Probing the Ligand-binding Domain of the mGluR4 Subtype of Metabotropic Glutamate Receptor*

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Metabotropic glutamate receptors (mGLuRs) are G-protein-coupled glutamate receptors that subserve a number of diverse functions in the central nervous system. The large extracellular amino-terminal domains (ATDs) of mGluRs are homologous to the periplasmic binding proteins in bacteria. In this study, a region in the ATD of the mGluR4 subtype of mGluR postulated to contain the ligand-binding pocket was explored by site-directed mutagenesis using a molecular model of the tertiary structure of the ATD as a guiding tool. Although the conversion of Arg78, Ser159, or Thr182 to Ala did not affect the level of protein expression or cell-surface expression, all three mutations severely impaired the ability of the receptor to bind the agonist L-[3H]amino-4-phosphonobutyric acid. Mutation of other residues within or in close proximity to the proposed binding pocket produced either no effect (Ser157 and Ser160) or a relatively modest effect (Ser181) on ligand affinity compared with the Arg78, Ser159, and Thr182 mutations. Based on these experimental findings, together with information obtained from the model in which the glutamate analog L-serine O-phosphate (L-SOP) was “docked” into the binding pocket, we suggest that the hydroxyl groups on the side chains of Ser159 and Thr182 of mGluR4 form hydrogen bonds with the α-carboxyl and α-amino groups on L-SOP, respectively, whereas Arg78 forms an electrostatic interaction with the acidic side chains of L-SOP or glutamate. The conservation of Arg78, Ser159, and Thr182 in all members of the mGluR family indicates that these amino acids may be fundamental recognition motifs for the binding of agonists to this class of receptors.

Metabotropic glutamate receptors (mGLuRs) are a family of eight G-protein-coupled receptors that are expressed throughout the central nervous system and in sensory cells of the retina and tongue. The mGluR family has been divided into three subgroups based on sequence homology, pharmacology, and signal transduction properties; in cell lines, group I mGluRs couple to phosphoinositide turnover, whereas group II and III receptors couple to the inhibition of forskolin-stimulated cAMP via Gi/Go proteins (1, 2). mGluR4 together with mGluR6, mGluR7, and mGluR8 constitute the group III subclass of mGluRs that are selectively sensitive to the phosphono derivative of L-glutamate, L-α-amino-4-phosphonobutyric acid (L-AP4), and the endogenous amino acid L-serine O-phosphate (L-SOP).

The group III mGluRs are important regulators of synaptic transmission in the central nervous system. Electrophysiological experiments have shown that activation of L-AP4-sensitive receptors causes a suppression of synaptic transmission by inhibiting neurotransmitter release from nerve terminals (3), and immunocytochemical studies have confirmed that group III mGluRs are localized presynaptically (4–6). The characterization of mutant mice lacking the mGluR4 subtype of mGluR has provided additional insight into the function of this receptor in the nervous system. For example, observations from electrophysiological analyses demonstrating impaired presynaptic functions in the mutant mice led to the suggestion that this receptor may be required for sustaining synaptic transmission during periods of high-frequency neurotransmission (7). Behavioral studies on mGluR4 mutant mice have shown that this receptor plays a role in motor and spatial learning (7, 8). The potential use of group III mGluR ligands as therapeutic agents in epilepsy and neurodegenerative disorders has provided a persuasive argument for conducting more detailed structural analyses of this class of neurotransmitter receptors (9, 10).

The amino acid sequences of the mGluRs are homologous to the periplasmic amino acid-binding proteins in bacteria (11), the calcium-sensing receptor of the parathyroid gland (12, 13), the GABA_β receptors (14–16), a group of mammalian pheromone receptors (17), and a class of taste receptors expressed in lingual tissue (18). The basic structural domains of mGluRs include a large extracellular amino-terminal domain (ATD), seven putative transmembrane domains, and an intracellular carboxyl terminus. The homology of the ATDs of mGluRs to the leucine/isoleucine/valine-binding protein (LIVBP) and other bacterial periplasmic binding proteins that mediate the transport of amino acids in prokaryotes is fortuitous because the mGluRs appear to possess a similar three-dimensional fold and the crystal structures of the bacterial proteins are known (11).

