Characteristics of Inhibitory Effects of Serotonin (5-HT)3-Receptor Antagonists, YM060 and YM114 (KAE-393), on the von Bezold-Jarisch Reflex Induced by 2-Methyl-5-HT, Veratridine and Electrical Stimulation of Vagus Nerves in Anesthetized Rats

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ABSTRACT—We evaluated the inhibitory effects of YM060 \((R)-5-[(1\text{-methyl}-1H\text{-indol-3-yl})\text{carbonyl}]4,5,6,7\text{-tetrahydro-1H-benzimidazole monohydrochloride}\) and YM114 (KAE-393) \((R)-5-[(1\text{-indolyl})\text{carbonyl}]4,5,6,7\text{-tetrahydro-1H-benzimidazole monohydrochloride}\) on the von Bezold-Jarisch reflex (BJR) induced by 2-methyl-5-HT, a selective serotonin (5-HT)3-receptor agonist; veratridine, which stimulates chemoreceptors and baroreceptors; and electrical stimulation of vagal efferent nerves in anesthetized rats. Results were compared with those of ondansetron and granisetron. 2-Methyl-5-HT (5–160 \(\mu g/kg\), i.v.) and veratridine (100–200 \(\mu g/kg\), i.v.) dose-dependently decreased the heart rate (BJR). YM060, YM114, ondansetron and granisetron dose-dependently inhibited 2-methyl-5-HT (40 \(\mu g/kg\), i.v.)-induced BJR, with ID50 values of 0.012, 0.060, 0.97 and 0.15 \(\mu g/kg\), i.v., respectively. Their 5-HT3 receptor blocking potencies against 2-methyl-5-HT-induced BJR were largely consistent with those against 5-HT-induced BJR. In contrast, higher doses (100 \(\mu g/kg\), i.v.) of YM060, YM114, ondansetron and granisetron did not inhibit veratridine (150 \(\mu g/kg\), i.v.)-induced BJR. Atropine (300 \(\mu g/kg\), i.v.) abolished bradycardia induced by electrical stimulation of vagal efferent nerves, whereas YM060, YM114, ondansetron and granisetron had no effect at a dose of 1000 \(\mu g/kg\), i.v. 5-HT (0.625–5.0 \(\mu g\)) injected into the left ventricle also caused a dose-dependent decrease in heart rate, an effect that was abolished by YM060 (0.1 \(\mu g/kg\), i.v.), atropine (100 \(\mu g/kg\), i.v.) and vagotomy. These results suggest that YM060 and YM114 are highly potent and selective 5-HT3-receptor antagonists that do not affect veratridine- or electrical stimulation-induced bradycardia in anesthetized rats. They also suggest that 5-HT-induced BJR in anesthetized rats originates from 5-HT3 receptors located on the endings of vagal afferent nerves in the heart.

Keywords: 5-HT3-receptor antagonist, von Bezold-Jarisch reflex, Vagus nerve, Veratridine

Serotonin (5-HT) is widely distributed and is implicated in a variety of physiological responses. Research into 5-HT-receptor subtypes, especially the 5-HT3 receptor, has advanced in recent years. 5-HT3 receptors are located on central, sensory and autonomic nerves and on the myenteric plexus (1, 2), and have been implicated in anxiety (3, 4), vomiting responses to cancer chemotherapeutic agents (5, 6) and stress-induced gastrointestinal disorders (7).

Bolus intravenous injection of 5-HT causes transient bradycardia (the von Bezold-Jarisch reflex; BJR) in many species including rats, cats, dogs and rabbits (8–11). Fozard (12) reported that this reflex in anesthetized rats is mediated through the activation of 5-HT3 receptors located on endings of vagal afferent nerves. Activation of these receptors lead to the stimulation of vagus nerves, resulting in the bradycardia. 5-HT1- and 5-HT2-receptor antagonists had no effects on BJR induced by 5-HT in anesthetized rats (13). BJR in anesthetized rats is therefore frequently utilized as an in vivo system for the evaluation of 5-HT3-receptor blocking effects. Bradycardiac reflex through the stimulation of vagus nerves is evoked not only by activation of 5-HT3 receptors but also by activation of chemoreceptors in the heart and electrical stimulation of vagus nerves. It was reported that veratridine-induced BJR was evoked by ACh released from
vagal efferent nerves through activation of chemoreceptors and baroreceptors located on the endings of vagal afferent nerves in the heart (14–17). Therefore, bradycardia induced by 5-HT, veratridine or electrical stimulation of vagus nerves is inhibited by anti-cholinergic agents, nicotine-receptor antagonists, local anesthetics and Na⁺ channel blockers.

