Role of Calcium and Calcium Channels in Progesterone Induced Acrosome Reaction in Caprine Spermatozoa

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ABSTRACT : There are several physiological and pharmacological evidences indicating that opening of voltage dependent Ca^{2+} channels play a critical role in induction of acrosome reaction in mammalian sperm. We determined the intracellular free Ca^{2-} concentration in ejaculated goat sperm using a fluorescent, Ca^{2-}-specific probe, Fura2/AM, after the suspension of sperm in KRB medium, capable of sustaining capacitation and the acrosome reaction. We used nifedipine, D-600 and diltiazem, the Ca^{2-} channel antagonists belonging to the classes of dihydropyridines, phenylalkylamines and benzothiazepines, to investigate the possibility that L-type voltage gated Ca^{2-} channels play a role in the progesterone-stimulated exocytotic response. Progesterone promoted a rise in intracellular Ca^{2-} in goat sperm and addition of nifedipine (100 nM) just prior to progesterone induction, significantly inhibited both intracellular Ca^{2+} rise and exocytosis suggesting that Ca^{2+} channels are involved in the process. However, the intracellular Ca^{2+} increase during the process of capacitation was not affected with the addition of nifedipine suggesting a role of focal channel for Ca^{2-} during capacitation. Studies using monensin and nigericin, two monovalent cation ionophores showed that an influx of Na^{-} also may play a role in the opening of Ca^{2-} channels. These results strongly suggests that the entry of Ca^{2+} channels with characteristics similar to those of L-type, voltage-sensitive Ca^{2-} channels found in cardiac and skeletal muscle, is a crucial step in the sequence of events leading to progesterone induced acrosome reaction in goat sperm. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 7 : 949-956)***

Key Words : Goat Sperm, Nifedipine, Monensin, Nigericin, Ca^{2-} Channels, Fura 2

INTRODUCTION

It has long been known that, upon release from the male reproductive tract, mammalian spermatozoa are incapable of fertilizing oocytes, but acquire functional ability during a species-dependent period of residence in the female reproductive tract (Austin, 1951; Chang, 1951). The changes in the spermatozoa that underlie this functional switching on are collectively called as `capacitation'. At the time of fertilization, capacitated mammalian spermatozoa undergo exocytosis of acrosomal granule in response to oocyte associated agonists. This essential process called `acrosome reaction' (AR) results in a release of enzymes necessary for the penetration of the egg vestments, and allows the spermatozoa to fuse with the oocyte plasma membrane after it penetrates the zona pellucida (ZP) (Yanagimachi, 1994). Two major inducers of AR have been identified in the oocyte vestments, progesterone (Meizel et al., 1990) which is trapped in the matrix of the cumulus oophorus and appears to be produced by the cumulus cells (Schuetz and Dubin, 1981) and ZP3 glycoprotein (Bleil and Wassarman, 1983).

It has been shown that progesterone can trigger exocytosis in a variety of species: human (Osman et al., 1989), golden hamster (Meizel et al., 1990), chinese hamster (Shi et al., 1992), mouse (Roldan et al., 1994), pig (Melendrez et al., 1994), horse (Meyers et al., 1995), guinea pig (Shi et al., 1996) and goats (Somanath et al., 2000). Progesterone effects are thought to be mediated by the membrane receptor(s) on the sperm head (Blackmore and Lattanzio, 1991; Sabeur et al., 1996).

Because Ca^{2-} is required for sperm capacitation and Ca^{2+} influx plays an essential role in initiation of sperm acrosomal exocytosis (Fraser, 1993; Gonzalez-Martinez et al., 2001), the existence of Ca^{2-} channels on sperm membrane could be anticipated. It was reported that there was a certain sequence of ionic changes involved in initiation of AR in capacitated sperm and Ca^{2+} influx was the pivotal step (Fraser, 1993). Entry of Ca^{2-} into voltage gated Ca^{2+} channels as a consequence of membrane depolarization is a key for the secretory activities of the many cell types. Relating the degree of depolarization, required for these activation, voltage gated Ca^{2+} channels have been classified into transient (T-type) or long acting (L-type) based on how long they remain under continuous depolarization (Reuter, 1983; Nilius et al., 1985). Not all cell types express voltage-gated Ca^{2+} channels, yet most of them do express more than one type of Ca^{2-} channels. Nifedipine sensitive Ca^{2-} channel has been reported in boar and human spermatozoa (Tiwari-Woodruff and Cox, 1995; O'Toole et al., 1996). The existence of a nifedipine and verapamil sensitive Ca^{2-} channel with unit conductance 40 pS has been reported on isolated human sperm membrane (Shi and Ma, 1998). Pharmacological studies suggest that the major channel in the sperm head plasma membrane

