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Bull sperm acrosome reaction induced by gamma-aminobutyric acid (GABA) is mediated by GABAergic receptors type A

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ABSTRACT

The effect of gamma-aminobutyric acid (GABA) on the bull sperm acrosome reaction was evaluated, and the interaction of progesterone, a physiologic inducer of the acrosome reaction, with the GABA receptor was explored. The acrosome reaction was stimulated by GABA in a dose-dependent manner. This effect was inhibited completely by bicuculline, a GABA A receptor antagonist, but GABA B and C receptor antagonists had no effect. Accordingly, muscimol, a GABA A receptor agonist, stimulated the acrosome reaction to the same extent as GABA, whereas baclofen (GABA B receptor agonist) and CACA (GABA C receptor agonist), had no effect. Preincubation with progesterone followed by the addition of GABA resulted in a significant increase in the percentage of acrosome reacted spermatozoa compared with progesterone or GABA alone. Taking into account that this increase was less than a simple addition of effects, it might be suggested that GABA and progesterone act through the same receptor and/or use the same mechanism of action. To test this hypothesis, the abilities of GABA and progesterone to induce acrosome reaction were tested in the presence of bicuculline, which suppressed both stimulatory effects. Given that the GABA A receptor is linked to the Cl^- channel, we tested whether picrotoxin, a blocker of this channel, could modulate the effects of progesterone or GABA. Cl^- channel blocker picrotoxin dramatically reduced the GABA and progesterone-initiated AR. In conclusion: GABA and progesterone stimulate the acrosome reaction in bull spermatozoa acting through a classical GABA A receptor. The mechanism of action requires the functional integrity of the Ca^{2+} Cl^- channel.

1. Introduction

Capacitated spermatozoa can undergo the acrosome reaction in response to appropriate stimuli involving multiple fusion of the outer acrosomal membrane with the overlying plasma membrane followed by vesiculation and exocytosis of the contents of the acrosomal cap (Yanagimachi, 1994). The stimuli initiating the mammalian sperm acrosome reaction *in vivo* are still undefined. The reaction can be provoked *in vitro* by some substances found in the vicinity of the oocyte such as follicular fluid (Mukherjee and Lippes, 1972; Tesarik, 1985), cumulus oophorus (Siiteri et al., 1988) and zona pellucida (Cross et al., 1988; Morales et al., 1989).

Undoubtedly, identification in female reproductive fluid factor or factors that regulate sperm capacitation would allow the design of chemically defined media to be used in *In Vitro* Fertilization (IVF) and related techniques. Among those factors, gamma-aminobutyric acid (GABA) emerged as a putative modulator of sperm function. As an inhibitory neurotransmitter in the central and peripheral nervous systems, the action of GABA is mediated by membrane receptors. GABA acts through at least three types of GABA receptors: A, B and C

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(Martin and Dunn, 2002). Particularly, GABA A receptor is a supramolecular receptor complex linked to the Cl^- channel whose activation produces a transmembrane Cl^- ion flux. GABA A receptors are also the major target for several clinically relevant drugs such as the barbiturates, benzodiazepines, neurosteroids and anesthetics (Rudolph et al., 2001). These compounds allosterically modulate GABA A receptor channel activities (Sieghart, 1992).

Specific GABA A receptors occur in bull sperm membranes and GABA exerts a capacitating effect on bull spermatozoa as assessed by chlortetracycline (CTC) technique (Ritta et al., 2004). Moreover, it was proposed that the mechanism of GABA action appears to involve at least two biochemical pathways: Ca^{2+} channels and cAMP production (Ritta et al., 2004). However, when human spermatozoa were evaluated, experimental evidence has shown that type A and type B GABAergic receptors mediate the stimulatory effects of exogenously added GABA on sperm kinematic parameters (Calogero et al., 1996) and the acrosome reaction (Shi et al., 1997; Calogero et al., 1999). Moreover, Burrello et al. (2004) demonstrated that the simultaneous blockade of GABA A and GABA B receptors suppressed the acrosome reaction induced by follicular fluid.

