REPRODUCTION
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Melatonin-enhanced hyperactivation of hamster sperm

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Abstract

The effects of melatonin on reproductive function were examined using hamster spermatozoa. When 1 pM to 1 μM melatonin was added to the mTALP medium, hyperactivation was significantly enhanced. Antagonists and agonists of the melatonin receptor (i.e., MT1 and MT2) were added to the medium. Luzindole, an MT1 and MT2 competitive antagonist, significantly inhibited melatonin-induced hyperactivation, whereas the MT2-specific antagonists, 4-phenyl-2-propionamidotetralin and *N*-pentanoyl-2-benzyltryptamine, had no effect. Moreover, hyperactivation was significantly enhanced when non-specific agonists, such as 6-chloromelatonin and 2-iodomelatonin, were added to the medium. 8-Methoxy-2-propionamidotetralin, which is a strong MT2 agonist and a weak MT1 agonist, significantly increased hyperactivation, although the effect was weak. Therefore, it is likely that melatonin enhances sperm hyperactivation via the MT1 receptor.

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Introduction

N-acetyl-5-methoxytryptamine (melatonin) is produced and secreted by the pineal gland and retina in a circadian rhythm, and it also regulates reproduction in seasonal breeders (Bronson & Heideman 1994, Turek & Van Cauter 1994). In the hamster, reproduction is inhibited by a long exposure to melatonin and stimulated by a short exposure, whereas in sheep, it is stimulated by a long exposure and inhibited by a short exposure. Moreover, acrosin activity is increased in spermatozoa obtained from rams with melatonin implants (Kokolis *et al*. 2000).

Melatonin exerts its effects via specific receptors. Two have been cloned, termed melatonin receptor type 1 (MT1) and type 2 (MT2), and they have seven transmembrane G-protein-coupled receptors (Reppert *et al*. 1994, 1995). The MT1 receptor is localized in the suprachiasmatic nucleus (SCN; Weaver & Reppert 1996), and the MT2 receptor exists in the retina and brain (Reppert *et al*. 1995). Both receptors bind melatonin with high affinity and are associated with the inhibition of cyclic AMP (cAMP) production (Reppert *et al*. 1994, 1995, Browning *et al*. 2000). The MT1 receptor has also been detected in the Leydig cells of the testis and linked to the inhibition of androgen production (Frungieri *et al*. 2005). In the rat SCN, melatonin inhibits the increase in cAMP induced by vasoactive intestinal peptide (Vanecek & Watanabe 1998) and is involved in the regulation of nitric oxide synthase (NOS) activity (Starkey 1996). In the hamster retina, melatonin decreases NOS activity and L-arginine influx, and inhibits the accumulation of cGMP induced by both L-arginine and sodium nitroprusside (Sáenz *et al*. 2002).

In order to fertilize the egg, mammalian spermatozoa have to be capacitated and then undergo the acrosome reaction and hyperactivation. The acrosome reaction is a modified exocytotic event involving the acrosome, a large secretory granule-like organelle in the sperm head, and the overlying sperm plasma membrane (Yudin *et al*. 1988). It is necessary for the penetration of the zona pellucida, the glycoprotein envelope of the ovum, and for sperm–egg plasma membrane fusion (Yanagimachi 1994). Hyperactivation is a specialized movement of the sperm flagellum that creates the propulsive force for the penetration of the zona pellucida. Hyperactivated spermatozoa exhibit large bend amplitude, whiplash, and frenzied flagellar movements (Morisawa 1994, Yanagimachi 1994, Suarez & Ho 2003). Albumin, Ca^{2+}, and HCO_3^- are essential in the process of capacitation of mammalian spermatozoa. Albumin promotes capacitation by removing cholesterol from the sperm plasma membrane (Langlais & Roberts 1985), Ca^{2+} is involved in the intracellular signaling (Visconti & Kopf 1998, Visconti *et al*. 1998, Ho & Suarez 2001, Ho *et al*. 2002), and HCO_3^- stimulates adenylate cyclase to increase cAMP (Okamura *et al*. 1985). Stimulation of these essential components leads to many sperm proteins being phosphorylated during capacitation (Fujinoki *et al*. 2006).

Bornman *et al*. (1989) reported that human seminal plasma contains melatonin, but that it did not affect sperm motility, whereas Van Vuuren *et al*. (1988) suggested that melatonin may have an effect, because

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they found melatonin receptors in human spermatozoa (Van Vuuren *et al*. 1992). A high concentration of melatonin inhibited the quality of sperm motility in rats, according to Gwayi & Bernard (2002), but the regulation of sperm function by melatonin is not yet understood.

Therefore, in the present study, the effects of melatonin on sperm hyperactivation in the hamster, which is a seasonally breeding mammal, were investigated.

Results

Effects of melatonin on sperm hyperactivation

When 1 nM melatonin was added to the mTALP medium, it significantly increased sperm hyperactivation after incubation for 0.5, 1, and 2 h, although it did not affect the % motility of spermatozoa (Fig. 1). At incubation for 0 h, there were no hyperactivated spermatozoa. After incubation for 3 and 4 h, there were no significant differences between 1 nM melatonin and vehicle.

Next, the dose-dependent effect of melatonin on hyperactivation was examined (Fig. 2). At incubation for 0 h, there were no hyperactivated spermatozoa (Fig. 2A). After incubation for only 0.5 h, 1 nM melatonin significantly increased sperm hyperactivation compared with vehicle or 1 fM, 10 fM, and 10 μM of melatonin (Fig. 2B). There were no significant differences between 1 nM melatonin and 100 fM to 1 μM melatonin. After incubation for 1 h (Fig. 2C) or 2 h (Fig. 2D), 1 pM to 1 μM melatonin significantly increased sperm hyperactivation compared with vehicle or 1–100 fM and 10 μM melatonin. Moreover, after incubation for 1 h (Fig. 2C), 10 μM melatonin significantly increased hyperactivation compared with vehicle (Fig. 2C). After incubation for 3 and 4 h, there were no significant differences among the various concentrations (Fig. 2A).

