Serotonin-enhanced hyperactivation of hamster sperm

Masakatsu Fujinoki

Department of Physiology, School of Medicine, Dokkyo Medical University, Mibu, Tochigi 321-0293, Japan

Correspondence should be addressed to M Fujinoki; Email: fujinoki@dokkyomed.ac.jp

Abstract

The effects of serotonin on reproductive function were examined using hamster spermatozoa. When serotonin at concentrations from 1 fmol/l to 1 μmol/l was added to modified Tyrode’s albumin lactate pyruvate (mTALP) medium, hyperactivation was significantly enhanced. Agonists and antagonists of 5-hydroxytryptamine hydrochloride (5-HT) receptors (5-HT2 and 5-HT4 receptors) were added to the medium. Both 5-HT2 and 5-HT4 receptor agonists significantly enhanced hyperactivation, although the effect was greater than the former. However, both 5-HT2 and 5-HT4 receptor antagonists significantly suppressed serotonin-enhanced hyperactivation, with the former suppressing stimulation by a lower concentration of serotonin than the latter. These results indicate that serotonin enhances hyperactivation via 5-HT2 and/or 5-HT4 receptors in a dose-dependent manner.

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Introduction

The neurotransmitter serotonin (5-hydroxytryptamine hydrochloride or 5-HT) is formed in the body by the hydroxylation and decarboxylation of tryptophan. After release from serotonergic neurons, serotonin is recaptured by an active reuptake mechanism and inactivated by monoamine oxidase to form 5-hydroxyindoleacetic acid, the principal urinary metabolite of serotonin. In the pineal gland, serotonin is converted to N-acetyl-5-methoxytryptamine (melatonin). Serotonin has multiple actions in diverse tissues. In general, serotonin actions are mediated by 5-HT receptors, characterized, and cloned as 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6, and 5-HT7 receptors. Most 5-HT receptors are G-protein-coupled receptors and act on adenylyl cyclase (AC) or phospholipase C (PLC). 5-HT3 receptors, however, are ion channel-coupled receptors (Noda et al. 2004, Ganong 2005).

Because serotonin and 5-HT receptors are found in oocytes, cumulus–oocyte complexes (COC), follicular fluid, and embryos in mammals (Dubé & Amireault 2007), some consider that serotonergic signals are associated with regulation of steroidogenesis, oocyte maturation, and embryonic development. Another study (Meizel & Turner 1983) demonstrated that serotonin and 5-methoxytryptamine, a 5-HT4 receptor agonist, induce an acrosome reaction (AR) in hamster spermatozoa. They also demonstrated that serotonin-induced and serotonin agonist-induced ARs were inhibited by 4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-1-methyl-1,2dihydrochloride hydrate (2:3) (cyproheptadine hydrochloride sesquihydrate or piperidine), a 5-HT2 receptor antagonist (Meizel & Turner 1983).

Mammalian spermatozoa are capacitated before fertilization. Capacitated spermatozoa exhibit the AR in their heads and hyperactivation of the flagella (Yanagimachi 1994, Fujinoki 2009). The AR is a modified exocytotic event involving an acrosome and is required for penetration of the zona pellucida (ZP) of the egg and sperm–egg plasma membrane fusion (Yudine et al. 1988, Yanagimachi 1994). Hyperactivation is a specialized movement of the sperm flagellum that creates the propulsive force for penetration of the ZP. Hyperactivated spermatozoa exhibit a high amplitude, asymmetrical beating pattern of the sperm flagellum (Yanagimachi 1994, Fujinoki et al. 2001a, Suarez & Ho 2003). Capacitation occurs artificially in a specific culture medium containing albumin, HCO₃⁻, and Ca²⁺. Albumin is an essential component for capacitation to occur (Fujinoki 2008, Noguchi et al. 2008), as it removes cholesterol from the sperm plasma membrane to change its fluidity (Langlais & Roberts 1985). HCO₃⁻ stimulates AC, thereby increasing cAMP levels (Okamura et al. 1985). cAMP activates protein kinase A (PKA), leading to protein serine/threonine phosphorylation and to sperm flagellar movement (Visconti & Kopf 1998, Visconti et al. 1999, Fujinoki et al. 2003, 2004a, 2004b, 2006). In many cases, protein tyrosine phosphorylation also occurs in a cAMP-dependent manner (Visconti et al. 1995, 1999, Visconti & Kopf 1998, Fujinoki et al. 2001b). Ca²⁺ is involved in many intracellular signal transductions, including regulation of AC, and phosphodiesterase and protein

Several studies have demonstrated that the AR is induced by ligands such as progesterone, the ZP, serotonin, and melatonin (Meizel & Turner 1983, Osman et al. 1989, Luconi et al. 2004, Baldi et al. 2009, Casao et al. 2009). However, hyperactivation is also enhanced by ligands such as progesterone and melatonin (Sueldo et al. 1993, Yang et al. 1994, Fujinoki 2008, 2009, Noguchi et al. 2008, du Plessis et al. 2010). Moreover, progesterone-enhanced hyperactivation was suppressed by 17β-estradiol (Fujinoki 2010). These ligands regulate the AR and hyperactivation via non-genomic regulation. Non-genomic regulation of the AR and hyperactivation by progesterone and the ZP are associated with Ca²⁺ signals via PLC (Luconi et al. 2004, Noguchi et al. 2008, Baldi et al. 2009, Fujinoki 2009). However, non-genomic regulation of the AR and hyperactivation by melatonin are associated with nitric oxide signals (Fujinoki 2008, 2009, Casao et al. 2009, du Plessis et al. 2010). However, regulation of the AR by serotonin is poorly understood. Moreover, it has yet to be determined whether serotonin enhances hyperactivation, although it is known to induce the AR (Meizel & Turner 1983). Accordingly, in this study, the effects of serotonin on hyperactivation using hamster spermatozoa were investigated.