Progesterone in human follicular fluid at concentrations capable of stimulating the acrosome reaction (Osman et al., 1989), and protein bound progesterone was identified as the acrosome reaction-inducing agent in human follicular fluid (Thomas and Meizel, 1988). Steroids can initiate exocytosis in a variety of species: human (Harper and Publicover, 2005), boar (Wu et al., 2006), hamster (Shi et al., 1992) and horse (Meyers et al., 1993). It has been suggested that the sperm progesterone receptor located on the cell surface may resemble a GABA A receptor (Wistrom and Meizel, 1993). Although the existence of GABA A receptors in spermatozoa has been revealed (Erdo and Wekerle, 1990; Wistrom and Meizel, 1993; Ritta et al., 1998, 2004), no clear evidence has been presented showing that the action of progesterone is in fact mediated by a GABA A receptor. In particular, a clear activation of this putative receptor by GABA A receptor agonists, and an interaction between the latter and steroids as observed in neuronal GABA A receptors (Robel and Baulieu, 1994), has been demonstrated by Calogero et al. (1999) in human spermatozoa. Moreover, Burrello et al. (2004) suggested that human follicular fluid stimulates the sperm acrosome reaction by interacting with GABA receptors; an effect mediated by both Cl^- and Ca^{2+} ions.

The present study was undertaken to examine the effects of GABA on bull sperm acrosomal reaction. Dose-dependency of sperm response was analyzed to GABA; GABA receptor subtype and ionic channels involved in the putative response and whether GABA A receptor allosteric modulators enhance the GABA effect.

2. Materials and methods

2.1. Reagents

GABA, progesterone, allopregnenolone (3α-hydroxy-5α-pregnan-20-one acid), baclofen, CTC, bicuculline, picrotoxin, saclofen (β-(aminomethyl)-4-chloro-benzenee thanesulfonic and other reagents for the preparation of buffers and media were obtained from Sigma Aldrich (St. Louis, MO, USA). 4-Amino-cis-2-butenoic acid (CACA) was purchased from Research Biochemicals International (MA, USA).

2.2. Sperm collection and preparation

Fresh semen obtained from 48 adult Aberdeen Angus bulls of proven fertility was collected using an artificial vagina. Sperm samples were individually assessed to ascertain live/dead relation (Eosin-Nigrosin vital stain) and sperm motility (visual microscopy examination). Eosin-Nigrosin staining has been used as a routine staining in order to evaluate sperm viability (World Health Organization, 1992; 1999; Rodríguez-Martínez, 2000). Briefly, after washing sperm samples in saline solution (154 mM NaCl) at 37 °C, one drop of the suspension containing 35×10^6 sperm/mL was placed on a tempered glass slide, which was mixed with one drop of Eosin-Nigrosin solution (0.2 g of eosin and 2 g of nigrosin, dissolved in a buffered saline solution). After 2 h, the mixture was smeared on the glass slide and let air dried and the samples were observed under a light microscope using pre-warmed slides (Mortimer, 1985, 1994).

Concentration and total number of spermatozoa were evaluated using a hemocytometer. The percentage of progressively motile spermatozoa was determined by visual microscopy examination at a magnification 400× using a Nikon (Tokyo, Japan) Diaphot 200 inverted fluorescence and phase contrast microscope. Cells were observed under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). After collection the semen was immediately diluted 1:1 in egg yolk-Tris extender (35–37 °C) without glycerol, 0.25 M Tris, 60 mM citric acid, 69 mM fructose, 25% (v/v) egg yolk and an antibiotic cocktail containing tylosin, gentamicin (Sigma–Aldrich, St. Louis, MO) and lincospectin (Pfizer SRL, Buenos Aires, Argentina). Motility was analyzed using pre-warmed (37 °C) slides with a stage warmer attached to the microscope.

Only samples with sperm concentrations > 1800×10^6 spermatozoa/mL, masal motility > 3, progressive motility > 60%, viability > 80% were used for subsequent manipulations.

