INTRODUCTION

Gamma-aminobutyric acid (GABA) is the main inhibitory transmitter in the central nervous system (CNS); it has been estimated that 20–50% of all synapses in the mammalian brain are GABAergic [1]. GABAergic systems have also been recognized outside the CNS, in tissues from the peripheral nervous system, the gastro-intestinal tract, the kidney, and the male and female genital tracts [2, 3]. GABA itself has been recognized in the testicle, epididymis, vas deferens, prostate, and seminal vesicle; and GABA receptors have been identified in vas deferens from mice and guinea pigs [4, 5]. The GABA-specific enzyme gamma-aminobutyric acid decarboxylase (GAD) has been shown to be present in spermatocytes from rat testis, and in the postacrosomal area of human spermatozoa [6–8]. Analysis of seminal plasma from human ejaculates has indicated that GABA is present in concentrations of >100 μM [9].

Recently, the role of GABA in the context of the mammalian sperm acrosome reaction (AR) has become subject to increasing attention. Several studies have reported that progesterone and 17α-OH progesterone may elicit human sperm AR and stimulate Ca<sup>2+</sup> influx [10]. Previous findings by several groups have established that both natural and synthetic steroids are capable of modifying GABAergic activity at the GABA<sub>A</sub> receptor [11–13]. Erdő and Wekerle [14] reported that GABA<sub>A</sub> receptors were present on the membranes of spermatozoa from rams, swine, and boars, and recently Wistrom and Meizel [15] reported that a unique sperm steroid receptor/Cl<sup>−</sup> channel complex, which resembles the neuronal GABA<sub>A</sub> receptor/Cl<sup>−</sup> channel complex, might induce human sperm AR when activated. In capacitated mouse spermatozoa, GABA at relatively low concentrations (0.5 μM) may mimic the effect of progesterone, increasing the fraction of acrosome-reacted cells [16]; progesterone has also been shown to enhance the effect of GABA on exocytosis of mouse spermatozoa [17]. Other authors, however, have demonstrated that the surface progesterone receptor leading to changes in Ca<sup>2+</sup> influx is unlike the steroid site on GABA<sub>A</sub> receptors [18].

Many transport proteins carrying various molecules across cell membranes in eucaryotic cells have been identified and molecularly sequenced during the past ten years. Several of the amino acid transport systems are typically coupled to co-transport of Na<sup>+</sup> ions [19, 20].

GABA transport proteins (GABA-Tp) belong to a family of amino acid transport proteins characterized structurally by a homology in amino acid sequence and functionally by their co-transporting of Na<sup>+</sup> and Cl<sup>−</sup> across the cell membrane [21].

Four GABA-Tp have been identified and cloned from mouse brain [21], and four high-affinity GABA-Tp have been identified from the human brain: GAT-1, GAT-2, GAT-3, and...
BGT-1, the latter transporting both betaine and GABA. Functionally, each of the transporters is unique in regard to transport kinetics and affinity for displacers [22].

A carrier-mediated transport of α-aminoisobutyric acid in fowl spermatozoa was proposed as early as 1962 [23], and the presence of an amino acid transport protein for glutamate on boar spermatozoa has recently been suggested [24]. Recent results have indicated that GABA-specific binding sites exist on human spermatozoa and that these sites might represent a GABA-Tp [25].

In the present study, GABA uptake by human spermatozoa was studied with regard to transport kinetics, time course, and requirements for extracellular Na⁺ and Cl⁻. Interindividual differences in GABA uptake in spermatozoa from different ejaculates was studied, and sperm motility as well as morphology in the same semen samples was analyzed.

**MATERIALS AND METHODS**

**Chemicals**

Earle’s balanced salt solution (Earle’s medium), BSA, luminol, KCl, choline chloride (choline Cl), LiCl, N-methyl-D-glucamine or D-glucuronic acid, GABA, nipecotic acid, and aminooxyacetic acid were all purchased from Sigma Chemical Co. (St. Louis, MO). [3H]GABA was purchased from Dupont (U.K.) Ltd. (Hertfordshire, UK). Tris-citrate buffer and dimethyl sulfoxide was purchased from Merck (Darmstadt, Germany).

