Negative cooperativity of glutamate binding in the dimeric metabotropic glutamate receptor subtype 1

1Yoshikazu Suzuki, 1Eiko Moriyoshi, 2Daisuke Tsuchiya, and 1Hisato Jingami*

Departments of 1Molecular Biology and 2Structural Biology, Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan

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*To whom correspondence should be addressed.
Phone: 81-6-6872-8214
Fax: 81-6-6872-8210
e-mail: jingami@beri.or.jp
Summary

Metabotropic glutamate receptor (mGluR) subtype 1 is a Class III G-protein coupled receptor, which is mainly expressed on the post-synaptic membrane of neuronal cells. The receptor has a large N-terminal extracellular ligand binding domain that forms a homodimer, however, the intersubunit communication of ligand binding in the dimer remains unknown. Here, using the intrinsic tryptophan fluorescence change as a probe for ligand binding events, we examined whether allosteric properties exist in the dimeric ligand binding domain of the receptor. The indole ring of the tryptophan 110, which resides on the upper surface of the ligand binding pocket, sensed the ligand binding events. From saturation binding curves, we have determined the apparent dissociation constants \( K_{0.5} \) of representative agonists and antagonists for this receptor (3.8, 0.46, 40 and 0.89 \( \mu \)M for glutamate, quisqualate, (S)-MCPG and LY367385, respectively). Calcium ions functioned as a positive modulator for agonist but not for antagonist binding \( K_{0.5} \) were 1.3, 0.21, 59 and 1.2 \( \mu \)M for glutamate, quisqualate, (S)-MCPG and LY367385, respectively, in the presence of 2.0 mM calcium ion). Moreover, a Hill analysis of the saturation binding curves revealed the strong negative cooperativity of glutamate binding between each subunit in the dimeric ligand binding domain. As far as we know, this is the first direct evidence that the dimeric ligand binding domain of mGluR exhibits intersubunit cooperativity of ligand binding.
Introduction

Glutamate is a major neurotransmitter in the excitatory synapses of the central nervous system, and two types of glutamate receptors are expressed in nerve cells: one is an ionotropic glutamate receptor and the other is a metabotropic glutamate receptor (mGluR) \(^1\). The former is a glutamate-gated ion channel, which induces a synaptic potential upon glutamate binding, while the latter is a G-protein coupled receptor (GPCR), which induces various cellular responses to glutamate stimulation, \textit{e.g.}, inositol triphosphate production and the subsequent elevation of intracellular calcium, or a cytoplasmic cyclic AMP concentration change caused by the modulation of adenylyl cyclase activity. Since these cellular responses modulate the degree of synaptic neurotransmission, mGluRs are believed to be involved in higher order neuronal activities such as memory, learning, and so on \((1, 2)\).

The mGluR belongs to the Class III GPCR and forms a subfamily consisting of eight subtypes (mGluR1 – 8) \((3 – 6)\). One outstanding feature of the receptor is the large extracellular ligand binding domain (LBD), which is characteristic of the Class III GPCRs. The mGluR1 LBD consists of ~ 520 amino acids and forms a clamshell-like bilobate domain (LBI and LBII) \((7, 8)\). Our previous biochemical and crystallographic studies demonstrated that the LBD forms a homodimer by not only an intersubunit disulfide bond but also hydrophobic interactions \((7 - 10)\). In the crystal structures, one protomer of the dimeric LBD adopts two different conformations: an open conformation and a closed conformation. A symmetric structure with both protomers in the open conformation is observed in the absence of glutamate, while the structure of the glutamate-binding state is asymmetric, because one protomer adopts the closed conformation and the other adopts the open conformation. Interestingly, even in the absence of glutamate, the asymmetric open-closed conformation is observed, implying that the open and closed conformations of the protomer are in equilibrium in an aqueous solution without ligands. Glutamate binding promotes the closing motion of the ligand binding pocket, so the closed conformation should be stabilized. Thus, the glutamate-
bound open conformation observed in the crystal structure is a fascinating puzzle. One attractive explanation for this is an allosteric property in the dimeric LBD: the closed conformation in one protomer would negatively affect the binding mode of the other protomer. However, such an allosteric effect on ligand binding has not been demonstrated yet for this receptor.

Some allosteric properties have been previously reported for several receptors. Extensive biochemical and crystallographic studies have been performed on the bacterial dimeric aspartate receptor, and the mechanism of negative cooperativity on aspartate binding has been elucidated on the basis of the structure (11 – 14). Allosteric properties are also inferred in several GPCRs (15, 16), however in these cases, the observed cooperativity seems to result from oligomerization of receptors on the membrane surface. Thus, the allosteric properties in terms of subunit-subunit communication are more obscure in the GPCRs than those in the bacterial aspartate receptor. Recently, dimer formation by GPCRs has been detected and ligand selectivity appears to be broader than previously expected (17 – 20). It was also reported that in the heterodimeric GABA\textsubscript{B} receptor, the GB2 subunit and its association with the GB1 subunit control the agonist affinity in GB1 subunit (21). Therefore, the cooperativity in dimeric GPCRs has become a current issue. In this context, our mGluR system provides a unique opportunity to decipher the cooperativity in the ligand binding event of Class III GPCR using a purified pre-formed dimer.

To investigate whether allostery functions in ligand binding, it is essential to analyze a saturation ligand binding curve with a wide range of ligand concentrations from sub-nanomolar to millimolar. [\textsuperscript{3}H]-labeled quisqualate, which is an agonist specific for mGluR1 and 5, is widely used in the ligand binding assay. However, this conventional assay cannot yield a saturation binding curve because of the upper limit of the applicable ligand concentration.

