J Pharmacol Sci **104**, 274 – 277 (2007)

Journal of Pharmacological Sciences

Short Communication

Identification of a Key Amino Acid of the Human 5-HT_{2B} Serotonin Receptor Important for Sarpogrelate Binding

Habib Abul Muntasir[1], Murad Hossain[1], Mohiuddin Ahmed Bhuiyan[1], Tadazumi Komiyama[2], Takashi Nakamura[1], Masanobu Ozaki[3], and Takafumi Nagatomo[1,*]

[1]Department of Pharmacology, [2]Department of Biochemistry, and [3]Department of Fundamental Pharmacology & Therapeutics, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima, Akihaku, Niigata 956-8603, Japan

Received December 11, 2006; Accepted May 15, 2007

Abstract. Based on radio-ligand binding and molecular modeling studies, sarpogrelate shows a moderate selectivity for 5-HT_{2B} versus 5-HT_{2A} receptors. To confirm the modeling data of sarpogrelate to 5-HT_{2B} receptors predicting interaction of sarpogrelate towards Asp135 in helix 3 of 5-HT_{2B} receptors, we constructed and characterized the mutation of this residue by site-directed mutagenesis. The Asp135Ala mutant did not exhibit any affinity for [^{3}H]rauwolscine. Therefore, it was not possible to find sarpogrelate affinity to the mutant using [^{3}H]rauwolscine. The mutation also abolished agonist-stimulated inositol phosphates formation. These results provide evidence that Asp135 is important for the interaction between 5-HT_{2B} receptors and sarpogrelate.

Keywords: 5-HT_{2B} receptor, sarpogrelate, site-directed mutagenesis

The 5-HT_{2B} receptor belongs to the G-protein-coupled seven transmembrane-spanning receptors superfamily and shares similar amino acid sequence (68% – 79% in the transmembrane segments), pharmacology, and signal transduction pathways with other 5-HT_2-receptor subtypes (5-HT_{2A} and 5-HT_{2C} receptors) (1). The 5-HT_{2B} receptor has been reported to play an important role in human embryonic development, especially at the cardiac level and in certain pharmacological cardiopathies (2 – 4). For instance, genetic ablation of 5-HT_{2B} receptor in mice leads to dilated cardiomyopathy. Overexpression of 5-HT_{2B} receptor in the heart also leads to abnormal mitochondrial function and hypertrophic cardiomyopathy (5). These findings promise to have important implications for understanding the pathogenesis of congenital heart disease and the development of potential therapeutic interventions for cardiovascular disease. Sarpogrelate, a 5-HT_2-receptor antagonist, was introduced as a therapeutic agent for the treatment of ischemic diseases associated with thrombosis (5).

With the help of molecular modeling and mutagenesis studies of 5-HT_{2B} receptors, we previously identified the important binding sites of sarpogrelate to 5-HT_{2A}- and 5-HT_{2C} receptors (6, 7). Molecular modeling of sarpogrelate to the 5-HT_{2B} receptor predicted that sarpogrelate makes strong electrostatic interactions towards aspartic acid (Asp) 135 in transmembrane helix (TMH) 3 of the 5-HT_{2B} receptor (8). Based on this data, our aim was to identify the sarpogrelate binding site of the 5-HT_{2B} receptor by site-directed mutagenesis.

A cDNA clone encoding human 5-HT_{2B} receptor (Clontech Laboratories, Mountain View, CA, USA) was subcloned into BamHI and XbaI sites of pcDNA 3.1(+) (Invitrogen Life Technologies, CA, USA) for expression. A 5-HT_{2B}-receptor mutant was constructed with the Quick Change Site-Directed Mutagenesis Kit (Stratagene U.S.A., La Jolla, CA, USA).

HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stably expressing cell lines were constructed in HEK293 cells by transfecting with Lipofectamine^{TM} 2000 reagent and selecting with 0.5 mg/ml G418-containing growth medium as detailed by the manufacturer's protocol (Invitrogen Life Technologies).

Suspensions of cells expressing human 5-HT_{2B} recep-

*Corresponding author. nagatomot@s4.dion.ne.jp
Published online in J-STAGE: July 3, 2007
doi: 10.1254/jphs.SC0060241

tors were harvested by centrifugation at 2,200 × *g* for 15 min at 4°C. All membrane preparation procedures were carried out as described by Wainscott et al. (9). Protein content was measured by the method of Lowry et al. (10). For the competition binding experiments, a 100 μg of membrane protein was incubated with 6.0 nM [^{3}H]rauwolscine (PerkinElmer Life Sciences, MA, USA) in buffer B (67 mM Tris-HCl, 3 mM $CaCl_2$, 10 μM pargyline, 0.1% ascorbic acid, pH 7.4) for 20 min at 37°C. For the saturation binding studies, concentrations of [^{3}H]rauwolscine (specific activity of 82 Ci/mmol) concentrations of 0.1 – 28 nM were used for both the wild type and mutant. Non-specific binding was defined with the use of 10 μM 1-1-napthyl piperazine (ICN Biomedicals Inc., Aurora, OH, USA). Analysis of the binding experiment data was performed by Prism software (GraphPad, San Diego, CA, USA). Binding affinities of unlabeled drugs are expressed as the negative logarithm of the equilibrium dissociation constant (pK_i) using the Cheng and Prusoff (11) equation.

