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# Activation switch in the transmembrane domain of metabotropic glutamate receptor

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**Running title page**

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**d) Abbreviations:**

  mGluR, Metabotropic glutamate receptor; TMD, transmembrane domain;

  ECD, extracellular domain; GPCR, G protein-coupled receptor;

  CAM, constitutively active mutation; CaSR, calcium-sensing receptor;

  L-AP4, $_L$-(+)-2-Amino-4-phosphonobutyric acid; LY341495,

  (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid

  MPEP, 2-Methyl-6(phenylethynyl)-pyridine; EM-TBPC,

  1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[d]azepin-3-yl)-

  1,6-dihydro-pyrimidine-5-carbonitrile

## Abstract

Metabotropic glutamate receptors (mGluRs), members of family 3 G protein-coupled receptors, play pivotal roles in the modulation of synaptic transmission and are important drug targets for various neurological diseases. The structures of the extracellular ligand-binding domain (ECD) of mGluRs and its changes upon ligand binding have been well studied by various techniques, including X-ray crystallography. In contrast, little is known about the structure and structural changes of the seven-transmembrane domain (TMD). Here we searched for constitutively active mutation (CAM) sites in the TMD of mGluR8 to get insight into the epicenter of the structural changes in TMD, a potential target for allosteric ligands. Mutational analyses based on the knowledge of activating mutations of calcium-sensing receptor showed the presence of several CAM sites in the TMD of mGluR8. Among them, the site at position 764 in helix V, where threonine is present, is unique in that any substitution resulted in elevation of the basal activity, and some substitutions caused a loss of responsiveness to agonist. Further comprehensive mutational analyses indicated that the additional mutation of the CAM site at position 663 in helix III, where a tyrosine residue is present, generated a revertant phenotype. Mutations at these sites also increased the agonist binding affinity, although these sites are far from the ECD. These results indicated that the specific pair of amino acids at these CAM sites forms an activation switch that stabilizes the inactive state of mGluR8 and mediates the signal flow from the ECD to the cytoplasmic G protein-interacting site.

**Introduction**

Metabotropic glutamate receptors (mGluRs), members of the family 3 G
protein-coupled receptors (GPCRs), play pivotal roles in the regulation of
synaptic plasticity in the cerebellum and hippocampus and in information
processing in the retina and olfactory bulb (Ferraguti and Shigemoto, 2006).
Eight subtypes of mGluRs have been identified and classified into three
subgroups based on their sequence similarity, transduction cascade and
pharmacology.   They exist as constitutive homodimers and possess a large
extracellular ligand-binding domain (ECD) on the N-terminal side of a
seven-transmembrane domain (TMD).    Upon agonist binding, they change the
structure of the ECD (Kunishima et al., 2000; Muto et al., 2007; Tsuchiya et al.,
2002), the helical arrangements of the TMD (Brock et al., 2007; Tateyama et al.,
2004; Yamashita et al., 2008) and the configuration of the cytoplasmic G
protein-interacting site (Francesconi and Duvoisin, 1998; Gomeza et al., 1996;
Yamashita et al., 2001), which eventually leads to the activation of G proteins.
Recently, mGluRs have received much attention as potential drug targets for a
wide variety of neurological disorders, and several artificial agonists, antagonists
and even allosteric modulators have been generated (Ossowska et al., 2007;
Patil et al., 2007).    In particular, allosteric modulators are expected to be
superior drugs with small side effects on ionotropic glutamate receptors and also
with high selectivity for each mGluR subtype (Marino et al., 2003; May et al.,
2007).    Thus, investigation of switch regions in the TMD, which are the potential
target sites of allosteric modulators, is of great interest.

It is generally accepted from the studies on family 1 GPCRs that a constitutively active mutation (CAM) site is one of the "epicenters" of active structural changes in GPCRs (Smit et al., 2007). In fact, it was reported that several CAM sites present in the transmembrane region in family 1 GPCRs act as an activation switch regulating the equilibrium between inactive and active states (Bakker et al., 2008; Cohen et al., 1993; Decaillot et al., 2003; Huang et al., 2001; Javitch et al., 1997; Kjelsberg et al., 1992; Prioleau et al., 2002). Recently, the crystal structures of rhodopsins and adrenergic receptors were revealed by X-ray crystallography studies (Okada et al., 2002; Palczewski et al., 2000; Park et al., 2008; Rasmussen et al., 2007; Rosenbaum et al., 2007; Scheerer et al., 2008), and provided substantial information about the CAM-induced conformational changes in family 1 GPCRs. Therefore, it can be speculated that if a CAM site is present in the TMD of mGluR, it should function in an activation switch of the TMD and could be a potential target site of allosteric modulators. The current findings clearly showed the presence of several CAM sites in the TMD of mGluR8. In addition, we obtained evidence that the CAM site at position 764 located in helix V forms a specific pair with that at position 663 in helix III. Destruction of this interaction by mutation of the amino acid residue at either site caused an elevation of constitutive activity. The importance of this pair of CAM sites in the TMD will be discussed in relation to the maintenance of the inactive state and the signal flow from the ECD to the cytoplasmic G protein-activating domain.