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responsible for modulating Ca^{2+} entry could be either an L-type or a T-type voltage gated Ca^{2+} channel (Publicover and Barrat, 1999). Patch clamp analysis of Ca^{2+} currents in immature spermatogenic cells demonstrate the presence of T-type currents (Benoff, 1998). Therefore, an argument has been putforth that the AR of ejaculated sperm is regulated by a T-type Ca^{2-} channel. However, indirect analysis of Ca^{2-} currents in mature sperm after transfer of ion channels to planar lipid bilayers detects three current types, including that similar but not identical to an L-type channel, but no T-type currents (Benoff, 1998).

Progesterone induced AR, a crucial step in the preparation of sperm cell for the fertilization of the egg is triggered by a rapid influx of Ca^{2-} that is mediated by Ca^{2-} channels located on the sperm plasma membrane (Florman et al., 1992; Blackmore, 1993). In addition to Ca^{2+} influx, a sequence of ionic changes such as Na^{-} influx and efflux of H^{+} and K^{+} has also been observed in association with the AR (Florman et al., 1992; Fraser et al., 1993). In goat, we have observed that Ca^{2-} and PO_4^{-} are important for the progesterone and ZP induced AR and a calcium-phosphate co-transporter is involved in the process. The involvement of a GABA/Cl^{-} channel for the entry of Ca^{2+} during progesterone induced AR has also been reported in goat sperm (Somanath et al., 2000).

In the present study, we used Fura 2/AM, a fluorescent dye for the measurement of intracellular Ca^{2-} ($[Ca^{2+}]_i$), during the course of capacitation and progesterone induced AR. In addition, we used the Ca^{2+} channel antagonists belonging to three different classes of dihydropyridines, phenyl aklylamines and benzothiazepines to check the involvement of different types of Ca^{2-} channels. Also, we used the monovalent cation ionophores, monensin and nigericin to investigate the possibility that an influx of Na^{-} might play a role in the exocytotic mechanism.

MATERIAL AND METHODS

Bovine serum albumin (Fraction V), sodium lactate, progesterone, chlortetracycline (CTC), Hoechst bis-benzimide 33258, 1,4-diazobiyclo [2.2.2] octane (DABCO), fura 2/AM, lanthanum chloride, monensin, nigericin, nifedipine, D-600, diltiazem, A23187, trizma and digitonin were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other reagents used in the study were of analytical grade.

Collection and preparation of spermatozoa

Semen was collected from the institute bucks using artificial vagina, washed twice with albumin saline (pH 7.4). Hundred percent motile cells were prepared by swim-up technique (Cross et al., 1988) and were suspended in Krebs-Ringer Bicarbonate (KRB) medium. The sperm concentration was adjusted to 5×10^6 cells/ml after counting in a hemocytometer. The sperm motility was assessed microscopically by examining uniform drops of semen under a coverslip 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 percent progressive motility were considered for experiments. Incubations were carried out at 37°C in a CO_2 incubator.

Reagent stock solutions

A 10 mg/ml progesterone stock solution was prepared daily in DMSO; this was diluted 10-fold in 1:1 DMSO: KRB medium and added into the sperm suspension to give a final concentration of 15 μM. Stock solution of nifedipine (500 μM) was prepared daily in absolute ethanol. Monensin (2 mM) and nigericin (10 mM) were prepared in absolute ethanol and stored at -20°C. All three were diluted in KRB medium as required before being added to sperm suspensions. Lanthanum chloride (50 mM), diltiazem (10 mM) and D-600 (10 mM) were prepared in PBS (pH 7.4) and diluted as required by the addition of KRB medium. Ca^{2+} (10 mM) was dissolved in PBS (pH 7.4) and Ca^{2+}-ionophore A23187 (150 mM) was prepared in DMSO and stored at -20°C.

Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

The relative fluorescent intensity was measured in a Cary3-varian Spectrophotometer with fluorescent accessory. The excitation wavelength set at 339 nm with 5 nm slits; emission was recorded at 500 nm with 10 nm slits.

Sperm suspensions were incubated with Fura 2/AM at a final concentration of 1 μM at 37°C for 1 h. Three ml of Fura 2 loaded sperm cells were transferred to a quartz cuvette (final concentration 1.5×10^7 cells/ml). The concentration of the DMSO in the Fura 2 loading solution was less than 0.5 percent. Control experiments showed no detectable influence of DMSO on sperm motility and $[Ca^{2+}]_i$ at the solvent concentrations used.

In dye loaded cells $[Ca^{2+}]_i$ concentration was determined using a modified procedure described by Malgaroli et al. (1987). Briefly, the presence of extracellular Fura 2 (including the leakage of Fura 2) was ascertained by the addition of EGTA (3 mM) and then Tris (20 mM) at pH 8.2. The fluorescence minimum (F_{min}) was obtained by dye release from sperm in the presence of digitonin (0.06 mg/ml). Following addition of HCl (20 mM), the fluorescence maximum (F_{max}) was measured in the presence of 3 mM Ca^{2+} added (pH 7.5) (figure 2). Auto fluorescence was substracted from the results. Generally, autofluorescence intensity was less than 20 percent to that of F_{max}; autofluorescence intensity remained constant regardless of the presence of EGTA, Tris, digitonin, HCl and Ca^{2+}. The $[Ca^{2-}]_i$ was calculated from the formula

$[Ca^{2-}]_i=K_d$ (F- F_{min})/(F_{max}-F). Where, K_d =224 nM, is the dissociation constant of Ca^{2-} binding to Fura 2.

Effect of La^{3+} on acrsome reaction induced by monovalent cation ionophores

Swim-up prepared sperm suspensions were incubated for 4 h at 37°C, then divided into different aliquotes, two of which received La^{3+} (500 μM). After incubating for 15 min, either monensin (100, 500 and 1.000 nM) or nigericin (10, 30 and 50 μM) were added. Both 50 μM nigericin and 1.000 nM monensin were added to two separate La^{3-} treated aliquotes. After 15 min further incubation, cells were stained with CTC and assessed.

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.

Statistical analysis

Percentage of AR obtained in different experiments are expressed as the mean±standard error of mean (SEM). Statistical differences involving multiple treatments were determined by one way ANOVA (Snedecor and Cochran, 1967). In instances where one treatment and control were compared, 'student t-test' was applied.

RESULTS

Role of Ca^{2+} in progesterone induced goat sperm acrosome reaction

Progesterone (15 μM), Ca^{2-} ionophore A21387 (15 mM), Ca^{2-} (10 mM), all significantly induced AR in capacitated goat spermatozoa ($p<0.01$). Addition of 2 mM EGTA or 0.5 mM Ca^{3+} inhibited progesterone induced AR ($p<0.01$) (figure 1). The solvent for progesterone and A23187 (DMSO) had no effect on AR.

Table 1 gives the results of experiments in which washed pre-capacitated sperm were exposed first to divalent cation (Ca^{2+}) and then to monovalent cations (Na^+ and K^+) (Expt. B) or in reverse order (Expt. C). In experiments B and C, many sperm thus treated lost motility, but were not dead (data not shown). It should be noted that the vast majority of spermatozoa were unable to acrosome react in response to Ca^{2+} in the absence of monovalent cations, but many of them acrosome reacted when they were transferred to Ca^{2-}-free medium containing Na^- (Expt. B). Virtually no AR took place when the sperm were first exposed to Ca^{2+}-free medium with monovalent cation, then transferred to Ca^{2-}-containing medium, without monovalent cations (Expt. C). Virtually, no inhibition of AR was noticed when K^+ alone was removed from the media (Expt. D).

