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Effects of Ketanserin and 3-[2-[4-(*o*-Methoxyphenyl)-1-piperazinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione Monohydrochloride (SGB-1534), Anti-hypertensive Agents, on ^{3}H-Serotonin and ^{3}H-Ketanserin Bindings to Serotonergic ($5HT_1$ and $5HT_2$) Receptors in Dog Brain and Aorta[1a)]

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Serotonin has an important role in the control of blood pressure, because serotonin$_2$ ($5HT_2$) antagonists ameliorate hypertension. The $5HT_2$ antagonistic action may be due in part to α_1-adrenoceptor blocking action. Thus, the present study was designed to examine the effects of $5HT_2$ antagonists (ketanserin and cinanserin) and α_1-antagonists (SGB-1534, 3-[2-[4-(*o*-methoxyphenyl)-1-piperazinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione monohydrochloride, and prazosin) on the ^{3}H-5-hydroxytryptamine creatine sulfate (^{3}H-serotonin) and ^{3}H-ketanserin bindings to serotonin$_1$ ($5HT_1$) receptors in the dog brain and $5HT_2$ receptors in the dog brain and aorta by using the radioligand binding assay method. The existence of ^{3}H-ketanserin binding sites was definitely observed in the dog brain and aorta. The K_d and B_{max} values of ^{3}H-ketanserin binding to $5HT_2$ receptors in the dog aorta were 1.20 nM and 36 fmol/mg protein, respectively. $5HT_1$ and $5HT_2$ receptors also existed in the dog brain. Inhibition of ^{3}H-ketanserin bindings by ketanserin and cinanserin in the dog brain and aorta was observed at very low concentrations, but inhibition by SGB-1534 and prazosin was observed only at very high concentrations. Furthermore, inhibitions of ^{3}H-serotonin binding by ketanserin and prazosin were observed at high concentrations, but the K_i value of SGB-1534 was found to be 371 nM. These results suggest that $5HT_2$ antagonists and α_1-adrenoceptor antagonists have different effects on the ^{3}H-ketanserin bindings to $5HT_2$ receptors in the aorta and that the hypotensive effect of $5HT_2$ antagonists may be largely concerned with $5HT_2$ receptors. On the other hand, that of α_1-antagonists may be due to α_1-adrenoceptor blocking action in vascular tissues as described previously.

Keywords——^{3}H-ketanserin; ^{3}H-serotonin; ketanserin; SGB-1534; dog aorta; dog brain; $5HT_2$-antagonist; α_1-adrenoceptor antagonist

Introduction

Serotonin is known to have many pharmacological actions on the cardiovascular system. In particular, the role of serotonin in hypertension has long been discussed, and it has been suggested that serotonin-induced vasoconstriction is mediated by serotonin$_2$ ($5HT_2$) receptors in the peripheral blood vessels. There is evidence that vascular contraction in response to serotonin appears to be associated with the binding of serotonin to $5HT_2$ receptors in the aorta and jugular vein.[2)] Peroutka and Snyder[3)] also defined two subtypes of serotonin receptors in the brain by radioligand binding techniques, using ^{3}H-5-hydroxytryptamine creatine sulfate (^{3}H-serotonin) and ^{3}H-ketanserin for serotonin$_1$ ($5HT_1$) and $5HT_2$ receptors, respectively. The $5HT_1$ receptors in the brain and $5HT_2$ receptors in the peripheral vascular vessels are important for the control of blood pressure in the body.

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It is well known that ketanserin and SGB-1534, 3-[2-[4-(*o*-methoxyphenyl)-1-piperazinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione monohydrochloride, antagonize the $5HT_2$ receptors and α_1-adrenoceptors, respectively, and that these drugs have similar anti-hypertensive effects.[1,2] It is now, however, evident that there is some functional overlap between serotonin receptor and α_1-adrenoceptors.[2d] The mechanism of this overlap is unknown. Thus, it is of interest to assess the inhibitory potencies of these compounds by using the radioligand binding assay method. The present study was designed to test the effects of ketanserin and SGB-1534 on the ^{3}H-ketanserin and ^{3}H-serotonin bindings to $5HT_1$ and $5HT_2$ receptors in the brain and aorta of dogs.