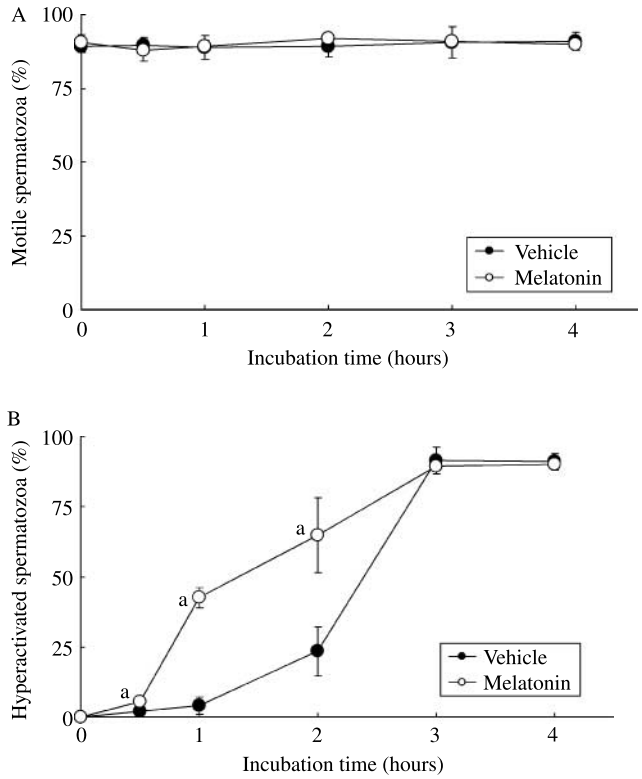

Figure 1 Enhancement of hyperactivation by melatonin. Percentages of (A) motile spermatozoa and (B) hyperactivated spermatozoa are shown, when 1 nM melatonin added to the mTALP medium. Data represent mean±S.D. Vehicle (●) and melatonin (○) indicate mTALP+0.1% ethanol and mTALP+1 nM melatonin+0.1% ethanol respectively. [a]Significant difference compared with vehicle ($P<0.05$).

Effects of melatonin receptor antagonists on sperm hyperactivation

Luzindole, 4-phenyl-2-propionamidotetralin (4P-PDOT), and *N*-pentanoyl-2-benzyltryptamine (DH97), which are melatonin receptor antagonists, were used to investigate the involvement of the melatonin receptors in the regulation of sperm hyperactivation. After incubation for 1 or 2 h, 1 nM and 1 μM luzindole, which is an MT1 and MT2 competitive antagonist, significantly inhibited the sperm hyperactivation induced by 1 nM melatonin, which was an average value of the effective concentrations (Fig. 3A). However, 1 nM melatonin significantly increased sperm hyperactivation after incubation for 1 or 2 h, even if luzindole was added to the medium. 4P-PDOT and DH97, which are MT2-specific antagonists, did not inhibit the % hyperactivated motility of spermatozoa when 1 nM melatonin was added to the medium (Fig. 3B and C). Neither of the melatonin receptor antagonists affected the % motility of spermatozoa (data not shown).

Effects of melatonin receptor agonists on sperm hyperactivation

8-Methoxy-2-propionamidotetralin (8M-PDOT), which is a strong MT2 agonist and a weak MT1 agonist, significantly increased sperm hyperactivation after incubation for 1 or 2 h (Fig. 4A), although it did not affect the % motility of spermatozoa (data not shown). It was observed that 1 pM significantly increased sperm hyperactivation after incubation for 1 h compared with vehicle or 10 fM, 10 pM, 100 pM, 1 μM, and 10 μM 8M-PDOT (Fig. 4B). After incubation for 2 h (Fig. 4C), 100 fM to 10 pM 8M-PDOT significantly increased sperm hyperactivation compared with vehicle and the other concentrations. Moreover, 10 fM 8M-PDOT also significantly increased sperm hyperactivation after incubation for 2 h compared with vehicle only (Fig. 4C).

The two non-specific melatonin agonists, 6-chloromelatonin and 2-iodomelatonin, also significantly increased sperm hyperactivation (Figs 5A and 6A), but did not affect the % motility of spermatozoa (data not shown). As shown in Fig. 5B, after incubation for 0.5 h, 1–100 fM 6-chloromelatonin significantly

increased sperm hyperactivation compared with vehicle or 10 pM to 10 μM, and 1 fM significantly increased sperm hyperactivation compared with 1 pM. Moreover, 1 pM 6-chloromelatonin significantly increased sperm hyperactivation compared with 1 nM, 100 nM, and 10 μM. After incubation for 1 h (Fig. 5C), 1 fM to 10 pM of 6-chloromelatonin significantly increased sperm hyperactivation compared with vehicle and the other concentrations. After incubation for 2 h (Fig. 5D), 1 fM to 10 nM significantly increased sperm hyperactivation compared with vehicle and the other concentrations. After incubation for 3 h (Fig. 5E), 100 nM to 10 μM 6-chloromelatonin significantly suppressed sperm hyperactivation compared with vehicle and the other concentrations.

As shown in Fig. 6B, 1–100 fM 2-iodomelatonin significantly increased hyperactivation after incubation for 0.5 h compared with vehicle or 1 μM and 10 μM. After incubation for 1 h (Fig. 6C), 1 fM to 10 pM 2-iodomelatonin significantly increased sperm hyperactivation compared with vehicle or 1 nM to 10 μM, 100 pM significantly increased sperm hyperactivation compared with vehicle or 10 nM to 10 μM, and 1 nM significantly increased sperm hyperactivation compared with vehicle. Moreover, 100 fM 2-iodomelatonin significantly increased sperm hyperactivation compared with 100 pM. After incubation for 2 h (Fig. 6D), 1 fM to 100 nM 2-iodomelatonin significantly increased sperm hyperactivation compared with vehicle or 1 μM and 10 μM 2-iodomelatonin, neither of which affected sperm hyperactivation at all.