Table 1. Acrosome reaction (AR) of precapacitated ejaculated goat sperm incubated consecutively in monovalent (Na^-/K^+)-free and divalent (Ca^{2+})-free media

Exp.	Incubation media contanining								AR after 15 min of incubation	
	1st treatment				2nd treatment					
	Na^+	K^+	Ca^{2-}	P	Na^+	K^-	Ca^{2+}	P	1st incubation	2nd incubation
A	+	+	+	+	+	+	+	+	70.76±2.17	70.26±4.03
B	-	-	+	+	+	+	-	+	21.66±1.71*	54.20±2.34*
C	+	+	-	+	-	-	+	+	19.33±1.39*	22.40±1.66*
D	+	-	+	+	+	-	+	+	72.96±0.98*	70.56±0.41**

In two treatments in four different experiments, cells were exposed to various ions in different combinations. Two separate sets of slide preparations were made for individual experiments after each treatments in order to ascertain the role of each ion and also to check which ion plays the initial role.

Results are mean±SEM (n=3). Concentration of Na^+, K^+ and Ca^{2-} were 119.37 mM, 1.20 mM and 1.71 mM, respectively. * $p<0.01$ compared with A. ** $p<0.001$ compared with A.

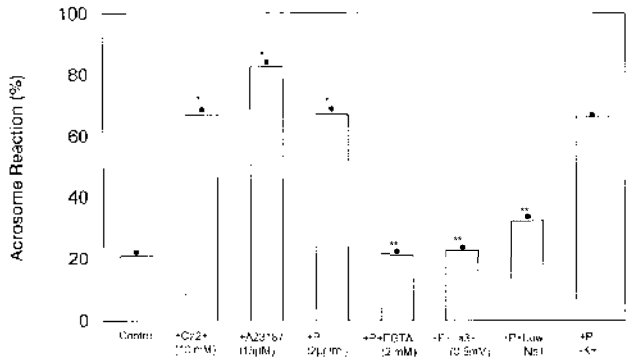

Figure 1. CTC fluorescence patterns in goat sperms suspension pre-incubated for 4 h in KRB medium and separately treated with Ca^{2+} (10 mM), A23187 (15 mM) and progesterone (15 μM). Patterns of separate experiments conducted to study the effects of La^{3+} (0.5 mM), EGTA (2 mM), low Na^{+} (25 mM) containing medium and K^{+} deficient medium on progesterone induced acrosome reaction are also shown. Results are mean±SEM (n=3). * $p<0.01$ compared with untreated control. ** $p<0.01$ compared with progesterone-only treated control.

Changes in $[Ca^{2+}]_i$ during the time course of capacitation and acrosome reaction

At the onset of incubation, $[Ca^{2+}]_i$ amounted to 180-210 nM. With an increase in incubation time, $[Ca^{2+}]_i$ increased concomitantly by reaching a peak of 1.428±36 nM after 3 h (figure 2). The addition of as little as 500 ng/ml (~1.5 μM) of progesterone in the capacitation media had a significant effect ($p<0.05$) on increase in $[Ca^{2+}]_i$ mounting up to 1,628± 118. When nifedipine (100 nM) was added prior to progesterone, it did not abolish the effect mediated by progesterone. The motility of the spermatozoa at the onset was approximately 90 percent which was reduced to approximately 60 percent. The suspension had above 90 percent cell viability in the beginning and above 70 percent towards the end of the experiment, as determined by the vital stain Hoechst 33258.

Addition of 15 μM progesterone to Fura 2-loaded capacitated sperm suspension caused an increase in $[Ca^{2+}]_i$ level mounting up to 8.7±1.79 μM after 6 min incubation. This rise in $[Ca^{2+}]_i$ in capacitated spermatozoa induced by progesterone was inhibited by 100 nM nifedipine ($p<0.05$) (not shown).

Effect of Ca^{2+} channel antagonists on progesterone induced goat sperm acrosome reaction

Swim-up prepared sperm suspensions were incubated for 4 h at 37°C with nifedipine (20-100 nM range) at 3 h 45 min. At 4 h, progesterone (15 nM) was added; after incubation for 30 min, cells were stained with chlortetracycline and assessed for the status of AR.

