

## Effects of progesterone on sperm function: mechanisms of action

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Progesterone stimulates sperm functions, e.g. hyperactivation, acrosome reaction, binding to oocyte zona pellucida and penetration rate into the hamster oocyte. The physiological relevance of these effects has been shown using female genital tract fluids which modulate sperm function according to their progesterone content. Progesterone interacts with specific sperm binding sites that, unlike the classic nuclear receptors, are located on the plasma membrane of the spermatozoon. Binding studies have revealed the presence of two classes of progesterone receptors in the human spermatozoon, one class has an elevated affinity constant (nanomolar) and is specific for progesterone, whereas the other class has an affinity constant in the micromolar range and binds equally well other hydroxylated progesterone derivatives. Following exposure to progesterone, the main event is a rapid (within seconds) increase of the intracellular free calcium concentration, followed by a sustained rise lasting for several minutes (plateau phase). Both these calcium transients are dependent upon entry of extracellular calcium. The nature of the calcium channel that mediates the effects of progesterone is, currently, unknown. It has been postulated that it may be: (i) part of the progesterone receptor; (ii) voltage-dependent; or (iii) operated by second messengers following activation of the progesterone receptor. Progesterone also modulates sperm function by stimulating a trypsin-like proteolytic activity, the biosynthesis of polyamine (putrescine and spermidine), phospholipase A<sub>2</sub> activity and protein tyrosine kinase activity in the sperm cell. Recent studies have shown that chloride ion efflux is vital for progesterone to promote the acrosome reaction. This effect is achieved by interaction with a sperm membrane receptor which resembles the neuronal GABA<sub>A</sub> receptor. Accordingly, GABA<sub>A</sub> receptors have been found in the spermatozoon plasma membrane and GABA stimulates hyperactivation and promotes the acrosome reaction.

*Key words:* GABA<sub>A</sub> receptor/intracellular calcium/progesterone/sperm function

## Introduction

Mammalian spermatozoa must spend some time in the female genital tract or under certain conditions *in vitro* before being able to fertilize the oocyte. During this time, spermatozoa undergo a not completely understood cellular process named capacitation which culminates with the acrosome reaction, a modified exocytotic event involving fusion followed by vesiculation of sperm head membranes (Yanagimachi, 1994).

It is widely accepted that a glycoprotein of the zona pellucida (ZP3) is the *in vivo* initiator of the acrosome reaction of a fertilizing spermatozoon (Kopf and Gerton, 1991). However, human spermatozoa can also undergo an acrosome reaction when they come into contact with the cumulus oophorus, a clump of ovarian follicular cells surrounding the ovulated oocyte (reviewed in Meizel *et al.*, 1990).

Progesterone, present around or secreted by cumulus oophorus cells, is capable of initiating the acrosome reaction *in vitro* in spermatozoa of several mammals, including humans. Hence, it has been postulated that this steroid, acting by itself and/or in synergy with the zona pellucida, is another physiological initiator of the acrosome reaction *in vivo* (Meizel *et al.*, 1990). Indeed, a synergic action between solubilized zona pellucida and progesterone on the acrosome reaction has been found in mouse spermatozoa *in vitro* (Roldan *et al.*, 1994). However, to act as a 'primer', progesterone must be added to capacitated spermatozoa a few minutes earlier than the addition of the zona pellucida extracts. Direct evidence for the existence of this type of mechanism is lacking in humans and there are conflicting reports on whether acrosome-reacted human spermatozoa retain unmodified their capacity to bind the zona pellucida (Morales *et al.*, 1989; Liu and Baker, 1990). Evidence supporting a physiological role for progesterone in the human sperm acrosome reaction comes from reports suggesting a relationship between male infertility and the inability of spermatozoa to respond to progesterone *in vitro* (Tesarik and Mendoza, 1992; Falsetti *et al.*, 1993; Oehninger *et al.*, 1994).

## Progesterone and sperm function

Progesterone induces sperm hyperactivation (Uhler *et al.*, 1992; Sueldo *et al.*, 1993; Calogero *et al.*, 1996), stimulates the acrosome reaction (Osman *et al.*, 1989; Foresta *et al.*, 1992; Parinaud *et al.*, 1992; Bronson *et al.*, 1999), increases the binding of spermatozoa to the oocyte zona pellucida (Sueldo *et al.*, 1993), and increases the sperm penetration rate into hamster oocytes (Margalioth *et al.*, 1988; Sueldo *et al.*, 1993) (Table I).

