Modeling of Amino-Terminal Domains of Group I Metabotropic Glutamate Receptors: Structural Motifs Affecting Ligand Selectivity

Gabriele Costantino, Antonio Macchiarulo, and Roberto Pellicciari*

Istituto di Chimica e Tecnologia del Farmaco, Università di Perugia, Via del Liceo, 1, I 06123 Perugia, Italy

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On the basis of a new sequence alignment between the amino terminal domains of mGlu1 and mGlu5 receptor subtypes and leucine/isoleucine/valine binding protein (LIVBP), three-dimensional models of the binding sites of the two group I metabotropic glutamate receptor subtypes were constructed. The 3D-models thus obtained showed a high degree of similarity. In the region of the putative binding site, identified by Ser165 and Thr188 (mGlu1) or Ser152 and Thr175 (mGlu5), the only nonconserved residue is Pro369 (mGlu1), which is substituted by Gln356 in mGlu5. Although not directly involved in ligand binding, these residues may provide a subtle difference in the steric environment of the two active sites that may account for the observed subgroup selectivity of recently reported ligands.

Introduction

In recent years there has been a growing interest in the metabotropic glutamate (mGlu) receptors family in view of their relevance on a variety of fundamental neuronal functions and their implication in a number of brain's diseases and disorders.[1]

Metabotropic glutamate receptors are characterized by a vast molecular diversity and heterogeneous localization. Indeed, at least eight different vertebrate mGlu receptor subtypes, termed mGlu1 to mGlu8, have so far been cloned and functionally expressed. On the basis of sequence homology, coupling to effector systems, and agonist pharmacology, the eight mGlu receptor subtypes have been classified into three groups. Group I includes mGlu1 and mGlu5, which share a very high sequence homology and are coupled to the phosphoinositide hydrolysis and intracellular calcium mobilization when expressed in heterologous systems. Group II, including mGlu2 and mGlu3, and group III, including mGlu4, mGlu6, mGlu7, and mGlu8, are both negatively coupled to the activity of adenylyl cyclase but are endowed with a different pharmacology.

The family of mGlu receptors constitutes an attractive target for the therapeutic control of a variety of neurological diseases and disorders associated with an abnormal glutamate neurotransmission. Selective group I antagonists and group II/III agonists have a potential role as neuroprotective agents.[2] In particular, group I receptor subtypes, namely, mGlu1 and mGlu5, have long been proposed to be involved in the propagation of the neuronal injury following glutamatergic excitotoxicity,[3] and selective group I antagonist, such as 4-CPG (**1**, Chart 1)[4] or AIDA (**2**),[5] have been demonstrated to significantly reduce neuronal death following ischemia or oxygen deprivation.[6] Several factors, however, indicate that mGlu1 and mGlu5 receptor subtypes may have different roles either in developmental processes[7] or in physiopathological states.[3] In particular, the recent discovery of the very potent, noncompetitive mGlu5 antagonists MPEP, (**3**)[8a] SIB-1757 (**4**), and SIB-1893 (**5**)[8b] has allowed to demonstrate the involvement of mGlu5 subtypes in the modulation of nociception. The availability of subtype-selective ligands is needed for clarifying the physiological roles mediated by these two receptor subtypes and, accordingly, the respective therapeutic opportunities. The discovery of mGlu1 or mGlu5 selective ligands, however, has been shown to be a difficult endeavor. So far, a few ligands, either agonists

Chart 1

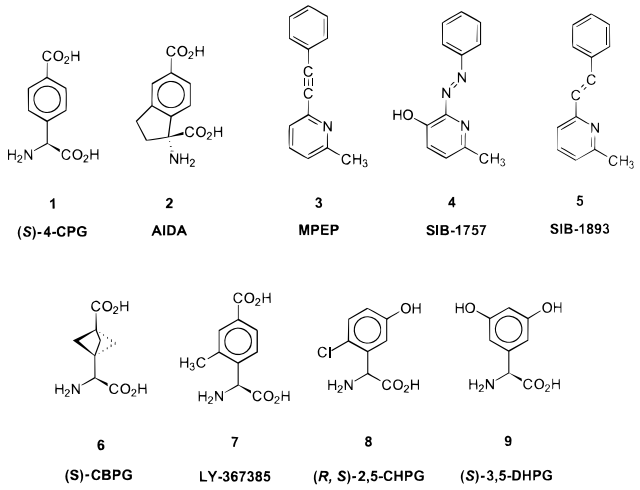

Table 1. Agonists and Antagonists Showing mGlu1/mGlu5 Selectivity

		mGlu1		mGlu5		
ligand	code	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	ref
AIDA	**2**		214		>1000	5
MPEP[a]	**3**				0.036	8a
SIB-1757[a]	**4**		>100		0.37	8b
SIB-1893[a]	**5**		>100		0.29	8b
(*S*)-CBPG	**6**		25	103		9
LY-367385	**7**		8.8		>100	10
(*R, S*)-2,5-CHPG	**8**			750		11
(*S*)-3,5-DHPG	**9**	6.6				12

[a] Noncompetitive antagonist.