Data obtained from experiments on chimeric constructs of the ATD of human mGluR4 with the transmembrane domains and carboxyl-terminal regions of mGluR1b (19) and constructs containing various segments of the ATD of rat mGluR2 and the...
transmembrane domain and carboxyl terminus of mGLuR1a (20) indicated that pharmacological selectivity is conferred by residues located in the ATDs of mGLuRs. More recent studies demonstrating that the ATDs of mGLuR1 (21) and mGLuR4 (22) can be expressed as soluble proteins that are secreted from transfected cells and that retain ligand-binding capabilities have corroborated the concept that the primary determinants of ligand binding to mGLuRs are contained within the ATDs. In this study, we have employed molecular modeling in conjunction with site-directed mutagenesis to probe the ligand-binding pocket of mGLuR4. Our results indicate that three conserved amino acids present in the ATDs may be key determinants of ligand binding to all members of the mGLuR family.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling**—The three-dimensional structure of the proposed ligand-binding domain of rat mGLuR4 was formulated by homology modeling using the experimentally determined structure of LIVBP from Escherichia coli and the strategy outlined by Blundell et al. (23). The atomic coordinates for the closed form of LIVBP with leucine in the binding pocket were kindly provided by Dr. F. A. Quiocho (Baylor College of Medicine). The QUANTA program (Version 97, MSI Corp.) and the SYBYL program (Version 6.4, Tripos Associates) were used to build the model that employed Gly47 to Lys199 in the ATD of mGLuR4. The sequence alignment used in the mGLuR4 model has been described previously (11). Backbone atom coordinates were assigned the corresponding residue coordinates from the crystal structure of LIVBP, and side chain atom coordinates were based on maximal side chain atom fitting to the LIVBP structure. Regions with insertions or deletions were modeled using known substructures identified by loop-searching techniques; regions 1–46, 125–149, 353–401, and 426–439, which are absent in LIVBP, were not included in the model. The L-SOP molecule was docked into the binding site of mGLuR4 in an orientation that corresponds to that observed for other mutants, the mGLuR4a cDNA in pBluescript SK I. Site-directed mutagenesis to probe the ligand-binding pocket of mGLuR4. Our results indicate that three conserved amino acids present in the ATDs may be key determinants of ligand binding to all members of the mGLuR family.

**RESULTS**

**Molecular Modeling**—The ATD of mGLuR4 extends from the amino terminus to the first putative transmembrane domain and encompasses the initial 66 kDa of the receptor protein (Fig. 1). The molecular model of the ATD of mGLuR4 retains the salient characteristics of the bacterial periplasmic binding proteins. These include two domains of similar shape connected by a hinge region made up of three interdomain crossover seg-
be indirectly involved in binding (Ser157 and Ser181). The model ligand (Arg78, Ser159, and Thr182) and at amino acids that may residues that were anticipated to interact directly with the alanine. Thus, a series of mutations were made at selected peptide backbone of the binding domain, these interactions can the exception of hydrogen bonds between the ligand and the complementary ionic interactions with charged residues. With the exception of hydrogen bonds between the ligand and the peptide backbone of the binding domain, these interactions can be disrupted by substituting the natural amino acids with alanine. Thus, a series of mutations were made at selected residues that were anticipated to interact directly with the ligand (Arg78, Ser159, and Thr182) and at amino acids that may be indirectly involved in binding (Ser157 and Ser181). The model predicted that Ser160 lies outside of the binding pocket, and therefore mutation of this residue to alanine was not likely to affect ligand binding.

Expression of Mutant Proteins—To determine whether any of the point mutations affected protein expression, immunoblots of cells transiently transfected with mGluR4a or with the R78A, S157A, S159A, S160A, S181A, or T182A mutant were probed with an antibody raised against the carboxyl terminus of mGluR4a. Labeled bands with relative molecular masses of ~96 and 100 kDa, which likely correspond to the non-glycosylated and glycosylated forms of mGluR4, respectively, were observed in samples of wild-type and c-Myc-tagged mGluR4, as indicated by the similar optical immunoreactive band at ~90 kDa; the nature of this band is not known. Nevertheless, the intensity of the monomer bands at 96 and 100 kDa was similar to that of the wild-type receptor in all mutants including R78A, demonstrating that none of the point mutations produced any substantial alterations in the level of protein expression. The similarity in the expression levels of wild-type mGluR4a and the S157A, S160A, and S181A mutants was also indicated by the similar B max values in the radioligand binding experiments (see below).