2.3. Sperm treatment

In vitro capacitated bull sperm were obtained as previously described (Ritta et al., 2004). Briefly, aliquots of 500 μL of sperm suspensions (10^7 cells/mL), corresponding to individual ejaculations, were mixed in 1 mL plastic wells with an equal volume of Biggers, Whitten and Whittingham (BWW) medium (Biggers et al., 1971). The samples were incubated at 38.5 °C in a humidified chamber under an atmosphere of 5% CO_2 in air. After 2 h, aliquots of each sample were assessed for capacitation by CTC assay (see below). At the end of that time, they were incubated with progesterone, GABA, allopregnanolone, GABAergic agonists or antagonists and/or ion channel blockers (picrotoxin, verapamil) for 20 min. Afterwards, samples were

incubated with medium (to evaluate the effect of GABA alone) or in the presence of the appropriate concentration of bicuculline, muscimol, baclofen, CACA, progesterone, allopregnenolone, diazepam, picrotoxin or verapamil for 30 min to give these compounds, on the basis of preliminary experiments, time enough to produce effects. Subsequently, GABA was added at the specified concentrations and the cell suspension was left to incubate for additional 30 min, according to preliminary experiments undertaken to determine the appropriate incubation conditions. For each experiment, a sperm aliquot was incubated in plain BWW and no GABA was added to evaluate the spontaneous rate of the acrosome reaction.

At the end of each incubation period sperm viability (live/dead relation, Eosin-Nigrosin vital stain), motility (visual microscopy examination at a magnification 400× using a phase contrast microscopy) and CTC staining patterns were recorded. Under all experimental conditions, only samples presenting viability and motility values greater than 80% after the incubation periods were considered.

2.4. CTC assay

CTC assay used to determine acrosome-reacted bovine spermatozoa was modified from Ward and Storey (1984) and Fraser et al. (1995). The CTC solution (750 mM CTC, 5 mM cysteine in 130 mM NaCl and 20 mM Tris–HCl, pH 7.4) was daily prepared and pH adjusted to 7.8. The solution was protected from light and maintained at 4 °C before using. Fifteen microliters of sperm suspension were mixed with equal volume of CTC solution on a slide at room temperature. After a few seconds 1.5 μL of glutaraldehyde (12.5% (v/v) in 20 mM Tris–HCl, pH 7.4) was added to samples. Finally, a drop of 0.22 M 1,4-diaza-bicyclo (2,2,2) octane dissolved in glycerol:phosphate-buffered saline (9:1) was added to retard the fading of CTC fluorescence. The slides were covered with coverslips and stored at 4 °C overnight in the dark. CTC fluorescence was observed with a Nikon microscope equipped with phase contrast and epifluorescent optics. All samples were processed in duplicate and the experiment was replicated four times. At least 200 spermatozoa per slide were classified according to one of three CTC staining patterns as described by Fraser et al. (1995): (1) uniform bright fluorescence over the whole head (characteristic of uncapacitated cells, pattern F), (2) fluorescence-free band in the post-acrosomal region (capacitated cells, pattern B) and (3) dull fluorescence over the whole head except for a thin punctate band of fluorescence along the equatorial segment (acrosome reacted cells, pattern AR). No fluorescence was observed when CTC was omitted from the preparation.

2.5. Statistical analysis

The percentage of acrosome-reacted spermatozoa was calculated for each experimental condition dividing the number of acrosome-reacted spermatozoa by the total number of spermatozoa scored (sum of acrosome-reacted and non acrosome-reacted) and multiplying this ratio by 100. Results are presented as Mean ± SD throughout the study. The software Infostat (School of Agricultural Sciences, University of Córdoba, Argentina) was used for statistical evaluation. Because the within-group variances are all the same (exhibit homoscedasticity), the data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test. A statistically significant difference was accepted when the P value was < 0.05.

Table 1
Effect of GABA on the acrosome reaction of bull spermatozoa.

GABA	Pattern AR (%)
Control	4.3 ± 0.8
1 μM	9.6 ± 1.1[a]
5 μM	13.7 ± 1.4[b]
10 μM	23.9 ± 1.5[b]
100 μM	25.2 ± 1.3[b]

Results are expressed as Mean ± SD for 43 individual ejaculates.
[a] $P < 0.05$.
[b] $P > 0.001$, compared to control.

