MOLECULAR REPRODUCTION AND DEVELOPMENT 67:478–486 (2004)

In Vitro Effect of Gamma-Aminobutyric Acid on Bovine Spermatozoa Capacitation

M.N. RITTA,[1,2]* D.E. BAS,[1] AND C.M. TARTAGLIONE[2]

[1]*Institute of Experimental Biology and Medicine, University of Lomas de Zamora, Buenos Aires, Argentina*
[2]*School of Agrarian Sciences, University of Lomas de Zamora, Buenos Aires, Argentina*

ABSTRACT Sperm capacitation is defined as the maturational changes that render a sperm competent for fertilization and occurs in the female reproductive tract. Identification of the factor/s that regulate sperm capacitation would allow the understanding of these phenomena. Among these factors, gamma-aminobutyric acid (GABA) has recently become as a putative modulator of sperm function. The aim of this study was to explore the presence of a GABAergic regulation of bovine sperm capacitation as well as the possible intracellular mechanisms involved. GABA was detected in fresh semen by a sensitive radioreceptor assay (spermatozoa, 0.064 ± 0.003 nmoles/10^6 cells; seminal plasma, 23.21 ± 1.16 nmoles/ml). Scatchard analysis of [^{3}H]-muscimol binding to sperm membranes yielded a linear plot consistent with a single population of binding sites ($K_d = 3.87$ nM, $B_{max} =$ 417 fmol/mg prot.). [^{3}H]-muscimol specific binding to sperm membranes was significantly inhibited by the GABA A receptor (GABA A-R) antagonist bicuculline and by the agonists muscimol and isoguvacine. Addition of GABA to the incubation medium resulted in a concentration-dependent increase in the percentage of capacitated spermatozoa (chlortetracycline assay). We observed a significant increment on intracellular calcium and cyclic 3′,5′ adenosine monophosphate (cAMP) concentrations induced by GABA, being the cation influx abolished when the cell suspensions were coincubated with the antagonists bicuculline or picrotoxin. It is concluded that GABA induces sperm capacitation through an intracellular mechanism dependent on calcium influx and cAMP accumulation mediated by a specific GABA A-R. *Mol. Reprod. Dev. 67: 478–486, 2004.* © 2004 Wiley-Liss, Inc.

Key Words: GABA; bovine; spermatozoa; capacitation; chlortetracycline

INTRODUCTION

Binding to the egg's zona pellucida stimulates the spermatozoon to undergo acrosome reaction, a process that enables the sperm to penetrate the egg. Prior to this binding, the spermatozoa undergo in the female reproductive tract a series of biochemical transformations, collectively called capacitation. The first event in capacitation is the elevation of intracellular calcium and bicarbonate to activate adenylyl cyclase to produce cyclic 3′,5′ adenosine monophosphate (cAMP), which activates protein kinase A to phosphorylate certain proteins (Breitbart and Naor, 1999; Guraya, 2000). Moreover, during capacitation there is also an increase in actin polymerization and in the membrane-bound phospholipase C, superoxide anion production, and protein tyrosine phosphorylation (Leclerc et al., 1998).

Depending on the species considered, capacitation occurs in different segments of the female reproductive tract. As a consequence, and for practical purposes, identification of the factor or factors that regulate sperm capacitation would allow to understand sperm physiology as well as the design of chemically defined media to be used in "in vitro" fertilization and related techniques.

Among those factors, gamma-aminobutyric acid (GABA) has emerged as a putative modulator of sperm function. In vertebrate central nervous system, GABA is the most widely distributed inhibitory neurotransmitter; it has been estimated that 20–50% of all synapses in the mammalian brain are GABAergic (Roberts and Frankel, 1950; Bormann, 1988; Sieghart, 1992).

The inhibitory action of GABA is mediated by membrane receptors. At least three types of GABA receptors have been characterized: A, B, and C. GABA A receptors (GABA A-R) are ligand-gated chloride channels and have a diverse molecular composition. Pharmacologically, these receptors are antagonized by bicuculline. GABA A-R are also the major targets for several clinically relevant drugs such as the barbiturates, benzodiazepines, neurosteroids, and anesthetics (Rudolph et al., 2001). These compounds allosterically modulate GABA A-R channel activities (Sieghart, 1995). GABA B-R belong to the G-protein coupled receptor superfamily (Bowery et al., 1983). The inhibition of

Grant sponsor: National Research Council of Argentina; Grant sponsor: Faculty of Agrarian Sciences, National University of Lomas de Zamora, Buenos Aires, Argentina.

M.N. Ritta's present address is Instituto de Biología y Medicina Experimental—CONICET, Obligado 2490, 1428-Buenos Aires, Argentina.

*Correspondence to: M.N. Ritta, Institute of Biology and Experimental Medicine, Obligado 2490-1428, Buenos Aires, Argentina. E-mail: mritta@dna.uba.ar

Received 11 August 2003; Accepted 28 October 2003
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20038

GABA B-R is mediated by indirect gating of either potassium or calcium channels, while they are activated by baclofen and antagonized by phaclofen (Kerr et al., 1987) and saclofen (Kerr et al., 1989). GABA C-R are the newly identified member of the GABA receptor family (Johnston, 1986). Pharmacologically, GABA C-R are bicuculline- and baclofen-insensitive, sensitive to the agonist 4-amino-cis-2-butenoic acid (CACA), and are not modulated by many GABA A-R modulators (such as benzodiazepines and barbiturates; Johnston, 1996).

GABAergic systems, the presence of GABA itself and GABA effects have also been recognized in many peripheral tissues such as the gastrointestinal tract, kidney, and male, female genital tracts, and human spermatozoa and seminal fluid (Taniyama et al., 1983; Erdö and Kiss, 1986; Ritta and Calandra, 1986; Ritta et al., 1987, 1991, 1998; Erdö and Wolf, 1990). Moreover, the rat oviduct has been found to be more than twice as rich in GABA than the brain (Martin del Rio, 1981). Immunohistochemical studies indicated that GABA was localized to the mucosal layer of the oviduct (Orensanz et al., 1986).

