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Progesterone action through aggregation of a receptor on the sperm plasma membrane

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Rapid steroid effects, reported in several cell types, have pointed out the possibility of non-genomic mechanisms of action, presumably on cell surface receptors. Here we analyzed the effects of antibody-mediated aggregation of a novel type of progesterone receptor on the plasma membrane of human sperm cells. We report that aggregation of hormone-receptor complexes induces Ca^{2+} influx and a Ca^{2+}-dependent exocytotic event in this system. These data suggest a possible mechanism for rapid steroid-induced events. Further research is warranted to examine if a similar mechanism is involved in rapid steroid effects in other cell types.

Progesterone receptor aggregation; Plasma membrane; Calcium influx; Acrosome reaction; Sperm; Human

1. INTRODUCTION

Steroid hormone action in most cellular systems studied so far involves hormone entry into the cell and binding to nuclear receptors with subsequent modulation of transcription, however, rapid steroid effects previously reported in several cell types, e.g. oocytes [1,2], hepatocytes [3], neurons [4], and spermatozoa [5–7], are hardly compatible with this classical scheme. Recent studies using human sperm cells as a model [8–10] have suggested that steroids can produce rapid cellular effects by acting at a novel type of steroid receptors on the plasma membrane, however, no explanation of the mechanism of function of this kind of receptor has yet been proposed.

One of the frequent mechanisms by which cell surface receptors transduce ligand-generated signals across the plasma membrane is receptor aggregation. We thus formulated a hypothesis that an aggregation mechanism is also instrumental in the coupling of steroid receptors on the cell surface to intracellular effectors of hormone action. In this study we tested this hypothesis by examining if a rapid Ca^{2+} influx followed by acrosomal exocytosis, phenomena previously described to occur in human spermatozoa in response to progesterone [5–7] or to its protein conjugates [8,10], can be induced by antibody-generated aggregation of hormone-receptor complexes on the cell surface.

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2. MATERIALS AND METHODS

2.1. *Study design*

To study the effect of receptor aggregation on progesterone-induced Ca^{2+} influx, we loaded sperm cells with a fluorescent indicator of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$), and exposed them, sequentially, to progesterone and a monoclonal antibody recognizing both free and protein-conjugated progesterone [11]. This antibody was used either intact or after enzymatic digestion to produce monovalent Fab fragments. In the latter case, anti-mouse IgG antiserum followed the incomplete anti-progesterone antibody to cross-link the fragments. Changes in $[Ca^{2+}]_i$ were recorded after individual additions.

To study the effect of receptor aggregation on the acrosomal exocytosis, sperm cells were treated as above, followed by evaluation of the acrosome reaction frequency using a fluorochrome-labelled acrosomal marker.

2.2. *Source and preparation of spermatozoa*

Sperm samples were obtained from 15 healthy donors with normal spermiogram parameters. Spermatozoa were capacitated in vitro as described previously [12]. Briefly, spermatozoa were washed from the seminal plasma in PBS followed by incubation for 3 h at 37°C in B2 culture medium (Bio Mérieux, Marcy l'Etoile, France) equilibrated with 5% carbon dioxide in air. These sperm suspensions were then used in individual experiments.

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

Sperm cells (5–10 × 10^6/ml) were incubated for 45 min at 37°C in B2 medium containing 4 μM indo 1-AM (indo; Sigma, La Verpillière, France). After washing in FM3B buffer [13], the cells were resuspended in the same buffer and analyzed in a Perkin-Elmer LS-5 luminescence spectrometer. The cells (1.5 × 10^6 in 1.5 ml buffer) were excited at 331 nm and emission was monitored at 410 nm. Aliquots of agents (5–15 μl) were added at different times (see Results). At the end of each measurement, cells were lysed with 0.05% Triton followed by chelating Ca^{2+} ions with 25 mM EGTA, and the corresponding values of emitted fluorescence were used, respectively, as F_{max} and F_{min} to estimate $[Ca^{2+}]_i$, as described by Grynkiewicz et al. [14].

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2.4. *Preparation of anti-progesterone monoclonal antibody*

Prog 53 monoclonal antibody [11] was purified from ascite fluid by caprylic acid precipitation; the IgG fraction was isolated by absorption on protein-A Sepharose (Sigma). The same treatment was used to purify non-relevant mouse IgG. Fab fragments of Prog 53 antibody were prepared as described [15] using immobilized papain (Sigma) followed by separation of Fab fragments from Fc and undigested IgG molecules on Protein-A Sepharose (Sigma). The purity of the preparation was checked by SDS-PAGE [16].