Results

Effects of serotonin on hyperactivation

In a previous study (Fujinoki 2008), hyperactivation was significantly enhanced when melatonin at concentrations between 1 fmol/l and 10 µmol/l was added to the Tyrode’s albumin lactate pyruvate (mTALP) medium. As melatonin is a metabolite of serotonin, it was examined whether serotonin enhanced hyperactivation. Serotonin at concentrations between 1 fmol/l and 1 µmol/l significantly enhanced hyperactivation (Fig. 1A), but serotonin did not affect the percentage of motile spermatozoa when added to the TALP medium at concentrations between 1 amol/l and 10 µmol/l (data not shown). With incubation for 0 h, no hyperactivated spermatozoa were seen (Fig. 1A). After incubation for 0.5 h, there were a few hyperactivated spermatozoa, but serotonin did not enhance hyperactivation (Fig. 1A). After incubation for 1 h (Fig. 1B), 100 nmol/l serotonin significantly enhanced hyperactivation in comparison with mTALP or 1 amol/l serotonin. Hyperactivation was significantly enhanced by 10 nmol/l serotonin in comparison with mTALP or serotonin at 1 or 10 amol/l. Similarly, hyperactivation was significantly enhanced by 10 pmol/l serotonin in comparison with mTALP or serotonin at 1 amol/l, 10 amol/l, 1 µmol/l, or 10 µmol/l. Hyperactivation was significantly enhanced by serotonin at concentrations between 1 fmol/l and 1 pmol/l, as well as 100 pmol/l and 1 nmol/l serotonin, in comparison with mTALP or serotonin at 1 amol/l, 10 amol/l, or 10 µmol/l. Although the increase was not significant,
serotonin at 100 amol/l enhanced hyperactivation. After incubation for 1.5 h (Fig. 1C), serotonin at 1 and 10 fmol/l significantly enhanced hyperactivation in comparison with mTALP or serotonin at 1 amol/l. Hyperactivation was significantly enhanced by 10 nmol/l serotonin in comparison with mTALP or serotonin at 1 amol/l, 10 amol/l, or 10 µmol/l. Moreover, serotonin at concentrations between 100 fmol/l and 1 nmol/l significantly enhanced hyperactivation in comparison with mTALP or serotonin at concentrations between 1 amol/l and 100 amol/l, 1 µmol/l, or 10 µmol/l. After incubation for 2 h (Fig. 1D), serotonin at concentrations between 10 pmol/l and 100 nmol/l significantly enhanced hyperactivation in comparison with mTALP or serotonin at concentrations between 1 amol/l and 100 amol/l, 1 µmol/l, or 10 µmol/l. Serotonin at 1 fmol/l and 1 µmol/l significantly enhanced hyperactivation in comparison with mTALP or serotonin at concentrations between 1 amol/l and 100 amol/l and 10 µmol/l serotonin. Moreover, serotonin at 100 amol/l significantly enhanced hyperactivation compared with serotonin at 1 amol/l or concentrations between 1 fmol/l and 1 µmol/l. After incubation for 2.5, 3, and 4 h, most motile spermatozoa were hyperactivated under all conditions (Fig. 1A).

Effects of 5-HT receptor agonists on hyperactivation

Quipazine is a nonspecific 5-HT receptor agonist, although Meizel & Turner (1983) reported that it was a 5-HT receptor antagonist and significantly suppressed serotonin-induced AR. Accordingly, it was examined whether quipazine acted as an antagonist to serotonin-enhanced hyperactivation using 100 pmol/l serotonin, an average value of the effective concentrations, and 1 µmol/l quipazine (Fig. 2). Although 1 µmol/l quipazine did not affect the percentage of motile spermatozoa (data not shown), it significantly enhanced hyperactivation (Fig. 2A). After incubation for 0 and 0.5 h, no hyperactivated spermatozoa were seen (Fig. 2A). After incubation for 1 (Fig. 2B), 1.5 (Fig. 2C), and 2 h (Fig. 2D), serotonin and quipazine significantly enhanced hyperactivation in comparison with mTALP. When spermatozoa were exposed to 100 pmol/l serotonin after exposure to 1 µmol/l quipazine, hyperactivation was significantly enhanced in comparison with mTALP. However, no significant difference was seen between ‘serotonin + quipazine’ and serotonin or quipazine respectively. After incubation for 2 h (Fig. 2D), serotonin significantly enhanced hyperactivation compared with quipazine. After incubation for 2.5, 3, and 4 h, most motile spermatozoa were hyperactivated under all conditions (Fig. 2A). These results indicate that quipazine acts as an agonist for hyperactivation.

In the next step, the dose-dependent effect of quipazine on hyperactivation was examined (Fig. 3). When quipazine concentrations between 1 amol/l and 10 µmol/l were added to mTALP medium, quipazine did not affect the percentage of motile spermatozoa (data not shown), but at concentrations between 100 amol/l and 1 µmol/l, quipazine affected the percentage of hyperactivated spermatozoa (Fig. 3A). After incubation for 0 h, no hyperactivated spermatozoa were seen (Fig. 3A). After incubation for 0.5 h, quipazine did not significantly enhance hyperactivation, with but a few spermatozoa hyperactivated (Fig. 3A). After incubation for 1 h (Fig. 3B), quipazine at a concentration of 1 fmol/l and between 1 pmol/l and 10 nmol/l significantly enhanced hyperactivation in comparison with mTALP or quipazine at 1 amol/l, 1 µmol/l, or 10 µmol/l. Quipazine at 10 and 100 fmol/l significantly enhanced hyperactivation in
comparison with mTALP or quipazine at 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l. Moreover, 100 amol/l quipazine significantly enhanced hyperactivation in comparison with mTALP or quipazine at 1 amol/l and 10 μmol/l. After incubation for 1.5 h (Fig. 3C), quipazine at concentrations between 1 and 100 fmol/l significantly enhanced hyperactivation in comparison with mTALP or quipazine at 1 or 10 μmol/l. Quipazine at 1 and 100 pmol/l significantly enhanced hyperactivation in comparison with mTALP or quipazine at concentrations of 1 amol/l, 10 amol/l, or between 100 nmol/l and 10 μmol/l. Quipazine at 10 pmol/l significantly enhanced hyperactivation in comparison with mTALP or quipazine at 10 amol/l, 1 μmol/l, or 10 μmol/l. Quipazine at 1 nmol/l quipazine significantly enhanced hyperactivation in comparison with mTALP or 10 μmol/l quipazine. Although the increase was not significant, quipazine at 100 amol/l and 10 nmol/l significantly enhanced hyperactivation in comparison with mTALP or quipazine at 1 and 10 amol/l. After incubation for 2.5, 3, and 4 h, most motile spermatozoa were hyperactivated under all conditions (Fig. 3A).

