Bangladesh Journal of Animal Science
Journal homepage: http://www.banglajol.info/index.php/BJAS

Effect of calmodulin on the stimulation of capacitation and acrosome reaction of frozen thawed bull spermatozoa

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Abstract

Capacitation and acrosome reaction (AR) are the prerequisites for successful fertilization by mammalian spermatozoa. Intracellular calcium (Ca^{2+}) has a regulatory role in sperm motility, capacitation, and AR. Calmodulin (CaM) antagonists calmidazolium (CZ) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) were used to investigate the possible role of CaM, a Ca^{2+} specific binding protein, on motility, capacitation and AR of frozen-thawed bovine spermatozoa. Capacitation and AR in sperm were evaluated by using chlortetracycline (CTC) staining technique. Addition of the 1 mM dibutyryl cAMP (dbcAMP) and 100 µM 1-methy l-3-isobutylxanthine (IBMX) to CaM antagonists treated sperm incubated in the presence of $NaHCO_3$ and $CaCl_2$ in media overcome the inhibitory effects of these antagonists to support capacitation and AR at 4 h of incubation period. In contrast, addition of dbcAMP with IBMX induced AR in spermatozoa incubated with $NaHCO_3$-free medium but these compounds did not induce AR in cells incubated in $CaCl_2$-free medium. However, the addition of dbcAMP and IBMX partially, but significantly ($p<0.01$) reversed the inhibitory effect of W7 and CZ on the sperm capacitation and AR. These results suggest that CaM may play an important role in the regulation of capacitation and AR in frozen-thawed bovine spermatozoa.

Key words: acrosome reaction, frozen thawed bull spermatozoa, capacitation, calmodulin

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Introduction

Artificial insemination is the most widely applied tool in modern animal breeding and thereby facilitating extensive utilization of cryopreserved spermatozoa. Mammalian spermatozoa undergo a variety of physiological and biochemical modifications during their transit through female reproductive tract (Harayama, 2013). These changes are collectively termed "capacitation" and regulated by sperm interaction with female reproductive tract (Kuo *et al*., 2016). However, sperm capacitation is associated with Ca^{2+} uptake (Handrow *et al.,* 1989), decreased binding of proteins to calmodulin (CaM) (Leclerc *et al.,* 1992), increased cyclic adenosine mono phosphate cAMP (White and Aitken, 1989), activation of cAMP dependent protein kinase-A (Visconti *et al.,* 1997), and changes in tyrosine phosphorylation levels (Tardif *et al.,* 2001). But, trans-membrane and intracellular signaling event regulating sperm capacitation are likewise, poorly understood.

In vitro capacitation and induction of AR can be accomplished using several treatments (Tanphaichitr and Hansen, 1994). After capacitation, one primary signal that initiates the AR is a change in sperm plasma membrane permeability for Ca^{+}-influx. Therefore, divalent cationic ionophores, such as A23187, artificially induce calcium entry into sperm, resulting in the AR in bovine (Triana *et al.,* 1980) and stallion (Farlin *et al.,* 1992) spermatozoa. However, it is not clear how sperm functions are affected by these media constituents. The onset of both capacitation and AR are dependent on the presence of bovine serum albumin (BSA), Ca^{2+} and $NaHCO_3$ in the culture medium, three components that have been demonstrated to be essential for *in vitro* capacitation of sperm from a variety of different mammals (Yanagimachi *et al.,*

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1994). The underlying Ca^{2+}-regulated pathways involved in the initiation and maintenance of sperm motility have not yet been fully understood. Ca^{2+} can directly bind to membrane phospholipids and to several enzymes, with the subsequent modification of membrane properties and enzymatic activity. This cation may also act through binding to CaM. The Ca^{2+}-binding protein CaM in somatic cells modulates the activity of key enzymes also identified in mammalian sperm cells, including adenylyl cyclases (Mons *et al.,* 1999), phosphatases (Rusnak and Mertz, 2000), phosphodiesterases (Wasco and Orr, 1984) and protein kinases (Hook and Means, 2001). Furthermore, immunocytochemical studies reveal that CaM is present in the head and flagellum of mammalian sperm (Moriya *et al.,* 1993), suggesting that CaM could be involved in functions occurring in the sperm head, tail, or both locations.