3. Results

In a first set of experiments, spermatozoa (10^7 cells/mL) were incubated for 2 h in medium BWW (control, C) or in the presence of 10 μM GABA or 10 μM progesterone. Addition of GABA to the incubation medium resulted in an increase of the percentage of CTC pattern corresponding to capacitated spermatozoa (pattern B) (C: 18.3 ± 1.6; GABA: 27.2 ± 1.8*, progesterone: 25.8 ± 2.1*, *$P > 0.05$ compared to control, $n = 12$). As previously observed, under these incubation conditions only capacitated gametes were detected in response to GABA, while pattern AR, corresponding to acrosome reacted cells, was undetectable. Taking into account these finding the possible stimulatory effect of GABA on the acrosome reaction was evaluated on capacitated spermatozoa.

GABA stimulated the acrosome reaction in a concentration dependent manner ($P < 0.001$, ANOVA) (Table 1). The effect occurred at a concentration of 1 μM ($P < 0.05$ as compared to the control), which doubled the percentage of spermatozoa undergoing spontaneous acrosome reaction. At the concentration of 10 μM, GABA was more effective ($P < 0.05$ vs. 10 μM, ANOVA followed by Duncan's test). Because the plateau was reached at 10 μM GABA, this concentration of GABA was chosen for the subsequent experiments.

When capacitated sperm suspensions were co-incubated in the presence of 10 μM GABA and the GABA A receptor antagonist bicuculline (100 μM), a significant suppression of the GABA-stimulated acrosome reaction was obtained (Table 2). These results suggest GABA stimulates the acrosome reaction by activating the previously described GABA A receptor. To substantiate this finding further, the effect of selective GABA A, GABA B and GABA C receptor agonists was evaluated. While muscimol mimicked GABA effect, neither the GABA B receptor agonist baclofen nor the GABA C receptor agonist CACA had an effect (Table 3).

When the progesterone effect was explored, the steroid stimulated the acrosome reaction in a dose dependent fash-

Table 2
Effects of bicuculline (100 μM) on the occurrence of spontaneous and 10 μM GABA induced acrosome reaction of bull spermatozoa.

GABA	Pattern AR (%)
Control	4.8 ± 0.9
GABA	22.9 ± 1.4[a]
Bicuculline	5.1 ± 1.1
GABA + bicuculline	4.9 ± 0.8

Results are expressed as Mean ± SD for 46 different ejaculates.
[a] $P < 0.001$.

Table 3
Effects of GABA, progesterone and GABAergic agonists on the occurrence of acrosome reaction of bull spermatozoa.

Treatment	Pattern AR (%)
Control	5 ± 1
GABA (10 μM)	24 ± 1[a]
Progesterone (10 μM)	23 ± 2[a]
Muscimol (10 μM)	21 ± 1[a]
Baclofen (10 μM)	9 ± 2
CACA (10 μM)	8 ± 1

Results are expressed as Mean ± SD for 34 different ejaculates.
[a] $P < 0.001$.

ion with a median effective dose (ED_{50}) of 29.4 ± 1.6 μM. In the presence of 10 μM progesterone, the percentages of acrosome-reacted spermatozoa were comparable to those obtained with 10 μM GABA (Table 4). When capacitated spermatozoa were incubated in the presence of 1 μM GABA and 1 μM progesterone there was an increment on the percentage of reacted spermatozoa, that was significantly different from that obtained with GABA alone. This increase was less than a simple addition of effects, suggesting that progesterone and GABA are acting through the same receptor and/or mechanism of action. Further evidence for the involvement of GABA A receptor in the effect of GABA on acrosomal reaction was obtained by incubating capacitated spermatozoa in the presence of 1 μM GABA, together with the neuroactive steroid allopregnanolone. As shown in Table 4, 1 μM GABA and 1 μM allopregnanolone, which had no effect *per se*, potentiated sperm exocytosis induced by 1 μM GABA. The effect of GABA on acrosome reaction was modified by other GABA A receptor modulator, i.e. the benzodiazepine diazepam. Diazepam had neither effect *per se* nor when spermatozoa were incubated in the presence of this compound and GABA.

