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—Original Article—

γ-Aminobutyric acid suppresses enhancement of hamster sperm hyperactivation by 5-hydroxytryptamine

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Abstract. Sperm hyperactivation is regulated by hormones present in the oviduct. In hamsters, 5-hydroxytryptamine (5HT) enhances hyperactivation associated with the $5HT_2$ receptor and $5HT_4$ receptor, while 17β-estradiol (E_2) and γ-aminobutyric acid (GABA) suppress the association of the estrogen receptor and $GABA_A$ receptor, respectively. In the present study, we examined the regulatory interactions among 5HT, GABA, and E_2 in the regulation of hamster sperm hyperactivation. When sperm were exposed to E_2 prior to 5HT exposure, E_2 did not affect 5HT-enhanced hyperactivation. In contrast, GABA partially suppressed 5HT-enhanced hyperactivation when sperm were exposed to GABA prior to 5HT. GABA suppressed 5HT-enhanced hyperactivation associated with the $5HT_2$ receptor although it did not suppress 5HT-enhanced hyperactivation associated with the $5HT_4$ receptor. These results demonstrate that hamster sperm hyperactivation is regulated by an interaction between the $5HT_2$ receptor-mediated action of 5HT and GABA.

Key words: γ-Aminobutyric acid, Capacitation, 5-Hydroxytryptamine, Hyperactivation, Sperm

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After ejaculation, mammalian sperm are transported to the female reproductive organs and begin to be capacitated. In the oviduct, sperm are capacitated and exhibit hyperactivation, during which flagella exhibit a large amplitude and asymmetric beating pattern of movement [1–3]. In hamsters and mice, hyperactivated sperm may writhe and swim in a figure 8 pattern. The pattern of hyperactivated sperm movement differs among mammals [1, 3]. Hyperactivation creates the propulsive force for swimming in the viscous oviductal fluid and for penetrating the zona pellucida [1, 2, 4, 5]. Finally, capacitated mammalian sperm undergo the acrosome reaction (AR), a specialized form of exocytosis that occurs at the sperm head [1]. AR is required for penetration of the zona pellucida and for binding to the egg [1, 4, 5].

Recently, it was shown that mammalian sperm hyperactivation is modulated by some oviductal molecules [3, 4], including progesterone (P_4) [6–11], 17β-estradiol (E_2) [8, 10, 12], melatonin (Mel) [12, 13], 5-hydroxytryptamine (5HT) [14], and γ-aminobutyric acid (GABA) [11, 15, 16]. P_4, Mel, and 5HT enhance sperm hyperactivation in hamsters [7–14]. In contrast, E_2 and GABA suppress the enhancement of hamster sperm hyperactivation by P_4 and Mel [8, 10–12]. In human sperm, P_4 and GABA induce sperm hyperactivation [6, 15]. Moreover, GABA also induces sperm hyperactivation in rams [16].

Although 5HT enhances hamster sperm hyperactivation, 5HT is generally a neurotransmitter with many functions and acts through various types of 5HT receptors in the tissues. Most 5HT receptors are G-protein coupled receptors and affect adenylyl cyclase or phospholipase C (PLC) [17, 18]. When adenylyl cyclase is stimulated by 5HT, cAMP concentration increases. PLC induces the production of inositol 1,4,5-tris-phosphate (IP_3) and increases intracellular Ca^{2+} levels. 5HT and 5HT receptors have been identified in oocytes, cumulus-oocyte complexes, follicular fluids, and embryos in mammals [19]. In hamster sperm, it has been suggested that 5HT induces AR and enhances hyperactivation through the $5HT_2$ and $5HT_4$ receptors in a dose-dependent manner [14, 20]. The $5HT_2$ receptor is a G-protein coupled receptor and stimulates PLC-induced Ca^{2+}-release from IP_3 receptor-gated Ca^{2+}-stores [17, 18]. The $5HT_4$ receptor is also a G-protein coupled receptor that stimulates adenylyl cyclase inducing cAMP production [17, 18]. Ca^{2+} and cAMP are important second messengers in the regulation of sperm hyperactivation [1–4].

As described above, *in vivo* hyperactivation is induced in capacitated sperm in the specific environment of the oviduct. Thus, it is important to examine the effects of oviductal molecules on the occurrence of hyperactivation in order to understand the regulatory mechanisms involved in modulating sperm functions in the female reproductive tracts. In our previous studies [7, 8, 10–13], we showed that *in vitro* hyperactivation of hamster sperm was modulated by a combination of enhancers and suppressors. P_4 enhances hyperactivation most effectively at 20 ng/ml [7]. E_2 and GABA suppressed P_4-enhanced hyperactivation [8, 10, 11]. Enhancement of hyperactivation by 20 ng/ml P_4 was suppressed by > 20 pg/ml E_2 [8, 10]. Moreover, 5 nM–5 μM GABA suppressed the enhancement of hyperactivation by 20 ng/ml P_4 [11]. Additionally, 1 nM Mel also enhanced hyperactivation [13]. We found that > 20 pg/ml E_2 suppressed 1 nM Mel-enhanced hyperactivation although GABA did not affect Mel-enhanced hyperactivation [12]. Low concentrations (fM to pM) of 5HT enhanced hyperactivation through the $5HT_2$ receptor and high concentrations (nM to μM) of 5HT enhanced hyperactivation through the $5HT_4$ receptor [14]. However, the suppressors of 5HT-enhanced

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hyperactivation have not been identified. Because the concentrations of molecules affecting hamster sperm hyperactivation change during the estrous cycle and/or ovulation [5], we hypothesized that sperm are hyperactivated by interactions between enhancers and suppressors [3]. In the present study, we examined whether hamster sperm hyperactivation is regulated by the interaction between 5HT (as an enhancer) and E_2 and/or GABA (as suppressors).