**Swim-Up Preparations of Human Spermatozoa**

Semen samples were obtained from men submitted for routine semen analysis to the Centre for Reproductive Medicine, Karolinska Hospital, Stockholm. Semen samples with normal progressive movement, sperm concentration, and morphology according to World Health Organization (WHO) standards were selected unless otherwise stated [26]. The ejaculates were allowed to liquefy for 30 min prior to the swim-up procedure. A highly motile fraction of spermatozoa was recovered after a 45-min swim-up in Earle’s medium supplemented with 1% BSA. The swim-up procedure was performed according to WHO guidelines, and all subsequent experiments were performed at 37°C unless otherwise stated. Swim-up fractions from several individuals were pooled and carefully mixed in order to obtain a sufficient amount of cells and to minimize possible interindividual differences in uptake in the studies on time-related accumulation, ion dependency, and transport kinetics. In the experimental design for studies on interindividual differences in [3H]GABA uptake, individual swim-up fractions were prepared and analyzed from each ejaculate. The final concentration was between 5 and 20 × 10⁶ spermatozoa/ml. The protocol was approved by the local Ethics Committee.

**Time-Related Accumulation of [3H]GABA in Human Spermatozoa**

Ejaculates from several individuals were pooled. The mixed ejaculates were divided into portions of 1 ml, and swim-up preparations were made according to procedures described above. The swim-up fractions were once again pooled, and cells were counted in Bürker chambers to determine cell concentrations prior to incubation. Incubations took place in vials, where 900 µl of the swim-up solution was mixed with 100 µl [3H]GABA diluted in Earle’s medium to a final concentration in the sample of 10 nM [3H]GABA. Unspecific binding was determined by preincubating parallel samples with unlabeled GABA (1 mM) or nipecotic acid (200 µM) 5 min before [3H]GABA was added. Samples were analyzed at 5, 30, 60, 180, and 1320 min after addition of [3H]GABA; aliquots were drawn from the incubating samples, and the incubation was terminated by centrifugation at 39500 × g for 15 min. Samples were subsequently washed three times with ice-cold Tris citrate buffer. The samples selected for analysis at each time point were subsequently placed in micro vials, and radioactivity was measured by a liquid scintillation counter (Wallac 1409 Liquid Scintillation Counter, Turku, Finland). Incubations and scintillation counts were carried out in triplicate. The experiment was repeated four times, each including cells from at least three ejaculates.

**Sodium and Chloride Dependence of [3H]GABA Uptake**

Swim-up preparations from several individuals were obtained and processed as described above. Earle’s balanced salt solution medium in which the sodium and chloride constituents were substituted was prepared in the laboratory according to the formulation described by Sigma, with the NaCl fraction varied by equimolar substitution. Earle’s medium of the following composition served as the initial swim-up medium (mM): CaCl₂ (1.80), KCl (5.36), MgSO₄ (0.81), NaCl (116.2), NaHCO₃ (26.19), NaH₂PO₄ (1.01), D-glucose (5.55). The swim-up preparation was thereafter centrifuged at 500 × g for 5 min. The spermatozoa were then resuspended and washed three times in modified Earle’s medium in which the NaCl fraction (116.2 mM) was replaced with either KCl, choline Cl, LiCl, N-methyl-D-glucamine (HCl), or D-glucuronic acid (sodium salt) in a stepwise fashion (25%, 50%, 75%, 100%) to explore the effect of ion replacement on [3H]GABA accumulation. Osmolarity and pH were measured prior to incubation. The original-formula Earle’s medium containing 116.2 mM NaCl served as a control, representing 100% uptake. [3H]GABA was added to a final concentration of 10 nM. After swim up, motility of all samples was > 85%, and reevaluation of motility after replacement of NaCl revealed no differences between controls and treated samples. Samples from each experimental series were assessed for viability using eosin staining fol-
lowing treatment. No differences between controls and treated samples were observed. The incubations were terminated as described above. Scintillation counts were performed after 2 and 3 h of incubation.

**Transport Kinetics**

After the swim-up procedure, \[^{3}H\]GABA transport was measured with GABA concentrations from 0.01 μM to 100 μM. Prior to incubations, a cell count was performed in a Bürker chamber to determine the cell concentration in each pooled batch of spermatozoa. In each vial, 700 μl of the swim-up preparation was mixed with 200 μl of Earle’s medium, and the transport reaction was started by adding 100 μl of Earle’s medium prepared with GABA in rising concentrations. After incubation for 30 min at 37°C, the incubations were terminated as described above, and scintillation counts were performed in duplicate at each concentration. The experiment was repeated three times. Each pooled swim-up preparation included cells from at least two ejaculates. The Eadie-Hofstee plot was obtained using data from the saturation curve, with GABA concentrations from 0.01 M to 50 M.

**Interindividual Differences in \[^{3}H\]GABA Uptake, Motility, and Morphology**

\[^{3}H\]GABA uptake. Ejaculates from 30 men undergoing investigation for infertility were explored to determine the capacity of their sperm for \[^{3}H\]GABA uptake. An initial cell count of the native ejaculate was performed to determine concentration. No ejaculate was excluded from analysis because of low concentration, subnormal motility, or presence of inflammatory cells in the native sample. Morphological assessment of the samples was performed according to WHO guidelines. Swim-up preparations were prepared from each individual ejaculate, and \[^{3}H\]GABA uptake in each sample was measured after 3 h of incubation with 10 nM \[^{3}H\]GABA.

