1991

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Repository Citation

Original Publication Citation

Cell Surface-binding Sites for Progesterone Mediate Calcium Uptake in Human Sperm*

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Recent studies (*e.g.* Blackmore, P. F., Beebe, S. J., Danforth, D. R., and Alexander, N.) (1990) *J. Biol. Chem.* 265, 1376–1380) have shown that in human sperm, progesterone produces a rapid increase in intracellular free calcium ($[Ca^{2+}]_i$) and an induction of the acrosome reaction (*e.g.* Osman, R. A., Andria, M. L., Jones, A. D., and Meizel, S. (1989) *Biochem. Biophys. Res. Commun.* 160, 828–833).

In this study, the location of progesterone receptors on the cell surface of human sperm was identified using progesterone immobilized on bovine serum albumin (BSA) (progesterone 3-(*O*-carboxymethyl)oxime:BSA) as well as progesterone and its 3-*O*-carboxymethyloxime derivative. Using fluorescence microscopy, BSA-fluorescein isothiocyanate was shown to be excluded from intact sperm, thus validating the use of progesterone 3-(*O*-carboxymethyl)oxime:BSA to identify cell surface-binding sites for progesterone. The immobilized progesterone and the 3-*O*-carboxymethyloxime derivative rapidly increased $[Ca^{2+}]_i$ and were full agonists, although they were approximately 1.5 orders of magnitude less potent than progesterone. They also displayed an identical time course to increase $[Ca^{2+}]_i$ as free progesterone, and the entire increase in $[Ca^{2+}]_i$ was due to the influx of Ca^{2+}. This progesterone-mediated response displayed different steroid receptor characteristics since the very potent inhibitors of genomic progesterone responses, RU38486 and ZK98.299, were very ineffective at inhibiting the progesterone-mediated increase in $[Ca^{2+}]_i$. Also the synthetic progestins megestrol, medroxyprogesterone acetate, norgestrel, norethynodrel, norethindrone, R5020, and cyproterone acetate did not mimic the effects of progesterone to increase $[Ca^{2+}]_i$. It is proposed that a distinct nongenomic cell surface receptor for progesterone exists in human sperm.

Studies have shown that progesterone can induce the acrosome reaction in capacitated human sperm (Osman *et al.*, 1989). It has also been demonstrated that progesterone and 17α-hydroxyprogesterone can induce a rapid influx of Ca^{2+} into either capacitated or noncapacitated human sperm (Blackmore *et al.*, 1990; Thomas and Meizel, 1989). The induction of the acrosome reaction is believed to follow this influx of Ca^{2+} (*e.g.* Yanagimachi, 1988; Sidhu and Guraya, 1990). This rapid effect of progesterone to stimulate Ca^{2+} influx indicates a nongenomic response and suggests that a cell surface receptor exists for progesterone (Blackmore *et al.*, 1990). Since progesterone can penetrate the plasma membrane, due to its hydrophobicity, one could invisage that the binding site for progesterone could conceivably be located intracellularly.

The best characterized cell surface progesterone receptor is from the *Xenopus* oocyte (Sadler and Maller, 1985), where progesterone reduces endogenous cAMP levels by inhibiting adenylylcyclase. There are other more recent reports of nongenomic effects of steroids. For example, progesterone causes the release of gonadotropin-releasing hormone from rat hypothalami via a plasma membrane receptor (Ke and Ramirez, 1987, 1990). Progesterone was also shown to enhance τ-aminobutyric acid effects on neuronal Cl^- ion channels (Wu *et al.*, 1990). The present evidence shows that progesterone as well as other steroids bind to a "distinct membrane-bound steroid site" (Gee *et al.*, 1987; Lan *et al.*, 1990). The rapid membrane effects of steroid hormones were recently reviewed by Schumacher (1990), Touchette (1990), and McEwen (1991).

To investigate the mechanism by which the progesterone receptor can regulate the uptake of Ca^{2+} by sperm and increase $[Ca^{2+}]_i$, it is imperative that the subcellular location of the progesterone receptor be identified. If it is suspected that the receptor is in the plasma membrane and the binding site for progesterone is on the extracellular surface, then use can be made of immobilized progesterone. Immobilized ligands, if coupled to large molecular weight molecules that cannot penetrate the plasma membrane and if the essential determinants for the ligands specificity and activity are still exposed, can stimulate cell surface receptors (*e.g.* Dehaye *et al.*, 1980). In the present study, we utilized progesterone (prog)[1] covalently linked at position 3 on the steroid nucleus via an *O*-carboxymethyloxime (CMO) to bovine serum albumin (BSA) (prog CMO BSA). It was found that prog CMO BSA was able to stimulate a rapid increase in $[Ca^{2+}]_i$.

