Caprine sperm acrosome reaction: promotion by progesterone and homologous zona pellucida

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Abstract

Experiments were designed to characterize the effect of progesterone and the zona pellucida (ZP) on the goat sperm acrosome reaction (AR) through a comparative study. Goat spermatozoa were incubated for 4 h in Krebs–Ringer bicarbonate media (KRB) for capacitation. Progesterone and ZP stimulated exocytosis of capacitated spermatozoa in a dose-dependent manner. EGTA and La$^{3+}$, added 10 min before the addition of the agonists, completely abolished the stimulatory effects. Ca$^{2+}$ influx was observed to occur through a calcium phosphate transporter. Picrotoxin and bicuculline, two GABA A/Cl$^{-}$ channel antagonists, also inhibited progesterone-induced AR when added 10 min before steroid addition. ZP-induced AR was unaffected by these antagonists. Studies using pertussis toxin (PTX) showed that, unlike ZP, progesterone acts without the involvement of a G-protein. Progesterone-3-(O-carboxymethyl) oxime: BSA conjugate (P-BSA) also induced AR in capacitated sperm suspension. Results suggest that progesterone and ZP induce AR via specific membrane receptors through different mechanisms, both requiring an influx of Ca$^{2+}$. It is assumed that both the mechanisms reconcile at some stages of the cascade and elicits a similar physiological response. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Goat; Spermatozoa; Acrosome reaction; Progesterone; Zona pellucida

1. Introduction

The acrosome of mammalian spermatozoa is a secretory granule akin to lysosomes, both sharing a common Golgi origin. Acrosome reaction (AR) is a process marked by multiple fusion (vesiculation) of the outer acrosomal membrane with the overlying plasma membrane (Yanagimachi and Usui, 1974) and resembles exocytosis in other cells, albeit with some unusual features. How the acrosome reaction is triggered under in vivo conditions is of great current interest. It is generally agreed that the zona pellucida (ZP) glycoprotein, ZP3 is the physiological initiator of acrosome reaction (Yanagimachi, 1988). Acrosome intact sperm bound to ZP shortly after mixing sperms and eggs have been successfully ’chased’ into acro- some reacted sperms (Bleil and Wassarman, 1983; Meyers et al., 1996). ZP-induced acrosome reaction has been confirmed in many species. There are reports that progesterone secreted by cumulus cells also induced acrosome reaction in humans (Osman et al., 1989), mouse (Melendrez et al., 1994), boar (Roldan et al., 1994), stallion (Meyers et al., 1995) and golden hamster (Meizel et al., 1990; Llanos and Anabalon, 1996) spermatozoa.
Initiation of acrosome reaction by progesterone has so far not been reported in domestic ruminants, namely bovines, caprines and buffalo. Incubation in Krebs–Ringer bicarbonate (KRB) medium converts >70% of fresh ejaculated goat spermatozoa into capacitated state in 4 h (Anand et al., 1989). This was confirmed by staining with chlortetracycline (Kaul et al., 1997). In this study, an attempt was made to investigate the similarities and differences in progesterone- and ZP-induced acrosome reaction in caprine spermatozoa.

2. Materials and methods

Bovine serum albumin (Fraction V), sodium lactate, progesterone, chlortetracycline (CTC) 1,4 diazobicyclo (2,2,2) octane (DABCO), progesterone-3-(O-carboxymethyl) oxime : BSA conjugate, ethylene glycol bis- (b-amino-ethyl ester) N,N'-tetra-acetic acid (EGTA), lanthanum chloride, picrotoxin, bicuculline, pertussis toxin and N-2-hydroxy ethyl piperazine-N0-2-ethane sulfonic acid (HEPES) (Sigma Chemicals, St. Louis, MO) were used in the study.

2.1. Collection and preparation of spermatozoa

Semen was collected from the bucks of the Jamnapari breed (n=10), housed at the National Dairy Research Institute, using artificial vagina. The semen was washed twice with albumin saline, pH 7.4 prior to usage. Motile cells were prepared by the swim-up technique (Cross et al., 1988). Motile sperm were suspended in Krebs–Ringer bicarbonate (KRB) medium. The sperm concentration was adjusted to 5×10^6 cells/ml after counting in a haemocytometer (Kaul et al., 1997). The sperm motility was assessed microscopically by examining a uniform drop of semen under a cover slip on a warm stage at 37°C, using two scales of 0 to 5 and 0 to 10 (Kaul et al., 1997). Only samples showing 90% progressive motility were considered for the experiments. Incubations were carried out at 37°C in a CO₂ incubator.