Results presented in Table 5 indicate the Cl^- channel blocker picrotoxine (10 μM) inhibited the ability of GABA

Table 4
Effects of GABA, progesterone, allopregnenolone and diazepam on the occurrence of acrosome reaction of bull spermatozoa.

Treatment	Pattern AR (%)
Control	6 ± 1
GABA (1 μM)	14 ± 2[a]
Progesterone (1 μM)	12 ± 1[a]
GABA (1 μM) + progesterone (1 μM)	22 ± 3[a]
Allopregnenolone (1 μM)	5 ± 2
GABA (1 μM) + allopregnenolone (1 μM)	21 ± 2[a]
Diazepam (1 μM)	5 ± 1
GABA (1 μM) + diazepam (1 μM)	7 ± 2[a]

Results are expressed as Mean ± SD for 38 different ejaculates.
[a] $P < 0.001$.

Table 5
Effect of GABA, picrotoxin and verapamil on the occurrence of acrosome reaction of bull spermatozoa.

Treatment	Pattern AR (%)
Control	6 ± 2
GABA (10 μM)	24 ± 3[a]
GABA (10 μM) + picotroxin (10 μM)	7 ± 1
GABA (10 μM) + verapamil (10 μM)	7 ± 1
Progesterone (10 μM)	21 ± 1[a]
Progesterone (10 μM) + picotroxin (10 μM)	7 ± 2[a]
Progesterone (10 μM) + verapamil (10 μM)	6 ± 1
Muscimol (10 μM)	26 ± 1[a]
Muscimol (10 μM) + picotroxin (10 μM)	7 ± 1
Muscimol (10 μM) + verapamil (10 μM)	8 ± 2
Picrotoxin (10 μM)	6 ± 1
Verapamil (10 μM)	5 ± 2

Results are expressed as Mean ± SD for 46 different ejaculates.
[a] $P < 0.001$.

or progesterone to stimulate exocytosis in bull spermatozoa.

To test whether Ca^{2+} channels are involved in GABA-and progesterone-stimulated exocytosis, preexposure to verapamil, the well-known Ca^{2+} channel blocker was examined, and this treatment affected the GABA and progesterone responses in capacitated spermatozoa. As can be seen in Table 5, verapamil (10 μM) reduced the percentage of capacitated cells undergoing exocytosis after GABA or progesterone treatment.

4. Discussion

Present results demonstrate that GABA stimulates acrosomal exocytosis in capacitated bull spermatozoa via the previously described GABA A receptor (Ritta et al., 2004). In addition, results of the present study indicate GABA effects on bull gametes are linked to Ca^{2+} and Cl^- entries via Ca^{2+} and Cl^- channels. A further proof of the involvement of GABA A receptor was the results demonstrating stimulation with GABA plus neuroactive steroid allopregnanolone leads to increased percentage of reacted gametes.

In the present study, the effect of GABA on the occurrence of acrosome reaction was investigated. Bull spermatozoa capacitated in BWW underwent exocytosis in response to GABA treatment. Maximal stimulation of exocytosis was observed with 10 μM GABA. It has been proposed that human spermatozoa preincubated for 2 h (presumed to be capacitated) are able to undergo exocytosis in response to progesterone treatment (Baldi et al., 1991, 1993). In the present study the same phenomenon was observed because only capacitated bull spermatozoa can undergo exocytosis in response to GABA and progesterone. In fact, the proportion of cells undergoing acrosomal exocytosis after treatment with 10 μM GABA was not different from that when spermatozoa were treated with 10 μM progesterone. These results are comparable to those obtained with mouse spermatozoa, where it was found that only capacitated cells were able to undergo an acrosome reaction in response to progesterone (Shi and Roldan, 1995).