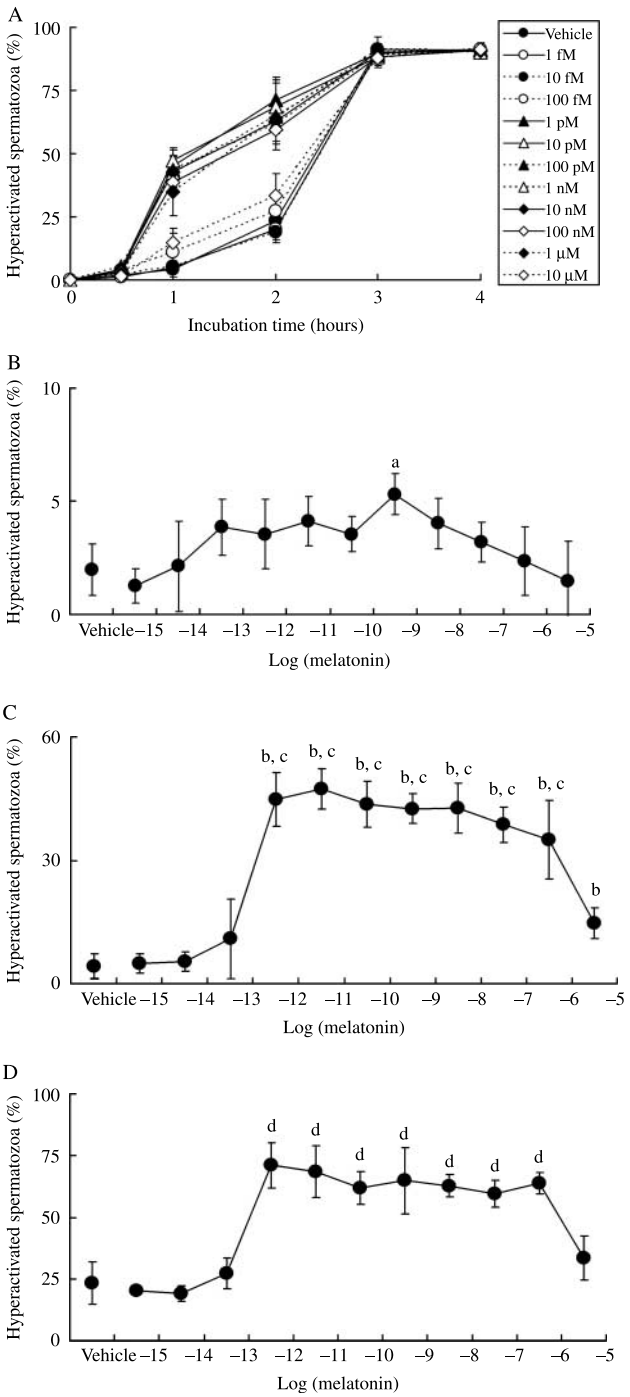

Figure 2 Dose-dependent effects of melatonin on hyperactivation. Percentage of hyperactivated spermatozoa is shown (A) as an overview of effects and after incubation for (B) 0.5, (C) 1, and (D) 2 h after melatonin was added to the mTALP medium. Data represent mean ± S.D. (vehicle), mTALP + 0.1% ethanol; (1 fM or −15), mTALP + 1 fM melatonin + 0.1% ethanol; (10 fM or −14), mTALP + 10 fM melatonin + 0.1% ethanol; (100 fM or −13), mTALP + 100 fM melatonin + 0.1% ethanol; (1 pM or −12), mTALP + 1 pM melatonin + 0.1% ethanol; (10 pM or −11), mTALP + 10 pM melatonin + 0.1% ethanol; (100 pM or −10), mTALP + 100 pM melatonin + 0.1% ethanol; (1 nM or −9), mTALP + 1 nM melatonin + 0.1% ethanol; (10 nM or −8), mTALP + 10 nM melatonin + 0.1% ethanol; (100 nM or −7), mTALP + 100 nM melatonin + 0.1% ethanol; (1 μM or −6), mTALP + 1 μM melatonin + 0.1% ethanol; and (10 μM or −5), mTALP + 10 μM melatonin + 0.1% ethanol. [a]Significant difference compared with vehicle, or 1 fM, 10 fM, and 1 nM–10 μM ($P<0.05$); [b]significant difference compared with vehicle ($P<0.05$); [c]significant difference compared with 1–100 fM and 10 μM ($P<0.05$); [d]significant difference compared with vehicle or 1–100 fM and 10 μM ($P<0.05$).

Effects of Ca^{2+} and albumin on sperm hyperactivation

As shown in Fig. 7A, % motility of spermatozoa was significantly decreased after incubation for 2 h in the mTALP medium without Ca^{2+}, and after incubation for 3 or 4 h, most spermatozoa were not motile. Melatonin did not affect % motility of spermatozoa in the mTALP medium without Ca^{2+}. As for hyperactivation, hamster spermatozoa could not be hyperactivated at all in the mTALP medium without Ca^{2+}, even if melatonin was added (Fig. 7B).

Hamster spermatozoa could not be hyperactivated at all in the mTALP medium without BSA, even if melatonin was added (Fig. 8), although the addition of BSA did not affect % motility of spermatozoa (data not shown).

