Role of Diacyl Glycerol (DAG) in Caprine Sperm Acrosomal Exocytosis Induced by Progesterone

P. R. Somanath* and K. K. Gandhi

Animal Biochemistry Division, National Dairy Research Institute, Karnal-132001 (Haryana), India

ABSTRACT : Capacitated goat spermatozoa generated diacyl glycerol (DAG) when suspended in Krebs-Ringer bicarbonate medium and induced by progesterone or Ca^{2+} ionophore A23187. We have added Sn-1-oleoyl-2-acetyl glycerol externally, to study the effect of DAG in goat sperm acrosomal exocytosis. Addition of neomycin abolished the DAG generating capacity of progesterone in a dose dependent manner, suggesting the involvement of a phosphoinositidase C activated phospholipase C system in the process. The level of increase in phosphatidic acid was considerably low and was produced well after the DAG generation thereby suggesting the involvement of a DAG kinase which phosphorylates DAG to produce PA. The inhibition of progesterone mediated effect by inhibitors of $GABA_A/Cl^-$ channel and Ca^{2+} channels further supports the evidence that the events of binding of agonist to the receptor(s), opening of Ca^{2+} channels and the activation of phospholipase C are reconciled to perform the function of acrosome reaction in capacitated goat spermatozoa. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 8 : 1091-1097)***

Key Words : Goat, Spermatozoa, Acrosome Reaction, Diacyl Glycerol, Phospholipase C

INTRODUCTION

In mammals, sperm must undergo post-testicular development stages first in the male and then in the female reproductive tract before it is ready to meet the female gamete for penetration. The post-ejaculatory modification of the sperm surface that render the sperm capable of undergoing acrosome reaction (AR), while in female reproductive tract is known as "capacitation" (Austin, 1951; Chang, 1951).

During its sojourn to the site of fertilization in the female reproductive tract, mammalian sperm interacts with the cellular and the humoral elements present therein. Some of these substances has shown to induce AR *in vitro* in humans, the most important among them being follicular fluid (Mukherjee and Lippes, 1972; Tesarik, 1985), cumulus oophorus (Siiteri et al., 1988). The ZP_3 glycoprotein of the egg has shown to bind to sperm and subsequently to induce AR in mammalian species. Both zona pellucida (ZP) and progesterone (secreted by cumulus oophorus), induced AR in goat sperm (Somanath et al., 2000).

In humans, progesterone has shown to induce a rapid influx of Ca^{2+}, causing a rise in the intracellular Ca^{2+} (Thomas and Meizel, 1989; Blackmore et al., 1990; Baldi et al., 1991) and is followed by the activation of polyphospho-inositide-specific phospho-inositidase C (PIC) and consequent hydrolysis of phosphatidyl inositol 4,5-bis phosphate to generate inositol 1,4,5-tri-phosphate (IP_3) and diacyl glycerol (DAG) (Roldan and Harrison, 1989; Thomas and Meizel, 1989; O'Toole et al., 1996a). Additional pathways for the generation of DAG include activation of phospholipase C (PLC) and phospholipase D (PLD) (Exton, 1990, 1994). The diglycerides generated as a result of hydrolysis of phospholipids other than the polyphospho-inositides (PPIs) by PLC are collectively called as diradyl glycerols or DRGs, where `radyl' refers to acyl, alkyl and alkenyl substituents (Exton, 1994). In addition, the hydrolysis of PPIs yields a small amount of DAG for a limited period of time, whereas the breakdown of other phospholipids such as phosphatidyl choline (PC), phosphatidyl serine (PS), etc. generates DAG over a longer time period, suggesting that the latter may be involved in cellular events requiring prolonged activation (Exton, 1990). Earlier studies using A23187 has shown that PIC-mediated hydrolysis of PPIs is an important early event during the exocytolic response in mammalian sperm (Roldan and Harrison, 1989; Thomas and Meizel, 1989). However, in ram, mouse and human sperm, it appears that the majority of DAG is generated via PLC pathway (Roldan and Dawes, 1993; Roldan and Murase, 1994; O'Toole et al., 1996b).