Since the fluorescence emitted from tryptophan is sensitive to the environment
surrounding the indole group, the intrinsic tryptophan fluorescence can be a good probe to sense ligand binding events (22 – 25). In the present study, we found that the fluorescence spectrum of the intrinsic tryptophans of purified mGluR1 LBD changed upon ligand binding. The system led us to obtain saturation binding curves by titration of the tryptophan fluorescence with glutamate, a native ligand, and other non-native ligands, such as quisqualate, (S)-α-methyl-4-carboxyphenylglycine ((S)-MCPG), and (+)-2-methyl-4-carboxyphenylglycine (LY367385). These binding curves allowed us to determine the apparent binding constants of these ligands and to demonstrate the positive effect of calcium ions on agonist binding. Furthermore, Hill analyses of the titration curves revealed that negative cooperativity of glutamate binding exists between each protomer of the dimeric mGluR1 LBD.
Experimental Procedures

Materials – L-quisqualate, (S)-MCPG and LY367385 were purchased from Tocris (UK). (R)-MCPG was a gift from Dr. D. Shunter (Tocris, U.K.). L-glutamate was purchased from Nacalai Tesque (Japan). Oligonucleotide primers were obtained from Proligo (Japan). All other reagents used in the present study were of molecular or analytical grade.

Construction of an expression vector for the FLAG-tagged LBD and its mutant – To obtain a C-terminal FLAG-tagged mGluR1 LBD, we performed PCR with pmGluR104 (9) as the template. The forward primer for the PCR was designed at the N-terminus of the LBD with a NotI site. The reverse primer was designed at the C-terminus of the LBD (at the 1566th thymine in the mGluR1 cDNA) with the DNA sequence for the FLAG epitope (DYKDDDDK) and a stop codon followed by an XbaI site. After verification by DNA sequencing, the PCR product was cloned into the pFastBac DUAL vector (Invitrogen) using the NotI and XbaI sites. The I120A and T188A point mutations were introduced into the FLAG-tagged LBD gene by replacing the NotI/PshAI region of the wild type with the same fragment containing the mutation, which was excised from the plasmid previously used in the mutation experiments (26). The W110V mutation was introduced by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), with pmGluR103 (9) as the template. After sequence verification, the NotI/PshAI fragment of this mutant was exchanged for that of the wild type on the pFastBac DUAL vector.

Production of baculoviruses for protein expression - Baculoviruses for protein expression were obtained by following the protocol of the Bac-To-Bac Baculovirus Expression System (Invitrogen). Briefly, the vector DNA was transformed into DH10Bac E. coli cells (Invitrogen). Then, the recombinant bacmid DNA purified from the DH10Bac cells was transfected into Sf9 insect cells, using the Cellfectin Reagent (Invitrogen). After an incubation for 72 hours at 27 °C, the viruses were harvested from the cell culture medium. Then, the
recombinant viruses were amplified by reinfecting Sf9 cells to enhance the viral titer. Finally, we checked the viral titer by a plaque formation assay using an immobilized monolayer culture of Sf9 cells.

Protein Expression and Purification – The wild-type and mutant FLAG-tagged LBD proteins were expressed by inoculating the baculoviruses into HighFive cells as previously described (9, 10). Purification of the protein was done by taking advantage of the FLAG tag. The cell culture medium (~ 500 ml) into which the target protein was sufficiently secreted was collected 4 – 5 days after the inoculation. After the addition of protease inhibitors (10 µg/ml, 2 µg/ml and 0.1 mM for leupeptin, pepstatin and PMSF, respectively), the cells were pelleted by centrifugation at 6, 700 x g for 15 min at 4 °C. Then, the supernatant was directly applied to ~ 1 ml of anti-FLAG M2 agarose (Sigma) packed in a disposable column (Bio Rad). After the column was washed with a low-salt buffer containing 10 mM Tris-HCl, pH 7.5, and 20 mM NaCl, the proteins bound to the beads were eluted by a high-salt buffer containing the FLAG peptide at a concentration of 150 µg/ml, dissolved in 10 mM HEPES, pH 7.4, and 300 mM NaCl. The eluted fractions were collected, and the protein was concentrated and buffer-exchanged to the assay buffer (20 mM HEPES, pH 7.4, and 50 mM NaCl) using an Ultra-free centrifugation filter unit (Millipore). The protein concentration was determined by the absorbance at 280 nm with a molar extinction coefficient of 126, 500 M⁻¹cm⁻¹ as a dimer, which was calculated from the number of tryptophans, tyrosines and cystines in the protein (27). The molecular weight of the FLAG-tagged protein was 119, 800 as calculated from the amino acid sequence including the C-terminal FLAG tag.

Purification of the wild-type LBD of mGluR1 (non-tagged) was done as previously described by using an immunoaffinity column conjugated with monoclonal antibodies (mG1Na-1) (9). The molecular weight of the wild-type LBD was estimated to be 117, 800 from the amino acid sequence.
Steady-state fluorescence measurement — Steady-state tryptophan fluorescence was measured by an F-4500 spectrofluorometer (HITACHI, Japan) with an excitation wavelength of 290 nm at 20 °C using a stirring cuvette. Protein concentration was 0.67 µM as the dimer. Measurements were performed in a buffer composed of 20 mM HEPES, pH 7.4, and 50 mM NaCl with or without 2.0 mM CaCl₂. Emission spectra from 300 to 400 nm were recorded. For the titration experiments, small aliquots of ligand (0.1 ~ 1.0 % of total volume) were sequentially added to the cuvette, and the fluorescence intensity at 350 nm was recorded. Volume changes in the titration experiments were corrected before analyzing the data. To obtain the titration curves, we also used the peak values of the fluorescence spectra instead of the values at 350 nm, because the peak shift of the fluorescence spectrum would affect the calculated values of the Hill coefficients. Both calculations yielded similar Hill coefficient values, and hence in this paper, we used the values calculated from the fluorescence intensity at 350 nm.

Transient kinetics measurement — Transient kinetics of the intrinsic tryptophan fluorescence change were measured with an SX18 stopped-flow spectrophotometer (Applied Photophysics, UK) at 20 °C. The excitation wavelength was 290 nm and the emission was monitored with a 335-nm-longpass optical filter. The protein and ligand were dissolved in a buffer consisting of 20 mM HEPES, pH 7.4, and 50 mM NaCl. Measurements were done with a fixed protein concentration of 0.84 µM (value after mixing).

