In Vitro Capacitating Effect of Gamma-Aminobutyric Acid in Ram Spermatozoa

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ABSTRACT

We have evaluated the capacitating effect of gamma-aminobutyric acid (GABA) in ram spermatozoa in vitro, in a chemically defined medium, by means of the chlortetracycline (CTC) binding assay. Semen from adult Australian Merino rams was collected in an artificial vagina; spermatozoa were washed once in modified Biggers, Whitten, and Wittingham medium (m-BWW), without BSA or serum, and incubated in m-BWW alone or in m-BWW containing GABA, GABA agonists, or antagonists for 2 h at 38.5°C under 5% CO₂ in air. Samples were taken for assessment of CTC binding pattern or were further incubated for 15 min in the presence of 5 μM calcium ionophore A23187. Acrosomal exocytosis was evaluated by Pisum sativum agglutinin binding. Addition of GABA to the incubation medium resulted in a concentration-dependent increase in the percentage of CTC forms II and III, corresponding to mid-capacitated and capacitated spermatozoa, respectively. The effect was marginally significant at 1 μM and maximal at 20 μM. The action of 20 μM GABA was mimicked by the GABAₐ-receptor agonist, muscimol, but not by the GABAₐ-receptor agonist, baclofen, and completely blocked by the GABAₐ-receptor antagonists, bicuculline and picrotoxin, which lacked effect per se. In a separate set of experiments, incubation of spermatozoa with GABA at a concentration of 1 μM, which was insufficient to stimulate sperm capacitation, together with the neurotransactive steroid allopregnanolone (1 μM) provoked a capacitating effect similar to that achieved by 20 μM GABA alone. These results show that GABA has a capacitating action on ram spermatozoa through a GABAₐ receptor-mediated mechanism.

INTRODUCTION

Before fertilization, mammalian spermatozoa must undergo a series of biochemical events that render them able to undergo the acrosome reaction, a specialized form of exocytosis that allows sperm to penetrate the oocyte’s investment and fuse with its plasma membrane. Depending on the species considered, these processes, collectively known as capacitation [1–3], occur in different segments of the female reproductive tract. The composition of cervical, uterine, and oviductal fluids varies through the estrous cycle [4], and this fact seems important for the regulation of the rate at which capacitation occurs, in order to optimize fertilization. As a consequence, and for practical purposes, identification in female reproductive fluids of the factor or factors that regulate sperm capacitation would allow the design of chemically defined media to be used in vivo fertilization and related techniques.

Among those factors, gamma-aminobutyric acid (GABA) has recently emerged as a putative modulator of sperm function. GABA is the main inhibitory neurotransmitter in the brain, but was also shown to be present at high concentrations in the rat oviduct [5] and human fallopian tube and ovary [6], being twice as abundant in these three locations as in the brain, at least in the diestrous rat [7]. The fallopian GABA content and the activity of its synthesizing enzyme, glutamic acid decarboxylase, are under the influence of ovarian activity [7]. Immunohistochemical studies indicated that GABA was localized to the mucosal layer of the oviduct [8], suggesting secretion to the oviductal lumen, where fertilization takes place.

Interest in the role of GABA in sperm function has been supported by numerous experiments involving progesterone and related compounds. In human spermatozoa, progesterone provokes an immediate increase in calcium influx [9, 10] and chloride efflux [11], stimulates protein tyrosine phosphorylation [12], induces the acrosome reaction [13–15], causes hyperactivation [16], and increases 3',5'-cyclic adenosine monophosphate levels [17]. An effect of progesterone on acrosome reaction has also been shown in spermatozoa from other species such as the mouse [18], hamster [19], stallion [20], and pig [21]. As the interaction of GABA with its A-type receptor (GABAₐ-R) from neural tissues is enhanced by progesterone metabolites, and a pregnancy binding site was identified on the GABAₐ-R [22], it was hypothesized that the action of progesterins on calcium influx and acrosome reaction might be mediated by their interaction with the GABAₐ-R. The presence of a GABAₐ-R α subunit on the equatorial segment of human spermatozoa was demonstrated by immunohistochemical techniques [23], and GABAₐ-R binding sites seem to occur in ram spermatozoa [24]. However, an effect of GABA on specific event(s) during capacitation and acrosome reaction has not been precisely defined so far. Moreover, interest has been focused mainly on GABA and/or progesterone or its metabolites, acting on the acrosome reaction of in vitro capacitated spermatozoa [18, 23]; the effect of progesterone on the acrosome reaction has been observed only after capacitation [18], whereas the effect of GABA on capacitation itself has not been thoroughly studied. In the present paper, we report the capacitating effect of GABA in ram spermatozoa in vitro, as assessed by chlortetracycline (CTC) assay [25, 26]. CTC binds membrane calcium and is readily visualized by fluorescence microscopy. The assay is based on changes in the CTC distribution pattern during the course of capacitation, and was successfully applied to human [27], monkey [28], mouse [18, 29], horse [30], bull [31], and ram [32] spermatozoa. We show that this effect of GABA is mediated through interaction with a GABAₐ-R.