YM060 and YM114 are 5-HT₃-receptor antagonists that inhibit 5-HT-induced BJR (18, 19), but only showed very low affinities for 5-HT₁-like, 5-HT₂, α₁, α₂, β₁, β₂, D₂, M₂, H₁, H₂, μ-opioid and benzodiazepine receptors in in vitro studies (20–22). However, the direct effects of YM060 and YM114 on vagus nerves, nicotine receptors and Na⁺ channels were not examined. In the present study, we evaluated the 5-HT₃ receptor blocking activities of YM060 and YM114 on BJR induced by 2-methyl-5-HT, a selective 5-HT₃-receptor agonist, in comparison with those of ondansetron and granisetron. We also investigated the characteristics and selectivity of these 5-HT₃-receptor antagonists by evaluating their inhibitory effects on bradycardia induced by veratridine, which stimulates chemoreceptors and baroreceptors, and electrical stimulation of vagal efferent nerves in anesthetized rats.

Various sensory receptors are located within the heart and the endings of vagal afferent nerve are distributed mainly in the left ventricle. To investigate the site of action of the 5-HT₃-receptor agonist and antagonist, we also evaluated the effect of intraventricular injection of 5-HT on the cardiovascular response in anesthetized rats.

MATERIALS AND METHODS

Animals
Male Wistar rats (220–310 g) were used (SLC, Hamamatsu). Food and water were available ad libitum. The rats were anesthetized with urethane (1.25 g/kg, i.p.) and a polyethylene tube was inserted into the trachea for artificial ventilation (60 respirations/min; tidal volume, 3.0 ml). The left common carotid artery was cannulated with a polyethylene catheter connected to a pressure transducer (MPU-0.5; Nihon Kohden, Tokyo). The pressure signal was amplified with a carrier amplifier (AP-621G, Nihon Kohden), and the heart rate was measured with a cardiotachometer (AT-600G, Nihon Kohden) triggered by the blood pressure pulse. The blood pressure and heart rate were monitored continuously and recorded (RMP-6008, Nihon Kohden). For intravenous drug administration, a catheter was inserted into the left femoral vein.

2-Methyl-5-HT-induced BJR
A dose-response curve for 2-methyl-5-HT-induced bradycardia was constructed by i.v. bolus injection of increasing doses of 2-methyl-5-HT (5, 10, 20, 30, 40 and 160 μg/kg) every 15 min. A submaximal dose (40 μg/kg, i.v.) of 2-methyl-5-HT was used to evaluate the inhibitory effects of antagonists. In a preliminary study, the time-dependency of changes in response to 2-methyl-5-HT was examined by injection of this submaximal dose 8 times at 20-min intervals. Because 2-methyl-5-HT under these conditions caused a constant decrease in heart rate beginning after the first injection, test drugs were injected after the 3rd or 4th dose. After a control response to 2-methyl-5-HT was obtained, the inhibitory effects of i.v. doses of test drugs on 2-methyl-5-HT-induced bradycardia were measured at 10 min after dosing. Test drugs were injected by threefold increasing doses.

Veratridine-induced BJR
A dose-response curve for veratridine-induced bradycardia was constructed by increasing i.v. bolus doses of veratridine (50, 100, 150, 200 and 400 μg/kg) every 20 min. A submaximal dose (150 μg/kg, i.v.) of veratridine was used to evaluate the inhibitory effects of antagonists. In the preliminary study, the time-dependency of changes in response to veratridine was examined by injection of this submaximal dose 6 times at 15-min intervals. Because veratridine under these conditions caused a constant decrease in heart rate beginning after the first injection, test drugs were injected after the 2nd to 4th dose. After a control response to veratridine was obtained, the inhibitory effects of i.v. doses of test drugs on veratridine-induced bradycardia were measured at 10 min after dosing.