* Corresponding author. Phone: ++39 075 46640. Fax: ++39 075 585 5124. E-mail: rp@unipg.it.

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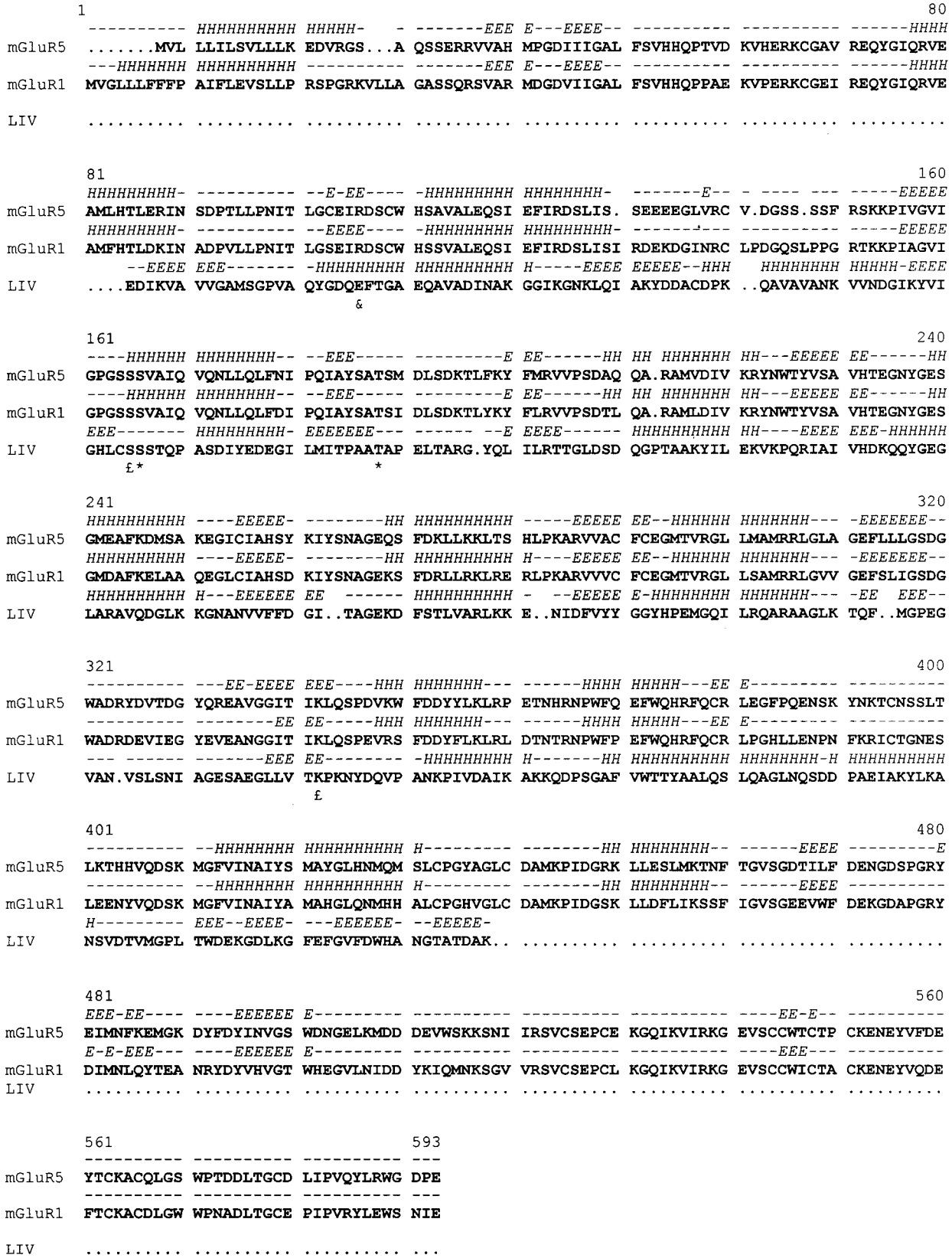