The role of GABA in the context of the mammalian sperm acrosome reaction has recently become subject of increasing attention. There is enough evidence supporting a role of GABA in some events involved in fertilization, such as capacitation and acrosome reaction.

Numerous reports support the role of GABA in sperm function involving progesterone and related compounds. In human spermatozoa progesterone provokes an immediate increase in calcium influx (Thomas and Meizel, 1989) and chloride influx (Turner and Meizel, 1995), stimulates protein tyrosine phosphorilation (Parinaud and Milhet, 1996; Calogero et al., 2000), induces the acrosome reaction (Calogero et al., 2000), causes hyperactivation (Calogero et al., 1996), and increases in cAMP levels (Parinaud and Milhet, 1996).

An effect of progesterone on acrosome reaction has also been shown in spermatozoa from other species such as mouse (Murase and Roldan, 1996), hamster (Llanos et al., 1993), stallion (Meyers et al., 1993), guinea pig (Zhang et al., 2000), goat (Somanath and Gandhi, 2002), boar (Barboni et al., 1995), dog (Sirivaidyapong et al., 2001), and pig (Martinez et al., 2002).

As the interaction of GABA with its A-type receptor (GABA A-R) from neural tissues is enhanced by progesterone metabolites, and a pregnane binding site was identified on the GABA A-R (Lan et al., 1991), it was hypothesized that the action of progestins on calcium influx and acrosome reaction might be mediated by their interaction with the GABA A-R.

GABA A-R in human spermatozoa was described by immunocytochemical techniques, using a monoclonal anti α-subunits of the GABA A-R (Wistrom and Meizel, 1993) and by biochemical techniques (Ritta et al., 1998). Moreover, the functional relationship between GABA A-R and GABA B-R in regulating acrosome reaction of rat spermatozoa has been recently explored (Hu et al., 2002). However, the effect of GABA on capacitation itself has not been thoroughly studied.

The current article was undertaken to explore the presence of GABA and GABA receptors in bovine semen, the "in vitro" capacitating effect of GABA on bovine spermatozoa and the role of GABA on two events associated with capacitation: calcium influx and cAMP increases.

MATERIALS AND METHODS

Reagents

[^{3}H]-muscimol (sp. act. 20 Ci/mmol) was purchased from New England Nuclear Co. (Boston, MA). GABA, baclofen, bicuculline, isoguvacine, picrotoxin, chlortetracicline, and other reagents for the preparation of buffers and media were obtained from Sigma Chemical Co. (St. Louis, MO). CACA was purchased from Research Biochemicals International (MA). Medium 199 from Difco (Detroit, MI) and Fura-2AM from Molecular Probes, Inc., Eugene, OR). Na^{125}I was obtained from Dupont-NEN (Boston, MA). cAMP was radiolabeled with Na^{125}I in our laboratory by the method of chloramine-T (sp. act. 600 Ci/mmol), as described by Birnbaumer (1980). The antibody anti-cAMP was a gift from NIDDKD and NHPP, University of Maryland, School of Medicine. All other reagents were of analytical grade.

Sperm Collection and Preparation

Fresh semen from 12 Aberdeen Angus bulls of proven fertility was collected using an artificial vagina. Sperm samples were assessed to ascertain live/dead relation (Eosin–Nigrosin vital stain) and sperm motility (visual microscopy examination). 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 phase contrast microscopy. 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, and incospectin). Only ejaculates with spermatozoal motility >60% and concentration $>3 \times 10^6$ spermatozoa/ml were used for subsequent manipulations.

GABA Radioreceptor Assay

The method is based on the competitive inhibition of [^{3}H]-muscimol binding to the GABA receptor of cerebellar membranes by GABA present in the sample (Bernasconi et al., 1980; Ritta et al., 1987, 1998). Sperm pellets were homogenized by ultra sonication in an appropriate volume of distilled water and centrifuged for 20 min at 12,000*g*, at 4°C. Aliquots of these supernatants or seminal plasma were incubated in Eppendorf tubes containing 100 μl of crude cerebellar membranes (70–100 μg of protein), 100 μl of a 40 nM [^{3}H]-muscimol solution, and Tris-citrate buffer (50 mM, pH 7.2) to give a final volume of 1 ml. After mixing, the samples were incubated at 4°C for 10 min and the assay terminated by

centrifugation at 12,000*g* for 10 min at 4°C. The supernatant was discarded and the pellet was rapidly rinsed with 1.5 ml of cold Tris-citrate buffer. After dissolving the pellets in 100 μl of hyamine hydroxide, 10 ml of acidified Aquasol was added and radioactivity was measured by liquid scintillation spectrometry.

Preparation of Sperm Membranes

For receptor binding experiments, semen was centrifuged at 500*g* for 10 min, and the sperm pellet was immediately frozen and kept at −20°C at least for 1 week, according to Enna and Snyder (1976). On the day of the experiment, the cells were thawed and homogenized in 15 volumes of ice-cold 0.32 M sucrose with an Ultraturrax (Janke and Kunkel, IKA, Labortechnik, FRG) at 2,500 rpm for 40 s. The homogenate was centrifuged at 1,000*g*, and the supernatant was collected and recentrifuged at 48,000*g* for 20 min. The final pellet was washed twice with distilled water, in order to remove endogenous GABA, and resuspended in 50 mM Tris-citrate buffer, pH 7.4 (Ritta et al., 1991, 1998).

[^{3}H]-Muscimol Binding Assay

Binding assay was performed as described by Bowery et al. (1983) and modified by Ritta et al. (1998). Membrane suspensions (100 μl) were incubated in glass tubes containing 10 nM [^{3}H]-muscimol (200 μl final volume) and increasing concentrations of unlabelled muscimol. Nonspecific binding was determined by the addition of a 1,000-fold excess of unlabelled muscimol. The reaction was stopped by rapidly filtering the suspensions through glass fiber (Whatman GF/C) under vacuum. The filters were washed three-times with ice-cold Tris-citrate buffer. Radioactivity in the filters was measured by liquid scintillation spectrometry at a counting efficiency of 60%.