Discussion

Melatonin is mainly produced and secreted by the pineal gland and retina (Turek & Van Cauter 1994), but Kvetnoy (1999) suggested that it was also produced in other tissues such as the Harderian gland, gut mucosa, cerebellum, airway epithelium, liver, kidney, adrenals, thymus,

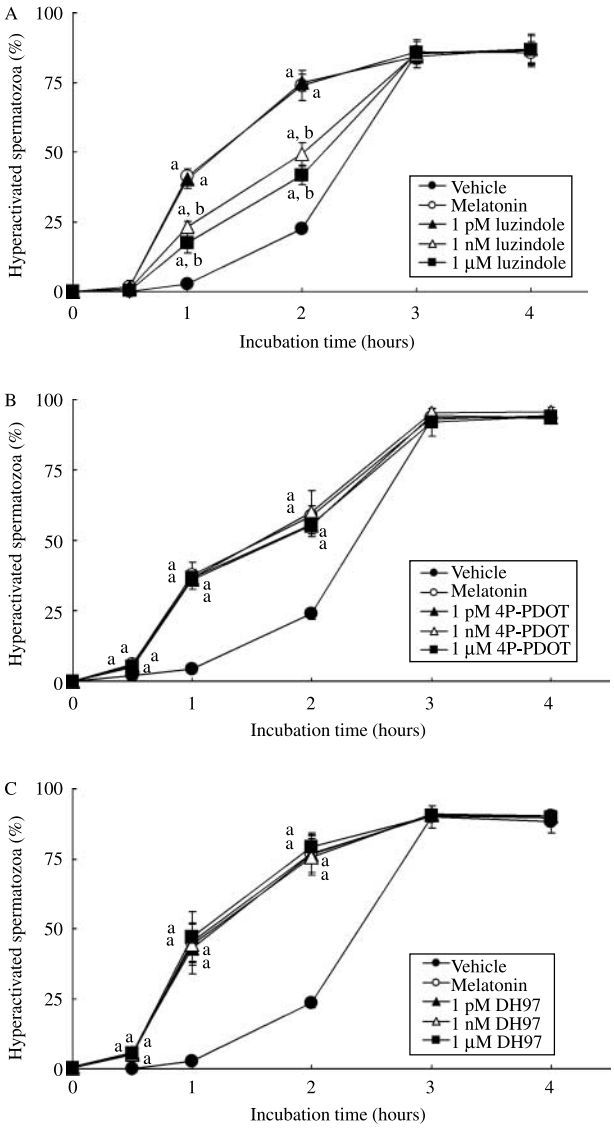

Figure 3 Effects of melatonin receptor antagonists on melatonin-enhanced hyperactivation. (A) Luzindole, which is an MT1 and MT2 competitive receptor antagonist, (B) 4P-PDOT, and (C) DH97, which are MT2-specific receptor antagonists, were respectively added to the mTALP medium before 1 nM melatonin was added to the mTALP medium. Data represent mean±S.D. Vehicle (●), melatonin (○), 1 pM antagonist (▲), 1 nM antagonist (△), and 1 µM antagonist (■) indicate mTALP+0.1% ethanol, mTALP+1 nM melatonin+0.1% ethanol, mTALP+1 nM melatonin+1 pM antagonist+0.1% ethanol, mTALP+1 nM melatonin+1 nM antagonist+0.1% ethanol, and mTALP+1 nM melatonin+1 µM antagonist +0.1% ethanol. [a]Significant difference compared with vehicle ($P<0.05$); [b]significant difference compared with melatonin and 1 pM antagonist ($P<0.05$).