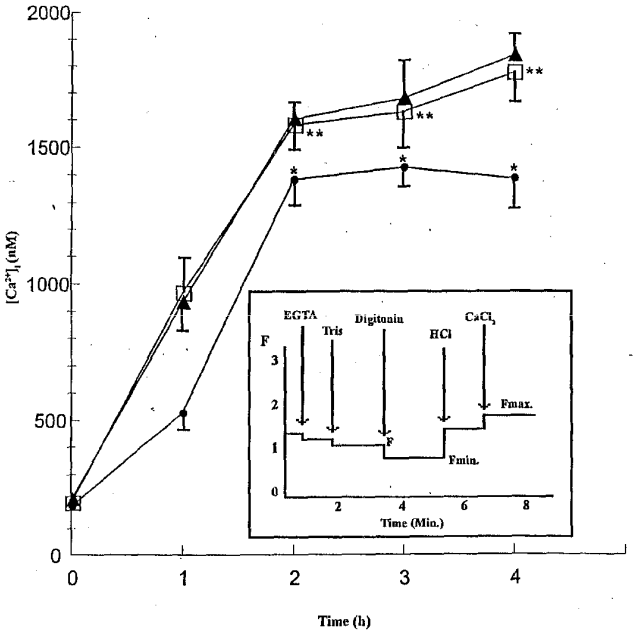

Figure 2. Dependence of intracellular free Ca^{2+} concentration in ejaculated goat sperm on incubation time in KRB medium in the presence and absence of progesterone and/or nifedipine. Following incubation, spectral measurements were performed on cells suspended in the same medium without BSA, as described in material and methods. Results are mean±SEM (n=4). * $p<0.05$ compared with the value of untreated control at 0 h. ** $p<0.05$ compared with the untreated controls of respective time periods.
Insert: Typical time dependence of changes in relative fluorescence intensity (F) of Fura 2-loaded sperm following addition of EGTA (3 mM), Tris (20 mM), digitonin (0.06 mM), HCl (20 mM) and $CaCl_2$ (3 mM). From these values, F_{max} and F_{min} of fluorescence intensity were derived. ( ) untreated control; (•) progesterone (500 ng/ml) only treated cells; ( ) progesterone (500 ng/ml) and nifedipine (100 nM) treated cells.

The addition of progesterone to sperm suspensions resulted in an increase in acrosomal exocytosis as evidenced by significantly more AR pattern cells ($p<0.01$) in the progesterone treated cells compared with the control group (figure 3). When 100 nM nifedipine was added first prior to progesterone, there was a significant inhibition of acrosomal exocytosis compared with the progesterone only treated cells. The presence of either verapamil or diltiazem also resulted in a significant inhibition ($p<0.01$) of acrosome loss, but at a very high concentration when compared to nifedipine (figure 4).

Effect of La^{3+} on acrosome reaction induced by monovalent cation ionophores

An alternative way of stimulation of AR is to induce an

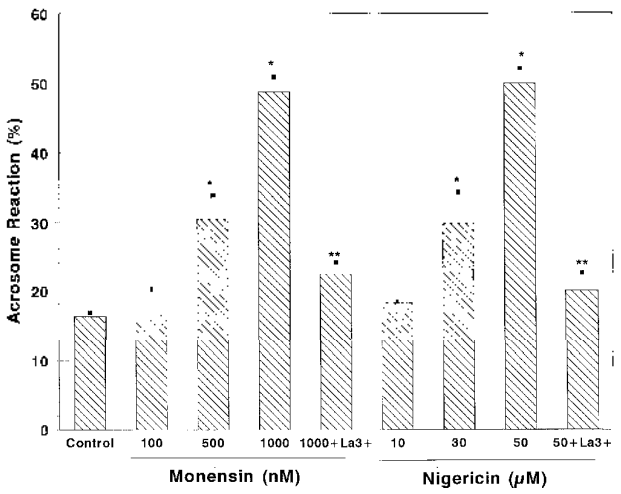

Figure 3. CTC fluorescence patterns in goat sperm suspensions pre-incubated for 4 h in KRB medium with nifedipine being added at 3 h 45 min. At 4 h, the sperm suspensions were exposed to 15 μM progesterone for 15 min. Results are mean±SEM (n=3). * $p<0.001$ compared with control. ** $p<0.001$ compared with progesterone-only treated control.