Another finding further substantiates that a GABA A receptor mediates GABA effect. In the present study, the

GABA-induced exocytosis was completely abolished by the specific GABA A receptor antagonist bicuculline, while GABA B and GABA C antagonists had no effect on GABA stimulatory action (data not shown). Calogero et al. (1999) demonstrated that GABA effects on human sperm acrosome reaction were completely antagonized by bicuculline. However, in the previous study it was proposed that GABA actions are mediated through GABA A and GABA B receptors, because the selective GABA B receptor antagonist saclofen blocked the stimulatory effect of GABA. In the present study, GABA induced the acrosome reaction by interacting exclusively with the uniquely described GABA A receptor located on the bull sperm crude membrane fraction (Ritta et al., 2004). Moreover, the GABA-like effect of the GABA A receptor agonist muscimol clearly indicates the involvement of the previously described GABA A receptor (Ritta et al., 2004), while the GABA B and GABA C receptor agonists (baclofen and CACA, respectively) had no effect.

The initiation of the acrosome reaction by progesterone is an example of a nongenomic activity of the steroid (Meizel et al., 1990; Thomas and Meizel, 1988). Shi and Roldan (1995) demonstrated that mouse spermatozoa capacitated in a modified Tyrode's medium underwent exocytosis in response to progesterone treatment, indicating that mouse spermatozoa share with other species (Meizel et al., 1990; Thérien and Manjunath, 2003) the ability to undergo acrosomal exocytosis in response to this steroid. In the present study, the proportions of gametes reacted were comparable when capacitated spermatozoa were incubated either in the presence of 1 μM GABA or 1 μM progesterone. Nevertheless, co-incubation with both inducers of exocytosis resulted in the same percentage of reacted cells as obtained when individual incubations were conducted.

Further evidence for the involvement of a classical GABA A receptor in the effect of GABA was obtained in the present study by incubating spermatozoa in the presence of 1 μM GABA together with the neuroactive steroid allopregnanolone. While progesterone failed to potentiate the acrosome reaction in the presence of GABA, allopregnanolone clearly exerted a potentiating effect. In fact, it has been reported that allopregnanolone caused the greatest increase in muscimol binding to uterine GABA A receptors (Fujii and Mellon, 2001). It is well known that GABA-mediated activation of the GABA A receptor supramolecular complex is modulated by various clinically important drugs acting on allosteric modulatory sites (MacDonald and Olsen, 1994; Lüddens and Korpi, 1995). Neurosteroids allosterically modulate GABA-activated Cl^- channels (Lambert et al., 1996). However, at greater concentrations, neuroactive steroids directly activate the GABA A receptor channels, showing the same property as barbiturates (Lambert et al., 1996). Several studies have suggested that steroid-induced allosteric modulation requires a specific steroid binding domain on the GABA A receptor channel complex separate from those for benzodiazepines and barbiturates (Gee et al., 1988; Turner et al., 1989). According to the present results, when bull spermatozoa were incubated in the presence of GABA and diazepam the benzodiazepine that had no effect *per se* did not modify the neurotransmitter stimulatory effect.

The mechanism of action leading to the observed responses in the present study when capacitated spermatozoa were incubated in the presence of GABA or progesterone and picrotoxin involves changes in Ca^{2+} influxes. It is well documented that progesterone and other progestogens cause a rapid increase in intracellular Ca^{2+} concentration in spermatozoa (Thomas and Meizel, 1989; Blackmore et al., 1990, Ritta et al., 2004), this response being mediated via cell surface receptors (Blackmore and Lattanzio, 1991; Osman et al., 1989; Tesarik and Mendoza, 1993; Ritta et al., 2004).