Ligand binding assay The [³H]-labeled ligand binding assay was performed using the polyethylene glycol (PEG) precipitation method (10) as previously described with minor modifications. Briefly, 20 nM [³H]-labeled quisqualate or glutamate (Pharmacia) and the protein solution (1 µg of protein) were mixed in 150 µl of binding buffer composed of 40 mM HEPES, pH 7.4, and 2.5 mM CaCl₂ at 4 °C for 1 h. Then, 6 kDa PEG was added to the
sample to a final concentration of 15% with 3 mg/ml of γ-globulin. After vortexing and centrifugation, the precipitated material was washed twice with 1 ml of the binding buffer containing 8% of 6 kDa PEG, and then it was dissolved in 1 ml of water. After the addition of 14 ml of Clearsol II (Nacalai Tesque), the radioactivity was measured using a scintillation counter.

Data analysis of the titration experiments – Titration curves for glutamate and quisqualate binding were made by plotting the values of \((F - F_0)/F_{\text{max}}\) against the ligand concentrations where \(F\) and \(F_0\) are the fluorescence intensities in the presence and absence of ligand, respectively, and \(F_{\text{max}}\) is the maximum value of the fluorescence change in the titration experiment. For the titration curves of (S)-MCPG and LY367385 binding, the values of \((F_0 - F)/F_{\text{max}}\) were used because the intrinsic tryptophan fluorescence decreased upon the addition of these antagonists. The titration curves were fitted to the following equation by the Kaleidagraph software (Synergy), \(F/F_{\text{max}} = S^nH / (K_{\text{app}} + S^nH)\) where \(F\) equals \(F - F_0\) or \(F_0\), \(S\) is the concentration of the ligand, \(K_{\text{app}}\) is the apparent dissociation constant and \(nH\) is the Hill coefficient. Hill coefficients were also calculated from the Hill plot, which yielded values similar to those derived from the curve fitting. With respect to \(K_{\text{app}}\), we also obtained similar values from the half maximum value of the titration curves. This value is denoted as \(K_{0.5}\) in the text, and we used it to estimate the affinity of the ligand in this paper.
Results

To elucidate intersubunit allostery in the dimeric ligand binding domain of mGluR1, we worked out a plan to utilize the intrinsic tryptophan fluorescence signal to detect a ligand binding event. For the first step, we constructed and purified the FLAG-tagged LBD of mGluR1 to test whether the intrinsic tryptophan fluorescence changes upon ligand binding.

Purification of the FLAG-tagged ligand binding domain – Since the protein from the anti-FLAG-antibody-conjugated agarose gel was eluted with the FLAG peptide at a neutral pH instead of with an acidic or alkaline buffer, the protein damage was minimized. As analyzed by SDS-PAGE followed by silver staining, the purified FLAG-tagged LBD was observed as almost a single band (Fig. 1A). It was previously reported that under the non-reduced conditions the two protomers of the mGluR1 LBD are crosslinked to form a dimer through an interprotomer disulfide bond even under the denaturing conditions on an SDS-polyacrylamide gel (9, 10). Like the non-tagged wild-type LBD, the band of the FLAG-tagged LBD under the reduced conditions (Fig. 1A, +DTT) was shifted to a position corresponding to twice the molecular weight under the non-reduced conditions (Fig. 1A, -DTT), indicating that the FLAG-tagged protein maintained the ability to form an interprotomer disulfide bond as demonstrated for the non-tagged LBD (9).

Next, we investigated the ligand binding ability of the FLAG-tagged LBD using a $[^3H]$-labeled quisqualate ($[^3H]$quisqualate) by the previously described PEG-precipitation method (10). The final concentration of $[^3H]$quisqualate was 20 nM. As shown in Fig. 1 B, the FLAG-tagged LBD bound the $[^3H]$quisqualate at the same level as that of the non-tagged LBD. This indicates that the additional eight amino acids at the C-terminus of the protein do not influence the ligand binding capacity. From these data, we concluded that the FLAG tag at the C-terminus did not perturb the activities of the protein, and therefore, we utilized the FLAG-tagged LBD for the following experiments described below.
Intrinsic tryptophan fluorescence changes of the FLAG-tagged LBD induced by ligand binding – We examined whether the intrinsic tryptophan fluorescence changed upon the addition of ligand. In the absence of ligand, the intrinsic tryptophans of the FLAG-tagged LBD exhibited an emission spectrum with a peak at ~ 345 nm when excited at 290 nm (Fig. 2A, dotted line). Upon the addition of excess glutamate (1.0 mM final concentration), a native agonist for mGluR, the fluorescence spectrum changed in a manner such that the fluorescence intensity was enhanced by about 18 % and the fluorescence maximum was slightly shifted by ~ 1 nm toward a longer wavelength (red shift). Upon the addition of excess quisqualate (100 µM at final concentration), a non-native strong agonist for group I mGluRs, the emission spectrum was enhanced, but amplitude of the spectral change was less than that observed for glutamate addition (Fig 2C). This result probably reflects some environmental differences between the glutamate and quisqualate binding states around the tryptophans that contribute toward sensing the ligand binding. As a control for these observations, we measured the emission spectrum of the T188A mutant. It has been demonstrated that this mutation dramatically reduced the binding affinity of agonists and that no cellular responses were detected in HEK293 cells expressing the full-length mGluR1 carrying the same mutation (26). As shown in Fig. 2E, the emission spectrum of the mutant did not exhibit any noticeable change upon the addition of excess glutamate. Although we also examined the spectrum upon the addition of quisqualate, no effects were observed as well (data not shown). Therefore, the enhanced emission spectra observed for the wild-type FLAG-tagged LBD resulted from agonist binding to the protein.

Fig. 2B shows fluorescence spectra in the presence and absence of (S)-MCPG (100 µM final concentration), an antagonist for mGluR1. In contrast to the results with the agonists, the fluorescence intensity of the intrinsic tryptophans decreased and the fluorescence maxima shifted toward a shorter wavelength (blue shift) upon the addition of (S)-MCPG. These changes were opposite to those observed for agonist binding. A similar spectral change was observed upon the addition of an excess of LY367385 (100 µM final concentration), another
antagonist (Fig 2D), suggesting that, in the ligand-binding states of the two antagonists, the environments around tryptophans involved in the fluorescence change were similar to each other. On the other hand, no spectral change was observed upon the addition of (R)-MCPG (Fig. 2F), a stereoisomer of (S)-MCPG that does not bind to the mGluR1 LBD (8). This result clearly indicates that the observed spectral changes are due to the binding of the antagonists.