2.5. *Cytochemical visualization of antibody binding to sperm*

The binding of intact Prog 53 and its Fab fragments to progesterone-treated sperm, and the absence of binding to sperm not exposed to steroids or to those exposed to 17β-estradiol, were checked by indirect immunofluorescence. In these experiments sperm were first incubated (15 min, 37°C) with steroid (3.2 μM progesterone or 3.7 μM 17β-estradiol), washed in PBS and incubated for an additional 15 min with the antibody (300 μg/ml) under the same conditions. After further washing sperm were exposed (30 min, 4°C) to anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma), smeared on microscope slides and examined by epifluorescence microscopy. The steroid was replaced with dimethylsulfoxide (DMSO) alone (the solvent for progesterone and 17β-estradiol) in control incubations. Some smears were counterstained with unconjugated tetramethylrhodamine isothiocyanate (TRITC) [12] to provide paired views showing sperm cell morphology.

2.6. *Evaluation of the acrosome reaction*

The percentage of living acrosome-reacted spermatozoa was determined by fluorescence microscopy after double fluorescent staining with fluorescein-labeled *Pisum sativum* agglutinin and Hoechst 33258 [17]. Both reagents were purchased from Sigma. Two hundred spermatozoa were evaluated in each replicate of individual experiments.

2.7. *Statistics*

Statistical analysis was performed using StatView II (Abacus Concepts, Berkeley, California, USA) statistical package. Percentages of acrosome-reacted spermatozoa in different treatment groups were compared, after arcsine transformation, by Dunnett's t-test for multiple comparisons.

3. RESULTS AND DISCUSSION

As a prelude to this study, emission spectra (from 360 to 520 nm) of indo only and of indo mixed with progesterone and Prog 53 antibody, at the same concentrations as used in cell suspensions, were analysed. Neither progesterone nor Prog 53 nor both together produced any detectable distortion of the spectra.

The addition of intact bivalent Prog 53 antibody to sperm previously exposed to different concentrations of progesterone resulted in a rapid increase in $[Ca^{2+}]_i$ which followed that provoked by the hormone alone (Fig. 1A,B,C). This antibody-generated increment was dependent on the concentration of previously added progesterone (Fig. 1A,B,C). Moreover, Prog 53 antibody produced an increase in $[Ca^{2+}]_i$ following the treatment with a sub-threshold progesterone concentration (3.2 nM) which did not produce any detectable effect by itself (Fig. 1D). No response to Prog 53 was observed following the addition of 17β-estradiol instead of progesterone (Fig. 1E), and $[Ca^{2+}]_i$ was not affected by exposure of progesterone-treated sperm to non-relevant IgG (Fig. 1F). When progesterone (3.2 μM) was mixed

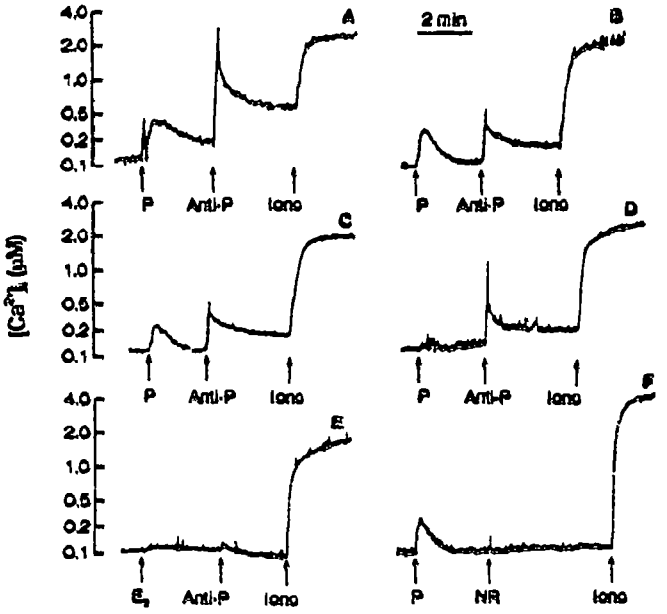

Fig. 1. Effects of Prog 53 anti-progesterone monoclonal antibody on $[Ca^{2+}]_i$ in human sperm measured using indo. The first addition was progesterone (P) at concentrations of 3.2 μM (panel A), 320 nM (panel B), 32 nM (panels C,F) and 3.2 nM (panel D), or 17β-estradiol (E_2) at a concentration of 37 nM (panel E). This was followed, sequentially, by 300 μg/ml Prog 53 antibody (anti-) (panels A–E) or the same concentration of non-relevant mouse IgG (NR)(F), and 10 μM ionomycin (Iono) (panels A–F). Results of a single experiment are presented which are representative of four independent determinations.