Experimental

Radioligands——^{3}H-Serotonin (20 Ci/mmol) and ^{3}H-ketanserin (60 Ci/mmol) were purchased from New England Nuclear Corporation, Ltd. and stored at −20 °C until used.

Isolation of Membrane Fraction——Mongrel dogs of either sex were used in these experiments. The brain and descending aorta were removed, immediately frozen in liquid nitrogen and stored in a deep freeze (−80 °C) until used. The homogenate fraction containing cell membrane was prepared by the method of Nagatomo *et al.*[1a] Briefly,

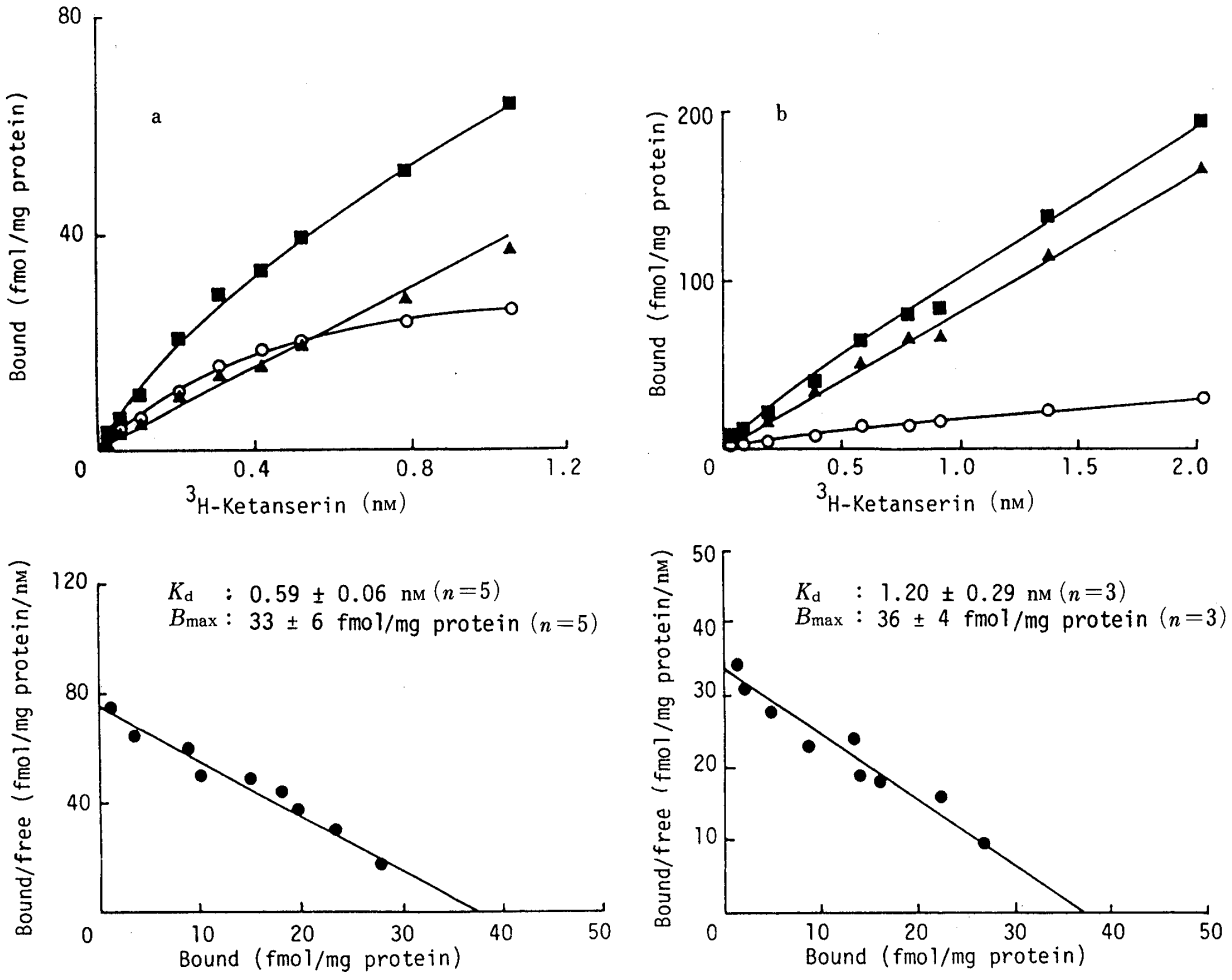

Fig. 1. Upper Half: Effect of ^{3}H-Ketanserin Concentration on the Amount of ^{3}H-Ketanserin Bound to the Dog Brain (a) and Aorta (b) in the Presence (▲) and Absence (■) of 0.1 mM Cinanserin
Lower Half: Scatchard Plots of the Same Data

The difference between the mean values of the total binding (■) and the non-specific binding (▲) was taken as the specific binding (○). Concentrations of ^{3}H-ketanserin ranged from 0.05 to 1.0 (brain) and 2.0 (aorta) nM, respectively.

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in the case of aorta, the vessel was crushed into a fine powder using a mortar and pestle, and then suspended in 10 volumes of 0.25 M sucrose, 5 mM Tris–HCl buffer containing 10 mM $MgCl_2$ (pH 7.5). The homogenate (Brinkmann Polytron homogenizer) was centrifuged at 1000 *g* for 10 min. The supernatant thus obtained was again centrifuged at 40000 *g* for 30 min. This pellet was incubated for 10 min in 50 mM Tris–HCl buffer (pH 7.4) at 37°C, again centrifuged at 40000 *g* for 20 min, resuspended in the incubation buffer, and used for the radioligand binding assay.

In the case of brain, defrosted brain tissue was minced with small scissors in 10 volumes of 0.25 M sucrose (10 mM Tris–HCl containing 10 mM $MgCl_2$, pH 7.5). The suspension was homogenized using glass-homogenizer and centrifuged at 40000*g* for 30 min. The pellet obtained was also incubated for 10 min at 37 °C in 50 mM Tris–HCl buffer (pH 7.4) and centrifuged at 40000*g* for 20 min. The pellet was resuspended in an incubation buffer and was used for the radioligand binding assay.

