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Pharmacological profiles of R-96544, the active form of a novel 5-HT_{2A} receptor antagonist R-102444

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Abstract

We examined the pharmacology of (2*R*,4*R*)-4-hydroxy-2-[2-[2-[2-(3-methoxy)phenyl]ethyl]phenoxy]ethyl-1-methylpyrrolidine hydrochloride (R-96544), the active form of a novel 5-HT_{2A} receptor antagonist, (2*R*,4*R*)-4-lauroyloxy-2-[2-[2-[2-(3-methoxy)phenyl]ethyl]phenoxy]ethyl-1-methylpyrrolidine hydrochloride (R-102444). R-96544 produced a concentration-dependent inhibition of platelet aggregation induced by serotonin (5-hydroxytryptamine, 5-HT) alone or in combination with ADP in platelets from humans, monkeys, cats, rabbits, rats and mice. An intravenous administration of R-96544 to rabbits significantly inhibited ex vivo platelet aggregation induced by 5-hydroxytryptamine (5-HT) combined with epinephrine. An oral administration of R-102444 to rats also resulted in significant inhibition of ex vivo platelet aggregation, whereas R-102444 was ineffective in an in vitro platelet aggregation assay. These antiplatelet effects of R-96544 and R-102444 were more potent than those of two other 5-HT_{2A} receptor antagonists, sarpogrelate and its active metabolite (±)-1-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propanol hydrochloride (M-1). A binding study using cat platelet membranes showed that R-96544 has high affinity for 5-HT_{2A} receptors but no effect on non-serotonergic [^{3}H]ketanserin-binding sites. R-96544 caused a parallel shift to the right of concentration–response curves for 5-HT in rat caudal artery contraction mediated by 5-HT_{2A} receptors. Schild plot analysis gave a pA_2 value of 10.4 with a slope near unity (1.04). R-96544 also inhibited 5-HT_{2A} receptor-mediated contraction of guinea pig trachea but not 5-HT_3 receptor-mediated contraction of guinea pig ileum and 5-HT_{2B} receptor-mediated contraction of rat fundus preparation. R-96544 (i.v.) attenuated the pressor responses evoked by 5-HT (15 μg/kg, i.v.) but not by phenylephrine (5 μg/kg, i.v.) and angiotensin II (0.1 μg/kg, i.v.), after ganglionic blockade in anesthetized spontaneously hypertensive rats. These results show that R-96544, the active form of R-102444, is a novel 5-HT receptor antagonist with potent, competitive, and 5-HT_{2A}-selective activity.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{2A} receptor antagonist; R-102444; R-96544

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) produces a wide array of activities on many organ systems including the central nervous, gastrointestinal and cardiovascular systems. The biological responses of 5-HT in these systems are mediated by receptors. To date, at least 14 receptors have been identified and classified into seven receptor subfamilies based on their sequence homology, pharmacology and signal transduction cascade (Hoyer et al., 1994). Among them, 5-HT_{2A} (formerly termed 5-HT_2) receptors on vascular smooth muscle and platelets (De Clerck et al., 1982) play important roles in the cardiovascular system (Nilsson et al., 1999; Wiernsperger, 1990). During platelet activation at the site of vessel injury, 5-HT in the dense granules is released from activated platelets. Released 5-HT contributes to thrombus formation, constriction of vascular smooth muscle and potentiation of platelet activation primarily induced by other agonists via 5-HT_{2A} receptors (Baumgartner and Born, 1968; Vanags et al., 1992; Ogawa et al., 1998). 5-HT_{2A} receptors are also involved in migration and proliferation of vascular smooth muscle cells (Tamura et al., 1997; Sharma et al., 1999). Thus, the development of 5-HT_{2A} receptor antagonists is of potential clinical interest.

A number of 5-HT_{2A} receptor antagonists including ketanserin (Van Nueten et al., 1981) and sarpogrelate (Hara et al., 1991) have been identified. We recently reported that (2*R*,4*R*)-4-lauroyloxy-2-[2-[2-[2-(3-methoxy)phenyl]

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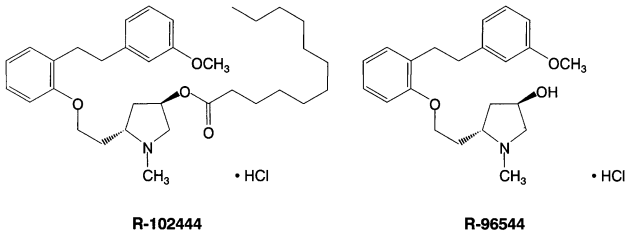

Fig. 1. Chemical structures of R-102444 and R-96544.

ethyl]phenoxy]ethyl-1-methylpyrrolidine hydrochloride (R-102444) (Fig. 1) is a prodrug-type 5-HT_{2A} receptor antagonist (Tanaka et al., 2000b) and that its active form is (2*R*,4*R*)-4-hydroxy-2-[2-[2-[2-(3-methoxy)phenyl]ethyl]-phenoxy]ethyl-1-methylpyrrolidine hydrochloride (R-96544) (Tanaka et al., 2000a). In the present study, the pharmacological profile of R-96544 was further examined in platelets and smooth muscle from various animal species.

