Determinants of Endogenous Ligand Specificity Divergence among Metabotropic Glutamate Receptors*

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Background: L-Glutamate and L-serine-O-phosphate (L-SOP) differentially regulate subtypes of metabotropic glutamate receptor (mGluR).

Results: Evolutionary trace identified residues whose swapping between mGluR1 and mGluR7 and mGluR4 altered responses as predicted.

Conclusion: Determinants for early and later functional divergence of mGluRs were identified.

Significance: The structural and evolutionary origins of divergent mGluR specificities may aid in design of new therapeutics.

To determine the structural origins of diverse ligand response specificities among metabotropic glutamate receptors (mGluRs), we combined computational approaches with mutagenesis and ligand response assays to identify specificity-determining residues in the group I receptor, mGluR1, and the group III receptors, mGluR4 and mGluR7. Among these, mGluR1 responds to L-glutamate effectively, whereas it binds weakly to another endogenous ligand, L-serine-O-phosphate (L-SOP), which antagonizes the effects of L-glutamate. In contrast, mGluR4 has in common with other group III mGluR that it is activated with higher potency and efficacy by L-SOP. mGluR7 differs from mGluR4 and other group III mGluR in that L-glutamate and L-SOP activate it with low potency and efficacy. Enhanced versions of the evolutionary trace (ET) algorithm were used to identify residues that when swapped between mGluR1 and mGluR4 increased the potency of L-SOP inhibition relative to the potency of L-glutamate activation in mGluR1 mutants and others that diminished the potency/efficacy of L-SOP for mGluR4 mutants. In addition, combining ET identified swaps from mGluR4 with one identified by computational docking produced mGluR7 mutants that respond with dramatically enhanced potency/efficacy to L-SOP. These results reveal that an early functional divergence between group I/II and group III involved variation at positions primarily at allosteric sites located outside of binding pockets, whereas a later divergence within group III occurred through sequence variation both at the ligand-binding pocket and at loops near the dimerization interface and interlobe hinge region. They also demonstrate the power of ET for identifying allosteric determinants of evolutionary importance.

Metabotropic glutamate receptors (mGluRs)2 are important for modulating signaling by glutamate, the main excitatory neurotransmitter in the central nervous system (1). The mGluRs have been implicated in a range of functions including protection from neuronal excitotoxicity, dim light phototransduction, and learning and memory. These homodimeric receptors have multiple functional domains and subdomains, including the extracellular N-terminal domain, which includes the “Venus flytrap” ligand-binding module, the cysteine-rich domains, the transmembrane segments and interhelical loops, and C-terminal intracellular domains. Ligand binding triggers a series of complex and poorly understood conformational changes, which include closing of the ligand-binding module and changes in interactions at the dimerization interfaces, ultimately leading to unknown conformational changes at intracellular interaction sites that allow activation of specific heterotrimetric G proteins and other cellular interaction partners.

Because of the immense therapeutic potential for drugs targeting these receptors, there has been considerable interest in trying to understand the structural basis of the specificity with which these receptors recognize ligands and generate functional responses upon binding. Many different synthetic ligands have been tested on native and mutant forms of the receptors in efforts to uncover the rules governing the potency and efficacy of such ligands. An alternative approach that complements such conventional approaches is to draw on the countless number of structure-function experiments carried out by nature in the course of evolution by extracting the record of such experiments residing in the ever growing mass of sequence data. A computational method for extracting such information, known as the evolutionary trace, has been applied to many different protein-ligand systems with consistent success (2–4) and has become even more effective as it has been refined and extended as sequence and structure databases have continued to grow larger. We have previously used these methods combined with site-directed mutagenesis and functional

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2 The abbreviations used are: mGluR, metabotropic glutamate receptor; ET, evolutionary trace; L-SOP, L-serine-O-phosphate; PDB, Protein Data Bank.
assays to identify functionally important residues in class A G protein-coupled receptors (5–8) and to identify specificity determinants in dopamine receptors (6). However, this approach has not been applied to ligand-binding domains of class C G protein-coupled receptors.