Materials and Methods

Chemicals

5HT, E_2, 5-methoxytryptamine (MT), and α-methylserotonin maleate salt (MS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin fraction V was purchased from Merck KGaA (Darmstadt, Germany). Other chemicals of reagent grade were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals

All golden hamsters (*Mesocricetus auratus*) used in the study were bred at the laboratory Animal Research Center of Dokkyo Medical University. The experiment was approved by the Animal Care and Use Committee of the Dokkyo Medical University (Experimental permission number: 0107) and carried out according to the Guidelines for Animal Experimentation of the university.

Preparation of hyperactivated sperm

Sperm were collected from the posterior epididymis of sexually mature (10–20-week-old) male hamsters. Hyperactivated sperm were prepared as described previously [12, 21]. Modified Tyrode's albumin lactate pyruvate (mTALP) medium [12, 22] was used as a capacitation medium. A drop (~ 5 µl) of posterior epididymal sperm was placed on a culture dish (35-mm dish) (Iwaki, Asahi Glass, Tokyo, Japan) and 3 ml of the medium was carefully added to the dish. Hamster sperm were incubated for 5 min at 37°C to allow them to swim into the medium. The supernatant containing motile sperm was collected, placed on new dish, and incubated for 4 h at 37°C under 5% CO_2 in air to induce hyperactivation. As a stock solution, 5HT (100 µM), MS (100 pM), bicuculline (Bic) (1 mM), and GABA (5 mM) were dissolved in pure water. MT (10 nM) and E_2 (20 µg/ml) were dissolved in ethanol. GABA, E_2, or vehicle was added to the mTALP medium after swim up, and after incubation for 5 min, 5HT, MS, MT, or vehicle was added to the medium (Figs. 1–3). Bic or vehicle was added to the medium after swim up, and after incubation for 5 min, GABA or vehicle was added to the medium. After additional incubation for 5 min, 5HT, MS, or vehicle was added to the medium again (Fig. 4). In all experiments, the maximal concentration of vehicle was 0.2%.

Measurement of motility and hyperactivation of sperm

Motility and hyperactivation measurements were performed as described previously [12, 21] with some modifications. Motile sperm were recorded on videotape via a CCD camera (Progressive 3CCD, Sony, Tokyo, Japan) attached to a microscope (IX70, Olympus, Tokyo, Japan) with phase-contrast illumination and a small CO_2 incubator (MI-IBC, Olympus). Each observation was performed at 37°C, recorded for 2 min, and analyzed by manually counting the numbers of total sperm, motile sperm, and hyperactivated sperm in 4 different fields of observation. Analyses were conducted in a blinded manner. Motile sperm exhibiting asymmetric and whiplash-like flagellar movement and a circular and/or octagonal swimming locus were defined as hyperactivated sperm [3, 23]. Motile sperm (%) and hyperactivated sperm (%) were respectively defined as the number of motile sperm/number of total sperm × 100 and the number of hyperactivated sperm/number of total sperm × 100. Experiments were performed four times using four hamsters. When the percentage of motile sperm was 80% or less, the experiment was re-performed. Statistical analysis was carried out using the post-hoc test of ANOVA using Microsoft Excel (Microsoft Japan, Tokyo, Japan) with the add-in software Statcel2 (OMS Publishing, Saitama, Japan). Differences were considered significant at $P < 0.05$.

Results

Effects of E_2 and GABA on 5HT-enhanced hyperactivation

We previously showed that 5HT-enhanced hyperactivation occurred through the $5HT_2$ and $5HT_4$ receptors in hamster sperm [14]. Low concentrations (1 fM–100 pM) of 5HT enhanced sperm hyperactivation through the $5HT_2$ receptor, while high concentrations (1 nM–1 µM) of 5HT enhanced sperm hyperactivation through the $5HT_4$ receptor. In the present study, we used 100 fM 5HT to stimulate the $5HT_2$ receptor, 100 nM 5HT to stimulate the $5HT_4$ receptor, and 100 pM 5HT as an average of effective concentration and probable stimulator of the $5HT_2$ receptor. In addition, we used two 5HT receptor-specific agonists: MS ($5HT_2$ receptor-specific agonist) and MT ($5HT_4$ receptor-specific agonist).

First, we examined whether hamster sperm hyperactivation was regulated by the interaction between 5HT and E_2. As shown in Fig. 1A, hamster sperm were exposed to 100 fM, 100 pM, and 100 nM 5HT after exposure to 20 ng/ml E_2. Although 20 ng/ml E_2 clearly suppressed P_4-enhanced and Mel-enhanced hyperactivation [8, 10, 12], the 5HT-dependent increases in the percentage of hyperactivated sperm after 1, 1.5, or 2 h incubation were not affected by exposure to 20 ng/ml E_2. In addition, when hamster sperm were exposed to 100 fM MS (Fig. 1B) or 10 pM MT (Fig. 1C) after exposure to 20 ng/ml E_2, the 5HT receptor agonist-dependent increases in the percentage of hyperactivated sperm after 1, 1.5, or 2 h incubation were not affected by exposure to 20 ng/ml E_2.

In the next step, sperm were exposed to 100 fM, 100 pM, and 100 nM 5HT after exposure to 5 nM or 5 µM GABA (Figs. 2A and 2B). GABA at range of 5 nM to 5 µM suppressed P_4-enahnced hyperactivation [11]. GABA at 5 nM significantly suppressed 100 fM 5HT-enhanced hyperactivation after incubation for 1 and 1.5 h. However, 5 nM GABA did not suppress the enhancement of hyperactivation by 100 pM and 100 nM 5HT. In addition, 5 µM GABA significantly suppressed 100 fM 5HT-enhanced hyperactivation after incubation for 1 and 1.5 h. After incubation for 1 h, 5 µM GABA significantly suppressed 100 pM 5HT-enhanced hyperactivation. However, 5 µM GABA did not suppress the enhancement of hyperactivation by 100 nM 5HT.