Because serotonin-induced AR is regulated via 5-HT2 and 5-HT4 receptors (Meizel & Turner 1983), it was examined whether 5-HT2 and 5-HT4 receptor agonists enhanced hyperactivation (Figs 4 and 5). When α-methylserotonin, a 5-HT2 receptor agonist, was added to the mTALP medium at concentrations between 1 amol/l and 10 μmol/l, it did not affect the percentage of motile spermatozoa (data not shown), although α-methylserotonin at concentrations between 1 fmol/l and 100 pmol/l did affect the percentage of hyperactivated spermatozoa (Fig. 4A). At incubation for 0 h, there were no hyperactivated spermatozoa (Fig. 4A). After incubation for 0.5 h (Fig. 4B), α-methylserotonin at 1 fmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations of 1 amol/l, 10 amol/l, or between 10 pmol/l and 10 μmol/l. α-Methylserotonin at 10 fmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations of 1 amol/l, 10 amol/l, or between 1 nmol/l and 10 μmol/l. Moreover, 100 fmol/l α-methylserotonin significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations of 1 amol/l, 10 amol/l, or between 100 pmol/l and 10 μmol/l. After incubation for 1 h (Fig. 4C), α-methylserotonin at 1 and 10 fmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations of 1 amol/l, 10 amol/l, or between 100 pmol/l and 10 μmol/l. α-Methylserotonin at 100 fmol/l significantly enhanced hyperactivation in comparison with mTALP or 1 amol/l α-methylserotonin. Although the increase was not significant, α-methylserotonin at concentrations between 1 and 100 nmol/l enhanced hyperactivation. After incubation for 1.5 h

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**Figure 3** Dose-dependent effects of quipazine on hyperactivation. The percentage of hyperactivated spermatozoa is shown (A) as an overview of effects after incubation for (B) 1, (C) 1.5, and (D) 2 h after quipazine was added to mTALP medium. Data are given as mean ± s.d. mTALP: mTALP only; (1 amol/l or 18); mTALP + 1 amol/l quipazine; (10 amol/l quipazine; (16): mTALP + 100 amol/l quipazine; (1 fmol/l or 15): mTALP + 100 fmol/l quipazine; (10 amol/l quipazine; (13): mTALP + 100 pmol/l quipazine; (1 pmol/l or 12): mTALP + 10 pmol/l quipazine; (10 pmol/l quipazine; (1: mTALP + 100 nmol/l quipazine; (1 nmol/l or 9): mTALP + 1 nmol/l quipazine; (8): mTALP + 10 nmol/l quipazine; (7): mTALP + 100 nmol/l quipazine; (1 μmol/l or 6): mTALP + 1 μmol/l quipazine; (5): mTALP + 10 μmol/l quipazine. *Significant difference in comparison with mTALP or 1 amol/l, 1 μmol/l, or 10 μmol/l quipazine; †Significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l quipazine; ‡Significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l quipazine; §Significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l quipazine; ††Significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l quipazine; †††Significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l quipazine; ††††Significant difference in comparison with mTALP or 10 pmol/l quipazine; †††††Significant difference in comparison with mTALP or 1 amol/l or 10 amol/l quipazine.
(Fig. 4D), α-methylserotonin at 1 and 10 fmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations between 1 and 100 amol/l or between 100 pmol/l and 10 μmol/l. α-Methylserotonin at 100 fmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations between 1 and 100 amol/l or between 1 nmol/l and 10 μmol/l. Moreover, α-methylserotonin at 1 and 10 pmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations between 1 nmol/l and 10 μmol/l. After incubation for 2 h (Fig. 4E), α-methylserotonin at concentrations between 1 and 100 fmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at concentrations of 1 amol/l, 10 amol/l, or between 1 nmol/l and 10 μmol/l. α-Methylserotonin at 1 pmol/l significantly enhanced hyperactivation in comparison with mTALP or α-methylserotonin at 1 amol/l, 10 amol/l, 1 μmol/l, or 10 μmol/l. Moreover, α-methylserotonin at 10 and 100 pmol/l significantly enhanced hyperactivation in comparison with 1 μmol/l α-methylserotonin. After incubation for 2.5, 3, and 4 h, most motile spermatozoa were hyperactivated under all conditions (Fig. 4A).