Moreover, CaM operates on various targets in sperm; the roles of CaM in sperm physiology are diverse and include the support of capacitation-related changes in plasma membrane properties, modulation of cAMP levels (Fournier *et al.,* 2003), and the ability to undergo the AR (Bendahmane *et al.,* 2001). Cyclic AMP is implicated in many sperm functions such as capacitation (Visconti *et al.,* 1995), AR (Kopf and Gerton, 1991) and initiation and maintenance of motility (Yanagimachi *et al.,* 1994). Recently, Zeng and Tulsiani *et al.,*(2003) demonstrated that elevated intracellular Ca^{2+} is required for the initiation of hyperactivated motility and capacitation suggesting that CaM and Ca/CaM-stimulated pathways are involved. Although, there are several studies that have shown the importance of CaM and Ca/CaM levels in sperm function, direct influence of CaM antagonists on capacitation and AR in bovine spermatozoa have not yet been definitively demonstrated.

In this study, we have investigated the role of CaM antagonists to understand its possible significance in multiple aspects of frozen-thawed bovine sperm capacitation and AR. Therefore, from these points of view the present study was designed to know the effect of several components including calcium ionophore, $CaCl_2$, $NaHCO_3$ and cAMP agonists on bovine sperm capacitation and AR.

Materials and Methods

Chemicals and media

All chemicals used in this experiment were purchased from Sigma Chemicals (St. Louis, MO) with the following exceptions: dimethyl-sulfoxide (DMSO), bovine serum albumin, glutaraldehyde, sodium pyruvate were obtained from Wako Pure Chemicals Industries and L-cysteine was obtained from Kanto Chemical Co., Inc, Japan. All chemicals were the highest analytical grade. Stock solutions of calmidazolium (CZ) and *N*-(6-aminohexyl)-5-chloro1- naphthalenesulfonamide (W7) were prepared in 1.5% dimethyl sulfoxide (DMSO) and stored at -20°C. The basic medium used throughout these experiments was a modified Bracket and Oliphant (BO; 1975) for semen dilution and washing.

Preparation of semen samples

Frozen semen of Holstein Friesian bull was obtained from the Nagano Livestock Improvement Association of Japan. The same pool of semen was used throughout this study. Frozen straws were thawed in a water bath at 37°C for 10 to 14 seconds. The frozen-thawed semen samples were suspended in BO media and washed thrice with BO-BSA through centrifugation (5 minutes at 2000 rpm) to remove the extra-cellular debris and egg-yolk particles. After centrifugation the supernatant was removed and then 0.5 ml BO-BSA added. The sperm suspension was gently mixed and again centrifuged, same procedure was performed for thrice for washing of bovine sperm. The concentration of sperm was estimated using a haemocytometer and adjusted with B0-BSA to 50×10^6 sperm/ml and incubated at 38.5°C, 5% CO_2 for 0-4 h incubation period.

Capacitating and AR inducing agent preparation

A solution of 0.5 μM calcium ionophore was prepared by diluting ionophore (1 mg) into EtOH-DMSO (955 μl). This solution was stored at -20°C until use. A working solution of calcium ionophore (2 mM) was made by diluting the stock solution into BO-Caffeine (1 ml). The stock of 1 mM cAMP and 100 μM IBMX were prepared by dissolving both cAMP and IBMX (0.005 g) in DMSO (15.18

ml and 2.25 ml, respectively) and was stored at -20°C until use.