2.2. Isolation and solubilization of zona pellucida

Zona pellucidas (ZP) were isolated and solubilized according to Hedrick and Wardship (1986). Goat ovaries were obtained from local abattoir, washed thoroughly and stored at −20°C until processed. Small antral follicles (3–5 mm in diameter) were aspirated using a 20-g needle syringe and transferred into a Petri dish containing isolation buffer (10 mm sodium phosphate (pH 7.4); 25 mm NaCl; 3 mm sodium citrate and 2 mm EDTA). The oocytes were collected using an oocyte picker under an inverted microscope. Cumulus-free oocytes were homogenized in a Potter–Elvehjem homogenizer (2 ml) using isolation buffer, and ZP ‘ghosts’ were recovered. The required number of ZPs were transferred to 1.5-ml microcentrifuge tubes containing 100-μl ammonium bicarbonate buffer (pH 8.0) and having 0.2 mm PMSF. ZPs were solubilized by keeping the tubes in a water bath at 72°C for 30 min. After solubilization, impurities were removed by centrifuging the tubes at 5000 rpm for 10 min. The tubes were stored at −40°C until used.

2.3. Effect of progesterone and solubilized ZP on acrosome reaction

Experiments were designed to test the acrosome reaction inducing ability of different concentrations of progesterone and ZP. The capacitated goat spermatozoa were independently treated with different concentrations of progesterone, its solvent (0.05% DMSO) and with different concentrations of ZPs.

The time-course study of progesterone and ZP-induced acrosome reactions was carried out as follows: aliquots of capacitated sperm suspension were treated with 2 μg/ml progesterone or solvent for different periods of time. The time-course study using ZP was carried out at a final concentration of 100 ZP/ml. At each time period, the suspensions were observed to determine percentage of sperm motility and acrosome reactions.

2.4. Role of Ca²⁺ and PO₄⁻ in the progesterone and ZP-induced acrosome reactions

After capacitation, spermatozoa were washed twice by centrifuging at 800 g for 10 min, and suspended in KRB medium without, or with 1.7 mM Ca²⁺ and 1.2 mM PO₄⁻ and stimulated with 15 mM A23187 or 2 μg/ml progesterone or 100 ZP/ml. EGTA (2 mm) or Ca²⁺ channel blocker La³⁺ (500 μM) was added in some experiments (La³⁺ did not affect sperm viabi-
The suspensions were inspected to determine the acrosomal status.

2.5. Effect of GABA<sub>α</sub>/Cl<sup>−</sup> channel antagonists and pertussis toxin on acrosome reactions

Bicuculline and picrotoxin, both GABA<sub>α</sub>/Cl<sup>−</sup> channel antagonists, were dissolved in DMSO and a final concentration of 10 μM bicuculline and 200 μM picrotoxin were used. These were added independently to capacitated sperm suspensions 10 min prior to the addition of progesterone or ZPs (incubated for 8 min and 30 min, respectively, before assessment).

Pertussis toxin was reconstituted in 30 mM HEPES buffer (pH 7.3) to make a stock solution of 50 μg/ml. A final concentration of 100 ng/ml was added to the sperm suspension. The suspensions were subjected for the assessment of acrosomal status.

2.6. Effect of progesterone-3-(O-carboxymethyl) oxime: BSA conjugate (P-BSA) on acrosome reaction

P-BSA was dissolved in phosphate buffered saline (pH 7.4), after which free progesterone was removed by treatment with a solution of charcoal-dextran (Meizel and Turner, 1991). Capacitated spermatozoa were incubated with 15 μg/ml of P-BSA (±2 μg of progesterone) or PBS (control) independently for 15 min. The suspensions were inspected to determine percentages of motile and acrosome reacted spermatozoa.

2.7. Assessment of acrosome reaction

The chlortetracycline (CTC) fluorescence assay was used to assess the functional status of cells, following the method described by Kaul et al. (1997). CTC solution was freshly prepared containing 750 μM CTC in a buffer of 130 mM NaCl, 5 mM cystine, 20 mM Tris-Hcl (final pH 7.4). The solution was wrapped in foil and kept at 4°C until required. To stain the cells, 50 μl of sperm suspension was added to 50 μl of CTC solution and thoroughly mixed. Cells were then fixed by adding 10 μl of 12.5% (w/v) paraformaldehyde in 0.5 M Tris-Hcl buffer (pH 7.4). Thereafter, 10 μl of stained suspension was placed on a clean glass slide and a drop of 0.22 M 1,4-diazobicyclo [2,2,2] octane dissolved in glycerol: PBS (9:1) was mixed carefully to retard fading of fluorescence. A coverslip was added and excess fluid was removed by compressing between tissues. Slides were sealed with colourless nail polish and assessed immediately.