At present, relatively little is known about the possible pathways involved in the generation of DAG in response to progesterone. In particular, there is no clear evidence as to PLD and/or PLC activation occur in goat sperms in response to natural agonists and whether these enzymes play a significant role in AR elicited under these conditions of stimulation. Therefore, in the present study, we have attempted to clarify mechanisms of DAG generation in goat sperm by comparing responses to progesterone and A23187, and to establish whether DAG may play a role in events leading to membrane fusion during acrosomal breakdown.

* Corresponding Author: P. R. Somanath. Department of Biological Sciences, Kent State University, P.O. Box 5190, Kent, Ohio-44242-0001, USA. Tel: +91-330-672-3613, Fax: +91-330-672-3713, E-mail: somanath_pr@hotmail.com

Received November 7, 2001; Accepted February 15, 2002

MAERIALS AND METHODS

Progesterone, nifedipine, picrotoxin, bicuculline, chlortetracycline, Hoechst bis-benzimide 33258, 1,4-diazobiyclo (2,2,2) octane (DABCO), diltiazem, D600, 1-oleoyl-2-acetyl-Sn-glycerol, bovine serum albumin (Fraction V), sodium lactate and A23187 were purchased from Sigma Chemical Co., St. Louis, MO, USA. Neomycin was purchased from SRL, Mumbai, India. 9,10[^{3}H] palmitic acid (specific activity 54 Ci/mmol) was purchased from Amersham International, Amersham, UK. All other reagents used in the study were of analytical grade.

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 37EC, using two scales of 0 to 5 and 0 to 10 (Kaul et al., 1997). Only samples showing 90 percent progressive motility were considered for the experiments. Incubations were carried out at 37EC in a CO_2 incubator.

Effect of exogenous DAG on goat sperm acrosome reaction

Goat sperm preincubated for 4 h in KRB medium, were incubated in the presence of exogenously administered synthetic form of DAG, Sn-1-oleoyl-2-acetyl-glycerol (OAG) for 30 min. The experiments were conducted in the presence and absence of various concentrations of nifedipine (a Ca^{2+} channel inhibitor), which was added 10 min before the addition of OAG. Sperm suspensions after the treatment were assessed for motility and acrosomal status.

Effect of neomycin on progesterone induced goat sperm acrosome reaction

Neomycin, a specific inhibitor of PIC was added to the capacitated goat sperm suspension in various concentrations of 1 μM, 10 μM, 100 μM and 1 mM. After incubating the suspensions for 15 min, they were subjected to 15 μM progesterone and were further incubated for 15 min. The suspensions were inspected to determine the motility and acrosomal status.

Assessment of acrosome reaction

The solution of supra vital stain Hoechst 33258 (which does not stain cells with intact plasma membranes) was prepared by dissolving the dye in tripple distilled water to a stock solution of 100 mg/ml. This was stored in a foil-wrapped vial at 4°C and used within one month. Final concentration of the dye when added to the sperm suspension was 1 μg/ml. Sperm was stained for 10 min and then washed through 45 percent percoll by centrifugation at 800 g for 10 min to remove free dye.

The chlortetracycline (CTC) fluorescence assay was used to assess the functional status of cells, following the modified method described by Kaul et al. (1997). CTC solution was prepared fresh containing 750 μM CTC in a buffer of 130 mM NaCl, 5 mM cysteine, 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 hoechst-treated sperm suspension was added to 50 μl of CTC solution and mixed thoroughly. Cells were then fixed by adding 6 μl of 12.5 percent (w/v) paraformaldehyde in 0.5 M Tris-HCl buffer (pH 7.4). Ten μl of stained suspension was placed on a clean glass slide and a drop of 0.22 M DABCO in glycerol : PBS (9:1) was mixed carefully to retard fading fluorescence. A covership was added and excess fluid was removed by compressing between tissues. Slides were sealed with colourless nail polish and assessed immediately, under an epifluorescence microscope.

