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Mechanism of Inverse Agonist Action of Sarpogrelate at the Constitutively Active Mutant of Human 5-HT_{2A} Receptor Revealed by Molecular Modeling

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We previously reported that sarpogrelate, a selective 5-HT_{2A} antagonist, showed a potent inverse agonist activity to constitutively active mutant (C322K) of human 5-HT_{2A} receptor (5-HT_{2A}R). However, it remains to be unknown about the actual mechanism of this mutant for its constitutive activation as well as inverse agonist activity of sarpogrelate. Our model shows that mutation (C322K) of 5-HT_{2A}R causes electronic repulsion between positively charged Arg173(3.50) and Lys322(6.34) residues resulting outward movement of the C-terminus of transmembrane helix (TMH) III. This motion of TMH III leads to a partially active structure of the receptor, which may be a key step in receptor activation. The structural model of the partially active receptor also indicates that the binding of sarpogrelate to the constitutively active receptor causes an inward swing of TMH III to an inactive receptor structure. Therefore, the present study may suggest that the electronic repulsion causing outward movement of the C-terminus of TMH III may be the key step for constitutive activation of mutant C322K of 5-HT_{2A}R and the inward movement of TMH III causes the inverse agonist activity of sarpogrelate.

Key words 5-HT_{2A} receptor; mutation; constitutive activity; molecular modeling; electronic repulsion

It has been established that many G-protein coupled receptors (GPCRs) can exist in a constitutively active form in the absence of agonist.^{1–3} Certain molecules could act as inverse agonists and reduce the levels of constitutive activity and functional cellular responses.^{3–6} Inverse agonism is very common among GPCR antagonists.⁷ It has become apparent over the past few years that a number of compounds that had been thought to be neutral antagonists at GPCRs possess negative efficacy and have been termed inverse agonists. This was first shown for μ -opiate and α_2 -adrenergic receptors^{1,8} but has subsequently been extended to many GPCRs.^{9,10} Considering the therapeutic implication, it is suggested that all new antagonists should be routinely tested for their potential inverse agonistic activity in future drug development programs.^{11,12}

This concept is of particular importance for the 5-HT_{2A} receptor (5-HT_{2A}R) where the drugs used to treat cardiovascular diseases had been assumed to be antagonists at this receptor. It has, however, been shown using potentiation of basal inositol phosphate activity¹³ that all of the drugs tested were inverse agonists and there was a good correlation between inverse agonist potency and ligand binding affinity. In the previous study, we demonstrated that sarpogrelate, a selective 5-HT_{2A} antagonist, had been shown to be a potent inverse agonist at the constitutive active human 5-HT_{2A}R.¹⁴ Patients are treated chronically with this drug and the effects of an inverse agonist on the activity of 5-HT_{2A}R may be quite different to those of an antagonist. Owing to the important therapeutic activity (*i.e.* to treat ischemic diseases) of sarpogrelate, it is very important to understand the mechanism of its inverse agonist activity at the 5-HT_{2A}R as the inverse agonism exhibited by

this drug may be of relevance to their therapeutic effects.

Mutations of critical residues at the cytoplasmic ends of transmembrane helix (TMH) III and VI can trigger a series of conformational changes that lead to the high constitutive activity of GPCRs.¹⁵ The availability of atomic-resolution crystal structure models of the inactive form of bovine rhodopsin^{16,17} facilitates computational modeling of such conformational changes, yet only few have been characterized to date. Moreover, so far there is no report on the level of activation (partial or full) by the constitutive active mutants of GPCRs for understanding the importance of their pathological state. In the present study, we have examined a partial agonist, dimethyl serotonin (DM 5-HT) for a constitutive active mutant by the substitution of Cys322(6.34)¹⁸ of TMH VI to the positively charged lysine (Lys), Cys322Lys (C322K). Thus, the purpose of this study is to investigate the mechanism of activation of the constitutive active mutant of 5-HT_{2A}R and examined whether this activation is partial or full by comparing with a partial agonist, DM 5-HT and a full agonist, 5-HT and thereby the inverse agonist activity of the antagonist with the help of molecular modeling studies.