2.8. Statistical analysis

Percentages of acrosome reaction obtained in different experiments were expressed as the mean±standard error of the mean (SEM). Statistical differences involving multiple treatments were determined by one-way ANOVA. In instances where one treatment and control were compared, the Student t-test was applied (Snedecor and Cochran, 1967).

3. Results and discussion

3.1. Concentration and time-course of incubation of progesterone and ZP induced acrosome reaction

Fig. 1 demonstrates how 1 mg/ml progesterone or 60 ZP/ml induced acrosome reaction (p<0.01). However, maximum acrosome reacted cells were obtained when incubated with 2 μg/ml progesterone or 100 ZP/ml, where DMSO (solvent) had no effect. Although the results obtained in this study are high, they correlate with the earlier reports that >70% of goat sperm can be capacitated in vitro (Anand et al., 1989; Kaul et al., 1997). Also, with the addition of 10 mM Ca<sup>2+</sup>, all the capacitated goat spermatozoa were observed to synchronously undergo acrosome reaction (Anand et al., 1989).

In Fig. 2, the time-course effect of progesterone and ZP in increasing the percentage of acrosome reacted spermatozoa is shown. Progesterone induced an increase within 4 min, reaching a maximal value at 8 min (p<0.01), whereas ZP induced an increase in 20 min (p<0.01), reaching a maximum in 30 min. This major difference in the time course of induction of acrosome reaction by progesterone or ZP indicates a difference in intracellular signaling events that follow.

3.2. Role of Ca<sup>2+</sup> and PO₄<sup>−</sup> in acrosome reaction

When capacitated goat spermatozoa were incubated with 10 mM Ca<sup>2+</sup>, a spontaneous acrosome reaction
Fig. 1. Dose-response study of the effect of progesterone (P) and zona pellucida (ZP) on goat sperm acrosome reaction (motility >60%; mean±SEM of three experiments; \( p<0.01 \)).

Fig. 2. Time-course effect of progesterone (P) and zona pellucida (ZP) in inducing goat sperm acrosome reaction (motility >60%; mean±SEM of three experiments; \( p<0.01 \)).
was observed (Anand et al., 1989). When goat sperm suspension was subjected to treatment with A23187 (Ca$^{2+}$ ionophore) acrosome reaction was induced without any stimulation by the physiological agonists (Table 1). This could be due to the impedance of extracellular Ca$^{2+}$ into the cytosol. 500 mM La$^{3+}$ (inhibitor of long lasting — L type — Ca$^{2+}$ channel) or 2 mM EGTA (a Ca$^{2+}$ chelator), when incubated with the sperm suspension prior to the addition of agonists, inhibited progesterone and ZP induced acrosome reaction. This indicates the major role of Ca$^{2+}$ in the process as a second messenger. The observation that acrosome reaction is prevented when PO$_4^{2-}$ is removed from the medium (Table 1) is a confirmation of an earlier report that Ca$^{2+}$ influx takes place through a calcium phosphate transporter (Kaul et al., 1997).

### 3.3. Effect of pertussis toxin and GABA A/Cl- channel antagonists on acrosome reaction

Pertussis toxin (PTX), an inhibitor of G protein activity, is an enzyme which covalently modifies specific G$\alpha$ subunits of a G protein by transferring ADP ribose moiety from NAD to cysteine of G$\alpha$ protein (ADP ribosylation reaction) (Birnbaumer et al., 1991). In these experiments, the sperm suspension was incubated along with 100 ng/ml PTX, and progesterone and ZP were added in separate units. The results (Table 2) indicate that PTX has a pronounced effect in inhibiting the ZP-induced acrosome reaction, whereas it has hardly any effect on acrosome reaction induced by progesterone. This suggests that a G-protein is not involved in progesterone-induced acrosome reaction which marks the major difference in the mechanism by which both the agonists act. This could be one of the reasons why progesterone-induced acrosome reaction is spontaneous.

In the absence of a G protein, the progesterone receptor might either be directly connected to a Ca$^{2+}$

### Table 1

<table>
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<th>Treatment</th>
<th>Ca$^{2+}$</th>
<th>PO$_4^{2-}$</th>
<th>EGTA</th>
<th>La$^{3+}$</th>
<th>Agonist</th>
<th>Acrosome reaction (%) Mean±SEM</th>
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<td>+</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
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<td>-</td>
<td>ZP</td>
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<td>P</td>
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<td>ZP</td>
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</tbody>
</table>

*aMotility >60%; Mean±SEM; n=3.
bDifferent from control Ca$^{2+}$/PO$_4^{2-}$ (p<0.01).
cDifferent from control Ca$^{2+}$/PO$_4^{2-}$/P (p<0.001).
dDifferent from control Ca$^{2+}$/PO$_4^{2-}$/ZP (p<0.001).