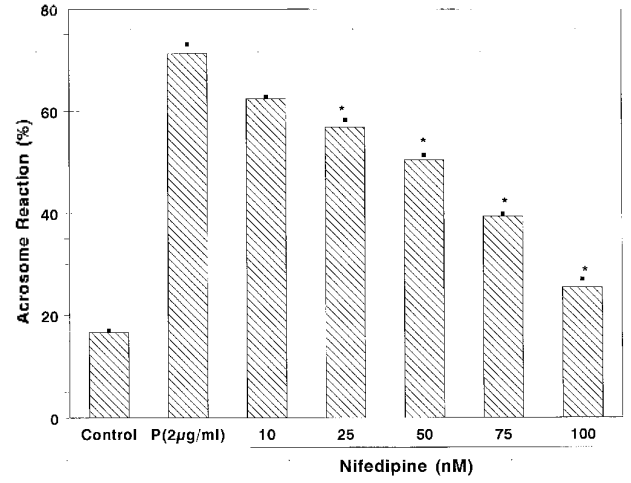

Figure 4. CTC fluorescence patterns in goat sperm suspensions pre-incubated for 4 h in KRB medium with D-600 and Diltiazem being added at 3 h 45 min. At 4 h, the sperm suspensions were exposed to 15 μM progesterone for 15 min. Results are mean±SEM (n=3). * $p<0.001$ compared with control. ** $p<0.05$ compared with progesterone-only treated control.

influx of Na^+ into sperm using monovalent cation ionophores (Fraser et al., 1993). Monensin has a greater specificity for Na^+ and would move Na^+ into the cell, under the conditions present in the experiments (high Na^+ outside and low Na^+ inside cells). Nigericin, another monovalent cation ionophore, has greater specificity for K^+. However, the K^+ gradient is the opposite of that of Na^+, i.e., low K^+ outside and high K^+ inside cells; therefore, the overall net effect of adding nigericin would be to move K^+ out and Na^+ into a cell, similar to the responses initiated by monensin (Hyne, 1984). Monensin and nigericin significantly induced AR in goat sperm ($p<0.01$). However, La^{3+} (0.5 mM) when added to individual sperm suspensions, separately treated with 50 μM nigericin and 1,000 nM monensin, abolished their effects.

DISCUSSION

Although capacitation and fertilization normally occur within the female reproductive tract, the fact that permissive conditions can be provided *in vitro* has allowed the analysis of specific requirements for these two distinct phases. Ca^{2+} is obligatory for both capacitation and AR, but the amount required for each differs markedly (Fraser, 1990). In the case of goat sperm, we observed an increase in $|Ca^{2+}|_i$ up to 1-2 μM, but maximum acrosome reacting ability is achieved only when the concentration increases up to 8-9 μM.

Figure 1 shows the importance of Ca^{2+} and Na^+ ions in the process of progesterone induced AR. K^+, being an intracellular cation and need to be effluxed out during the entry of Na^+ into the cells does not seem to have a role in AR when it is present in the extracellular medium. When progesterone was added to a low Na^+ (25 mM) containing medium, it did not induce the effect showing the importance of Na^+ in the process.

As reported previously (Murphy et al., 1986; Fraser, 1990) and confirmed in this study, both Na^+ and Ca^{2+} must be in the medium to ensure AR. But to find out the ion which acts first on the sperm, we incubated capacitated spermatozoa first in Ca^{2+}-containing monovalent-free medium and then in Na^+-containing Ca^{2+}-free medium and vice-versa. According to our results (table 1), Ca^{2+} apparently enters first. This support the findings of Fraser (1990) that Ca^{2+} passing through the plasma membrane of the capacitated sperm inactivates a membrane-bound Na^+/K^+-ATPase. This inactivated ATPase would no longer pump Na^+ out, resulting in a rapid accumulation of intracellular Na^+ that in turn would cause H^+ efflux via a plasma membrane-associated Na^+/H^+ anti-porter. As suggested by Hyne et al. (1984), an increase in intracellular Na^+ concentration could reverse a plasma membrane associated Na^+/Ca^{2+} anti-porter, allowing more Ca^{2+} to enter the sperm.

Figure 2 shows the increase in $|Ca^{2+}|_i$ during the course of capacitation. Progesterone showed a significant effect in increasing $|Ca^{2+}|_i$ when added at 0 h which was not inhibited by the addition of 100 nM nifedipine. However,

the increase in $[Ca^{2+}]_i$ with the addition of 15 µM progesterone during the process of AR was completely abolished by prior incubation with 100 nM nifedipine. This shows the difference in Ca^{2+} channels involved in the process of capacitation and AR. This is in agreement with the report of Walensky and Snyder (1995) that, capacitative Ca^{2+} entry occurs through focal voltage insensitive channels which on later stages helps in depolarization, and, in turn, activates L-type Ca^{2+} channels to mediate AR.