In Vitro Capacitation

Semen aliquots (100 μl) were washed once in 4 ml of Biggers, Whitten, and Wittingham (BWW) medium (Biggers et al., 1971) without BSA or serum, supplemented with 10 mM Hepes, and adjusted to pH 7.4 under 5% CO_2 with 0.1 N NaOH. The sperm suspension was centrifuged in 15-ml conical plastic tubes, at 500*g* for 10 min, in a swinging-bucket centrifuge at room temperature. The loose pellet was resuspended in BWW so as to get a final sperm count of 10^7 cells/ml. Aliquots (500 μl) of this suspension were mixed in 1-ml plastic wells with an equal volume of BWW alone or BWW containing GABA, GABA agonists, or antagonists at the final concentrations specified in each experiment. The samples were incubated at 38.5°C in a humidified chamber under an atmosphere of 5% CO_2 in air. After 2 hr, aliquots of each sample were assessed for capacitation by CTC assay (see below). Sperm motility was monitored before and after incubations as previously described.

CTC Assay

The CTC method used to determine bovine sperm capacitation 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 acid, 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 microliter of spermatozoal 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. Chlortetracycline 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 1 of 3 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.

Fura-2AM Assays of $[Ca^{2+}]_i$

The intracellular concentration of free Ca^{2+} was assessed using the fluorescence indicator Fura-2AM (Grynkiewicz et al., 1985). Washed spermatozoa were incubated with 1 μM Fura for 30 min al 37°C. The loaded cells were then washed by centrifugation (780*g*, 10 min at 25°C) in buffer containing 110 mM NaCl, 5 mM KCl, and 10 mM 3-(N-morpholino)propanesulfonic acid, pH 7.4 to remove extracellular Fura-2AM, incubated for another 30 min, and washed twice as described above. Fluorescence measurements were performed in a Jacso spectrofluorometer at excitation wavelength 340/380 nm and emission wavelength 505 nm. During fluorescence measurements sperm suspensions were continuously stirred in a thermostated cuvette holder (37°C). The different compounds to be tested (GABA, picrotoxin, baclofen, CACA) were added to the cuvette containing 5×10^5 cells 200 s after data collection was started. The ratio 340/380 was converted to $[Ca^{2+}]_i$ according to the formula described by Grynkiewicz et al. (1985). R_{max} and R_{min} were determined in the presence of 0.1% Triton X-100 and 2 mM ethylene-bis (oxyethilenenitriolo) tetraacetate acid.

Determination of Intracellular cAMP

Washed sperm pellets were resuspended in 10 mM Hepes-Tris buffer, pH 7.4, containing (mM): NaCl 145, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, and glucose 1.1. One milliliter aliquots (0.5×10^6 cells) were incubated at 37°C in the presence of GABA (1 μM). After the incubation period, tubes were placed on ice, centrifuged in an Eppendorf

microfuge (Brinkmann Instruments, NY), and the pellets were resuspended in 500 μl H_2O heated for 1 min in boiling water. After centrifugation the pellets were resuspended in 50 mM sodium acetate buffer (pH 6.0). Samples and standards were acetylated and mixed with ^{125}I-cAMP (15,000–20,000 dpm, 140 mCi/mmol) and rabbit antiserum (kindly supplied by the National Institute of Diabetes and Digestive and Kidney Disease) diluted 1:5,000 and incubated overnight at 4°C. At the end of the incubation, the antigen–antibody complexes were precipitated by the addition of 50 μl 2% BSA and 2 ml cold ethanol and centrifuged at 2,000 rpm for 20 min, supernatants were aspirated, and pellets counted in a Packard Auto-Gamma (Packard Instrument Co., Downers Grove, IL; Steiner et al., 1969).

Statistical Analysis

All data were analyzed by analysis of variance using mixed models that contained fixed treatments and random effects due to individual bulls (Snedecor and Cochran, 1967). A random × fixed effect interaction was considered random. Planned linear contrasts were used to test differences among means.

Prior to statistical comparisons an arc sine, square root transformation was applied to the individual percentages of spermatozoa showing pattern B to homogenize the residual variances.

RESULTS

GABA was measured by radioreceptor assay in both seminal plasma and washed spermatozoa from bovine fresh semen. As shown in Table 1, GABA was detected in both fractions. This was verified as GABA through the observation that it was almost completely (>85%) degraded by the treatment of the samples with the metabolizing enzyme, GABAase (Grahan and Aprison, 1966; data not shown).

Specific [^{3}H]-muscimol binding to bovine sperm membranes increased linearly with protein concentration (200–1,000 μg), and was maximal al 37°C, occurring the highest binding between 20 and 30 min of incubation. [^{3}H]-muscimol binding to bovine sperm membranes was inhibited by GABA and to a less degree by the GABA A antagonist bicuculline. In contrast, the GABA B agonist baclofen and the GABA C agonist CACA were unable to compete with [^{3}H]-muscimol for sperm binding sites (Table 2). Scatchard analysis of [^{3}H]-muscimol binding yielded a linear plot consistent with a single, homogeneous population of binding sites, with $K_d = 2.87$ nM and $B_{max} = 399$ fmol/mg protein (Fig. 1).

TABLE 1. Presence of GABA in Bovine Semen

	GABA
Spermatozoa	0.064 ± 0.003 nmoles/10^6 cells
Seminal plasma	23.210 ± 1.160 nmoles/ml

Each value represents mean ± SEM (n = 12).

TABLE 2. Inhibition of [^{3}H]-Muscimol Bound to Bovine Sperm Membranes

Compound	IC_{50} (μM)
GABA	0.063 ± 0.060
Muscimol	0.036 ± 0.009
Bicuculline	16.000 ± 1.370
Isoguvacine	21.000 ± 4.800
Baclofen	>400
CACA	>400

IC_{50} values (concentrations producing 50% displacement of the specific binding), given in μM, were determined by probit analysis of three experiments. Each compound was tested in triplicate (concentration range: 10 nM to 10 mM).