Progesterone Receptors

MATERIALS AND METHODS

Human sperm, either capacitated or noncapacitated, were isolated as previously described (Blackmore *et al.*, 1990). The same results were obtained with either sperm preparation (data not shown). Sperm were loaded with fura-2 and fluorescence measurements made as detailed before (Blackmore *et al.*, 1990). Prog CMO BSA, BSA-fluorescein isothiocyanate (FITC-BSA), and Prog CMO were from Sigma. The particular preparation of prog CMO BSA had 31 mol of progesterone/mol of BSA. RU38486 was from Roussel-Uclaf, France, and ZK98.299 was from Schering Ag (F.R.G.). Other steroids were from Sigma.

Free progesterone was removed from various solutions as follows. A solution (100 μl) of prog CMO BSA (100 μM) was incubated at room temperature with a slurry (20 μl) of activated charcoal (charcoal 0.5 g/ml, BSA 4 mg/ml). After removal of the charcoal by centrifugation (2000 $\times$ g for 5 min), the supernatant solution should only contain progesterone covalently bound to BSA (see Fig. 6 for results). To confirm that charcoal did remove free progesterone and CMO progesterone from aqueous solution, charcoal was added, as above, to a solution of free progesterone (100 μM) or CMO progesterone containing 100 μM BSA (see Fig. 6 for results).

Fluorescence and light microscopy were performed using a Nikon microphot-FX. Suspensions of sperm were mixed with 10 μM FITC-BSA for 1 h in the presence (0.05% w/v) of digitonin or in its absence. Images were recorded on VHS videotape using a MTI CCD72 camera and a videoscope image intensifier. Images were printed on a Sony video printer UP-5000W.

RESULTS

Fig. 1 shows the temporal effects of progesterone, prog CMO, and prog CMO BSA (10 μM progesterone covalently bound) to increase $[Ca^2]_i$. This concentration of prog CMO BSA was equivalent to the effect observed with 10 μM progesterone and was the maximum effect seen with prog CMO BSA. Thus, prog CMO BSA acted as a full agonist. The time course of prog CMO BSA to elevate $[Ca^{2+}]_i$ was very similar to that seen with progesterone; all agents elevated $[Ca^{2+}]_i$ without apparent delay. The BSA control did not elevate $[Ca^{2+}]_i$, which is not surprising since the media already contained 3.0 mg/ml BSA (data not shown). Fig. 2 shows the concentration response curve for prog CMO BSA to elevate $[Ca^{2+}]_i$ compared with the effect of progesterone and prog CMO. Progesterone was more potent than either prog CMO or prog CMO BSA. The EC_{50} for progesterone was approximately 50 nM, whereas the EC_{50} for prog CMO BSA was approximately 1 μM. However, prog CMO and prog CMO BSA were essentially equipotent, indicating that the BSA
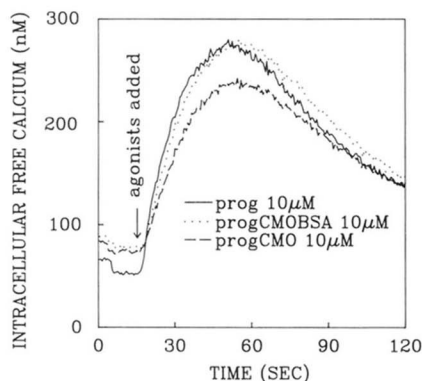
FIG. 2. **Concentration response curves of progesterone, prog CMO, and prog CMO BSA to increase $[Ca^{2+}]_i$ in sperm.** The various agents were added at the indicated concentrations and the peak $[Ca^{2+}]_i$ measured usually 30 s after the addition of the agent. See Fig. 1 legend for other details. Data are means ± S.E. from three separate experiments.

FIG. 3. **Light and fluorescence microscopy of sperm in the presence of FITC-BSA (10 μM) and the effect of digitonin on the fluorescence.** Sperm were mixed with 10 μM FITC-BSA then placed on a microscope slide coverslip. Cells were observed using a Fluor 100× objective. Images of sperm were recorded on videotape. Representative sperm are shown for each condition. For other details, see "Materials and Methods."

added was not in itself responsible for the reduced potency, even though prog CMO BSA cannot cross the plasma membrane. Consequently, the actions of prog CMO and prog CMO BSA can be accounted for by their extracellular actions.