There are a number of possible mechanism of actions that could result in the opening of Ca^{2+} channels (Reviewed by Revelli et al., 1994), but experimental evidences do not support all the possibilities. Although the mechanisms involved in the sperm cell's exocytotic response to progesterone have yet to be fully elucidated, our study with Ca^{2+} channel antagonists fully support a role of Ca^{2+} channels with characteristics similar to L-type, voltage gated channels identified in somatic cells. The addition of nifedipine, a dihydropyridine Ca^{2+} channel antagonist, which is most specific to L-type Ca^{2+} channels, inhibited progesterone initiated AR (figure 3). The addition of D-600 and Diltiazem, two Ca^{2+} channel inhibitors belonging to the classes of phenylakylamines and benzothiozepines, respectively, also inhibited AR; but at a higher concentrations (figure 4). Moreover, the report that progesterone does not activate T-type Ca^{2+} channels in human sperm (Blackmore and Eisoldt, 1999), further confirms the involvement of L-type channel for the entry of Ca^{2+}.

The stimulation of acrosome loss elicited by monovalent cation ionophores, monensin and nigericin, and the inhibition of both the responses by the prior inclusion of La^{3+} (500 µM) suggests that an influx of Na^+ also play a role in the same pathway (figure 5). These ionophores bypass the role of initial Ca^{2+} entry which inactivates the membrane-bound Na^+/K^+-ATPase, thereby accumulating Na^+ inside the sperm. This in turn, increases the alkalinity thereby causing a depolarization in the membrane and leads to the opening of Ca^{2+} channel. Electrophysiological studies of other tissues also have showed that in alkaline media, less depolarization is required to activate Ca^{2+} channels, probably as a consequence of localized surface charge effects (Ijima et al., 1986). The addition of La^{3+} blocks the opening of voltage gated Ca^{2+} channels, thereby inhibiting the AR initiated by the monensin and nigericin.

CONCLUSION

The fact that the nifedipine is inhibitory at nanomolar concentrations suggests that the channels involved in the caprine sperm responses are similar to L-type voltage gated channels. There is now considerable evidence that such Ca^{2+} channels exists in many, if not all, mammalian

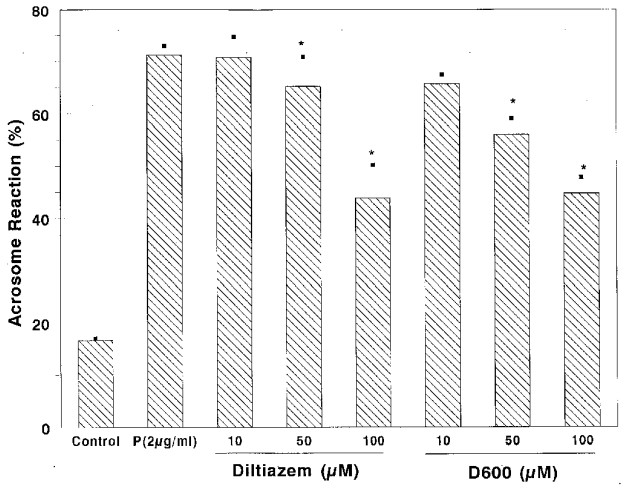

Figure 5. CTC fluorescence patterns in goat sperm suspensions pre-incubated for 4 h in KRB medium and exposed to monensin and nigericin for 15 min. Results are mean±SEM (n=3). * $p<0.01$ compared with untreated control. ** $p<0.01$ compared with monensin (1.0 µM)/nigericin (50 µM) treated control.

spermatozoa. Binding sites for various Ca^{2+} channel antagonists, including the dihydropyridines and phenylalkylamines have been identified in ram and bull sperm (Florman et al., 1992). In mouse spermatozoa, nifedipine and verapamil have been shown to inhibit both spontaneous AR and *in vitro* fertilization (Fraser and McIntyre, 1989) and AR stimulated by progesterone (Shi and Roldan, 1995). Therefore, the existence of similar Ca^{2+} channels in caprine sperm would be consistent with the evidence from many other mammalian species. Further study to analyse the channel properties and behaviours as well as to compare the channel activity in normal and pathologic sperm may reveal the ionic basis underlying sperm physiology and some cases of male infertility.

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