Stimulation of goat sperm suspensions with progesterone and A23187

Washed sperm suspensions were incubated for 3 h at 37°C in KRB medium and then labelled by incubating them with 5 μCi [^{3}H]-palmitic acid/ml for 1 h at 37°C. Suspensions were washed by centrifuging at 800 g for 10 min and resuspended in fresh KRB medium. The suspensions were then separately stimulated by the addition of 15 μM progesterone and 15 μM A23187, and the reactions were stopped at various intervals for the quantification of lipid changes.

Effect of inhibitors of Ca^{2+} channels and $GABA_A$/ Cl^- channel on progesterone induced DAG generation in goat sperm

Goat sperm suspensions labelled using [^{3}H]-palmitic acid were subjected to treatment with various concentrations of nifedipine, D600, diltiazem, picrotoxin and bicuculline, and incubated for 15 min. After further incubating the suspension for 7.5 min in the presence of 15 μM progesterone, the reaction was stopped, the lipids were extracted and quantified.

Lipid analysis

After the addition of 15 μM progesterone, the reactions were stopped at selected intervals by adding chloroform/ methanol (1:2 v/v) for the quantification of DAG and phosphatidic acid (PA). Lipids were extracted as described by Bligh and Dryer (1959) including corresponding lipids which are used as carriers. Lipids were then separated using thin layer chromatography (TLC) on self prepared silica gel-G coated plates (0.25 mm thickness). DAG was separated in the solvent system, n-hexane/diethyl ether/ acetic acid (70:30:1 v/v). PA was separated in the upper phase of the solvent system ethyl acetate/2,2,4 tri-methyl pentane/acetic acid/H_2O (110:50:20:100 v/v). Lipid spots were visualized by exposing the TLC plates to iodine vapours by keeping the plates in a glass chamber previously saturated with iodine. The spots were identified by comparison with standards run in parallel on the same plate. Spots were marked by pencil and were scraped off after drying the plates. The scrapings were separately dissolved in 4 ml scintillation cocktails and each vial were counted in a Packard Tricarb Scintillation Counter (Bray, 1960).

Statistical analysis

Percentage of acrosome reaction obtained in different experiments were expressed as the mean " standard error of 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).

RESULTS

Effect of exogenous DAG on goat sperm acrosome reaction

A 10 mM stock solution of permeant DAG, Sn-1-oleoyl-2-acetyl glycerol (OAG) was prepared in n-hexane; when required, an aliquot was dried with nitrogen flux and redissolved in similar volume of DMSO. This was diluted in KRB medium to give a final concentration of 1 μM OAG. Treatment of goat sperm with OAG resulted in an increase ($p<0.05$) in the proportion of acrosome reacted cells (figure 1). There was no significant change noticed when DAG was added to uncapacitated sperm suspensions, suggesting that only capacitated cells could respond to OAG. These results suggest that DAG is an important metabolite involved in the acrosomal exocytosis of capacitated goat sperm.

Effect of neomycin on progesterone induced goat sperm acrosome reaction

Neomycin, an aminoglycoside antibiotic has the ability to bind specifically to polyphosphoinositides (PPIs)

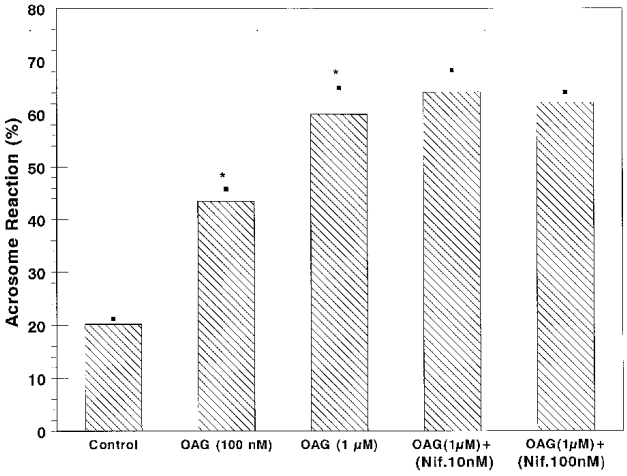

Figure 1. Effect of permeant diacyl glycerol, 1-oleoyl-2-acetyl-Sn-glycerol (OAG) on acrosome reaction [Mean±S.E.M. (n=3); * $p<0.05$ compared with control].