To confirm that BSA was confined to the extracellular space, FITC-BSA was added to sperm suspensions in the presence (Fig. 3, *panels C and D*) and absence (*panels A and B*) of digitonin (0.05% w/v). The data in Fig. 3 show a representative experiment in which sperm in the presence of FITC-BSA were examined under light microscopy (*panels A and C*) and fluorescence microscopy (*panels B and D*). *Panel A* shows an intact sperm under light microscopy; however, the same sperm under fluorescence microscopy (*panel B*) demonstrate that FITC-BSA is excluded. Conversely, when sperm were treated with digitonin to render them permeable, FITC-BSA was localized intracellularly (*panel D*). This result confirms the rationale for using the progesterone BSA conjugate to localize cell surface progesterone receptors.

It was previously shown that progesterone was able to promote an increase in $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx rather than a release of intracellular Ca^{2+} (Blackmore *et al.*, 1990). The prog CMO BSA-stimulated increase in $[Ca^{2+}]_i$ was also due to an influx of extracellular Ca^{2+}, since the response was completely inhibited when extracellular Ca^{2+} was removed by chelation with the Ca^{2+} chelator EGTA (Fig. 4). The readdition of Ca^{2+} to the EGTA-treated cells that were previously exposed to prog CMO BSA resulted in an immediate increase in $[Ca^{2+}]_i$ similar to that observed when Ca^{2+}

FIG. 1. **Time course of progesterone, prog CMO BSA, and prog CMO to increase $[Ca^{2+}]_i$ in sperm.** To fura-2-loaded sperm, 10 μM progesterone, 10 μM prog CMO BSA, or 10 μM prog CMO were added at 15 s (*arrow*). The sperm were loaded with fura-2 as described under "Materials and Methods." The prog CMO BSA was dissolved in 0.1% BSA, 150 mM NaCl, while the progesterone and prog CMO were dissolved in dimethyl sulfoxide. Each agonist produced an immediate increase in $[Ca^{2+}]_i$, which reached a maximum value after approximately 30 s. A representative experiment is shown.
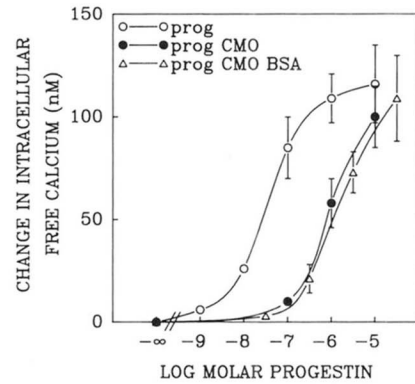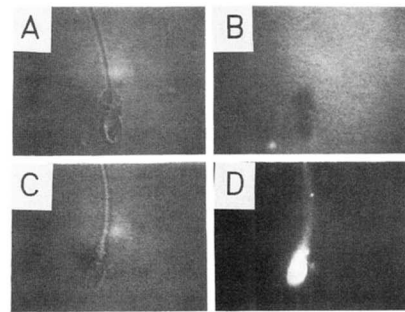
FIG. 4. **Effect of EGTA and La³⁺ on prog CMO BSA (1 μM) induced increase in [Ca²⁺]ᵢ.** Fura-2-loaded sperm were treated with prog CMO BSA at 15 s (*arrow*) in the presence of Ca²⁺ (2.5 mM), in the presence of 3.0 mM EGTA (added at zero time), or 200 μM La³⁺ (added at zero time). To the EGA-treated cells at 50 s, 3.0 mM Ca²⁺ was added back. A representative experiment of three is shown.
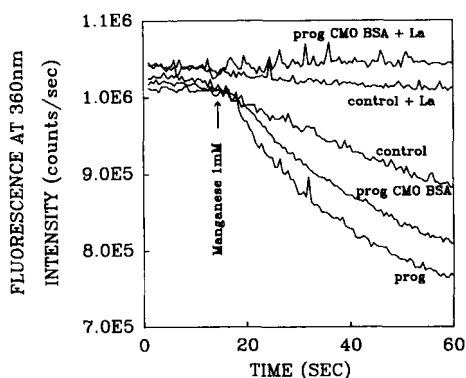

FIG. 5. **Effect of prog CMO BSA (10 μM) and prog (10 μM) to induce Ca²⁺ influx measured by observing the ability of Mn²⁺ added extracellularly to quench fura-2.** To sperm loaded with fura-2, 1 mM Mn²⁺ was added together with the various agents at 15 s (*arrow*). The excitation fluorescence used was 360 nm, the isosbestic point of fura-2. The specificity of the Mn²⁺ for the Ca²⁺ channel was verified by observing that the Ca²⁺ channel antagonist La³⁺ (200 μM) effectively blocked the Mn²⁺-induced quenching of fura-2.

was initially present. Also shown in Fig. 4 is the effect of the nonselective Ca²⁺ channel blocker, La³⁺, which also inhibited the prog CMO BSA-induced increase in [Ca²⁺]ᵢ. These data show that immobilized prog CMO BSA promotes an increase in [Ca²⁺]ᵢ by stimulating Ca²⁺ influx.