In the next step, when 5-methoxytryptamine, a 5-HT4 receptor agonist, was added to the mTALP medium at concentrations between 1 amol/l and 10 μmol/l, it did not affect the percentage of motile spermatozoa (data not shown), whereas 5-methoxytryptamine at concentrations between 1 fmol/l and 100 nmol/l enhanced hyperactivation, although the increase was not significant (Fig. 5A). After incubation for 0 h, no hyperactivated spermatozoa were seen (Fig. 5A). After incubation for 0.5 h, 5-methoxytryptamine at concentrations between 1 fmol/l and 100 nmol/l enhanced hyperactivation, although the increase was not significant (Fig. 5A). After incubation for 1 h (Fig. 5B), 5-methoxytryptamine at 10 and 100 fmol/l significantly enhanced hyperactivation in comparison with mTALP. Although the increase was not significant, 5-methoxytryptamine at concentrations of 1 fmol/l and between 1 pmol/l and 100 nmol/l enhanced hyperactivation. After incubation for 1.5 h (Fig. 5C), 5-methoxytryptamine at 1 and 10 fmol/l significantly enhanced hyperactivation in comparison with mTALP or 5-methoxytryptamine at concentrations between 1 amol/l and 100 amol/l or 1 μmol/l or

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**Figure 4** Dose-dependent effects of α-methylserotonin on hyperactivation. The percentage of hyperactivated spermatozoa is shown (A) as an overview of the effects after incubation for (B) 0.5, (C) 1, (D) 1.5, and (E) 2 h when α-methylserotonin was added to mTALP medium. Data represent mean ± s.d. (mTALP): mTALP only; (1 amol/l or −18): mTALP + 1 amol/l α-methylserotonin; (−17): mTALP + 10 amol/l α-methylserotonin; (−16): mTALP + 100 amol/l α-methylserotonin; (1 fmol/l or −15): mTALP + 1 fmol/l α-methylserotonin; (−14): mTALP + 10 fmol/l α-methylserotonin; (−13): mTALP + 100 fmol/l α-methylserotonin; (1 pmol/l or −12): mTALP + 1 pmol/l α-methylserotonin; (−11): mTALP + 10 pmol/l α-methylserotonin; (−10): mTALP + 100 pmol/l α-methylserotonin; (1 nmol/l or −9): mTALP + 1 nmol/l α-methylserotonin; (−8): mTALP + 10 nmol/l α-methylserotonin; (−7): mTALP + 100 nmol/l α-methylserotonin; (1 μmol/l or −6): mTALP + 1 μmol/l α-methylserotonin; (−5): mTALP + 10 μmol/l α-methylserotonin. *Significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or between 10 pmol/l and 10 μmol/l; †significant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or between 100 pmol/l and 10 μmol/l; ‡significant difference in comparison with mTALP or 10 pmol/l, 100 pmol/l, or between 100 nmol/l and 10 μmol/l; §significant difference in comparison with mTALP or 100 pmol/l and 10 μmol/l; ††significant difference in comparison with 1 pmol/l or 10 amol/l; †‡significant difference in comparison with mTALP or 1 pmol/l, 10 amol/l, or between 100 pmol/l and 10 μmol/l; †§significant difference in comparison with mTALP or 10 pmol/l, 100 pmol/l, or between 100 nmol/l and 10 μmol/l; †‖significant difference in comparison with mTALP or 100 pmol/l and 10 μmol/l; †††significant difference in comparison with mTALP or between 1 and 100 amol/l or between 1 nmol/l and 10 μmol/l; ††‡significant difference in comparison with mTALP or between 1 and 10 amol/l or between 1 nmol/l and 10 μmol/l; ††§significant difference in comparison with mTALP or between 1 and 100 amol/l or between 1 nmol/l and 10 μmol/l; ††‖significant difference in comparison with only 1 μmol/l.
Dose-dependent effects of 5-methoxytryptamine on hyperactivation. The percentage of hyperactivated spermatozoa is shown (A) as an overview of effects after incubation for (B) 1, (C) 1.5, and (D) 2 h when 5-methoxytryptamine was added to mTALP medium. Data are given as mean ± S.D. (Vehicle): mTALP + 0.1% EtOH (1 amol/l or ~18): mTALP + 1 amol/l 5-methoxytryptamine + 0.1% EtOH; (–17): mTALP + 10 amol/l 5-methoxytryptamine + 0.1% EtOH; (–16): mTALP + 100 amol/l 5-methoxytryptamine + 0.1% EtOH: (1 fmol/l or –15): mTALP + 1 fmol/l 5-methoxytryptamine + 0.1% EtOH; (–14): mTALP + 10 fmol/l 5-methoxytryptamine + 0.1% EtOH: (–13): mTALP + 100 fmol/l 5-methoxytryptamine + 0.1% EtOH; (1 pmol/l or –12): mTALP + 1 pmol/l 5-methoxytryptamine + 0.1% EtOH: (–11): mTALP + 10 pmol/l 5-methoxytryptamine + 0.1% EtOH; (–10): mTALP + 100 pmol/l 5-methoxytryptamine + 0.1% EtOH: (1 nmol/l or –9): mTALP + 1 nmol/l 5-methoxytryptamine + 0.1% EtOH; (–8): mTALP + 10 nmol/l 5-methoxytryptamine + 0.1% EtOH; (–7): mTALP + 100 nmol/l 5-methoxytryptamine + 0.1% EtOH: (1 µmol/l or –6): mTALP + 1 µmol/l 5-methoxytryptamine + 0.1% EtOH; (–5): mTALP + 10 µmol/l 5-methoxytryptamine + 0.1% EtOH. #Significant difference in comparison with mTALP; $^\ddagger$significant difference in comparison with mTALP or between 1 amol/l and 100 amol/l, 1 µmol/l, or 10 µmol/l. 5-Methoxytryptamine at 100 fmol/l and 1 pmol/l significantly enhanced hyperactivation in comparison with mTALP or 5-methoxytryptamine at concentrations between 1 and 100 amol/l or between 100 nmol/l and 10 µmol/l 5-methoxytryptamine. 5-Methoxytryptamine at concentrations of 10 pmol/l and between 1 and 100 nmol/l significantly enhanced hyperactivation in comparison with mTALP or 5-methoxytryptamine at 1 amol/l, 10 amol/l, 1 µmol/l, or 10 µmol/l. After incubation for 2.5, 3, and 4 h, most motile spermatozoa were hyperactivated under all conditions (Fig. 5A).