Pharmacological Analyses of Epitope-tagged and Mutant Receptors—Saturation analyses of L-[3H]AP4 binding to membranes prepared from HEK cells transfected with the wild-type mGluR4a expression plasmid showed a dissociation constant (K D) and maximum number of binding sites (B max) of 504 nM and 8.6 pmol/mg, respectively (Fig. 3A and Table I). The dissociation constant for mGluR4a expressed in HEK cells was similar to that reported previously for mGluR4a expressed in hamster kidney cells (K D = 441 nM) (25) and in insect Sf9 cells (K D = 480 nM) (30). A modified expression vector was also constructed in which a c-Myc epitope tag was inserted immediately downstream of the proposed signal peptide (Fig. 1). The insertion of the c-Myc tag at this position was done (a) to provide an extracellular antibody epitope to facilitate immunocytochemical labeling (see below) and (b) to ensure that the tag would not be cleaved by signal peptidases. c-Myc-tagged mGluR4a displayed K D and B max values of 404 nM and 8.7 pmol/mg, respectively (Fig. 3B and Table I); neither value was significantly different (p > 0.05, one-way analysis of variance and Dunnett’s multiple comparison test) from that of the untagged receptor, indicating that the insertion of the epitope at this site did not affect ligand affinity or the level of expression of mGluR4a.

The molecular model of the ATD of mGluR4 suggests that Arg78, Ser159, and Thr182 interact directly with the glutamate ligand. When mutated to alanine, all three residues produced receptors that were nearly devoid of the ability to bind l-[3H]AP4 (Fig. 4). The R78A, S159A, and T182A mutants displayed 2 ± 0.8, 5 ± 1, and 4 ± 2% (mean ± S.E. of three experiments) of control (wild-type mGluR4a) binding, respectively. Due to the very low level of binding of the radioligand, it was not possible to obtain estimates of affinities for these two mutants in saturation or competition experiments. To further probe the ligand-binding domain of mGluR4a, several additional mutations were made at amino acid residues that were predicted to be in or very near the binding pocket, but not directly involved in ligand binding. Saturation experiments showed that neither the dissociation constants nor the maximum numbers of binding sites of the S157A, S160A, and S181A mutants were significantly different from those of the wild-type receptor (p > 0.05, one-way analysis of variance and Dunnett’s multiple comparison test) (Table I).

To assess the pharmacological profile of these mutants, competition experiments were conducted using the agonists L-glutamate, L-SOP, and L-CCG-1 and the group III antagonist CPPG (31). The rank order of potency in the S157A, S160A, and S181A mutants was similar to that observed in the wild-type receptor (L-SOP > L-CCG-1 > L-glutamate > CPPG) (Fig. 5). The inhibition constants for these drugs with the S157A and S160A mutants were also similar to those seen with the wild-type receptor (Table II). However, the inhibition constants for the S181A mutant were ~3–5 times higher than those for the wild-type receptor, indicating that this mutation produced a moderate decrease in affinity for the series of compounds tested.

Immunocytochemical Analysis—Although the results from the immunoblot experiments indicated that the R78A, S159A, and T182 mutant polypeptides were translated and expressed at levels comparable to those of the wild-type receptor, it is possible that the very low level of ligand binding of the mutants was caused by misfolding and/or lack of cell-surface expression. To investigate this possibility, an immunocytochemical analysis was carried out on the c-Myc-tagged wild-type receptor, the R78A and T182A mutant receptors, and the untagged S159A
conducted at 30 nM L-[3H]AP4. Each R78A, S159A, and T182A mGluR4a mutant receptor was labeled with the anti-mGluR4a antibody (Fig. 6D) and in Triton X-100-permeabilized cells labeled with the anti-mGluR4a antibody in permeabilized cells (data not shown). In unpermeabilized cells expressing the c-Myc-tagged wild-type mGluR4a (WT) and labeled with the anti-c-Myc antibody, followed by a biotinylated anti-rabbit or anti-mouse secondary antibody and a lightly fixed HEK cells (4% paraformaldehyde for 10 min) with the anti-mGluR4a or anti-c-Myc antibody, followed by a biotinylated anti-rabbit or anti-mouse secondary antibody and a fluorescein isothiocyanate-avidin conjugate.