Another sensitive measure of Ca²⁺ influx is to determine the rate of intracellular fura-2 quenching by Mn²⁺ (Hallam and Rink, 1985). The divalent cation Mn²⁺ is carried by Ca²⁺ channels, hence its influx into cells is a measure of Ca²⁺ influx activity (Rink, 1990; Merritt *et al.*, 1989). The data in Fig. 5 show that prog CMO BSA and progesterone cause an immediate decline in fura-2 fluorescence induced by Mn²⁺. Although not shown, the dose response for the Mn²⁺-induced quenching of fura-2 elicited by prog CMO BSA correlates very closely with the rise in [Ca²⁺]ᵢ measured with fura-2 (Fig. 2).

To eliminate the possibility that there was no free progesterone or prog CMO in the prog CMO BSA preparation that was eliciting the increase in [Ca²⁺]ᵢ, the prog CMO BSA preparation was treated with charcoal to remove noncovalently bound progesterone or prog CMO. The data in Fig. 6 show that the charcoal treatment had no effect on the ability of 1 μM prog CMO BSA to increase [Ca²⁺]ᵢ. Charcoal was, however, capable of removing noncovalently bound progester-

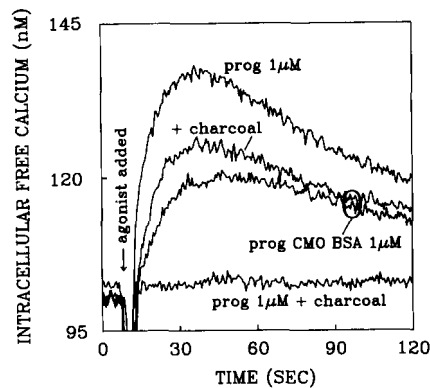FIG. 6. **Effect of charcoal treatment on the ability of prog CMO BSA and progesterone to elevate [Ca²⁺]ᵢ.** A solution of 100 μM prog CMO BSA was treated with charcoal to remove traces of free progesterone. The prog CMO BSA solution was diluted 100-fold into the sperm suspension to give a final concentration of 1 μM. Also, a solution of 100 μM free progesterone in the presence of 100 μM BSA was treated with charcoal. See "Materials and Methods" for other details. A representative experiment is shown.
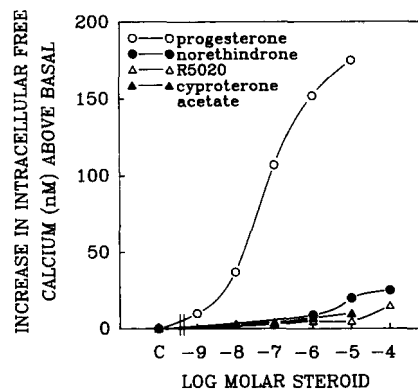

FIG. 7. **Concentration response curves for progesterone, norethindrone, R5020, and cyproterone acetate to increase [Ca²⁺]ᵢ.** See legend to Fig. 2 for details.

one from albumin (Fig. 6) and prog CMO (data not shown). These data show that the prog CMO BSA did not contain any free progesterone or prog CMO. Thus, the observed effects of prog CMO BSA on [Ca²⁺]ᵢ were due to covalently bound progesterone and not free progesterone or prog CMO in the prog CMO BSA.