MATERIALS AND METHODS

Materials Mianserin was obtained from RBI (Research Biochemical Inc., Natick, MA, U.S.A.) and sarpogrelate from Mitsubishi Tanabe Pharma, Tokyo, Japan. Di-methyl serotonin was synthesized in the department of Organic Chemistry, Niigata University of Pharmacy and Applied Life Sciences. Serotonin was obtained from Merck, Germany. [³H]Ketanserin was purchased from NEN Life Sciences, MA, U.S.A.

Ligand Binding DNA plasmid encoding the mutant

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C322K 5-HT_{2A}R gene was stably transfected in HEK293 cells. After harvesting the cell pellets were stored at -80°C until use. All membrane preparation procedures were carried out at 4°C. Cell pellets were thawed and homogenized in 1 mL of 50 mM Tris-HCl (pH 7.4 at room temperature) with a Polytron homogenizer (Kinematica, Switzerland). The homogenates were centrifuged at 35000×*g* for 15 min. The membrane pellets were resuspended in the same buffer with a Teflon glass homogenizer. Protein content was measured by the method of Lowry.¹⁹ Each binding incubation tube contained *ca.* 50 µg of membrane protein, [³H]ketanserin, unlabeled drug as required, and binding buffer in a final volume of 250 µL. Competition binding assays were carried out as described previously.¹⁴ Briefly, for competition binding studies, the concentration of [³H]ketanserin for both wild-type and mutant receptors was 1.0 nM and 5 concentrations of competing ligands were used in duplicate. Nonspecific binding was defined with the use of 10 µM mianserin. For radioligand, incubations were carried out for 30 min at 37°C and were terminated by rapid filtration through Whatman GF/C filters that had been presoaked in 0.3% polyethyleneimine followed by washing with 10 mL ice-cold buffer. The radioactivity retained on the filters was quantitated by liquid scintillation spectrophotometry.

Inositol Phosphate Assay Accumulation of total [³H]-inositol phosphates (IP) was assayed as described previously.¹⁴ Stably transfected HEK293 cells at about 90% confluent in 10-cm dishes were seeded into 24-well plates. Twenty-four hours after seeding, cells were washed with serum-free Dulbecco's modified Eagle's medium (DMEM) and labeled with 1 µCi/mL [³H]myo-inositol in serum-free DMEM for 18–20 h. After labeling, the medium was replaced with the assay medium (Hank's buffered salt solution containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and 20 mM LiCl, pH 7.4, 37°C). The cells were incubated for 15 min at 37°C by floating the plates in a temperature-controlled water bath. Agonists and antagonist in assay medium were added to each well and incubation continued for additional 30 min. Assay medium was removed and the reaction was stopped by adding 1 mL of 10 mM formic acid (previously stored at 4°C) to each well. The plates were stored at 4°C for 24 h and cells were neutralized by adding 1 mL 500 mM KOH and 9 mM sodium tetraborate per well. The contents of each well were extracted and centrifuged for 5 min at 3000 rpm and the upper layer loaded onto a 1-mL AG1-X8 resin (100–200 mesh, Assist Co., Japan) column. Columns were washed 2 times with 5 mL 60 mM sodium formate and 5 mM borax. Total IPs were eluted with 5 mL 1 M ammonium formate and 0.1 M formic acid. Radioactivity was measured by liquid scintillation spectrophotometry.

Computational Methods Three-dimensional structural models of metarhodopsins Ib and I₃₈₀ constructed previously²⁰ were used for the homology modeling of antagonist-bound and partial agonist-bound structures of human 5-HT_{2A}R, respectively. Construction of the GPCR structural models was described previously.²¹ The structures of the N-terminal residues and the C-terminal residues, which are not available in the crystal structure of rhodopsin were not constructed for the present receptor models.