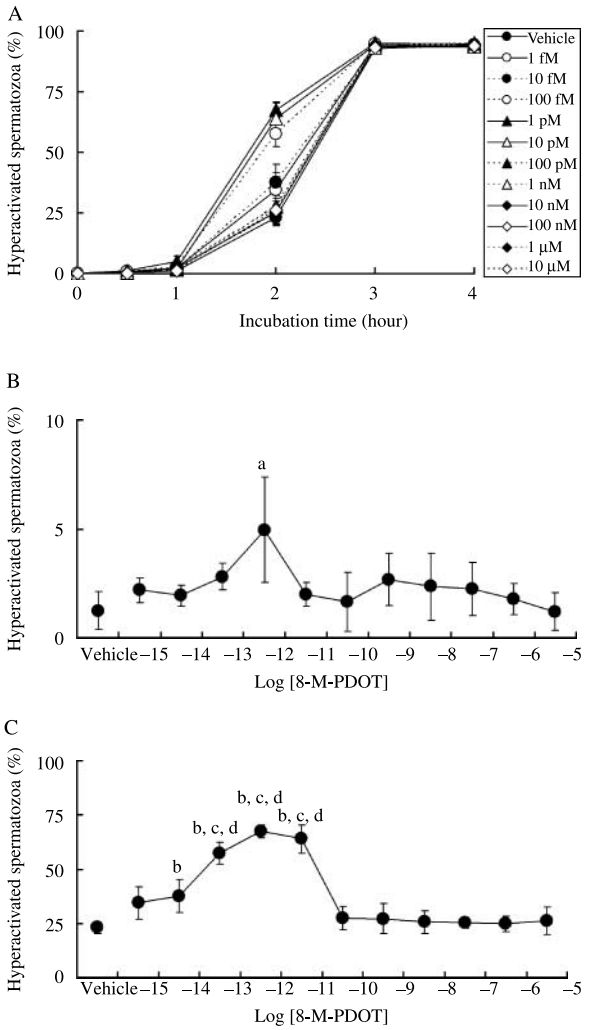

Figure 4 Effects of 8M-PDOT, which is a strong MT2 receptor agonist and a weak MT1 receptor agonist, on melatonin-enhanced hyperactivation. Percentage of hyperactivated spermatozoa is shown (A) as an overview of effects and after incubation for (B) 1 and (C) 2 h after 8M-PDOT was added to the mTALP medium. Data represent mean± S.D. (vehicle), mTALP+0.1% ethanol; (1 fM or −15), mTALP+1 fM 8M-PDOT+0.1% ethanol; (10 fM or −14), mTALP+10 fM 8M-PDOT+0.1% ethanol; (100 fM or −13), mTALP+100 fM 8M-PDOT+0.1% ethanol; (1 pM or −12), mTALP+1 pM 8M-PDOT+ 0.1% ethanol; (10 pM or −11), mTALP+10 pM 8M-PDOT+0.1% ethanol; (100 pM or −10), mTALP+100 pM 8M-PDOT+0.1% ethanol; (1 nM or −9), mTALP+1 nM 8M-PDOT+0.1% ethanol; (10 nM or −8), mTALP+10 nM 8M-PDOT+0.1% ethanol; (100 nM or −7), mTALP+100 nM 8M-PDOT+0.1% ethanol; (1 µM or −6), mTALP+1 µM 8M-PDOT+0.1% ethanol; and (10 µM or −5), mTALP+10 µM 8M-PDOT+0.1% ethanol. [a]Significant difference compared with vehicle or 10 fM, 10 pM, 100 pM, 1 µM, and 10 µM 8M-PDOT ($P<0.05$); [b]significant difference compared with vehicle ($P<0.05$); [c]significant difference compared with 1 fM and 10 fM 8M-PDOT ($P<0.05$); [d]significant difference compared with 100 pM to 10 µM 8M-PDOT ($P<0.05$).

thyroid, pancreas, ovaries, carotid body, placenta and endometrium, and non-neuroendocrine cells, such as mast cells, natural killer cells, eosinophilic leukocytes, ovarian Leydig cells, platelets, and endothelial cells. Therefore, it is likely that melatonin is a key molecule in many functions in many tissues and cells.

Capacitated mammalian spermatozoa exhibit the acrosome reaction and hyperactivation. The acrosome reaction occurs in the sperm head in a ligand-dependent manner (Yudin *et al*. 1988, Yanagimachi 1994) and it has been reported that progesterone, the zona pellucida, epidermal growth factor, and serotonin are ligands that induce the acrosome reaction (Meizel & Turner 1983, Yudin *et al*. 1988, Osman *et al*. 1989, Furuya *et al*. 1994, Fukami *et al*. 2001). A recent study has indicated that progesterone enhanced hyperactivation in a concentration-dependent manner (Noguchi *et al*. 2008), and the results of the present study suggest that melatonin is a new ligand to be hyperactivated on hamster spermatozoa (Fig. 1). Because spermatozoa are hyperactivated in the female reproductive tract, it is probably the melatonin produced and secreted by the ovaries that acts as the ligand in the modulation of sperm hyperactivation.

In the present study, the effective concentration of melatonin for hyperactivation ranged from 1 pM to 1 μM (Fig. 2). In rat spermatozoa, 10 μM melatonin significantly decreased the quality of sperm motility (Gwayi & Bernard 2002), but in hamster spermatozoa, 10 μM melatonin was not effective (Fig. 2). In a human study, the serum melatonin concentration was 10.0±1.4 pg/ml and the follicular fluid melatonin concentration was 36.5±4.8 pg/ml (Brzezinski *et al*. 1987), but in another study Rönnberg *et al*. (1990) reported that the serum melatonin concentration was 38.6±1.8 pM and the follicular fluid melatonin concentration was 98.1±8.9 pM. Moreover, they reported that the follicular fluid melatonin concentration in the