Figure 1. Multiple alignment between the amino terminal domain (ATD) of mGlu1 and mGlu5 receptor subtypes and LIVBP, resulting from MULTALIN by setting a zero gap-end penalty. The secondary structures (H: α-helix; E: β-sheet) of ATDs of group I receptors were predicted using PHDsec (Rost, B; Sander, C. Prediction of Protein Secondary Structure at Better than 70% Accuracy. *J. Mol. Biol.* **1993**, *232*, 584−599). The secondary structure of LIVBP is the experimentally determined one. Residues involved in ligand binding, according to mutagenesis experiments, are indicated by an asterisk. Residues involved in agonist binding are marked with "&". Residues involved in antagonist binding are marked with "£".

or antagonists, display significant selectivity between mGlu1 and mGlu5 (Table 1). In 1995, we reported AIDA (**2**)[5] as a selective mGlu1 antagonist with no activity at mGlu5, and in 1996 S-CBPG (**6**),[9] as a competitive

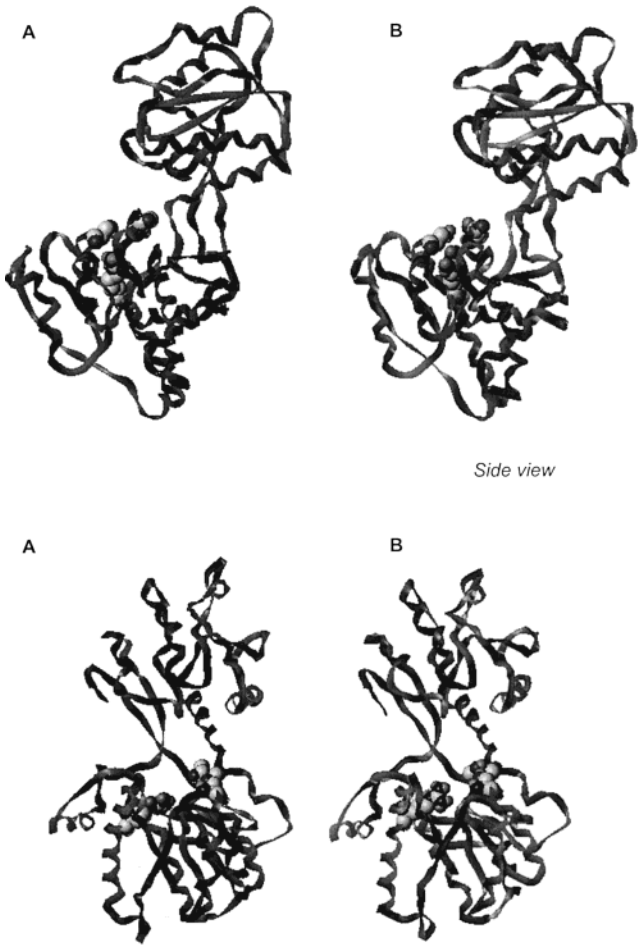

Figure 2. Top: Side views of the 3D-models of the amino terminal domain of mGlu1 (A) and mGlu5 (B); Bottom: Front views of the 3D-models of the amino terminal domain of mGlu1 (A) and mGlu5 (B). Residues involved in glutamate binding are showed as Van der Waals spheres.

mGlu1 antagonist with partial agonist character at mGlu5. More recently, LY-367385 (**7**) was reported as a potent and selective mGlu1 antagonist.[10] As far as agonists are concerned, 2-chloro-5-hydroxyphenylglycine (**8**) is a selective mGlu5 agonist,[11] while 3,5-dihydroxyphenylglycine (**9**) is a selective mGlu1 agonist.[12]

The apparent difficulty to design and synthesize mGlu1/mGlu5 selective ligands can be associated to the characteristic of these two receptor subtypes that share a very high percentage of sequence homology. It can be, therefore, be expected that only subtle differences in the overall steric environments of the binding sites are responsible for the selectivity. Central to the understanding of such differences, which may have a valuable impact in the design of new, more selective ligands, is the possibility to construct heuristic receptor models by taking advantage of the increasing knowledge on structural details of the mGlu receptor neurotransmitter binding sites coupled with the potentiality of the homology modeling techniques. This may have a valuable impact in suggesting site-directed mutagenesis experiments or in the design of new ligands. We have, therefore, initiated a project aimed at deriving homology models of group I receptors. In 1996, we reported an homology model of the amino terminal domain (ATD) of mGluR1 based on sequence alignment with members of family of periplasmic binding proteins (vide infra).[13]