Because 100 fM 5HT-enhanced hyperactivation was strongly suppressed by GABA (Figs. 2A and 2B), we examined whether GABA suppressed the enhancement of hyperactivation via stimulation

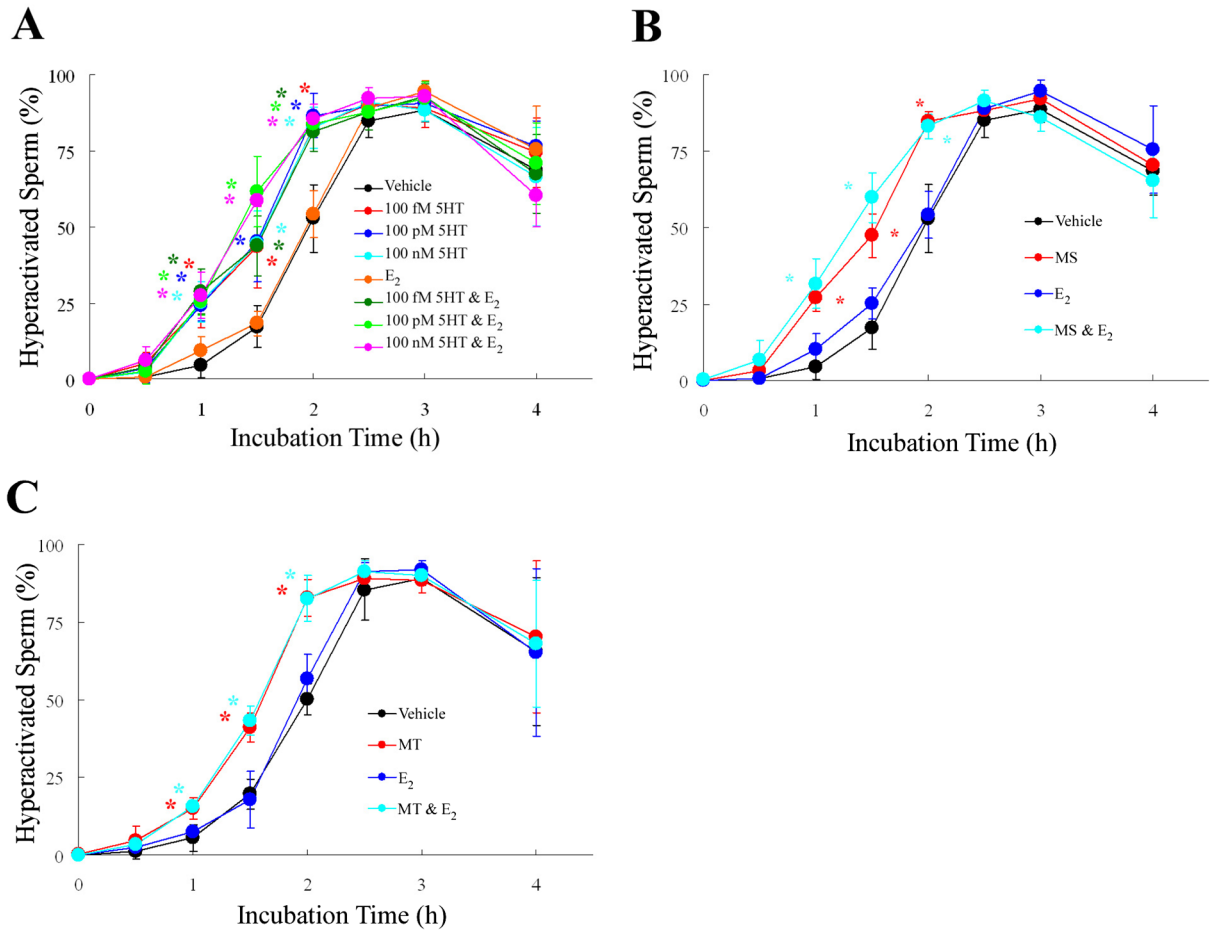


Fig. 1. Effects of 17β-estradiol (E_2) on enhancement of hyperactivation by 5-hydroxytryptamine (5HT), α-methylserotonin maleate salt (MS), and 5-methoxytryptamine (MT). Percentages of hyperactivated sperm are shown when sperm were exposed to various concentrations of 5HT (A), 100 fM MS (B), or 10 pM MT (C) after exposure to 20 ng/ml E_2. Data represent the mean ± SD. (A) (Vehicle): medium containing 0.1% (v/v) pure water and 0.1% (v/v) ethanol as a vehicle; (100 fM 5HT): medium containing 100 fM 5HT and vehicle; (100 pM 5HT): medium containing 100 pM 5HT and vehicle; (100 nM 5HT): medium containing 100 nM 5HT and vehicle; (E_2): medium containing E_2 and vehicle; (100 fM 5HT & E_2): medium containing 100 fM 5HT, E_2 and vehicle; (100 pM 5HT & E_2): medium containing 100 pM 5HT, E_2 and vehicle; (100 nM 5HT & E_2): medium containing 100 nM 5HT, E_2, and vehicle. (B) (Vehicle): same as above; (MS): medium containing MS and vehicle; (E_2): medium containing E_2 and vehicle; (MS & E_2): medium containing MS, E_2, and vehicle. (C) (Vehicle): medium containing 0.2% (v/v) ethanol as vehicle; (MT): medium containing MT and vehicle; (E_2): medium containing E_2 and vehicle; (MT & E_2): medium containing MT, E_2 and vehicle. *Significant difference compared with "Vehicle" or "E_2" ($P < 0.05$).

of the $5HT_2$ receptor. When sperm were exposed to 100 fM MS ($5HT_2$ receptor agonist) after exposure to 5 nM or 5 μM GABA, MS-enhanced hyperactivation was significantly suppressed by only 5 μM GABA after incubation for 1 and 1.5 h although it was not suppressed by 5 nM GABA (Fig. 2C). When hamster sperm were exposed to 10 pM MT ($5HT_4$ receptor agonist) after exposure 5 nM or 5 μM GABA, the percentage of hyperactivated sperm and MT-enhanced hyperactivated sperm were not affected (Fig. 2D).