Electrical stimulation of vagal efferent nerve-induced bradycardia
After insertion of a polyethylene catheter into the left common carotid artery, the right vagus nerve was detached and cut at the cervical part. The peripheral endings of the sectioned nerves were electrically stimulated (30 V, 1 msec, for 5 sec) with an electrical stimulator (SEN-3301, Nihon Kohden). A frequency-response curve for electrical stimulation-induced bradycardia was constructed by increasing the frequency of electrical stimulation (5, 10, 20 and 40 Hz) every 5 min. Because the frequency-response curve for electrical stimulation was reproducible in the same animal, the inhibitory effects of the test drugs were examined by i.v. injection 10 min before the second frequency-response curve was constructed.

BJR induced by 5-HT injected into the left ventricle
After insertion of a polyethylene catheter into the left common carotid artery, laparotomy was carried out. The xiphoid was raised, and a microsyringe was inserted into the left ventricle through the diaphragmatic surface for
5-HT administration. The experimental protocol for i.v. injection of 5-HT was the same as that for the 2-methyl-5-HT-induced Bezold-Jarisch reflex. A dose-response curve for 5-HT-induced bradycardia was constructed by increasing doses of intraventricular injection of 5-HT (0.625, 1.25, 2.5 and 5.0 μg) every 15 min. Intravenous injection of 5-HT at a dose of 30 μg/kg and intraventricular injection at 2.5 μg/rat were used to evaluate the influence of i.v.-injected YM060 and atropine, and bilateral vagotomy. After control responses to intravenous and intraventricular injection of 5-HT were obtained, the inhibitory effects of i.v. doses of YM060 and atropine, and bilateral vagotomy on 5-HT-induced bradycardia were measured at 10 min after dosing or vagotomy. YM060 was injected by three-fold increasing doses. Bilateral vagotomy was carried out at the cervical level.

Statistical analyses
All values are expressed as the mean ± S.E.M. or as the mean with 95% confidence limits. Probit analysis was used to obtain ID50 values. Statistical significance of values for the inhibitory effect after i.v. dosing was determined by the paired Student’s t-test. Probabilities of < 5% (P < 0.05) were considered significant.

Drugs
YM060 {(R)-5-[(1-methyl-1H-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole monohydrochloride}, YM114 (KAE-393) {(R)-5-[(1-indolinyl)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole monohydrochloride}, ondansetron, granisetron and 2-methyl-5-HT were prepared by Yamanouchi Pharmaceutical Co., Ltd. 5-HT creatinine sulfate, veratridine and atropine sulfate were purchased from E. Merck (Darmstadt, FRG), Sigma Chemical Co. (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka), respectively. All drug doses are expressed as the free base. Veratridine was dissolved in a minimal amount of 0.1 N HCl solution and diluted with physiological saline. Other drugs were dissolved in physiological saline. 5-HT was injected into the left ventricle at a volume of 50 μl/rat. Other drugs were intravenously administered to rats at a volume of 1 ml/kg.

RESULTS

Inhibitory effects of 5-HT3-receptor antagonists on 2-methyl-5-HT-induced BJR
The basal heart rate in anesthetized rats was 306.6 ± 8.1 beats/min (n = 23). Bolus i.v. injection of 2-methyl-5-HT (5, 10, 20, 30, 40, 80 and 160 μg/kg) dose-dependently produced transient bradycardia (Fig. 1A). A submaximal response to 2-methyl-5-HT was obtained at a dose of 40 μg/kg, i.v., while the maximum decrease in heart rate was obtained at 160 μg/kg, i.v. (253.0 ± 18.5 beats/min). In the following experiments, we evaluated the antagonistic activity of the test drugs in blocking the response to a standard dose of 2-methyl-5-HT, 40 μg/kg, i.v. After i.v.
injection, YM060 (0.003–0.03 μg/kg), YM114 (0.03–0.3 μg/kg), ondansetron (0.3–3 μg/kg) and granisetron (0.03–1 μg/kg) dose-dependently inhibited bradycardia induced by 2-methyl-5-HT, with ID₅₀ values (95% confidence limits) of 0.012 (0.007–0.023), 0.060 (0.050–0.073), 0.97 (0.80–1.17) and 0.15 (0.10–0.22) μg/kg, i.v., respectively (Fig. 1B, Table 1). YM060, YM114, ondansetron and granisetron had no effects on the resting heart rate up to 1000 μg/kg, i.v.