The initial models of antagonist-bound and partial agonist-bound 5-HT_{2A}Rs were energy-minimized and then optimized using the molecular dynamics/minimization procedure

by tethering the α -carbons of the receptor structures at the original position. The lowest-energy structure for each receptor model was selected as an energy-refined receptor model.

The ligand-binding space in the antagonist-bound receptor model was estimated by the Binding-Site module installed in InsightII (Accelrys Inc., San Diego, CA, U.S.A.). The ligands were then docked into the ligand-binding cleft within a distance (<3.0 Å) between the cationic amine of the ligand and the carboxylate oxygen of the conserved Asp173 (3.50) residue at the extracellular site of TMH III of the 5-HT_{2A}R. An initial energy-minimization of the complex structure was performed by using Discover 3 (Accelrys Inc.), followed by optimizing the structure with the molecular dynamics procedure. The lowest-energy structure for the receptor model was selected as an energy-refined complex model.

Data Analysis Nonlinear regression analysis of competition binding assay was performed using GraphPad Prism software (San Diego, CA, U.S.A.). In competition binding experiments, the values of inhibition constants (K_i) were calculated by the following equation.²²

$$K_i = IC_{50} / \{1 + ([L] / K_d)\}$$

where, the inhibition concentrations (IC_{50}) were determined as the concentrations of ligands that inhibited [³H]ketanserin binding by 50%; $[L]$ =the concentration of [³H]ketanserin used and K_d =the dissociation constant of [³H]ketanserin for the receptors. Results are represented as mean±S.E.M. Statistical analyses were performed by the Student's unpaired *t*-test or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison analysis. A *p* value of less than 0.05 was taken as significant.

RESULTS

Ligand Binding Affinities To compare the binding affinities of DM 5-HT, 5-HT and sarpogrelate in between the wild type and C322K mutant 5-HT_{2A} receptors, we examined their K_i values in competition binding experiments for sites labeled with [³H]ketanserin by radioligand binding assay and the results were shown in Table 1. The K_i values for DM 5-HT remained unchanged for both wild type and mutant receptors. The C322K mutant exhibited almost 100-fold increase in 5-HT affinity in comparison with their wild type receptors. The results also showed that there was a significant decrease in the binding affinities of sarpogrelate and it showed almost 2-fold lower binding affinity for the C322K mutant receptors compared with the wild type.

Inositol Phosphate Assay HEK293 Cells stably transfected with the C322K mutant receptor displayed a 4-fold increase in basal levels of inositol triphosphate (IP₃) production compared with the cells expressing wild type receptors (Figs. 1A,B). These results demonstrate that mutations of Cys322(6.34) to lysine can be rendered constitutively active. Partial agonist (DM 5-HT) increased IP₃ production 4-fold the basal level of wild type receptor (Fig. 1A). In contrast, C322K mutant receptor showed no further increase of IP₃ production after addition of 10 µM DM 5-HT and showed almost similar stimulation with the basal level of C322K mutant receptor (Fig. 1A). On the other hand, both the wild type and the C322K mutant receptors produced a further increase of IP₃ production after stimulation of 10 µM 5-HT (Fig. 1A). In

Table 1. Binding Affinities (pK_i) of Agonists and Antagonists to Wild-Type and Mutant 5-HT_{2A} Receptors

	5-HT _{2A} Receptors	
	Wild type K_i (nM)	Mutant (C322K) K_i (nM)
Partial agonist		
DM 5-HT	4.0±0.2	4.0±0.1
Full agonist		
5-HT	4.8±0.5	0.5±0.04**
Antagonists		
Sarpogrelate	23.0±2.0	44.0±3.0**