Dose-dependent suppression of $5HT_2$ receptor-mediated enhancement of hyperactivation by GABA

Because 5 nM or 5 μM GABA suppressed 5HT-enhanced hyperactivation associated with $5HT_2$ receptor (Fig. 2), we examined the dose-dependent suppression of $5HT_2$ receptor-mediated enhancement of hyperactivation by GABA (Fig. 3). When sperm were exposed to 100 fM 5HT after exposure to 5 pM–5 μM GABA, GABA significantly suppressed 100 fM 5HT-enhanced hyperactivation in a dose-dependent manner (Fig. 3A). After incubation for 1 h, 5 nM–5 μM GABA significantly suppressed 100 fM 5HT-enhanced hyperactivation. After incubation for 1.5 h, 50 nM–5 μM GABA significantly suppressed 100 fM 5HT-enhanced hyperactivation. GABA at 500 pM and 5 nM slightly suppressed 100 fM 5HT-enhanced hyperactivation although their suppression was not significant compared with the vehicle and 100 fM 5HT. After incubation for 2 h, 5 μM GABA only significantly suppressed 100 fM 5HT-enhanced hyperactivation.

As shown in Fig. 3B, GABA significantly suppressed 100 pM 5HT-enhanced hyperactivation in a dose-dependent manner when sperm were exposed to 100 pM 5HT after exposure to 5 pM–5

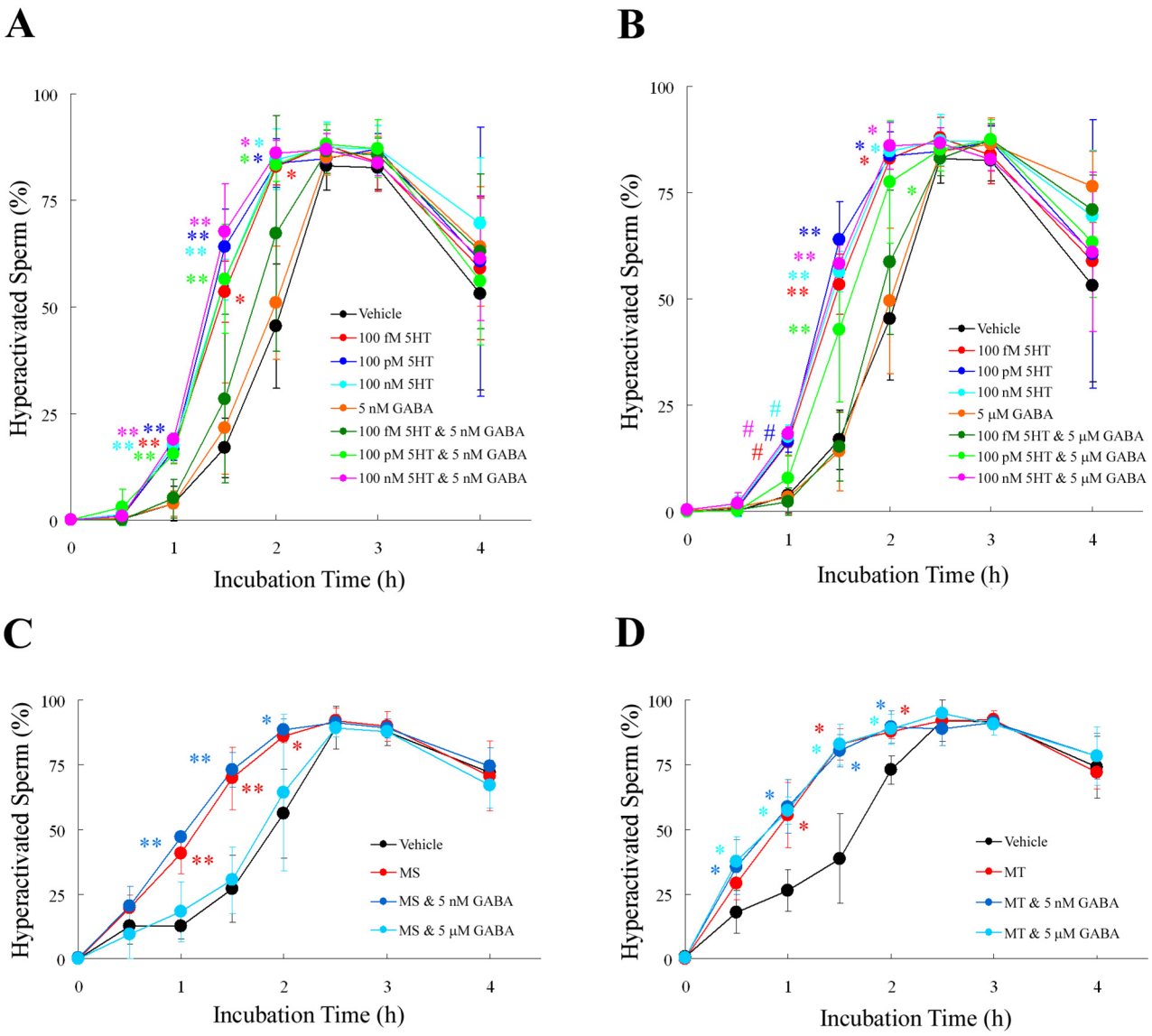
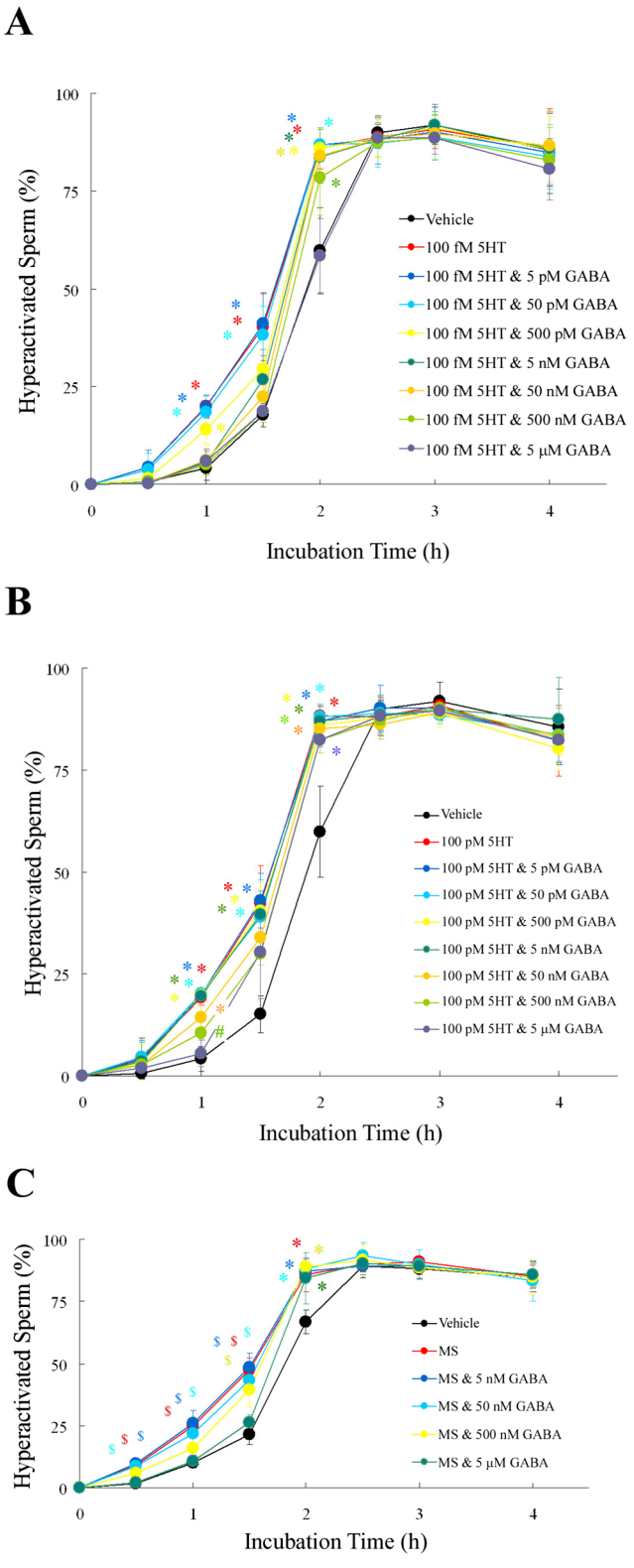


