Serotonin-enhanced hyperactivation of hamster sperm

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Abstract

The effects of serotonin on reproductive function were examined using hamster spermatozoa. When serotonin at concentrations from 1 fmol/l to 1 μ mol/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-(5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-1-methyl-,hydrochloride,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.* 2001*a*, 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, 2004*a*, 2004*b*, 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.* 2001*b*). Ca²⁺ is involved in many intracellular signal transductions, including regulation of AC, and phosphodiesterase and protein

phosphorylation (Visconti & Kopf 1998, Visconti *et al.* 1998, Ho & Suarez 2001, Breitbart 2002, Ho *et al.* 2002, Marín-Briggiler *et al.* 2005).

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 17 β -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 μ mol/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 μ mol/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 μ mol/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 μ mol/l, or 10 μ mol/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 μ mol/l. Although the increase was not significant,

Figure 1 Dose-dependent effects of serotonin 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 serotonin was added to mTALP medium. Data are given as mean $\pm$ s.d. (mTALP): mTALP; (1 amol/l or -18): mTALP+1 amol/l serotonin; (-17): mTALP+10 amol/l serotonin; (-16): mTALP+100 amol/l serotonin; (1 fmol/l or -15): mTALP+1 fmol/l serotonin; (-14): mTALP+10 fmol/l serotonin; (-13): mTALP+100 fmol/l serotonin; (1 pmol/l or -12): mTALP+1 pmol/l serotonin; (-11): mTALP+10 pmol/l serotonin; (-10): mTALP+100 pmol/l serotonin; (1 nmol/l or -9): mTALP+1 nmol/l serotonin; (-8): mTALP+10 nmol/l serotonin; (-7): mTALP+100 nmol/l serotonin; (1 μ mol/l or -6): mTALP+1 μ mol/l serotonin; (-5): mTALP+10 μ mol/l serotonin. ^aSignificant difference in comparison with mTALP or 1 amol/l serotonin; ^bsignificant difference in comparison with mTALP or 1 amol/l or 10 amol/l serotonin; ^csignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μ mol/l, or 10 μ mol/l serotonin; ^dsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or 10 μ mol/l serotonin; ^esignificant difference in comparison with mTALP or between 1 amol/l and 100 amol/l, 1 μ mol/l, and 10 μ mol/l serotonin; ^fsignificant difference in comparison with mTALP or between 1 amol/l and 100 amol/l or 10 μ mol/l serotonin; ^gsignificant difference in comparison with 1 amol/l or between 1 fmol/l and 1 μ mol/l serotonin.

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 μ mol/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 μ mol/l, or 10 μ mol/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 μ mol/l, or 10 μ mol/l. Serotonin at 1 fmol/l and 1 μ mol/l significantly enhanced hyperactivation in comparison with mTALP or serotonin at concentrations between 1 amol/l and 100 amol/l and 10 μ mol/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 μ mol/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 μ mol/l quipazine (Fig. 2). Although 1 μ mol/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 h (Fig. 2B), 1.5 h (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 μ mol/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 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

Figure 2 Effects of quipazine on serotonin-enhanced hyperactivation. The serotonin-induced AR was reportedly suppressed by quipazine (Meizel & Turner 1983), although quipazine is a non-specific 5-HT receptor agonist. After spermatozoa were exposed to 1 μ mol/l quipazine for 5 min, they were exposed to 100 pmol/l serotonin. 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 serotonin and quipazine were added to mTALP medium. Data are given as mean $\pm$ s.d. 'mTALP', 'serotonin', 'quipazine', and 'serotonin + quipazine' indicate mTALP, mTALP + 100 pmol/l serotonin, mTALP + 1 μ mol/l quipazine, and mTALP + 100 pmol/l serotonin + 1 μ mol/l quipazine. ^aSignificant difference in comparison with mTALP. ^bSignificant difference in comparison with quipazine.