(Schacht, 1978) and inhibit PPI hydrolysis (Carney et al., 1985; Cochroft and Gompertes, 1985). We used neomycin to examine the possibility of a phosphoinositidase C pathway, which might be a pre-requisite for the stimulation of PLC pathway by which the majority of the DAG being generated. Results (figure 2) indicate that the addition of neomycin resulted in a dose dependent decrease in the percentage of AR patterned cells after stimulation with progesterone, compared with progesterone only treated controls ($p<0.01$).

Role of DAG in agonist induced acrosome reaction in goat sperm

Results (figure 3A) shows that treatment with progesterone led to an increase in labelled DAG, which

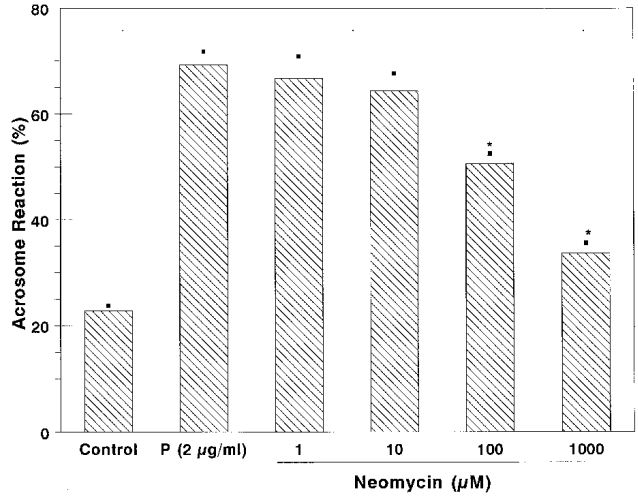

Figure 2. Effect of neomycin on acrosome reaction induced by progesterone [Mean±S.E.M. (n=4); * $p<0.01$].

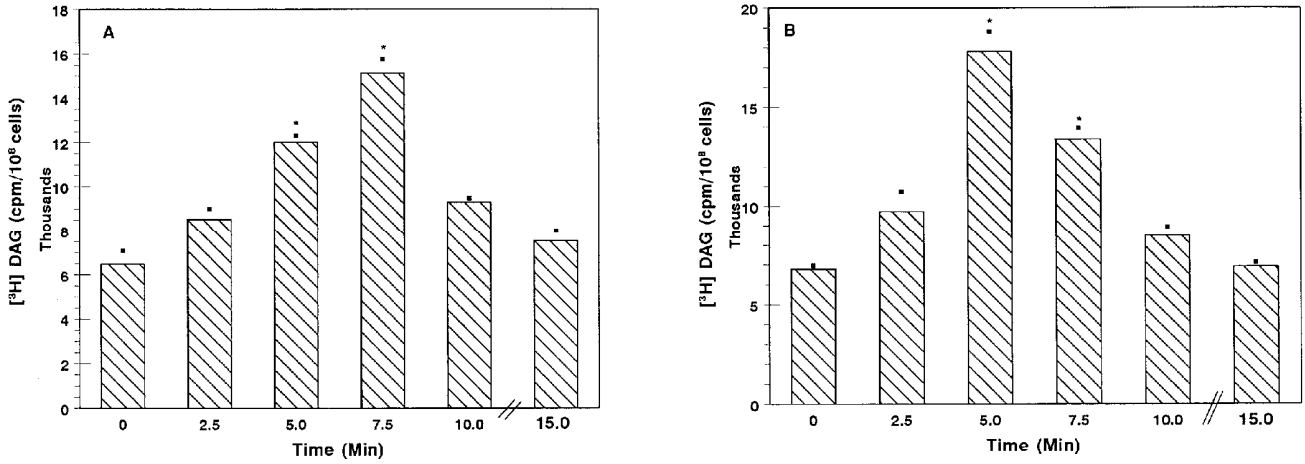

Figure 3. Changes in accumulation of diacyl glycerol after stimulation with A) progesterone [Mean±S.E.M. (n=3); * p<0.01] and B) A23187 [Mean±S.E.M. (n=3); * p<0.05].

peaked at 7.5 min (p<0.01) and then declined. The level of phosphatidic acid (PA) increased to a maximum after 10 min incubation (p<0.01). However, the amount of labelled PA generated was considerably lower than that of DAG (figure 4A).