As shown in Table 3, incubation of bovine spermatozoa for 2 hr in medium BWW (control) did not alter the percentage of CTC form corresponding to capacitated cells with respect to nonincubated samples. Addition of GABA to the incubation medium resulted in a concentration-dependent increase in the percentage of this CTC pattern corresponding to capacitated spermatozoa (pattern B). The effect of GABA was marginal but statistically significant at 1 μM and was maximal at 20 μM.

To determine if GABA A-R was involved in the action of GABA on sperm capacitation, spermatozoa were incubated, under the same conditions as described above, in the presence of 20 μM GABA, the GABA A-R agonist muscimol, the GABA B-R agonist baclofen, and the GABA C-R agonist CACA. Muscimol at 10 or 20 μM had a capacitating effect similar to that of 20 μM GABA. In contrast, baclofen (1 or 10 μM) and CACA (1 or 10 μM) were ineffective (Table 4).

We evaluated the effect of GABA, picrotoxin, and baclofen on intracellular Ca^{2+} concentrations in spermatozoa loaded with Fura-2AM. GABA (10 μM) induced an increment on $[Ca^{2+}]_i$ (Fig. 2), while baclofen 100 μM

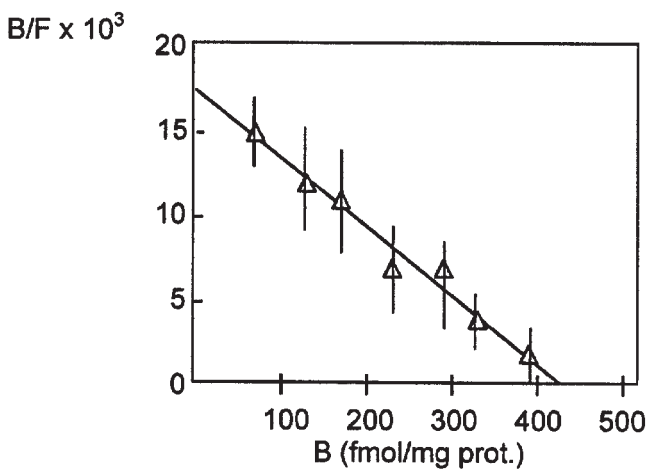

Fig. 1. Scatchard analysis of [^{3}H]-muscimol bound to bovine sperm membranes. Each point represents the mean ± SEM of three independent experiments. B, bound; F, free. The dissociation constant (K_d) and maximal binding (B_{max}) are 3.74 ± 0.19 nM and 417.6 ± 22.6 fmol/mg protein, respectively.

TABLE 3. Effect of GABA on Bovine Sperm Capacitation

GABA	Pattern B (%)
Control	19.3 ± 1.8
Nonincubated	18.4 ± 1.9^a
1 μM	26.6 ± 2.8^b
5 μM	36.7 ± 1.4^b
20 μM	39.2 ± 1.5^b
50 μM	38.5 ± 1.3^b

Spermatozoa were incubated as described in "Materials and Methods," in BWW medium alone (control) or containing GABA. Capacitation was evaluated by percentage of pattern B of CTC staining. Nonincubated, washed samples without incubation; control, samples washed and incubated in BWW medium, in the absence of GABA. Results are expressed as percentage data transformed to the arc sine of their square roots (mean ± SD for 12 different ejaculates).
$^aP < 0.001$. $^bP > 0.05$ vs. control.

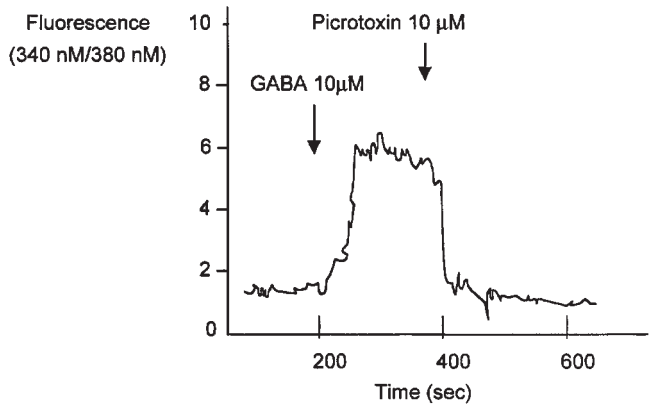

Fig. 2. Effect of gamma-aminobutyric acid (GABA) and picrotoxin on intracellular $[Ca^{2+}]_i$ concentrations. Intact sperm cells (5×10^5 cells/ml) were loaded with Fura-2AM and the changes in $[Ca^{2+}]_i$ were recorded using a spectrofluorometer (see "Materials and Methods"). At the time indicated by the arrows, GABA and picrotoxin were added. The data represent one typical experiment of repetitions performed with samples obtained from 12 different animals.

had no effect on calcium influx (data not shown), These results suggest that the GABAergic receptor involved might be type A since the GABA B-R agonist baclofen had no effect. Furthermore, the GABA A-R antagonist picrotoxin prevented the effect triggered by 100 μM GABA (Fig. 2). Moreover, the GABA C agonist CACA had no effect on calcium influx (data not shown). The measurements of changes in $[Ca^{2+}]_i$ are summarized graphically in Figure 3.

When intracellular cAMP concentration was evaluated in the presence or absence of 2.5 mM Ca^{2+}, GABA only induced an increment on cAMP concentration in the presence of 2.5 μM Ca^{2+} (Table 5). To ascertain whether GABA acts through the GABA A-R, the effect of GABA and the GABA B-R and GABA C-R agonistis baclofen and CACA on the generation of cAMP were examined. It was found (Table 6) that GABA triggered cAMP formation while the agonists were ineffective. Moreover, GABA-induced generation of cAMP was inhibited by bicuculline, the GABA A-R antagonist.