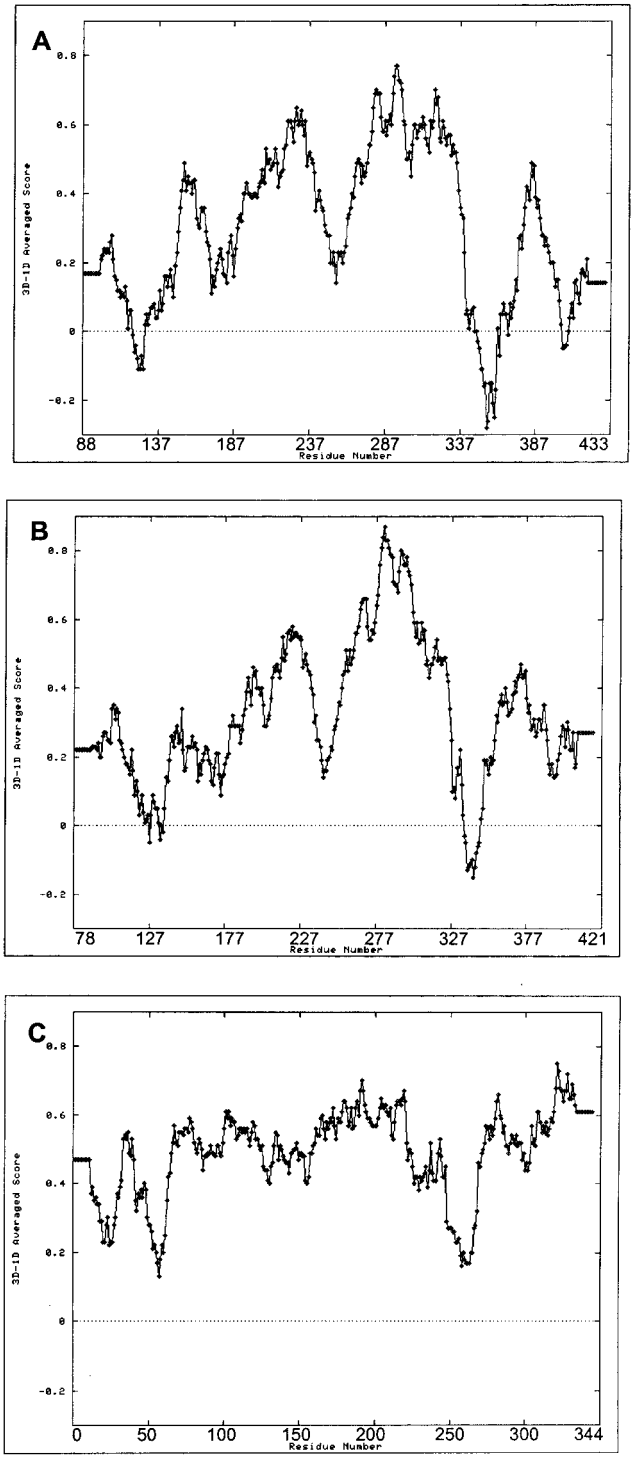

Figure 3. Verify 3D plots of the amino terminal domain models of mGlu1 (A), mGlu5 (B), and LIVBP (C). Although negative score regions are present (120−127, 349−364, 403−408, mGlu1 numbering, and 134−136, 336−346, mGlu5 numbering), they are localized on the surface of both models and far away from the proposed binding sites. Thus, they should not affect the validity of the structural motifs affecting ligand selectivity in the amino terminal domain models of mGlu1 and mGlu5.