MATERIALS AND METHODS

Chemicals and Samples

Chlortetracycline, Tris, L-cysteine, BSA (fraction V), glutaraldehyde, and other reagents for the preparation of buffers and media were obtained from Sigma Chemical Company (St. Louis, MO). Dimethylsulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany).

Semen from adult Australian Merino rams was collected using an artificial vagina and assessed for volume, appear-
ance, sperm concentration, and subjective motility. Samples showing less than 70% motile cells were discarded.

In Vitro Capacitation

Semen aliquots (100 μl) were washed once in 4 ml of Biggers, Whitten and Wittingham (BWW) medium [33] without BSA or serum, supplemented with 10 mM Hepes, and adjusted to pH 7.4 under 5% CO₂ with 0.1 N sodium hydroxide. This medium is hereafter referred to as modified BWW (m-BWW). 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 m-BWW so as to get a final sperm count of 10⁸ cells/ml. Aliquots (500 μl) of this suspension were mixed in 1-ml plastic wells with an equal volume of m-BWW alone or m-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% CO₂ in air. After 2 h, aliquots of each sample were assessed for capacitation by CTC assay (see below) or incubated with the calcium ionophore A23187 for induction of acrosomal exocytosis (see below). As controls, all samples were also assayed for CTC binding pattern after washing but without incubation. Sperm motility was monitored before and after incubations by means of a computer-assisted semen analyzer (CellTrak; Motion Analysis, Santa Rosa, CA) calibrated in our laboratory for ram sperm [34].

Induction of Acrosomal Exocytosis by A23187

Aliquots (450 μl) of sperm suspensions, incubated as above, were transferred to 1-ml wells containing 50 μl of 50 μM A23187 in m-BWW, prepared from a stock 5 mM solution in DMSO, to obtain a final A23187 concentration of 5 μM. The samples were incubated in the same conditions as described above for 15 min and immediately processed for evaluation of acrosomal integrity after staining with fluoresceinated Pisum sativum agglutinin (PSA). Acrosomal integrity was confirmed by differential interference contrast microscopy as described by Watson et al. [35]. Three categories were established for scoring of acrosomal integrity: intact, intermediate (characterized by prominence of the anterior margin of the equatorial segment), and acrosomeless. At least 200 spermatozoa per sample, in duplicate, were observed.

CTC Assay

A modification of the technique described by Ward and Storey [26] was used. A CTC solution (1 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM cysteine, pH 7.8. CTC staining was initiated by rapidly mixing 10 μl of sperm suspension with 10 μl of CTC solution on a low-fluorescence glass slide at room temperature. After 15 sec, the reaction was stopped by addition of 5 μl of a 12.5% (v:v) glutaraldehyde solution in 2 M Tris-HCl, pH 7.8. The samples were covered with a 24 × 48-mm coverslip, sealed with colorless enamel, and stored at 4°C in the dark. For the evaluation of CTC patterns, the samples were observed within 24 h under a Nikon Optiphot microscope (Nikon Co., Tokyo, Japan) under epifluorescence illumination using a V-2A filter. All samples were processed in duplicate, and at least 150 spermatozoa per slide were scored. In ram spermatozoa, four CTC distribution forms, accounting for more than 95% of those found immediately after ejaculation, are normally observed [32]: form I, noncapacitated (fresh); form II, intermediate stage of capacitation; form III, capacitated; form IV, acrosome reacted. No fluorescence was observed when CTC was omitted from the preparation.