2. Materials and methods

2.1. Materials

The following agents and chemicals were used in this study: 5-HT creatinine sulphate (Merck, Darmstadt, Germany), ketanserin tartrate (Research Biochemicals International, Natick, MA, USA), [^{3}H]ketanserin (DuPont NEN, Boston, MA, USA, 60–90 Ci/mmol), tetrabenazine (Fluka, Buchs, Germany), angiotensin II acetate salt (Peptide Institute, Osaka, Japan), ADP sodium salt, epinephrine bitartrate salt, mecamylamine hydrochloride and phenylephrine hydrochloride (Sigma, St. Louis, MO, USA). Inactin was obtained from Chemtech Labo (Tokyo, Japan). R-102444 ((2*R*,4*R*)-4-lauroyloxy-2-[2-[2-[2-(3-methoxy)phenyl]ethyl]phenoxy]ethyl-1-methylpyrrolidine hydrochloride), R-96544 ((2*R*,4*R*)-4-hydroxy-2-[2-[2-[2-(3-methoxy)phenyl]ethyl]phenoxy]ethyl-1-methylpyrrolidine hydrochloride), sarpogrelate hydrochloride ((±)-1-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propyl hydrogen succinate hydrochloride), and M-1 ((±)-1-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propanol hydrochloride) were chemically synthesized at Sankyo (Tokyo, Japan). Agents were dissolved in saline for in vitro studies and intravenous administration and suspended in 0.5% gum tragacanth (Sigma) for oral administration.

2.2. Animals

All animal procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at Sankyo Research Laboratories (Tokyo, Japan). Male cynomolgus monkeys (5–7 kg) were purchased from Clea Japan (Tokyo, Japan). Male Sprague–Dawley rats (260–330 g) and male DDY mice (31–37 g) were purchased from Japan SLC (Shizuoka, Japan). Male American Domestic Short Hair cats (2.5–4.0 kg, Kasho, Tokyo, Japan), male Japanese White rabbits (2.5–3.5 kg, Shiraishi Laboratory Animals, Tokyo, Japan) and male spontaneously hypertensive rats (SHR, 380–420 g, Hoshino Laboratory Animals, Saitama, Japan) were also used. Animals were housed in animal quarters at 24–27 °C, and 50–60% humidity, in a 12-h light/dark cycle.

2.3. Preparation of platelets

Platelets were prepared as previously described (Ogawa et al., 2000; Sugidachi et al., 2001) with slight modifications. Blood was collected from male human volunteers (26–38 years old) who had not taken any medication for more than 1 week, monkeys, cats, rabbits, rats and mice using 3.8% sodium citrate (1/9 volume of blood) as anticoagulant. The blood was centrifuged at 100–230 × *g* for 15–20 min to obtain platelet-rich plasma (PRP). The remaining blood was further centrifuged at 2000 × *g* for 10 min to obtain platelet-poor plasma (PPP). Platelet counts in PRP were adjusted to 3×10^8/ml for human, monkey, cat, rabbit and mouse platelets and 5×10^8/ml for rat platelets by adding PPP.

2.4. Measurement of platelet aggregation

Platelet aggregation studies were performed in aggregometers (PAM-6C and PAM-8C, Mebanix, Tokyo, Japan). PRP was incubated at 37 °C for 1.5 min in aggregometer with continuous stirring and then stimulated with agonists. Changes in light transmittance were recorded for 5 min using PPP as 100%. The area under the curve (AUC) for the aggregation tracing occurring during 5 min was determined to calculate platelet aggregation. The potentiation by 5-HT of ADP-induced platelet aggregation was calculated by subtracting 5-HT- and ADP-induced aggregation AUC from 5-HT + ADP-induced platelet aggregation AUC as reported by Cohen et al. (1992). In humans, cats and rabbits, the extent of platelet aggregation induced by 5-HT + ADP, 5-HT or 5-HT + epinephrine was estimated quantitatively by measuring maximum curve height above baseline level (%).