Figure 3B shows that treatment with A23187 led to an increase in labelled DAG, reaching a maximal value at 5.0 min and then declined (p<0.05). A maximum increase in labelled PA was noticed at 7.5 min (p<0.01) (figure 4B). Thus, with both the agonists, the rise in DAG preceded the rise in PA. These results suggest that progesterone and A23187 stimulate generation of DAG via a mechanism involving mainly PLC activation, rather than PLD and PA-phosphohydrolase. In addition, they also suggest that the DAG generated in this way could, at least in part, be converted to PA via the action by DAG kinase (Berridge et al., 1987).

Effect of inhibitors of Ca^{2+} channels and $GABA_A$/ Cl^- channel in progesterone induced DAG generation by goat sperm

The addition of progesterone to sperm suspension resulted in an increase in DAG production (p<0.01). However, the inclusion of 100 nM nifedipine just prior to progesterone addition, there was a significant inhibition in labelled DAG generation (p<0.01) compared with progesterone only treated controls (figure 5). $GABA_A$/ Cl^- channel antagonists picrotoxin (200 μM) and bicuculline (10 μM) when added 15 min prior to the addition of progesterone have also shown to inhibit DAG generating ability of progesterone (figure 5). These results suggests that the activation of the Ca^{2+} channels as well as $GABA_A$/

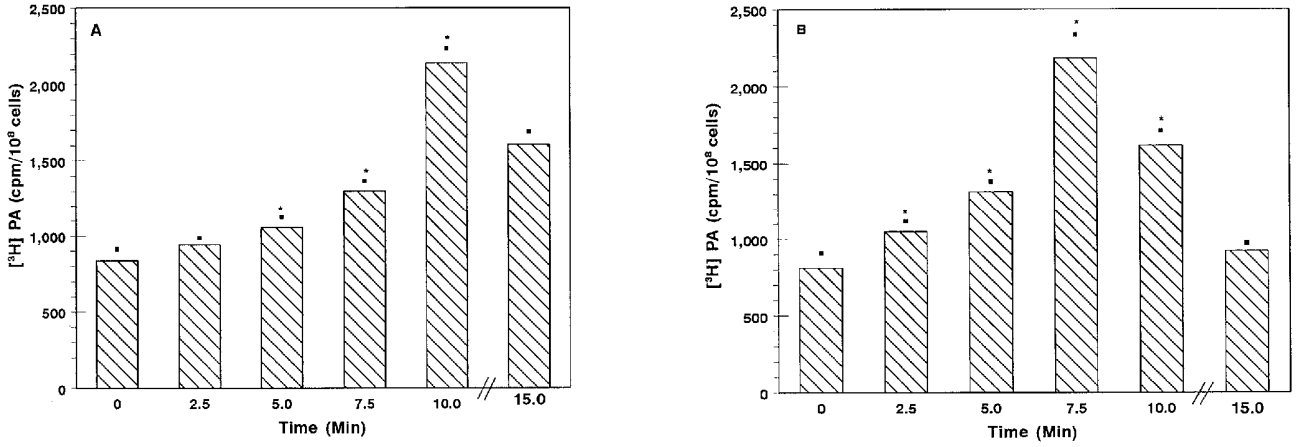

Figure 4. Changes in accumulation of Phosphatidic acid (PA) after stimulation with A) progesterone [Mean±S.E.M. (n=3); * p<0.01] and B) A23187 [Mean±S.E.M. (n=3); * p<0.01].