Fig. 2. Effects of γ-aminobutyric acid (GABA) on enhancement of hyperactivation by 5-hydroxytryptamine (5HT), α-methylserotonin maleate salt (MS), and 5-methoxytryptamine (MT). Percentages of hyperactivated sperm are shown when sperm were exposed to various concentration of 5HT (A, B), 100 fM MS (C), or 10 pM MT (D) after exposure to 5 nM or 5 μM GABA. Data represent mean ± SD. (A) (Vehicle): medium containing 0.2% (v/v) pure water as vehicle; (100 fM 5HT): medium containing 100 fM 5HT and vehicle; (100 pM 5HT): medium containing 100 pM 5HT and vehicle; (100 nM 5HT): medium containing 100 nM 5HT and vehicle; (5 nM GABA): medium containing 5 nM GABA and vehicle; (100 fM 5HT & 5 nM GABA): medium containing 100 fM 5HT, 5 nM GABA, and vehicle; (100 pM 5HT & 5 nM GABA): medium containing 100 pM 5HT, 5 nM GABA, and vehicle; (100 nM 5HT & 5 nM GABA): medium containing 100 nM 5HT, 5 nM GABA, and vehicle. * Significant difference compared to "Vehicle" or "5 nM GABA" ($P < 0.05$). ** Significant difference compared to "Vehicle", "5 nM GABA", or "100 fM 5HT & 5 nM GABA" ($P < 0.05$). (B) (Vehicle): medium containing 0.2% (v/v) pure water as vehicle; (100 fM 5HT): medium containing 100 fM 5HT and vehicle; (100 pM 5HT): medium containing 100 pM 5HT and vehicle; (100 nM 5HT): medium containing 100 nM 5HT and vehicle; (5 μM GABA): medium containing 5 μM GABA and vehicle; (100 fM 5HT & 5 μM GABA): medium containing 100 fM 5HT, 5 μM GABA, and vehicle; (100 pM 5HT & 5 μM GABA): medium containing 100 pM 5HT, 5 μM GABA, and vehicle; (100 nM 5HT & 5 μM GABA): medium containing 100 nM 5HT, 5 μM GABA, and vehicle. *Significant difference compared to "Vehicle" or "5 μM GABA" ($P < 0.05$). **Significant difference compared to "Vehicle", "5 μM GABA", or "100 fM 5HT & 5 μM GABA" ($P < 0.05$). #Significant difference compared to "Vehicle", "5 μM GABA", "100 fM 5HT & 5 μM GABA", or "100 pM 5HT & 5 μM GABA" ($P < 0.05$). (C) (Vehicle): same as above; (MS): medium containing MS and vehicle; (MS & 5 nM GABA): medium containing MS, 5 nM GABA, and vehicle; (MS & 5 μM GABA): medium containing MS, 5 μM GABA, and vehicle. *Significant difference compared to "Vehicle" ($P < 0.05$). **Significant difference compared to "Vehicle" or "MS & 5 μM GABA" ($P < 0.05$). (D) (Vehicle): medium containing 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (MT): medium containing MT and vehicle; (MT & 5 nM GABA): medium containing MT, 5 nM GABA, and vehicle; (MT & 5 μM GABA): medium containing MT, 5 μM GABA, and vehicle. *Significant difference compared to "Vehicle" ($P < 0.05$).