Binding Assay——Routine binding assays were performed in duplicate. Binding assays were carried out by the method reported previously by us.[4] The concentrations of ^{3}H-ketanserin in the incubation medium were 0.05—1.0 nM (brain) and 0.05—2.0 nM (aorta), and those of ^{3}H-serotonin were 0.05–17 nM (brain) for the Scatchard analysis. The concentrations of ^{3}H-ketanserin and ^{3}H-serotonin for the displacement experiment were 0.6 and 1.0 nM, respectively. Individual tubes containing ^{3}H-serotonin or ^{3}H-ketanserin along with 50 mM Tris–HCl buffer (pH 7.4) and 10 mM $MgCl_2$ in a total volume of 0.5 ml (brain) or 1 ml (aorta) were incubated at 23 °C for 30 min in a shaking water bath. In the case of ^{3}H-serotonin binding, the incubation medium contained 0.1% ascorbic acid and 1 μM pargyline. The reaction was initiated by the addition of membrane protein (0.5 mg). At the end of the incubation period the reaction mixture was filtered through a glass fiber filter (Whatman GF/C) using an improved automatic cell harvester, LM-101 (Labo Science, Tokyo). The filters were washed with a continuous flow for 2 s. The radioactivity remaining on the filters was counted using an Aloka scintillation counter. Specific binding is defined as the differences of binding in the presence or absence of 10 mM unlabelled serotonin (^{3}H-serotonin binding) and cinanserin (^{3}H-ketanserin binding). The equilibrium dissociation constants (K_d values) and the maximal number of binding sites (B_{max} values) were obtained from Scatchard plots. Linear regression lines were calculated according to the least-squares method. The values of inhibition constant (K_i) were calculated by the methods previously described.[5] Protein was determined by using the method of Lowry *et al.*[6]