Evaluation of motility

After completion of the 0-4 h incubation period, the subsamples (10 μL) of each sample were transferred to an examination chamber (Sekisui, Tokyo, Japan), placed on a warmer set (MP-10DM; Kitazato, Tokyo, Japan) kept at 38°C, and the motility was examined under a light microscope (100×). Sperm motility was assessed by determining the percentage of spermatozoa in each of the following five categories of movement: (a) rapid progressive motility; (b) slow or sluggish progressive motility; (c) non-progressive motility; (d) immotility and e) dead. Motility was then expressed as a progressive motility percentage. The percentage of spermatozoa with progressive motility was determined subjectively by scoring 400 individual sperm population in each sample at 0, 1, 2 and 4 h of incubations.

Evaluation of viability

Sperm viability was determined by Hoechst 33258 staining (Sigma, B-2883). Fifty-microliter sample was mixed with 1 μl of modified Hoechst 33258 solution and sperm was incubated with the dye Hoechst 33258 for 3 minutes. After incubation, the slides were covered with 10 μl of sperm suspension and mounted on a glass slide under a coverslip. At least 100 sperms per slide were counted by fluorescent microscopy (FL-microscopy, 420 nm) and spermatozoa were classified as dead when nuclei showed bright blue fluorescence over sperm head and live when sperm head was not strongly fluorescent.

Evaluation of Capacitation and AR by the CTC Assay

The fluorescent antibiotic CTC was used to assess sperm capacitation. We used a modified version of the CTC assay described by Ward and Storey (Ward and Storey, 1994). In addition to simultaneous evaluation of cell viability and capacitation state, CTC staining was coupled with Hoechst 33258 staining Storey (Ward and Storey, 1994). CTC (750 μM) was prepared in 20 mM tris buffer containing 130 mM NaCl and 5 mM DL-cysteine and the pH was adjusted to 7.8. At room temperature, 50 μl of Hoechst stained sperm sample was mixed with 450 μl of Polyvinylpyrrolidone-PBS. After 5 min centrifugation at 1500 rpm, the supernatant was discarded. Then 0.3% BO-BSA added and finally the sperm sample was mixed with 100 μl CTC solution. After 30 sec, 5 μl of 12.5% glutaraldehyde (pH 7.4), was added. Finally, 5 μl of 0.22 M 1, 4-diaza-byciclo (2.2.2) octane *(*DABCO*,* Sigma, USA*)* in 50% glycerol was added to retard fluorescence fading and 10 μl of this suspension was placed on a clean slide. After mounting with a coverslip, the slides were stored in dark at 4°C until counting within 24 h. The slides were assessed with microscope (Olympus BX40) equipped with epifluorescence optics; cells were observed under blue-violet illumination (excitation at 400-440 nm and emission at 470 nm). In addition, we assessed the HO-positive sperm (dead sperm) in the same field, and thus, the percentage of each CTC pattern in the live sperm population was determined. One hundred spermatozoa per slide were counted and classified according to the CTC patterns: F, showing uniform fluorescence on the head, indicating incapacitated, acrosome intact spermatozoa; B, represented by a fluorescence-free band on the post acrosomal region, indicating capacitated, acrosome intact spermatozoa; and AR, represented by a uniformly fluorescence-free head and with a fluorescence band in the equatorial region, indicating AR as shown in Figure 1.

Experimental layout

In experiment 1, the sperms were treated with various concentrations of CaM antagonists, CZ (5, 10, 15 μM) and W7 (50, 75, 100 μM). Then sperms were incubated up to 4 h at 38°C, 5% CO_2. The inhibitor treatments were added to the sperm suspension immediately before incubation. Control without inhibitors received an equivalent amount of DMSO. At 0, 1, 2, and 4 h, 10 μl aliquots were removed for evaluation of motility. Viability, capacitation and AR were assessed at 0 and 4 h of incubation.

In experiment 2, the sperms were then treated in separate analyses with, IA and dibutyryl cAMP (dbcAMP) plus 3-isobutyl-1-methylxanthine (IBMX) in the absence (control) or presence of CaM antagonists (CZ and W7). The concentration of CZ (5 μM) and W7 (50 μM) was selected from

the first experiment. The mixture was then incubated in conditions (38°C, 5% CO_2 in air) for 4 h.