The physiological relevance of these effects have been proven by studies involving incubation of spermatozoa with female genital tract fluids which modulate sperm function in a manner related to their steroid content. For example, the follicular fluid exerts a stimulating effect on human sperm motility (Calvo

**Table I.** Effects of progesterone on sperm function

Induction of sperm hyperactivation
Induction of the acrosome reaction
Enhancement of the binding of spermatozoa to the oocyte zona pellucida
Increment of the sperm penetration rate into the hamster oocyte

*et al.*, 1989; Falcone *et al.*, 1991; Revelli *et al.*, 1992) and acrosome reaction (Tesarik, 1985; Suarez *et al.*, 1986; Calvo *et al.*, 1989; Stock *et al.*, 1989; Siegel *et al.*, 1990; Revelli *et al.*, 1992) and these effects disappear when follicular fluid samples are stripped of steroids.

High performance liquid chromatography of human follicular fluid revealed two peaks with acrosome reaction-stimulating activity and these peaks were identified as progesterone and 17 $\alpha$ -hydroxy-progesterone (Osman *et al.*, 1989). Another study (Morales *et al.*, 1992) further supported the hypothesis that progesterone is the compound which mediates the biological activity of the follicular fluid on sperm function. These authors observed a significant correlation between progesterone concentration of individual follicular fluid samples and their competence to stimulate the acrosome reaction. In contrast, no correlation was observed between the rate of acrosome reaction and the follicular fluid content of other steroids, such as 17 $\beta$ -oestradiol, testosterone, or 17 $\alpha$ -hydroxy-progesterone. Furthermore, the biological activity of the follicular fluid could be removed by treatment with activated charcoal, which extracts steroids, and replaced by the addition of exogenous progesterone, but not of 17 $\beta$ -oestradiol.

We found that progesterone induces the acrosome reaction in a concentration-dependent manner with a half-maximal stimulatory concentration of 32.4  $\pm$  1.97  $\mu$ g/ml (Calogero *et al.*, 1999). Even at concentrations ~50-fold higher than those found in the follicular fluid, progesterone retains its ability to promote the acrosome reaction without affecting sperm viability (Parinaud *et al.*, 1992). Although its biological effect is lower than that of the calcium ionophore A23187, a non-physiological acrosome reaction inducer, its lower cytotoxic effect resulted in a similar percentage of live, acrosome-reacted spermatozoa. In addition, progesterone did not significantly affect total sperm motility, whereas A23187 had a detrimental effect.

Progesterone has been found able to increase significantly the number of spermatozoa moving with hyperactivated motility even at very low concentrations (~3 ng/ml). This effect was observed on both capacitated or capacitating cells and appeared after 10 min of incubation. Higher concentrations did not result in a further increase of the percentage of hyperactivated cells (Uhler *et al.*, 1992). We examined the effects of progesterone on sperm kinematic parameters evaluated by computer-assisted semen analysis (CASA) (Calogero *et al.*, 1996). Progesterone increased beat cross frequency and curvilinear velocity, and decreased linearity of progression and straightness of motile spermatozoa. No significant effect was seen on the other parameters. This resulted in an almost three-fold increase of the percentage of spermatozoa moving with hyperactivated motility (Table II).

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**Table II.** Effects of progesterone on sperm kinematic parameters and hyperactivation (modified from Calogero *et al.*, 1996)

	Control (n = 12)	Progesterone (n = 12)
Beat cross frequency (Hz)	4.93 ± 0.15	5.27 ± 0.14**
Curvilinear velocity (µm/s)	44.7 ± 0.57	48.5 ± 1.39*
Linearity of progression (%)	46.8 ± 2.0	41.0 ± 2.1**
Straight line velocity (µm/s)	21.3 ± 0.93	20.3 ± 1.32
Straightness (%)	77.4 ± 2.0	72.5 ± 2.2**
Lateral head displacement (µm)	3.72 ± 0.23	3.71 ± 0.13
Average path velocity (µm/s)	27.7 ± 0.96	27.7 ± 1.18
Hyperactivation <sup>a</sup> (%)	1.45 ± 0.32	3.82 ± 0.84**

\* $P < 0.05$ ; \*\* $P < 0.005$  compared with control (Student's paired *t*-test).

<sup>a</sup>Spermatozoa moving with a curvilinear velocity  $\geq 70$  µm/s, linearity of progression  $\leq 65\%$ , and with lateral head displacement  $\geq 5$  µm.

### Sperm progesterone receptor

Like other steroids, progesterone acts to induce transcriptional events mediated by an intracellular receptor belonging to the superfamily of nuclear transactivators. However, in the case of the spermatozoon, progesterone acts on an almost completely transcriptionally inactive cell, with mechanisms that are not responsive to antagonists of the classic progesterone receptor (Blackmore *et al.*, 1991). Therefore, the mechanisms by which progesterone elicits a biological response from human spermatozoa involve interactions with a cell surface receptor and this is further substantiated by the rapid onset of its biological effects (Thomas and Meizel, 1989; Blackmore *et al.*, 1990, 1991). A demonstration of the presence of a surface receptor comes from studies showing that progesterone covalently conjugated to serum albumin, a molecule which does not allow the steroid to cross the sperm membrane, is able to increase the intracellular concentrations of free calcium and to induce the acrosome reaction (Blackmore *et al.*, 1991; Meizel and Turner, 1991). More recently, the presence of masked progesterone receptors has been shown on human sperm membranes treated with surfactants (Ambhaikar and Puri, 1998). These binding sites seem to have a structural homology with the nuclear progesterone receptor, but differ in terms of ligand specificity and location.