Results from the present study demonstrate that GABA stimulates acrosomal exocytosis in capacitated bovine spermatozoa through its interaction with a GABA A receptor. In addition, progesterone action on the GABA A receptor is linked to Ca^{2+} entry via Ca^{2+} channels. Mouse spermatozoa capacitated in a modified Tyrode's medium (Fraser, 1983) underwent exocytosis in response to progesterone treatment; this indicates that mouse spermatozoa share with other species (Meizel et al., 1990; Melendez et al., 1993; Shi and Roldan, 1995) the ability to undergo acrosomal exocytosis in response to this agonist. In human spermatozoa, progesterone can stimulate an increase in intracellular Ca^{2+}, regardless of whether sperm cells have been preincubated and, presumably, have become capacitated (Blackmore et al., 1991; Thomas and Meizel, 1989). It appears, however, that human spermatozoa preincubated for 2 h (presumed to be capacitated) are able to undergo exocytosis in response to progesterone challenge (Baldi et al., 1991, 1993). Although several observations strongly indicate that the progesterone effect may be mediated by a GABA A receptor, evidence gathered in this and previous studies also suggests that this GABA A receptor may be somewhat different from that present in neuronal cells. In addition, it is also possible that not all progesterone effects are mediated by a GABA A receptor (Robel and Baulieu, 1994; Turner et al., 1994). Therefore, the suggestion has been made that progesterone acts via a novel steroid receptor that is similar, but not identical, to the neuronal GABA A receptor (Wistrom and Meizel, 1993). It is possible that tissue-specific differences in subunit composition of the GABA A receptor may be important in determining the modulation by steroids (Puia et al., 1990; Ragan et al., 1993). A final distinction could be drawn in relation to the type of effect caused by the activation of these GABA receptors. Thus, whereas activation of neuronal GABA A receptor leads to a reduction in neuronal excitability and hence mediates an inhibitory action, activation of the sperm GABA A receptor results in stimulation of a cellular response, i.e., exocytosis of the acrosomal granule.

It seems clear now that the action of progesterone on spermatozoa results in entry of Ca^{2+} from the extracellular space. The increase in intracellular Ca^{2+} prompted by progesterone in human spermatozoa does not occur if extracellular Ca^{2+} is chelated with EGTA or if entry is prevented by inclusion of La^{3+} (Blackmore et al., 1991). Likewise, progesterone is not able to stimulate acrosomal exocytosis in hamster spermatozoa if EGTA or La^{3+} is present (Thomas and Meizel, 1988). However, the mechanism underlying Ca^{2+} entry is not clearly understood. It has been speculated that the intracellular Ca^{2+} increase

could take place as a result of the interaction between progesterone and a Ca^{2+}-ATPase (Fraser and McDermott, 1992) or that the sperm progesterone receptor could be coupled to a Ca^{2+} channel (Blackmore et al., 1990); the fact that La^{3+} blocks the progesterone-induced increase in intracellular Ca^{2+} lends support to the latter idea. Results in the present study indicate that progesterone-, GABA-, and muscimol-triggered activation of a GABA A receptor is indeed related to entry of Ca^{2+} via Ca^{2+} channels, because verapamil blocked the acrosomal exocytosis stimulated by these agonists. Evidence gathered in previous studies on the effects of verapamil is rather contradictory. Whereas in some studies it was found that verapamil was able to inhibit completely the transient increase in intracellular Ca^{2+} triggered by progesterone in human spermatozoa (McLaughlin and Ford, 1994), in other previous studies it was found that verapamil exerted little inhibitory action (Foresta et al., 1993). Likewise, stimulation of Ca^{2+} influx into human spermatozoa triggered by human follicular fluid (Osman et al., 1989; Morales et al., 1992) was somewhat inhibited by verapamil (Blackmore et al., 1990) or was unaffected by this Ca^{2+} channel antagonist (Thomas and Meizel, 1989). Despite these contradictory observations, results in the present study showed that not only verapamil, but also nifedipine (data not shown) clearly inhibited the exocytosis triggered by GABA-related agonists.

5. Conclusions

In the present study GABA, in a concentration-dependent manner, as well as progesterone induced bull sperm acrosome reaction as determined by CTC staining. Moreover, GABA effect seems to be mediated by the GABA A receptor previously described because acrosome reaction is allosterically modulated by the neurosteroid allopregnenolone. Therefore, these results strongly suggest the existence of a link between the activation of a GABA A receptor and a Ca^{2+} channel involved in the development of the acrosome reaction. These observations provide further evidence for the involvement of a classical GABA A receptor as mediator of the GABA effect on bull spermatozoa.

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