Accumulation of total [^{3}H]inositol phosphates (IP) was assayed as described previously (7). Briefly, cells were split into 24-well plates at a density of 10^5 cells/well and labeled with 3 μCi/ml [^{3}H]myo-inositol in serum-free DMEM for 24 h. Then the cells were washed with the assay medium (20 mM LiCl, 130 mM NaCl, 900 μM NaH_2PO_4, 5.4 mM KCl, 1.8 mM $CaCl_2$, and 25 mM glucose in 20 mM HEPES, pH 7.4) and incubated with both agonists (10^{-9} – 10^{-4} M) and antagonists at 37°C for 1 h. Cell extracts, in 10 mM formic acid, were applied to a 1-ml AG1-X8 resin (100 – 200 mesh; Assist Co., Tokyo) column before elution by buffer containing 1 M ammonium formate and 0.1 M formic acid. Radioactivity was measured by a liquid scintillation spectrophotometer. Single concentrations of the antagonists tested at 60 – 75 times higher than the binding affinity (pK_i) were used to clearly detect the shift in concentration-response curve for the agonist 5-HT. The pK_b values were calculated as described by Furchgott (12).

Western blotting analysis was done as described previously (6). Stably transfected HEK 293 cells expressing 5-HT_{2A} wild type and mutant receptors were constructed according to the methods described above. Equal amounts of protein samples were resolved by SDS-polyacrylamide gel electrophoresis (12% gradient gels) and transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences, USA) using a semi dry system in immunotransfer buffer. The membranes were blocked in blocking buffer (PBS with 10% nonfat dried milk) overnight at 4°C and incubated for 1 h at room temperature with anti-rabbit 5-HT_{2B}R polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:1000 dilutions in blocking buffer. The membranes were washed three times with PBS containing 0.1% Tween-20 and incubated with alkaline phosphatase-conjugated anti-rabbit IgG for 1 h at room temperature at 1:5000 dilution in blocking buffer. The membranes were washed and then the proteins on the membranes were visualized by adding Western blue (Promega) as a substrate.

Table 1. Binding and functional affinities of several 5-HT_2 antagonists for the wild-type 5-HT_{2B} receptor

Drugs	Wild-type 5-HT_{2B}	
	pK_i	pK_b
Sarpogrelate	6.11 ± 0.10 (3)	5.44 ± 0.10 (4)
Cyproheptadine	8.22 ± 0.03 (3)	7.77 ± 0.14 (3)
SB 206553	7.86 ± 0.19 (3)	7.72 ± 0.12 (3)

The binding affinities expressed as pK_i were determined using [^{3}H]rauwolscine in cell membranes and the functional affinities expressed as pK_b were estimated from the inhibitory effects of the antagonists on the concentration-dependent total inositol phosphate accumulation curve for serotonin according to the literature by Furchgott (12). The data shown indicate the mean ± S.E.M. and numbers in parentheses indicate number of experiments.

Both wild type and Asp135Ala mutant receptor genes were stably expressed in HEK 293 cells. Radioligand binding with [^{3}H]rauwolscine showed that the wild type receptor bound as expected, with a K_d value of 5.50 ± 0.25 nM, whereas the mutant receptor failed to bind [^{3}H]rauwolscine (data not shown). On the other hand, the B_{max} value for the wild type receptor, calculated from the maximal specific binding of [^{3}H]rauwolscine, was 292 ± 41 fmol/mg protein, whereas no B_{max} value was obtained with the mutant. The competition binding studies showed that the wild type receptor bound serotonin (Merck, Germany) with high affinity. The affinities (pK_i values) of two 5-HT_{2B} antagonists, cyproheptadine and SB 206553 (Research Biochemical Incorporated, Natick, MA, USA), for the wild type receptor were determined for sites labeled with [^{3}H]rauwolscine and compared with that of sarpogrelate (Mitsubishi Pharma Corporation, Tokyo). Both cyproheptadine and SB 206553 showed high binding affinities to the wild type receptor (Table 1). On the other hand, the binding affinity for sarpogrelate was significantly lower than those for cyproheptadine and SB 206553. In the case of the mutant receptor, it was not possible to find any 5-HT and sarpogrelate affinities to the mutant as the mutant was not able to bind [^{3}H]rauwolscine (Table 2). Such a factor underlies the crucial role of Asp135 in both 5-HT and sarpogrelate binding.