Since one of the mechanisms by which cell surface receptors transduce ligand-generated signals across the plasma membrane is receptor aggregation, other authors (Tesarik *et al.*, 1992b) evaluated whether this mechanism also occurs in spermatozoa. The addition of non-stimulatory concentrations of progesterone followed by antibody anti-progesterone, which causes receptor aggregation, results in an increase of the intracellular free calcium concentrations and an induction of the acrosome reaction. The presence of this mechanism is of particular interest in light of the fact that steroids are complexed with proteins in many biological systems. If one protein carries several molecules of the steroid, the protein can bridge plasma membrane receptors recognized specifically by the steroid, thus promoting receptor aggregation which, in turn, will trigger

a cascade of biochemical events culminating in the activation of the intracellular effectors of the hormone response.

In this regard, a progesterone-binding protein is an essential component of the acrosome reaction-inducing capacity of the follicular fluid. In fact, treatment of human follicular fluid with proteases results in a loss of biological activity that cannot be restored by the addition of progesterone. Preliminary biochemical characterization of this substance revealed a concentration of activity in a peak spanning the molecular mass of 37–53 kDa (Fehl *et al.*, 1995). Since the acrosome-inducing competence of the human follicular fluid is suppressed by the addition of antibodies against corticosteroid-binding globulin (CBG) and the addition of CBG to progesterone significantly increases its ability to promote the acrosome reaction (Miska *et al.*, 1994), the progesterone-binding protein seems to be similar to the 50 kDa CBG. A lack of CBG may be one of the reasons why some studies have failed to find any effect by progesterone on the acrosome reaction (Carver-Ward *et al.*, 1992).

A conjugate of fluorescein isothiocyanate (FITC)-labelled bovine serum albumin (BSA) and progesterone has been used to localize progesterone receptors in the sperm plasma membrane. The results of these studies showed that only a relatively small percentage of spermatozoa (~10–30%) binds this hormone and that the binding has a marked topographical selectivity, being restricted to the whole or the equatorial acrosomal region (Blackmore *et al.*, 1991; Tesarik *et al.*, 1992a). Using an antibody directed against the C-terminal portion of the genomic progesterone receptor and able to inhibit the steroid-promoted acrosome reaction activity, a 52 kDa antigen has been found in the equatorial segment of ~50% of sperm heads (Sabeur *et al.*, 1996). These findings suggested that the expression of the progesterone receptor on human spermatozoa is the major factor determining the biological responsiveness of individual sperm samples to progesterone stimulation. Such results led some authors to propose that the low number of spermatozoa that bind FITC-labelled BSA–progesterone may be due to the possibility that only capacitated spermatozoa exhibit the receptor (Mendoza and Tesarik, 1993).

The paucity of cells exhibiting the progesterone receptor is, however, in contrast with recent data employing image analysis techniques to monitor the size of the sperm population that responds to progesterone, which indicate that >90% of all spermatozoa do so (Plant *et al.*, 1995). This evidence suggests that the binding of FITC-labelled BSA–progesterone to human spermatozoa does not reflect the true size of the progesterone-responsive cell population.

Computer analysis of binding competition curves with different steroids revealed the presence of two distinct binding sites for progesterone in human spermatozoa. The high affinity site ( $K_d$  in the nanomolar range) appears to be specific for progesterone, whereas the low affinity one ( $K_d$  in the micromolar range) binds with equal affinity  $11\beta$ -hydroxyprogesterone and  $17\alpha$ -hydroxyprogesterone. A significant correlation exists among the affinity constants of these binding sites and the potency of these steroids to increase the intracellular concentration of free calcium. In particular, dose–response curves for progesterone

**Table III.** Mechanisms

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**Table III.** Mechanisms of progesterone action on sperm function

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Increase of the intracellular free calcium concentration
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Depolarization of the plasma membrane (due to sodium influx)
Stimulation of a trypsin-like proteolytic activity
Activation of polyamine biosynthesis pathway
Activation of protein tyrosine kinase
Stimulation of protein kinase C
Increase of the intracellular concentration of cAMP
Activation and redistribution of extracellular signal-regulated kinases
Activation of phospholipase A <sub>2</sub> and increased biosynthesis of platelet-activating factor
Maintenance of a basic intracellular pH.