### Table 2

<table>
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<tr>
<th>Treatment</th>
<th>P</th>
<th>ZP</th>
<th>bicuculline (10 μM)</th>
<th>picrotoxin (200 μM)</th>
<th>pertussis toxin (200 ng/ml)</th>
<th>Acrosome reaction (%) Mean±SEM</th>
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<td>23.9±0.93</td>
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<td>72.81±0.78</td>
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<td>+</td>
<td>42.26±0.45</td>
</tr>
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</table>

*aMotility >60%; mean±SEM of at least four experiments; p<0.01.
channel or the receptor itself is a Ca\textsuperscript{2+} channel as hypothesized by Revelli et al. (1994). In one of the reports, GABA was shown to induce acrosome reaction in human spermatozoa (Shi et al., 1997). Based on the observation made by Wistrom and Meizel (1993), who revealed the presence of a GABAA receptor on the surface of human spermatozoa, the involvement of a GABAA/Cl\textsuperscript{−} channel in the acrosome reaction induced by progesterone and ZP was tested. The results indicate that 200 µM picrotoxin- and 10 µM bicuculline-inhibited progesterone induced acrosome reaction. However, ZP-induced acrosome reaction was unaffected by these antagonists. Hence, it is clear that, binding of progesterone to the GABAA/Cl\textsuperscript{−} channel induces a Ca\textsuperscript{2+} influx which bypasses the role of G-protein and leads to a spontaneous acrosome reaction.

3.4. Effect of protein bound progesterone on acrosome reaction

The inhibition of progesterone-induced acrosome reaction by picrotoxin and bicuculline has strengthened the hypothesis that progesterone acts through plasma membrane receptor(s), one of them being a GABAA/Cl\textsuperscript{−} channel. Progesterone in the female genital tract is found in a protein-bound form (Fehl et al., 1995). Also, the progesterone-induced acrosome reaction was not inhibited by RU486, an inhibitor of genomic receptor for progesterone (Blackmore et al., 1991; Uhler et al., 1992). Progesterone-3-(O-carboxymethyl) oxime:BSA conjugate (P-BSA) induced a rapid and transient increase in intracellular free Ca\textsuperscript{2+} which was followed by an acrosome reaction (Turner and Meizel, 1995; Blackmore et al., 1991). In the current incubation conditions, spermatozoa incubated for, at least, 4 h. were able to undergo acrosome reaction when incubated with P-BSA (Fig. 3). P-BSA, being a large molecule, cannot penetrate the plasma membrane and the sperm cell does not exhibit endocytotic activity. Apart from this, epifluorescence microscopy and flow cytometry experiments showed that P-BSA–fluorescein isothiocyanate conjugate (P-BSA-FITC), binds to >90% of the goat sperm population. Although these values are very high in comparison with humans, where only 30% of the sperm population is reported to have progesterone receptors (Blackmore and Lattanzio, 1991), it is in agreement with the report of Cheng et al., (1998) where 95% of the percoll washed equine spermatozoa binds to P-BSA-FITC. The main cause of this might be due to the fact that the receptors for progesterone are not exposed before capacitation. In goat spermatozoa, it was observed that >80% of the motile sperm population gets capacitated.
4. Conclusion

Our study showed that both, progesterone and ZP bind to different plasma membrane receptors and follow different cascades in inducing the acrosome reaction. However, the possibility whether progesterone and ZP offer irreconcilable alternatives or rather that both interact sequentially during sperm activation remains to be tested. Progesterone is a steroid present in the vicinity of spermatozoa starting from its entry into the female reproductive tract until it reaches the site of fertilization, the source being follicular fluid and cumulus cells. The cumulus oophorus acts as a barrier for the spermatozoa to reach the ZP. It is hypothesized that the hydrolytic enzymes released as a result of the acrosome reaction induced by progesterone secreted by cumulus cells, opens the route for the entry of the most potent and viable spermatozoa to reach the ZP, which ultimately gets acrosome reacted and fertilized. It is, therefore, assumed that both the agonists induced acrosome reaction independently in the normal process of fertilization. The absence of any one of these lead to infertility. However, further experiments should be conducted to trace the complete mechanism by which both the agonists induce the acrosome reaction and to characterize the progesterone receptors.

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