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## Materials and Methods

## Materials

[³H] LY341495 (1.28 TBq/mmol) and L-AP4 were purchased from Tocris Cookson;

[³⁵S]guanosine 5'-3-$O$-(thio)triphosphate (GTPγS) (37 TBq/mmol) from PerkinElmer Life

Sciences.

## Preparation of mGluR mutants

Expression of mGluR8 in HEK293S cells was performed by the method previously reported

(Yamashita et al., 2008).  In order to detect the expression of mGluRs by immunoblotting

analysis, the cDNAs of mGluR were tagged with the epitope sequence of the anti-bovine

rhodopsin monoclonal antibody Rho1D4 at the C-terminus.  The wild-type and mutant

cDNAs of mGluR were introduced into the mammalian expression vector pcDNA3.1

(Invitrogen).  HEK293S cells were grown to ~ 40 % confluency in DMEM/F12

supplemented with 10 % fetal bovine serum and were transfected with wild-type or mutant

mGluR8 plasmid DNA (10 μg / 100-mm dish) using the calcium-phosphate method.  The

cells were collected by centrifugation 48 hr after transfection.  The cell membranes were

homogenized in 50 % sucrose in buffer A [50 mM HEPES (pH 6.5) and 140 mM NaCl] and

collected by centrifugation.  The supernatant was diluted in two volumes of buffer A and

re-centrifuged.  The membrane pellets were washed with buffer A and suspended in buffer

B [50 mM HEPES (pH 8.0) and 140 mM NaCl, and 3 mM MgCl₂] for the ligand binding assay

and the GTPγS binding assay.

## Ligand binding assay of mGluR8

The ligand binding assay was performed according to our previous report (Yamashita

et al., 2008).   [$^3$H]LY341495 binding to membranes expressing mGluR8 was measured at

room temperature.   A mixture of membranes and 0 - 400 nM [$^3$H]LY341495 in buffer B was

incubated for 30 min (final assay volume: 20 µl).   After incubation, bound and free

radioligands were separated by centrifugation.   The membrane pellets were washed with

buffer B and solubilized in 1 M NaOH.   The amount of bound [$^3$H]LY341495 in the

solubilized membranes was quantified using a liquid scintillation counter.   Non-specific

binding was defined with 1 mM L-AP4.   Kd and Bmax values were calculated using the

nonlinear one-site binding equation: y=(Bmax)(x)/(Kd+x).   Protein concentration in the

membranes was determined by the Bradford method.   Displacement by L-AP4 of

[$^3$H]LY341495 binding to membranes expressing mGluR8 was also measured at room

temperature.   A mixture of membranes (30 µg total protein), 100 nM [$^3$H]LY341495 and 0 ~

100 µM L-AP4 in buffer B was incubated for 30 min (final assay volume: 20 µl).   After

incubation, bound and free radioligands were separated by centrifugation.   The membrane

pellets were washed with buffer B and solubilized in 1 M NaOH.   The specific binding,

defined using 1 mM L-AP4 as displacer, was ~93 % of total binding using 100 nM

[$^3$H]LY341495 and 30 µg of tissue protein.   Competition binding curves were fitted to the

one-site binding model: y=(Max-Basal)/(1+x/IC50)+Basal.


**GTPγS binding assay of mGluR8**

The assay of G protein activation by mGluR8 was carried out according to our previous

report (Yamashita et al., 2008).   The mGluR8-expressing membranes (final concentration,

2 nM) after sucrose flotation were suspended in 0.03 % dodecylmaltoside (DM) in buffer C

(50 mM HEPES (pH 7.2), 140 mM NaCl, and 3 mM MgCl$_2$) and preincubated with Go-type G

protein (final concentration, 200 nM) purified from pig cerebral cortex and the agonist, L-AP4.