Here we ask whether this approach can be applied to the ligand-binding domain of mGluR to unravel the amino acid residues that determine their ligand specificities. Because our approach relies on evolutionary information, we have focused on specificity toward the competing ligands to which the receptors of interest are naturally exposed to and must discriminate for or against. In a recent study (9), we conducted a thorough re-examination of the responses of mGluR to amino acids present at significant levels in cerebrospinal fluid and found evidence that group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) receptors all bind to and are activated by L-Glu, albeit with varying efficacies and potencies and that the only other endogenous ligand that can activate or inhibit them is 2-D-serine-O-phosphate (D-SOP). Whereas group III mGluRs are activated by D-SOP, groups I and II are not, but L-SOP does bind to and inhibit mGluR1 and mGluR2 (9). Even among group III mGluRs, there are substantial differences in ligand affinities and responses. In particular, mGluR7 has much lower affinity for L-Glu and L-SOP than the other group III receptors and also displays lower efficacy for both ligands. The work described here tests the hypothesis that these differences in ligand binding and responsiveness between and among subgroups of mGluR have conferred selection advantages over the course of evolution and that the records of those evolutionary processes are embodied in the patterns of sequence variation among the mGluR family members.

In particular, we have focused on the hypotheses that it has been important for mGluR1 to develop and maintain weak binding and no activation in response to L-SOP, while maintaining strong binding and activation in response to L-Glu, for mGluR7 to display weak binding and efficacy but low selectivity for both L-Glu and L-SOP and for mGluR4 to maintain strong binding and activation in response to both ligands. We have tested these hypotheses by using the evolutionary trace, in conjunction with computational structure analysis of the ligand-binding pocket, to identify important amino acid positions within the ligand-binding domain and then exchanging residue identities at those positions between mGluR4 and mGluR7 on the one hand and either mGluR1 or mGluR7 on the other to test for functional changes.

Recently we tested 10 amino acids that are known to be present at substantial concentrations in cerebrospinal fluid, as well as D-serine-O-phosphate, on mGluR from groups I, II, and III and found that only L-Glu and L-SOP displayed measurable interactions (9), despite similar structures and/or charge distributions of others. Therefore, in this study, we restricted the ligands analyzed to those two.

**MATERIALS AND METHODS**

**Evolutionary Trace Analysis**—We used a combination of sequence and structure analysis to determine the functionally important residues that differentiate group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8). Evolutionary trace analysis was performed with rvET and piET algorithms (10, 11).

The rvET algorithm measures the importance of a residue based on its variation pattern in the context of the phylogenetic tree. The piET algorithm differs from rvET in that it considers structure and common variation patterns between contacting residues. We used the structure of the glutamate-bound extracellular ligand-binding domain of mGluR1 (PDB code 1EWK) (12) to perform piET calculations on the ligand-binding domain sequences. The submitted protein sequence of rat mGluR4 is NP_073157.1.

To construct a multiple sequence alignment, protein sequences were gathered from a BLAST search of the NCBI reference sequence database and aligned with PROMALS3D (13). To identify functional homologues, the proteins sequences were filtered based on protein length (90% of the query protein) and sequence identity (>20%). Evolutionary trace analysis was performed on 311 mGluR homologues.

To identify the key residues that differentiate group III from group I/group II, we constructed a set of target substitutions. First, we determined the key residues using rvET analysis of the mGluR subfamily. The residues ranked in the top 30% were selected. We then determined the residues that varied in amino acid type in the respective positions between group III and group I/II. To focus on the targets most likely relevant to ligand response specificity, we eliminated from consideration any residue greater than 20 Å from the bound ligand glutamate in PDB code 1EWK. These steps led to a unique set of six targets that can be found in Table 1 and Fig. 2. Each residue number refers to the corresponding position in the alignments of mGluR4 and mGluR7 to mGluR1, *i.e.* the numbering in mGluR1 (PDB code 1EWK) is used throughout.

To identify the key residues that differentiate mGluR7 from the rest of group III, we constructed a second set of substitutions. We found that rvET led to no targets in the top 30%, and therefore we used the updated piET algorithm and expanded the set to include the residues in the top 45%. We then determined at which of these residue positions the amino acid type differs between mGluR7 and the rest of group III. We again eliminated any targets greater than 20 Å from the bound ligand glutamate in PDB code 1EWK. These steps led to a unique set of targets that can be found in Table 1 and Fig. 2.