Inhibitory effects of 5-HT₃-receptor antagonists on veratridine-induced BJR

Bolus i.v. injection of veratridine (50, 100, 150, 200 and 400 μg/kg) dose-dependently produced transient bradycardia in anesthetized rats. A submaximal response to veratridine was obtained at a dose of 150 μg/kg, i.v., while the maximum decrease in heart rate was obtained at 200 μg/kg, i.v. (199.0±26.0 beats/min). In the following experiments, we evaluated the effect of the test drugs in blocking the response to a standard dose of veratridine, 150 μg/kg, i.v. YM060, YM114, ondansetron and granisetron did not inhibit veratridine-induced BJR at a dose of 100 μg/kg, i.v.

Inhibitory effects of 5-HT₃-receptor antagonists on electrical stimulation-induced bradycardia

Electrical stimulation (5, 10, 20 and 40 Hz) of right vagal efferent nerves induced frequency-dependent and transient bradycardia in anesthetized rats. Atropine (300 μg/kg, i.v.) abolished this electrical stimulation-induced bradycardia, whereas YM060 had no effect at a dose of 1000 μg/kg, i.v. (Fig. 2). YM114, ondansetron and granisetron also failed to inhibit electrical stimulation-induced bradycardia (data not shown).

BJR induced by 5-HT injected into the left ventricle

To investigate the site of action of the 5-HT₃-receptor agonist and antagonist, 5-HT was injected into the left ventricle in anesthetized rats. Intraventricular 5-HT (0.625, 1.25, 2.5 and 5.0 μg) caused dose-dependent decreases in heart rate (Fig. 3A). After i.v. injection, YM060 (0.01–0.1 μg/kg) dose-dependently inhibited bradycardia induced by intravenous (30 μg/kg, n=3–4) and intraventricular (2.5 μg/rat, n=3–4) injections of 5-HT, with ID₅₀ values (95% confidence limits) of 0.036 (0.031–0.041) and 0.026 (0.024–0.030) μg/kg, i.v., respectively (Fig. 3B). Bilateral vagotomy and i.v. application of atropine completely abolished the bradycardia induced by intravenous (30 μg/kg, n=3–4) and intraventricular (2.5 μg/rat, n=3) injections of 5-HT.

| Compounds    | ID₅₀ (μg/kg, i.v.) | n  |
|--------------|--------------------|----|
| YM060        | 0.012 (0.007–0.023) | 4  |
| YM114        | 0.060 (0.050–0.073) | 3–5|
| Ondansetron  | 0.97 (0.80–1.17)    | 3–4|
| Granisetron  | 0.15 (0.10–0.22)    | 3–4|

Figures in parentheses represent 95% confidence limits.

Table 1. Inhibitory effects of YM060, YM114, ondansetron and granisetron on 2-methyl-5-HT-induced bradycardia in anesthetized rats

| Frequency (Hz) | Decrease in heart rate (beats/min) |
|---------------|-----------------------------------|
| 0             | 0                                 |
| 5             | 200                               |
| 10            | 300                               |
| 20            | 300                               |
| 30            | 300                               |
| 40            | 300                               |
| 50            | 300                               |

Fig. 2. Inhibitory effects of atropine (○: control, ●: after 300 μg/kg, i.v.) (A) and YM060 (○: control, ●: after 1000 μg/kg, i.v.) (B) on bradycardia induced by electrical stimulation of vagal efferent nerves in anesthetized rats. Antagonists were injected i.v. 10 min before electrical stimulation. Each point represents the mean ±S.E.M. from 4 animals. *P<0.05, **P<0.01 vs control.
DISCUSSION

