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Activation Switch in the Transmembrane Domain of Metabotropic Glutamate Receptor[S]

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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.

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; Tsuchiya et al., 2002; Muto et al., 2007), the helical arrangements of the TMD (Tateyama et al., 2004; Brock et al., 2007; Yamashita et al., 2008), and the configuration of the cytoplasmic G protein-interacting site (Gomeza et al., 1996; Francesconi and Duvoisin, 1998; Yamashita et al., 2001), which eventually leads to the activation of G proteins. 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

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; ECD, extracellular ligand-binding domain; TMD, transmembrane domain; CAM, constitutively active mutation; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DM, dodecylmaltoside; CaSR, calcium-sensing receptor.

GPCRs act as an activation switch regulating the equilibrium between inactive and active states (Kjelsberg et al., 1992; Cohen et al., 1993; Javitch et al., 1997; Huang et al., 2001; Prioleau et al., 2002; Décaillot et al., 2003; Bakker et al., 2008). The crystal structures of rhodopsins and adrenergic receptors have been revealed by X-ray crystallography studies (Palczewski et al., 2000; Okada et al., 2002; Rasmussen et al., 2007; Rosenbaum et al., 2007; Park et al., 2008; 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.

Materials and Methods

Materials. [^{3}H]LY341495 (1.28 TBq/mmol) and L-AP4 were purchased from Tocris Bioscience (Ellisville, MO); [^{35}S]guanosine 5′-3-*O*-(thio)triphosphate (GTPγS; 37 TBq/mmol) was from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Preparation of mGluR Mutants. Expression of mGluR8 in HEK293S cells was performed by a method reported previously (Yamashita et al., 2008). 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% confluence in Dulbecco's modified Eagle's medium/Ham's F12 medium 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 h 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 recentrifuged. The membrane pellets were washed with buffer A and suspended in buffer B (50 mM HEPES, pH 8.0, 140 mM NaCl, and 3 mM $MgCl_2$) 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 to 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. Nonspecific binding was defined with 1 mM L-AP4. K_d and B_{max} values were calculated using the nonlinear one-site binding equation: $y = (B_{max})(x)/(K_d + x)$. 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 of 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: $y = (\text{Max} - \text{Basal})/(1 + x/IC_{50}) + \text{Basal}$.

GTPγS Binding Assay of mGluR8. The assay of G protein activation by mGluR8 was carried out as described in our previous

TABLE 1

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

Relative Go activation abilities were estimated using the [^{35}S]GTPγS binding assay on transfected HEK293 cell membranes. Data were normalized to wild-type basal activity and expressed as the mean ± S.D. of more than two independent experiments performed in duplicate.

mGluR8 Mutation	Known CaSR Activating Mutation	Receptor Domain	Go Activation	
			0 mM L-AP4	1 mM 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

N ter, amino terminal; e2, extracellular loop 2; e3, extracellular loop 3; i3, intracellular loop 3; N.D., not detectable.
* Significant increase of constitutive activity compared with wild-type. ($P < 0.05$; Student's t test, two-tailed).

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).

Results

Identification of Novel CAMs in TMD of mGluR8. To search for the key amino acid residues responsible for the structural changes in the TMD of mGluR8, we used 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]GTPγS binding assay in the absence or presence of the agonist L-AP4 (Table 1). The [^{35}S]GTPγS binding assay was performed by counting the amount of [^{35}S]GTPγS bound to Go protein in 3 min, as described under *Materials and Methods*. Among 21 mutants, 7 showed elevation of the constitutive activity. Six mutations (F797L, I798A, I800S, F801A, Y812L, and T819E), all of which are 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. Thr764 is located in helix V and is completely conserved in all mGluR subtypes, whereas the six residues mutated in the connecting region exhibit moderate conservation among mGluR subtypes.