After preincubation for 30 min at 10 °C, the GDP/GTPγS exchange reaction was started by

adding GTPγS solution.    The assay mixture (20 μl) consisted of 50 mM HEPES (pH 7.2),

140 mM NaCl, 5 mM MgCl$_2$, 0.015% DM, 0.03% sodium cholate, 0.8 mg/ml

L-α-phosphatidylcholine, 0.1 μM GTPγS, and 3 μM GDP.    After incubation for 3 min, the

reaction was terminated by adding stop solution (200 μl: 20 mM Tris/Cl (pH 7.4), 100 mM

NaCl, 25 mM MgCl$_2$, 0.1 μM GTPγS and 3 μM GDP) and immediately filtering the sample

through a nitrocellulose membrane to trap [$^{35}$S]GTPγS bound to G proteins.

## Immunoblotting

Wild-type and mGluR mutants were detected with the Rho1D4 monoclonal antibody against

the tag sequence at the C-terminus.    The cell membrane suspension in the buffer

described above containing no β-mercaptoethanol was subjected to SDS-PAGE (5.5%),

and then transferred onto a polyvinylidene difluoride membrane and probed with Rho1D4

antibody.    Immunoreactive proteins were detected by the ABC method and were visualized

using the horseradish peroxidase-diaminobenzidine reaction.    Visualization was carried out

according to (Terakita et al., 1996).

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## Results

### Identification of novel CAMs in TMD of mGluR8

In order to search for the key amino acid residues responsible for the structural changes in the TMD of mGluR8, we utilized previously obtained information about the activating mutations identified in the calcium-sensing receptor (CaSR), which also belongs to family 3.   More than 30 naturally occurring activating mutations of CaSR have been genetically identified as a cause of autosomal dominant hypocalcemia (Hu and Spiegel, 2003).   These mutations result in a severalfold increase of sensitivity to $Ca^{2+}$ or elevation of the constitutive activity of CaSR (Hu et al., 2005).   We expected that some of these mutation sites would also be involved in the activation of other family 3 GPCRs.

We therefore introduced amino acid substitutions that caused modification of the CaSR phenotype into the corresponding positions of mGluR8 according to their sequence alignment (Table 1, Fig.1) (Duvoisin et al., 1995).   The wild-type and mutants of mGluR8 were expressed in HEK293 cells.   The receptor expression level was quantified by the antagonist [$^3$H]LY341495 binding assay and was also examined by immunoblotting analysis (data not shown).   We then obtained HEK293 cell membranes containing active proteins of all the mutants except I750R, and measured their Go activation abilities by the [$^{35}$S]GTP$\gamma$S binding assay in the absence or presence of the agonist L-AP4 (Table 1).   The [$^{35}$S]GTP$\gamma$S binding assay was performed by counting the amount of [$^{35}$S]GTP$\gamma$S bound to Go protein in 3 min, as described in "Materials and Methods".   Among 21 mutants, 7 mutants showed elevation of the constitutive activity.   Six

mutations (F797L, I798A, I800S, F801A, Y812L and T819E), which are all located in the region connecting helices VI and VII, exhibited a slight elevation of constitutive activity and a decrease of the agonist-induced activity.   In contrast, T764C showed remarkably high basal and agonist-induced activities.   T764 is located in helix V and is completely conserved in all mGluR subtypes, while the six residues mutated in the connecting region exhibit moderate conservation among mGluR subtypes.

**Characterization of mGluR8 T764 Mutants**

Reasoning that the characterization of T764 mutants should provide some novel insights about the role of helix V in the activation path of mGluR, next we prepared 19 single-replacement mutants of mGluR8 at this position and investigated their Go activation abilities.   Extremely low expression of the functional protein was detected when T764 was substituted with charged amino acids or proline.   All the other mutants showed a remarkable increase in constitutive activity, which was monitored by the $[^{35}S]GTP\gamma S$ binding assay (Fig. 2a).   The agonist-induced activities of the mutant proteins were also elevated over the maximal activity of wild-type, but there was no relationship between the level of basal activity and that of agonist-induced activity.   More intriguingly, several substitutions (N, Q, Y, W) resulted in a loss of ability to respond to L-AP4. Because of their high expression levels, we selected T764A and T764W as representative mutants that do and do not respond to agonist, respectively, and analyzed the mutant profiles in more detail.

The agonist binding affinities of the wild-type and the T764 mutants were estimated based on the inhibition of [$^3$H]LY341495 binding by L-AP4 in a concentration-dependent manner. Although the Kd of antagonist LY341495 was constant (Table 2), T764A exhibited about a 2-fold higher affinity to L-AP4 than the wild-type (Fig. 2b). In addition, it exhibited a left-shifted EC$_{50}$ value in the measurements of L-AP4-dependent Go activation (Fig. 2c). Like T764A, T764W showed higher affinity to L-AP4, but it exhibited no L-AP4-dependent Go activation. Thus, introduction of a bulky amino acid into the 764 position breaks down the relay of structural changes from ECD to the cytoplasmic region, although it induces a structural change of the ECD that produces a high affinity state.