Statistical analysis

Results are presented as mean ± SEM of the number of samples indicated in each experiment. To determine whether there were significant differences among treatments and between conditions of incubation, means were compared using two tailed ANOVA. Multiple comparisons were made using Tukey's test, differences were considered to be statistically significant when P<0.01.

Results

Effects of calmidazolium（CZ; a）and *N*-(6-aminohexyl)-5-chloro-1 Naphthalenesulfonamide (W7; b) on the percentages of sperm progressive motility are shown in Figure 1 (a, b). Progressive motility were decreased in the sperm treated by the concentrations of CZ (5-15 μM) and W7 (50-100 μM) than that of the control sperm after 1 h of incubation. The mean percentage of motile sperm of CZ and W7 treated group was less than 20% at 4 h of incubation. However, the effect of CZ and W7 was more severe at higher concentrations compared with the control in all incubation periods.

CaM antagonists, both CZ and W7 inhibited the sperm capacitation (B pattern) and AR pattern in a concentration-dependent manner. CZ, at 10 μM significantly suppressed the frequency of the B pattern, and inhibition was even greater at higher concentrations, 15 μM CZ compared with the control (Figure 2, a). Another CaM antagonist, W7 also decreased the percentage of B pattern sperm in a concentration-dependent manner, with maximal inhibition at 75-100 μM, the results is shown in Figure 2, (a). In contrast of two CaM antagonists, W7 did not significantly affect the B pattern even at 50 μM. Sperm were incubated for 4h in BO medium, the percentage of AR sperm to about 20-25% in all treated group. Sperm treated with 5 μM CZ and 50 μM W7 showed similar percentages of AR compared with the control (Figure. 2, b). But at higher concentrations of CZ and W7 were significantly shown to inhibit the acrosome reaction.

In the concentration range of 5-15 μM for CZ and 50-100 μM for W7, none of these CaM antagonists showed significant inhibitory effect on sperm viability. The proportion of live and dead sperm was separated by Hoechst Staining. The viability was around 61.7±1.7% at 0 h of incubation and 52±1.1% at 4 h of incubation, in both control and all treated groups (data not shown).

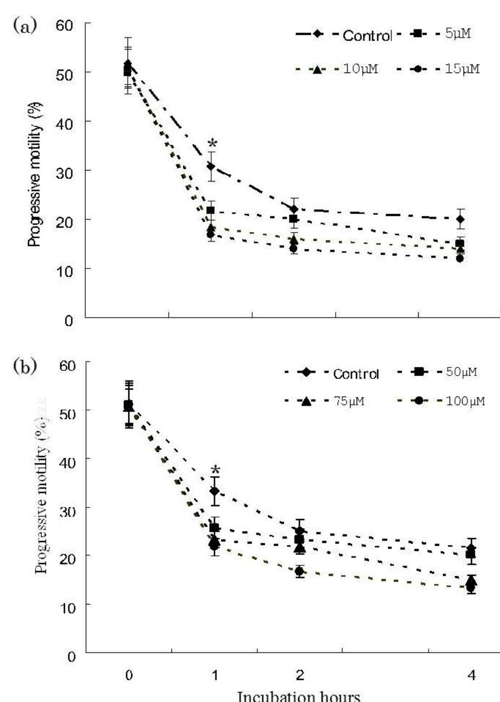

Figure 1. Effects of calmidazolium (CZ; a) and *N*-(6-aminohexyl)-5-chloro-1 Naphthalene sulfonamide (W7; b) on the percentages of sperm progressive motility. Spermatozoa incubated with various concentrations of CZ, W7 and control sperm receiving no added antagonist. Sperm motility observed at 0, 1, 2 and 4 h of incubation periods. Results are the mean±standard error (n=3). * Significantly different (P<0.01) from control.