Characterization of mGluR8 Thr764 Mutants. Reasoning that the characterization of Thr764 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 Thr764 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]GTPγS 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 (Asn, Gln, Tyr, Trp) 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 Thr764 mutants were estimated based on the inhibition of [^{3}H]LY341495 binding by L-AP4 in a concentration-dependent manner. Although the K_d of antagonist LY341495 was constant (Table 2), T764A exhibited approximately 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 Tyr663 as a Partner Residue with Thr764. To understand the detailed mechanism of structural

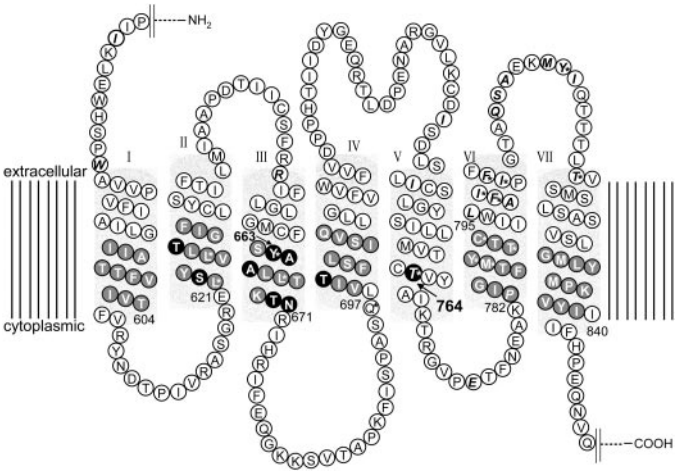

Fig. 1. Two-dimensional diagram of mGluR8. The mutated residues corresponding to the activating mutation sites of CaSR are indicated by bold italic letters. Thr764 and the residues that were replaced by alanine (or serine) in the double mutation analysis are indicated by either black or gray shading. 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, 2008).

changes around Thr764, it is necessary to identify the residue(s) interacting with Thr764. Because 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 Thr764 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]GTPγS 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, similar to the Thr764 mutants (Table 2).

Next, we prepared double mutants combining these novel CAMs and T764W to narrow down candidate residue(s) interacting with Thr764. 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]GTPγS 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 Tyr663 interacts with Thr764 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 to obtain more information about the environment around Tyr663. In the [^{35}S]GTPγS binding assay, four mutants (Ala, Ser, Thr, Trp) showed increases of the constitutive activity