**Identification of Y663 as a partner residue with T764**

To understand the detailed mechanism of structural changes around T764, it is necessary to identify the residue(s) interacting with T764. Since all mutations at position 764 resulted in elevation of the basal activity, a mutation at the partner residue should also cause some elevation of basal activity by disturbing the microenvironment constituted by T764 and this partner. Consequently, we searched for novel CAM sites in other helices by comprehensive alanine-scanning mutagenesis. We introduced alanine mutations into 10 residues in each helix (a total of 60 residues) (Fig. 1). The original alanine residues were substituted with serine. Then the G protein activation abilities of these mutants were monitored by the [$^{35}$S]GTP$\gamma$S binding

assay (Supplemental fig. 1).　Among the 60 mutants, 7 mutants in helices II (L625A), III (Y663A and L667A) and VI (M787A, Y788A, T789A and C791A) showed an elevation of the basal activity (Fig. 3a).　Competition ligand binding analysis indicated that some of them (Y663A, L667A, M787A and C791A) showed an increase in affinity to L-AP4, like T764 mutants (Table 2).

Next, we prepared double mutants combining these novel CAMs and T764W to narrow down candidate residue(s) interacting with T764.　If an interacting residue were replaced by alanine, some hindrance introduced by T764W should be relieved in the mutants, resulting in suppression of the elevated basal activity and recovery of the responsiveness to agonist.　The results of the $[^{35}S]GTP\gamma S$ binding assay revealed that only the double mutant Y663A/ T764W showed lower basal activity than that of the T764W single mutant (Fig. 3b).　In contrast, all the other double mutants exhibited higher basal activity.　Furthermore, Y663A/T764W showed recovery of the responsiveness to agonist (Fig. 3c).　These results strongly suggest that Y663 interacts with T764 to form an activation switch in the TMD of mGluR.

We therefore prepared a set of site-directed mutants at position 663 and analyzed their G protein activation abilities in order to obtain more information about the environment around Y663.　In the $[^{35}S]GTP\gamma S$ binding assay, 4 mutants (A, S, T, W) showed increases of the constitutive activity (Fig. 3d). Especially, introduction of a tryptophan at residue 663 enlarged the side chain volume and markedly increased the activity to a level comparable to that of T764A.　Y663W also displayed 2-fold higher affinity to L-AP4 than the wild-type

in the ligand binding analysis (Table 2).

To test whether or not other residues are involved in this activation switch,

we also analyzed 53 other double mutants combining T764W and alanine

mutations that did not elevate the basal activity.    The results of the screening

revealed that 7 mutations in helices II (S622A and T627A), III (A664S, A665S,

T670A and N671A) and IV (T699A) partially abrogated the loss of

responsiveness to agonist caused by T764W (Supplemental fig. 1).    Thus,

these residues are also involved in the modulation of the microenvironment

around T764.

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## Discussion

In the present study, we identified several novel CAM sites in the TMD of mGluR8 by systematic point mutation analyses based on the known activating variants of CaSR (Hu et al., 2005). Among the CAM sites, the site at position 764 was remarkable, because mutation at this site caused prominent elevation of the basal activity (Table 1). In addition, we found that the amino acid residue at this position forms a specific pair with that at position 663, which is also a CAM site. In this section, we will discuss the mechanism of activation of mGluR deduced from the characterization of these CAMs.

T764 is presumably located in the middle of helix V, according to the 2D diagram of mGluR8 (Fig.1). As might be expected from the position of T764, replacements of this residue with charged amino acids or proline (an alpha-helix breaker) caused loss of expression of functional proteins, whereas all the other mutants showed reasonable levels of expression. It was a surprise for us to find that all the expressed mutants showed significant elevation of the basal activity (Fig.2a), because the replacements included a wide variety of amino acids, from the smallest (glycine) to the largest (tryptophan). Even replacement by serine, which differs from threonine only by lacking one methyl group, resulted in a marked increase of constitutive activity (Fig.2a). These results strongly suggested that the threonine residue at position 764 in helix V takes part in a specific interaction with amino acid residue(s) situated in a proximal transmembrane helix, thereby resulting in stabilization of the inactive state of mGluR8.