[³H]Ketanserin (1.0 nM) was used to label 5-HT_{2A} wild type and mutant receptors expressed in HEK293 cells. Data represent the mean±S.E.M. $n=3-4$, each experiment performed in duplicate. ** $p<0.01$ vs. wild type.

cells transfected with wild type receptors, 5-HT stimulated IP₃ production 11-fold over the basal level. In addition, the C322K mutant showed 3-fold increase of IP₃ production after stimulation of 5-HT. However, there was no significant difference in maximal 5-HT stimulation of the wild type and mutant 5-HT_{2A} receptors. 5-HT_{2A}R selective antagonist, sarpogrelate used in this study showed inverse agonist activity at the C322K mutant by significantly reducing the basal IP₃ production (Fig. 1B). Maximal inhibition of basal activity was 70%. Although this drug tested had robust inverse agonist activity, it did not produce a complete inhibition of the basal IP₃ production. On the other hand, they had no effects on basal activity of the wild type receptors (data not shown).

Molecular Modeling The molecular model of the 5-HT_{2A}R based on the three-dimensional structural model of metarhodopsins Ib (an antagonist-bound form) (Ishiguro *et al.*²⁰) suggests that the most conserved residue, Arg173(3.50) in TMH III and Cys322(6.34) in TMH VI are in close proximity (Fig. 2). The residues are well packed and no atomic clashes are observed here. In contrast, the replacement of Lysine (Lys) residue for Cys322(6.34) yielded an electronic repulsion against the positively charged Arg173(3.50) residue and sterically unfavorable interactions (Fig. 3). As a result, the electronically and sterically repulsive forces cause the outward swing of the C-terminal end of TMH III, leading

to an open-state (partially active) structure of the receptor at the intracellular site (Fig. 3). In the open-state structure of the mutant receptor, Lys322(6.34) formed a salt bond with Glu318(6.30). This interaction may also disturb the ionic lock between Arg173(3.50) and Glu318(6.30) to render the TMH III more labile. This open-state structure would lead to an activated conformation state, which can couple to the G-protein to form the functionally activated state, thus providing an explanation for the observed constitutive activity on a structural level. These results are in good agreement with the proposed metarhodopsin I₃₈₀ model that the outward swing of the C-terminal end of TMH III leads to a conformational change in Arg173(3.50), which facilitates the GDP-GTP exchange in G proteins (G-protein activation).²¹

Our structural model also showed that sarpogrelate docking into the ligand-binding cleft at the extracellular site of TMH III of the receptor caused the inward swing of TMH III to an inactive state (Fig. 4). However, the reversion to the native inactive state (the antagonist bound form) was not complete (Fig. 1B).

DISCUSSION

We previously showed that mutation of Cys322(6.34) of 5-HT_{2A} receptors might lead to constitutive receptor activation.¹⁴ In the present study, we made a substitution of Cys322(6.34) of TMH VI to the positively charged lysine residue that evoked an active form displaying the same level of activation induced by partial agonist in wild type receptors. It is well established that TMH III plays an important role in GPCR activation. The DRY motif at the C-terminus of TMH III is highly conserved throughout the subfamily of rhodopsin-like GPCR family. Mutation of Arg173(3.50) has established an important role of this residue in G-protein activation in many GPCRs, including 5-HT_{2A}.²³⁻²⁵ The crystal structures of rhodopsin, β -adrenergic and adenosine receptors suggest that the Arg173(3.50) residue would face to the Glu318(6.30) residue in TMH VI, forming an ionic bond. The Cys322(6.34) residue is located proximal to Glu318(6.30) as well as Arg173(3.50) (Fig. 2) and thus we could expect