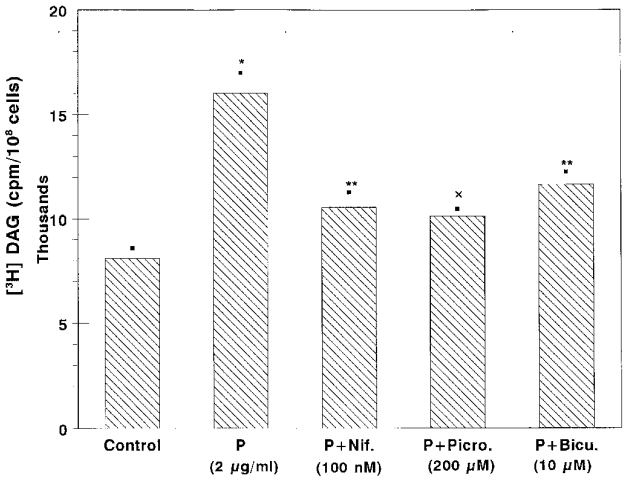

Figure 5. Effect of Ca^{2+} channel and $GABA_A/Cl^-$ channel antagonists on diacyl glycerol generation by progesterone [Mean±S.E.M. (n=3); * $p<0.01$ compared with untreated control; ** $p<0.01$ compared with progesterone only treated cells; ^{x} $p<0.05$ compared with progesterone only treated cells].

Cl^- channel and the generation of DAG in goat sperm progesterone take place in the same pathway which mediates acrosomal exocytosis.

DISCUSSION

In this study, progesterone or A23187 has clearly shown to generate DAG in goat sperm via similar pathways. Majority of the DAG was seen to be produced by PLC, with essentially no contribution from PLD route. The fact that OAG induced AR in goat sperm indicates that DAG thus generated plays an important role in acrosomal exocytosis in capacitated sperms.

Extracellular Ca^{2+} has shown to play an important role in goat sperm AR (Somanath et al., 2000) which is mediated through plasma membrane associated L-type voltage gated Ca^{2+} channels (Somanath and Gandhi, 2002). The fact that nifedipine, D600 and diltiazem inhibited progesterone induced DAG generation (figure 5) and that nifedipine failed to inhibit AR induced by OAG (figure 1) indicates that the essential involvement of voltage gated Ca^{2+} channels in goat sperm AR is after the binding of progesterone to its receptor(s) but before the generation of DAG. Moreover, the ability of Ca^{2+} ionophore A23187 to generate DAG more rapidly support the above findings. The inhibition of DAG rise with the addition of picrotoxin and bicuculline (figure 5) not only indicates the involvement of a $GABA_A/Cl^-$ channel in the process, but also shows that all the three events of progesterone receptor binding, activate Ca^{2+} channels and $GABA_A/Cl^-$ channel as well as generation of DAG follows the same route.

Phosphoinositidase C mediated hydrolysis of polyphospho inositides (PPIs) is an important early event during the exocytotic response in mammalian sperm (Roldan and Harrison, 1989; Thomas and Meizel, 1989). The presence of PIC has been reported in goat sperm (Bansal and Atreja, 1991). The ability of neomycin - an aminoglycoside antibiotic which binds to PPIs and inhibit PPI hydrolysis (Carney et al., 1985; Cockcroft and Gonsperts, 1985)-to inhibit progesterone induced AR in goat sperm in a dose dependent manner (figure 2) confirms the role of PIC in the process. Moreover, it has been reported by O'Toole et al. (1996b) that PIC generated DAG plays a role in the activation of other types of PLC which then produces majority of the DAG necessary for AR.

Induction of goat sperm prelabelled with [^{3}H]-palmitic acid with progesterone (figure 3B) and A23187 (figure 4B) resulted a rise in phosphatidic acid. But, this temporal pattern suggests that rise in labelled DAG preceded the rise in PA; furthermore, the amount of labelled PA generated was considerably lower that it was not sufficient to play its fusogenic role. These results suggest that progesterone stimulation led to generation of DAG via a mechanism involving mainly PLC activation rather than PLD. In addition, the reason that the occurrence of a PLD has been reported only in invertebrate sperm (Domino et al., 1989) and not in mammals suggests that PA generation may be as a result of phosphorylation of DAG by DAG kinase (deChaffoy de Courcelles et al., 1989).