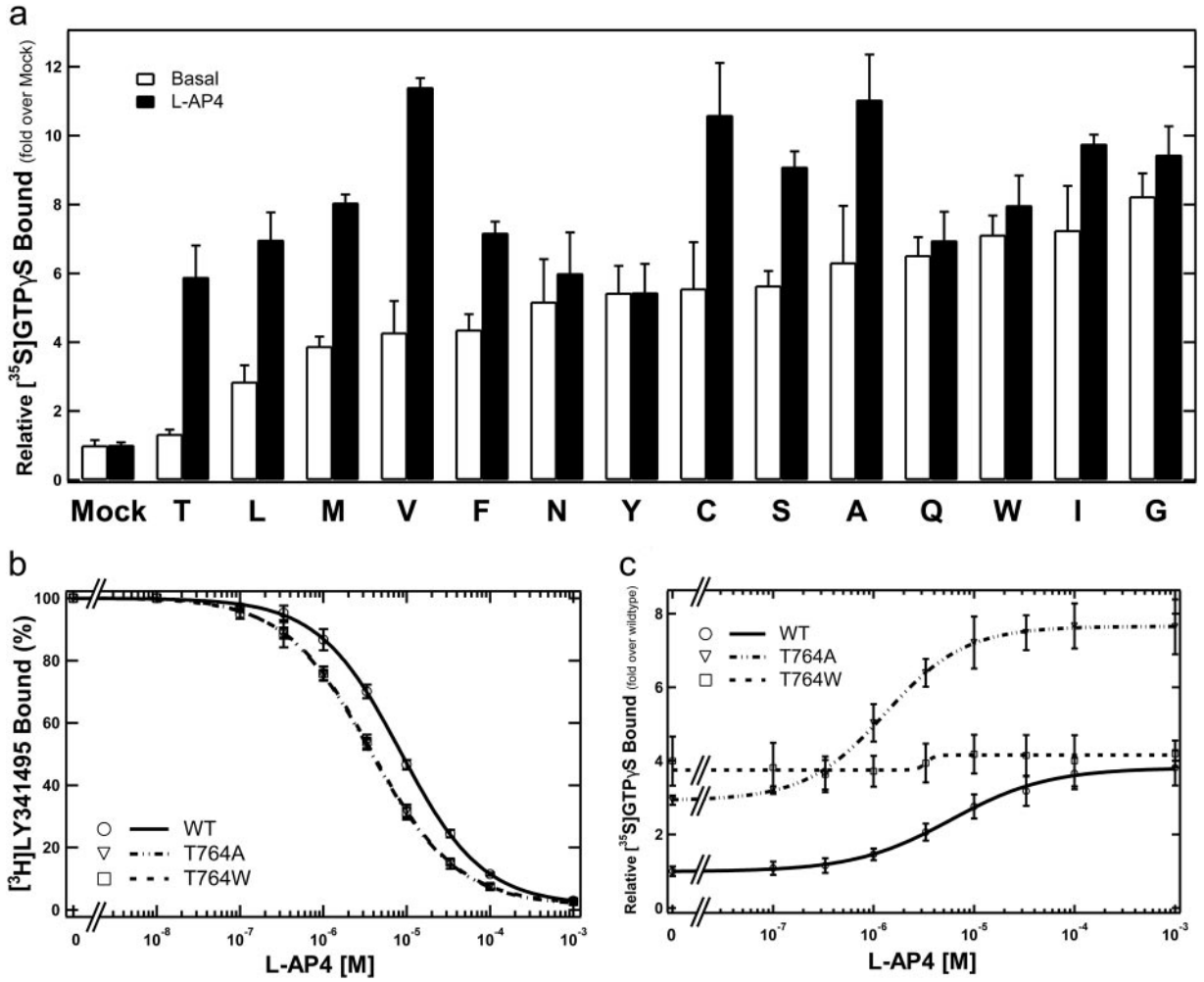

Fig. 2. Characterization of the mutations at Thr764 in mGluR8. a, a series of site-directed mutations at Thr764 in mGluR8. Initial rates of the Go activation abilities were compared among wild-type and Thr764 mutants of mGluR8 using the [^{35}S]GTPγS binding assay on transfected HEK293 cell membranes. 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 Thr764 mutants. [^{3}H]LY341495 binding to wild-type and Thr764 mutant-expressing HEK293 cell membranes was measured (○, wild-type; ▽, T764A; □, T764W). The IC_{50} values (wild-type, 8.5 ± 0.5 μM; T764A, 3.6 ± 0.1 μM; T764W, 3.8 ± 0.2 μM) 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; ▽, T764A; □, T764W). 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. 3d). In particular, introduction of a tryptophan at residue 663 enlarged the side chain volume and markedly increased the activity to a level comparable with 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 seven 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 Thr764.

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.

Thr764 is presumably located in the middle of helix V, according to the two-dimensional diagram of mGluR8 (Fig. 1). As might be expected from the position of Thr764, replacements of this residue with charged amino acids or proline (an α-helix breaker) caused loss of expression of functional pro-

TABLE 2

Ligand-binding affinities of wild-type and mutants of mGluR8

Specific [^{3}H]LY341495 binding and competition by L-AP4 were measured as described under *Materials and Methods*. Each binding was expressed as the mean ± range of two independent experiments performed in duplicate.