with Prog 53 antibody (300 μg/ml) before the addition to sperm, or when the antibody was added first followed by progesterone, no effects on $[Ca^{2+}]_i$ were detected. The effect of Prog 53 on $[Ca^{2+}]_i$ was entirely due to extracellular Ca^{2+} influx, and not to intracellular Ca^{2+} mobilization, since it was completely abolished by chelating extracellular Ca^{2+} with 5 mM EGTA (data not shown).

When monovalent Fab fragments of Prog 53 antibody were used instead of the intact bivalent antibody, no increase in $[Ca^{2+}]_i$ followed immediately the antibody addition, but it did occur after subsequent addition of anti-mouse IgG antiserum (Fig. 2B). This antiserum had no effect when added after intact Prog 53 (Fig. 2A), probably because all competent cells had already responded to the previous treatments. When added to sperm suspensions that had not undergone any previous antibody treatment, anti-mouse IgG antiserum did not produce any measurable change in $[Ca^{2+}]_i$.

The reaction of intact Prog 53 and its Fab fragments with sperm-bound progesterone was confirmed by immunocytochemistry. When added to progesterone-treated sperm, the antibody selectively bound the equatorial acrosomal region of some sperm (Fig. 3.). No antibody binding was observed when sperm had not been pretreated with progesterone.

To evaluate the physiological significance of the Ca^{2+} influx induced by progesterone receptor aggregation,

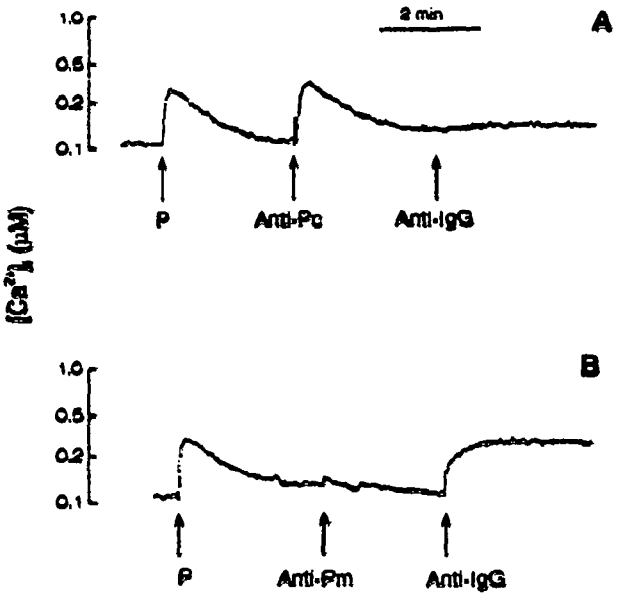

Fig. 2. Comparison of the effects of intact bivalent Prog 53 antibody and its monovalent Fab fragments on $[Ca^{2+}]_i$ in human sperm measured using indo. The first addition of 320 nM progesterone (P) was followed by either complete Prog 53 antibody (anti-Pc) (panel A) or its monovalent Fab fragments (anti-Pm) (panel B), both at a concentration of 200 μg/ml. Sheep antiserum to mouse IgG (Sigma) followed as a third addition (anti-IgG) in both cases. It was added at a concentration of 50 μg/ml.

we treated sperm cells with a sub-threshold concentration of progesterone (see Fig. 1D) and examined the effect of the subsequent antibody addition on the acrosomal exocytosis (Fig. 4). The data obtained show that bivalent Prog 53 antibody, but not its monovalent Fab fragments, significantly increase the percentage of acrosome-reacted sperm when added after progesterone treatment.