In order to identify residue(s) interacting with T764, we first searched for CAM sites by means of comprehensive alanine-scanning mutagenesis. We expected that the replacement of the residue(s) interacting with T764 would also cause elevation of the basal activity because it would disrupt the specific interaction with T764. Among the seven CAM sites we newly identified, we found that Y663 in the middle of helix III is the residue interacting with T764. Considering the molecular characteristics of the tyrosine and threonine located at positions 764 and 663, it is expected that these two residues would have steric and electrostatic interactions with each other. Figure 4 shows a model in which T764 and Y663 constitute a signal mediator in the TMD of mGluR8. In the wild-type, these residues would form an activation switch which relays the agonist-induced structural change in the ECD to the cytoplasmic surface responsible for G protein activation (Fig.4a). Thus, a drastic mutation such as T764W would disrupt the relay so as to make mGluR8 insensitive to agonist binding (Fig.4b). In Y663A/T764W, the alanine substitution of Y663 relieves the steric and electrostatic hindrance caused by T764W, and as a result, the activation profile of the double mutant becomes similar to that of the wild-type.

The model in Figure 4 is the most straightforward interpretation of the present results, but we cannot exclude the possibility that mutations at the switch region such as T764W could induce a global conformational change throughout the receptor molecule which might break the functional coupling between ECD and the cytoplasmic G protein-interacting site. Moreover, other residues neighboring T764 or Y663 might be involved in this activation switch secondarily.

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In fact, some of the replacements of tyrosine at position 663 do not cause

elevation of the basal activity, whereas all of the T764 replacements do (Fig.3d).

These results suggest that, although Y663 is the primary residue interacting with

T764, there are other residues that optimize the interaction between Y663 and

T764. Regarding this point, it should be noted that the residues in mGluR5 and

mGluR1 corresponding to Y663 form binding sites of MPEP and EM-TBPC,

which are allosteric modulators of mGluR5 and mGluR1, respectively (Malherbe

et al., 2003a; Malherbe et al., 2003b). These facts emphasize the physiological

significance of the activation switch identified in this study.

Several CAMs (T764A/W, Y663A/W, L667A, M787A and C791A) in TMD

identified in this study resulted in an increase of affinity to agonist in ECD (Fig.

2b, Table 2), although these domains are separated. This could be explained

by a simple two-state model (Samama et al., 1993), where mGluR8 would be in

thermal equilibrium between inactive and active states and affinity to agonist

would be increased to some extent by CAMs, which shift the equilibrium to the

active state. In contrast, no significant elevation was detected for other CAMs

identified in this study (L625A, Y788A or T789A) (Table 2) or CAMs in our

previous studies (L621 or Q695) (Yamashita et al., 2004; Yamashita et al., 2008).

Thus, mGluR could function via the equilibrium of not two but multiple

conformational states.

In conclusion, the present results provide some novel insights about

amino acid residues responsible for the regulation of mGluR activity. Especially,

the interaction between Y663 in helix III and T764 in helix V constitutes one of

the switch regions regulating the equilibrium between the active and inactive

states of mGluR TMD.    Further double mutation analyses of other CAMs will

clarify the structure-function relationship and the activation path of mGluR.

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## References

Bakker RA, Jongejan A, Sansuk K, Hacksell U, Timmerman H, Brann MR, Weiner DM, Pardo L and Leurs R (2008) Constitutively active mutants of the histamine H1 receptor suggest a conserved hydrophobic asparagine-cage that constrains the activation of class A G protein-coupled receptors. *Mol Pharmacol* **73**:94-103.

Brock C, Oueslati N, Soler S, Boudier L, Rondard P and Pin JP (2007) Activation of a dimeric metabotropic glutamate receptor by intersubunit rearrangement. *J Biol Chem* **282**:33000-8.

Cohen GB, Yang T, Robinson PR and Oprian DD (1993) Constitutive activation of opsin: influence of charge at position 134 and size at position 296. *Biochemistry* **32**:6111-5.

Decaillot FM, Befort K, Filliol D, Yue S, Walker P and Kieffer BL (2003) Opioid receptor random mutagenesis reveals a mechanism for G protein-coupled receptor activation. *Nat Struct Biol* **10**:629-36.

Duvoisin RM, Zhang C and Ramonell K (1995) A novel metabotropic glutamate receptor expressed in the retina and olfactory bulb. *J Neurosci* **15**:3075-83.

Ferraguti F and Shigemoto R (2006) Metabotropic glutamate receptors. *Cell Tissue Res* **326**:483-504.

Francesconi A and Duvoisin RM (1998) Role of the second and third intracellular loops of metabotropic glutamate receptors in mediating dual signal transduction activation. *J Biol Chem* **273**:5615-24.

Gomeza J, Joly C, Kuhn R, Knopfel T, Bockaert J and Pin JP (1996) The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G-proteins. *J Biol Chem* **271**:2199-205.