YM060 and YM114, selective 5-HT3-receptor antagonists, have been shown to potently inhibit BJR induced by bolus i.v. injection of 5-HT in anesthetized rats (18, 19). In addition to 5-HT, 2-methyl-5-HT, a selective 5-HT3-receptor agonist, has also been reported to evoke BJR (13). Paintal (14) reported that 5-HT-sensitive receptors (chemoreceptor-like tissues) and veratridine-sensitive chemoreceptors are located in the same area in the heart, indicating that 5-HT might act not only 5-HT3 receptors but also on chemoreceptors on the endings of afferent vagus nerves. In the present study, therefore, we evaluated the inhibitory effects of YM060 and YM114 on 5-HT-induced BJR in anesthetized rats and compared the results with those of ondansetron and granisetron. Intravenous injection of YM060, YM114, ondansetron and granisetron dose-dependently inhibited 2-methyl-5-HT-induced BJR at doses similar to those that inhibited 5-HT-induced BJR in rats (18, 19). On the basis of ID50 values, the rank order of 5-HT3-receptor blocking potency of these antagonists was YM060 > YM114 > granisetron > ondansetron. These results indicate that their 5-HT3-receptor blocking potencies against 2-methyl-5-HT-induced BJR were consistent with those against 5-HT-induced BJR.

Transient bradycardia may be evoked not only by activation of 5-HT3 receptors but also by activation of chemoreceptors and baroreceptors and electrical stimulation of vagus nerves. These responses are mediated by ACh released from the endings of vagal efferent nerves. In anesthetized cats, dogs and rats, intraventricular and intravenous injection of veratridine stimulates the vagus nerves through activation of chemoreceptors and baroreceptors located in the heart, resulting in bradycardia and hypotension (14-17). In the present study, intravenous veratridine, like 5-HT and 2-methyl-5-HT, dose-dependently produced transient bradycardia in anesthetized rats. We examined the inhibitory effects of YM060, YM114, ondansetron and granisetron on bradycardia induced by veratridine and electrical stimulation of vagal efferent nerves in anesthetized rats to investigate the characteristics and selectivity of these 5-HT3-receptor antagonists. YM060, YM114, ondansetron and granisetron did not inhibit veratridine- or electrical stimulation-induced BJR at doses sufficient to block 5-HT3 receptors, indicating that they have no effect on chemoreceptors, baroreceptors, muscarine receptors, nicotine receptors or vagal discharge. In contrast, the muscarine-receptor antagonist atropine abolished electrical stimulation-induced bradycardia. These results suggest that YM060, YM114, ondansetron and granisetron are selective 5-HT3-receptor antagonists in the BJR of anesthetized rats.

A number of authors have examined the mechanism of 5-HT-induced BJR. Evans et al. (10) reported that intratrial injection of phenylbiguanide, a 5-HT3-receptor agonist, induced a dose-dependent decrease in heart rate.
in conscious rabbits and that this response was inhibited by atropine and bilateral vagotomy. Bradycardia induced by intraatrial and intraventricular injections of 5-HT was also inhibited by both atropine and vagotomy in conscious and anesthetized dogs (8). Chianca et al. (17) also examined the effect of 5-HT injected into the left ventricle in anesthetized rats. Detailed investigation in anesthetized rats, however, has not been carried out. In the present study, we found that intraventricular injection of 5-HT (0.625–5.0 𝜇g) dose-dependently caused bradycardia, and this response was abolished by YM060, atropine and bilateral vagotomy. Intraventricular doses of 5-HT from 0.625 to 5.0 𝜇g corresponded doses of 2.5 to 20 𝜇g/kg. In a previous study, i.v. injection of 5-HT produced a dose-dependent BJR at doses of 5 to 80 𝜇g/kg in anesthetized rats (18). Taken together, these results indicate that 5-HT-induced BJR in anesthetized rats originates from 5-HT3 receptors located on the endings of vagal afferent nerves in the heart, and they are consistent with previous reports (12, 17).

In conclusion, we suggest that YM060 and YM114 are highly potent and selective 5-HT3-receptor antagonists in the BJR of rats. We also suggest that 5-HT-induced BJR in anesthetized rats is evoked by ACh released from vagal efferent nerves through activation of 5-HT3 receptors located on the endings of vagal afferent nerves in the heart.

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