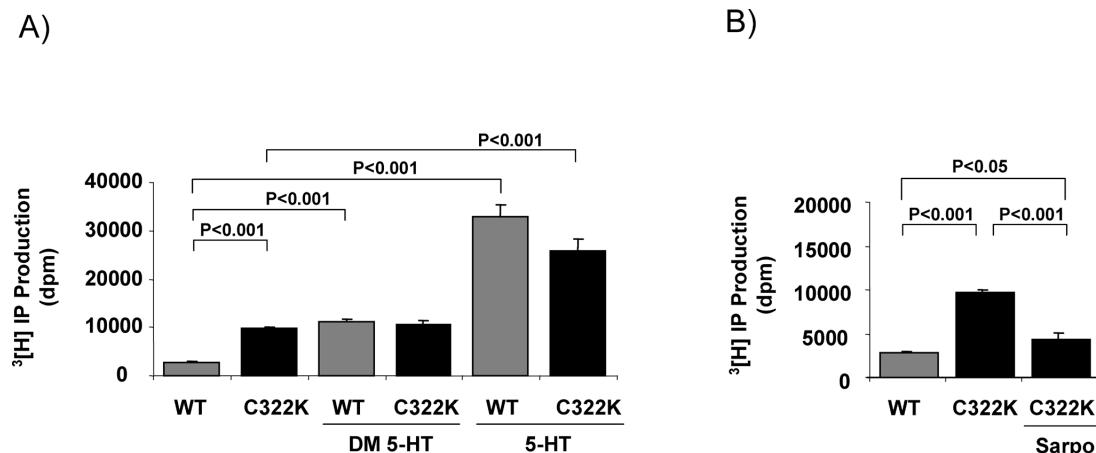


Fig. 1. Effect of Partial and Full Agonists Stimulation of IP Production with 5-HT_{2A} Wild Type and Mutant (C322K) Receptors (A) and Inverse Agonist Activity of Sarpogrelate with C322K Mutant of 5-HT_{2A} Receptors (B)

HEK 293 cells were incubated for 30 min with the indicated compounds, and inositol phosphate accumulation was measured as described under Materials and Methods. The concentration used for each drug was 10 μ M. Data are the mean±S.E.M. of four to six separate experiments performed in duplicate. The means were calculated by using one-way ANOVA with Bonferroni's multiple comparison analysis with $p\leq 0.001$.

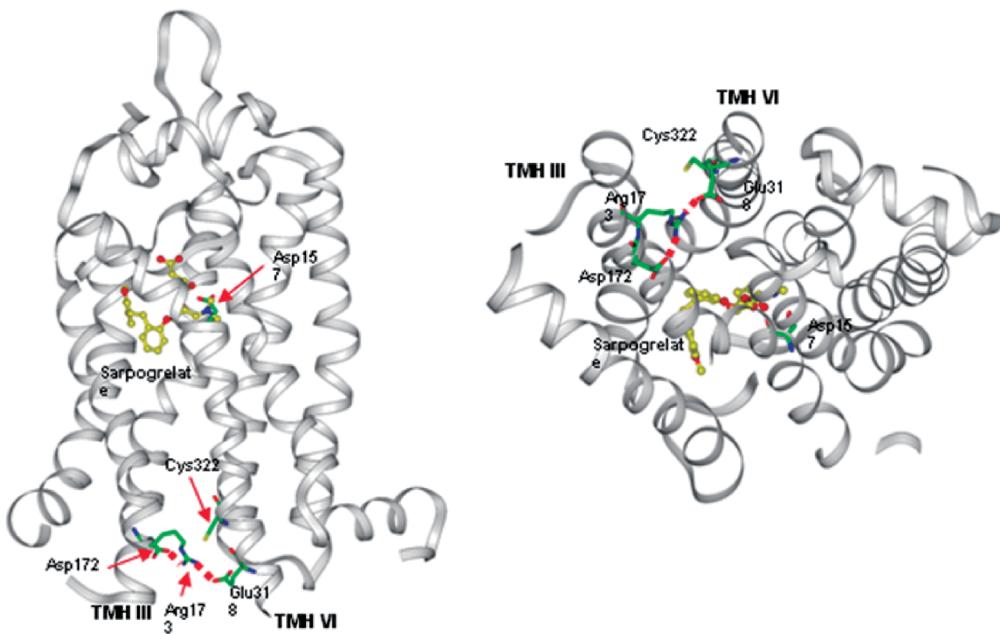


Fig. 2. Computational Model of the 5-HT_{2A}R Based on the Metarhodopsin Crystal Structure Showing the Close Proximity between Arg173(3.50) of TMH III and Cys322(6.34) of TMH VI

Left: lateral view, right: bottom view. Sarpogrelate (yellow) and important amino acid residues are shown and hydrogen bonds are shown by dotted red lines.