To display the locations of residues, we used the structure of the extracellular domain of mGluR3 (PDB code 2E4U) or the structures of the N-terminal domains of mGluR1 with bound L-Glu (PDB code 1EWK), the structure of mGluR7 (PDB code 2E4Z) computationally docked with L-SOP (9), or a homology model of mGluR4 calculated using the automated homology modeling tool, Swiss-Model server (14, 15), with the N-terminal domain of mGluR7 (2E4Z) as a template.

**Compounds and cDNA Clones**—L-Glutamate and L-serine-O-phosphate were purchased from Sigma-Aldrich. L-Serine was purchased from Spectrum Chemicals Corp. The rat mGluR1a cDNA was tagged at the N-terminal with triple Myc epitope (a gift from Dr. Anna Francesconi, Albert Einstein College of Medicine). The N-terminal Myc-tagged rat mGluR1a construct was a gift from Hans Brünger-Osborne (University of
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Copenhagen, Copenhagen, Denmark). The rat mGluR7 cDNA construct was a gift from Dr. Shigetada Nakanishi (Osaka Bioscience Institute, Osaka, Japan). cDNA encoding mGluR7 was subcloned into the backbone of the mGluR4 plasmid with cDNA encoding mGluR4 excised out and replaced by cDNA of mGluR7. mGluR1 mutants, mGluR4 mutants and mGluR7 mutants were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. cDNA for chimeric G protein G

Cell Culture and Transfection—HEK-293 cells were maintained with complete DMEM, which is composed of 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin at 37°C in the presence of CO2. Cells were plated in 96-well, black-walled, clear-bottomed, poly-D-lysine-coated plates at a density of 75,000 cells/well and transiently with Lipofectamine 2000 as recommended by the manufacturer. mGluR-expressing plasmids were transfected with or without co-transfection with plasmids directing expression of promiscuous G proteins.

Calcium Mobilization Assay—After 36 h following transfection, cells were washed with Krebs/Ringer/Hepes buffer (120 mM NaCl, 4.7 mM KCl, 1.1 mM CaCl2, 10 mM Hepes, 1.2 mM KH2PO4, 1.2 mM MgSO4, pH 7.4) supplemented with 1.8 g/liter glucose and 1 mM probenecid to inhibit dye efflux. Cells were incubated with 2.5 μM Fluo-4 AM (Invitrogen) for 1 h at room temperature. Afterward, cells were washed twice and then incubated with 80 μl of buffer at room temperature for 30 min. Ligand plates were prepared with different concentrations of ligands to be tested at three times the desired final concentration, and 40 μl of ligand solution was injected after 20 s of recording (to determine baseline signal). Ca2+-enhanced fluorescence (excitation, 485 nm; emission, 525 nm) was detected using a FlexStation 3 microplate reader (Molecular Devices). As controls for background signals, cells transfected with vector lacking an expression construct were challenged with drugs in parallel.

Surface Expression—Surface expression was measured by an ELISA using chemiluminescence detection. Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were incubated with PBS supplemented with 2% BSA (Sigma) followed by incubations with a rabbit anti-Myc epitope (Santa Cruz Biotechnologies, Santa Cruz, CA), and HRP-conjugated goat anti-rabbit antibody and Supersignal West Femto Substrate (Pierce) were used for development of the signal. Baseline signal from mock transfected cells was subtracted from each detected signal.

RESULTS

ET identified positions important for l-SOP recognition for mGluR4 through (1) intergroup receptor comparison (group I (mGluR1 and mGluR5) and group II (mGluR2 and mGluR3) versus group III (mGluR4, mGluR6, mGluR7, and mGluR8)) and (2) intermember comparison (mGluR4 versus mGluR7) among group III mGluRs (Figs. 1 and 2 and Table 1). These comparisons were made between subsets of mGluR that respond oppositely to l-SOP (groups I/II versus group III) or respond to l-SOP and l-Glu in ways that differ quantitatively (mGluR4/6 versus mGluR7). In addition, the residues selected were all within 20 Å of the bound ligand and ranked highly in evolutionary importance. Therefore they represented logical targets for altering ligand response specificity, based on the hypothesis that the evolution of receptors with divergent responses to l-SOP and l-Glu has conferred selective advantage. The residue sets identified by ET are indicated in Figs. 1 and 2 and Table 1.