Table 2. Competition studies of 5-HT and sarpogrelate for [^{3}H]rauwolscine binding (pK_i) and 5-HT efficacy (EC_{50}) and functional affinity of sarpogrelate (pK_b) to 5-HT_{2B} wild-type and mutant receptors

Receptors	5-HT		Sarpogrelate	
	pK_i	EC_{50} (nM)	pK_i	pK_b
5-HT_{2B}-WT	8.04 ± 0.18 (3)	74.83 ± 2.25 (3)	6.11 ± 0.10 (3)	5.44 ± 0.10 (4)
Asp135Ala	N.D.	N.D.	N.D.	N.D.

The data shown indicate the mean ± S.E.M. and numbers in parentheses indicate number of experiments. N.D., not detectable.

The concentration-response curves for 5-HT-stimulated IP accumulation were determined. The wild type receptor showed an EC_{50} value of 74.83 ± 2.25 nM for 5-HT. Inhibition of 5-HT-stimulated IP formation by the antagonists was qualitatively similar to the binding assay results. Both cyproheptadine (4×10^{-7} M) and SB 206553 (10^{-6} M) showed high potencies in inhibiting 5-HT-stimulated IP formation (Table 1). Sarpogrelate (5×10^{-5} M) showed decreased sensitivity to the wild type receptor in inhibiting 5-HT-stimulated IP formation. On the other hand, the mutation in the receptor eliminated detectable IP accumulation. Therefore, it was not possible to determine 5-HT potency and inhibitory effects of sarpogrelate on 5-HT-stimulated IP accumulation for the mutant (Table 2).

As the Asp135Ala mutant did not exhibit detectable affinity for the [^{3}H]rawolscine and eliminated detectable 5-HT-stimulated IP accumulation, Western blotting was performed for both 5-HT_{2B} wild-type and mutant receptors. Western blots probed with the anti-5-HT_{2B} receptor antibody detected an immunoreactive band of about 54 kDa in both wild-type and mutant receptor-expressing cells (Fig. 1), as reported earlier for this antibody in NIH-3T3 cells (13), suggesting that the mutations in TMH 3 did not cause any abnormality in the receptor proteins. It is also suggested that the mutant proteins are inactive but are still expressed and detectable by antibody.

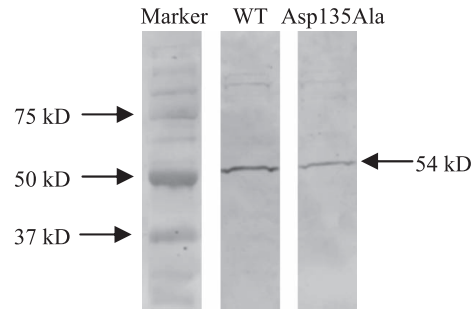

Fig. 1. Western blot analysis of Asp135Ala mutant receptor and comparison with the wild-type 5-HT_{2B} receptor in stably transfected HEK293 cells. Lane 1: human 5-HT_{2B} wild-type; lane 2: Asp135Ala mutant.

The main finding reported herein is that Asp135 in the 5-HT_{2B} receptor contributes to sarpogrelate binding through electrostatic interactions between the acidic function oxygen of Asp135 and the hydrogen atom of the trimethyl ammonium of the ligand. In the present study, Asp135 was mutated to alanine. The mutant receptor was expressed in HEK293 cells and assessed for the ligand binding affinity and 5-HT-induced IP production. From the binding data, it is evident that the Asp135Ala mutant eliminated both 5-HT and sarpogrelate binding. The mutant also abolished 5-HT-stimulated IP accumulation. These effects observed upon mutation confirm its interaction with both 5-HT and sarpogrelate in the activated state of the native form of human 5-HT_{2B} receptor. These data also correlated fully with expectations from the computational modeling of sarpogrelate with 5-HT_{2B} receptor (8). It has been reported that the interaction of ligands with the highly conserved residue aspartate of many neurotransmitter receptors, including serotonin receptors, provides a major component of the binding affinity (14). A previous study on the Asp135Ala mutant of the 5-HT_{2B} receptor revealed that the Asp135Ala receptor markedly decreased both 5-HT binding affinity and transduction efficacy (15). A much simpler explanation for the effect of the Asp135Ala mutation on ligand binding to the 5-HT_{2B} receptor is that the mutation results in disruption of the function of the receptor due to a change in the specific physicochemical properties of the side chain of the receptor. Moreover, the mutant may affect the ligand-receptor interactions by distortion of the neighboring amino acid residues, which are responsible for the interaction of the ligand.