Acrosomal Evaluation by PSA Binding Assay

A modification of the procedure described by Cross et al. [36] was used for acrosomal evaluation [37]. Sperm suspensions were centrifuged at 2000 × g for 90 sec in an Eppendorf (Hamburg, Germany) Microcentrifuge Model 5414, and the resultant pellet was fixed with 95% ethanol at 4°C for 5 min. The suspensions were centrifuged, washed in 1 ml PBS at room temperature, and resuspended in 90 μl PBS. Labeling was carried out by addition of 10 μl of 100 μg/ml PSA and incubation at 37°C for 12 min in the dark. Labeled spermatozoa were washed in 1 ml PBS, resuspended in 50 μl of 90% (v:v) glycerol in PBS, and mounted on low-fluorescence glass slides. At least 150 spermatozoa per slide were assessed for binding pattern in a Nikon Optiphot epifluorescence microscope with a B-2A filter. Spermatozoa showing partial or complete loss of the acrosome were considered acrosome exocytosed.

Statistical Analysis

ANOVA and Tukey-Kramer multiple comparisons test were performed after angular transformation of the data [38].

RESULTS

As seen in Table 1, incubation of ram spermatozoa for 2 h in m-BWW (control) did not alter the percentage of each CTC form with respect to nonincubated samples. Addition of GABA to the incubation medium resulted in a concentration-dependent increase in the percentage of those CTC forms corresponding to mid-capacitated or capacitated spermatozoa (i.e., forms II and III). The effect of GABA was marginal but statistically significant at 1 μM and was maximal at 20 μM.

To determine which receptor is 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, muscimol (a GABA₄ agonist), or baclofen (a GABA₈ agonist), both of the latter at 1 or 10 μM final concentration. Muscimol at 10 μM had a capacitating effect similar to that of 20 μM GABA. In contrast, baclofen at 1 μM or 10 μM was ineffective (Table 2).

<table>
<thead>
<tr>
<th>Form</th>
<th>Nonincubated</th>
<th>Control</th>
<th>GABA, 1 μM</th>
<th>GABA, 5 μM</th>
<th>GABA, 20 μM</th>
<th>GABA, 50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form I (%)</td>
<td>77 ± 3a</td>
<td>72 ± 5b</td>
<td>57 ± 2b</td>
<td>54 ± 1c</td>
<td>48 ± 1c</td>
<td>48 ± 1c</td>
</tr>
<tr>
<td>Form II (%)</td>
<td>13 ± 2a</td>
<td>20 ± 3a</td>
<td>26 ± 3b</td>
<td>20 ± 2b</td>
<td>24 ± 3b</td>
<td>25 ± 3b</td>
</tr>
<tr>
<td>Form III (%)</td>
<td>9 ± 2a</td>
<td>7 ± 2a</td>
<td>15 ± 2b</td>
<td>24 ± 3b</td>
<td>26 ± 3b</td>
<td>24 ± 3b</td>
</tr>
<tr>
<td>Form IV (%)</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

* Spermatozoa were incubated as described in Materials and Methods, in m-BWW medium alone or containing GABA at the concentrations indicated. Capacitation was evaluated by the percentage of forms I to IV of CTC binding. Nonincubated, washed samples without incubation; control, samples washed and incubated in m-BWW medium, in the absence of GABA; results are expressed as mean ± SD for three different ejaculates.

+ Values with different superscripts differ (p < 0.05).
The capacitating effect of 20 μM GABA was completely blocked by coincubation in the presence of the GABA_A-R antagonists, bicuculline and picrotoxin, which had no effect per se (Table 3).

Further evidence for the involvement of a classical GABA_A receptor in the effect of GABA was obtained by incubating spermatozoa in the presence of GABA, at 1 and 20 μM, together with the neuroactive steroid, 5α-pregnane-3α-ol-20-one (allopregnanolone). This compound is known to interact with the GABA_A receptor from neural tissues at a site distinct from the GABA binding site, and to potentiate its effect [39]. As seen in Table 4, both 1 μM GABA and 1 μM allopregnanolone, which lacked effect per se, induced sperm capacitation to a degree similar to that achieved by 20 μM GABA alone.