ET Positions 319, 342, 344, and 415 Are Important for Ligand Responses in mGluR4 and mGluR1—When the residues at these positions in mGluR1 were substituted, one at a time, for those at the equivalent positions in mGluR4, all mGluR4 single-point mutants were able to reach the membrane and respond to
FIGURE 2. Sequence alignment of metabotropic glutamate receptor members. ET positions studied for comparison between group I/II and group III are indicated with #, whereas ET positions differing between mGluR4 and mGluR7 are indicated with *. Amino acids are highlighted with different colors according to their specific functional properties: blue, hydrophobic; white, basic; darkest gray, acidic; pink, N-H bond donors; lightest gray, other polar. The mGluR1 sequence colored by evolutionary importance is represented at the bottom of the alignment.
Evolutionary Trace of mGluR Ligand-binding Domain

TABLE 1
Mutations and positions in sequence and structure

| Mutant          | Number in mGluR1 | Number in mGluR4 | Number in mGluR7 | Rank | Distance from ligand |
|-----------------|------------------|------------------|------------------|------|----------------------|
| Groups I/II (mGluR1) vs. Group III (mGluR4) |
| Q170M (M170Q)   | 170              | 164              | 160              | 11.0 |
| S189A (A189S)   | 189              | 183              | 20.0             | 4.2  |
| G319S (S319G)   | 319              | 313              | 14.0             | 3.7  |
| L342P (P342L)   | 342              | 336              | 16.0             | 7.5  |
| S344R (R344S)   | 344              | 338              | 30.0             | 11.1 |
| N415D (D415N)   | 415              | 411              | 28.0             | 14.5 |
| mGluR4 vs. mGluR7 |
| N74K            | 74               | 74               | 45.3             | 2.5  |
| E191D           | 191              | 185              | 31.9             | 9.3  |
| D195N           | 195              | 189              | 29.9             | 13.2 |
| P207S           | 207              | 201              | 10.7             | 8.6  |
| S209T           | 209              | 203              | 40.4             | 10.6 |
| F210Y           | 210              | 204              | 40.6             | 12.2 |
| K239S           | 239              | 233              | 24.8             | 8.7  |
| V288I           | 288              | 282              | 49.6             | 14.1 |
| D293E           | 293              | 287              | 35.7             | 5.1  |
| E294D           | 294              | 288              | 44.4             | 7.2  |
| I338V           | 338              | 332              | 29.0             | 13.8 |
| Q341L           | 341              | 335              | 25.9             | 9.6  |

ligands but displayed significant changes in potency or efficacy in our cell-based Ca\(^{2+}\) release assay. At 100 \(\mu\)M \(\text{L-SOP}\), the assay demonstrated the hypothesized importance of these residues, because the residue-swapped versions of mGluR4 displayed significantly decreased function in efficacy or potency. Activation of \(G_{\text{q0}}\) in response to \(\text{L-SOP}\) was diminished for four of the residue swaps: P342L, D415N, R344S, and S319G. Dose-response curves revealed that \(\text{L-SOP}\) potency was diminished for P342L, D415N, and R344S (Fig. 2). The maximal response was decreased for P342L and S319G (Fig. 2). Therefore, four of six mGluR4 mutants showed impaired interaction with \(\text{L-SOP}\). These mutants showed similar effects with \(\text{L-Glu}\). P342L, R344S, and D415N (Fig. 2) showed lowered \(\text{L-Glu}\) potency compared with WT, and P342L and S319G showed lowered efficacy for \(\text{L-Glu}\) (Fig. 2), indicating that the corresponding residues in mGluR4 are involved in recognition of not only \(\text{L-SOP}\), but also \(\text{L-Glu}\).