The present study showed that the binding and the functional affinity of sarpogrelate to the 5-HT_{2B} receptor are significantly lower than those of other antagonists used. We reported that sarpogrelate shows very high binding and functional affinity to 5-HT_{2A} receptors (6). Molecular modeling of sarpogrelate to 5-HT_{2A} and 5-HT_{2B} receptors also showed the participation of a higher number of interacting residues in the 5-HT_{2A} receptor

than in the 5-HT_{2B} receptor (8). In the present study, we propose that the Asp135Ala mutant of the 5-HT_{2B} receptor markedly reduces the affinity of sarpogrelate, which indicates that this amino acid residue is the major determinant of sarpogrelate affinity and confirms the molecular modeling data. The modeling and the mutagenesis data for 5-HT_{2A} receptors we reported earlier and for 5-HT_{2B} receptors we have reported here are in good agreement with experimentally determined binding and functional affinity of sarpogrelate for receptors expressed in transfected cells.

In conclusion, our results indicate that Asp135 in helix 3 of the 5-HT_{2B} receptor is the most important site for sarpogrelate binding.

References

1 Barnes NM, Sharp T. A review of central 5-HT receptors and their function. Neuropharmacology. 1999;38:1083–1152.

2 Nebigil CG, Launay JM, Hickel P, Tournois C, Maroteaux L. 5-Hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways. Proc Natl Acad Sci U S A. 2000;97:2591–2596.

3 Nebigil CG, Etienne N, Scaerlinger B, Launay JM, Hickel P, Maroteaux L. Developmentally regulated serotonin 5-HT_{2B} receptors. Int J Dev Neurosci. 2001;19:365–372.

4 Fitzgerald LW, Burn TC, Brown BS, Patterson JP, Corjay MH, Valentine P, et al. Possible role of valvular serotonin 5-HT_{2B} receptors in the cardiopathy associated with fenfluramine. Mol Pharmacol. 2000;57:75–81.

5 Ito K, Notsu T. Effect of sarpogrelate hydrochloride (MCI-9042) on peripheral circulation of chronic arterial occlusive diseases. J Clin Ther Med. 1991;7:1243–1251.

6 Muntasir HA, Rashid M, Komiyama T, Kawakami J, Nagatomo T. Identification of amino acid residues important for sarpogrelate binding to the human 5-hydroxytryptamine$_{2A}$ serotonin receptor. J Pharmacol Sci. 2006;102;55–63.

7 Muntasir HA, Takahashi J, Rashid M, Ahmed M, Komiyama T, Hossain M, et al. Site-directed mutagenesis of the serotonin 5-hydroxytryptamine$_{2C}$ receptor: identification of amino acids responsible for sarpogrelate binding. Biol Pharm Bull. 2006;29:1645–1650.

8 Rashid M, Manivet P, Nishio H, Pratuangdejkul J, Rajab M, Ishiguro M, et al. Identification of the binding sites and selectivity of sarpogrelate to human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor subtypes by molecular modeling. Life Sci. 2003; 73:193–207.

9 Wainscott DB, Marlene LC, Kathryn WS, James EA, Jeffrey SN, Melvyn B, et al. Pharmacological characteristics of the newly cloned rat 5-hydroxytryptamine$_{2F}$ receptor. Mol Pharmacol. 1992;43:419–426.

10 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–275.

11 Cheng Y-C, Prusoff WH. Relationship between the inhibition (Ki) and concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacol. 1973;22:3099–3108.

12 Furchgott RF. The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In: Blaschko H, Muscholl E, editors. Catecholamines. Handbook of experimental pharmacology. Berlin: Springer; 1972: p. 283–335.

13 Wu C, Yoder EJ, Shih J, Chen K, Dias P, Shi LR, et al. Development and characterization of monoclonal antibodies specific to the serotonin 5-HT2A receptor. J Histochem Cytochem. 1998;46:811–824.

14 Wang CD, Gallaher TK, Shih JC. Site-directed mutagenesis of the serotonin 5-hydroxytryptamine 2 receptors: identification of amino acids necessary for ligand binding and receptor activation. Mol Pharmacol. 1993;43:931–940.

15 Manivet P, Schneider B, Smith JC, Choi D, Maroteaux L, Kellermann O, et al. The serotonin binding site of human and murine 5-HT_{2B} receptors; molecular modeling and site-directed mutagenesis. J Biol Chem. 2002;277:17170–17178.