When dbcAMP plus IBMX (a nonspecific inhibitor of cAMP and cGMP phosphodiesterases) was added to the incubation medium containing either 5 μM or CZ 50 μM W7 and incubated for 4 h, the sperm displayed motility almost similar to that obtained when cAMP analogues were not added (data not shown). Thus, addition of cAMP analogues was unable to reverse the effects of CaM antagonists on motility.

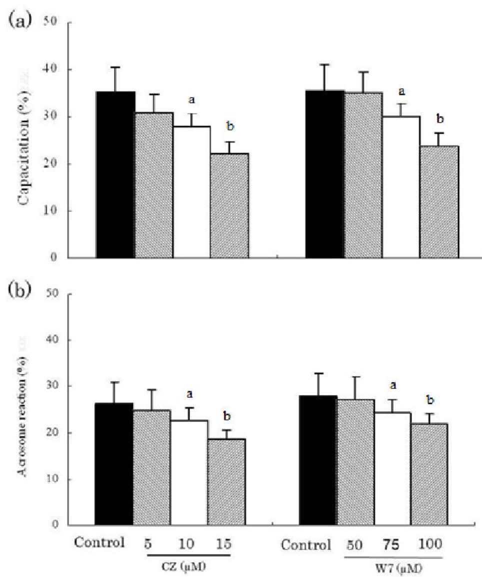

Figure 2. Effects of calmidazolium (CZ) and *N*-(6-aminohexyl)-5-chloro-1 naphthalene sulfonamide (W7) on the percentage of sperm showing capacitation (a) acrosome reaction (b) after CTC staining at 4 h of incubation periods with various concentrations of CZ and W7. Results are the mean±standard error (n=3). a-b values with different letter superscripts are significantly different (P<0.01) from control.

The effect of cAMP-elevating agents (dbcAMP and IBMX) on sperm capacitation and AR of CaM inhibitors treated sperm samples containing with or without bicarbonate the results is shown in Figure 3(a, b). The capacitation pattern analysis (CTC/HO staining) revealed that the addition of dbcAMP and IBMX to samples in presence of bicarbonate produced a significant increase in sperm displaying CTC capacitation pattern (along with a decrease in not-capacitated pattern) relative to control samples at 4 h incubation, shown in Figure 3(a). CaM antagonists treated sperm incubated in medium devoid of $NaHCO_3$ also did not display the CTC fluorescence B pattern. In contrast, the addition of 1 mM dbcAMP plus 100 μM IBMX to medium devoid of $NaHCO_3$ could substitute for $NaHCO_3$ in supporting the ability of the sperm to undergo the Zona pellucida-induced acrosome reaction (Figure 3 b). In addition, in the presence of bicarbonate, a significantly higher value of the acrosome-reacted population was found in case both agents.

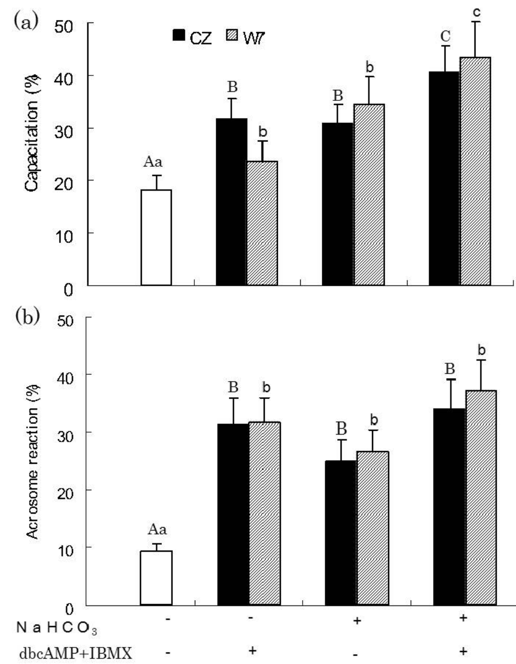

Figure 3. Effects of 1mM dibutyryl cAMP plus 100μM 3-isobutyl-1-methylxanthine (IBMX) in presence of CZ (5μM) and W7 (50μM) on the percentage of sperm showing (a) capacitation (B pattern) and (b) acrosome reacted (AR pattern) after chlortetracycline fluorescence (CTC) staining in media containing $NaHCO_3$ or in medium devoid of $NaHCO_3$ at 4 h of incubation period. Results are expressed mean±standard error (n=3). a-c; A-B values with different letter superscripts are significantly different(P<0.01) from each other.