10 μ mol/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 μ mol/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 μ mol/l, or 10 μ mol/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 μ mol/l, or 10 μ mol/l. Moreover, 100 amol/l quipazine significantly enhanced hyperactivation in comparison with mTALP or quipazine at 1 amol/l and

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 $\pm$ S.D. (mTALP): mTALP only; (–18): mTALP + 1 amol/l quipazine; (–17): mTALP + 10 amol/l quipazine; (–16): mTALP + 100 amol/l quipazine; (1 fmol/l or –15): mTALP + 1 fmol/l quipazine; (–14): mTALP + 10 fmol/l quipazine; (–13): mTALP + 100 fmol/l quipazine; (1 pmol/l or –12): mTALP + 1 pmol/l quipazine; (–11): mTALP + 10 pmol/l quipazine; (–10): mTALP + 100 pmol/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 μ mol/l or –6): mTALP + 1 μ mol/l quipazine; (–5): mTALP + 10 μ mol/l quipazine. ^aSignificant difference in comparison with mTALP or 1 amol/l, 1 μ mol/l, or 10 μ mol/l quipazine; ^bsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μ mol/l, or 10 μ mol/l quipazine; ^csignificant difference in comparison with mTALP or 1 amol/l or 10 μ mol/l quipazine; ^dsignificant difference in comparison with mTALP or 1 μ mol/l or 10 μ mol/l quipazine; ^esignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 100 nmol/l, 1 μ mol/l, or 10 μ mol/l quipazine; ^fsignificant difference in comparison with mTALP or 10 amol/l, 1 μ mol/l, or 10 μ mol/l quipazine; ^gsignificant difference in comparison with mTALP or 10 μ mol/l quipazine; ^hsignificant difference in comparison with mTALP; ⁱsignificant difference in comparison with mTALP or 1 amol/l or 10 amol/l quipazine.

10 μ mol/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 μ mol/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 μ mol/l. Quipazine at 10 pmol/l significantly enhanced hyperactivation in comparison with mTALP or quipazine at 10 amol/l, 1 μ mol/l, or 10 μ mol/l. Quipazine at 1 nmol/l quipazine significantly enhanced hyperactivation in comparison with mTALP or 10 μ mol/l quipazine. Although the increase was not significant, quipazine at 100 amol/l and 10 nmol/l enhanced hyperactivation. After incubation for 2 h (Fig. 3D), quipazine at 100 amol/l and 1 μ mol/l significantly enhanced hyperactivation in comparison with mTALP. Quipazine at concentrations between 1 fmol/l and 100 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 μ mol/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 μ mol/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 μ mol/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 μ mol/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 nmol/l and 10 μ mol/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

(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 μ mol/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 μ mol/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 μ mol/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 μ mol/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 μ mol/l, or 10 μ mol/l. Moreover, α -methylserotonin at 10 and 100 pmol/l significantly enhanced hyperactivation in comparison with 1 μ mol/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 μ mol/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 affected the percentage of hyperactivated spermatozoa (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 μ mol/l or

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 $\pm$ s.d. (mTALP): mTALP only; (–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 μ mol/l or –6): mTALP + 1 μ mol/l α -methylserotonin; (–5): mTALP + 10 μ mol/l α -methylserotonin. ^aSignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or between 10 pmol/l and 10 μ mol/l; ^bsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or between 1 nmol/l and 10 μ mol/l; ^csignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or between 100 pmol/l and 10 μ mol/l; ^dsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or between 100 nmol/l and 10 μ mol/l; ^esignificant difference in comparison with mTALP or between 1 and 100 amol/l or between 100 pmol/l and 10 μ mol/l; ^fsignificant difference in comparison with mTALP or between 1 and 100 amol/l or between 1 nmol/l and 10 μ mol/l; ^gsignificant difference in comparison with mTALP or between 1 nmol/l and 10 μ mol/l; ^hsignificant difference in comparison with mTALP or between 1 nmol/l and 10 μ mol/l; ⁱsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μ mol/l, or 10 μ mol/l; ^jsignificant difference in comparison with only 1 μ mol/l.