2.5. [^{3}H]ketanserin-binding assay

Blood was drawn from the carotid artery of anesthetized cats with pentobarbital (40 mg/kg, i.p.), and platelets were collected by centrifugation as described above. Platelet membranes were prepared according to the method described by Leysen et al. (1983). Binding assay was performed in a total volume of 1 ml of buffer (50 mM Tris–HCl–0.3% BSA, pH 7.4) containing 0.5 mg of membrane protein. The concentration of [^{3}H]ketanserin ranged between 0.5 and 10 nM for the saturation binding experiments and a concentration of 2 nM was used for the competition experiments. After 30-min incubation at 30 °C, radioligand bound to the membrane was separated from free radioligand by filtration using a Whatman GF/B glass–fiber

filter. The filter was washed three times with 5 ml of ice-cold buffer and the radioactivity counted in a scintillation spectrometer. Nonspecific binding was determined in the presence of 3000-fold of unlabeled ketanserin; specific binding was calculated as the difference between total binding and nonspecific binding. The equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) were determined by Scatchard analysis. The inhibition constant (K_i) was calculated from the following equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1+[L]/K_d)$, where IC_{50} is the 50% inhibitory concentration calculated from the displacement curve and [L] is the concentration of [^{3}H]ketanserin in the tube.

2.6. 5-HT-induced contraction in isolated tissues

Contractile studies were performed by established procedures for rat caudal artery (Van Nueten et al., 1981), guinea pig trachea (Van Nueten et al., 1982), guinea pig ileum (Cohen et al., 1985) and rat stomach fundus (Cohen et al., 1985). The pA_2 value, as index of the potency of antagonism, was determined using the equation of Arunlakshana and Schild (1956).

2.7. Vasopressor responses in SHR

SHR were anesthetized with inactin (100 mg/kg, i.p.), and a catheter was placed in a left femoral artery and left femoral vein of each for measurement of vasopressor and drug administration, respectively. The rats were subjected to ganglionic blockade with mecamylamine (10 mg/kg, i.v.), and 5-HT (15 μg/kg, i.v.), phenylephrine (5 μg/kg, i.v.), or angiotensin II (0.1 μg/kg, i.v.) was injected 15 and 30 min after mecamylamine treatment. Increases in mean blood pressure (ΔMBP) were recorded. The average of two pressor responses was used as the control. The pressor responses to 5-HT, phenylephrine, or angiotensin II were measured again 5 min after intravenous administration of R-96544 and the percentages of inhibition from the control responses calculated for each rat.

2.8. Statistics

Results are expressed as means ± S.E.M. unless otherwise stated. Differences among multiple groups were assessed by Dunnett's multiple comparison test using an SAS statistical computer package (SAS Institute, Cary, NC). *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. In vitro antiplatelet activity

In cat platelets, 5-HT alone was used as agonist because of the high sensitivity of cat platelets to 5-HT (De Clerck and Herman, 1983; Seggel et al., 1987). R-96544 (10–100 nM) inhibited 5-HT-induced cat platelet aggregation in a concentration-dependent manner (Fig. 2A). In human and rat platelets, R-96544 also showed inhibitory effects of platelet aggregation induced by a combined use of 5-HT and ADP at a similar concentration range (Fig. 2B and C). The IC_{50} values of R-96544 from all animal species tested ranged from 7 to 27 nM (Table 1). In contrast, R-102444 had no anti-aggregatory effect on platelets from any animal species tested, even at 1 μM (data not shown). Sarpogrelate and M-1 showed inhibitory effects on platelet aggregation, but higher concentration was required to achieve the inhibition equivalent to that of R-96544.

3.2. Ex vivo antiplatelet activity

The ex vivo effect of intravenously administered R-96544 on platelet aggregation induced by 5-HT (3 μM) + epinephrine (5 μM) was measured in anaesthetized rabbits. R-96544 (0.03 mg/kg, i.v.) showed more than 80% inhibition 0.5 h after dosing, and this was sustained for at least 6