We measured the rate constants of ligand binding to the LBD with a stopped-flow apparatus. As shown in Fig. 3A, the tryptophan fluorescence was abruptly enhanced upon mixing with glutamate (0.5 mM after mixing), and it almost reached a plateau within one second. The time course of the fluorescence intensity change was roughly fitted to a single exponential curve. From the fitting curve, the observed rate constant ($k_{obs}$) of glutamate binding to the LBD was estimated to be $22.2 \text{ s}^{-1}$. On the other hand, upon mixing with (S)-MCPG (0.5 mM after mixing), the intrinsic tryptophan fluorescence was suddenly quenched and completely reached a plateau within one second (Fig. 3B). The time course of the fluorescence change was fitted well to a single exponential curve and the observed rate constant was estimated to be $38.6 \text{ s}^{-1}$. It should be noted that these rate constants of ligand binding are apparent rates measured only at the saturating concentration of ligands. They do not represent actual association rate constants of ligand.

**Identification of the tryptophan residue contributing to the spectral change of the intrinsic tryptophan fluorescence** – Tryptophan fluorescence in a protein is generally influenced by the surrounding environment. Water molecules attacking the indole moiety of tryptophan, and polar side chains and peptide bonds in a close proximity of the indole ring are major origin to quench tryptophan fluorescence (28). There are seven tryptophan residues in one protomer (W110, W224, W320, W367, W372, W468 and W500). To predict which tryptophan residue contributes to the observed spectral changes, we examined the environment around each tryptophan side chain using the atomic models.
We first considered solvent accessibility of each tryptophan. To quantify it, we calculated the solvent accessible area of each of the tryptophan side chains (indole rings) for the two distinct conformations of the protomer, i.e., the open conformation and the closed conformation, by the program SURFACE (29) (Table I). Of the seven tryptophans, only the values for one tryptophan, W110, which is located on the upper surface of the ligand binding pocket (Fig. 4), dramatically changed between the open and closed conformations: the solvent accessible area markedly decreased in the closed conformation. In addition, the W110 indole ring and the bound glutamate also contact with each other through a hydrophobic interaction (Fig. 4 and ref. 8). These observations suggest that the closed conformation shields the W110 indole ring from water attack, implying that the fluorescence intensity increases in the transition from the open to the closed conformations. We next examined the polar side chains and the peptide bonds around tryptophans. The side chain of E502 approaches the side chain of W468 in a structural transition from the closed conformation to the open conformation. However, E502 is fully exposed to the solvent and the temperature factors of its side chain in the crystal structures are extremely high (86 ~ 107 Å²), indicating that it rotates freely in random orientations in solution. Thus it was unlikely that this side chain affects the fluorescence of W468 through electrostatic interactions. On the other hand, the carboxyl group of E292 side chain forms a hydrogen bond with a nitrogen atom of the W110 indole ring in the closed conformation. In addition, the carboxyl group of the bound glutamate is also near to the indole ring. Thus, these carboxyl groups might quench the emission from W110 with the excited-state electron transfer (28).

These considerations from crystal structures led us to predict that the observed fluorescence changes mainly resulted from environmental changes around W110. To confirm this prediction, we altered the tryptophan residue to valine by site-directed mutagenesis and measured the fluorescence spectra of the W110V mutant in the presence and absence of glutamate. This mutant maintained the agonist binding ability as judged from the ligand binding
assay (data not shown). Nonetheless, no changes were detected in the fluorescence spectrum upon the addition of a sufficient amount of glutamate (Fig. 2G). Therefore we concluded that the observed spectral changes of the intrinsic tryptophan fluorescence upon ligand binding were due to environmental changes around the W110 indole ring. The fluorescence spectra exhibited the enhanced intensity rather than decreased intensity upon the addition of glutamate (Fig. 2A). This fact suggests that the two carboxyl groups of E292 and the bound glutamate in the closed conformation slightly affect, if any, the emission from W110. In this case, the solvent effects would predominate over the effects of these carboxyl groups. Hence the main mechanism of the spectral changes on ligand binding is possibly due to the solvent quench of the W110 fluorescence.

Titration of the intrinsic tryptophan fluorescence with ligands and effects of calcium ions on binding affinity – Using the intrinsic tryptophan fluorescence change, we produced saturation binding curves by ligand titration and evaluated the apparent binding constants from the titration curves. We also examined the effects of calcium ions on the ligand binding affinity by carrying out titrations in the presence and absence of calcium ions. Figs. 5A – D show the titration curves for four different ligands. In the titration with glutamate without calcium ions, we estimated the apparent dissociation constant (K_{0.5}) of glutamate to be 3.8 μM from the half-maximal change of the titration curve (Fig. 5A and Table II). In the presence of 2.0 mM calcium ion, the curve was shifted leftward as compared to that in the absence of calcium ions (Fig. 5A). In this case, the apparent dissociation constant was estimated to be 1.3 μM, which was about three-fold higher affinity than that without calcium ions. These results indicate that calcium ions act to increase the affinity of glutamate for the LBD. In the titration with quisqualate, the K_{0.5} values were shifted to lower ones by about one order in comparison with those of glutamate (0.21 μM and 0.46 μM in the presence and absence of calcium ion, respectively), indicating that quisqualate binds to the LBD more tightly than glutamate (Fig.
This was consistent with the previously-reported pharmacological data, in which the relative binding affinities of these agonists were examined by inhibiting the binding of \[^3\text{H}\]quisqualate in the conventional ligand binding assay (9, 10). We further tested the positive effect of calcium ions by the ligand binding assay using \[^3\text{H}\]quisqualate and \[^3\text{H}\]glutamate (Table III). As expected, the amount of bound ligand in the absence of calcium ions was smaller than that in the presence of calcium ions in both cases.