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were biphasic, with an EC<sub>50</sub> in the nm range and another in the micromolar range. Conversely, curves for the other two steroids were monophasic, with an EC<sub>50</sub> in the μm range (Luconi *et al.*, 1998b).

### Progesterone mechanisms of action

A number of mechanisms of action may occur when spermatozoa are exposed to progesterone (Table III). Among them, the increase of the intracellular concentration of free calcium plays a critical role and the activation of other pathways seems to increase the availability of this ion.

#### *Calcium ion transmembrane flux*

An increase of the intracellular free calcium concentration is observed when progesterone activates human spermatozoa (Thomas and Meizel, 1989; Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Foresta *et al.*, 1993; Aitken *et al.*, 1996). During incubation with progesterone, there is an immediate increase of free calcium which takes place within seconds after the addition of the steroid, followed by a sustained increase (plateau phase) which may last for minutes. A similar increment is also observed in non-capacitated human spermatozoa, although they do not acrosome react (Thomas and Meizel, 1989; Meizel *et al.*, 1990; Blackmore *et al.*, 1990), suggesting that capacitation involves more than just an increase of intracellular free calcium concentration. Using video-image analysis, it has been shown that progesterone treatment of capacitated spermatozoa resulted in a wave-like increase in sperm head cytosolic free calcium that appears to increase fastest in a region near the equatorial segment and then spreads throughout the rest of the head (Meizel *et al.*, 1997). Single cell analysis showed the presence of two increases of intracellular free calcium (Tesarik *et al.*, 1996).

The transient of the intracellular free calcium after progesterone exposure is dependent upon the presence of extracellular free calcium (Thomas and Meizel, 1989; Blackmore *et al.*, 1990). Even in the presence of compounds able to mobilize the intracellular free calcium stores (inhibitors of the endoplasmic

reticulum calcium-ATPase pump), the acrosome reaction requires an influx of extracellular free calcium (Meizel and Turner, 1993a). In many cells, the endoplasmic reticulum is the site of calcium stores, but the endoplasmic reticulum in the cytoplasm of mature spermatozoa is almost completely absent. Hence, hypothetical calcium storage sites in the spermatozoon are the nucleus and the outer acrosomal membrane (Blackmore, 1993; Meizel and Turner, 1993a). Interestingly, receptors for inositol trisphosphate, a physiological releaser of intracellular calcium stores, have been detected in the acrosomal region of rat spermatozoa (Walensky and Snyder, 1995).

The nature of the calcium channel that mediates the effects of progesterone on sperm function is not known and there are conflicting reports concerning the effects of various calcium channel blockers (for review, see Shi and Roldan, 1995). The calcium channel may be: (i) part of the progesterone receptor; (ii) voltage-dependent and hence opened by depolarization of the plasma membrane; or (iii) operated by second messengers following activation of the progesterone receptor. Inhibitor studies indicate that neither voltage-sensitive nor second messenger-operated channels are involved in the generation of the primary calcium transient in response to progesterone (Aitken *et al.*, 1996).

### *Sodium ion transmembrane flux*

In an attempt to clarify the mechanism through which progesterone raises intracellular free calcium, one study (Foresta *et al.*, 1993) found that the intracellular free calcium content in response to progesterone was potentiated in the absence of sodium ions. The acrosome reaction and plasma membrane depolarization (due to sodium influx) were not affected by sodium ions, whereas the absence of calcium caused a larger depolarization following progesterone exposure. These findings led the authors (Foresta *et al.*, 1993) to postulate that sodium and calcium ions penetrate into the cell through the same channel. In a subsequent study, however, they gated experimental information to suggest the presence of two distinct channels activated by progesterone: one for calcium and the other for sodium (Foresta *et al.*, 1995). A study by another laboratory partially contradicted these findings by showing that progesterone loses its acrosome reaction-inducing effect in the absence of sodium ions (Garcia *et al.*, 1996).

### *Proteolytic activity*

Trypsin-like activity in spermatozoa seems to play a role in the progesterone-mediated increase of intracellular free calcium concentration. Pre-incubation of capacitated human spermatozoa with inhibitors of trypsin-like enzymes suppresses almost completely the increase of intracellular free calcium and the acrosome reaction induced by exposure to progesterone. Transmission electron microscopic examination of spermatozoa after progesterone treatment confirmed that the inhibitors blocked the calcium-dependent membrane fusion events of the acrosome reaction (Pillai and Meizel, 1991). Since such inhibitors do not modulate the

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binding of progesterone to its receptor (Tesarik and Mendoza, 1993), these results suggest that some sperm trypsin-like activity is directly or indirectly involved in increasing sperm intracellular free calcium concentrations during stimulation with progesterone.