The results described in this study suggest that antibody-mediated aggregation of progesterone-receptor complexes on the sperm surface elicit a rapid Ca^{2+} influx. In human follicular fluid, which is a physiological inducer of the acrosomal exocytosis [18], progesterone is non-covalently bound to a protein to form a complex of 50 kDa [5]. Thus, the protein backbone of this complex may help to aggregate sperm surface components recognized by progesterone. On the other hand, free progesterone is also capable of inducing Ca^{2+} influx in human sperm ([5–7], this study). It is possible that free progesterone added to sperm suspensions forms complexes with proteins in the incubation medium, which may mimic the action of the natural steroid–protein complex found in the follicular fluid. It should be noted that direct progesterone effects on human sperm require relatively high hormone concentrations. In this respect, the observation of an antibody-induced Ca^{2+} influx following treatment with sub-threshold progesterone concentrations is a strong piece of evidence in favour of the receptor aggregation hypothesis.

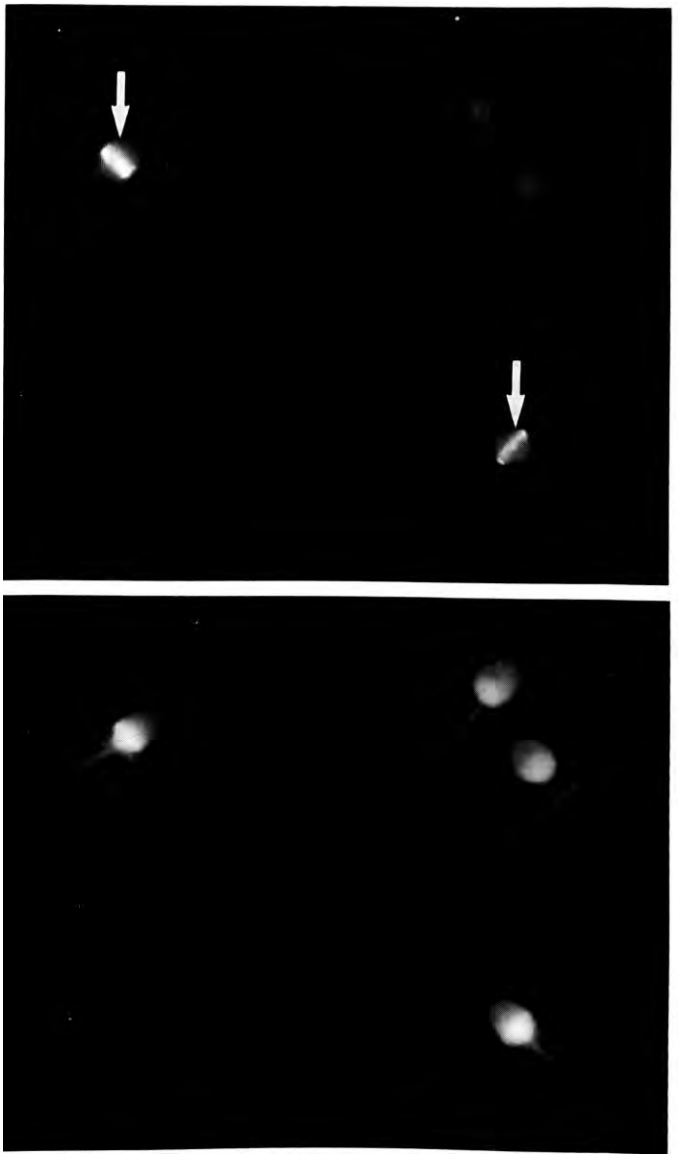

Fig. 3. Cytochemical demonstration of intact Prog 53 antibody binding (visualized by FITC-labelled anti-mouse IgG) to sperm pre-incubated with progesterone (upper panel) and a paired view showing the morphology of the same sperm cells counterstained with unconjugated TRITC (lower panel). Two sperm cells show a heavy FITC fluorescence in the equatorial acrosomal region (arrows). A similar fluorescent pattern was obtained with Fab fragments of Prog 53 antibody. Magnification: ×500.