Figure 5 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 $\pm$ 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 μ mol/l or -6): mTALP + 1 μ mol/l 5-methoxytryptamine + 0.1% EtOH; (-5): mTALP + 10 μ mol/l 5-methoxytryptamine + 0.1% EtOH. ^aSignificant difference in comparison with mTALP; ^bsignificant difference in comparison with mTALP or between 1 amol/l and 100 amol/l, 1 μ mol/l, or 10 μ mol/l; ^csignificant difference in comparison with mTALP or between 1 and 100 amol/l or between 100 nmol/l and 10 μ mol/l; ^dsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, 1 μ mol/l, or 10 μ mol/l; ^esignificant difference in comparison with mTALP or between 1 amol/l and 100 amol/l, 1 μ mol/l, or 10 μ mol/l; ^fsignificant difference in comparison with mTALP or 1 amol/l, 10 amol/l, or 10 μ mol/l.

10 μ mol/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 μ mol/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 μ mol/l, or 10 μ mol/l. 5-Methoxytryptamine at 100 pmol/l significantly enhanced hyperactivation in comparison with mTALP or 5-methoxytryptamine at concentrations between 1 amol/l and 100 amol/l or 1 μ mol/l or 10 μ mol/l 5-methoxytryptamine. After incubation for 2 h (Fig. 5D), 5-methoxytryptamine at concentrations between 1 fmol/l and 100 nmol/l significantly enhanced hyperactivation in comparison with mTALP or 5-methoxytryptamine at 1 amol/l, 10 amol/l, or 10 μ mol/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 μ mol/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 μ mol/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 μ mol/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 μ mol/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

Figure 6 Effects of cyproheptadine on serotonin-enhanced and quipazine-enhanced hyperactivation. After spermatozoa were exposed to cyproheptadine for 5 min, they were exposed to serotonin or quipazine. The percentages of hyperactivated spermatozoa (A–D) are shown when 100 pmol/l serotonin, 100 nmol/l serotonin, 100 pmol/l quipazine, 100 nmol/l quipazine, and 1 μ mol/l cyproheptadine were added to mTALP medium. Data are given as mean $\pm$ s.d. In (A), ‘vehicle’, ‘100 pmol/l serotonin’, ‘cyproheptadine’, and ‘100 pmol/l serotonin + cyproheptadine’ indicate mTALP + 0.1% MeOH, mTALP + 100 pmol/l serotonin + 0.1% MeOH, mTALP + 1 μ mol/l cyproheptadine + 0.1% MeOH and mTALP + 100 pmol/l serotonin + 1 μ mol/l cyproheptadine + 0.1% MeOH. In (B), ‘vehicle’, ‘100 nmol/l serotonin’, ‘cyproheptadine’, and ‘100 nmol/l serotonin + cyproheptadine’ indicate mTALP + 0.1% MeOH, mTALP + 100 nmol/l serotonin + 0.1% MeOH, mTALP + 1 μ mol/l cyproheptadine + 0.1% MeOH and mTALP + 100 nmol/l serotonin + 1 μ mol/l cyproheptadine + 0.1% MeOH. In (C), ‘vehicle’, ‘100 pmol/l quipazine’, ‘cyproheptadine’, and ‘100 pmol/l quipazine + cyproheptadine’ indicate mTALP + 0.1% MeOH, mTALP + 100 pmol/l quipazine + 0.1% MeOH, mTALP + 1 μ mol/l cyproheptadine + 0.1% MeOH and mTALP + 100 pmol/l quipazine + 1 μ mol/l cyproheptadine + 0.1% MeOH. In (D), ‘vehicle’, ‘100 nmol/l quipazine’, ‘cyproheptadine’, and ‘100 nmol/l quipazine + cyproheptadine’ indicate mTALP + 0.1% MeOH, mTALP + 100 nmol/l quipazine + 0.1% MeOH, mTALP + 1 μ mol/l cyproheptadine + 0.1% MeOH and mTALP + 100 nmol/l quipazine + 1 μ mol/l cyproheptadine + 0.1% MeOH. *Significant difference in comparison with ‘vehicle’ or ‘cyproheptadine’ or ‘100 pmol/l serotonin + cyproheptadine’. ^bSignificant difference in comparison with ‘vehicle’ or ‘cyproheptadine’ or ‘100 nmol/l serotonin + cyproheptadine’. ^cSignificant difference in comparison with ‘vehicle’ or ‘cyproheptadine’ or ‘100 pmol/l quipazine + cyproheptadine’. ^dSignificant difference in comparison with ‘vehicle’ or ‘cyproheptadine’ or ‘100 nmol/l quipazine + cyproheptadine’.

were exposed to 10 pmol/l 5-methoxytryptamine, the average effective concentration, after exposure to 1 μ mol/l cyproheptadine for 5 min (Fig. 7B).