### *Polyamines*

The polyamine putrescine and its derivative spermidine and spermine are present in mammalian spermatozoa (Breitbart *et al.*, 1997). In an attempt to further clarify the mechanism of progesterone action of sperm function, Meizel and Turner (1993b) studied the effects of DFMO, an inhibitor of putrescine synthesis, and MDL-73811, an inhibitor of spermidine and spermine synthesis, on progesterone-induced sperm function. DFMO inhibited the acrosome reaction induced by progesterone and preincubation with putrescine or with spermidine counteracted this inhibition. MDL-73811 also inhibited the progesterone-induced acrosome reaction and preincubation with spermidine, but not with putrescine or spermine, reversed this inhibition. Both DFMO and MDL-73811 partially inhibited the rapid calcium transient produced by progesterone which was partially reversed by putrescine and spermidine, respectively. Putrescine or spermidine alone did not have any effect on acrosome reaction or intracellular free calcium concentrations. Neither inhibitor was able to inhibit the acrosome reaction initiated by the calcium ionophore ionomycin. These data suggest that the sperm polyamine biosynthetic pathway, leading to a rise of spermidine, mediates the rapid increase of intracellular free calcium concentrations and the subsequent acrosome reaction induced by progesterone. Although polyamines can activate a number of mammalian cell enzymes and can enhance the ligand binding affinity of several brain receptors (Williams, 1994), the precise mechanisms involved in the sperm function are not known.

### *Protein tyrosine kinase*

Progesterone stimulation triggers changes in the tyrosine phosphorylation status of spermatozoa (Tesarik *et al.*, 1993). The target for this signal transduction pathway is not known, but it could relate to phosphoinositidase C-mediated hydrolysis of the polyphosphoinositides seen after stimulation with progesterone (Thomas and Meizel, 1989). It has been proposed that one of the progesterone binding sites on the human spermatozoon is itself a tyrosine kinase receptor, that can be activated by a progesterone-induced oligomerization process (Tesarik *et al.*, 1993). Indeed, the progesterone-induced opening of the plasma membrane calcium channel and the activation of protein tyrosine kinase are two distinct reactions triggered by the steroid which can be experimentally dissected (Mendoza *et al.*, 1995). These effects of progesterone, however, influence each other. A recent study, using a single cell analysis protocol, showed that inhibition of protein tyrosine kinase does not influence the initial progesterone-induced calcium transient, but inhibits the secondary free calcium increase, and the resulting



acrosome reaction. The initial protein tyrosine kinase-independent calcium response can be induced by progesterone in both non-capacitated and capacitated spermatozoa, whereas the ability to generate a secondary, protein tyrosine kinase-dependent response developed during in-vitro capacitation (Tesarik *et al.*, 1996).

### *Protein kinase C*

Indirect studies suggest that protein kinase C has a role in sperm motility and acrosome reaction. It may be involved in progesterone-stimulated acrosome reaction, although controversial results have been obtained concerning the effect of protein kinase C inhibition of progesterone-stimulated intracellular free calcium increase and tyrosine phosphorylation of proteins. Recently, it has been reported that progesterone stimulates protein kinase C and that its inhibition partially reverses the effects of progesterone on the acrosome reaction, whereas it was devoid of effect on progesterone-stimulated calcium transient. In addition, preincubation with protein kinase C inhibitors have a suppressive effect on tyrosine phosphorylation of sperm proteins induced by progesterone (Bonaccorsi *et al.*, 1998). These results suggest that protein kinase C plays a role in progesterone-induced acrosome reaction and that progesterone-stimulated activation of protein kinase C is downstream to stimulation of calcium influx by the steroid.

### *cAMP*

Progesterone action does not appear to involve activation of G proteins (Foresta *et al.*, 1993), whereas it may involve generation of cAMP. Indeed, Parinaud and Milhet (1996) found that progesterone increases sperm cAMP content in a concentration-dependent fashion. Kinetic study revealed the presence of two peaks, a smaller one occurring after 30 min and a more robust peak detected after 120 min of incubation. These effects of progesterone on intracellular cAMP content were partially inhibited by the tyrosine kinase inhibitor genistein and correlated positively with the percentage of hyperactivated spermatozoa occurring at the same time.

### *Extracellular signal-regulated kinases*

Extracellular signal-regulated kinases (ERKs) are cytoplasmic and nuclear serine/threonine kinases present in spermatozoa which seem to be involved in the regulation of the capacitation process (Luconi *et al.*, 1998a). It has been shown that progesterone modulated the activity of ERKs. Indeed, short-term incubation of spermatozoa with progesterone induces phosphorylation and activation of these kinases, resulting in a redistribution of the proteins from the post-acrosomal region to the equatorial segment of the sperm head. Interestingly, the ERK cascade inhibitor PD-098099 suppresses progesterone-induced ERK-2 activation, prevents the redistribution of the enzyme to the equatorial region of the sperm

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head, but does not have any effect on progesterone-induced acrosome reaction (Luconi *et al.*, 1998c).