Pharmacological Observation: $5HT_2$-Blocking actions were studied by using modifications of the method of Van Nueten *et al.*[7] and Cohen *et al.*[8] Male Wistar rats (300—450 g) were killed by a blow to the head. The descending aorta was cut into rings approximately 2 mm in width and the preparations were mounted in a 12 ml organ bath. The contractile tension of these preparations was recorded on a potentiometric recorder using a strain gauge transducer

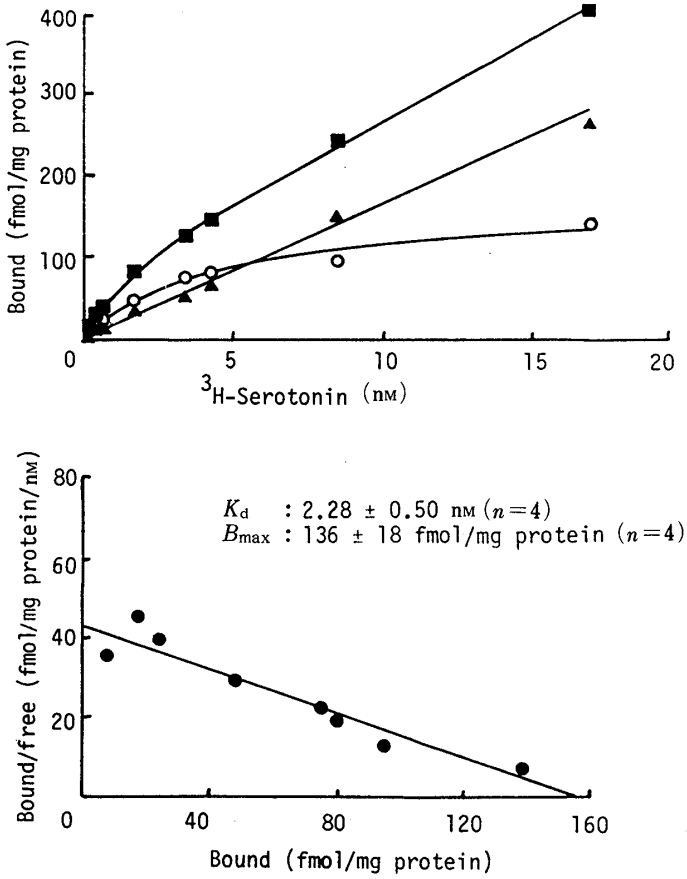

Fig. 2. Upper Half: Effect of ^{3}H-Serotonin Concentration on the Amount of ^{3}H-Serotonin Bound to the Dog Brain in the Presence (▲) and Absence (■) of 0.1 mM Serotonin

Lower Half: Scatchard Plots of the Same Data

The difference between the mean values of the total binding (■) and the non-specific binding (▲) was taken as the specific binding (○). Concentrations of ^{3}H-serotonin ranged from 0.05 to 17 nM.

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and a carrier-amplifier. The bathing solution was Krebs–Henseleit's solution (containing in mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 12) aerated with a mixture of 95% O_2 and 5% CO_2. The temperature of the solution was maintained at 37 ± 1 °C.

An initial resting force of 1 g was applied to the aorta. The preparations were allowed to equilibrate for 1 or 2 h before exposure to drugs. Cumulative contractile concentration-response curves to serotonin were obtained by injection of appropriate amounts dissolved in a constant volume (12 μl) of double-distilled water.

Chemicals: Ketanserin and SGB-1534 used in the present study were donated by Kyowa Hakko Kogyo Co., Ltd. and Chugai Pharmaceutical Co., Ltd., respectively.