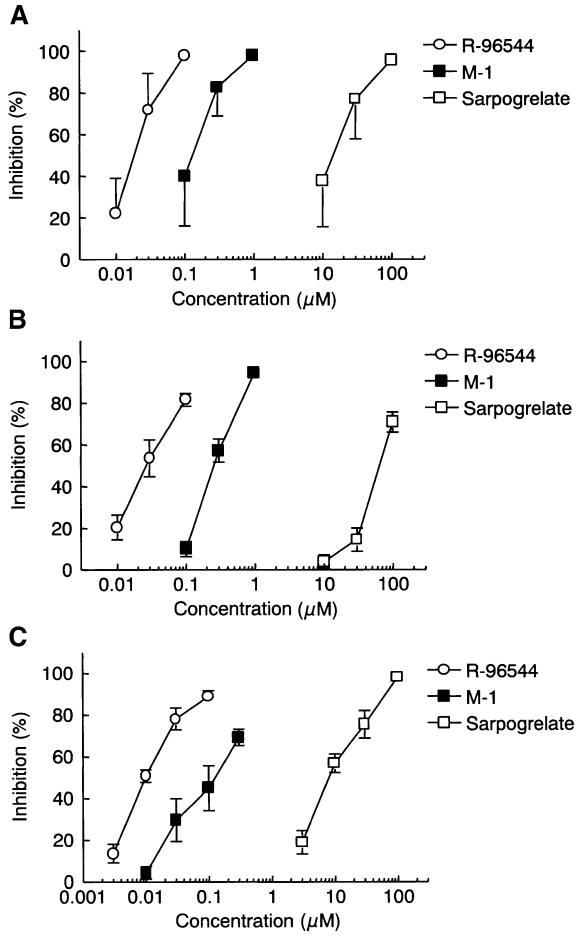

Fig. 2. In vitro effects of R-96544, sarpogrelate and M-1 on platelet aggregation in PRP from cats (A), humans (B) and rats (C). Platelet aggregation was induced by 5-HT (cat) and 5-HT + ADP (human and rat). Results are expressed as the mean ± S.E.M. ($n = 5–6$).

Table 1
IC_{50} values (nM) of R-96544, sarpogrelate and M-1 on in vitro platelet aggregation in PRP from various animal species

Species	Agonists	R-96544	Sarpogrelate	M-1
Human	5-HT (10) + ADP (1)	27	64,000	2,500
Monkey	5-HT (10) + ADP (2)	12	N.D.	N.D.
Cat	5-HT (6–8)	20	14,000	120
Rabbit	5-HT (10) + ADP (3)	7	12,000	510
	5-HT (3) + Epi (5)	5	N.D.	570
Rat	5-HT (10) + ADP (2)	10	8,100	290
Mouse	5-HT (10) + ADP (1)	13	N.D.	270

Concentrations (μM) of agonists are shown in parentheses. N.D.: not determined. Epi: epinephrine.

h after dosing (Fig. 3). Sarpogrelate and M-1 also showed a significant inhibition of platelet aggregation at 0.5 h, but the inhibition of platelet aggregation by both agents even at 3 mg/kg (i.v.) was less than that of R-96544 at 0.03 mg/kg (i.v.).

The ex vivo effects of orally administered R-102444, sarpogrelate and M-1 on platelet aggregation were measured 1 h after dosing in rats. R-102444 (0.01–1 mg/kg, p.o.) inhibited platelet aggregation induced by 5-HT (10 μM) + ADP (2 μM) in a dose-dependent manner (Fig. 4). Orally administered sarpogrelate (10 and 100 mg/kg, p.o.) and M-1 (10 mg/kg, p.o.) also inhibited rat platelet aggregation, but higher doses of each agent compared with R-102444 were required to produce statistically significant inhibition.

3.3. [^{3}H]ketanserin binding to cat platelet membranes

The effect of R-96544 against platelet 5-HT_{2A} receptors was evaluated in [^{3}H]ketanserin-binding assay using cat platelet membranes. The [^{3}H]ketanserin binding to cat platelet membranes was saturable and Scatchard plot analysis resulted that B_{max} and K_d of [^{3}H]ketanserin to cat platelets were 81 fmol/mg protein and 2.7 nM, respectively, which are similar to previously reported values (Leysen et al., 1983).

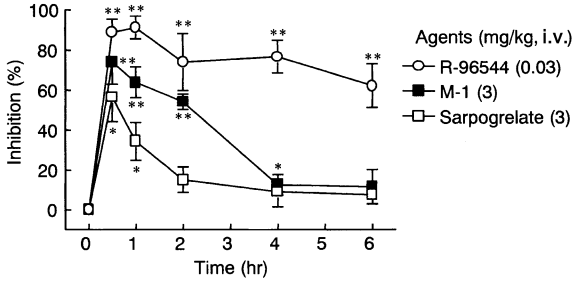

Fig. 3. Ex vivo effects of intravenously administered R-96544 (0.03 mg/kg), sarpogrelate (3 mg/kg) and M-1 (3 mg/kg) on platelet aggregation in rabbits. Platelet aggregation induced by 5-HT (3 μM) + epinephrine (5 μM) was measured before (time 0), and 0.5, 1, 2, 4 and 6 h after the administration. Results are presented as the mean ± S.E.M. ($n = 6$). *$P < 0.05$, **$P < 0.01$ compared to time 0.