To test whether these same positions are also important for ligand responses in mGluR1, we generated reciprocal mutations by swapping the corresponding amino acids from mGluR4 into mGluR1. For the reciprocal mGluR1 mutants, three of six residue swaps showed increased affinity or selectivity for \(\text{L-SOP}\). Recently, we found that \(\text{L-SOP}\) behaves as an antagonist for mGluR1 at submillimolar concentrations (9). To test the antagonistic effect of \(\text{L-SOP}\) on mGluR1 mutants, varying concentrations of \(\text{L-Glu}\) and a fixed concentration of \(\text{L-SOP}\) were applied to mGluR1 or mGluR1 mutant-expressing cells. Then the ratio of the EC\(_{50}\) values derived from \(\text{L-Glu}\) dose-response curves from calcium release assays in the presence and absence of \(\text{L-SOP}\) was used as an indication of the potency of the antagonistic effect. Of the residues tested, mGluR1-N415D showed a 50% enhancement of \(\text{L-SOP}\) potency \((p < 0.05)\), with an EC\(_{50}\) value of 0.5 ± 0.1 mM (mean ± S.E., \(n = 3\)) as compared with the WT-mGluR1 EC\(_{50}\) value of 1.2 ± 0.12 mM (mean ± S.E., \(n = 6\)) (see Fig. 4, A–C). This gain of \(\text{L-SOP}\) potency by mGluR1-N415D is consistent with the loss of potency by its reciprocal mutant mGluR4-D415N (Fig. 3C). It is striking that the residue is 15 Å away from the bound ligand (based on an \(\text{L-Glu}\) bound x-ray structure of mGluR1 and \(\text{L-SOP}\) computationally docked in the x-ray structure of mGluR7, 2E4Z (9)), indicating that this position located outside of the binding pocket affects recognition of \(\text{L-SOP}\) through allosteric interactions (see Fig. 3, A and D) in both mGluR1 and mGluR4.

We also investigated \(\text{L-Glu}\) responses for six mGluR1 mutants. Interestingly, there are two mutants, mGluR1-S344R and mGluR1-L342P, that significantly decreased the \(\text{L-Glu}\) response (for mGluR1-S344R, \(p < 0.05\), \(n = 6\); for mGluR1-L342P, \(p < 0.005\), \(n = 4\)) but retained the WT potency of \(\text{L-SOP}\)
inhibition, providing greatly enhanced selectivity toward L-SOP versus L-Glu (Fig. 4, C and D, and Table 2). The reciprocal mutants mGluR4-R344S and mGluR4-P342L decreased L-SOP potency, confirming that these positions are involved in L-SOP recognition/binding (Fig. 3C). These results suggest an evolutionary divergence corresponding to the functional separation of group III receptors from group I and group II, in which mGluR1 acquired or retained residues that eliminate physiological L-SOP responses while retaining high sensitivity to L-Glu, whereas mGluR4 retained or acquired residues that allowed sensitivity to physiological levels of both ligands.

**Evolutionary Trace of mGluR Ligand-binding Domain**

**Swaps of mGluR4 Residues into mGluR7 Reveal That Positions 191, 195, and 74 Are Important for L-SOP and L-Glu Recognition**—To find residues important for the functional divergence of mGluR7, for which both L-Glu and L-SOP have low potency and efficacy, from mGluR4, which is much more sensitive to both ligands, we swapped multiple ET residues from mGluR4 into mGluR7. We also combined these mutations with one suggested by previous computational docking and mutagenesis studies (9), which demonstrated that a lysine at position 74 increased the potency of L-SOP interactions with mGluR7. As shown in Fig. 5 (A and B), the maximal response to activation of G_{max} by L-SOP or L-Glu was increased for two multiple ET swaps: P2075/S2097/F210Y (R1) and E191D/D195N (R2) (p < 0.005, n = 6–8). These double or triple mutations are located at positions outside of the ligand-binding pocket, near the hinge region between the two lobes of the ligand-binding domain/dimerization interface (Fig. 6A), suggesting that their effect is propagated through conformational and dynamic effects, i.e., by allostery. Dose-response curves revealed that these mutants did not significantly alter potency for L-SOP or L-Glu (Fig. 5, A and B). The most dramatic functional effects were observed for the combined swaps R2 + N74K, which increased potency for L-SOP by 16-fold compared with WT (Fig. 5C and Table 3). On the other hand, N74K, which is located at the ligand-binding pocket and was suggested by previous docking results, did increase potency for L-SOP by 5-fold compared with WT (Fig. 5C and Table 3; p < 0.05, n = 3) without increasing efficacy (maximal response) for L-SOP (Fig. 5A). To determine whether there are synergistic effects of the potency-altering residues and activation-altering residues, we generated a series of three combined mutants: R2 + N74K, R1 + R2 + N74K, and R1 + R2 + R3 + N74K. The combined swaps R2 + N74K, R1 + R2 + N74K, and R1 + R2 + R3 + N74K increased efficacy for L-SOP, and R1 + R2 + N74K showed slightly increased efficacy for L-Glu more than the individual component swaps (Fig. 5, A and B). The most dramatic functional effects were observed for the combined swaps R2 + N74K and R1 + R2 + N74K, which increased potency for L-SOP by 16-fold (p < 0.05, n = 3) and 25-fold (p < 0.05, n = 3), respectively, over WT mGluR7 (Fig. 5C and D, and Table 3), without significantly increasing L-Glu potency (Fig. 5D). Thus these residues work together to confer potent activation specifically by L-SOP.