Sperm motility was not affected by either treatment throughout the study (data not shown).

To evaluate whether the effect of GABA would also increase the ability of spermatozoa to undergo acrosomal exocytosis in response to calcium entry, samples from the experiments described above, treated as indicated for 2 h, were challenged with 5 μM A23187 for 15 min and assessed for acrosomal status. Figure 1 shows that the response of spermatozoa to GABA or GABA agonists/antagonists, in terms of A23187-induced acrosomal exocytosis, closely parallels the changes observed in the percentage of CTC form III under various conditions. Data on acrosomal status obtained with PSA agglutinin closely paralleled those obtained by differential interference contrast microscopy (data not shown).

**TABLE 2. Effect of muscimol and baclofen on capacitation stage of ram spermatozoa.**

<table>
<thead>
<tr>
<th>Form I (%)</th>
<th>Form II (%)</th>
<th>Form III (%)</th>
<th>Form IV (%)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78 ± 1</td>
<td>13 ± 2</td>
<td>8 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>GABA, 20 μM</td>
<td>64 ± 5^b</td>
<td>17 ± 2</td>
<td>13 ± 4^a</td>
<td>6 ± 3^c</td>
</tr>
<tr>
<td>Muscimol, 1 μM</td>
<td>64 ± 6^b</td>
<td>19 ± 5</td>
<td>11 ± 2</td>
<td>6 ± 1^b</td>
</tr>
<tr>
<td>Muscimol, 10 μM</td>
<td>61 ± 4^b</td>
<td>17 ± 6</td>
<td>15 ± 3^c</td>
<td>7 ± 3^b</td>
</tr>
<tr>
<td>Baclofen, 1 μM</td>
<td>79 ± 3</td>
<td>14 ± 2</td>
<td>6 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Baclofen, 10 μM</td>
<td>76 ± 4</td>
<td>14 ± 3</td>
<td>8 ± 2</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

* Spermatozoa were incubated as described in Materials and Methods, in m-BWW medium alone (Control) or containing GABA or one of the agonists indicated; capacitation was evaluated by the percentage of forms I to IV of CTC binding; results are expressed as mean ± SD for five different ejaculates.

DISCUSSION

The foregoing results show that GABA exerts a capacitating effect on ram spermatozoa as assessed by the increase in the percentage of spermatozoa showing a chlorotetracycline binding pattern compatible with a capacitated state [32]. The GABA-like effect of muscimol (a GABA_A-receptor agonist) but not of baclofen (a GABA_B-receptor agonist), as well as complete abolition by bicuculline and picrotoxin and potentiation by allopregnanolone, clearly indicate the involvement of a classical GABA_A receptor in the observed effect; these observations also indicate that a GABA_B receptor, or at least an entity similar to a GABA_B receptor with respect to its sensitivity to bicuculline, is lacking in ram sperm.

The study of a possible role of GABA in sperm function was impelled by the high concentrations of this compound, usually regarded as a classical neurotransmitter acting at the synaptic cleft, in male and female reproductive tracts in other species. The high GABA concentration found in the oviduct [5, 6] points to a role in the fertilization process, acting directly on the oocyte, on sperm, or both. Alternatively, GABA could modulate oviducal and uterine contractility to facilitate sperm transport [40, 41] or even serve an immuno modulatory function [42, 43]. Notwithstanding, the presence of an immunoreactive GABA_A-R [23] and of GABA_A binding sites [24] in spermatozoa strongly suggest a direct action at this level.