**Motility**

After the swim-up procedure and before incubation with \[^{3}H\]GABA, the motility characteristics of each ejaculate were analyzed by a Hamilton-Thorne Motility Analyzer (HTM-S, Ver 7.2; Hamilton Thorn Research, Inc., Danvers, MA). The parameter settings applied were as follows: frames–16 at 25 Hz, minimum contrast 9, minimum size 6; Lo/Hi size gates–0.5 to 2.0; Lo/Hi intensity gates–0.8 to 1.2; nonmotile head size–10; nonmotile intensity–280; medium velocity of average path (VAP) value–50; slow cells motile–no; threshold str–90. The sort criteria used to define hyperactivated cells were set according to Burkman [27]: curvilinear velocity (VCL) ≥ 100 μm/sec, amplitude of lateral head movement (ALH) ≥ 7.5 μm, and linearity (LIN) ≤ 65%. A minimum of 200 motile cells were analyzed per ejaculate.

**RESULTS**

**Time-Dependent Uptake**

Accumulation of GABA continued for more than 3 h after incubation with 10 nM \[^{3}H\]GABA (Fig. 1). Up to 22 h, a further increase in uptake was noted, indicating that the spermatozoa were capable of transporting and accumulating GABA for more than the 3 initial hours during capacitation.

**Kinetics**

GABA uptake increased in a dose-dependent manner at concentrations from 0.01 μM to 100 μM (Fig. 2). The GABA uptake approached saturation at values > 200 μM. Values for $K_m$ (14 μM) and $V_{max}$ (134 fmol/million cells/min) were calculated by using an Eadie-Hofstee plot based on data from three different experiments with substrate concentrations from 0.01 μM to 50 μM (Fig. 2, inset). Incubations in the presence of 100 μM aminooxyacetic acid did not influence the results. Linearity of the plot used to calculate $K_m$ and $V_{max}$ was tested using Kendall’s rank correlation, $p < 0.0001$, $r^2 = 0.749$.

**Ion Dependency of GABA Uptake**

GABA uptake was dependent on both sodium and chloride in the extracellular environment. Replacement of these
ions led to a dose-dependent reduction in GABA uptake as illustrated in Figure 3. Total replacement of the NaCl fraction of Earle’s medium with KCl, choline Cl, LiCl, N-methyl-D-glucamine (HCl), or D-glucuronic acid (sodium salt) reduced the uptake of GABA by 99%, 93%, 91%, 88%, and 71%, respectively, after 2 h. Further incubation for a total period of 3 h did not change the results significantly (data not shown).

\[ ^{3}H \text{GABA} \text{Uptake, Motility, and Morphology} \]

Individual \(^{3}H\)GABA uptake varied within a wide range (Fig. 4). All samples, however, did accumulate \(^{3}H\)GABA intracellularly at levels above controls preincubated with nipecotic acid or unlabeled GABA. Motility parameters and morphology did not correlate with \(^{3}H\)GABA uptake (data not shown).

\[ \text{DISCUSSION} \]

The results from our studies on GABA uptake depicted in Figures 1 and 2 indicate that a GABA transport mediated by specific transport proteins does take place in human spermatozoa and that the process can be inhibited by nipecotic acid, a competitive inhibitor of several GABA-Tp [22]. Kinetic analysis of the GABA transport activity in human spermatozoa at increasing GABA concentrations indicated that the uptake is carrier-mediated. A \(K_m\) value for GABA of 14 \(\mu M\) was calculated using Eadie-Hofstee plots based on data from three different experiments. This result is in agreement with kinetic analysis of several high-affinity GABA-Tp [28–31]. Addition of aminooxyacetic acid to the swim-up medium prior to the addition of radiolabeled GABA did not change the amount of internalized GABA as measured by scintillation counts; hence the radioactivity measured most likely represents accumulated, unmetabolized \(^{3}H\)GABA.