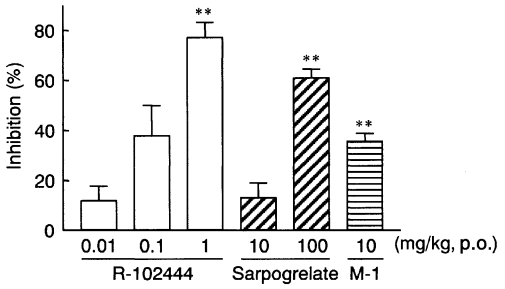

Fig. 4. Ex vivo effects of orally administered R-102444, sarpogrelate and M-1 on platelet aggregation in rats. Platelet aggregation induced by 5-HT + ADP was measured 1 h after the administration of these agents. Results are presented as the mean ± S.E.M. ($n = 5–6$). **$P < 0.01$ compared to vehicle-treated control.

The inhibitory effects of R-96544 on [^{3}H]ketanserin (2 nM) binding to platelet membranes were compared with those of ketanserin (Fig. 5A). R-96544 and ketanserin decreased [^{3}H]ketanserin binding to cat platelet membranes in a concentration-dependent manner, with K_i values of 1.6 and 5.1 nM for each, respectively. However, there was a difference in the maximal effects of R-96544 and ketanserin; maximal inhibition of R-96544 was 74.5 ± 4.6% ($n = 6$) vs. 98.3 ± 0.9% ($n = 6$) for ketanserin.

To further characterize the inhibitory effect of R-96544 on [^{3}H]ketanserin binding to cat platelet membranes, the combined effect of R-96544 with tetrabenazine, a monoamine-depleting agent (Leysen et al., 1991; Leysen et al., 1988), was examined. Specific [^{3}H]ketanserin binding in the presence of R-96544 (1 μM) was 23.9 ± 0.9% ($n = 3$, Fig. 5B). Tetrabenazine (1 nM–1 μM) produced a concentration-dependent inhibition of [^{3}H]ketanserin binding (data not shown); 78.2 ± 0.9% ($n = 3$) of [^{3}H]ketanserin binding

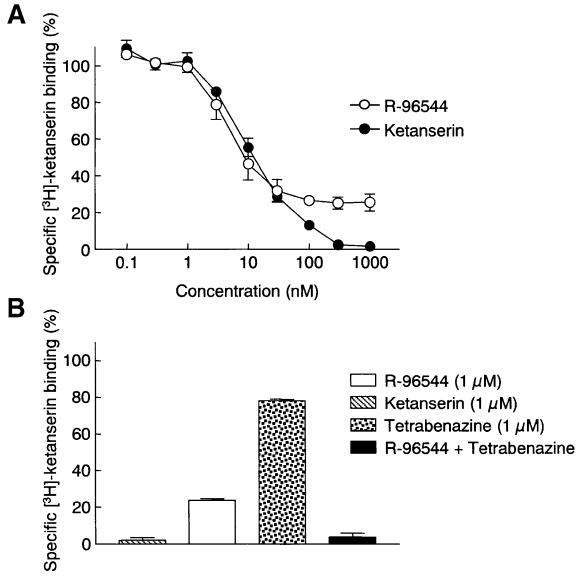

Fig. 5. (A) Displacement curves of [^{3}H]ketanserin (2 nM) binding to cat platelet membranes by R-96544 and ketanserin. (B) Effect of R-96544 (1 μM) combined with tetrabenazine (1 μM) on [^{3}H]ketanserin binding to cat platelet membranes. Results are presented as the mean ± S.E.M. ($n = 3–6$).

unblocked at a concentration of 1 μM. The remaining [^{3}H]ketanserin binding in the presence of R-96544 (1 μM) was abolished by combined use of tetrabenazine (1 μM).

3.4. In vitro 5-HT receptor antagonism in isolated tissues

Addition of 5-HT to rat caudal artery produced contractile responses mediated by 5-HT$_{2A}$ receptors, which was concentration dependent. Pretreatment with R-96544 (0.1–10 nM) caused a parallel shift to the right of the concentration–response curve for 5-HT. No significant suppression of the maximum response was observed even at 10 nM, the highest concentration tested (Fig. 6A). Schild plot analysis gave a unity slope (1.04) and pA$_2$ value of 10.5 ± 0.1 (n = 7, Fig. 6B). Ketanserin and sarpogrelate also gave a unity slope (0.95 and 1.04, respectively); the pA$_2$ values for ketanserin and sarpogrelate were 9.2 ± 0.1 (n = 6) and 8.2 ± 0.0 (n = 6), respectively.