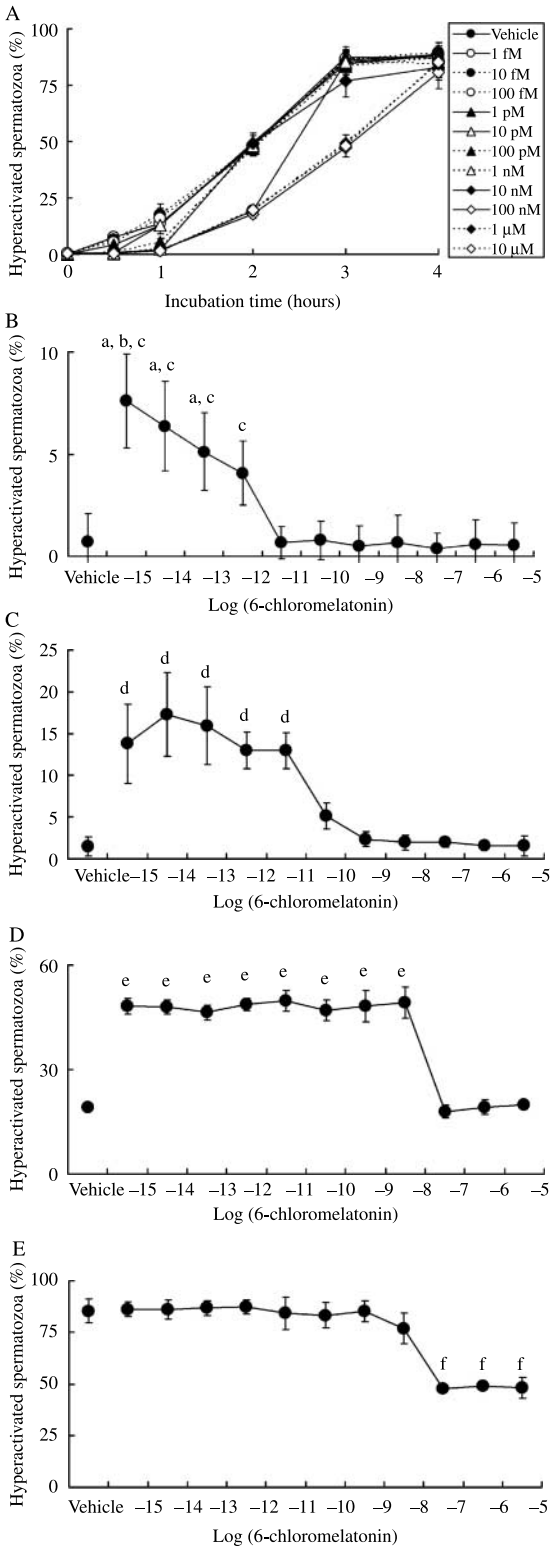

Figure 5 Effects of 6-chloromelatonin, which is a non-specific agonist, on melatonin-enhanced hyperactivation. Percentage of hyperactivated spermatozoa is shown (A) as an overview of effects and after incubation for (B) 0.5, (C) 1, (D) 2, and (E) 3 h after 6-chloromelatonin was added to the mTALP medium. Data represent mean±S.D. (vehicle), mTALP+0.1% ethanol; (1 fM or −15), mTALP+1 fM 6-chloromelatonin+0.1% ethanol; (10 fM or −14), mTALP+10 fM 6-chloromelatonin+0.1% ethanol; (100 fM or −13), mTALP+100 fM 6-chloromelatonin+0.1% ethanol; (1 pM or −12), mTALP+1 pM 6-chloromelatonin+0.1% ethanol; (10 pM or −11), mTALP+10 pM 6-chloromelatonin+0.1% ethanol; (100 pM or −10), mTALP+100 pM 6-chloromelatonin+0.1% ethanol; (1 nM or −9), mTALP+1 nM 6-chloromelatonin+0.1% ethanol; (10 nM or −8), mTALP+10 nM 6-chloromelatonin+0.1% ethanol; (100 nM or −7), mTALP+100 nM 6-chloromelatonin+0.1% ethanol; (1 μM or −6), mTALP+1 μM 6-chloromelatonin+0.1% ethanol; and (10 μM or −5), mTALP+10 μM 6-chloromelatonin+0.1% ethanol. [a]Significant difference compared with vehicle or 10 pM, 100 pM, 10 nM, and 1 μM 6-chloromelatonin ($P<0.05$); [b]significant difference compared with 1 pM 6-chloromelatonin ($P<0.05$); [c]significant difference compared with 1 nM, 100 nM, and 10 μM 6-chloromelatonin ($P<0.05$); [d]significant difference compared with vehicle or 100 pM to 10 μM 6-chloromelatonin ($P<0.05$); [e]significant difference compared with vehicle or 100 nM to 10 μM 6-chloromelatonin ($P<0.05$); [f]significant difference compared with vehicle or 1 fM to 10 nM 6-chloromelatonin ($P<0.05$).

morning was 58.9±3.8 and 23.2±0.8 pM during the day. Although the follicular fluid melatonin concentration in rodents is not known, it is likely to be the same as in humans because serum melatonin concentration in rodents and humans is the same (Brzezinski *et al*. 1987, Rönnberg *et al*. 1990, Bronson & Heideman 1994). In the present study, 1 pM to 1 μM melatonin enhanced sperm hyperactivation (Fig. 2), so it is likely that melatonin in both serum and follicular fluid enhances sperm hyperactivation.

Melatonin exerts its effects via receptors, such as MT1 and MT2 (Reppert *et al*. 1994, 1995), which are seven transmembrane G-protein-coupled receptors (Reppert *et al*. 1994, 1995). Dubocovich (1988) reported that MT1 was of high-affinity type (picomolar range) and MT2 was of low-affinity type (nanomolar range) in the rodent brain and the chicken retina. Moreover, a melatonin receptor has been found in human spermatozoa and classified as MT2 because it had low affinity in a saturation assay (Van Vuuren *et al*. 1992). It has been suggested that the melatonin receptor is localized in the sperm midpiece (Pitout *et al*. 1991, du Toit *et al*. 1994), although Browning *et al*. (2000) recently reported that the affinities of human recombinant MT1 and MT2 were essentially similar. Therefore, the type of melatonin receptor in sperm is yet to be determined.

Three melatonin receptor antagonists (luzindole, 4P-PDOT, and DH97; Fig. 3) and three melatonin receptor agonists (8M-PDOT, 6-chloromelatonin, and 2-iodomelatonin; Figs 4–6) were used to investigate the type of sperm melatonin receptor associated with melatonin-enhanced hyperactivation. Luzindole is an MT1 and MT2 competitive antagonist and has a higher affinity for the MT2 receptor (11-fold; Browning *et al*. 2000). Both 4P-PDOT and DH97 are MT2-specific antagonists. 8M-PDOT is a strong MT2 agonist and a weak MT1 agonist, whereas 6-chloromelatonin and 2-iodomelatonin are non-specific agonists. Data obtained after the addition of melatonin receptor antagonists and agonists to the medium suggest that stimulation and inhibition of the MT1 receptor regulates melatonin-enhanced hyperactivation of hamster sperm, so it is likely that the receptor is MT1, although there is a