In conclusion, we have shown that capacitated goat sperm when stimulated by progesterone or A23187 leads to an increase in endogenous DAG, which plays a central role in acrosomal exocytosis. The generation of DAG is followed by a rise in PA, but was considerably lower than the DAG rise, and is considered to be produced as a result of phosphorylation of DAG by DAG kinase. Inhibition of L-type voltage gated Ca^{2+} channels by inhibitors belonging to the different classes of dihydropyridines, phenyl alkylamines and benzothiazepines abolished the effects mediated by progesterone. The ability of neomycin to block acrosome reaction confirms the fact that DNA generated through a PIC initiated PLC system is essential in mediating progesterone induced AR in goat sperm.

ACKNOWLEDGEMENTS

The authors are grateful to the Director, National Dairy Research Institute, Karnal, for providing the facilities and financial assistance. Also, the financial assistance provided by the Council of Scientific and Industrial Research (CSIR), New Delhi, to one of the authors (P. R. Somanath) in the form of Senior Research Fellowship is duly acknowledged.

REFERENCES

Austin, C. R. 1951. Observation on the penetration of sperm into the mammalian egg. Aust. J. Sci. Res. B4:697-698.

Baldi, E., R. Casano, C. Falcetti, C. Krausz, M. Maggi and G. Forti. 1991. Intracellular Ca^{2+} accumulation and responsiveness to progesterone in capacitating human spermatozoa. J. Androl. 12:323-330.

Bansal, P. and S. K. Atreja. 1991. Phosphoinositide specific phospholipase C activity of goat spermatozoa in transit from the caput to the Cauda epididymis. Indian J. Biochem. Biophys. 28:307-311.

Berridge, M. J. 1987. Inositol triphosphate and diacyl glycerol : Two interacting second messengers. Rev. Biochem. 56:159-193.

Blackmore, P., S. Beebe, D. Danforth and N. Alexander. 1990. Progesterone and 17 hydroxy progesterone, novel stimulators of calcium influx in human sperm. J. Biol. Chem. 265: 1376-1380.

Bligh, E. G. and W. J. Dryer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Bray, G. A. 1960. A simple efficient liquid scintillation for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279-285.

Carney, D. H., D. L. Scott, E. A. Gordon and E. F. La Belle. 1985. Phosphoinositides in mitogenesis : neomycin inhibits thrombin -stimulated phosphoinositide turnover and initiation of cell proliferation. Cell. 42:479-488.

Chang, M. C. 1951. Fertilizing capacity of spermatozoa deposited in the fallopian tubes. Nature 168:697-689.

Cockcroft, S. and B. D. Gomperts. 1985. Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature 314:534-536.

Cross, N. L., P. Morales, J. M. Overstreet and F. W. Hanson. 1988. Induction of acrosome reaction by human zona pellucida. Biol. Reprod. 38:235-244.

De Chaffong de Courcellas, D., P. Rocvens and H. Van Belle. 1989. The role of endogenously formed diacyl glycerol in the propagation and termination of platelet activation. A biochemical and functional analysis using the novel diacyl glycerol kinase inhibitor R59949. J. Biol. Chem. 264: 3247-3285.

Domino, S. E., S. B. Bockino and D. L. Garbers. 1989. Activation of phospholipase D by the fucose-sulfate glyco conjugate that induces an acrosome reaction in spermatozoa. J. Biol. Chem. 264:9412-9419.

Exton, J. H. 1990. Signalling through phosphatidylcholine breakdown. J. Biol Chem. 265:1-4.

Exton, J. H. 1994. Phosphatidyl choline breakdown and signal transduction. Biochim. Biophys. Acta. 1212:26-42.

Kaul, G., S. Singh, K. K. Gandhi and S. R. Anand. 1997. Calcium requirement and time course of capacitation of goat spermatozoa assessed by chlortetracycline assay. Andrologia 24:243-251.

Meizel, S. and K. O. Turner. 1991. Progesterone acts at the plasma membrane of human sperm. Mol. Cell. Endocrinol. 11:R1-R5.