In that work, a cluster of amino acids responsible for agonist recognition as well as a mechanistic model for antagonist functioning were also proposed. This work describes the inclusion of mGlu5 in the sequence alignment and the use of an alternative alignment procedure

```
          1                                                                                                  80
          ---HHHHHHHH HHHHHHHHHH ---------- -------EEE E---EEEE-- ---------- ---------- ------HHHH
mGluR1    MVGLLLFFFP AIFLEVSLLP RSPGRKVLLA GASSQRSVAR MDGDVIIGAL FSVHHQPPAE KVPERKCGEI REQYGIQRVE
          ---------- ---------- ---------- ---------- ----EEEEEE E--------- ---------- ---HHHHHHH
LIV       .......... .......... .......... .......... ..EDIKVAVV GAMS...... .......GPV A.QYGDQEFT

          81                                                                                                160
          HHHHHHHHHH- ---------- --EEEE---- -HHHHHHHHH HHHHHHHHH- ---------- ---------- -----EEEEE
mGluR1    AMFHTLDKIN ADPVLLP.NI TLGSEIRDSC .WHSSVALEQ SIEFIRDSLI SIRDEKDGIN RCLPDGQSLP PGRTKKPIAG
          HHHHHHHHHH HHH-----EE EEEEEE-E-- HHHHHHHHHH HHHHHH---- ---------- ---------- --------EE
LIV       GAEQAVADIN AKGGIKGNKL QIAKYD.DAC DPKQAVAVAN KVVNDG.... .......... .......... .......IKY

          161                                                                                               240
          ----HHHHHH HHHHHHHH-- --EEE----- ---------E EE------HH HH HHHHHHH HH---EEEEE EE------HH
mGluR1    VIGPGSSSVA IQVQNLLQLF DIPQIAYSAT SIDLSDKTLY KYFLRVVPSD TLQARAMLDI VKRYNW.TYV SAVHTEGNYG
          EEEEE----- --HHHHHHHH H-EEEEEEE- ---------- -EEEEE---- --HHHHHHH HHHH----EE EEEEE-HHHH
LIV       VIGHLCSSST QPASDIYEDE GILMITPAAT APELTARG.Y QLILRTTGLD SDQGPTAAKY ILEKVKPQRI AIVHDKQQYG

          241                                                                                               320
          HHHHHHHHHH ----EEEEE- --------HH HHHHHHHHHH H----EEEEE EE--HHHHHH HHHHHHH--- -EEEEEEE--
mGluR1    ESGMDAFKEL AAQEGLCIAH SDKIYSNAGE KSFDRLLRKL RERLPKARVV V.CFCEGMTV RGLLSAMRRL GVVGEFSLIG
          HHHHHHHHHH HHH---EEEE EEEE------ --HHHHHHH HH------EE EEEE-HHHHH HHHHHHHHHH ------EEEE
LIV       EGLARAVQDG LKKGNANVVF FDGI..TAGE KDFSTLVARL KKE...NIDF VYYGGYHPEM GQILRQARAA GL..KTQFMG

          321                                                                                               400
          ---------- --------EE EE-----HHH HHHHHHH--- ------HHHH HHHHH---EE E--------- ----------
mGluR1    SDGWADRDEV IEGYEVEANG GITIKLQSPE ....VRSFDD YFLKLRLDTN TRNPWFPEFW QHRFQCRLPG HLLENPNFKR
          E--------- ---------- EEEEE----- ---HHHHHHH HHHH------ ---------- ---------- ----------
LIV       PEGVAN.VSL SNIAGESAEG LLVTKPKNYD QVPANKPIVD AIKAKK.... .......... .......... ..........