Table I

| mGluR4a            | $K_D$ (nM) | $B_{max}$ (pmol/mg) |
|--------------------|------------|---------------------|
| WT                 | 504 ± 99   | 8.6 ± 2.9           |
| c-Myc-WT           | 404 ± 64   | 8.7 ± 1.3           |
| S157A              | 683 ± 52   | 6.3 ± 1.0           |
| S160A              | 470 ± 72   | 5.0 ± 1.6           |
| S181A              | 570 ± 52   | 4.2 ± 1.2           |

*Wild-type.

![Fig. 3. Saturation analysis of L-[3H]AP4 binding. Membranes from HEK cells expressing wild-type mGluR4a (A) or c-Myc-tagged mGluR4a (B) were analyzed in the membrane binding assay. Each experiment was repeated three to four times. Insets, Scatchard plots of the saturation data. The $K_D$ and $B_{max}$ values are summarized in Table I.](image)

![Fig. 4. Comparison of L-[3H]AP4 binding to membranes prepared from HEK cells expressing wild-type mGluR4a and the R78A, S159A, and T182A mGluR4a mutants. L-[3H]AP4 binding was conducted at 30 nM L-[3H]AP4. Each bar represents the mean ± S.E. of five experiments. WT, wild-type mGluR4a.](image)

**Discussion**

The amino-terminal portions of the ATD of mGluRs are homologous to prokaryotic LIVBP, whereas two discontinuous segments of the ionotropic glutamate receptors are homologous to the bacterial lysine/arginine/ornithine-binding protein (11, 32, 33). Our model of the ATD of mGluR4 maintains the general structural characteristics of the bacterial periplasmic binding proteins. It consists of two lobes connected by a hinge region, which, in the open configuration, forms a cleft where the ligand can enter. After ligand binding, the cleft closes to form a binding pocket, where the ligand is sequestered from the surrounding solvent (Fig. 8). The amino acids mutated in this study were all located within a region of the ATD of mGluR4 that forms part of the amino-terminal segment of the bilobed
either Ser165 or Thr188 in the ATD of mGluR1 caused substantial reductions in the agonist-evoked stimulation of phosphatidylinositol hydrolysis and in the binding of L-[^3H]glutamate, suggesting that these amino acids may be involved in ligand recognition. Ser165 and Thr188 of mGluR4 align with Ser159 and Thr182 of mGluR1 (see Fig. 9B for a compilation of equivalent mutations in mGluR1, mGluR4, GABA<sub>A</sub> receptors, and LIVBP). Although the amino acid sequence of rat mGluR4 is only 43% identical to that of rat mGluR1 and the two receptors display different pharmacological and biochemical profiles, our results indicate that at least three conserved amino acids in the ATDs of mGluRs may be key determinants of ligand binding to all members of the mGluR family.

In the molecular model of the ATD of mGluR4, mutations at Arg<sup>78</sup>, Ser<sup>159</sup>, and Thr<sup>182</sup> were predicted to have a major impact on L-[^3H]AP4 binding, whereas mutations at Ser<sup>157</sup>, Ser<sup>160</sup>, and Ser<sup>181</sup> were predicted to have less dramatic effects on binding; our experimental results have corroborated the predictions of the molecular model of mGluR4. The substantial reductions in L-[^3H]AP4 binding in the R78A, S159A, and T182A mutants suggest that these amino acids are directly involved in ligand recognition. It is unlikely that the large decrease in binding was caused by a reduction in protein expression and/or misfolding of the mutant receptors because immunoblot and immunocytochemical analyses demonstrated that both mutants were expressed at similar levels and showed similar cell-surface expression patterns compared with the wild-type receptor.