μM GABA. After incubation for 1 h, 5 μM GABA significantly suppressed 100 pM 5HT-enhanced hyperactivation. GABA at 500 nM significantly suppressed 100 pM 5HT-enhanced hyperactivation, while 100 pM 5HT significantly enhanced hyperactivation following exposure to 500 nM GABA. GABA at 50 nM slightly suppressed 100 pM 5HT-enhanced hyperactivation although its suppression was not significant. After incubation for 1.5 h, 50 nM–5 μM GABA slightly suppressed 100 pM 5HT-enhanced hyperactivation although their suppression were not significant compared with vehicle and 100 pM 5HT. After incubation for 2 h, GABA did not suppress 100 pM 5HT-enhanced hyperactivation.

When sperm were exposed 100 fM MS after exposure to 5 nM–5 μM GABA, GABA significantly suppressed MS-enhanced hyperactivation in a dose-dependent manner (Fig. 3C). After incubation for 0.5 and 1 h, 5 μM GABA significantly suppressed MS-enhanced hyperactivation. GABA at 500 nM slightly suppressed MS-enhanced hyperactivation although its suppression was not significant. After incubation for 1.5 h, 5 μM GABA significantly suppressed MS-enhanced hyperactivation. After incubation for 2 h, GABA did not suppress MS-enhanced hyperactivation.

Involvement of the $GABA_A$ receptor in suppression of 5HT-enhanced hyperactivation by GABA

Previously, we showed that GABA suppressed P_4-enhanced hyperactivation through the $GABA_A$ receptor [11]. Because GABA suppressed 5HT-enhanced hyperactivation associated with the $5HT_2$ receptor (Figs. 2 and 3), we examined the involvement of the $GABA_A$ receptor in the suppression of 5HT-enhanced hyperactivation by GABA (Fig. 4). As shown in Fig. 4A, 100 fM 5HT-enhanced hyperactivation was significantly suppressed by 5 nM GABA, while this suppression was negated by 1 μM Bic ($GABA_A$ receptor inhibitor) after incubation for 1 h. Moreover, 100 fM 5HT-enhanced hyperactivation was suppressed by 5 μM GABA, but this suppression was also negated by Bic after incubation for 1, 1.5, and 2 h. As shown in Fig. 4B, 5 μM GABA suppressed 100 pM 5HT-enhanced sperm

Fig. 3. Dose-dependent negative effects of γ-aminobutyric acid (GABA) on enhancement of hyperactivation by 5-hydroxytryptamine (5HT) and α-methylserotonin maleate salt (MS). Percentages of hyperactivated sperm are shown when sperm were exposed to 100 fM 5HT (A), 100 pM (B), 5HT or 100 fM MS (C) after exposure to various concentrations of GABA. Data represent the mean ± SD. (A) (Vehicle): medium containing 0.2% (v/v) pure water as vehicle; (100 fM 5HT): medium containing 100 fM 5HT and vehicle; (100 fM 5HT & all concentrations of GABA): medium containing 100 fM 5HT, respective concentrations of GABA and vehicle. (B) (Vehicle): same as above; (100 pM 5HT): medium containing 100 pM 5HT and vehicle; (100 pM 5HT & all concentrations of GABA): medium containing 100 pM 5HT, respective concentrations of GABA and vehicle. (C) (Vehicle): same as above; (MS): medium containing MS and vehicle; (MS & all concentrations of GABA): medium containing 100 fM MS, respective concentrations of GABA and vehicle. * Significant difference compared to "Vehicle" ($P<0.05$). # Significant difference compared to "Vehicle" or "100 pM 5HT" ($P < 0.05$). $ Significant difference compared to "Vehicle" or "MS" ($P < 0.05$).