The percentage of B and AR pattern significantly increased when CaM treated spermatozoa supplemented with dbcAMP plus IBMX in presence of Ca^{2+}. When 5 μM CZ and 50 μM W7 treated sperm were incubated for 4 h with 1mM dbcAMP and 100 μM IBMX in presence of $CaCl_2$ about 40–45% of the sperm displayed the B pattern (Figure 4.a). The combination of dbcAMP and IBMX increased the percentage of acrosome-reacted spermatozoa to about 35% in the presence of calcium in 5 μM CZ treated sperm. Similar results were observed when the spontaneous acrosome reaction was inhibited by W7, about 38% AR observed after treated with dbcAMP at 4 h of incubation (Figure 4.b). The

addition of 1mM dbcAMP plus 100 μM IBMX to medium devoid of $CaCl_2$ could not substitute for $CaCl_2$ in supporting the ability of the sperm to display the CTC fluorescence B pattern in both CZ and W7 treated groups.

The calcium ionophore has been shown to accelerate the spontaneous AR. In order to investigate whether A23187 can induce the AR, CaM treated spermatozoa were supplemented with 0.5 μM IA. When sperm were incubated for 4 h with 5 μM CZ and calcium ionophore about 35% of the sperm displayed the AR pattern (Figure 5 b). However, when sperm were incubated with 50 μM W7 and ionophore, the level of sperm with the AR pattern was increased from that of sperm incubated in W7 alone. In case of capacitation state, similarly an increased rate of B pattern was also observed with these groups; the result is shown Figure 5a. The IA treated sperm did not alter the inhibition of CZ and W7 on sperm motility. The percentage of motile sperm was reduced significantly to less than 20% of motile cells when both CZ were incubated with IA at 4h incubation (data not shown).

Discussion

The roles of CaM in sperm physiology are diverse and include the support of capacitation-related changes in plasma membrane properties (Bendahmane *et al.,* 2001), modulation of cAMP levels (Fournier *et al.,* 2003), and the ability to undergo the AR (Fournier *et al.,* 2003). To identify the targets of Ca^{2+}/CaM in sperm motility, capacitation and AR, we examined CaM antagonists (CZ and W7) for their ability to block CaM-dependent capacitation. In our study; both CZ and W7 depressed motility relative to that of control sperm. W7, a CaM antagonist was previously shown to reduce the proportion of hyperactivation among intact epididymal mouse sperm (Si and Olds-Clarke, 2000). These findings indicate that CaM plays an essential role in activating bovine sperm motility.

This study shows that inhibition of CaM inhibitors, CZ and W7 decreased capacitation and AR of bovine sperm, reported by the CTC assay. The B pattern after CTC staining was used as a probe for monitoring capacitation-dependent sperm membrane changes, since it is correlated with the sperm's ability to undergo the ZP-induced AR (Ward and Storey, 1994). Both W7 and CZ inhibited the B pattern in a concentration-dependent manner (is shown in Figure 3). Furthermore, both CZ and W7 inhibited the sperm's ability to undergo the AR.