The fact that such a relatively small molecule as progesterone can bind two macromolecules at the same time, the presumptive progesterone receptor and the anti-progesterone antibody, is somewhat surprising. Nevertheless, the cytochemical visualization of sperm-bound progesterone using Prog 53 antibody provides additional evidence that these three elements can form rather durable complexes. The observed confinement of the antibody binding to the equatorial acrosomal region of sperm cells may have been a sequela of a primary progesterone binding to the whole acrosomal region

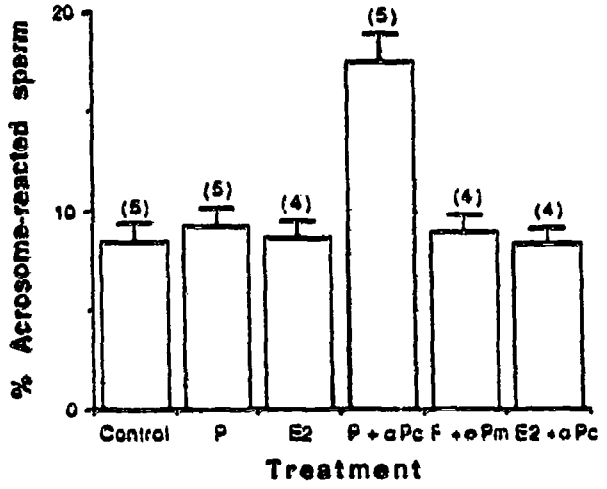

Fig. 4. Effect of Prog 53 monoclonal antibody on the acrosomal exocytosis. Capacitated sperm were incubated for 30 min with 3.2 nM progesterone (P) or with 3.7 nM 17β-estradiol (E2) or with DMSO (solvent for both steroids) alone (control). In some treatment groups, complete Prog 53 antibody (P + αPc) or its monovalent Fab fragments (P + αPm) were added at a concentration of 300 μg/ml for the last 15 min to sperm incubated with progesterone. Complete Prog 53 antibody was added, under the same conditions, to sperm pretreated with 17β-estradiol instead of progesterone (E2 + αPc). Only progesterone followed by Prog 53 increased the percentage of acrosome-reacted sperm above control values ($P < 0.001$). Values are mean ± S.E.M. for the number of replicates in parentheses.

with subsequent loss of the plasma membrane overlying the anterior part of the acrosome during the exocytotic reaction. Alternatively, the plasma membrane may still have been intact over the entire acrosome, at least in some of these spermatozoa, but hormone-bound receptors may have migrated to the equatorial region as a result of aggregation, a phenomenon which may not be dissimilar to 'capping' of aggregated receptors on B lymphocytes [19]. Studies are under way in our laboratory to decide which of the two mechanisms is actually involved.

On the other hand, previous formation of progesterone–antibody complexes (when both were pre-incubated before addition to sperm or when excess antibody was already present in sperm incubation medium before progesterone addition) prevented the hormone effect. It is possible that the reaction of progesterone with its receptor activates other elements in the plasma membrane which stabilize the hormone–receptor complex so as to endure the subsequent binding of the anti-progesterone antibody. The integration of the receptor in the plasma membrane structure may also cause constraints to the receptor interaction with antibody-bound progesterone while still allowing the recognition of receptor-bound progesterone by the antibody. These aspects of progesterone interaction with its plasma membrane receptor are currently under investigation.

Previously published data [9,10] have indicated that the progesterone effect on human sperm is mediated by a true receptor and not merely by steroid interaction with the lipid bilayer of the plasma membrane. The present findings suggest that this ligand–receptor system may transduce the ligand-generated signal across the plasma membrane using a mechanism similar to that employed by peptide hormones, such as epidermal growth factor [20], insulin [21], or insulin-like growth factor I [22]. Receptor aggregation is instrumental in the induction of the acrosomal exocytosis in mouse sperm by a zona pellucida glycoprotein [23], and antibody-mediated crosslinking of cell surface antigens has been reported to induce exocytosis in ram [24] and sea urchin [25] sperm. This study is the first to suggest that a similar mechanism can mediate the action of a steroid hormone.

Steroids are complexed with proteins in many biological systems. If one protein molecule carries several molecules of steroid, the protein can bridge plasma membrane receptors recognized specifically by the steroid thus promoting aggregation which, in turn, will trigger a cascade of biochemical events culminating in activation of intracellular effectors of hormone response. The present findings warrant further investigation into the molecular identity of cell surface steroid receptors and the mechanisms of cellular signalling downstream of receptor aggregation. Since the sperm acrosome reaction is a key event in fertilization [26], these data may be applied in reproductive medicine and contraception research. If the receptor aggregation hypothesis is confirmed in other cell types showed rapid response to steroids (e.g. nerve cells, liver cells), new applications in neurology, psychiatry, anaesthesiology and internal medicine can be envisaged.

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