Hu J, McLarnon SJ, Mora S, Jiang J, Thomas C, Jacobson KA and Spiegel AM (2005) A region in the seven-transmembrane domain of the human Ca2+ receptor critical for response to Ca2+. *J Biol Chem* **280**:5113-20.

Hu J and Spiegel AM (2003) Naturally occurring mutations of the extracellular Ca2+-sensing receptor: implications for its structure and function. *Trends Endocrinol Metab* **14**:282-8.

Huang P, Li J, Chen C, Visiers I, Weinstein H and Liu-Chen LY (2001) Functional role of a conserved motif in TM6 of the rat mu opioid receptor: constitutively active and inactive receptors result from substitutions of Thr6.34(279) with Lys and Asp. *Biochemistry* **40**:13501-9.

Javitch JA, Fu D, Liapakis G and Chen J (1997) Constitutive activation of the beta2 adrenergic receptor alters the orientation of its sixth membrane-spanning segment. *J Biol Chem* **272**:18546-9.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG and Lefkowitz RJ (1992) Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J Biol Chem* **267**:1430-3.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic

glutamate receptor. *Nature* **407**:971-7.

Malherbe P, Kratochwil N, Knoflach F, Zenner MT, Kew JN, Kratzeisen C, Maerki HP, Adam G and Mutel V (2003a) Mutational analysis and molecular modeling of the allosteric binding site of a novel, selective, noncompetitive antagonist of the metabotropic glutamate 1 receptor. *J Biol Chem* **278**:8340-7.

Malherbe P, Kratochwil N, Zenner MT, Piussi J, Diener C, Kratzeisen C, Fischer C and Porter RH (2003b) Mutational analysis and molecular modeling of the binding pocket of the metabotropic glutamate 5 receptor negative modulator 2-methyl-6-(phenylethynyl)-pyridine. *Mol Pharmacol* **64**:823-32.

Marino MJ, Williams DL, Jr., O'Brien JA, Valenti O, McDonald TP, Clements MK, Wang R, DiLella AG, Hess JF, Kinney GG and Conn PJ (2003) Allosteric modulation of group III metabotropic glutamate receptor 4: a potential approach to Parkinson's disease treatment. *Proc Natl Acad Sci U S A* **100**:13668-73.

May LT, Leach K, Sexton PM and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**:1-51.

Muto T, Tsuchiya D, Morikawa K and Jingami H (2007) Structures of the extracellular regions of the group II/III metabotropic glutamate receptors. *Proc Natl Acad Sci U S A* **104**:3759-64.

Okada T, Fujiyoshi Y, Silow M, Navarro J, Landau EM and Shichida Y (2002) Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proc Natl Acad Sci U S A* **99**:5982-7.

Ossowska K, Konieczny J, Wardas J, Pietraszek M, Kuter K, Wolfarth S and Pilc A (2007) An influence of ligands of metabotropic glutamate receptor subtypes on parkinsonian-like symptoms and the striatopallidal pathway in rats. *Amino Acids* **32**:179-88.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739-45.

Park JH, Scheerer P, Hofmann KP, Choe HW and Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* **454**:183-7.

Patil ST, Zhang L, Martenyi F, Lowe SL, Jackson KA, Andreev BV, Avedisova AS, Bardenstein LM, Gurovich IY, Morozova MA, Mosolov SN, Neznanov NG, Reznik AM, Smulevich AB, Tochilov VA, Johnson BG, Monn JA and Schoepp DD (2007) Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat Med* **13**:1102-7.

Prioleau C, Visiers I, Ebersole BJ, Weinstein H and Sealfon SC (2002) Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel "locked-on" phenotype and double revertant mutations. *J Biol Chem* **277**:36577-84.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI and Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* **450**:383-7.

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC and Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* **318**:1266-73.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268**:4625-36.

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP and Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**:497-502.

Smit MJ, Vischer HF, Bakker RA, Jongejan A, Timmerman H, Pardo L and Leurs R (2007) Pharmacogenomic and structural analysis of constitutive g protein-coupled receptor activity. *Annu Rev Pharmacol Toxicol* **47**:53-87.

Tateyama M, Abe H, Nakata H, Saito O and Kubo Y (2004) Ligand-induced rearrangement of the dimeric metabotropic glutamate receptor 1alpha. *Nat Struct Mol Biol* **11**:637-42.

Terakita A, Takahama H, Tamotsu S, Suzuki T, Hariyama T and Tsukahara Y (1996) Light-modulated subcellular localization of the alpha-subunit of GTP-binding protein Gq in crayfish photoreceptors. *Vis Neurosci* **13**:539-47.