Results

The saturation curves for total and specific binding and Scatchard plots of specific binding are shown in Figs. 1 and 2. Specific ^{3}H-serotonin and ^{3}H-ketanserin binding in dog brain and aorta gave linear Scatchard plots. The K_d and B_{max} values of ^{3}H-ketanserin binding to $5HT_2$ receptors in dog brain and aorta were 0.59 nM and 33 fmol/mg protein (brain), and

Table I. Inhibition of ^{3}H-Ketanserin Binding to $5HT_2$ Receptor Sites in Brain and Aorta by Drugs

	Brain K_i (nM)	Aorta K_i (nM)
Ketanserin	3.72 ± 1.32 (5)	368 ± 290 (4)
Cinanserin	10.8 ± 5.90 (5)	228 ± 108 (6)
SGB-1534	58300 ± 53800 (4)	15700 ± 5470 (4)
Prazosin	21800 ± 9310 (3)	51100 ± 49200 (4)

Values are each the mean ± S.E. The numbers in parenthesis are the numbers of experiments. The concentration of ^{3}H-ketanserin used was 0.6 nM. Values of the inhibition constant (K_i) were calculated according to the following equation of Cheng and Prusoff (*Biochem. Pharmacol.*, **22**, 3099 (1973)): $K_i = IC_{50}/(1 + D^*/K_d^*)$, where IC_{50} = the concentration of non-radioactive drug that inhibits 50% of the radioactive drug binding. D^* = free concentration of the radioactive drug in the incubation solution, and K_d^* = equilibrium dissociation constant of the radioactive drug for the binding site.

Table II. Inhibition of ^{3}H-Serotonin Binding to $5HT_1$ Receptor Sites in Brain by Drugs

	K_i (nM)
Serotonin (4)	11.0 ± 5.0
Ketanserin (5)	13500 ± 6850
SGB-1534 (4)	371 ± 233
Prazosin (3)	183000 ± 95700

Values are each the mean ± S.E. Numbers in parenthesis are the numbers of experiments. The concentration of ^{3}H-serotonin used was 1.0 nM.

Table III. pA_2 Values of Ketanserin, Cinanserin, SGB-1534 and Prazosin

	pA_2
Ketanserin	8.57 ± 0.12 (5)
Cinanserin	7.67 ± 0.17 (5)
SGB-1534	4.69 ± 0.05 (5)
Prazosin	—

Values are each the mean ± S.E. The numbers in parentheses are the numbers of experiments. Prazosin (10^{-4} M) did not inhibit contraction induced by serotonin. The pD_2 value of serotonin was 5.80 ± 0.06 ($n = 27$).

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1.20 nM and 36 fmol/mg protein (aorta), respectively. The K_d and B_{max} values of ^{3}H-serotonin in the dog brain were 2.28 nM and 136 fmol/mg protein, respectively.

Tables I and II summarize the K_i values of ketanserin, cinanserin, prazosin and SGB-1534 derived from displacement experiments using ^{3}H-serotonin and ^{3}H-ketanserin as radioligands, respectively. Ketanserin exhibited low values of K_i to $5HT_2$ receptors in dog brain and aorta, but SGB-1534 and prazosin had high values of K_i (Table I). On the other hand, the effect of ketanserin on ^{3}H-serotonin binding to $5HT_1$ receptors in dog brain showed a high K_i value (Table II). Table III shows the results of the pharmacological assessment of antagonistic effects of ketanserin, cinanserin and SGB-1534 against $5HT_2$ receptors. Ketanserin and cinanserin showed high pA_2 values, but prazosin and SGB-1534 did not.

Discussion

Serotonin has a stimulant effect on the aorta and heart both *in vivo* and *in vitro*, and there is convincing evidence that the role of serotonin in the peripheral vasculature contributes to the development and maintenance of hypertension.[9] Ketanserin antagonizes the vascular effect of serotonin *in vivo*. In addition, although the presence of serotonin receptors has been demonstrated in various tissues including cardiac tissues, the central nervous system and platelets, little information is available on $5HT_2$ receptors in the vascular tissues. Thus, it is of interest to study the existence and role of serotonin receptors in vascular tissues and the effect of drugs on the serotonin receptors in those tissues.