We also carried out titration experiments for two antagonists, (S)-MCPG and LY367385 (Figs. 5B and 5D). In the absence of calcium ions, the apparent dissociation constants were 40 \(\mu\text{M}\) and 0.89 \(\mu\text{M}\), respectively, for (S)-MCPG and LY367385 (Table II). The higher affinity of LY367385 than (S)-MCPG was consistent with the previous pharmacological data (30 - 32). Interestingly, the positive effects of calcium ions observed for the agonist were not detected for these antagonists, suggesting that the positive effect of the calcium ions is specific for agonist binding.

**Hill analysis of ligand binding to the LBD** – To investigate the allosteric properties of ligand binding, we analyzed the titration curves by a Hill analysis. It is well known that a Hill coefficient calculated from the slope of a Hill plot represents the cooperativity of ligand binding in oligomeric proteins. If the Hill coefficient is larger than 1, then the ligand binding has positive cooperativity, whereas the Hill coefficient smaller than 1 indicates negative cooperativity.

Typical Hill plots of the titration curves for glutamate and (S)-MCPG are shown in Fig. 6. With respect to glutamate binding, the Hill plots at low and high concentrations of the ligand below 1.0 \(\mu\text{M}\) and above 100 \(\mu\text{M}\), respectively, showed no (or low) cooperativity as judged from the Hill coefficients, which were close to 1 (0.88 and 0.89, respectively). However, at ligand concentrations between 1.0 \(\mu\text{M}\) and 100 \(\mu\text{M}\), the Hill plot exhibited strong negative cooperativity with a Hill coefficient of 0.48. This result is reasonable because the
cooperativity of ligand binding generally does not appear at the upper and lower limits of the ligand concentration and emerges at the middle range of ligand concentrations. On the other hand, with respect to (S)-MCPG, the Hill plot showed one straight line over all ligand concentrations with a Hill coefficient of \( \sim 0.9 \), suggesting that the negative cooperativity observed for glutamate tends to disappear upon (S)-MCPG binding. The inset in Fig. 6 shows the Hill plots at the middle range of ligand concentrations (around \( K_{0.5} \)), where the averaged values of three independent experiments were plotted. The Hill coefficients calculated from the plots of the inset were 0.55 and 0.83 for glutamate and (S)-MCPG, respectively. Taking the errors denoted in the graph into account, these Hill coefficient values were convincing enough to conclude the negative cooperativity. Similarly, we calculated the Hill coefficients for other ligands in the presence and absence of calcium ions. These values are summarized in Table IV. For quisqualate, the Hill coefficient was close to 1, indicating the lack of cooperativity between each protomer on quisqualate binding. For LY367385, the Hill coefficient was also close to 1. Calcium ions seemed to slightly affect the Hill coefficient of glutamate binding because it changed from 0.55 to 0.70. Among the four different ligands examined, only the Hill coefficient for glutamate showed remarkable negative cooperativity.

**Reduced cooperativity in the I120A mutant** – The negative cooperativity in mGluR1 LBD indicates that the conformational change of one subunit is transmitted into the other through the dimer interface. Thus, we carried out the same experiments using a purified FLAG-tagged I120A LBD, which has a mutation in the subunit interface. This mutation led to the uncoupling of ligand binding and signal transduction induced by mGluR1 (26). This mutant still maintained the capacity of ligand binding as previously demonstrated (Fig. 7A). The intrinsic tryptophan fluorescence of this mutant was enhanced and reduced upon the addition of excess glutamate and (S)-MCPG, respectively, as observed for the wild type (Fig. 7B). We then obtained the titration curves for glutamate and (S)-MCPG binding (Fig. 7C). From the
half-maximum value of the titration curve, we estimated the apparent dissociation constants 
\( K_{0.5} \) to be 16 µM and 39 µM for glutamate and (S)-MCPG, respectively (Table II). These 
results indicate that only the glutamate binding affinity is negatively affected by the mutation. 
We also obtained the Hill coefficient value from the titration curves. For glutamate binding, 
the Hill coefficient was calculated to be 0.80, while it was 0.83 for (S)-MCPG binding. In 
comparison with the wild type, the Hill coefficient for glutamate binding was significantly 
increased in this mutant, whereas the value for (S)-MCPG binding was similar to that of wild 
type. These results indicate that the negative cooperativity of glutamate binding observed for 
the wild type tends to disappear with the mutation. Hence, the negative cooperativity possibly 
operates through the hydrophobic subunit interface.
Discussion

This study demonstrated that the intrinsic tryptophan fluorescence of the mGluR1 LBD changed upon ligand binding, and that this intrinsic fluorescence yielded a saturation binding curve with an extensive range of ligand concentrations. Consequently, the Hill analysis revealed the negative cooperativity of glutamate binding between each protomer of the homodimeric mGluR LBD.

Relationship between the structure and the intrinsic tryptophan fluorescence change of the mGluR1 LBD – Interestingly the directions of the fluorescence change were opposite between agonist and antagonist binding, for not only the emission intensity but also the peak position of the emission spectrum (see Fig. 2 and summarized in Fig. 8). With respect to the emission intensity, agonist binding enhanced the emission, whereas antagonist binding reduced it. Since the tryptophan fluorescence intensity change of the protein is attributed to the emission intensity of W110, the opposite changes in the spectral intensity between agonist and antagonist are reasonable for the following reasons. In the agonist-induced closed conformation, the solvent accessible area decreases. As a result, the indole ring of W110 would be protected from attacks by water molecules. This should lead to the observed enhanced emission upon agonist binding. By contrast, in the antagonist-induced open conformation, the water molecules could attack the indole ring more easily than in the closed conformation. This would result in the decreased emission.

With respect to the peak position of the emission spectrum, agonist binding yielded a spectrum with a slightly red-shifted peak, whereas antagonist binding provides a spectrum with a markedly blue-shifted peak (Fig. 8). These spectral shifts can be also explained by the emission intensity of W110. The fluorescence spectrum of W110V was significantly blue-shifted with emission maximum was at ~ 335 nm (Fig. 2G). The blue-shifted spectrum induced by the lack of the W110 indicates that the W110 itself has a red-shifted fluorescence spectrum with a peak maximum longer than 335 nm. This is reasonable, because the indole
ring of W110 is not buried inside the protein, but instead exposed to the solvent in the open conformation (7, 8, 25). If the red-shifted emission of W110 is quenched upon antagonist binding, the total emission would be apparently blue-shifted. Indeed, the fluorescence spectrum was blue-shifted upon the addition of antagonist (Figs. 2B and 2D), indicating that this consideration is valid. Based on this notion, we can correlate the position of the emission maximum with the conformational states of the protomer: A blue-shifted spectrum indicates an open cleft conformation, whereas a red-shifted spectrum indicates a closed conformation.