**Effects of 5-HT receptor antagonists on serotonin-enhanced hyperactivation**

As both the 5-HT₂ receptor agonist (α-methylserotonin) and the 5-HT₄ receptor agonist (5-methoxytryptamine) enhanced sperm hyperactivation, it was examined whether a 5-HT₂ receptor antagonist (cyproheptadine) and 5-HT₄ receptor antagonist (GR113808) suppressed serotonin-enhanced and agonist-enhanced hyperactivation.

Cyproheptadine at 1 µmol/l significantly suppressed serotonin-enhanced (Fig. 6A), quipazine-enhanced (Fig. 6C), and α-methylserotonin-enhanced hyperactivation (Fig. 7A), although it did not affect the percentages of motile (data not shown) or hyperactivated spermatozoa (Figs 6 and 7), or of 5-methoxytryptamine-enhanced hyperactivation (Fig. 7B). Cyproheptadine significantly suppressed serotonin-enhanced and quipazine-enhanced hyperactivation when spermatozoa were exposed to 100 pmol/l serotonin or 100 pmol/l quipazine, the respective average effective concentrations, after exposure to 1 µmol/l cyproheptadine for 5 min (Fig. 6A and C). However, cyproheptadine did not suppress serotonin-enhanced and quipazine-enhanced hyperactivation when spermatozoa were exposed to 100 nmol/l serotonin and 100 nmol/l quipazine, the respective maximum effective concentrations, after exposure to 1 µmol/l cyproheptadine for 5 min (Fig. 6B and D). When spermatozoa were exposed to 100 fmol/l α-methylserotonin, the average effective concentration, after exposure to 1 µmol/l cyproheptadine for 5 min, cyproheptadine significantly suppressed α-methylserotonin-enhanced hyperactivation (Fig. 7A). However, 5-methoxytryptamine-enhanced hyperactivation was not suppressed by cyproheptadine when spermatozoa...
were exposed to 10 pmol/l 5-methoxytryptamine, the average effective concentration, after exposure to 1 µmol/l cyproheptadine for 5 min (Fig. 7B).

GR113808 at 1 µmol/l also significantly suppressed serotonin-enhanced (Fig. 8A), quipazine-enhanced (Fig. 8C), and 5-methoxytryptamine-enhanced hyperactivation (Fig. 9B), although GR113808 did not affect the percentages of motile (data not shown) or hyperactivated spermatozoa (Figs 8 and 9). GR113808 significantly suppressed serotonin-enhanced and quipazine-enhanced hyperactivation when spermatozoa were exposed to 100 nmol/l serotonin and 100 nmol/l quipazine after exposure to 1 µmol/l GR113808 for 5 min (Fig. 8A and C). When spermatozoa were exposed to 100 pmol/l serotonin and 100 pmol/l quipazine after exposure to 1 µmol/l GR113808 for 5 min, however, GR113808 did not suppress serotonin-enhanced and quipazine-enhanced hyperactivation (Fig. 8B and D). When spermatozoa were exposed to 100 pmol/l α-methylserotonin after exposure to 1 µmol/l GR113808 for 5 min, α-methylserotonin-enhanced hyperactivation was not suppressed by GR113808 (Fig. 9A). However, 5-methoxytryptamine-enhanced hyperactivation was suppressed by 1 µmol/l GR113808 when spermatozoa were exposed to 10 pmol/l 5-methoxytryptamine after exposure to 1 µmol/l GR113808 for 5 min (Fig. 9B).

Effects of Ca²⁺ and albumin on hyperactivation

Progesterone and melatonin significantly enhance hyperactivation, although they are not associated with the spontaneous regulatory mechanisms of hyperactivation (Fujinoki 2008, Noguchi et al. 2008). Accordingly, it was examined whether serotonin-enhanced hyperactivation is associated with the spontaneous regulatory mechanisms of hyperactivation.

As shown in Fig. 10A, the percentage of motile spermatozoa was significantly decreased after incubation for 1 h in mTALP medium without Ca²⁺, and after incubation for 2.5, 3, or 4 h, most spermatozoa were not motile. Serotonin did not affect the percentage of motile spermatozoa in mTALP medium without Ca²⁺. Spermatozoa were not hyperactivated in mTALP medium without Ca²⁺, even if serotonin was added (Fig. 10B).

In mTALP medium without BSA, most spermatozoa were not hyperactivated even if serotonin was added (Fig. 10C). However, the addition of BSA did not affect the percentage of motile spermatozoa (data not shown).

Discussion

Recent studies have been suggested that hyperactivation is regulated in a ligand-dependent manner, with ligands including progesterone, 17β-estradiol, and melatonin (Sueldo et al. 1993, Yang et al. 1994, Fujinoki
serotonin-induced and 5-methoxytryptamine-induced AR are inhibited by cyproheptadine. Therefore, it is likely that serotonin induces the AR via 5-HT$_2$ and 5-HT$_4$ receptors. Both $\alpha$-methylserotonin and 5-methoxytryptamine significantly enhanced sperm hyperactivation in a dose-dependent manner (see Figs 4 and 5). The effective concentration ranges of $\alpha$-methylserotonin and 5-methoxytryptamine were 1 fmol/l to 100 pmol/l and 1 fmol/l to 100 nmol/l respectively. Moreover, cyproheptadine and GR113808 suppressed $\alpha$-methylserotonin-enhanced and 5-methoxytryptamine-enhanced hyperactivation respectively (see Figs 7 and 9). Interestingly, enhancement of hyperactivation by 100 pmol/l serotonin and 100 pmol/l quipazine were significantly suppressed by cyproheptadine (see Fig. 6A and C), although enhancement of hyperactivation by 100 nmol/l serotonin and 100 nmol/l quipazine were not suppressed by cyproheptadine (see Fig. 6B and D). However, enhancement of hyperactivation by 100 nmol/l serotonin and 100 nmol/l quipazine were significantly suppressed by GR113808 (see Fig. 8A and C), whereas enhancement of hyperactivation by 100 pmol/l serotonin and 100 pmol/l quipazine were not suppressed by GR113808 (see Fig. 8B and D). These results suggest that serotonin enhances hyperactivation via the 5-HT$_2$ and 5-HT$_4$ receptors in a dose-dependent manner.