The drastic reduction in L-[^3H]AP4 binding in the S159A mutant agrees with the loss of activity seen in the analogous mutation in mGluR1 (Ser<sup>165</sup>) (11). Our molecular model suggests that the hydroxyl group on the side chain of this serine forms a hydrogen bond with the α-carboxylic acid group on the glutamate ligand (Fig. 8). The nearly complete loss of L-[^3H]AP4 binding in the Arg<sup>78</sup> mutant indicates that this amino acid is another crucial feature of the ligand recognition motif in mGluR4. Although no equivalent mutation has been made in other mGluRs, this arginine is also conserved in all mGluRs, and it is well positioned for such an interaction. The orientation of the ligand in the binding pocket places the γ-carboxy group on the side chain of L-glutamate in close proximity to the positive charge on the side chain of Arg<sup>78</sup> (Fig. 8). We postulate that an ion pair between the γ-carboxyl group on the side chain of L-glutamate and the γ-phosphonate group on L-SOP or L-AP4 and the amino group on the side chain of Arg<sup>78</sup> is an essential component of the ligand-binding pocket of mGluRs. This suggestion is supported by the fact that this arginine is conserved in all members of the mammalian mGluR family, the salmon brain mGluR, and the Drosophila mGluR, but not in the bacterial binding proteins such as LIVBP that mediate the transport of amino acids lacking an acidic side chain.

Mutation of Thr<sup>182</sup> to alanine in mGluR4 produced a 96% decrease in L-[^3H]AP4 binding compared with the wild-type receptor. In mGluR1a, conversion of the analogous threonine (Thr<sup>186</sup>) to alanine virtually eliminated [^3H]glutamate binding (11). The threonine at position 182 of mGluR4 is conserved in 18 homologous proteins, including all eight members of the mammalian mGluR family, an mGluR1 homolog from salmon brain, an mGluR from Drosophila, the calcium-sensing receptor, the GBR2 GABA<sub>A</sub> receptor subunit, LIVBP, and the leucine-binding proteins from E. coli and Salmonella typhi-murium, and an amide-binding protein (AmiC) from Pseudomonas aeruginosa. AmiC has been subclassified with LIVBP and the leucine-binding proteins in “cluster 4” of the bacterial periplasmic binding proteins (34). As is the case with other periplasmic binding proteins, AmiC has low sequence identity to LIVBP (17%), but the overall fold of the protein appears to be "clamshell" part of the ATD. The rationale for targeting selected amino acids for mutagenesis was guided by the model of the ATD of mGluR4, which is, in turn, based on the known three-dimensional structure of LIVBP determined by x-ray diffraction studies (29).

Based on the sequence homology and structural data from crystallographic studies on the bacterial amino acid-binding proteins, O’Hara et al. (11) formulated and tested a molecular model of the ATD of mGluR1, mGluR1. Mutation of either Ser<sup>165</sup> or Thr<sup>188</sup> in the ATD of mGluR1 caused substantial reductions in the agonist-evoked stimulation of phosphatidylinositol hydrolysis and in the binding of L-[^3H]glutamate, suggesting that these amino acids may be involved in ligand recognition. Ser<sup>165</sup> and Thr<sup>188</sup> of mGluR1 align with Ser<sup>159</sup> and Thr<sup>182</sup> of mGluR4 (see Fig. 9B for a compilation of equivalent residues mutated in mGluR1, mGluR4, GABA<sub>A</sub> receptors, and LIVBP). Although the amino acid sequence of rat mGluR4 is only 43% identical to that of rat mGluR1 and the two receptors display different pharmacological and biochemical profiles, our results indicate that at least three conserved amino acids in the ATDs of mGluRs may be key determinants of ligand binding to all members of the mGluR family.

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similar to that of LIVBP and other members of this subclass of binding proteins (35). Thus, based on these experimental findings, the molecular model of mGluR4, and the high degree of amino acid conservation in the related proteins noted above, we suggest that ligand binding in mGluRs is stabilized by a hydrogen bond formed between the oxygen of the hydroxyl group on the side chain of Thr182 and the \( \alpha \)-amino group of the ligand (Fig. 8).