TABLE 4. Effect of Muscimol, Baclofen, and CACA on Bovine Sperm Capacitation

Compound	Pattern B (%)
Control	20.9 ± 1.5
GABA 20 μM	37.8 ± 1.1^a
Muscimol 1 μM	38.1 ± 0.9^a
Muscimol 10 μM	39.3 ± 1.1^b
Baclofen 1 μM	22.2 ± 1.1^b
Baclofen 10 μM	22.5 ± 1.8^b
CACA 1 μM	20.1 ± 1.6^b
CACA 10 μM	21.0 ± 1.6^b

Spermatozoa were incubated as described in "Materials and Methods," in BWW medium alone (control) or containing GABA or one of the agonist or antagonist indicated. Capacitation was evaluated by percentage of pattern B of CTC staining. Results are expressed as percentage data transformed to the arc sine of their square roots (mean ± SD for 12 different ejaculates).
$^aP < 0.001$. $^bP > 0.05$ vs. control.

DISCUSSION

In this study we explored different aspects of the role of GABA in modulating sperm function: 1) the presence of GABA in both bovine seminal plasma and washed spermatozoa; 2) the existence of a specific population of GABA R in sperm membranes; 3) the capacitating effect of GABA; 4) the effect of GABA on Ca^{2+} influx into spermatozoa; and 5) the effect of GABA on cAMP accumulation into sperm cells.

It is well known that in order to fertilize the egg, spermatozoa must go through the capacitation process where they experience increased Ca^{2+} influx, cAMP and superoxide anion production, and protein tyrosine phosphorilation (Leclerc et al., 1998).

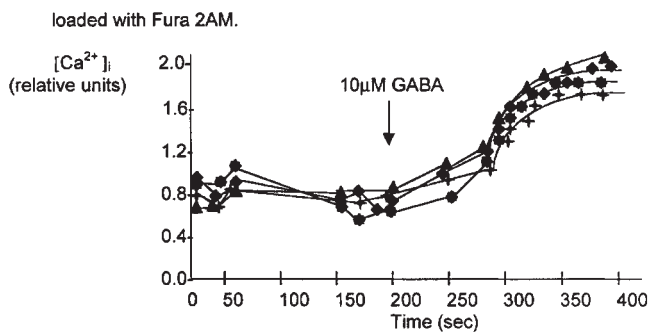

Fig. 3. Graphic representation of $[Ca^{2+}]_i$ in noncapacitated bovine sperm cells loaded with Fura-2AM. Results correspond to four randomly chosen bovine sperm cells samples loaded with Fura-2AM. Changes in $[Ca^{2+}]_i$ were measured after the addition of 10 μM GABA. The Ca^{2+} concentration scale is expressed in relative units.

TABLE 5. Effect of GABA on cAMP Accumulation in Spermatozoa in the Presence or Absence of Calcium

	cAMP (pmol/10^6 cells)	
	—	2.5 mM $CaCl_2$
None	$2.37+0.70^a$	$2.07 + 0.53^a$
GABA 1 μM	2.28 ± 0.38^a	4.18 ± 0.83^b

Data represent one of three similar experiments which gave essentially the same results. Each value represents the mean ± SEM (n = 8, number of ejaculates).
[a]Values with different superscripts within the same column are significantly different ($P > 0.01$).
[b]Values with different superscripts within the same column are significantly different ($P > 0.01$).

In fact, mammalian spermatozoa require extracellular Ca^{2+}, some of which must be internalized to undergo complete capacitation. Moreover, at a critical threshold, a rise in intracellular Ca^{2+} will trigger acrosomal exocytosis, primary induced by the egg zona pellucida glycoprotein ZP3 or by progesterone (Roldan et al., 1994).

Shi and Roldan (1995) suggested that acrosomal exocytosis involves activation of a GABA A-R apparently linked to Ca^{2+} channels. As it has been mentioned, GABA has been considered among the factors that regulate sperm capacitation and acrosomal reaction. In the present study we report the presence of GABA in bovine semen at concentrations similar to or higher than those reported in numerous peripheral organs of mammals (Erdö and Kiss, 1986). Particularly, we have reported the presence of GABA in human semen (Ritta et al., 1998) at concentrations comparable to those described herein. Since the enzymes for GABA synthesis were not measured in this study, the source(s) of GABA in spermatozoa remain to be elucidated. GABA could be locally synthesized in spermatozoa or, alternatively, taken up from seminal plasma by an active transport mechanism as suggested by Aanesen et al. (1995) to be present in human spermatozoa.

In order to investigate the presence of possible recognition sites for GABA in bovine spermatozoa we investigated the association of [^{3}H]-muscimol to crude membranes. We provide a biochemical and pharmacological description of GABA A-R in bovine sperm membranes. According to our results the K_d for (^{3}H(-muscimol calculated by Scatchard analysis is in agreement with values found in other peripheral tissues (Erdö, 1986).

TABLE 6. Effect of GABA, Baclofen, and CACA on cAMP Accumulation

Compound	cAMP (pmol/10^6 cells)
None	1.76 ± 0.16 (9)
GABA 1 μM	3.26 ± 0.05 (8)[a]
Baclofen 1 μM	1.71 ± 0.09 (8)
CACA 1 μM	1.82 ± 0.10 (8)

Data represent one of three similar experiments which gave essentially the same results. Each value represents the mean ± SEM; numbers in parentheses indicate number of ejaculates. [a]$P < 0.001$, vs. control.

It is widely accepted that sperm capacitation (and acrosome reaction) can be indirectly visualized by incubation of spermatozoa with the antibiotic (CTC), which fluoresces while monitoring Ca^{2+} displacement in the sperm head membrane (Fraser et al., 1995). The foregoing results show that GABA exerts a capacitating effect on bovine spermatozoa as assessed by the increase in the percentage of spermatozoa showing a CTC pattern compatible with a capacitated state (Fraser et al., 1995). The GABA-like effect of the GABA A-R agonist muscimol clearly indicates the involvement of the described GABA A-R in the observed effect, while the GABA B-R and GABA C-R agonists (baclofen and CACA, respectively) had no effect. Moreover, the GABA-induced capacitation was completely abolished by the specific GABA A-R antagonist bicuculline. The high GABA levels found in the oviduct (Erdö and Kiss, 1986; Erdö and Wolf, 1990) points to a role in the fertilization process, acting directly on the oocyte, on sperm, or both. Notwithstanding, the presence of GABA in bull semen and the existence of GABA A-R reported herein strongly suggest a direct action at sperm level.