### *Platelet-activating factor*

Stimulation of spermatozoa with progesterone also leads to activation of phospholipase A<sub>2</sub> and synthesis of alkyl-acetyl-glycerophosphocholine (platelet-activating factor) (Baldi *et al.*, 1993), but mechanisms regulating these processes are also unknown.

### *Intracellular pH*

The absence of extracellular bicarbonate partially suppresses the progesterone-induced acrosome reaction in human spermatozoa (Sauber and Meizel, 1995). Recently, a study was published (Aitken *et al.*, 1998) which evaluated the mechanism(s) of this interaction. They found bicarbonate to exert a dose-dependent impact on the ability of progesterone to promote sperm-oocyte fusion. The loss of sperm function in bicarbonate-free medium was found associated with a failure to produce reactive oxygen species, an impaired capacity to exhibit redox-associated changes in tyrosine phosphorylation, and an apparent incapacity to generate normal calcium transients in response to progesterone. These defects were not related to a cAMP deficiency, but were associated with a significant fall in intracellular pH, emphasizing the importance of an alkaline intracellular milieu for the extragenomic action of progesterone.

### **Progesterone and the GABA<sub>A</sub> receptor**

A growing body of literature has shown that progesterone modulates sperm function by interacting with a membrane-binding site resembling the  $\gamma$ -aminobutyric acid (GABA<sub>A</sub>) receptor. It has been shown that the progesterone-initiated acrosome reaction could be prevented by preincubation of capacitated spermatozoa with chloride channel blockers or incubation in medium devoid of chloride ions (Wistrom and Meizel, 1993). Our findings further substantiate this hypothesis. In fact, blockade of the GABA<sub>A</sub> receptor with picrotoxin, a chloride channel blocker, or bicuculline did not allow progesterone to induce the acrosome reaction (Calogero *et al.*, 1999). The efficacy of bicuculline in suppressing progesterone-initiated acrosome reaction is probably due to its ability to block the chloride channel (Aprison and Lipkowitz, 1989) where the steroid appears to have a binding site (Turner *et al.*, 1989). Recently, the presence of chloride channels has been revealed in mouse spermatozoa by patch-clamp recording (Espinosa *et al.*, 1998).

### *Neuronal GABA<sub>A</sub> receptor*

The GABA<sub>A</sub> receptor is a plasma membrane multisubunit receptor complex constituting a chloride channel. The binding site for GABA on the GABA<sub>A</sub>

receptor is identified by the binding of the antagonist bicuculline, whereas muscimol is the classic competitive agonist for this site. Activation of the GABA<sub>A</sub> receptor results in the opening of the chloride channel, leading to an influx of chloride ions and cell hyperpolarization in the neuron. Both benzodiazepines and barbiturates modulate GABA<sub>A</sub> receptors allosterically by interacting with specific binding sites present on this molecule (Bowery *et al.*, 1984). More recently, progesterone and its metabolites have been shown to enhance the interaction of GABA with the GABA<sub>A</sub> receptor in the central nervous system (Lan *et al.*, 1991). Indeed, progesterone amplifies GABA-induced chloride currents in the spinal cord (Majewska *et al.*, 1986).

### Sperm GABA<sub>A</sub> receptor

Progesterone and its metabolites with a hydroxyl group (OH) in position 3 $\alpha$  (anaesthetically active) potentiate GABA activation of neuronal GABA<sub>A</sub> receptors and some of them are able to activate this receptor by themselves, whereas progesterone metabolites with a hydroxyl group in position 3 $\beta$  (anaesthetically inactive) are devoid of this effect. Using a number of these metabolites, one study (Wistrom and Meizel, 1993) determined that anaesthetically-active metabolites initiated the acrosome reaction, whereas the 3 $\beta$ -OH isomers were without effect. The greater effectiveness of progesterone, a progestin which is not able to activate the neuronal GABA<sub>A</sub> in the absence of GABA, suggested that the GABA<sub>A</sub> receptor present in the human spermatozoon is not identical to that present in the neuron. The difference with the neuronal GABA<sub>A</sub> receptor is further illustrated by the fact that activation of the sperm GABA<sub>A</sub>-like receptor results in an efflux of chloride ions rather than an influx as reported in the neuronal tissue. The stimulation of a chloride efflux is not simply a matter of opening a channel because the chloride concentration in the extracellular space is significantly higher than the sperm cytoplasm. Chloride ions have therefore to be pumped out of the cell against a concentration gradient. In this context, it may be significant that induction of chloride efflux by progesterone is tyrosine phosphorylation-dependent (Meizel and Turner, 1996).