In light of the sequence homology between the mGluRs and the GABA\(_B\) receptors and the fact that both classes of receptors are activated by amino acids, it is conceivable that some of the determinants of ligand binding to mGluRs may extend to the GABA\(_B\) receptor. A sequence alignment of the mGluRs with the GABA\(_B\) receptor subunits shows that Ser\(^{159}\) of mGluR4 is conserved in GBR1a/b, whereas Thr\(^{182}\) of mGluR4 is conserved in the GBR2 protein; in the GBR1a and GBR1b subunits, there is a serine at this position (Fig. 9).

Galvez et al. (37) have examined several sites in the GBR1a protein using site-directed mutagenesis; the amino acids mutated included Ser\(^{246}\) and Ser\(^{259}\), which align with Ser\(^{159}\) and Thr\(^{182}\) of mGluR4. Mutation of Ser\(^{246}\) completely eliminated antagonist binding to GBR1a. Thus, this serine residue appears to be critical for ligand binding to both mGluRs and GABA\(_B\) receptors. Analogous to mGluRs, Ser\(^{246}\) of GBR1a/b may form a hydrogen bond with the \( \alpha \)-carboxyl group of the ligand. The position of the hydroxyl group on the side chain of Thr\(^{182}\) close to the side chain of Ser\(^{159}\) of mGluR4 suggests the possibility that the precise positioning of Ser\(^{159}\) might be dependent upon hydrogen bonding between the side chains of the two amino acids. The data from the competition experiments, in which mutation of Ser\(^{181}\) to alanine resulted in an \( \sim 4 \)-fold increase in the IC\(_{50}\) values for the series of drugs tested, support this idea and indicate that Ser\(^{181}\) may be indirectly involved in ligand binding through the formation of a hydrogen bond with Ser\(^{159}\).

The model of the ATD indicates that Ser\(^{160}\) is situated just outside the binding cavity and is not likely to be involved in ligand recognition, whereas Ser\(^{157}\) could be indirectly involved in ligand recognition due to hydrogen bonding to Arg\(^{97}\). In both cases, mutation to alanine produced no discernible effects on \( \sim 3\)H[AP4] binding. These results indicate that Ser\(^{160}\) is likely located outside of the ligand-binding pocket and that if a hydrogen bond between Ser\(^{157}\) and Arg\(^{97}\) does exist, it is not critical for ligand binding. Ser\(^{160}\) of mGluR4 is conserved in all other members of the mGluR family except mGluR2, which has...
The absence of L-glutamate and that the serine residues at this position (equivalent to Ser160 of mGluR4) in wild-type mGluR1, mGluR3, and mGluR5 expressed in oocytes (36). Our data indicating that the S160A mutation in mGluR4 did not affect ligand binding, the mutations affecting calcium activation in mGluR1, mGluR3, and mGluR5 did not affect the EC50 values for glutamate activation of mGluR1, mGluR3, and mGluR5 expressed in oocytes (36).

The endogenous ligand for mGluRs is generally assumed to be L-glutamate. However, other amino acids that are present in brain tissue may also act as activators of mGluRs. Although L-AP4 does not exist in the brain, L-SOP is present in microdialysates of brain tissue (38). The possibility that substances other than L-glutamate may act as endogenous ligands for mGluRs has been supported by recent findings indicating that the neuropeptide N-acetylaspartylglutamate may be a selective ligand for the mGluR3 subtype of mGluR (39). Our data indicating that mGluR4 has an ~2–3-fold higher affinity for L-SOP compared with L-glutamate suggest that L-SOP could act as an endogenous ligand for mGluR4 and other group III mGluRs. The higher affinity of L-SOP for mGluR4 compared with L-glutamate together with the relative selectivity of L-SOP for group III mGluRs suggest that this subclass of mGluRs might be preferentially activated by L-SOP over L-glutamate in vivo. Future modeling and mutagenesis studies will likely provide more detailed insight into the molecular basis of the selective activation of group III mGluRs by phosphate-containing amino acids such as L-AP4 and L-SOP.

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