          401                                                                                               480
          ---------- --HHHHHHHH HHHHHHHHHH H--------- --------HH HHHHHHHH-- ------EEEE ----------
mGluR1    ICTGNESLEE NYVQDSKMGF VINAIYAMAH GLQN.MHHAL CPGHVGLCDA MKPIDGSKLL DFLIKSSFIG VSGEEVWFDE
          ---------- -------HHH HHHH-HHHHH HHHHHHHHH- ---------- ----HHHHHH HHHHHH---- ---- -EEE-
LIV       .......... ...QDPSGAF VWTT.YAALQ SLQAGLNQS. .......... ...DDPAEIA KYLKANSVDT VMGP.LTWDE

          481                                                                                               560
          E-E-EEE--- ----EEEEEE E--------- ---------- ---------- ---------- ----EEE--- ----------
mGluR1    KGDAPGRYDI MNLQYTEANR YDYVHVGTWH EGVLNIDDYK IQMNKSGVVR SVCSEPCLKG QIKVIRKGEV SCCWICTACK
          -EEEE----- EEEEEE---- EEEEE----- ---------- ---------- ---------- ---------- ----------
LIV       KGDLKG.FEF GVFDWH.ANG TATDAK.... .......... .......... .......... .......... ..........

          593
          ---------- ---------- ---------- ---------- -
mGluR1    ENEYVQDEFT CKACDLGWWP NADLTGCEPI PVRYLEWSNI E
          ---------- ---------- ---------- ---------- -
LIV       .......... .......... .......... .......... .
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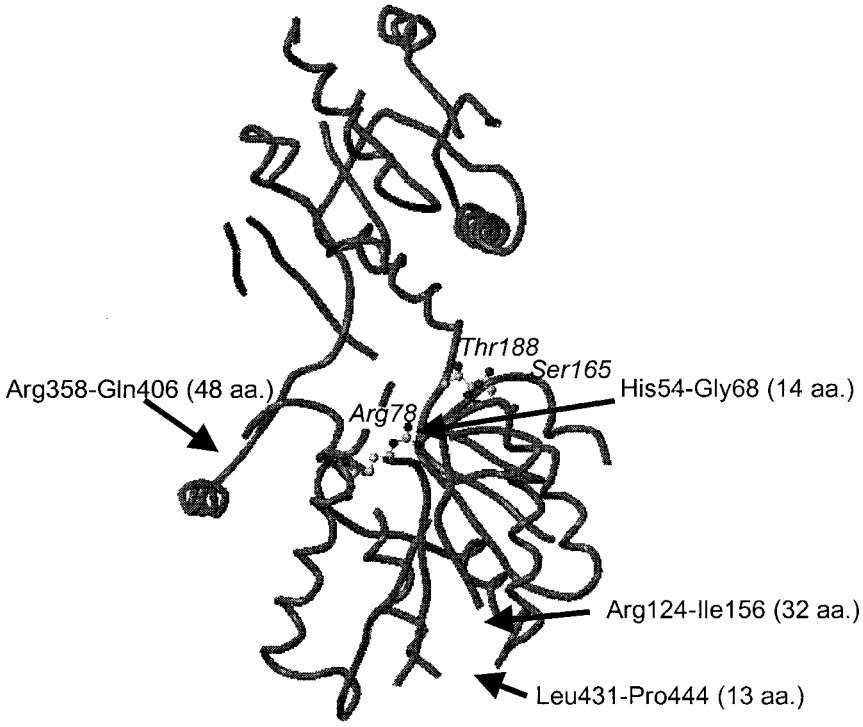


Figure 4. Original alignment proposed by O'Hara et al.14 (top). 3D-translation of the above alignment. Arg78 occupies the same region of Arg106, but the presence of large insertion regions may affect the global folding pattern of the protein (bottom).

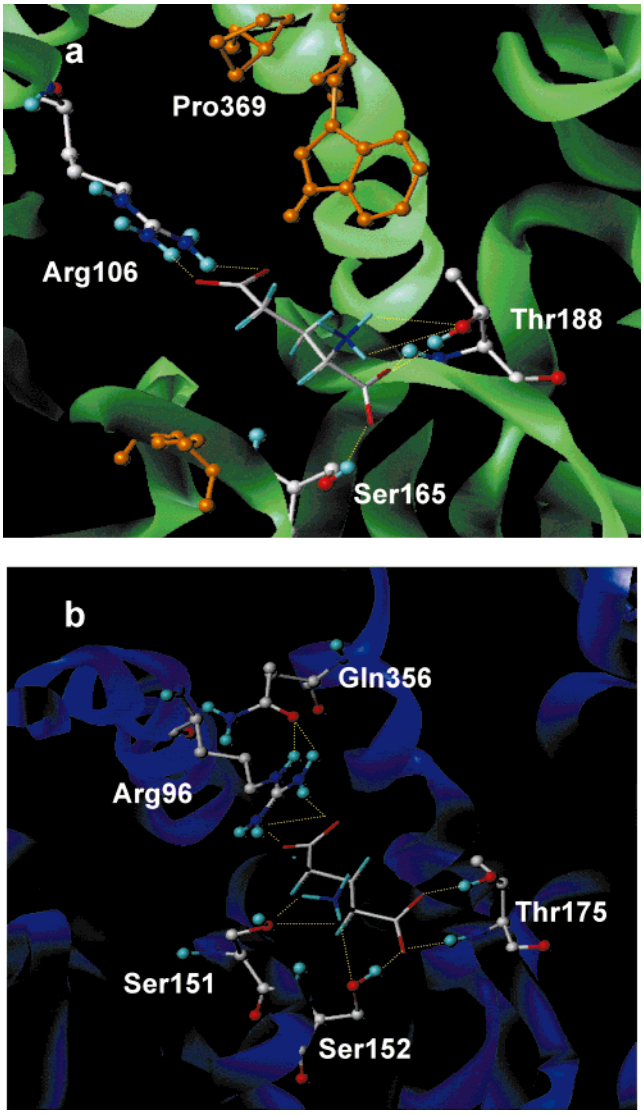


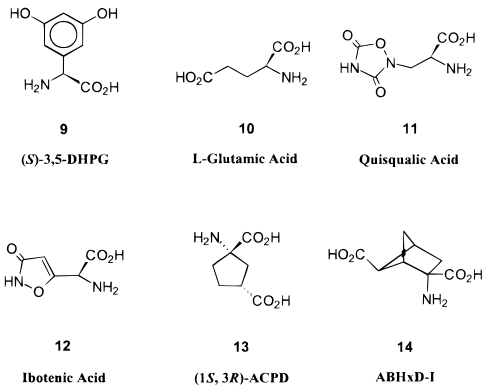
Figure 5. L-Glutamic acid (**9**) docked into binding site of mGlu1 (A) and mGlu5 (B). Hydrogen bonds are showed with yellow dashed lines.

that allowed us to revise the old mGlu1 model and to construct three-dimensional frameworks of the active site regions of mGlu1 and mGlu5 upon which ligand selectivity can be discussed. Specifically, the perturbation induced by mGlu1/mGlu5 agonist and responsible for receptor closure will be discussed on qualitative grounds. The results are reported herein.

Structural Properties of Metabotropic Glutamate Receptors. Since their first cloning and functional expression, mGlu receptors have been recognized as bearing a number of peculiar structural features among the G-protein coupled receptor (GPCR) families. Indeed, it is now generally accepted that mGlu receptors constitute, together the Ca^{2+}-sensing receptor, a putative pheromone receptor and $GABA_B$ receptors a distinct family (type C family) of GPCRs. Distinctive features of type C family are: (i) an unusually large extracellular ATD, (ii) no homology with other GPCR families at the level of the transmembrane regions, and (iii) the coupling with G proteins localized at the level of the second and not the third intracellular loop.