GR113808 at 1 μ mol/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 μ mol/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 μ mol/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 fmol/l α -methylserotonin after exposure to 1 μ mol/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 μ mol/l GR113808 when spermatozoa were exposed to 10 pmol/l 5-methoxytryptamine after exposure to 1 μ mol/l GR113808 for 5 min (Fig. 9B).

Effects of Ca^{2+} 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 Ca^{2+} , 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 Ca^{2+} . Spermatozoa were not hyperactivated in mTALP medium without Ca^{2+} , 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, 17 β -estradiol, and melatonin (Suelo *et al.* 1993, Yang *et al.* 1994, Fujinoki

Figure 7 Effects of cyproheptadine on α -methylserotonin-enhanced and 5-methoxytryptamine-enhanced hyperactivation. After spermatozoa were exposed to cyproheptadine 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 μ mol/l cyproheptadine were added to mTALP medium. Data are given as mean $\pm$ s.d. In (A), 'vehicle', ' α -methylserotonin', 'cyproheptadine', and ' α -methylserotonin + cyproheptadine' indicate mTALP + 0.1% MeOH, mTALP + 100 fmol/l α -methylserotonin, + 0.1% MeOH, mTALP + 1 μ mol/l cyproheptadine + 0.1% MeOH and mTALP + 100 fmol/l α -methylserotonin, + 1 μ mol/l cyproheptadine + 0.1% MeOH. In (B), 'vehicle', '5-methoxytryptamine', 'cyproheptadine', and '5-methoxytryptamine + cyproheptadine' indicate mTALP + 0.1% MeOH + 0.1% EtOH, mTALP + 10 pmol/l 5-methoxytryptamine + 0.1% MeOH + 0.1% EtOH, mTALP + 1 μ mol/l cyproheptadine + 0.1% MeOH + 0.1% EtOH and mTALP + 10 pmol/l 5-methoxytryptamine + 1 μ mol/l cyproheptadine + 0.1% MeOH + 0.1% EtOH. ^aSignificant difference in comparison with 'vehicle' or 'cyproheptadine' or ' α -methylserotonin + cyproheptadine'. ^bSignificant difference in comparison with 'vehicle' or 'cyproheptadine' or '5-methoxytryptamine + cyproheptadine'.

2008, 2010, Noguchi *et al.* 2008, du Plessis *et al.* 2010). Those ligands are also associated with regulation of the AR (Osman *et al.* 1989, Luconi *et al.* 2004, Baldi *et al.* 2009, Casao *et al.* 2009).

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 μ mol/l and 100 amol/l to 1 μ mol/l respectively. As the effective concentration range of melatonin for enhancement of hyperactivation is 1 pmol/l to 1 μ mol/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-induced and 5-methoxytryptamine-induced AR are inhibited by cyproheptadine. Therefore, it is likely that serotonin induces the AR via 5-HT₂ and 5-HT₄ receptors. Both α -methylserotonin and 5-methoxytryptamine significantly enhanced sperm hyperactivation in a dose-dependent manner (see Figs 4 and 5). The effective concentration ranges of α -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 α -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-HT₂ and 5-HT₄ receptors in a dose-dependent manner.

Generally, 5-HT₂ receptor and 5-HT₄ 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 (IP₃) and diacylglycerol, increases intracellular Ca²⁺, 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 IP₃ is also associated with regulation of hyperactivation (Ho & Suarez 2001). Because serotonin-enhanced hyperactivation was regulated via 5-HT₂ receptors (see Figs 4, 6 and 7), it is likely that 100 pmol/l serotonin and α -methylserotonin enhance hyperactivation through 5-HT₂ receptors and PLC-IP₃ 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-HT₄ receptors (see Figs 5, 8 and 9). As 5-HT₄ receptor induces activation of AC and cAMP production, it is likely that 100 nmol/l serotonin and 5-methoxytryptamine enhance hyperactivation through