Generally, 5-HT$_2$ receptor and 5-HT$_4$ receptors are activated by PLC and AC respectively (Noda et al. 2004, Ganong 2005). Activation of PLC induces production of inositol 1,4,5-tris-phosphate (IP$_3$) and diacylglycerol, increases intracellular Ca$^{2+}$, and activates protein kinase C. In mammalian spermatozoa, PLC is associated with the regulation of progesterone-induced AR and progesterone-enhanced hyperactivation (Luconi et al. 2004, Noguchi et al. 2008, Baldi et al. 2009, Fujinoki 2009). It has been suggested that IP$_3$ is also associated with regulation of hyperactivation (Ho & Suarez 2001). Because serotonin-enhanced hyperactivation was regulated via 5-HT$_2$ receptors (see Figs 4, 6 and 7), it is likely that 100 pmol/l serotonin and $\alpha$-methylserotonin enhance hyperactivation through 5-HT$_2$ receptors and PLC-IP$_3$ signals. In progesterone-enhanced hyperactivation, tyrosine phosphorylation of sperm proteins is enhanced and increased after activation of PLC (Noguchi et al. 2008, Fujinoki 2009, 2010). Although it is not clear whether serotonin enhances and increases tyrosine phosphorylation of sperm proteins together with enhancement of hyperactivation, it is my understanding that tyrosine phosphorylation is enhanced and increased when serotonin enhances hyperactivation. However, serotonin also enhanced hyperactivation via 5-HT$_4$ receptors (see Figs 5, 8 and 9). As 5-HT$_4$ receptor induces activation of AC and cAMP production, it is likely that 100 nmol/l serotonin and 5-methoxytryptamine enhance hyperactivation through

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**Figure 7** Effects of cyproheptadine on $\alpha$-methylserotonin-enhanced and 5-methoxytryptamine-enhanced hyperactivation. After spermatozoa were exposed to cyproheptadine for 5 min, they were exposed to $\alpha$-methylserotonin or 5-methoxytryptamine. The percentages of hyperactivated spermatozoa (A and B) are shown when 100 fmol/l $\alpha$-methylserotonin, 10 pmol/l 5-methoxytryptamine, and 1 pmol/l cyproheptadine were added to mTALP medium. Data are given as mean±s.d. In (A), ‘vehicle’, ‘$\alpha$-methylserotonin’, ‘$\alpha$-methylserotonin + cyproheptadine’ and ‘$\alpha$-methylserotonin + cyproheptadine’ indicate mTALP + 0.1% MeOH, mTALP + 100 fmol/l $\alpha$-methylserotonin, +0.1% MeOH, mTALP + 1 pmol/l cyproheptadine +0.1% MeOH and mTALP + 100 pmol/l $\alpha$-methylserotonin, +1 pmol/l cyproheptadine +0.1% MeOH. In (B), ‘vehicle’, ‘5-methoxytryptamine’, ‘cyproheptadine’, and ‘5-methoxytryptamine + cyproheptadine’ indicate mTALP + 0.1% MeOH+0.1% EOH, mTALP + 10 pmol/l 5-methoxytryptamine +0.1% MeOH +0.1% EOH, mTALP + 1 pmol/l cyproheptadine +0.1% MeOH +0.1% EOH and mTALP + 10 pmol/l 5-methoxytryptamine +1 pmol/l cyproheptadine +0.1% MeOH +0.1% EOH. Significant difference in comparison with ‘vehicle’ or ‘cyproheptadine’ or ‘$\alpha$-methylserotonin + cyproheptadine’. Significant difference in comparison with ‘vehicle’ or ‘cyproheptadine’ or ‘5-methoxytryptamine + cyproheptadine’.

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In this study, it was examined whether serotonin and its agonists are associated with regulation of hyperactivation, because a previous study (Meizel & Turner 1983) demonstrated that serotonin and its agonists induce the AR in hamster spermatozoa. Serotonin and quipazine significantly enhanced hyperactivation in a dose-dependent manner (see Figs 1–3). For hamster spermatozoa, the effective concentration ranges of serotonin and quipazine were 1 fmol/l to 1 pmol/l and 100 amol/l to 1 pmol/l respectively. As the effective concentration range of melatonin for enhancement of hyperactivation is 1 pmol/l to 1 pmol/l (Fujinoki 2008), serotonin is more potent than melatonin. In general, the actions of serotonin are mediated by 5-HT receptors (Noda et al. 2004, Ganong 2005). A previous study (Meizel & Turner 1983) demonstrated that serotonin and 5-methoxytryptamine induce the AR and that both
Serotonin-enhanced hyperactivation

Serotonin and 5-HT receptors and AC-cAMP signals. In general, hyperactivation is regulated by cAMP-PKA signals (Yanagimachi 1994, Visconti et al. 1995). In addition, tyrosine phosphorylation is also regulated by cAMP/PKA signals (Visconti et al. 1995, 1999, Fujinoki et al. 2006). Therefore, it is reasonable to assume that serotonin also enhances and increases tyrosine phosphorylation together with hyperactivation through 5-HT4 receptors and AC-cAMP-PKA signals.