Although the K_d for [^{3}H]-muscimol was in the nanomolar range, the effect of GABA on sperm capacitation was only evident al micromolar concentrations. Considering the occurrence of an active uptake GABA mechanism in spermatozoa (Aanesen et al., 1995), it is possible that GABA is taken up, resulting in a decreased GABA concentration. On the other hand, since GABA concentrations in the oviduct have been reported to be comparable to those in the brain, the effect described herein on sperm capacitation, even at micromolar concentrations, could be of physiological relevance. Moreover, it should be taken into account that neuroactive steroids or endogenous compounds with benzodiazepine activity could enhance the in vivo effect of GABA.

An interesting data is the identification of another neurotransmitter, norepinephrine, within the microenvironment of the bovine oviduct (Way et al., 2001). Moreover, it was reported that this catecholamine capacitates and induces the acrosome reaction in freshly ejaculated bovine spermatozoa at the physiological concentrations previously determined for bovine oviductal fluid (Way and Killian, 2002).

As it has been mentioned, Hu et al. (2002) reported that the induction of acrosome reaction in rat sperm by GABA was found to be a biphasic phenomenon. According to their observations, acrosome reaction rates increased with increasing GABA concentrations up to 5 μM and at higher concentrations of the neurotransmitter (>5 μM) there was a reduction in the acrosome reaction rates. This biphasic phenomenon was proposed to be apparently due to the differential interaction of the neurotransmitter with GABA receptor subtypes in a dose-dependent manner. These authors postulate that

the induction of acrosome reaction in rat sperm by GABA is regulated by the proportionality of activated GABA A-Rs and GABA B-Rs acting as a yin–yang control. However, in the present study we provide a biochemical and pharmacological description of GABA A-Rs in bull sperm membranes and the lack of GABA B recognition sites.

It is generally accepted that the first event in capacitation is the increase in intracellular Ca^{2+} bicarbonate and hydrogen peroxide, which collectively activate adenylyl ciclase to produce cAMP, which activates protein kinase A to phosphorylate certain proteins (Breitbart and Naor, 1999). Moreover, Galantino-Homer et al. (1997) reported an interrelationship between tyrosine kinase/phosphatase and cAMP signaling pathways at the level of protein kinase A in bovine sperm capacitation.

To ascertain a possible rise in intracellular Ca^{2+} triggered by GABA, we evaluated the effect of GABA, the antagonist of GABA A-R picrotoxin and the agonists baclofen and CACA on $[Ca^{2+}]_i$ in bull sperm cells loaded with Fura-2AM. While baclofen and CACA had no effect on Ca^{2+} influx, GABA induced an increment on $[Ca^{2+}]_i$. These results suggest that the GABA receptor involved in Ca^{2+} mobilization is the described type A recognition site. Moreover, the GABA A-R antagonist picrotoxin prevented the effect triggered by GABA demonstrating the activation of the GABA A-R induces Ca^{2+} response.

Since the ATP-dependent Ca^{2+} pump [Ca^{2+}–Mg^{2+}-ATPase] is auto-phosphorylated by ATP, it can be classified as a protein kinase. It is hypothesized that the plasma membrane Ca^{2+} pump is involved in the regulation of $[Ca^{2+}]_i$ during sperm capacitation (Adeoya-Osiguwa and Fraser, 1993; DasGupta et al., 1994); however, there is no direct evidence to support this. The mechanism of regulation of $[Ca^{2+}]_i$ within the spermatozoon involves the plasma membrane, intracellular Ca^{2+} stores, most likely in the acrosome, and the mitochondria. In bull spermatozoa it was reported 6-fold increment in $[Ca^{2+}]_i$ during incubation under capacitating conditions (Breitbart and Naor, 1999). The entry of Ca^{2+} results in an increase in $[Ca^{2+}]_i$ which stimulates the ATP-dependent Ca^{2+} pump (Breitbart et al., 1983) and the plasma membrane $3Na^{+}.Ca^{2+}$ exchanger (Rufo et al., 1984) to excrete Ca^{2+} from the cell. Moreover, it has been proposed the presence of either a cAMP-gated Ca^{2+} channel or a channel opened upon phosphorylation by cAMP-dependent protein kinase A (Spungin and Breitbart, 1996). Since cAMP concentrations and protein kinase A-dependent protein tyrosine phosphorylation are enhanced during capacitation, and this enzyme can activate Ca^{2+} channels in the acrosomal membrane, it is suggested that the acrosomal calcium is mobilized during capacitation (Breitbart and Naor, 1999).

Parinaud and Milhet (1996) and other authors reported the effect of progesterone on cAMP levels in human sperm. These authors propose that the progesterone-induced cAMP increase appears to be Ca^{2+}-dependent and related to the Ca^{2+} influx, because the ionophore A23187 has the same effect as the steroid. This progesterone-induced Ca^{2+} influx has been well described by Thomas and Meizel (1989) and by Blackmore (1993), and it is proposed to be related to cell surface progesterone receptor. Furthermore, Parinaud and Milhet (1996) reported that hyperactivation, the major measurable phenomenon linked to capacitation (Cohen-Dayag and Eisenbach, 1994), is induced by progesterone. The effect of the steroid is partially reproduced by GABA and decreased by the GABA A-R antagonist picrotoxin. A GABA A-like receptor has been shown to be involved in progesterone-induced acrosome reaction (Shi and Roldan, 1995) but not in progesterone-induced Ca^{2+} influx (Blackmore et al., 1994; Turner et al., 1994). This could explain why GABA does not totally abolish the effect of progesterone on cAMP levels. According to the present results, 10 μM GABA was able to induce an increment on intracellular cAMP concentration only in the presence of Ca^{2+}. Parinaud and Milhet (1996) reported a not statistically significant increase of cAMP when human sperm were incubated in the presence of 5 μM GABA. However, they observed that picrotoxin (10 μM) abolished the effect of 100 μM progesterone on cAMP. According to the present results, incubation of bovine sperm cells in the presence of GABA B-R and GABA C-R agonist failed to induce cAMP accumulation, while the GABA A-R antagonist bicuculline inhibited GABA-induced cAMP production. The intracellular mechanism of GABA-induced cAMP increase remains to be elucidated. Indeed, GABA could act either by stimulating adenylyl cyclase or by inhibiting phosphodiesterase.