The effects of R-96544 on 5-HT-induced contractions of guinea pig trachea, guinea pig ileum and rat fundus are shown in Fig. 7. 5-HT produced a concentration-dependent contraction in all the tissues used. In guinea pig trachea, R-96544 (1–100 nM) caused a rightward shift of the 5-HT concentration–response curve, but with suppression of the maximum response (Fig. 7A). Ketanserin and sarpogrelate also induced depression of the maximum response (data not shown). In guinea pig ileum and rat fundus, R-96544 had no significant effects on 5-HT-induced contraction up to 100 nM (Fig. 7B and C). However, at 1000 nM, the highest concentration tested, R-96544 significantly reduced 5-HT-induced responses of these tissues. Ketanserin and sarpogrelate also showed similar responses (data not shown).

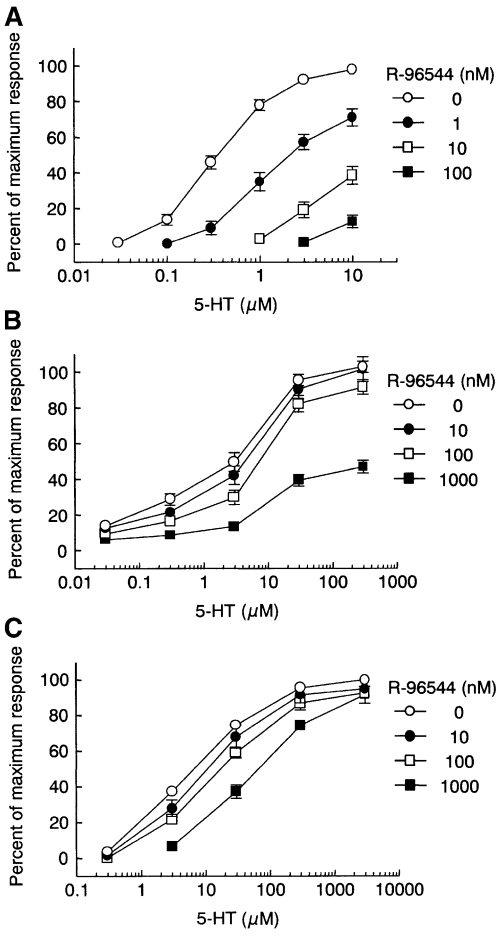

Fig. 7. Effect of R-96544 on response of guinea pig trachea (A), ileum (B) and rat fundus (C) to increasing concentrations of 5-HT. Results are expressed as the mean ± S.E.M. (n = 4–8).

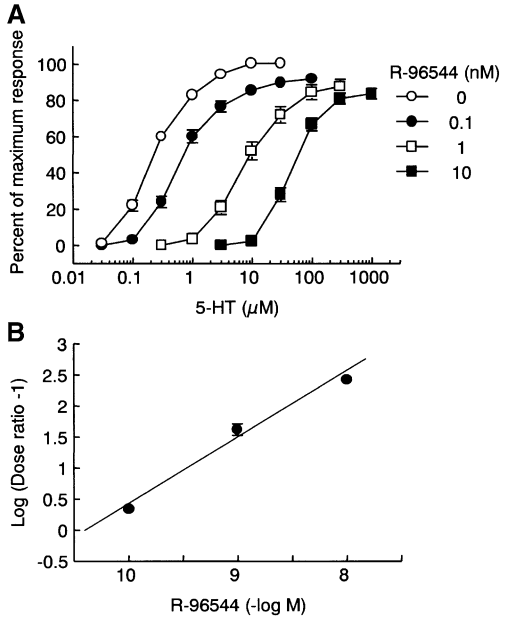

Fig. 6. (A) Effect of R-96544 on contractile response of rat caudal artery to increasing concentrations of 5-HT. (B) Schild plot analysis of the effect of R-96544 on the contractile response to 5-HT in rat caudal artery. Results are expressed as the mean ± S.E.M. (n = 7).