The data presented above clearly show that the progesterone receptor responsible for mediating Ca²⁺ influx resides on the extracellular surface of the sperm and not on the intracellular surface. It was of interest to compare this cell surface receptor with the intracellular genomic receptor and the *Xenopus* oocyte surface progesterone receptor regarding pharmacological characteristics. The synthetic progestin agonists R5020 and norethindrone elicited a small increase in intracellular free Ca²⁺ but only at high concentrations and to only approximately 10% of the maximum effect elicited by progesterone and prog CMO BSA (Fig. 7). The antiandrogen cyproterone acetate, which is also a potent progestin (Neri, 1976), was also without effect on [Ca²⁺]ᵢ (Fig. 7). In addition to the data shown in Fig. 7, the data in Table I show the effect of several other progestins on [Ca²⁺]ᵢ. It can be seen that only 17α-hydroxyprogesterone and 11α-hydroxyprogesterone had effects on [Ca²⁺]ᵢ similar to that observed with progesterone. On the other hand, the potent synthetic genomic progestins megestrol, medroxyprogesterone acetate, norgestrel, norethindrone, and norethynodrel (Gilman *et al.*, 1990) were very

TABLE I

Effect of various progestins on $[Ca^{2+}]_i$ in human sperm

The data shown are the means ± S.E. from three separate experiments. Each steroid was dissolved in dimethyl sulfoxide and was added at a final concentration of 10 μM. The peak responses on $[Ca^{2+}]_i$ were expressed as % of the maximum progesterone effect observed.

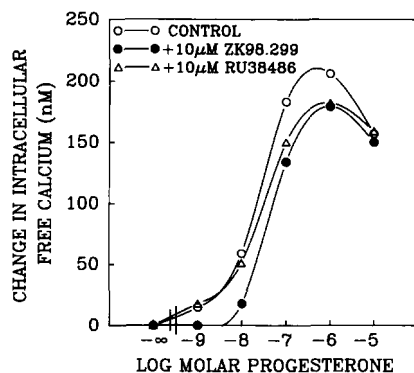| Progestin	Increase in $[Ca^{2+}]_i$ expressed as % of maximum progesterone effect
Progesterone	100
17α-Hydroxyprogesterone	133 ± 10
11α-Hydroxyprogesterone	102 ± 7
21-Hydroxyprogesterone or (11-deoxycorticosterone)	32 ± 6.9
Megestrol	5 ± 5
Medroxyprogesterone acetate	14 ± 3
Norgestrel	1 ± 1.3
Norethindrone	8 ± 4
Norethynodrel	12 ± 2

FIG. 8. **Concentration response curves of progesterone to increase $[Ca^{2+}]_i$ in the presence and absence of 10 μM ZK98.299 or 10 μM RU38486.** To fura-2-loaded sperm, 10 μM ZK98.299 or 10 μM RU38486 was added 15 s before the addition of progesterone. The peak responses were measured, which usually occurred 20–30 s after the progesterone additions. The data shown are the mean from three separate experiments.

weak agonists on the elevation of $[Ca^{2+}]_i$ in sperm. The addition of a hydroxyl group on the 21-position of progesterone (11-deoxycorticosterone or 21-hydroxyprogesterone) profoundly reduced the ability of the compound to elevate $[Ca^{2+}]_i$.

The effect of the two potent genomic antiprogestins ZK98.299 and RU38486 (Baulieu, 1989) was also examined to see whether they would block progesterone-mediated increases $[Ca^{2+}]_i$. The data in Fig. 8 show that either antagonists at 10 μM had small inhibitory effects over a wide range of progesterone concentrations. In another set of experiments, various concentrations of both antiprogestins RU38486 and ZK98.299 were examined to see if they would inhibit a submaximal (~80% of maximum) concentration of progesterone (0.1 μM). The data in Fig. 9 show that equimolar doses of ZK98.299 and RU38486 (*i.e.* 0.1 μM) had a small inhibitory effect. However, when a large 1000-fold excess of each antagonist was added, nearly complete inhibition was observed. This is in contrast to the antagonism of genomic progesterone actions that requires a 10-fold lower concentration of RU38486 to completely block progesterone effects. RU38486 and many synthetic progestins have a higher affinity for the genomic progesterone receptor than progesterone with a K_d < 1 nM (Baulieu 1989). These data indicate that the cell surface progesterone receptor responsible for eliciting an increase in $[Ca^{2+}]_i$ is distinct from the intracellular genomic
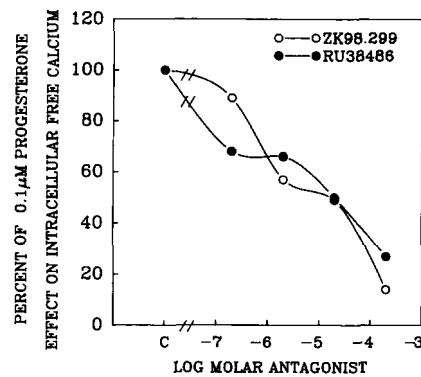

FIG. 9. **Concentration response curves of ZK98.299 or RU38486 to inhibit 0.1 μM progesterone-mediated increases in $[Ca^{2+}]_i$.** See legend to Fig. 8 for details. The data shown are the mean from three separate experiments.

progesterone receptor from the standpoint of agonist and antagonist specificity.