Particularly intriguing is the role played by the long (>500 amino acids) ATD. In 1993, O'Hara et al.[14] found

Chart 2



a low but significant sequence homology between the ATD of mGlu1 and the Leucine (LBP) and Leu/Iso/Val (LIVBP) binding proteins,[15] members of the bacterial periplasmic binding protein (PBP) family, a well-known class of proteins involved in the transport into bacterial cells of nutrients such as amino acids, metals, and sugars. On the basis of the sequence homology, a conceptual three-dimensional model of amino terminal domain of mGlu1 receptor was proposed. Like periplasmic bacterial proteins, the amino terminal domain of mGlu1 is thought to fold into two lobes, constituted by α-helices alternated with *β*-sheets, connected by an hinge region. Two amino acids, involved in the binding of leucine, isoleucine, or valine in LBP and LIVBP are conserved in mGlu1 as Ser165 and Thr188, thus suggesting that they can be involved in glutamate binding. Site-directed mutagenesis confirmed this hypothesis,[14] thus supporting the notion that the neurotransmitter binding site is located in the amino-terminal domain. In subsequent years, a number of elegant experiments, including the construction of chimeric receptors,[16] the production of monoclonal antibodies against the mGlu1's ATD,[17] and very recently the expression of a soluble form of mGlu1's ATD, which keeps the binding profile of the native receptors,[18] have brought substantial evidence for an extracellular localization of the agonist binding site. The active role of the ATD in ligand binding is not confined to mGlu receptors but, rather, is a peculiar characteristic of type C GPCR family shared for example by the $GABA_B$ receptors[19] and also kept by the ionotropic family of glutamate receptors.[20] In particular, based on the mechanism of action of LBPs, a Venus-flytrap mechanism[21] of signal transduction has been proposed to be operative for all the above receptor families.[19] This mechanism implies the equilibrium between an open (functionally inactive) and a closed (functionally active) form of the ATD. The neurotransmitter is recognized by the open form of the receptor and the shift of the equilibrium toward the closed form gives rise to the transduction of the signal.

Methods

The primary sequence of LIVBP, mGlu1's ATD, and mGlu5's ATD were aligned by hierarchical clustering analysis. MULTALIN algorithm was used.[22] Briefly, this multiple alignment algorithm performs a hierarchical clustering of the sequences using the matrix of the pairwise alignment scores. Then, the closest sequences are aligned creating groups of aligned sequences. Close groups are aligned until all sequences are aligned giving a score for the multiple alignment. A new