The presence of GABA receptors on human spermatozoa has been evaluated by using a monoclonal antibody to the bovine cerebral cortex GABA<sub>A</sub> receptor  $\alpha$ -subunit. Immunoreactivity was observed in live and fixed spermatozoa as a fluorescent band in the plasma membrane overlying or near the narrow equatorial segment region of the acrosome. Immunoblotting using this antibody detected two major bands; one with an apparent molecular weight of 50 kDa, as reported in other cell types, and a second of 75 kDa, which has not been reported from other cells (Wistrom and Meizel, 1993). Accordingly, Aanesen *et al.* (1995) found the presence of GABA binding sites on the surface of human spermatozoa, and they seem to be the GABA<sub>A</sub> receptor (Erdö and Wekerle, 1990; Ritta *et al.*, 1998).

**Table IV.** Effects of the selected GABA<sub>B</sub> receptor antagonist reaction of capacitated human spermatozoa

Experiment	n	C
Experiment 1	(n = 21)	(
Experiment 2	(n = 11)	(
Experiment 3	(n = 9)	(
Experiment 4	(n = 21)	(
Experiment 5	(n = 12)	(
Experiment 6	(n = 9)	(

n = number of replicates; C  
\*P < 0.05 compared with control

### Sperm

The acrosome reaction is affected by chloride ion blockers or exposure to the increase of intracellular calcium (Turner *et al.*, 1991; Turner *et al.*, 1991). Chloride ions are involved in the regulation of the biological expression of the biological clock. The chloride efflux from the sperm membrane and not the opening of the post-synaptic chloride channel by neurotransmitter GABA<sub>A</sub> receptors is an additional set of channels that regulate calcium transient and tyrosine phosphorylation. Evidence for the involvement of extragenomic actions of progesterone, GABA<sub>A</sub> receptors, and their blocked by EGTA and

The presence of GABA<sub>A</sub> receptors on human spermatozoa is substantiated by a number of studies (Aanesen *et al.*, 1995; Shi *et al.*, 1995). We have recently demonstrated that the GABA<sub>A</sub> receptor mediates its stimulation (Ritta *et al.*, 1999). GABA<sub>A</sub> receptors seem to be involved in the regulation of the GABA<sub>A</sub> receptor

**Table IV.** Effects of the selective GABA<sub>A</sub> receptor antagonist bicuculline or the selective GABA<sub>B</sub> receptor antagonist saclofen on the basal and GABA-stimulated rate of acrosome reaction of capacitated human spermatozoa

	GABA ( $\mu$ M)	Bicuculline ( $\mu$ M)	Saclofen ( $\mu$ M)	Acrosome-reacted spermatozoa ( $\mu$ M)
Experiment 1 ( $n = 21$ )	0	0	0	3.6 $\pm$ 0.3
Experiment 2 ( $n = 11$ )	0	100	0	3.1 $\pm$ 0.5
Experiment 3 ( $n = 9$ )	0	0	100	5.7 $\pm$ 0.6
Experiment 4 ( $n = 21$ )	10	0	0	10.6 $\pm$ 1.3*
Experiment 5 ( $n = 12$ )	10	100	0	4.7 $\pm$ 0.6**
Experiment 6 ( $n = 9$ )	10	0	100	7.2 $\pm$ 0.8***

$n$  = number of replicates; GABA =  $\gamma$ -aminobutyric acid.

\* $P < 0.05$  compared with experiment 1; \*\* $P < 0.05$  compared with experiment 4.

### *Sperm GABA<sub>A</sub> receptor and calcium influx*

The acrosome reaction initiated by the calcium ionophore ionomycin is not affected by chloride ions (Wistrom and Meizel, 1993). Similarly, chloride channel blockers or exposure to GABA<sub>A</sub> receptor antagonists do not have any effect on the increase of intracellular free calcium induced by progesterone (Baldi *et al.*, 1991; Turner *et al.*, 1994; Aitken *et al.*, 1996). However, the chloride flux may be involved in the regulation of secondary calcium transient essential for the expression of the biological response (Tesarik *et al.*, 1996; Meizel *et al.*, 1997). The chloride efflux from the spermatozoon induces depolarization of the plasma membrane and not the hyperpolarization observed when chloride channels are opened in the post-synaptic membranes of neurons in response to the inhibitory neurotransmitter GABA. This chloride-induced depolarization may then open an additional set of channels that are voltage-regulated, leading to the secondary calcium transient and the induction of acrosome reaction (Tesarik *et al.*, 1996). Evidence for the involvement of voltage sensitive channels in mediating the extragenomic actions of progesterone has come from studies on murine (Shi and Roldan, 1995) and human (Shi *et al.*, 1997) spermatozoa in which the abilities of progesterone, GABA and muscimol to induce the acrosome reaction were blocked by EGTA and calcium channel antagonists.