**DISCUSSION**

Our results lead to five major general conclusions: 1) The evolutionary trace can identify residues important for functional responses and specificity for endogenous ligands within the ligand-binding domains of class C G protein-coupled receptors, such as mGluR, just as previous studies demonstrated such predictive power for the transmembrane domain of class A G protein-coupled receptors (6). 2) An early and more extensive functional
divergence between group I/group II and group III involved variation at positions primarily in allosteric sites located outside of binding pockets. 3) A later divergence in quantitative responses within group III occurred through sequence variation at both the ligand-binding pocket and at loops near the dimerization interface and interlobe hinge region. 4) Within group III, ligand affinity or potency is determined by ligand-contacting residues, and responsiveness or efficacy is determined by residues located outside of binding pocket, such as the dimerization interface or interlobe hinge region. 5) Within group III, residues that do not contact the binding pocket can also modulate the specificity and potency of ligands when coupled with a mutation in the ligand-binding domain.

The first conclusion is based on the findings that three of six mGluR1 mutants gained L-SOP affinity or L-SOP specificity over L-Glu and that eight of nine mGluR7 multiple mutants improved efficacy or potency for L-SOP or L-Glu. The second conclusion derives from the observation that mGluR1 mutants N415D and S344R that resulted in increased potency for L-SOP are found at positions that are 15.4 and 11 Å away from the bound ligand, respectively (based on an L-Glu bound x-ray structure of mGluR1 and an L-SOP-docked x-ray structure of mGluR7; Fig. 4D), indi-
teric interactions. This increased potency was observed for L-SOP
communicating with the distant ligand-binding site through allos-
of the ligand-binding pocket, docked L-SOP in mGluR7 (Fig. 6
(Figs. 5
(E191D/D195N), which do not increase potency of L-SOP by
from the observation that R1 (P207S/S209T/F210Y) or R2
be L-SOP-specific. These results have important implications for
be L-SOP-specific. These results have important implications for
structure of the dimer interface in different functional states, as
structural changes in the cysteine-rich, transmembrane, and cytoplasmic
domains (17–19). The first step in activation has been proposed to be the
relocation of helix B and helix C in the dimerization inter-
face of mGluR (12, 17). Several hydrophobic residues located in
helix B and helix C, such as Leu-116, Ile-120, Leu-174,
and-Leu-177, are conserved across the mGluR groups, suggesting
that a short stretch of hydrophobic core residues in the dimeriza-
tion interface might be critical for oligomerization of mGluRs (17, 18).
Another potentially important feature of mGluR activation
involves the interlobe hinge region, which connects two N-termi-
nal lobes and includes Asp-208, an invariant residue across the
mGluR groups. A D208I mutation in mGluR1 abolished not only
L-Glu responses but also responses to the synthetic ligand L-
quisqualic acid (20), showing that this residue is very important for
receptor activation or potency. This ligand nonspecific result can be
explained by a salt bridge between Asp-208 and the
amino group of bound ligand.