3.5. In vivo antagonism on vasopressor responses to 5-HT in SHR

An intravenous administration of 5-HT (50 μg/kg) to SHR without ganglion blockade caused a significant reduction in heart rate (Bezold–Jarish reflex). R-96544 (3 μg/kg, i.v.) had no effect on Bezold-Jarish reflex (data not shown). 5-HT (15 μg/kg) intravenously administered to anesthetized SHR pretreated with mecamylamine-evoked pressor responses (ΔMBP = 43.9 ± 1.9 mm Hg, n = 15). No significant changes in MBP occurred after injection of R-96544 (0.3–100 μg/kg) alone. R-96544 (0.3–3 μg/kg, i.v.) dose-dependently antagonized 5-HT-evoked pressor responses (Fig. 8). Phenylephrine (5 μg/kg, i.v.) and angiotensin II (0.1 μg/kg, i.v.) also evoked pressor responses (ΔMBP = 65.4 ± 2.0 mm Hg, n = 5 and 42.8 ± 2.9 mm Hg, n = 5), but a high dose (100 μg/kg, i.v.) of R-96544 showed minimal inhibitory effects on phenylephrine- and angiotensin II-induced responses (2.1 ± 1.4% and 10.2 ± 3.4% for each,

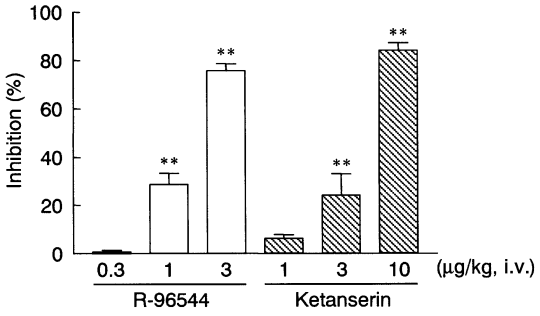

Fig. 8. In vivo effects of R-96544 and ketanserin on pressor responses to 5-HT. Results are presented as the mean ± S.E.M. ($n=5$). **$P<0.01$ compared to vehicle-treated control.

respectively, $n=5$ for each). Likewise, ketanserin (1–10 μg/kg, i.v.) dose-dependently inhibited 5-HT-induced pressor responses. In contrast to R-96544, a high dose of ketanserin (100 μg/kg) significantly ($P<0.01$) inhibited phenylephrine-induced pressor responses (inhibition = 36.8 ± 5.4%, $n=5$).

4. Discussion

R-102444, a prodrug of R-96544, is an orally active 5-HT_{2A} receptor antagonist (Tanaka et al., 2000b). Previous radioligand binding study demonstrated that R-96544 has high affinity for 5-HT_{2A} receptor rather than 5-HT_1, 5-HT_3, dopamine D_2 and α- and β-adrenoceptors, whereas R-102444 is essentially inactive in vitro (Tanaka et al., 2000a). We performed the present study to examine the pharmacology of R-96544 in greater details, and found that it is a potent, competitive and selective 5-HT_{2A} receptor antagonist.

Platelets possess 5-HT_{2A} receptors (Drummond and Gordon, 1974), but 5-HT-induced platelet aggregation is highly variable among animal species (De Clerck and Herman, 1983). Indeed, irreversible aggregation is induced by 5-HT in cat platelets (Seggel et al., 1987; Ogawa et al., 1998) but 5-HT alone causes only slight, reversible platelet aggregation in platelets from most other species including humans. In these species, 5-HT itself is weak as an aggregatory factor but strongly potentiates the aggregatory responses of other agonists such as ADP, epinephrine and collagen. In the present study, R-96544 inhibited both 5-HT-induced and -potentiated platelet aggregation in all animals tested, and the anti-aggregatory potency of R-96544 was higher than those of both sarpogrelate and its metabolite M-1 (Table 1). In contrast, R-96544 had no effects on platelet aggregation induced by ADP alone (data not shown). Thus, these in vitro results indicate that R-96544 is a potent and specific inhibitor on 5-HT-induced platelet activation with no obvious species differences.

Ketanserin is widely used as a specific ligand to 5-HT_{2A} receptors, but it has been reported that platelets have two classes of [^{3}H]ketanserin-binding sites, the high-affinity 5-HT_{2A} receptors and a low-affinity (20 times lower than that of 5-HT_{2A}) non-serotonergic site related to uptake and release of intraplatelet 5-HT (Leysen et al., 1991; Leysen et al., 1988). To evaluate the effects of R-96544 on these two classes of [^{3}H]ketanserin-binding sites, the effects of R-96544 on [^{3}H]ketanserin-specific binding in the presence of excess unlabeled ketanserin was examined. R-96544 displaced [^{3}H]ketanserin binding with a similarly high affinity to that of ketanserin. However, the maximal inhibition achieved by R-96544 was smaller than that achieved by ketanserin, suggesting a difference in the site of action between the two drugs. To demonstrate the specificity of R-96544 to 5-HT_{2A} receptors, the effect of R-96544 combined with tetrabenazine, which acts on the non-serotonergic ketanserin-binding site, was investigated, with the result that tetrabenazine showed an inhibition additive to that of R-96544. Thus, these radioligand-binding studies clearly show that R-96544 has high affinity to platelet 5-HT_{2A} receptors but not to the non-serotonergic [^{3}H]ketanserin-binding site.