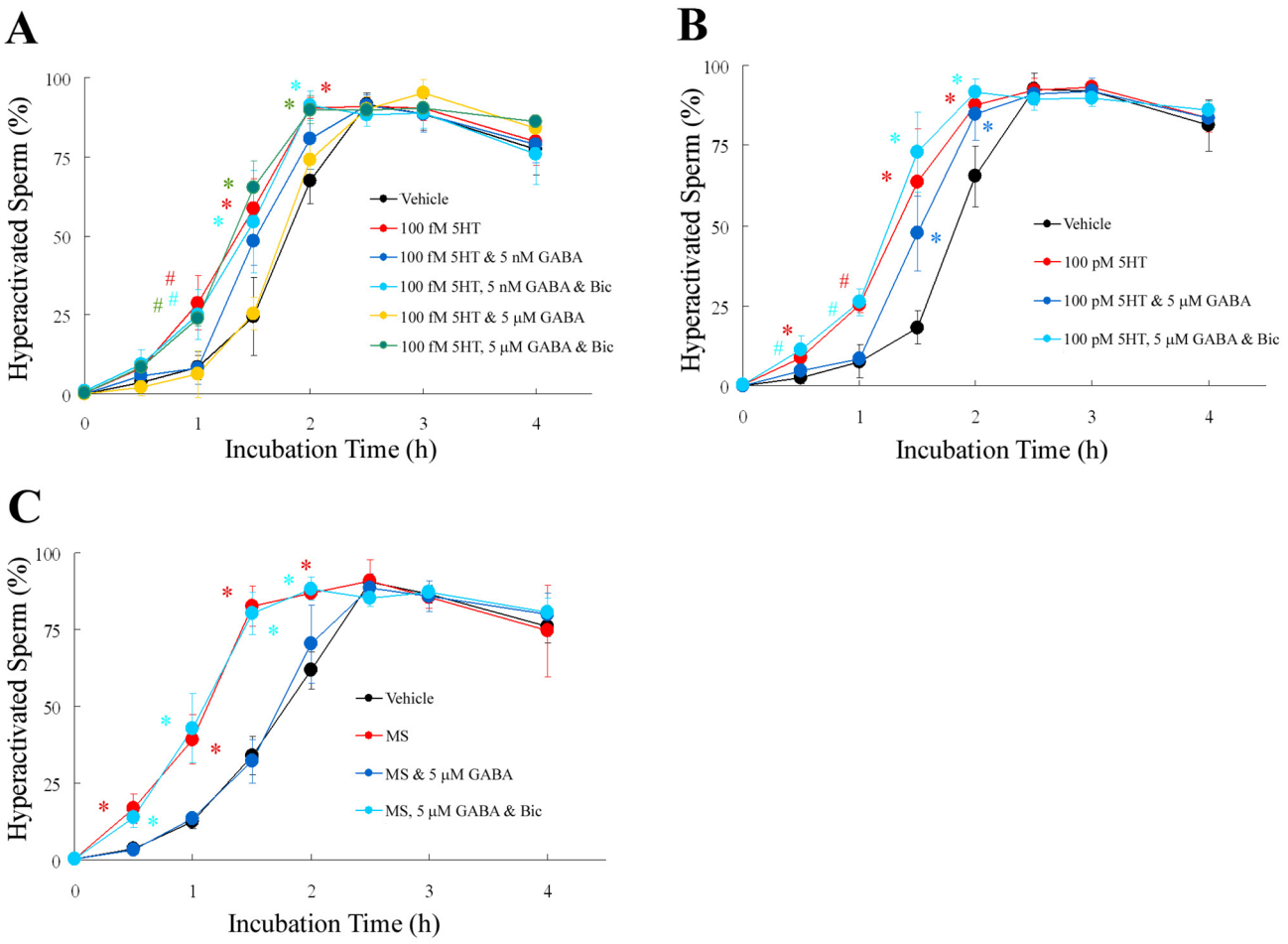


Fig. 4. Involvement of $GABA_A$ receptor in regulation of hyperactivation by γ-aminobutyric acid (GABA), 5-hydroxytryptamine (5HT), and α-methylserotonin maleate salt (MS). Percentage of hyperactivated sperm are shown when sperm were exposed to 5HT (A, B) or 100 fM MS (C) after exposure to GABA and 1 μM bicuculline (Bic). Data represent the mean ± SD. (A) (Vehicle): medium containing 0.3% (v/v) pure water as vehicle; (100 fM 5HT): medium containing 100 fM 5HT and vehicle; (100 fM 5HT & 5 nM GABA): medium containing 100 fM 5HT, 5 nM GABA, and vehicle: (100 fM 5HT, 5 nM GABA, & Bic): medium containing 100 fM 5HT, 5 nM GABA, Bic, and vehicle; (100 fM 5HT & 5 μM GABA): medium containing 100 fM 5HT, 5 μM GABA, and vehicle: (100 fM 5HT, 5 μM GABA, & Bic): medium containing 100 fM 5HT, 5 μM GABA, Bic, and vehicle. * Significant difference compared to "Vehicle" or "100 fM 5HT & 5 μM GABA" ($P < 0.05$). # Significant difference compared to "Vehicle", "100 fM 5HT & 5 nM GABA" or "100 fM 5HT & 5 μM GABA" ($P < 0.05$). (B) (Vehicle): same as above; (100 pM 5HT): medium containing 100 pM 5HT and vehicle; (100 pM 5HT & 5 μM GABA): medium containing 100 pM 5HT, 5 μM GABA, and vehicle: (100 pM 5HT, 5 μM GABA, & Bic): medium containing 100 pM 5HT, 5 μM GABA, Bic, and vehicle. * Significant difference compared to "Vehicle" ($P < 0.05$). #Significant difference compared to "Vehicle" or "100 pM 5HT & 5 μM GABA" ($P < 0.05$). (C) (Vehicle): medium containing 0.3% (v/v) pure water as vehicle; (MS): medium containing MS and vehicle; (MS & 5 μM GABA): medium containing MS, 5 μM GABA, and vehicle: (MS, 5 μM GABA, & Bic): medium containing MS, 5 μM GABA, Bic, and vehicle. * Significant difference compared to "Vehicle" or "MS & 5 μM GABA" ($P < 0.05$).

hyperactivation, which was negated by Bic after incubation for 0.5 and 1 h. As shown in Fig. 4C, 100 fM MS-enhanced hyperactivation was significantly suppressed by 5 μM GABA, and its suppression was negated by 1 μM Bic after incubation for 0.5, 1, 1.5, and 2 h.

Discussion

Capacitation is a necessary event that occurs in the female reproductive tract. The observable phenotype associated with capacitation consists of AR and hyperactivation. Some hormones and neurotransmitters are known to induce AR. P_4, Mel, 5HT, and GABA induce AR in hamsters, humans, mice, rams, and rats [20, 24–33]. P_4-induced AR is suppressed by E_2 in human [24, 34]. Additionally, hyperactivation is also enhanced by some hormones and neurotransmitters in a dose-dependent manner. P_4, Mel, and 5HT enhance hyperactivation in hamsters and humans [6, 7, 13, 14]. GABA also enhances hyperactivation in rams and humans, but not in hamsters, [11, 15, 16]. P_4-enhanced hyperactivation is dose-dependently suppressed by E_2 and GABA in hamsters [8, 10, 11]. In addition, Mel-enhanced hyperactivation of hamster sperm is

suppressed by E_2 in a dose-dependent manner [12]. In humans and hamsters, GABA binds to the $GABA_A$ receptor [11, 15]. However, P_4 also binds to the $GABA_A$ receptor in humans [15] although in hamsters P_4 binds to its receptor [7, 8, 11]. Generally, the $GABA_A$ receptor is a receptor-coupled Cl^- channel and induces Cl^- influx and hyperpolarization [39]. Therefore, in humans, P_4 and GABA may bind to the $GABA_A$ receptor and induce hyperactivation through Cl^- influx and hyperpolarization. In contrast, in hamsters, P_4 likely binds to its receptor and enhances hyperactivation. In addition, GABA binds to the $GABA_A$ receptor and suppresses P_4-enhanced hyperactivation. Previous studies [7, 8, 11, 15] suggested that P_4 and GABA regulate hyperactivation through a species-specific regulatory mechanism in humans and hamsters.