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 μ mol/l GR113808 were added to mTALP medium. Data are given as mean $\pm$ 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 μ mol/l GR113808 + 0.1% DMSO, and mTALP + 100 nmol/l serotonin + 1 μ mol/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 μ mol/l GR113808 + 0.1% DMSO, and mTALP + 100 pmol/l serotonin + 1 μ mol/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 μ mol/l GR113808 + 0.1% DMSO, and mTALP + 100 nmol/l quipazine + 1 μ mol/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 μ mol/l GR113808 + 0.1% DMSO, and mTALP + 100 pmol/l quipazine + 1 μ mol/l GR113808 + 0.1% DMSO. ^aSignificant difference in comparison with ‘vehicle’ or ‘GR113808’ or ‘100 nmol/l serotonin + GR113808’. ^bSignificant difference in comparison with ‘vehicle’. ^cSignificant difference in comparison with ‘vehicle’ or ‘GR113808’. ^dSignificant difference in comparison with ‘vehicle’ or ‘GR113808’ or ‘100 nmol/l quipazine + GR113808’. ^eSignificant difference in comparison with ‘vehicle’ or ‘GR113808’ or ‘100 pmol/l quipazine + GR113808’. ^fSignificant difference in comparison with ‘GR113808’.

5-HT₄ 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 μ g/g fresh tissue (Juorio *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 μ g/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 granulosa 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 μ mol/l and between 1 fmol/l and 1 μ mol/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, 17 β -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.

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 μ mol/l GR113808 were added to mTALP medium. Data are given as mean $\pm$ 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 μ mol/l GR113808+0.1% DMSO, and mTALP+100 fmol/l α -methylserotonin, +1 μ mol/l GR113808+0.1% DMSO. In (B), 'vehicle', '5-methoxytryptamine', 'GR113808', and '5-methoxytryptamine+GR113808' indicate mTALP+0.1% DMSO+0.1% EtOH, mTALP+10 pmol/l 5-methoxytryptamine+0.1% DMSO+0.1% EtOH, mTALP+1 μ mol/l GR113808+0.1% DMSO+0.1% EtOH, and mTALP+10 pmol/l 5-methoxytryptamine+1 μ mol/l GR113808+0.1% DMSO+0.1% EtOH. ^aSignificant difference in comparison with 'vehicle' or 'GR113808'. ^bSignificant difference in comparison with 'vehicle' or '5-methoxytryptamine+GR113808'.

Materials and Methods

Chemicals

Serotonin, 2-(1-piperazinyl)quinoline maleate salt (quipazine maleate salt), 5-methoxytryptamine, cyproheptadine hydrochloride sesquihydrate, ($\pm$)-3-(2-aminopropyl)indol-5-ol maleate salt (α -methylserotonin maleate salt or α -methyl-5-hydroxytryptamine maleate salt), and 1-[2-[(methylsulfonyl)-amino]ethyl]-4-piperidinyl)methyl 1-methyl-1*H*-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 CaCl_2 , 1.5 mmol/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.36 mmol/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 35.70 mmol/l NaHCO_3 , 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 μ mol/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) CO_2 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% CO_2 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 10 Effects of Ca^{2+} and albumin on serotonin-enhanced hyperactivation. The percentages of motile spermatozoa (A) and hyperactivated spermatozoa (B) are shown when Ca^{2+} was removed from the mTALP medium using 1 mmol/l EGTA. Data are given as mean $\pm$ s.d. 'mTALP', 'serotonin', 'mTALP (EGTA)', and 'serotonin (EGTA)' indicate mTALP only, mTALP+100 pmol/l serotonin, mTALP without Ca^{2+} +1 mmol/l EGTA, and mTALP without Ca^{2+} +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 $\pm$ 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. ^aSignificant difference in comparison with 'mTALP'; ^bsignificant difference in comparison with 'serotonin'.

of serotonin antagonists, spermatozoa were exposed to serotonin and agonists after exposure to antagonists for 5 min. Cyproheptadine, 5-methoxytryptamine, and GR113808 were dissolved in methanol (MeOH), ethanol (EtOH), and DMSO. In all experiments, the maximal concentration of vehicle was 0.2%.

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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