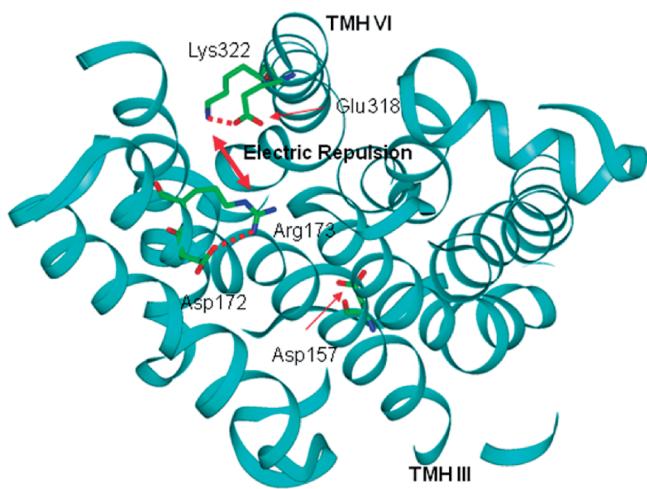


Fig. 3. The Electronic Repulsion between Arg173(3.50) of TMH III and Lys322(6.34) of TMH VI (Thick Red Arrow) Leading to Partial Agonist Bound Form of Cys322(6.34)Lys Mutant of 5-HT_{2A}R

A putative hydrogen bond between Lys322(6.34) and Glu318(6.30) is shown by a dotted red line.

that the mutation of the positively charged Lys residue for the Cys322(6.34) residue would influence the role of the Arg173(3.50) residue in the receptor activation to produce a constitutive activity of the receptor. A G protein-bound structure of β -adrenergic receptor²⁶⁾ and a structure of metarhodopsin II binding a peptide fragment of transducin, a G protein,²⁷⁾ are reported. Although these structures would be candidates for the active structures, it still remains unclear whether they are active structures in the physiologically conditions and whether they are partial agonist bound structures. Thus, we have used a structural model for a partially active receptor model derived from the rhodopsin structure.^{20,21)}

To investigate how much the level of activation produced by

the introduction of the mutation of Lys for Cys322(6.34) of the 5-HT_{2A} receptor, we examined the binding affinities and IP₃ production of the C322K mutant after addition of partial and full agonists and a 5-HT_{2A}R selective antagonist. The most striking findings of the present study are that the constitutive activation of the C322K mutant showed the same level of activation induced by partial agonist in wild type receptors and that the activity level of the mutant receptor was not changed by the partial agonist (Fig. 1A). On the other hand, 5-HT produced same level of activation for both wild type and mutant receptors and thus produced higher activation than DM 5-HT. In addition, sarpogrelate, an antagonist for 5HT_{2A}R, inhibited the activation of this constitutive active mutant and rendered it to a similar inactive state of the wild type (Fig. 1B). Thus, the present study indicates that the C322K mutant of human 5-HT_{2A}R constitutively produces partial activation of the receptor. On the other hand, the binding affinities of DM 5-HT were unchanged for both the wild type and the mutant 5-HT_{2A}R, while the affinity of 5-HT was increased at the C322K mutant compared with the wild type 5-HT_{2A} receptor. This result suggests that the free energy of the conformational change of the receptor from the partially activated state to the fully activated state is much lower than that from the inactive state to the fully active state. It has also been suggested that constitutively active receptor mutants display increased affinity for agonists as compared to wild type GPCRs²⁸⁾ and thus the increase of affinity for agonists appears to be common in constitutively active GPCRs.