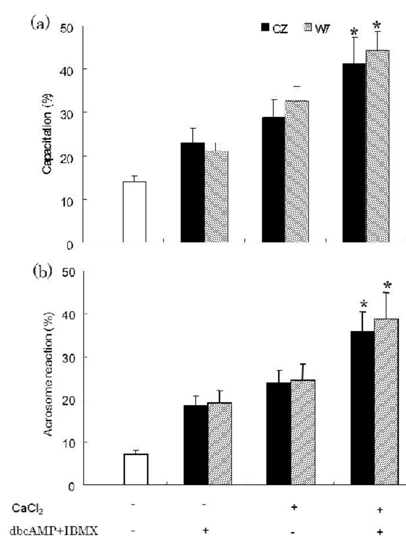

Figure 4. Effects of 1mM dibutyryl cAMP plus 100 μM 3-isobutyl-1-methylxanthine (IBMX) in presence of CZ (5μM) and W7 (50μM) on the percentage of sperm showing (a) capacitation (B pattern) and (b) acrosome reacted (AR pattern) after chlortetracycline fluorescence (CTC) staining in media containing $CaCl_2$ or in medium devoid of $CaCl_2$ at 4 h of incubation period Results are expressed mean±standard error (n=3). * Significantly different ($P<0.01$) from all other treatments.

However, examination of the percentage of viable sperm indicated that the antagonist-induced inhibition was not due to nonspecific toxic effects (data not shown). These results indicate that CaM antagonists inhibit capacitation dependent membrane changes, and suggest that this pathway involves CaM. CaM can also make use of the calcium stores in the endoplasmic reticulum, and the sarcoplasmic reticulum. CaM undergoes a conformational change upon binding to calcium, which enables it to bind to specific proteins for a specific response. CaM is present in both the acrosomal region and the flagellum of sperm (Leclerc and Goupil, 2000), supporting the idea that CaM is in an appropriate location to regulate sperm membrane changes important for fertilization, and thus suggests that CaM plays a role in bovine sperm capacitation.

Changes in sperm cyclic nucleotide metabolism have been implicated in a variety of sperm functions, including the initiation and maintenance of motility (Yanagimachi *et al.,* 1994), induction of the AR (Kopf and Gerton, 1991), and capacitation (Stein and Fraser, 1984). Since the mammalian sperm enzyme has been demonstrated to be regulated directly/indirectly by Ca^{2+} (Hyne and Garbers, 1979), CaM (Gross *et al.,* 1987) and $NaHCO_3$ (Visconti *et al.,* 1990). Ca^{2+} and $NaHCO_3$ also appear to be required for capacitation (Boatman and Robbins, 1991) and the induction of the AR (Spira and Breitbart, 1992). Moreover, changes in the activity of adenylyl cyclase and cAMP have been postulated in some species to play a role in capacitation (Yanagimachi *et al.,* 1994).

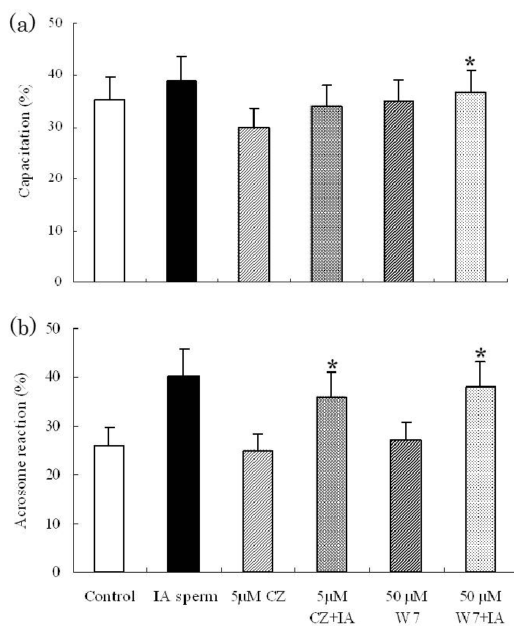

Figure 5. Effects of calcium ionophore A23187 in presence of CaM antagonists CZ (5 μM) and W7 (50μM) on the percentage of sperm showing (a) capacitation (B pattern) and (b) acrosome reacted (AR pattern) after CTC staining at 4 h of incubation periods. Results are expressed mean±standard error (n=3). * Significantly different ($P<0.01$) from the treatments where ionophore was not added with CZ and W7.