In this context, the blue-shifted emission spectrum of the I120A mutant in the ligand-free state (Fig. 8) indicates that the ligand binding cleft of this mutant opens more easily than that of the wild type even in the absence of antagonist. This is consistent with the reduced affinity of glutamate in this mutant (Table II).

The opposite directions of the spectral changes between the agonist and antagonist binding states imply that, in the ligand-free state, the LBDs exist in a mixed population of the open and closed conformations. This observation suggests that, in the ligand-free state, the LBD protomers are in dynamic equilibrium between the two conformations. This notion is consistent with the activation model previously proposed by crystallographic studies (7, 8).

Positive effect of calcium ions on agonist binding – From the titration curves based on the ligand-dependent fluorescence change, we evaluated the apparent dissociation constant for several ligands from the titration binding curves, and found that calcium ions increased the affinity of agonists. This calcium effect was specific for agonists, and was not observed for antagonists, indicating that calcium ions stabilize the closed conformation of the protomer.

Positive effects of calcium ions on physiological mGluR activity have been reported. For example, extracellular calcium ions potentiate the phosphoinositide signaling generated by receptor activation (33), and extracellular calcium ions were required for a persistent response to glutamate stimulation (34). Thus, our finding may provide one of the molecular mechanisms for the positive effects of calcium ions described in these previous reports.
Negative cooperativity of glutamate binding in the dimeric ligand binding domain – The Hill analysis of glutamate binding yielded the Hill coefficient which was smaller than 1 ($n^H$, a Hill coefficient, = 0.55 without Ca$^{2+}$). Although one interpretation of the result is the negative cooperativity between the two binding sites in the dimer, an alternative interpretation is also possible: it is due to the presence of two independent non-interacting sites which have different affinities for glutamate. However, this is not plausible because glutamate does not bind to any other portion besides the each ligand binding cleft of the homodimer in the crystal structures even at the saturating concentration of glutamate (1 mM). Therefore, we concluded that the Hill coefficient smaller than 1 was due to the negative cooperativity of glutamate binding. Contrary to glutamate, the negative cooperativity was completely abolished for the other agonist, quisqualate ($n^H = 1.04$ without Ca$^{2+}$). This might be due to the higher affinity of quisqualate for the receptor than glutamate. That is, the strong affinity of quisqualate may overcome the weak-binding conformation of the protomer. As a result, quisqualate could also bind to the second protomer, and the ligand binding cleft of the second protomer would close. The notion that the negative cooperativity tends to disappear for a high affinity agonist is consistent with the observation that calcium ions, which raise the agonist affinity, slightly increased the Hill coefficient (Table IV). Thus, the possibility remains that calcium ions are partly involved in the cooperativity through affinity modulation.

For the I120A mutant, the negative cooperativity upon glutamate binding had a tendency to disappear in comparison with that of the wild type because the Hill coefficient was closer to 1 with this mutation ($n^H = 0.80$ without Ca$^{2+}$). I120 is located at the center of the dimer interface formed by the hydrophobic interaction between each LB I, and hence, it is likely that the cooperativity would come out by the interaction between each protomer through the hydrophobic dimer interface. It should be noted that this mutation reduced the affinity for glutamate, despite the relatively long distance between I120 and the ligand binding site. The reduced affinity only for glutamate indicates that the closing motion of the
protomer of this mutant would be restricted. This is consistent with the blue-shifted fluorescence spectrum of this mutant, which implies that the ligand binding cleft of this mutant prefers the open conformation as discussed above. The reduced affinity of I120A also suggests that the modulation of the hydrophobic dimer interface can indirectly affect the affinity of the ligand. Taken altogether, the conformational changes of one protomer induced by ligand binding, such as the closing motion of the ligand binding pocket, will modulate the interaction between the two protomers, resulting in suppression of the closing motion in the second protomer.

The fact that the negative cooperativity was obliterated in the I120A mutant reminds us of the behaviors of mutants of the bacterial homodimeric aspartate receptor, whose structures have been solved in the apo-form, one aspartate-bound dimer (half site occupied) and two aspartate-bound dimeric states (11, 12). Mutations at a single residue (serine 68), which is located in the subunit interface and within the aspartate binding pocket, altered the original negative cooperativity to non or positive cooperativity (13). These results, together with our present mutation study, indicate that the cooperativity of ligand binding in a dimeric receptor would be maintained by a subtle force balance in the dimer interface.

What is the role for the negative cooperativity in receptor functions? In general, negative cooperativity extends the ligand concentration range over which the protein can work (14). Thus one possible role for the negative cooperativity is to extend the glutamate concentration range to which the receptor can respond. This mechanism will be useful in the situations where continuous stimulation takes place at the synapse. Even in such a situation, the receptor will be able to respond because the ligand binding sites of the receptors on the cell surface would not be completely saturated by glutamate. The other advantage for the negative cooperativity is a greater sensitivity for low ligand concentration (14). This effect would also work favorably at the glutamatergic synapse in neuronal activity.

Each protomer of the mGluR LBD seems to close easily at the hinge without structural constraints and a closed protomer was actually observed in the ligand-free crystal.
Nevertheless, the crystallization with glutamate disclosed the asymmetric open-closed conformation. This is probably due to the negative cooperativity of glutamate binding between each protomer. If a closed-closed conformation actually exists in vivo, then it might perform a distinct function from that of the open-closed conformation, although the closed-closed symmetric crystal structure has been obtained only with co-existence of glutamate and possibly non-physiological gadolinium ions. The rank order in the signal strength between the open-closed and closed-closed states has not been examined, however, the release of the negative cooperativity with aid of metal ions, such as calcium ions, might trigger the shift to the closed-closed form. If the negative cooperativity defines the proportion between the open-closed and closed-closed forms, and they differ in their signal strength or pathway, then the negative cooperativity may discriminate between the associations with interacting proteins, including G proteins, in the cytoplasmic milieu and define the receptor destinies. In this context, an intriguing phenomenon is receptor desensitization. A receptor conformation with both ligand binding sites fully occupied by agonists might be a desensitization signal, and the frequency of desensitization events would be suppressed by the feature of the negative cooperativity. Further analysis will be needed to clarify these possibilities.