	[^{3}H]LY341495		
	Binding		Inhibition of Binding by L-AP4 (IC_{50})
	B_{max}	K_d	
	pmol/mg	*nM*	*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*

* Significant decrease of IC_{50} compared with wild-type ($P < 0.05$; Student's t test, two-tailed).

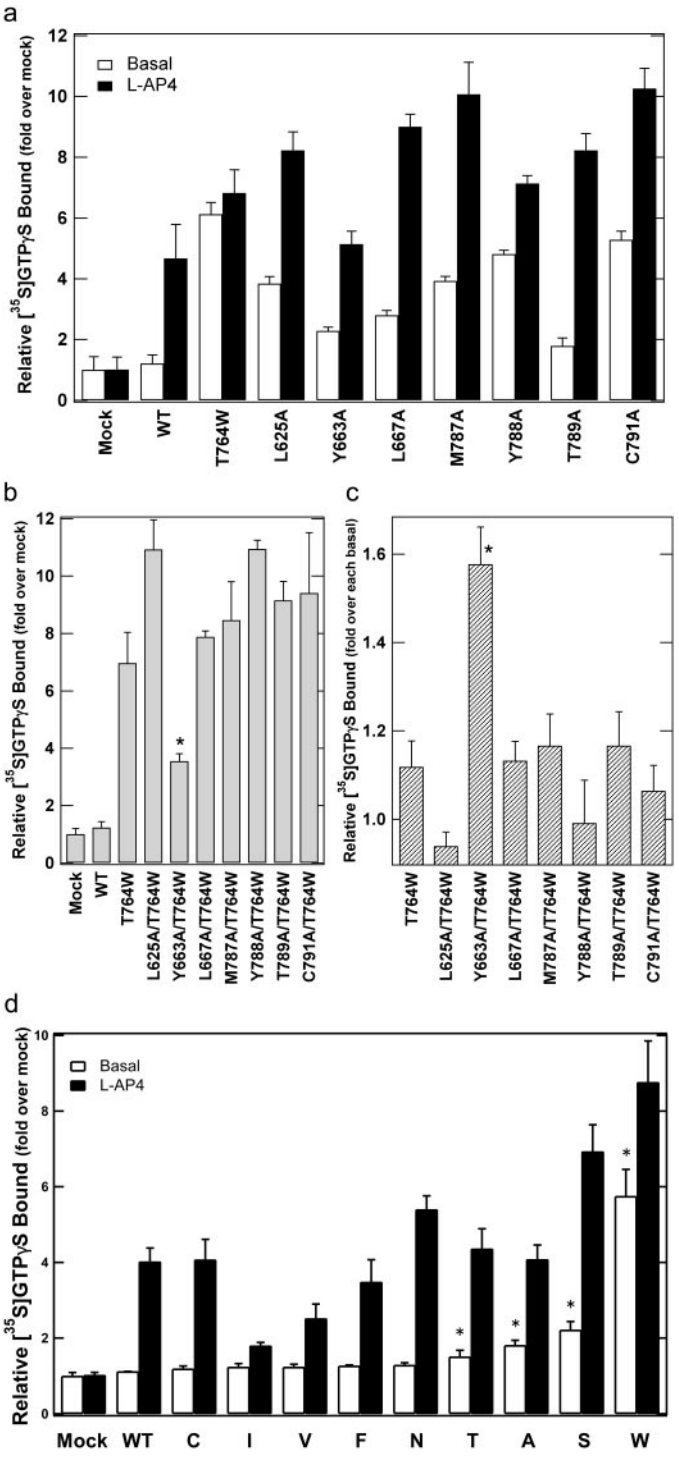

Fig. 3. Analysis of a series of double mutants of Thr764. a, novel constitutively active mutations identified by alanine-scanning mutagenesis. Initial rates of the G protein activation abilities of single mutants were compared with those of wild-type and T764W using the [^{35}S]GTPγS binding assay on 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 Tyr663 mutants of mGluR8. Experiments were performed with or without 1 mM 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).

teins, 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 in 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.