Progesterone-enhanced and melatonin-enhanced hyperactivation are not associated with the spontaneous regulatory mechanisms, because these enhancements only occur in complete media in which spermatozoa can be hyperactivated (Fujinoki 2008, Noguchi et al. 2008). Serotonin-enhanced hyperactivation also occurred in the complete medium in which spermatozoa could be hyperactivated and did not occur in media without Ca2+ or albumin (see Fig. 10). Therefore, it is likely that serotonin-enhanced hyperactivation also occurs through a modulatory mechanism, as with progesterone-enhanced and melatonin-enhanced hyperactivation.

Serotonin and 5-HT receptors are found in oocytes, COC, follicular fluid, and embryos in mammals (Dubé & Amireault 2007). The serotonin content in the rat oviduct ranged from 2.06 to 3.34 μg/g fresh tissue (Jurio et al. 1989). In human, serotonin in the preovulatory follicles and the cystically degenerated follicles were 14.3 ± 8.9 and 12.2 ± 6.2 μg/100 ml respectively (Bódis et al. 1992). In the female reproductive organs, serotonin is mainly released from cumulus cells and is associated with the regulation of steroidogenesis and oocyte maturation (Dubé & Amireault 2007). Serotonin at 100 pmol/l induced progesterone released from human granulose cells (Bódis et al. 1993). The results of a previous study (Meizel & Turner 1983) and this study both suggest that serotonin directly induces the AR and enhances hyperactivation. The effective concentrations of serotonin to induce the AR and to enhance hyperactivation are several μmol/l and between 1 fmol/l and 1 μmol/l respectively (Meizel & Turner 1983, see Fig. 1). The AR occurs near or at oocytes and the COC. In contrast, hyperactivation occurs at a distance from oocytes and the COC. Therefore, it is likely that serotonin regulates sperm functions depending on the distance between the spermatozoa and the oocytes. Spermatozoa have various systems to regulate sperm functions depending on the distance between spermatozoa and oocytes. Progesterone enhances hyperactivation (Noguchi et al. 2008, Fujinoki 2009). However, 17β-estradiol suppresses enhancement of hyperactivation by progesterone (Fujinoki 2010). Melatonin also enhances hyperactivation (Fujinoki 2008). Those hormones are contained in follicular fluid and are found in reproductive fluids. Therefore, it seems that spermatozoa have several regulatory systems to regulate the timing of fertilization using hormones released from oocytes, COC, or follicular fluid.

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Figure 8 Effects of GR113808 on serotonin-enhanced and quipazine-enhanced hyperactivation. After spermatozoa were exposed to GR113808 for 5 min, they were exposed to serotonin or quipazine. The percentages of hyperactivated spermatozoa (A–D) are shown when 100 nmol/l serotonin, 100 pmol/l serotonin, 100 nmol/l quipazine, 100 pmol/l quipazine, and 1 μmol/l GR113808 were added to mTALP medium. Data are given as mean ± s.d. In (A), ‘vehicle’, 100 nmol/l serotonin, ‘GR113808’, and ‘100 nmol/l serotonin + GR113808’ indicate mTALP + 0.1% DMSO, mTALP + 100 nmol/l serotonin + 0.1% DMSO, mTALP + 1 μmol/l GR113808 + 0.1% DMSO, and mTALP + 100 nmol/l serotonin + 1 μmol/l GR113808 + 0.1% DMSO. In (B), ‘vehicle’, ‘100 pmol/l serotonin’, ‘GR113808’, and ‘100 pmol/l serotonin + GR113808’ indicate mTALP + 0.1% DMSO, mTALP + 100 pmol/l serotonin + 0.1% DMSO, mTALP + 1 μmol/l GR113808 + 0.1% DMSO, and mTALP + 100 pmol/l serotonin + 1 μmol/l GR113808 + 0.1% DMSO. In (C), ‘vehicle’, ‘100 nmol/l quipazine’, ‘GR113808’, and ‘100 nmol/l quipazine + GR113808’ indicate mTALP + 0.1% DMSO, mTALP + 100 nmol/l quipazine + 0.1% DMSO, mTALP + 1 μmol/l GR113808 + 0.1% DMSO, and mTALP + 100 pmol/l quipazine + 1 μmol/l GR113808 + 0.1% DMSO. In (D), ‘vehicle’, ‘100 pmol/l quipazine’, ‘GR113808’, and ‘100 pmol/l quipazine + GR113808’ indicate mTALP + 0.1% DMSO, mTALP + 100 pmol/l quipazine + 0.1% DMSO, mTALP + 1 μmol/l GR113808 + 0.1% DMSO, and mTALP + 100 pmol/l quipazine + 1 μmol/l GR113808 + 0.1% DMSO. Significant difference in comparison with ‘vehicle’ or ‘GR113808’ or ‘100 nmol/l serotonin’ or ‘100 pmol/l quipazine’ or ‘GR113808’. Significant difference in comparison with ‘vehicle’ or ‘GR113808’ or ‘100 nmol/l quipazine + GR113808’. Significant difference in comparison with ‘vehicle’ or ‘GR113808’ or ‘100 pmol/l quipazine + GR113808’. Significant difference in comparison with ‘vehicle’ or ‘100 nmol/l quipazine’ or ‘GR113808’. Significant difference in comparison with ‘vehicle’ or ‘100 pmol/l quipazine’.
Materials and Methods

Chemicals

Serotonin, 2-(1-piperazinyl)quinoline maleate salt (quipazine maleate salt), 5-methoxytryptamine, cyproheptadine hydrochloride sesquihydrate, (+)-3-(2-aminopropyl)indol-5-ol maleate salt (α-methylserotonin maleate salt or α-methyl-5-methoxyhydroxytryptamine maleate salt), and 1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinylmethyl 1-methyl-1H-indole-3-carboxylate (GR113808) were purchased from Sigma Chemical Company. BSA fraction V was purchased from Merck KGaA. Other reagent grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of hyperactivated spermatozoa

Spermatozoa were obtained from the caudal epididymis of sexually mature (10–20 week old) male golden hamsters (Mesocricetus auratus), which were housed in accordance with the Dokkyo Medical University and Laboratory Animal Research Center in Dokkyo Medical University guidelines for the care and use of laboratory animals.