The crystal structures of bovine and squid rhodopsins^{16,29,30)} suggested that the Arg3.50 in the inactive state forms salt bond with the adjacent Glu or Asp3.49 residue of TMH III. Upon mutation of the adjacent Glu or Asp3.49, the arginine3.50 residue will be released from the electronic constraint leading to a conformational change (Fig. 3) which enables the guanosine 5'-diphosphate (GDP)-guanosine 5'-triphosphate (GTP)

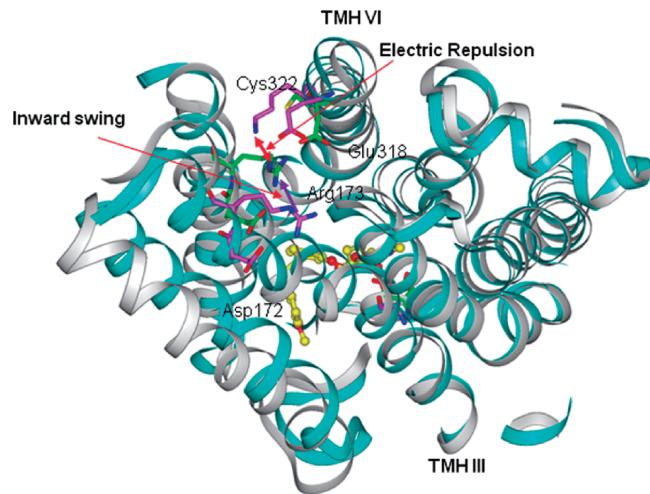


Fig. 4. The Inward Swing of TMH III (Purple) by Antagonist to Inactive Receptor Structure

White ribbon represents the partial agonist-bound form and cyan color ribbon represents antagonist-bound form of Cys322(6.34)Lys mutant of 5-HT_{2A}R.

exchange in G protein. It has also been observed that Arg3.50 and Glu6.30 participate in an ionic lock between TMH III and TMH VI at the intracellular end. It is assumed that 5-HT_{2A} and β_2 -adrenergic receptors also maintain the receptors in the inactive state through the ionic lock and that disruption of this ionic lock leads to the receptors in an active state.^{24,31} Therefore, we have hypothesized from the structural models of the native and mutant 5HT_{2A} receptor that the ionic interaction of Cys322(6.34)Lys with Glu318(6.30) weaken the ionic bond between Arg173(3.50) and Glu318(6.30) and the electronic repulsion between Arg173(3.50) and Cys322(6.34)Lys results in the interruption of the ionic interactions between Arg173(3.50) and Glu318(6.30), leading to the outbound movement of the C-terminus of TMH III which leads to a constitutive activity.

It has been reported that changes of the side chain to larger residues at the interface between TMH VI and III promoted constitutive receptor activation.²⁴ In human thyrotropin receptor, steric repulsion of Met325(6.37) caused by the side chain of Ile154(3.46) in TMH III provides evidence for the constitutive activity.³² The present mutagenesis and modeling studies suggest that steric hindrance caused by the bulkier side chain of the Lys322(6.34) residue is also a likely basis of the constitutive activity, although the contribution to the constitutive activity is smaller than the mutant at the residue 6.37, which is one turn inside of the TMH VI. The introduced steric repulsion between TMH III and TMH VI would shift the C-terminus of TMH III of the receptor in a more active conformation.

A higher residual activity of the mutant than that of the wild type receptor was observed upon response to the antagonist (Fig 1B). The bulkiness of the Lys residue may be a steric factor for inhibiting an inward motion of TMH III. It is more likely that there are two states equilibrating between antagonist-bound and -unbound states. In the native receptor, the bound and unbound forms are equally inactive whereas antagonist-unbound receptor is constitutively active in the constitutively active mutant. Thus, the mutant receptor should be more active than the native receptor when interacting with antagonists.