In the present study, we examined whether 5HT-enhanced hyperactivation was suppressed by E_2 and/or GABA in hamster sperm. E_2 at 20 ng/ml, which suppressed P_4- and Mel-enhanced hyperactivation [8, 10, 12], did not affect 5HT-enhanced hyperactivation (Fig. 1). In addition, we also found that 5HT-dependent enhancement of hyperactivation in the sperm after 1, 1.5, or 2 h incubation was not affected by exposure to higher concentrations of E_2 (20 μg/ml) (data not shown). In contrast, GABA suppressed the enhanced hyperactivation by 100 fM and 100 pM 5HT (Fig. 2). Previously, we demonstrated that low concentrations (fM–pM order) of 5HT enhanced hyperactivation through the $5HT_2$ receptor, while high concentrations (nM–μM order) of 5HT enhanced hyperactivation through the $5HT_4$ receptor [14]. Thus, GABA likely suppressed 5HT-enhanced hyperactivation through the $5HT_2$ receptor. Using the $5HT_2$ receptor agonist (MS) and $5HT_4$ receptor agonist (MT) (Fig. 2), we found that MS-enhanced hyperactivation was suppressed by GABA, while MT-enhanced hyperactivation was not. These observations suggest that GABA suppressed $5HT_2$ receptor-mediated enhancement of hyperactivation, but not $5HT_4$ receptor-mediated enhancement. Moreover, the inhibitory effect of GABA on 5HT-enhanced hyperactivation was concentration-dependent (Figs. 2 and 3). GABA at 5 nM (nM order) suppressed 100 fM 5HT-enhanced hyperactivation although it did not suppress 100 pM 5HT-enhanced hyperactivation and MS-enhanced hyperactivation. In contrast, GABA at 5 μM suppressed 100 fM 5HT-enhanced hyperactivation, 100 pM 5HT-enhanced hyperactivation, and MS-enhanced hyperactivation. Based on those results, suppression of 5HT-enhanced hyperactivation by 5 μM GABA was stronger than that by 5 nM GABA. Moreover, stimulation by 100 pM 5HT was stronger that that by 100 fM 5HT because stimulation by 100 pM 5HT was not suppressed by 5 nM GABA, while stimulation by 100 fM 5HT was suppressed by 5 nM GABA. Therefore, our previous study [14] and the present results suggest that 5HT dose-dependently enhances sperm hyperactivation through the $5HT_2$ and $5HT_4$ receptors, while enhancement via the $5HT_2$ receptor is suppressed by GABA. We did not identify molecules that suppressed the enhancement involved in $5HT_4$ receptor activity.

We previously showed that GABA suppressed P_4-enhanced hyperactivation of hamster sperm through the $GABA_A$ receptor [11]. In the present study, GABA also suppressed the enhancement of hyperactivation via the $5HT_2$ receptor through the $GABA_A$ receptor (Fig. 4). The $GABA_A$ receptor is a ligand-gated Cl^- channel that induces hyperpolarization [39]. Because GABA inhibited the binding of P_4 to its receptor through the $GABA_A$ receptor, we predict that GABA bound to the $GABA_A$ receptor induces Cl^- influx and hyperpolarization to inhibit the binding of P_4 to its receptor [11]. However, the mechanisms of the negative effects of GABA on P_4-enhanced hyperactivation remain unclear. In the present study, GABA suppressed the enhancement of hyperactivation via the $5HT_2$ receptor through the $GABA_A$ receptor. GABA may bind to the $GABA_A$ receptor to induce Cl^- influx and hyperpolarization, which suppresses the enhancement of hyperactivation via the $5HT_2$ receptor although the mechanisms of the negative effects of GABA on enhanced hyperactivation via the $5HT_2$ receptor remain unclear. A common regulatory mechanism exists between P_4-enhanced hyperactivation and enhanced hyperactivation via the $5HT_2$ receptor. P_4 enhances sperm hyperactivation through Ca^{2+}-signaling associated with PLC, IP_3 receptor, and protein kinase C [9]. The enhancement of hyperactivation via $5HT_2$ receptor may be regulated through Ca^{2+}-signaling associated with PLC and the IP_3 receptor [11] because the $5HT_2$ receptor in neurons stimulates PLC-induced Ca^{2+}-release form IP_3 receptor-gated Ca^{2+}-stores [17, 18]. In future studies, we will examine the inhibitory mechanisms by which GABA suppresses P_4-enhanced hyperactivation and enhances hyperactivation via the $5HT_2$ receptor.

5HT is mainly released from cumulus cells in female reproductive organs [19]. The 5HT content in the rat oviduct ranges from 2.06 to 3.34 μg/g fresh tissue [35]. In human, the concentrations of 5HT in preovulatory follicles and cystic degenerated follicles are 14.3 ± 8.9 and 12.2 ± 6.2 μg/100 ml, respectively [36]. In contrast, greater than 2.5-fold greater levels of GABA are found in the rat oviduct than in the rat brain [37]. In addition, the GABA concentration changes in the rat female genital tract with the estrous cycle [37, 38]. GABA concentration at the pro-estrous stage was found to be higher than at the estrous stage [37, 38]. Therefore, the concentration of 5HT and GABA appear to change during the estrous cycle. Based on the results of our previous study [14] and the present study, regulation of the hyperactivation by 5HT (enhancer) and GABA (suppressor) may be associated with the estrous cycle.

Recently, we reported that several hormones and neurotransmitters regulate hamster sperm hyperactivation [7, 8, 10–14]. P_4, Mel, and 5HT enhance hyperactivation [7, 13, 14]. P_4-enahnced hyperactivation is suppressed by E_2 [8, 10] and GABA [11] and Mel-enhanced hyperactivation is suppressed by E_2 [12]. In the present study, we showed that the 5HT-enhanced hyperactivation involved in the $5HT_2$ receptor was suppressed by GABA. Thus, hamster sperm hyperactivation is regulated by at least three enhancers and two suppressor. Moreover, the regulation of hyperactivation by these hormones and neurotransmitters may be associated with the estrous cycle and the interaction between the sperm and oocyte (or cumulus-oocytes complexes).

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