Previous reports have demonstrated an absolute demand for sodium and chloride ions in the external medium for GABA-Tp to function; GABA accumulation in synaptic vesicles from rat brain is dependent on external sodium and chloride [19, 20, 32], and studies on cloned GAT-1 isolated from rat brain and expressed in Xenopus oocytes demonstrated a reduction of transport to background levels as sodium chloride was replaced with lithium chloride or choline Cl [33]. The experiment depicted in Figure 3 demonstrates a similar ion dependency for GABA uptake in human spermatozoa. Isoosmotic replacement of the NaCl component of Earle’s medium with KCl, choline Cl, LiCl, or N-methyl-D-glucamine (HCl) reduced GABA uptake 99%, 93%, 98%, and 88%, respectively. The most dramatic reduction in GABA uptake was observed when NaCl was replaced by KCl. High extracellular potassium concentrations cause depolarization of the sperm membrane [34], and the combined effect of sodium depletion and depolarization may explain the rapid decrease in GABA uptake when NaCl was replaced by KCl. The results are in accordance with several other studies on the ion dependency of GABA transport [35–37]. Replacement of Cl\(^-\) ions was limited to the chloride fraction of Earle’s medium contributed by NaCl. By totally replacing
NaCl with D-glucuronate (sodium salt), GABA uptake was reduced by 71%. NaCl contributes to 93% of the total [Cl−] in Earle’s medium; it was not shown whether a further reduction would result in a total replacement of the Cl− ions in the medium. A total exchange of Cl− ions would require a more extensive change, altering other ionic constituents in Earle’s medium. The observed reduction in GABA uptake following reduction of [Cl−] agrees well with other studies reporting that Cl− ions stimulate GABA influx approximately 100-fold but is not absolutely required for GABA transport [30].

The 30 ejaculates included in the study on interindividual differences in GABA uptake represented an unselected sample of men undergoing investigation for infertility; consequently, the sperm samples demonstrated wide variation in the parameters traditionally describing the human ejaculate (Table 1). We have previously described a lack of effect of GABA on sperm motility in vitro [25], and the present results indicate a lack of correlation between GABA uptake and traditional semen parameters. The possible role of GABA in the context of human fertility thus remains to be explored further by investigating other aspects of sperm function than those traditionally describing the human ejaculate.

In neuronal tissues, the role of a GABA transporter in the context of sperm function and physiology is not clear from the present knowledge about the function of similar transport proteins in neuronal and kidney tissues. The abundance of GABA in both seminal plasma [9] and the female genital tract [2] suggest a dynamic interaction between the capacitating sperm cell and the extracellular environment, involving GABA. Further studies are required to establish the role of GABA in this context. Such studies should address the possible release of accumulated GABA by either exocytosis or non-exocytotic processes, and the possibility that GABA might act as a regulator of the osmotic pressure of the environment in several biological systems [39].

The role of a GABA transporter in the context of sperm function and physiology is not clear from the present knowledge about the function of similar transport proteins in neuronal and kidney tissues. The abundance of GABA in both seminal plasma [9] and the female genital tract [2] suggest a dynamic interaction between the capacitating sperm cell and the extracellular environment, involving GABA. Further studies are required to establish the role of GABA in this context. Such studies should address the possible release of accumulated GABA by either exocytosis or non-exocytotic processes, and the possibility that GABA might act as a regulator of the intracellular environment in the human sperm. In addition, new antiepileptic drugs are being developed by synthesis of novel GABA uptake inhibitors [40], and the rapid development of GABA-Tp inhibitors as anticonvulsant drug candidates makes the issue of possible side effects on the reproductive system important.

**REFERENCES**


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**TABLE 1. Semen characteristics of native ejaculates (n=30) obtained from men undergoing routine semen analysis.**

<table>
<thead>
<tr>
<th>Semen parameter</th>
<th>Median (25th–75th percentile) range</th>
</tr>
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<tbody>
<tr>
<td>Concentration in native sample (%)</td>
<td>65 (33.0–90.0) 6.8–175.5</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>29 (15–33) 9–46</td>
</tr>
<tr>
<td>Motility*</td>
<td>81 (73–97) 47–96</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>91(92–105) 82–117</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>75 (72–83) 80–93</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>79 (76–84) 68–90</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>4.3(3.8–4.9) 3.2–6.0</td>
</tr>
<tr>
<td>BCF</td>
<td>13(13–14) 10–16</td>
</tr>
<tr>
<td>Hyperactivated cells (%)</td>
<td>8.5(5.0–14.0) 1–20</td>
</tr>
<tr>
<td>([3H]GABA uptake (fmol/mill cells)</td>
<td>8.9(5.7–15.5) 1.8–48.0</td>
</tr>
</tbody>
</table>

*Motility analysis of swim-up preparations of the ejaculates was performed using a Hamilton-Thorne computerized semen analyzer. The swim-up preparations were incubated with 10 nM [3H]GABA for 3 h to determine GABA uptake in each sample.


23. Anuah, GA, Buckland, RB. The uptake of glyceraldehyde, a-malobutyric acid and 2-deoxy-d-glucose by spermatozoa of a line of chickens selected for fertility of frozen-thawed semen and a control line. Theriogenology 1982; 17:401-408.