Compared with the wild type receptor, sarpogrelate showed almost 2-fold lower affinity for the C322K mutant receptor.

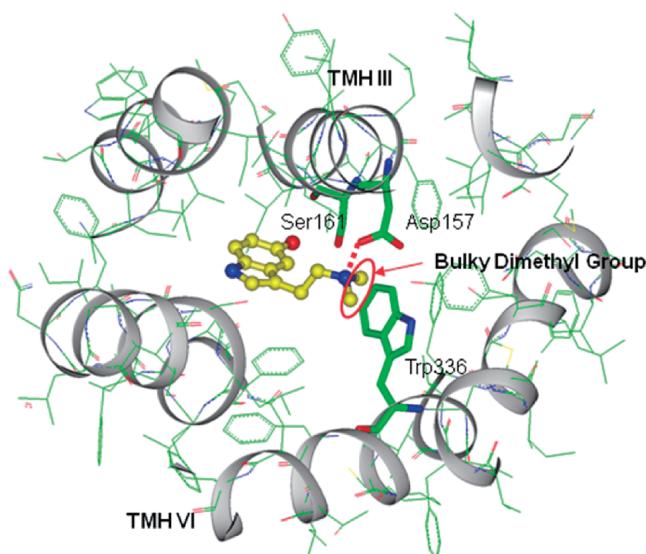


Fig. 5. A Complex Model of *N,N*-Dimethyl Serotonin (Yellow), a Partial Agonist, at the Ligand-Binding Cleft of the Partial Agonist-Bound Form of 5-HT_{2A}R

The ligand is shown by ball-and-stick model and the residues (green) are shown by stick model. Helices are shown by gray ribbon model. *N,N*-Dimethyl group shown by an arrow fits into the space generated by the motion of TMH III.

Our structural model of the antagonist-bound form of the receptor indicates that binding of sarpogrelate would cause the inward movement of the receptor (Fig. 4) for reversion to the inactive state structure, which have been evident from its lower binding affinity for the mutant receptors. On the other hand, the partial agonist, DM 5-HT, fits into the partial agonist-bound structure model. The *N,N*-dimethylamino group appears to require the larger ligand-binding space which formed by TMH III and TMH VI (Fig. 5).

As discussed above, the full agonist, 5-HT, showed a higher affinity in the binding to the constitutively active mutant receptor than the native receptor, although the both receptors show the same activation level. Furthermore, the constitutive activity of the mutant receptor is same as the activity produced by the partial agonist in the native receptor. This

implies that the constitutively active mutant receptor has the same structure as the partial agonist-bound structure. Thus, the partial agonist behaves as an antagonist on the mutant receptor. The present study suggests that the partially active receptor structure is different from the inactive structure which binds antagonists and also different from the fully activated structure bound by full agonists.

In conclusion, this study explored how a defect in packing of the cytoplasmic layer triggers a significant increase in basal 5-HT_{2A}R activity. Repulsion of the cytoplasmic portions of TMH III and TMH VI provides the molecular basis for the structural change in the Cys322Lys mutant. The present study suggests that the interruption of the ionic lock by the positively charged Lys322(6.34) and the electronic repulsion between the positively charged Arg173(3.50) residue at the C-terminus of TMH III and the positively charged Lys322(6.34) residue of TMH VI of the mutant receptor causes an outward motion of the C-terminus of TMH III. This motion of TMH III may lead to a partially active structure of the receptor, thus causing the constitutive activation of the receptor. The structural model of the partially active receptor indicates that the binding of sarpogrelate to the mutant receptor causes an inward swing of TMH III to an inactive receptor structure. Thus, the antagonist, sarpogrelate becomes an inverse agonist at the constitutively active receptor.

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