CONCLUSIONS

In conclusion, GABA is present in bovine seminal plasma and washed spermatozoa, specific GABA A-R occurs in sperm membranes and GABA exerts a capacitating effect on bull spermatozoa as assessed by CTC technique. According to the foregoing results, the mechanism of GABA action appears to involve at least two biochemical pathways: calcium channels and cAMP production.

ACKNOWLEDGMENTS

The authors thank Lic. Amalia Boto Ardouin for assistance and review.

REFERENCES

Aanesen A, Fried G, Andersson E, Gottlieb C. 1995. Evidence for gamma-aminobutyric acid specific binding sites on human spermatozoa. Hum Reprod 10:1885–1890.

Adeoya-Osiguya SA, Faser LR. 1993. A biphasic pattern of $^{45}Ca^{2+}$ uptake by mouse spermatozoa in vitrocorrelates with changing functional potential. J Reprod Fertil 99:187–194.

Barboni B, Mattioli M, Seren E. 1995. Influence of progesterone on boar sperm capacitation. J Endocrinol 144:13–18.

Bernasconi R, Bittiger H, Heid J, Martin PJ. 1980. Determination of GABA levels by a [3H]Muscimol radioreceptor assay. J. Neurochem 34:614–619.

Biggers JD, Whitten WK, Whittingham DG. 1971. The culture of mouse embryos in vitro. In: Daniel JC, editor. Methods of mammalian embryology. San Francisco: WH Freeman & Co. pp 86–116.

Birnbaumer L. 1980. Techniques in cyclic nucleotides research. In: Schrader WT, O'Malley BW, editors. Laboratory methods manual for