Meizel, S., M. Pillai, E. Diaz-Perez and P. Thomas. 1990. Initiation of human sperm acrosome reaction by components of human follicular fluid and cumulus secretions including steroids. In: Fertilization in Mammals (Ed. B. D. Bavister, J. M. Cummins, E. R. S. Roldan). Serona Symposia, Norwell MA, pp. 205-222.

Melendrez, C., S. Meizel and R. Berger. 1994. Comparison of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction. Mol. Reprod. Dev. 39:433-438.

Meyers, S. A., K. M. Irwain, J. W. Overstreet, S. Wadas and E. Z. Drobniz. 1996. Zona pellucida binding and zona induced acrosome reaction in horse spermatozoa : comparison between fertile and infertile stallions. Theriogenology 46:1277-1288.

Meyers, S. A., J. W. Overstreet, I. K. M. Liu and E. Z. Drobniz. 1995. Capacitation *in vitro* of stallion spermatozoa : comparisons of progesterone induced acrosome reactions in fertile and subfertile males. J. Androl. 16:47-54.

Mukerjee, A. B. and J. Lippes. 1972. Effect of human follicular and tubal fluids on human, mouse and rat spermatozoa *in vitro*. Can. J. Genet. Cytol. 14:167-174.

O'Toole, C. M. B., E. R. S. Roldan and L. R. Fraser. 1996a. Role of Ca^{2+} channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. Mol. Reprod. Dev. 45:204-211.

O'Toole, C. M. B., E. R. S. Roldan, P. Hampton and L. R. Fraser. 1996b. A role of diacyl glycerol in human sperm acrosomal exocytosis. Mol. Hum. Reprod. 2:317-326.

Osman, R. A., M. L. Andria, A. D. Jones and S. Meizel. 1989. Steroid induced exocytosis : the human sperm acrosome reaction. Biochem. Biophys. Res. Commun. 160:828-833.

Revelli, A., M. Modotti, A. Pittaretti-Yanez, M. Massobrio and M. Balerna. 1994. Steroid receptors in human spermatozoa. Hum. Reprod. 9:760-766.

Roldan, E. R. S. and E. Dawes. 1993. Phospholipase D and exocytosis of the ram sperm acrosome. Biochim. Biophys. Acta. 1210:48-54.

Roldan, E. R. S. and R. A. P. Harrison. 1989. Polyphosphoinositide breakdown and subsequent exocytosis in the Ca^{2+} ionophore induced acrosome reaction of mammalian spermatozoa. Biochem. J. 259:397-406.

Roldan, E. R. S. and T. Murase. 1994. Polyphosphoinositide-derived diglycerol stimulates the hydrolysis of phosphatidyl choline by phospholipase C during exocytosis of the ram sperm acrosome. J. Biol. Chem. 269:23583-23589.

Schacht, J. 1978. Purification of polyphosphoinositides by chromatography or immobilized neomycin. J. Lipid Res. 19:1063-1067.

Siiteri, J. E., P. Dandekar and S. Meizel. 1988. Human sperm acrosome reaction - initiating activity associated with the human cumulus oophorus and murine granulosa cells. J. Exp. Zool. 246:71-80.

Snedecor, G. W. and W. G. Cochran. 1967. Statistical methods. Oxford & IBH Publishing Co., Calcutta.

Somanath, P. R. and K. K. Gandhi. 2002. Role of calcium and calcium channels in progesterone induced acrosome reaction in caprine spermatozoa. Asia-Aust. J. Anim. Sci. 15(7):949-956.

Somanath, P. R., K. Suraj and K. K. Gandhi. 2000. Caprine sperm

acrosome reaction: Promotion by progesterone and homologous zona pellucida. Small Rum. Res. 37:279-286.

Tesarik, J. 1985. Comparison of acrosome reaction - inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertility ability *in vitro*. J. Reprod. Fertil. 74:383-388.

Thomas, P. and S. Meizel. 1989. Phosphatidyl inositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. Biochem. J. 264:539-546.