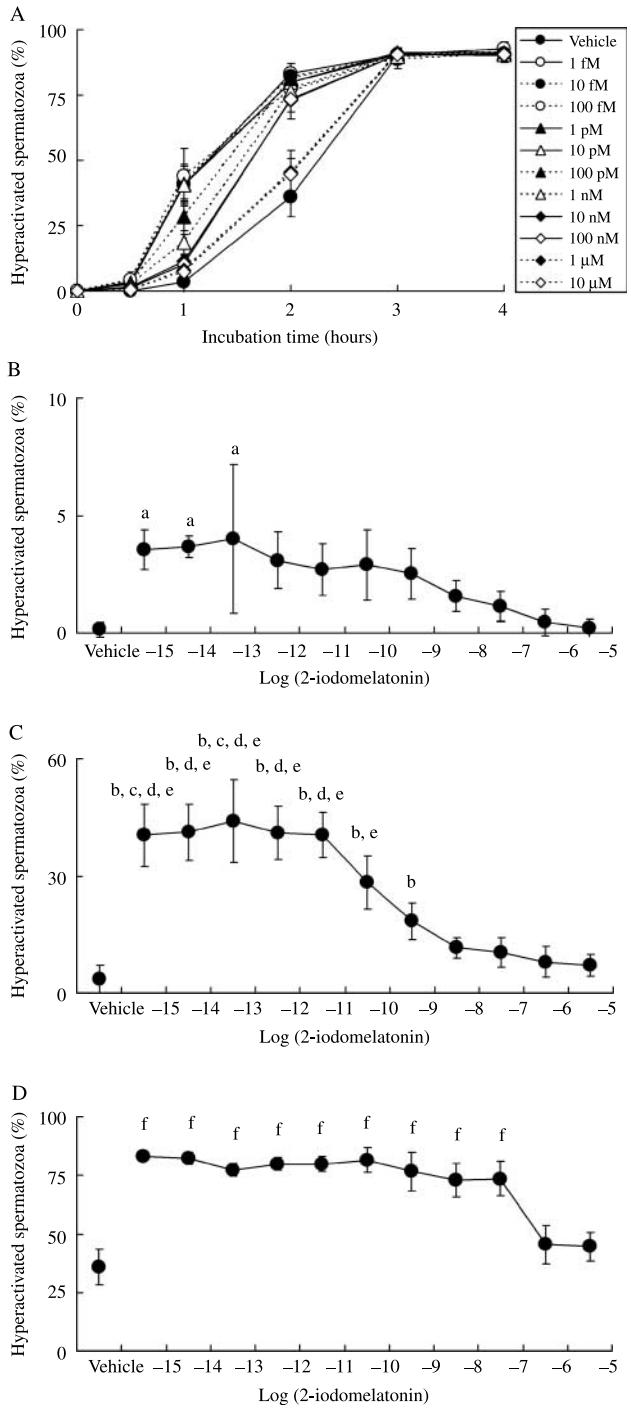

Figure 6 Effects of 2-iodomelatonin, which is a non-specific agonist, on melatonin-enhanced hyperactivation. Percentage of hyperactivated spermatozoa is shown (A) as an overview of effects and after incubation for (B) 0.5, (C) 1, and (D) 2 h after 2-iodomelatonin was added to the mTALP medium. Data represent mean±S.D. (vehicle), mTALP+0.1% ethanol; (1 fM or −15), mTALP+1 fM 2-iodomelatonin+0.1% ethanol; (10 fM or −14), mTALP+10 fM 2-iodomelatonin+0.1% ethanol; (100 fM or −13), mTALP +100 fM 2-iodomelatonin+0.1% ethanol; (1 pM or −12), mTALP+1 pM 2-iodomelatonin+0.1% ethanol; (10 pM or −11), mTALP+10 pM 2-iodomelatonin+0.1% ethanol; (100 pM or −10), mTALP+100 pM 2-iodomelatonin+0.1% ethanol; (1 nM or −9), mTALP+1 nM 2-iodomelatonin+0.1% ethanol; (10 nM or −8), mTALP+10 nM 2-iodomelatonin+0.1% ethanol; (100 nM or −7), mTALP+100 nM 2-iodomelatonin+0.1% ethanol; (1 μM or −6), mTALP+1 μM 2-iodomelatonin+0.1% ethanol; and (10 μM or −5), mTALP+10 μM 2-iodomelatonin+0.1% ethanol. [a]Significant difference compared with vehicle or 1 μM and 10 μM 2-iodomelatonin ($P<0.05$); [b]significant difference compared with vehicle ($P<0.05$); [c]significant difference compared with 100 pM 2-iodomelatonin ($P<0.05$); [d]significant difference compared with 100 nM 2-iodomelatonin ($P<0.05$); [e]significant difference compared with 10 nM to 10 μM 2-iodomelatonin ($P<0.05$); [f]significant difference compared with vehicle or 1 and 10 μM 2-iodomelatonin ($P<0.05$).

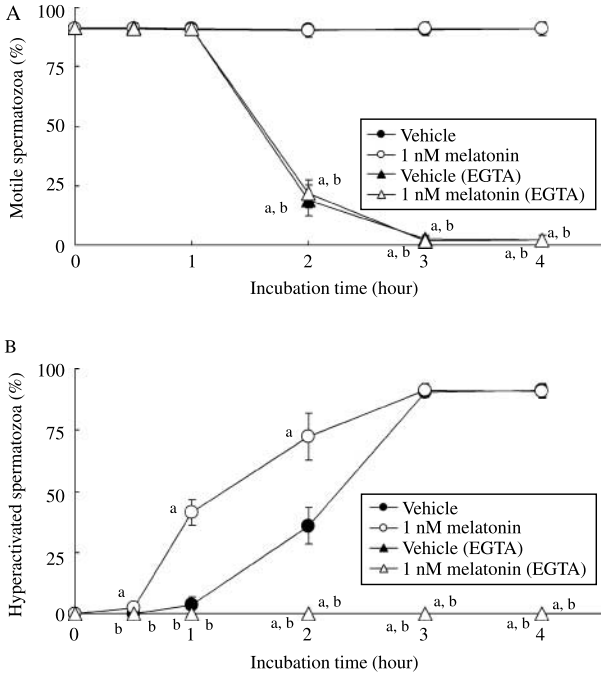

Figure 7 Effects of Ca^{2+} on melatonin-enhanced hyperactivation. Percentages of (A) motile and (B) hyperactivated spermatozoa are shown, when Ca^{2+} was removed from the mTALP medium using 1 mM EGTA. Data represent mean±S.D. Vehicle (●), 1 nM melatonin (○), vehicle (EGTA) (▲), and 1 nM melatonin (EGTA) (△) indicate mTALP+0.1% ethanol, mTALP+1 nM melatonin+0.1% ethanol, mTALP without Ca^{2+}+1 mM EGTA+0.1% ethanol, and mTALP without Ca^{2+}+1 nM melatonin+1 mM EGTA+0.1% ethanol. [a]Significant difference compared with vehicle; [b]significant difference compared with 1 nM melatonin.