Acknowledgements

We are grateful to Dr. K. Morikawa (Biomolecular Engineering Research Institute) and Dr. S. Nakanishi (Kyoto University) for critical reading of the manuscript and discussions. We also thank Dr. K. Sutoh (University of Tokyo) for kindly sharing the stopped-flow apparatus.
Footnotes

1 Abbreviations: mGluR1, metabotropic glutamate receptor subtype 1; GPCR, G-protein coupled receptor; LBD, ligand binding domain; MCPG, α-methyl-4-carboxyphenylglycine; LY367385, (+)-2-methyl-4-carboxyphenylglycine; PEG, polyethylene glycol

*Corresponding author: Hisato Jingami, M. D. & Ph. D.
The Department of Molecular Biology, Biomolecular Engineering Research Institute (BERI), 6-2-3, Furuedai, Suita, Osaka 565-0874 JAPAN. Tel: +81-6-6872-8214. Fax: +81-6-6872-8210. E-mail: jingami@beri.or.jp
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Figure Legends

Fig. 1. Purification and ligand binding activity of the FLAG-tagged mGluR1 LBD.

A, silver stained polyacrylamide gel of the FLAG-tagged mGluR1 LBD, purified with the anti-FLAG M2 agarose gel. Samples were run in the presence (left, +DTT) and absence (right, -DTT) of 20 mM DTT on a 9 % SDS-PAGE gel. The interprotomer disulfide bond was formed under the nonreduced condition (-DTT), resulting in an upward shift of the target band. B, ligand binding ability of the FLAG-tagged LBD. The amount of the bound \[^3\text{H}]\text{quisqualate}\) in equilibrium with 20 nM \[^3\text{H}]\text{quisqualate}\) was measured in the presence and absence of 1.0 mM non-labeled glutamate. The difference between the two bars represents the specific binding of \[^3\text{H}]\text{quisqualate}\). Each binding experiment was performed in triplicate, and each bar is shown as mean ± s.d..

Fig. 2. Intrinsic tryptophan fluorescence spectra of wild-type and mutant FLAG-tagged LBDs.

Steady-state fluorescence spectra of intrinsic tryptophan were measured by a fluorophotometer. The excitation wavelength was 290 nm. A, fluorescence spectra of wild-type FLAG-tagged LBD with and without 1.0 mM glutamate. B, fluorescence spectra of wild-type FLAG-tagged LBD with and without 0.1 mM (S)-MCPG. C, fluorescence spectra of wild type FLAG-tagged LBD with and without 0.1 mM quisqualate. D, fluorescence spectra of wild-type FLAG-tagged LBD with and without 0.1 mM LY367385. E, fluorescence spectra of T188A FLAG-tagged LBD with and without 1.0 mM glutamate. F, fluorescence spectra of wild-type FLAG-tagged LBD with and without 0.1 mM (R)-MCPG. G, fluorescence spectra of W110V FLAG-tagged LBD with and without 1.0 mM glutamate. In all spectra, the \textit{solid} and \textit{dotted lines} show the spectra with and without the denoted ligand, respectively. Conditions were 20 mM HEPES pH 7.4 and 50 mM NaCl at 20°C. The protein concentration was 0.67 µM.
Fig. 3. Transient kinetic measurements of glutamate and (S)-MCPG binding to the FLAG-tagged LBD. Binding rate constants of glutamate and (S)-MCPG were measured with a stopped-flow apparatus in 20 mM HEPES pH 7.4 and 50 mM NaCl. The protein concentration was 0.84 µM (value after mixing). A, transient tryptophan fluorescence change upon mixing with excess glutamate (0.5 mM after mixing). The dotted line shows a single exponential fitting curve. From the fitting curve, the observed rate constant was estimated to be 22.2 s⁻¹. B, transient tryptophan fluorescence change upon mixing with excess (S)-MCPG (0.5 mM after mixing). The dotted line shows a single exponential fitting curve. From the fitting curve, the observed rate constant was estimated to be 38.6 s⁻¹.

Fig. 4. Structure around W110.

The open conformation (light blue) of the protomer superimposes onto the glutamate-bound closed conformation (ivory) by their LB1 domains. The side chain of W110 is colored green (the closed conformation) and orange (the open conformation). The glutamate in the closed conformation is colored yellow.

Fig. 5. Titration curves of the intrinsic tryptophan fluorescence change with ligand binding.

Titration curves with (open squares) and without (closed circles) calcium ion were generated by monitoring the fluorescence intensity at 350 nm with sequential additions of a small aliquot of ligand solution as described in the ‘Experimental Procedures’. A, titration curves for glutamate binding. B, titration curves for (S)-MCPG binding. C, titration curves for quisqualate binding. D, titration curves for LY367385 binding. Data were fitted by a hyperbola as described in the ‘Experimental Procedures’ with the Hill coefficients shown in Table IV.

Fig. 6. Hill plot for the titration curves of glutamate and (S)-MCPG binding.
Typical Hill plots derived from the titration curves are shown for glutamate (closed circles) and (S)-MCPG (open squares) binding. $Y$ means the degree of saturation of the ligand binding sites with bound ligand. Thus, $Y$ equals $|F - F_0|/F_{max}$, where $F$ and $F_0$ are the fluorescence intensities in the presence and absence of ligand, respectively, and $F_{max}$ is the maximum of the fluorescence change in the titration experiment. $n^H$ is the Hill coefficient estimated from the slope of the plot. Inset shows Hill plots at the middle range of ligand concentrations, with values representing the mean of at least three independent experiments.