Hyperactivated spermatozoa were prepared according to the method described by Fujinoki et al. (2006) using a modified mTALP medium containing 101.02 mmol/l NaCl, 2.68 mmol/l KCl, 2 mmol/l CaCl2, 1.5 mmol/l MgCl26H2O, 0.36 mmol/l Na2HPO4·2H2O, 35.70 mmol/l NaHCO3, 4.5 mmol/l D-glucose, 0.09 mmol/l sodium pyruvate, 9 mmol/l sodium lactate, 0.5 mmol/l hypotaurine, 0.05 mmol/l (−)-epinephrine, 0.2 mmol/l sodium taurocholic acid, 5.26 µmol/l sodium metabisulfite, 0.05% (w/v) streptomycin sulfate, 0.05% (w/v) potassium penicillin G, and 15 mg/ml BSA (pH 7.4 at 37 °C under 5% (v/v) CO2 in air). An aliquot of caudal epididymal spermatozoa was placed on a culture plate (35 mm dish) and several milliliters of the medium were carefully added before incubation for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on the culture plate, and incubated for 4 h at 37 °C under 5% (v/v) CO2 in air to accomplish hyperactivation. Serotonin and agonists were added to the medium after placing motile spermatozoa on the culture plate. For examination of the effects

Figure 9 Effects of GR113808 on α-methylserotonin-enhanced and 5-methoxytryptamine-enhanced hyperactivation. After spermatozoa were exposed to GR113808 for 5 min, they were exposed to α-methylserotonin or 5-methoxytryptamine. The percentages of hyperactivated spermatozoa (A and B) are shown when 100 fmol/l α-methylserotonin, 10 pmol/l 5-methoxytryptamine, and 1 µmol/l GR113808 were added to mTALP medium. Data are given as mean ± s.d. In (A), ‘vehicle’, ‘α-methylserotonin’, ‘GR113808’, and ‘α-methylserotonin + GR113808’ indicate mTALP + 0.1% DMSO, mTALP + 100 fmol/l α-methylserotonin, + 0.1% DMSO, mTALP + 1 µmol/l GR113808 + 0.1% DMSO, and mTALP + 100 pmol/l α-methylserotonin, + 1 µmol/l GR113808 + 0.1% DMSO. In (B), ‘vehicle’, ‘5-methoxytryptamine’, ‘GR113808’, and ‘5-methoxytryptamine + GR113808’ indicate mTALP + 0.1% DMSO, mTALP + 10 pmol/l 5-methoxytryptamine + 0.1% DMSO + 0.1% EtOH, mTALP + 1 µmol/l 5-methoxytryptamine + 0.1% DMSO + 0.1% EtOH, mTALP + 1 µmol/l GR113808 + 0.1% DMSO + 0.1% EtOH, and mTALP + 10 pmol/l 5-methoxytryptamine + 1 µmol/l GR113808 + 0.1% DMSO + 0.1% EtOH. ‘Significant difference in comparison with ‘vehicle’ or ‘GR113808’.

Figure 10 Effects of Ca2+ and albumin on serotonin-enhanced hyperactivation. The percentages of motile spermatozoa (A) and hyperactivated spermatozoa (B) are shown when Ca2+ was removed from the mTALP medium using 1 mmol/l EGTA. Data are given as mean ± s.d. ‘mTALP’, ‘serotonin’, ‘mTALP (EGTA)’, and ‘serotonin (EGTA)’ indicate mTALP only, mTALP + 100 pmol/l serotonin, mTALP without Ca2+, + 1 mmol/l EGTA, and mTALP without Ca2+ + 100 pmol/l serotonin + 1 mmol/l EGTA. The percentages of hyperactivated spermatozoa (C) are shown when albumin was removed from the mTALP medium. Data are given as mean ± s.d. ‘mTALP’, ‘serotonin’, ‘mTALP (− BSA)’, and ‘serotonin (− BSA)’ indicate mTALP, mTALP + 100 pmol/l serotonin, mTALP without BSA, and mTALP without BSA + 100 pmol/l serotonin. Significant difference in comparison with ‘mTALP’; ‘Significant difference in comparison with ‘serotonin’.
Measurement of spermatozoa motility and hyperactivation

Motility and hyperactivation measurements were performed according to the method described by Fujinoki et al. (2006) with some modifications. Spermatozoa suspended in the mTALP medium were diluted tenfold and placed on a culture plate. Motility and hyperactivation were recorded on VHS via a CCD camera (Progressive 3CCD, Sony Corp., Tokyo, Japan) attached to a microscope (IX70, Olympus Corp., Tokyo, Japan) with phase-contrast illumination and a small CO₂ incubator (MI-IBC, Olympus). Each observation was performed at 37 °C, recorded for 2 min, and analyzed by manually counting the number of total spermatozoa, motile spermatozoa, and hyperactivated spermatozoa in ten different fields. Motile spermatozoa that exhibited asymmetric and whiplash flagellar movement and a circular and/or octagonal swimming locus were defined as hyperactivated spermatozoa (Fujinoki et al. 2001a). Motile spermatozoa (%) and hyperactivated spermatozoa (%) were, respectively, defined as the number of motile spermatozoa/number of total spermatozoa×100, and the number of hyperactivated spermatozoa/number of total spermatozoa×100. Experiments were performed four times using four hamsters. Statistical analyses were performed using the post hoc ANOVA test. P<0.05 was considered significant.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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