Tsuchiya D, Kunishima N, Kamiya N, Jingami H and Morikawa K (2002) Structural views of the ligand-binding cores of a metabotropic glutamate receptor complexed with an antagonist and both glutamate and Gd3+. *Proc Natl Acad Sci U S A* **99**:2660-5.

Yamashita T, Kai T, Terakita A and Shichida Y (2004) A novel constitutively active mutation in the second cytoplasmic loop of metabotropic glutamate receptor. *J Neurochem* **91**:484-92.

Yamashita T, Terakita A, Kai T and Shichida Y (2008) Conformational change of the transmembrane helices II and IV of metabotropic glutamate receptor involved in G protein activation. *J Neurochem*.

Yamashita T, Terakita A and Shichida Y (2001) The second cytoplasmic loop of metabotropic glutamate receptor functions at the third loop position of rhodopsin. *J Biochem* **130**:149-55.

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**Footnotes**

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**Figure Legends**

**Fig. 1   2D diagram of mGluR8.**

The mutated residues corresponding to the activating mutation sites of CaSR

are indicated by bold italic letters.   T764 and the residues which were replaced

by alanine (or serine) in the double mutation analysis are indicated by either

black or gray shading.   The residues whose substitution resulted in the

recovery of responsiveness to agonist (which was abolished by T764W) are

indicated by black shading.   * indicates the residues whose substitution

resulted in an increase of basal activity in our present and previous studies

(Yamashita et al., 2004; Yamashita et al., 2008).

**Fig. 2   Characterization of the mutations at T764 in mGluR8**

(a) A series of site-directed mutations at T764 in mGluR8.   Initial rates of the Go

activation abilities were compared among wild-type and T764 mutants of

mGluR8 using the [$^{35}$S]GTP$\gamma$S binding assay on transfected HEK293 cell

membranes.   Experiments were performed with or without 1 mM L-AP4 (black

and white bars, respectively).   The activities were normalized to that of

mock-transfected membranes.   (b) Ligand-binding potency of T764 mutants.

[$^{3}$H]LY341495 binding to wild-type and T764 mutant-expressing HEK293 cell

membranes was measured (wild-type: open circles, T764A: open triangles,

T764W: open squares).   The IC$_{50}$ values (wild-type: 8.5 ± 0.5 $\mu$M, T764A: 3.6 ±

0.1 $\mu$M, T764W: 3.8 ± 0.2 $\mu$M) were calculated from the respective competition

curves.    Results are presented as the percentage of total specific [$^3$H]LY341495

binding.    (c) Concentration-dependent effect of L-AP4 on Go activation

efficiency.    The L-AP4-stimulated activities of wild-type- and

T764A/W-expressing HEK293 cell membranes were measured (wild-type: open

circles, T764A: open triangles, T764W: open squares).    The $EC_{50}$ values

(wild-type: 6.6 ± 1.8 μM, T764A: 1.3 ± 0.1 μM) were calculated from the

respective competition curves.    Results were normalized to the basal activity of

wild-type.    All data are expressed as mean ±S.D. of more than two independent

experiments done in duplicate.

**Fig. 3    Analysis of a series of double mutants of T764**

(a) Novel constitutively active mutations identified by alanine-scanning

mutagenesis.    Initial rates of the G protein activation abilities of single mutants

were compared with those of wild-type and T764W using the [$^{35}$S]GTPγS binding

assay on transfected HEK293 cell membranes.    All mutants showed a

significant increase of basal activity compared with that of wild-type (p<0.05;

Student's t- test, two-tailed).    Experiments were performed with or without 1 mM

L-AP4 (black and white bars, respectively).    The activities were normalized to

that of mock-transfected membranes.    (b) Initial rates of G protein activation

abilities of double mutants without L-AP4.    * indicates a significant decrease of

basal activity compared with that of T764W (p<0.05; Student's t- test, two-tailed).

(c)    The ability of double mutants to respond to agonist.    Data are expressed

as the rate of G protein activation ability with L-AP4 relative to that without it.    *

indicates a significant increase of the rate compared with that of T764W (p<0.05; Student's t- test, two-tailed).    For comparison, the rate of wild-type was 4.43 ± 0.69.    All data were expressed as mean ± S.D. of more than two independent experiments done in duplicate.    (d) Comparison of Go activation abilities among wild-type and Y663 mutants of mGluR8.    Experiments were performed with or without 1mM L-AP4 (black and white bars, respectively).    The activities of the wild-type and mutants were normalized to that of the mock-transfected membranes.    * indicates a significant increase of constitutive activity compared with wild-type (p<0.05; Student's t- test, two-tailed).