To identify residue(s) interacting with Thr764, we first searched for CAM sites by means of comprehensive alanine-scanning mutagenesis. We expected that the replacement of the residue(s) interacting with Thr764 would also cause elevation of the basal activity because it would disrupt the specific interaction with Thr764. Among the seven CAM sites we newly identified, we found that Tyr663 in the middle of helix III is the residue interacting with Thr764. 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 Thr764 and Tyr663 constitute a signal mediator in the TMD of mGluR8. In the wild-type, these residues would form an activation switch that 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 Tyr663 relieves the steric and electrostatic hindrance caused by T764W; as a result, the activation profile of the double mutant becomes similar to that of the wild-type.

The model in Fig. 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 that might break the functional coupling between ECD and the cytoplasmic G protein-interacting site. Moreover, other residues neighboring Thr764 or Tyr663 might be involved in this activation switch secondarily. In fact, some of the replacements of tyrosine at position 663 do not cause elevation of the basal activity, whereas all of the Thr764 replacements do (Fig. 3d). These results suggest that, although Tyr663 is the primary residue interacting with Thr764, there are other residues that optimize the interaction between Tyr663 and Thr764. Regarding this point, it should be noted that the residues in mGluR5 and mGluR1 corresponding to Tyr663 form binding sites of 2-methyl-6(phenylethynyl)-pyridine and 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[*d*]azepin-3-yl)- 1,6-dihydro-pyrimidine-5-carbonitrile, which are allosteric modulators of mGluR5 and mGluR1, respectively (Malherbe et al., 2003a,b). 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), in which 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 (Leu621 or Gln695) (Yamashita et al., 2004, 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. In particular, the interaction between

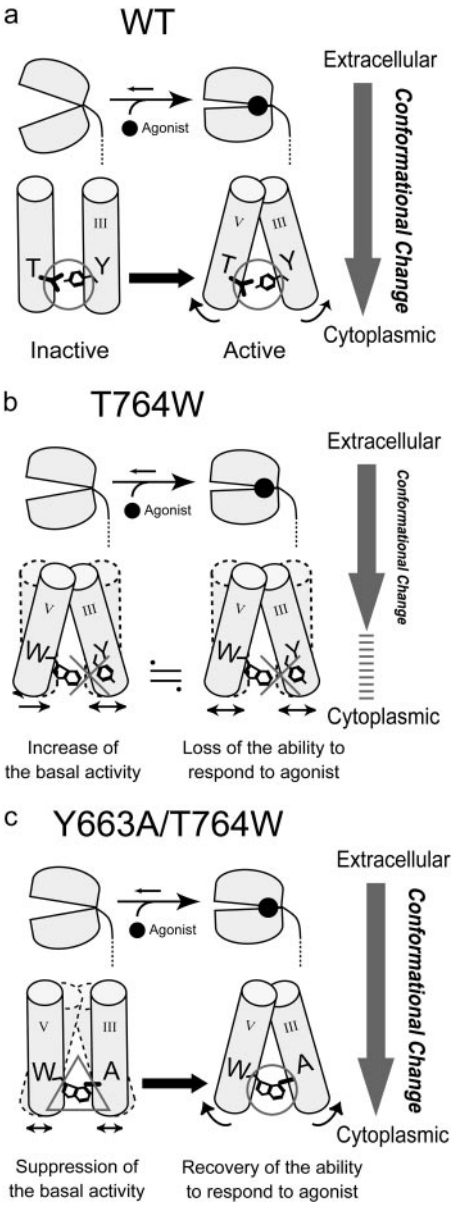

Fig. 4. Proposed functional model of the interaction between Tyr663 and Thr764. Schematic model of the effect of the mutations on the activation of mGluR deduced from the experimental data. a, in wild-type, the interaction between Tyr663 and Thr764 stabilizes the inactive state and transmits the agonist-induced 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.

Tyr663 in helix III and Thr764 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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