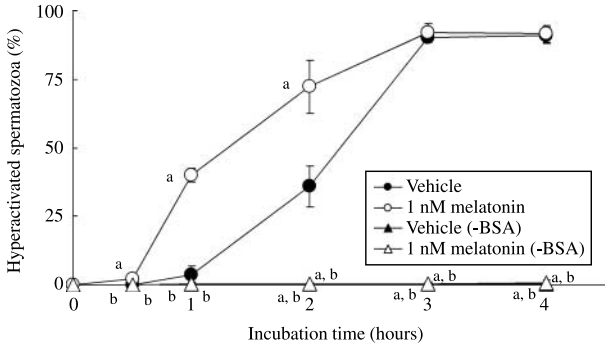

Figure 8 Effect of albumin on melatonin-enhanced hyperactivation. Percentage of hyperactivated spermatozoa is shown when albumin was removed from the mTALP medium. Data represent mean±S.D. Vehicle (●), 1 nM melatonin (○), vehicle (-BSA) (▲), and 1 nM melatonin (-BSA) (△) indicate mTALP+0.1% ethanol, mTALP+1 nM melatonin+0.1% ethanol, mTALP without albumin+0.1% ethanol, and mTALP without albumin+1 nM melatonin+0.1% ethanol. [a]Significant difference compared with vehicle; [b]significant difference compared with 1 nM melatonin.

need to detect and identify melatonin receptor protein from spermatozoa in future studies.

In order to be hyperactivated, hamster spermatozoa need extracellular Ca^{2+} and albumin (Noguchi *et al.* 2008). Because melatonin significantly increased sperm hyperactivation only under these conditions (Figs 7 and 8), it is likely that melatonin modulates sperm hyperactivation and controls the timing of it.

Melatonin signals negatively affect cAMP production and NO production in cells and tissues (Reppert *et al.* 1994, 1995, Starkey 1996, Vanecek & Watanabe 1998, Browning *et al.* 2000, Sáenz *et al.* 2002, Frungieri *et al.* 2005). cAMP is very important for sperm function, because sperm flagellar movement and capacitation occur in a cAMP-dependent manner (Morisawa 1994, Visconti & Kopf 1998, Visconti *et al.* 1998, Fujinoki *et al.* 2001*a*, 2001*b*). It has been suggested that NOS is positively involved in the regulation of capacitation under low concentrations (O'Flaherty *et al.* 2006, Agarwal *et al.* 2008, de Lamirande & O'Flaherty 2008), because high concentrations of NO negatively affects sperm function (Iwasaki & Gagnon 1992, Agarwal *et al.* 2008). Low concentrations of NO stimulate a MAP kinase cascade and tyrosine phosphorylation during sperm capacitation (de Lamirande & O'Flaherty 2008). Although it is not well known whether a MAP kinase cascade regulates sperm hyperactivation, it is widely accepted that tyrosine phosphorylation is associated with sperm hyperactivation (Visconti & Kopf 1998, Visconti *et al.* 1998, Fujinoki *et al.* 2001*b*, 2006). Moreover, it has been suggested that tyrosine phosphorylation is regulated through a MAP kinase cascade (de Lamirande & O'Flaherty 2008). Therefore, it is likely that a negative effect of melatonin on NO production, but not on cAMP production, is involved in the modulation of sperm hyperactivation.

Materials and Methods

Chemicals

Melatonin was purchased from Sigma Chemical Company. 4P-PDOT, 8M-PDOT, *N*-acetyl-2-benzyltryptamine (luzindole), DH97, 6-chloromelatonin (*N*-[2-(6-chloro-5-methoxyindol-3-yl)ethyl]acetamide), and 2-iodomelatonin (*N*-[2-(2-iodo-5-methoxyindol-3-yl)ethyl]acetamide) were purchased from Tocris Cookson Ltd (Hung Rd, Bristol, UK). BSA fraction V was purchased from Merck KGaA. Other chemicals of reagent grade were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of hyperactivated spermatozoa

Spermatozoa were obtained from the caudal epididymis of sexually mature (12 to 20 weeks old) male golden hamsters (*Mesocricetus auratus*) that were housed in accordance with the guidelines of the Dokkyo Medical University and the Laboratory Animal Research Center in Dokkyo Medical University for the care and use of laboratory animals.

Hyperactivated spermatozoa were prepared according to the method described by Si & Okuno (1999) using the mTALP

(modified Tyrode's albumin lactate pyruvate) medium. An aliquot of caudal epididymal spermatozoa was placed at the bottom of a test tube and several milliliters of medium were carefully added before incubation for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on a culture plate (35 mm dish), and incubated for 4 h at 37 °C under 5% CO_2 in air to accomplish hyperactivation. Melatonin, its agonists and melatonin receptor antagonist dissolved in ethanol (EtOH) were added to the medium after placing motile spermatozoa on the culture plate. In all experiments, the final concentration of EtOH was 0.1%.

Measurement of the motility and hyperactivation of spermatozoa

Motility and hyperactivation measurements were performed according to the method of 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 S-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 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 spermatozoa, motile spermatozoa, and hyperactivated spermatozoa in 20 different fields. Motile spermatozoa that exhibited asymmetric and whiplash flagellar movement (Fujinoki *et al.* 2001*a*) and a circular and/or octagonal swimming locus were defined as hyperactivated spermatozoa. Motile (%) 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 analysis was carried out using the Student's *t*-test or the *post hoc* test of ANOVA. $P<0.05$ 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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