**Fig. 4   Proposed functional model of the interaction between Y663 and T764**

Schematic model of the effect of the mutations on the activation of mGluR deduced from the experimental data.    (a) In wild-type, the interaction between Y663 and T764 stabilizes the inactive state and transmits the agonist-induced structural change to the cytoplasmic region.    (b) In T764W, the hindrance causes an increase of the constitutive activity and a loss of responsiveness to agonist.    (c) In Y663A/T764W, the basal activity is suppressed and the loss of responsiveness to agonist is abrogated because of relaxation of the hindrance.

*Table 1.   Mutations introduced into mGluR8 that correspond to known CaSR activating mutations, and the Go activation abilities of wild-type and mutants*

| mGluR8 Mutation | Known CaSR Activating Mutation | Receptor Domain | Go Activation # 0mM L-AP4 | Go Activation # 1mM L-AP4 |
|---|---|---|---|---|
| WT | | | 1.00±0.05 | 4.11±0.69 |
| I575K | E604K | N ter | 1.34±0.16 | 2.22±0.37 |
| W583S | F612S | Helix I | 1.11±0.12 | 1.62±0.27 |
| R652H | Q681H | Helix III | 1.30±0.03 | 2.28±0.49 |
| I744K | E767K | e2 | 1.10±0.07 | 2.71±0.20 |
| I750R | L773R | Helix V | N.D. | N.D. |
| T764C | F788C | Helix V | *4.17±1.01 | 7.94±1.14 |
| E775K | E799K | i3 | 1.15±0.08 | 2.84±0.14 |
| P782S | F806S | Helix VI | 1.28±0.08 | 2.82±0.27 |
| L795A | I819A | Helix VI | 0.97±0.08 | 1.33±0.20 |
| A796F | S820F | Helix VI | 1.08±0.24 | 1.28±0.17 |
| F797L | F821L | Helix VI | *1.50±0.19 | 2.39±0.24 |
| I798A | I822A | Helix VI | *1.35±0.13 | 2.18±0.16 |
| I800S | A824S | Helix VI | *1.76±0.24 | 2.33±0.04 |
| F801A | Y825A | Helix VI | *1.60±0.16 | 2.81±0.17 |
| Q806A | G830A | e3 | 1.27±0.12 | 2.61±0.22 |
| S807A | K831A | e3 | 1.24±0.17 | 3.49±0.61 |
| A808S | F832S | e3 | 1.18±0.18 | 3.08±0.38 |
| M811T | A835T | e3 | 1.21±0.23 | 2.10±0.23 |
| Y812L | V836L | e3 | *1.72±0.17 | 2.79±0.34 |
| I813K | E837K | e3 | 1.21±0.16 | 1.59±0.09 |
| T819E | A843E | Helix VII | *1.40±0.19 | 2.29±0.19 |

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# Relative Go activation abilities were estimated using the [$^{35}$S]GTP$\gamma$S binding assay on transfected HEK293 cell membranes.   Data were normalized to wild-type basal activity and expressed as the mean $\pm$ SD of more than two independent experiments done in duplicate. N.D., not detectable

* indicates a significant increase of constitutive activity compared with wild-type. (p<0.05; Student's t- test, two-tailed)

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Table 2.   *Ligand-binding affinities of wild-type and mutants of mGluR8*

| | [$^3$H]LY341495 binding | | Inhibition of [$^3$H]LY341495 binding by L-AP4 |
|---|---|---|---|
| | (Bmax; pmol/mg) | (Kd; nM) | (IC$_{50}$; μM) |
| WT | 63±20 | 33±4 | 8.5±0.5 |
| L625A | 60±13 | 40±7 | 7.7±0.6 |
| Y663A | 59±7 | 32±1 | 4.7±1.3* |
| Y663W | 55±6 | 35±7 | 4.2±0.2* |
| L667A | 49±7 | 30±8 | 5.8±0.6* |
| T764A | 57±5 | 34±1 | 3.6±0.1* |
| T764W | 40±10 | 42±3 | 3.8±0.2* |
| M787A | 77±3 | 40±1 | 4.5±1.1* |
| Y788A | 59±12 | 35±8 | 8.1±0.9 |
| T789A | 71±3 | 32±6 | 7.3±0.1 |
| C791A | 60±11 | 35±1 | 5.2±0.7* |

Specific [$^3$H]LY341495 binding and competition by L-AP4 were measured as described in "Experimental Procedures".   Each binding was expressed as the mean ± range of two independent experiments done in duplicate.

* indicates a significant decrease of IC$_{50}$ compared with wild-type ($p<0.05$; Student's t-test, two-tailed)

## Fig.1

## Fig.2

### (a)



### (b)



### (c)

# Fig.3

Fig.4