated with structures of the sperm flagellum and are resistent to detergent extraction (29). Finally, an increase in in-situ tyrosine phosphorylation of the human sperm head region corresponding to the localization of Hu9 has been demonstrated in human spermatozoa treated with P4 in Ca^{2+}-free medium (13). Interestingly, no steroid binding domain can be predicted from the amino acid sequence of Hu9 (28). Consequently, Hu9 appears to be able to act as both a receptor PTK (with regard to ZP3) and as a nonreceptor PTK (with regard to P4). In the latter case, physical association of Hu9 with a yet unknown receptor (see below) would be necessary.

With regard to the current knowledge of steroid-activatable PTKs present in mammalian spermatozoa

(13–16), steroids may first interact with a receptor in the sperm plasma membrane which subsequently co-aggregates with Hu9, leading to Hu9 autophosphorylation. Tyrosine phosphorylation of a protein whose electrophoretic mobility corresponded to Hu9, and which was in all probability identical to the major Triton-soluble sperm phosphoprotein previously shown to be phosphorylated on tyrosine in response to P4 (13) and solubilized zona pellucida proteins (30), could actually be stimulated by all the three steroids tested in this study. This early response is Ca^{2+}-independent. Because binding sites for several Scr homology 2-containing proteins are present in the intracellular region of Hu9 (28), the autophosphorylated Hu9 can subsequently trigger a cascade reaction involving the phosphorylation of Shc (31) and the activation of the Src/p21^{ras}/Erk pathway (32). This cascade reaction appears to require a concomitant increase in the cytosolic free Ca^{2+} concentration, mediated by an idependent steroid action at a plasma membrane Ca^{2+} channel. Both components of this compound response are needed for the physiological response – the sperm acrosome reaction (19).

A recent study has demonstrated the presence of two pharmacologically distinct types of steroid receptor on the human sperm surface (33). It is tempting to speculate that one of these receptors is instrumental in the Ca^{2+} response and the other mediates the PTK-initiated pathway. The present identification of selective agonists of each of the two responses opens a new perspective to testing this hypothesis. This research is expected to explain the relationship between steroid structure and nongenomic activity and to enable the identification of specific agonists and antagonists of individual nongenomic effects with potential therapeutical use in the treatment of steroid-dependent diseases.

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