Recently, radioligand binding assay methods with ^{3}H-lysergic acid dimethylamide (LSD),[3,10] ^{3}H-spiperone,[11] ^{3}H-mianserin[12] and ^{3}H-ketanserin[13] as radioligands have been used to demonstrate the existence of $5HT_1$ and $5HT_2$ receptors in the various tissues mentioned above. In the present study, binding techniques using ^{3}H-ketanserin as a radioligand have demonstrated the existence of $5HT_2$ receptors in the aorta as well as the brain. In addition, K_d and B_{max} values obtained here from homogenate fractions prepared from dog brain coincided exactly with those of membrane fraction from rat frontal cortex.[13] It is clear that vascular tissues used in the present study contained $5HT_2$ receptors, although the ketanserin binding showed higher affinity in the dog brain than in the aorta. In addition, K_i values of ketanserin, cinancerin, SGB-1534 and prazosin were different between the brain and aorta. Hoyer *et al.*[14] reported that ^{3}H-ketanserin bound to both $5HT_2$ receptors and α_1-adrenoceptors and the displacement analysis in human and pig brain membranes showed biphasic curves. The different affinity of ^{3}H-ketanserin in brain and aorta may be due to the membrane composition, enzyme activity in the membrane, membrane fluidity or different environments of receptors in membrane preparation from the two tissues.

It is well known that both $5HT_2$ antagonists and α_1-adrenoceptor antagonists ameliorate hypertension, and these two different types of chemicals have different types of hypotensive mechanism. As shown in Table I, SGB-1534 and prazosin showed a weak displacing effect on ^{3}H-ketanserin binding to $5HT_2$ receptors in the brain and aorta. Ketanserin, which is the prototype of a new chemical series of selective, pure and potent $5HT_2$ receptor-blocking agents, is a basic 4-substituted piperidine derivative.[15] Ketanserin also showed potent inhibition of ^{3}H-ketanserin binding to $5HT_2$ receptors in the dog brain and aorta (Table I). However, we showed in our previous report[1a] that prazosin and SGB-1534 markedly inhibited ^{3}H-prazosin binding to α_1-adrenoceptors in the aorta, suggesting that the hypotensive effect of this drug may be due to α_1-blocking action in vascular tissues. Ketanserin and SGB-1534 both have basic 3-ethyl-2,4(1*H*, 3*H*)-quinazolinedione moieties, but piperidine and the fluorobenzoyl group of ketanserin are different from those of SGB-1534. The different structures may be important for the activity of $5HT_2$ binding receptor blocking. Prazosin, which is a potent α_1-adrenoceptor blocker, also lacks this structure and showed weak

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inhibition of ^{3}H-ketanserin binding, like SGB-1534.

Not only do radioligand studies provide an accurate measure of receptor density and drug affinity for specific membrane recognition sites, but they can also assess the potencies of the anti-serotonergic action of newly synthesized chemicals. Highly significant correlations were found between the half-maximal contraction of vessels (ED_{50}) and the binding affinity of serotonin for $5HT_2$ binding sites.[16] Also, the potencies of drugs to antagonize serotonin-induced vasoconstriction matched the binding affinities of drugs for the serotonin-S_2 ($5HT_2$) site. Hence, although the present study used only two kinds of methods (radioligand binding assay and pharmacological methods) to assess the $5HT_2$ blocking activity, we can conclude that the potencies of $5HT_2$ receptor blocking activity by SGB-1534 and prazosin are weak.

Central serotonergic neurons have important influences on blood pressure,[17] although the nature of the regulatory function of serotonin in the brain is uncertain. Furthermore, there are contradictory reports in the literature as to whether a change in brain serotonin level causes a blood pressure change. However, the results presented here suggest that SGB-1534 has a weak effect on the serotonergic receptors in the brain, because the serotonergic neurons are largely concerned in the hypotensive action of drugs.

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