In the study the effect of CaM antagonists (CZ and W7) on motility were not reversed by exogenous cAMP analogues. This finding is in agreement with a study of hamster sperm in which addition of IBMX to CZ-treated sperm increased the intracellular cAMP concentration to a level higher than that in capacitated sperm but did not restore hyperactivated motility [White and Aitken, 1989). Since both Ca^{2+} and $NaHCO_3$ have been linked to capacitation and the regulation of mammalian sperm cAMP metabolism (Kopf and Gerton, 1991) and this intracellular second messenger has been implicated in both mammalian sperm capacitation and the AR, we were also interested in determining whether these membrane permeable cAMP analogues could support capacitation in CaM antagonists treated sperm in the absence of these ions. Figure 4 shows the experiments performed in media absence of $NaHCO_3$. However, in the case of the Ca^{2+} depletion experiments, the membrane permeable cAMP analogues do not completely restore the ZP-induced AR. This could be due to the fact that final stages of capacitation, presumably either independent of or downstream of the protein tyrosine phosphorylations, are not being completed, and/or Ca^{2+} is important components for the induction of the AR (Yanagimachi *et al.,* 1994).

The role of cAMP in the capacitation process as well as in the AR is still unknown (Aitken et al., 1998; Yanagimachi *et al.,* 1994). It would be predicted that cAMP concentrations should rise during capacitation. Although some investigators have observed elevations in cAMP concentrations when spermatozoa were incubated under conditions that support capacitation (White and Aitken, 1989), others have reported decreasing concentrations of cAMP under these conditions (Stein and Fraser, 1984). In this study, when the sperm are incubated in the absence of $NaHCO_3$, does not support capacitation. Regarding the possible participation of cAMP in the AR, it was observed in this present study that cAMP accumulation in $NaHCO_3$-containing media was significantly higher than in $NaHCO_3$-free medium. When the combination of dbcAMP and IBMX were added to $NaHCO_3$-free media, the percentage of AR spermatozoa rose significantly, in comparison with the AR in the $NaHCO_3$-containing medium. These results suggest that the ability of $NaHCO_3$ to stimulate the AR is mediated by a cAMP pathway. Using a biochemical approach for measuring the AR, Spira and Breitbart (Spira and Breitbart, 1992) reached similar conclusions in

bovine spermatozoa, observing that $NaHCO_3$ is no longer required when the cAMP levels are artificially elevated by the use of dbcAMP. The addition of dbcAMP and IBMX partially, but significantly, reversed the effect of CaM antagonists on the capacitation and AR. This study also supports the role of a cAMP dependent signaling pathway in capacitation (Aitken *et al.,* 1998) via increased cAMP levels. Furthermore, these results reinforce the hypothesis that a loss of CaM accompanies capacitation of bovine sperm (Leclerc *et al.,* 1992), thereby favoring the increased cAMP levels necessary to drive the signaling events associated with this phenomenon.

The calcium ionophore A23187 has been shown to induce the AR in the sperm of almost all species tested, suggesting that Ca^{2+} is a primary mediator of this exocytotic reaction. Our results shows that CaM inhibitors have no observable effect on the calcium ionophore induced AR. When CZ and W7 were supplemented with ionophore, the percentage of AR spermatozoa significantly increased compared with the control and these antagonists treated sperm that were not supplemented with ionophore (Figure 6). The AI induces the AR by incorporating into the plasma membrane of sperm and transporting calcium, resulting in high intracellular calcium concentrations necessary for sperm capacitation and AR (Talbot *et al.,* 1976).

Conclusion

The present study indicates that CaM is important for the control of bovine sperm motility and the inhibition of CaM antagonists on sperm motility might be due to uncontrolled influx of calcium ion of frozen-thawed bovine spermatozoa. The inhibition of CaM antagonists on capacitation and AR could partially overcome by supplementing cAMP analogues that were re-suspended in a capacitation supporting factors such as calcium and bicarbonate in the medium. These findings support the hypothesis that CaM antagonists can prevent capacitation and AR by interfering with multiple regulatory pathways. Thus, The results suggest that CaM may play an important role in the regulation of capacitation and AR in frozen-thawed bovine spermatozoa.

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