R-96544 (i.v.) showed potent and long-lasting inhibition of ex vivo platelet aggregation, and this potent efficacy correlates well with its in vitro activity in rabbit PRP. Although pharmacokinetic evaluation remains to be done, the long duration of antiaggregatory action of R-96544 observed in the ex vivo study might be due to its high affinity to platelet 5-HT_{2A} receptors. As previously reported (Tanaka et al., 2000b), oral administration of R-96544 to rats causes gastric irritation. We examined the oral antiplatelet potency of R-102444, an ester-type prodrug that is devoid of gastric irritation effects, in rats. Orally administered R-102444 to rats produced significant inhibition of ex vivo platelet aggregation, whereas R-102444 was ineffective in in vitro study (data not shown). The ex vivo effects of R-102444 were greater than those of sarpogrelate and M-1. Taken together, the results suggest that R-102444 should be an orally available and potent 5-HT_{2A} receptor antagonist with long duration of action and without gastric irritation.

Simultaneous blockade of 5-HT_{2A} receptors on platelets and vascular smooth muscles is believed to contribute to the clinical effects of 5-HT_{2A} receptor antagonists. R-96544 caused a parallel shift to the right of the concentration–contraction curves in 5-HT-induced rat caudal artery, in which contraction is mediated by 5-HT_{2A} receptors (Docherty, 1988). Schild plot analysis for the inhibition by R-96544 gave a pA_2 value of 10.5 ± 0.1 and a slope near unity, suggesting high potency and competitive antagonistic action against 5-HT_{2A} receptors in vascular smooth muscle. The potent antagonistic effects of R-96544 on the vascular 5-HT_{2A} receptor were also confirmed in vivo. R-96544 inhibited the pressor response to 5-HT in SHR treated with a ganglion blocker without affecting the pressor responses to phenylephrine or angiotensin II. In contrast, ketanserin, a dual inhibitor of 5-HT_{2A} receptors and α_1-adrenoceptors (Fozard, 1982; Cohen et al., 1983), prevented pressor responses evoked by both phenylephrine and 5-HT. In addition, the 5-HT-induced Bezold–Jarish reflex, a 5-HT_3 receptor-

mediated response (Nagakura et al., 1993) in anesthetized rats without ganglion blockade, was not affected by R-96544 (data not shown). Thus, the present results provide clear evidence that R-96544 exhibits potent, selective and competitive 5-HT_{2A} receptor antagonistic activities on the vascular wall.

5-HT_{2A} receptors are reported to be widely distributed on tissues other than platelets and the vasculature. For instance, the guinea pig trachea is reported to be contracted by low concentrations of 5-HT via 5-HT_{2A} receptors (Baumgartner et al., 1990). R-96544 competitively blocked 5-HT-induced contraction in this tissue when low concentrations were used; however, increasing concentrations suppressed the maximum response to 5-HT. In trachea, similar results have been observed with the 5-HT_{2A} receptor antagonists SR 46349B, ketanserin and ritanserin (Rinaldi-Carmona et al., 1992; Lemoine and Kaumann, 1986; Kameda et al., 1988). These observations were interpreted as competitive antagonism and allosteric regulation of the contractile serotonergic binding site (Kameda et al., 1988). In contrast, 5-HT-induced contractions in guinea pig ileum mediated by 5-HT_3 receptors and rat fundus mediated by 5-HT_{2B} receptors (Cohen et al., 1990; Baxter et al., 1994) were hardly affected by R-96544 even at high concentrations up to 100 nM. In preliminary radiobinding studies using cells expressing human 5-HT receptors, R-96544 showed 100-fold higher affinity for the human 5-HT_{2A} receptors than 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{5A}, 5-HT_6, 5-HT_7 receptors and transporter, although R-96544 had relatively high affinity for 5-HT_{2C} receptors (fourfold less compared to 5-HT_{2A} receptors). Taken together, these results suggest that R-96544 is a specific 5-HT_{2A} receptor antagonist.

In summary, the present study showed that R-96544, an active form of R-102444 is a highly potent and selective 5-HT_{2A} receptor antagonist in platelets and vascular smooth muscle. R-102444 and R-96544 should be useful for evaluating the possible role of 5-HT_{2A} receptors in several 5-HT-mediated responses in cardiovascular systems.

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