hormone action and molecular endocrinology, Vol. 4. Houston, Texas: Department of Cell Biology, Baylor College of Medicine. pp 9–16.
Blackmore PF. 1993. Rapid non-genomic actions of progesterone stimulate Ca^{2+} influx and the acrosome reaction in human sperm. Cell Signal 5:531–538.
Blackmore PF, Im WB, Bleasdale JE. 1994. The cell surface progesterone receptor which stimulates calcium influx in human sperm is unlike the A ring reduced steroid site on the GABA A receptor/chloride channel. Mol Cell Endocrinol 104:237–243.
Bormann J. 1988. Electrophysiology of GABA A and GABA B receptor subtypes. Trends Neurosci 11:112–116.
Bowery NG, Hill DR, Hudson AL. 1983. Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. Br J Pharmacol 78:191–206.
Breitbart H, Naor Z. 1999. Protein kinases in mammalian sperm capacitation and the acrosome reaction. Rev Reprod 4:151–159.
Breitbart H, Stern B, Rubinstein S. 1983. Calcium transport and Ca2+-ATPase activity in ram spermatozoa plasma membrane vesicles. Biochim Biophys Acta 728:349–355.
Calogero AE, Hall J, Fishel S, Green S, Hunter A, D'Agata R. 1996. Effects of gamma-aminobutyric acid on human sperm motility and hyperactivation. Mol Hum Reprod 2:733–738.
Calogero AE, Burrello N, Barone N, Palermo I, Grasso U, D'Agata R. 2000. Effects of progesterone on sperm function: Mechanisms of action. Hum Reprod 1(suppl):28–45.
Cohen-Dayag A, Eisenbach M. 1994. Potential assays for sperm capacitation in mammals. Am J Physiol 267:1167–1176.
Das Gupta S, Toole CO, Mills CL, Fraser LR. 1994. Effect of pentoxifylline and progesterone on human sperm capacitation and acrosomal exocytosis. Hum Reprod 9:2103–2109.
Enna SJ, Snyder SH. 1976. A simple, sensitive and specific radioreceptor assay for endogenous GABA in brain tissue. J Neurochem 26:221–224.
Erdö SL. 1986. GABA receptor binding sites in peripheral tissues of mammals. In: Erdö SL, Bowery N, editors. GABAergic mechanisms in the mammalian periphery. New York: Raven Press. pp 77–86.
Erdö SL, Kiss B. 1986. Presence of GABA, glutamate decarboxilase and GABA transaminase in peripheral tissues: A collection of quantitative data. In: Erdö SL, Bowery N, editors. GABAergic mechanisms in the mammalian periphery. New York: Raven Press. pp 5–17.
Erdö SL, Wolf JR. 1990. γ-Aminobutyric acid outside the mammalian brain. J Neurochem 54:363–372.
Fraser LR, Abeydeera LR, Niwa K. 1995. Ca(2+)-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev 40: 233–241.
Galantino-Homer HL, Visconti PE, Kopf GS. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3′5′-monophosphate-dependent pathway. Biol Reprod 56:707–719.
Grahan L, Aprison M. 1966. Fluorometric determination of aspartate, glutamate and gamma-aminobutyrate in nerve tissue using enzymic methods. Anal Biochem 15:487–497.
Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.
Guraya SS. 2000. Cellular and molecular biology of capacitation and acrosome reaction in spermatozoa. Int Rev Cytol 199:1–64.
Hu JH, He XB, Wu Q, Yan YC, Koide SS. 2002. Biphasic effect of GABA on rat sperm acrosome reaction: Involvement of GABA (A) and GABA (B) receptors. Arch Androl 48:369–378.
Johnston GAR. 1986. Multiplicity of GABA receptors. In: Olsen RW, Venter JC, editors. Receptor biochemistry and methodology, Vol. 5. Alan R: Liss, Inc. pp 57–71.
Johnston GAR. 1996. GABAc receptors: Relatively simple transmitter-gated ion channels? Trends Pharmacol Sci 17:319–323.
Kerr DI, Ong J, Prager RH, Gynther BD, Curtis DR. 1987. Phaclofen: A peripheral and central baclofen antagonist. Brain Res 405: 150–154.
Kerr DI, Ong J, Johnston GA, Abbenante J, Prager RH. 1989. Antagonism at GABAB receptors by saclofen and related sulphonic analogues of baclofen and GABA. Neurosci Lett 107:239–244.
Lan NC, Bolger MB, Gee KW. 1991. Identification and characterization of a pregnane steroid recognition site that is functionally coupled to an expressed GABA A receptor. Neurochem Res 16:347–356.
Leclerc P, de Lamirande E, Gagnon C. 1998. Interaction between Ca^{2+}, cyclic 3′,5′ adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. J Androl 19:434–443.
Llanos MN, Morales PJ, Anabalon MC. 1993. Characterization of the progesterone induced hamster sperm acrosome reaction. Biol Reprod 48:107.
Martin del Rio R. 1981. Gamma-aminobutyric acid system in rat oviduct. J Biol Chem 256:9816–9819.
Martinez G, Zayas H, Ducolomb Y, Garcia GA, Betancourt M, Castro I. 2002. Effect of norethisterone and its A-ring reduced metabolites on the acrosome reaction in porcine spermatozoa. Andrologia 34: 296–300.
Meyers SA, Liu IKM, Overstreet JW, Drobnis EZ. 1993. Capacitation of stallion sperm in vitro essesed by progesterone-induced acrosome reaction. Biol Reprod 48:107.
Murase T, Roldan ER. 1996. Progesterone and the zona pellucida activate different transducing pathways in the sequence of events leading to diacylglycerol generation during mouse sperm acrosomal exocytosis. Biochem J 320:1017–1023.
Orensanz ML, Fernandez I, Martín del Río R, Storm-Mathisen J. 1986. Gamma-aminobutyric acid in the rat oviduct. Adv Biochem Psychopharmacol 42:265–274.
Parinaud J, Milhet P. 1996. Progesterone induces Ca^{2+}-dependent 3′,5′-cyclic adenosine monophosphate increase in human sperm. J Clin Endocrinol Metab 81:1357–1360.
Ritta MN, Calandra RS. 1986. Occurrence of GABA in rat testis and its effect on androgen production. In: Racagni G, Donoso A, editors. GABA and endocrine function, advances in biochemical psychopharmacology. New York: Raven Press. pp 291–297.
Ritta MN, Campos MB, Calandra RS. 1987. Effect of GABA and benzodiazepines on testicular androgen production. Life Sci 40:791–798.
Ritta MN, Campos MB, Calandra RS. 1991. Coexistence of gamma-aminobutyric acid type A and type B receptors in testicular interstitial cells. J Neurochem 56:1236–1240.
Ritta MN, Bas DE, Calamera JC. 1998. Occurrence of GABA and GABAergic receptors in human spermatozoa. Mol Human Reprod 4:769–773.
Roberts E, Frankel S. 1950. Gamma-aminobutyric acid in brain. Fed Proc 9:219–224.
Roldan ER, Murase T, Shi QX. 1994. Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science 266:1578–1581.
Rudolph U, Crestani F, Mohler H. 2001. GABA (A) receptor subtypes: Dissecting their pharmacological functions. Trends Pharmacol Sci 22:402–403.
Rufo GA Jr, Schoff PK, Lardy HA. 1984. Regulation of calcium content in bovine spermatozoa. J Biol Chem 259:2547–2552.
Shi QX, Roldan ER. 1995. Evidence that a GABA A-R like is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Biol Reprod 52:373–381.
Sieghart W. 1992. GABA A receptors: Ligand-gated Cl^- ion channels modulated by multiple drug-binding sites. Trends Pharmacol 13: 446–450.
Sieghart W. 1995. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. Pharmacol Rev 47:181–234.
Sirivaidyapong S, Bevers MM, Gadella BM, Colenbrander B. 2001. Induction of the acrosome reaction in dog sperm cells is dependent on epididymal maturation: The generation of a functional progesterone receptor is involved. Mol Reprod Dev 58:451–459.
Snedecor GW, Cochran WG. 1967. Statistical methods, 6th edn. Ames: Iowa University Press.
Somanath PR, Gandhi KK. 2002. Expression of membrane associated non-genomic progesterone receptor(s) in caprine spermatozoa. Anim Reprod Sci 74:195–205.
Spungin B, Breitbart H. 1996. Calcium mobilization and influx during sperm exocytosis. J Cell Sci 109(pt 7):1947–1955.

Steiner AL, Kipnis DM, Utiger R, Parker C. 1969. Radioimmunoassay for the measurement of adenosine 3′,5′-cyclic phosphate. Proc Natl Acad Sci USA 64:367–373.

Taniyama K, Kusunoki M, Saito N, Tanaka T. 1983. GABA-evoked acetylcholine release from isolated guinea pig ileum. Life Sci 32:2342–2353.

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

Turner KO, Meizel S. 1995. Progesterone-mediated efflux of cytosolic chloride during the human sperm acrosome reaction. Biochem Biophys Res Commun 213:774–780.

Turner KO, Garcia MA, Meizel S. 1994. Progesterone initiation of the human sperm acrosome reaction: The obligatory increase in intracellular calcium is independent of the chloride requirement. Mol Cell Endocrinol 101:221–225.

Ward CR, Storey BT. 1984. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol 104:287–296.

Way AL, Killian GJ. 2002. Capacitation and induction of the acrosome reaction in bull spermatozoa with norepinephrine. J Androl 23: 352–357.

Way AL, Barbato GF, Killian GJ. 2001. Identification of norepinephrine in bovine oviductal fluid by high performance liquid chromatography. Life Sci 70:567–576.

Wistrom CA, Meizel S. 1993. Evidence suggesting involvement of a unique human sperm steroid receptor/Cl-channel complex in the progesterone-initiated acrosome reaction. Dev Biol 159: 679–690.

Zhang ZH, Chen WY, Shi QX, Yuan YY, Lou YC. 2000. Capacitating action of GABA and progesterone in spermatozoa of human and guinea pig in vitro. Sheng Li Xue Bao 52:179–184.