### *GABA and sperm function*

The presence of GABA receptors on spermatozoa and their biological competence is substantiated by a number of studies showing that GABA is, by itself, able to initiate the acrosome reaction in human (Wistrom and Meizel, 1993; Aanesen *et al.*, 1995; Shi *et al.*, 1997) and murine spermatozoa (Shi and Roldan, 1995). We have recently conducted a study in an attempt to establish which GABA receptor mediates its stimulatory effect on the acrosome reaction (Calogero *et al.*, 1999). GABA seems to stimulate acrosome reaction of human spermatozoa through both GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Table IV). However, the involvement of the GABA<sub>A</sub> receptor is more pronounced since muscimol, a selective

agonist for this receptor, mimicked almost entirely the effects of GABA. Although no data are available on the presence of the GABA<sub>B</sub> receptor in human spermatozoa, the findings that baclofen (a selective agonist for this receptor), induced the acrosome reaction and that the stimulatory effect of GABA was not completely overridden by the chloride channel blocker picrotoxin, shed some light on a possible functional role of this receptor. This is more relevant if we consider that other biological actions of GABA on human sperm function, such as modulation of kinematic parameters and hyperactivation, are not mediated exclusively by the GABA<sub>A</sub>, but also by the GABA<sub>B</sub> receptor (Calogero *et al.*, 1996).

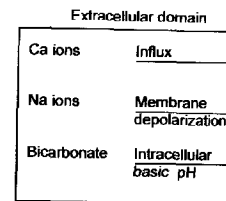
Since GABA receptors and the GABA uptake system are present in the human ovary, uterus, and Fallopian tubes and the concentration of GABA in the epithelial tissue of the Fallopian tube increases towards the ampulla (Erdö, 1989; László *et al.*, 1992), these findings suggest that this neurotransmitter, alone or in conjunction with progesterone, may be regarded as another physiological modulator of the sperm function.

### Conclusions

Progesterone modulates many aspects of the sperm function and it is the compound which mimics almost entirely the acrosome reaction-inducing properties of the follicular fluid. The effects of progesterone on sperm function are mediated by receptors located on the plasma membrane. Albeit their exact nature is not known, experimental evidence suggests that there may be at least three types of receptors: a plasma membrane calcium channel, a GABA<sub>A</sub>-like receptor and a membrane-associated protein tyrosine kinase (Tesarik, 1996).

The main mechanism through which progesterone modulates the sperm function is the increase of the intracellular free calcium concentration. Indeed, following exposure to progesterone, there is a rapid (within seconds) increase of the concentration of this ion, followed a secondary transient which requires an influx of extracellular calcium. Many other mechanisms activated by progesterone contribute to its determination, e.g. stimulation of a trypsin-like activity, biosynthesis of polyamines (putrescine and spermidine) and platelet-activating factor (PAF), and protein tyrosine kinase (PTK) activity (Figure 1).

However, not all the biological effects of progesterone are mediated by an increase of intracellular calcium. Experimental evidence suggests that chloride ion transmembrane flux is vital for progesterone to promote the acrosome reaction. This efflux is activated by interaction with the GABA<sub>A</sub>-like receptor and seems to require PTK mediation since it happens against a chemical gradient. The ensuing membrane depolarization causes biological effects. Recent observations, however, suggest that these two pathways are not independent, but influence each other. Indeed, membrane depolarization, induced by sodium ion influx or by chloride ion efflux, seems to increase the concentration of intracellular free calcium (Figure 1).

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**Figure 1.** Simplified scheme showing that the increase of the intracellular free calcium concentration is dependent upon an influx of extracellular calcium. The increase of the intracellular free calcium concentration is dependent upon an influx of extracellular calcium. This increase is dependent upon an influx of extracellular calcium. Also chloride ion efflux seems to play an important role in this process. PLA<sub>2</sub> = phospholipase A<sub>2</sub>; PTK = protein tyrosine kinase.

To this already known effect of progesterone, it is added that progesterone increases the activity of the protein tyrosine kinase, which in turn causes biological effects.

- Ambhaikar, M. and Purdy, R. (1991) Progesterone and human spermatozoa. *Mol. Hum. Reprod.*, **1**, 186-196.
- Aprison, M.H. and Lipkowitz, D. (1987) A novel approach. *J. Neurosci.*, **7**, 1885-1890.
- Aanesen, A., Fried, G., and Lippman, S. (1985) Progesterone: an acid specific binding site. *J. Biol. Chem.*, **260**, 1885-1890.
- Aitken, R.J., Buckingham, P., and Harkiss, D. (1987) Progesterone on human spermatozoa. *Endocrinology*, **137**, 39-44.
- Aitken, R.J., Harkiss, D., and Walters D.E. (1987) Bicarbonate ion mediated activation of human spermatozoa. *Reprod.*, **58**, 186-196.