**Fig. 7. Characterization of the I120A mutant.**

A, the ligand binding activity of the FLAG-tagged I120A LBD was measured by the PEG precipitation method with $[^3]$H]quisqualate. 20 nM $[^3]$H]quisqualate was used in the binding experiment. The difference between the two bars represents the specific binding of $[^3]$H]quisqualate as shown in Fig. 1B. B, intrinsic tryptophan fluorescence spectra of the I120A LBD in the presence and absence of ligand. The dotted line is a fluorescence spectrum in the absence of ligand. Solid and broken lines show fluorescence spectra in the presence of 1.0 mM glutamate and 0.1 mM (S)-MCPG, respectively. C, titration curves for glutamate (closed circles) and (S)-MCPG (open squares) binding to the I120A LBD. Data were fitted by a hyperbola as described in the 'Experimental Procedures' with the Hill coefficients shown in Table IV. These data were generated using the averaged values of three independent experiments.

**Fig. 8. Correlation between the peak wavelength and the relative fluorescence level of the intrinsic tryptophan fluorescence spectrum.**

Peak wavelength versus relative fluorescence level in the tryptophan fluorescence spectrum was plotted for the wild-type (closed circles) and I120A (open circles) FLAG-tagged LBDs. Each point in the graph is an averaged value, and the errors are derived from three
independent fluorescence spectra. The left and right ordinates indicate ligand-binding states and fluorescence intensity levels, respectively. The fluorescence intensities of the emission spectra in each ligand-binding state are divided into three levels. It should be noted here that the right ordinate represents the direction of fluorescence change rather than the actual fluorescence intensity.

Table I

*Solvent accessible area of tryptophan side chains in mGluR1 LBD*

Solvent accessible area of each tryptophan side chain in mGluR1 LBD was estimated by a program SURFACE (29) with a sphere probe whose radius was 1.4 Å. $S_{\text{open}}$ is an average of three values for each tryptophan calculated from the two open conformations in 1ISS (PDB ID code) and one open conformation in 1EWK. $S_{\text{closed}}$ is an average of two values calculated from one closed conformation in 1EWK and the other closed conformation in 1ISR. These values except for those in parentheses were calculated from atomic coordinates whose coordinates of bound ligands were excluded. The values in parentheses were calculated from the coordinates including the bound ligands.

<table>
<thead>
<tr>
<th></th>
<th>$S_{\text{open}}$</th>
<th>$S_{\text{closed}}$</th>
<th>$S_{\text{open}} - S_{\text{closed}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W110</td>
<td>74.7 (52.3)</td>
<td>46.1 (27.9)</td>
<td>28.6 (24.4)</td>
</tr>
<tr>
<td>W224</td>
<td>72.1</td>
<td>66.2</td>
<td>5.9</td>
</tr>
<tr>
<td>W320</td>
<td>0.9</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>W367</td>
<td>8.2</td>
<td>10.0</td>
<td>-1.8</td>
</tr>
</tbody>
</table>
Table II

*Apparent dissociation constant (K_{0.5}) of ligands for the FLAG-tagged mGluR1 LBD*

These values were calculated from the half maximum of the titration curves shown in Fig. 5.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>0 mM Ca^{2+}</th>
<th>2.0 mM Ca^{2+}</th>
<th>0 mM Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>W372</td>
<td>4.0</td>
<td>2.8</td>
<td>1.2</td>
</tr>
<tr>
<td>W468</td>
<td>109.7</td>
<td>118.0</td>
<td>-8.3</td>
</tr>
<tr>
<td>W500</td>
<td>4.9</td>
<td>4.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table III

*Calcium effect on ligand binding activity*

These values were obtained from ligand binding assay using the PEG precipitation method.
Final concentration of the \(^{3}\text{H}\)-labeled ligand in the assay mixture was 20 nM. Each assay mixture contained 1 µg of protein. These were averaged values obtained from at least three independent experiments (mean ± S.D.). Units of the values are expressed as bound ligand molecule per one protein molecule.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>0 mM Ca(^{2+})</th>
<th>2.0 mM Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{3}\text{H})quisqualate</td>
<td>0.057 ± 0.003</td>
<td>0.17 ± 0.002</td>
</tr>
<tr>
<td>(^{3}\text{H})glutamate</td>
<td>0.029 ± 0.002</td>
<td>0.092 ± 0.004</td>
</tr>
</tbody>
</table>

Table IV

*Hill coefficients calculated from titration curves measured by intrinsic tryptophan fluorescence change*

These values were obtained from the slope in the middle section (around \(K_d\) of the ligand) of Hill plots of the titration curves shown in Fig. 5 and Fig. 7. These are mean values and standard deviations derived from at least three independent titration experiments (mean ± S.D.).

Statistical significance of some pairs of these values was examined by the Student’s t-test.
The differences are statistically significant ($P < 0.05$).

The $P$ value was 0.058.

n.d., not determined.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Wild type</th>
<th>I120A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM Ca$^{2+}$</td>
<td>2.0 mM Ca$^{2+}$</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.55 ± 0.07 $a$, $b$, $c$, $d$</td>
<td>0.70 ± 0.07 $d$</td>
</tr>
<tr>
<td>Quisqualate</td>
<td>1.04 ± 0.06 $b$</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>(S)-MCPG</td>
<td>0.83 ± 0.09 $a$</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>LY367385</td>
<td>1.06 ± 0.08</td>
<td>0.92 ± 0.07</td>
</tr>
</tbody>
</table>
The figure shows two lines on a log-log scale, each with a different slope.

- The line on the right with the slope labeled as $n_H = 0.79$.
- The line on the left with the slope labeled as $n_H = 0.48$.

Both lines are described by the equation $\log(Y/(1-Y)) = n_H \log[\text{ligand}] + b$.

In the inset, the dotted line represents another relationship, but the slope is not explicitly labeled.
Glutamate -  
No ligand -  
S-MCPG -  

- Enhanced level  
- Middle level  
- Decreased level  

Peak wavelength in the tryptophan fluorescence spectrum (nm)
Negative cooperativity of glutamate binding in the dimeric metabotropic glutamate receptor subtype 1
Yoshikazu Suzuki, Eiko Moriyoshi, Daisuke Tsuchiya and Hisato Jingami

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