DISCUSSION

The data presented in this study clearly show that progesterone immobilized by covalent linkage to BSA is capable of eliciting Ca^{2+} influx in human sperm. The most likely conclusion to be drawn from the present results is that there is a receptor that is present in the plasma membrane of the spermatozoa and that the progesterone-binding site resides in that portion of the receptor that is exposed to the extracellular space.

The presence of a carboxymethyloxime group on carbon 3 of progesterone reduced its sensitivity for inducing the Ca^{2+} influx (Fig. 2). However, this is not surprising since relatively small modifications of the progesterone molecule result in a loss of potency for this response (Blackmore *et al.*, 1990). Since prog CMO and prog CMO BSA were equipotent, the presence of BSA esterified to the carboxymethyloxime linker had no influence on the activity of prog CMO to elicit its effects at the progesterone-binding site. Furthermore, both prog CMO and prog CMO BSA produced the same time action (Fig. 1) and maximal responses (Fig. 2) as progesterone, suggesting that it was the CMO derivative rather than the exclusion from the sperm cytoplasm that was responsible for the reduced potency. These data also show that the increase in $[Ca^{2+}]_i$ induced by prog CMO BSA was not due to progesterone or prog CMO being liberated during the incubation, since prog CMO BSA, prog CMO, and progesterone had identical time courses to elevate $[Ca^{2+}]_i$. One would expect a slower time course or lag with the prog CMO BSA if the progesterone or prog CMO had to be liberated by hydrolysis first before it could stimulate Ca^{2+} influx. However, this was not observed.

Evidence has been presented to support the notion that cell surface steroid receptors do exist in several other systems (*e.g.* Godeau *et al.*, 1978; Ishikawa *et al.*, 1977; Ke and Ramirez, 1990). Having identified that the progesterone-binding moiety is present in the plasma membrane of the sperm and that it is distinct from the cytosolic nuclear progesterone receptor, then one can consider various possibilities whereby this receptor can couple to the mechanism that is responsible for the Ca^{2+} entry process. Some possibilities are as follows. 1) The generation of an inositol lipid breakdown product may promote Ca^{2+} influx. However, evidence suggests that inositol lipid breakdown occurs following an elevation in $[Ca^{2+}]_i$ (Blackmore *et al.*, 1990; Roldan and Harrison, 1989; Thomas and Meizel, 1989). 2) The progesterone receptor activates a G

protein (Birnbaumer *et al.*, 1990) that then activates the Ca^{2+} channel. The latter possibility does not appear to be operative since the nonselective G protein activator AlF_4 (*e.g.* Blackmore *et al.*, 1985) failed to promote Ca^{2+} influx (Blackmore *et al.*, 1990). 3) The progesterone receptor may stimulate a protease that activates the Ca^{2+} influx process. In support of this possibility is the observation that the human sperm acrosome reaction is inhibited by proteinase inhibitors (De-Jonge *et al.*, 1989). 4) The progesterone receptor activates some other second messenger system, such as phospholipase D, which if activated would elevate phosphatidic acid levels (*e.g.* Bocckino *et al.*, 1987). This activity has been demonstrated in sea urchin sperm (Domino *et al.*, 1989). Perhaps phosphatidate acts as a second messenger to elevate $[Ca^{2+}]_i$ (see Bocckino *et al.* (1987) for references). 5) The progesterone receptor is a Ca^{2+} channel or a binding site on a Ca^{2+} channel analogous to the steroid-binding site on the neuronal Cl^- ion channel (Wu *et al.*, 1990). 6) The progesterone receptor couples directly to a Ca^{2+} channel by some unknown mechanism.

Another feature of the data presented is the lack of similarity of this receptor to the genomic progesterone receptor in regard to steroid selectivity. The synthetic genomic agonists R5020, norgestrel, norethynodrel, medroxyprogesterone acetate, megestral, and norethindrone were very weak partial agonists, and the potent genomic antiprogestins RU38486 and ZK98.299 were very weak antagonists of the progesterone effects in sperm (Figs. 8 and 9) compared with their potency in affecting the genomic receptor.

Addendum—After the submission of this manuscript, another study was submitted showing that prog CMO BSA stimulated the sperm acrosome reaction (Meizel and Turner, 1991).

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