The concentrations of GABA found to exert a capacitating effect in vitro are compatible with its concentrations in ovine oviducal fluid (as indicated by preliminary results from our laboratory), supporting a physiological basis for its action. Moreover, it should be taken into account that in vivo, the effect of GABA could be enhanced by species known to act at the GABA_A-R, such as neuroactive steroids or endogenous compounds with benzodiazepine activity. Progesterone and 17α-hydroxyprogesterone have been identified as responsible for the acrosome reaction-inducing activity of human follicular fluid [13]. That progesterone acts at the sperm plasma membrane level is supported by several authors [44, 45], but the occurrence of a GABA_A-like receptor does not fully account for the differential requirements for stimulation of Ca^{2+} influx and for induction of the acrosome reaction [46, 47]. Kholkute et al. [48] showed that progesterone induced the acrosome reaction and increased fertilization rate in mice. Remarkably, in this species, the effect of progesterone on the acrosome reaction of capacitated spermatozoa was inhibited by bicuculline but not by picrotoxin, suggesting the involvement of a partici-
GABA AND CAPACITATION OF RAM SPERMATOZOA

FIG. 1. Effect of GABA, GABA agonists and antagonists, and allopregnanolone on A23187-induced acrosomal exocytosis. In all cases, spermatozoa were incubated for 2 h in the conditions specified in each experiment and subsequently challenged with 5 μM A23187 for 15 min. Acrosomal status was assessed by PSA binding. a) Effect of GABA at concentrations of 1 μM (G1), 5 μM (G5), 20 μM (G20), or 50 μM (G50). b) Effect of 20 μM GABA (G), muscimol at 1 μM (M1) or 10 μM (M10), and baclofen at 1 μM (B1) or 10 μM (B10). c) Effect of 20 μM GABA (G), alone or in combination with 1 μM bicuculline (B) or 1 μM picrotoxin (P). d) Effect of 1 μM allopregnanolone (allo) alone or in combination with 1 μM GABA (G1). Bars represent mean ± SD; * p < 0.05; ** p < 0.001 vs. control; n = 4, 8, 9, and 8, respectively.

ular type of GABA receptor [18]. In this same study, GABA was shown to induce acrosome reaction of capacitated spermatozoa in a bicuculline- and picrotoxin-sensitive fashion. In our model, progesterone failed to accelerate capacitation even in the presence of GABA (data not shown), whereas allopregnanolone clearly exerted a potentiating effect (Table 4). On the other hand, there does not appear to be a direct relationship between the ability of several progestins to potentiate the action of GABA at an A-type receptor and their ability to induce the acrosome reaction of human spermatozoa [24]. Taken together, these results prompt us to hypothesize that classical GABA<sub>A</sub> receptors may coexist with a distinct GABA receptor, showing unique pharmacological properties and perhaps serving different functions at specific steps of fertilization; species differences appear to exist as well. It is well documented that the combination of different forms of the GABA<sub>A</sub>-R subunits determines the sensitivity of the supramolecular complex to different agonists and antagonists [49]. The recent findings of GABA action at the sperm level warrant more detailed studies on the molecular characterization and pharmacological properties of the receptor involved in the regulation of sperm function.

The experiments carried out in this study showed that the percentage of spermatozoa reaching CTC form III by the action of GABA never exceeded 20%, suggesting that only a small sperm subpopulation in a semen sample is responsive to GABA. For comparison, about 70% of spermatozoa from the same rams, obtained in the same season, exhibit CTC form III upon incubation in serum-containing synthetic oviductal fluid [32, 50]. The low response to GABA is supported by Wistrom and Meizel [23], who demonstrated than in human semen, only a subpopulation (approximately one half) of spermatozoa were positive to immunofluorescent staining with a monoclonal anti-GABA<sub>A</sub>-receptor α subunit antibody. Nonetheless, there exists the possibility that in vivo, endogenous modulators of GABA action at the GABA<sub>A</sub>-receptor level (such as endogenous benzodiazepines or progesterone metabolites) may contribute to increasing the efficacy of GABA on sperm capacitation.

From a practical point of view, the discovery of novel compounds having a capacitating effect on spermatozoa may aid in the development of chemically defined media for in vitro fertilization. This might contribute to minimizing the uncertainty in the outcome of results derived from the use of biological fluids such as serum. Since the GABA<sub>A</sub>-R from neural tissues is a target for a variety of compounds acting at different sites to regulate the action of GABA, studies are currently being carried out to optimize their use in chemically defined media for in vitro sperm capacitation.

REFERENCES