TABLE 3
Activation profiles of mGluR7 mutants for L-Glu or L-SOP

|                | EC50 for L-SOP | EC50 ratio to WT | EC50 for L-Glu | EC50 ratio to WT |
|----------------|----------------|------------------|----------------|------------------|
| mGluR7-WT      | 23.2 ± 2.8     | 1.00 ± 0.17      | 1.2 ± 0.4      | 1.00 ± 0.45      |
| P207S/S209T/F210Y (R1) | 24.3 ± 4.6     | 1.04 ± 0.23      | 0.61 ± 0.1     | 0.50 ± 0.18      |
| E191D/D195N (R2) | 12.7 ± 2.2     | 0.54 ± 0.11      | 0.55 ± 0.07    | 0.45 ± 0.15      |
| D293E/E294D (R3) | 22.0 ± 0.65    | 0.95 ± 0.11      | 0.54 ± 0.14    | 0.45 ± 0.18      |
| V288I          | 22.1 ± 7.9     | 0.95 ± 0.36      | 0.43 ± 0.02    | 0.36 ± 0.12      |
| K239S          | 25.6 ± 3.2     | 1.10 ± 0.19      | 0.86 ± 0.57    | 0.47 ± 0.28      |
| N74K           | 4.5 ± 0.87     | 0.19 ± 0.04      | 1.85 ± 1.14    | 1.53 ± 1.0       |
| R2 + N74K      | 1.3 ± 0.18     | 0.05 ± 0.01      | 0.75 ± 0.42    | 0.6 ± 0.40       |
| R1 + R2 + N74K | 0.87 ± 0.11    | 0.04 ± 0.01      | 1.54 ± 1.28    | 1.27 ± 1.13      |

In mGluRs, a signal is initiated by ligand binding to the extracel-
lar domain, which induces closing of the domain and rearrange-
ment of the dimer interface, followed by a relay of conformational
changes in the cysteine-rich, transmembrane, and cytoplasmic
domains (17–19). The first step in activation has been proposed to be
the relocation of helix B and helix C in the dimerization inter-
face of mGluR (12, 17). Several hydrophobic residues located in
helix B and helix C, such as Leu-116, Ile-120, Leu-174,
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quisqualic acid (20), showing that this residue is very important for
receptor activation or potency. This ligand nonspecific result can be
explained by a salt bridge between Asp-208 and the
amino group of bound ligand.

The third and fourth conclusions are based on the observation that mGluR7 to mGluR4 mutant N74K that increased
potency for L-SOP is located within the ligand-binding pocket
(Figs. 5D and 6, A and B), whereas P207S/S209T/F210Y (R1)
and E191D/D195N (R2), which enhanced efficacy for both
L-SOP and L-Glu without affecting potency, are located outside
of the ligand-binding pocket, i.e. more than 8–16 Å from the
docked L-SOP in mGluR7 (Fig. 6A). The fifth conclusion comes from the observation that R1 (P207S/S209T/F210Y) or R2
(E191D/D195N), which do not increase potency of L-SOP by
themselves, increased potency of L-SOP by 16- and 25-fold
when combined with N74K (Fig. 5D).

Thus, residues near the hinge region/dimerization interface can
communicate with the distant ligand-binding site through allos-
teric interactions. This increased potency was observed for L-SOP
but not L-Glu, indicating that this allosteric pathway has evolved to
be L-SOP-specific. These results have important implications for
structure-based drug design, because they suggest that drug design
targeting the dimerization area or hinge, as well as specificity-de-
dtermining residues like N74K, may be able to generate potent and
effective drugs with subtype specificity. They also have implica-
tions for the possibility that residues in this region may regulate the
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the α amino group of bound amino acid glutamate like Asp-208 does (Fig. 6) rather than the side chain of the amino acid ligand, and swapping them improved activation for both l-Glu and l-SOP. These results show that computation-guided mutation can identify new allosteric pathways, which generate mGluR subtype-specific conformational coupling, ultimately leading to G protein activation. A deepening understanding of allosterism is emerging in signaling proteins, including mGluRs (21–25). The recently determined x-ray structure of the transmembrane domain of mGluR1 reveals a strong interaction between the linker region (Ile-581–Glu-592) and the extracellular loop 2 β-sheet, suggesting a potential coupling site between the extracellular domain and the transmembrane domain (26). It has been suggested that there can be allosteric control of the extracellular domain by the transmembrane domain, because the active conformation of the transmembrane domain, induced either by constitutively activating mutations or by application of a positive allosteric modulator, can induce relative movements of the ligand-binding domains as measured by cell surface labeling and fluorescence resonance energy transfer (27). There is evidence that application of an allosteric modulator to mGluR1 in the absence of agonist had no effect on Ca²⁺ signaling, but modulated ERK1/2 phosphorylation, suggesting that biased signaling of mGluRs can be achieved by allosteric modulators (28). Together with the results presented here, these previous structural and mechanistic studies suggest that evolution-guided mutagenesis of the cysteine-rich and transmembrane domains could be used to understand not only allosteric coupling between domains but also to develop signaling-biased therapeutics, potentially offering a new strategy for development of new subtype-selective and allosteric drugs targeting mGluRs.

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