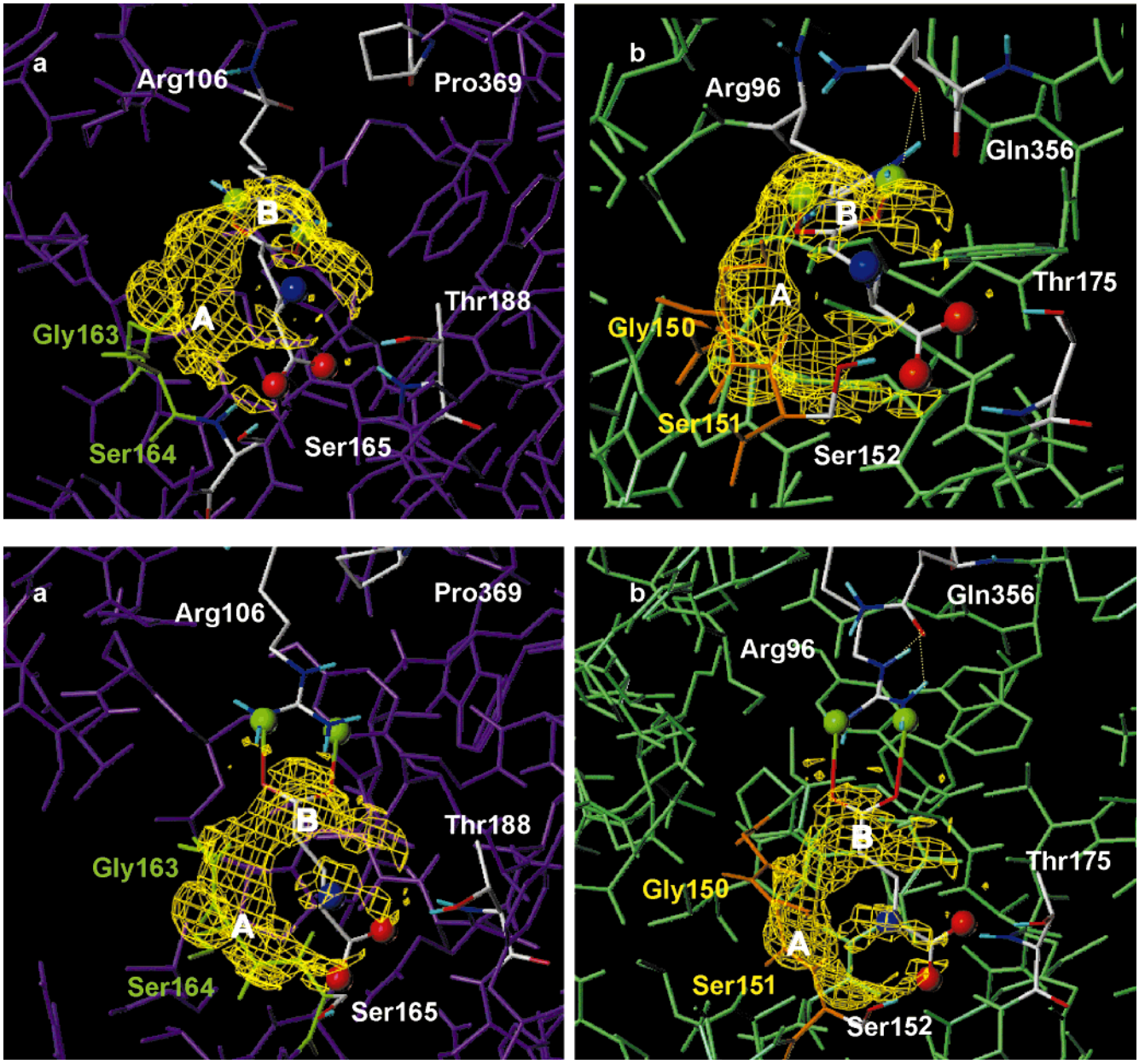


Figure 6. Group I pharmacophores inserted into the binding sites of mGlu1 (A) and mGlu5 (B). Top: Backside view. Bottom: Upper side view. Residues overlapping region A are labeled in green and orange, respectively, for mGlu1 and mGlu5 (see text for details).

hierarchical clustering is performed with this new score. Finally, if the new clustering is different from the old one, new multiple alignments are performed until the clustering of the sequences is unchanged. The matrix used for the pairwise alignment score was BLOSUM62.[23] Moreover, a zero-value was set for end-gap penalty. In this way, the presence of large gaps in the core of the alignment was avoided. The translation of such gap-regions into 3D-models usually results in the insertion of large loops in the space of a few angstroms. These loops destroy the model during minimization processes because of their large bad van der Waals contacts. The resulting alignment was checked with AMAS[24] and the regions with average high scores (>7) were chosen as structural conserved regions (SCRs). The initial 3D-coordinate generation was accomplished by the COMPOSER module of Sybyl, by using LIVBP (PDB code: 2LIV) as template. SCRs were manually defined as described above. This operation was conducted in such a way to avoid the presence of short gaps in regions with high scores. The variable regions of the resulting models were locally minimized with 1000 iterations of conjugate gradient ignoring electrostatic contributions. The whole protein models were then minimized using the conjugate gradient method with the united atom scheme of AMBER-UNI force field (Tripos implementation) until a convergence gradient of 1.0 kcal/mol·A^2 was reached. Atomic charges were retrieved from AMBER-UNI dictionary and a distance-dependent dielectric function was used throughout the minimization processes. A cut off of 8 Å was used for nonbonding interactions. The resulting folding of both models was checked with Verify 3D[25] while their geometry was checked using PROCHECK.[26] Amino acids with distorted geometry were manually optimized following this operation by minimization procedures of the whole protein as described above. The operation of fixing these amino acids did not change the overall folding pattern of the proteins.

Docking of mGlu1 and mGlu5 agonists and antagonists was manually performed exploring different possible spatial dispositions of the compounds inside the active site with continuous energy monitoring by using the DOCK module of Sybyl. All the dispositions of the compounds were studied by anchoring their amino acidic groups near the residues indicated by mutagenesis experiments as involved in the binding of the α-amino acidic group of ligands (i.e., Ser165 and Thr188 for

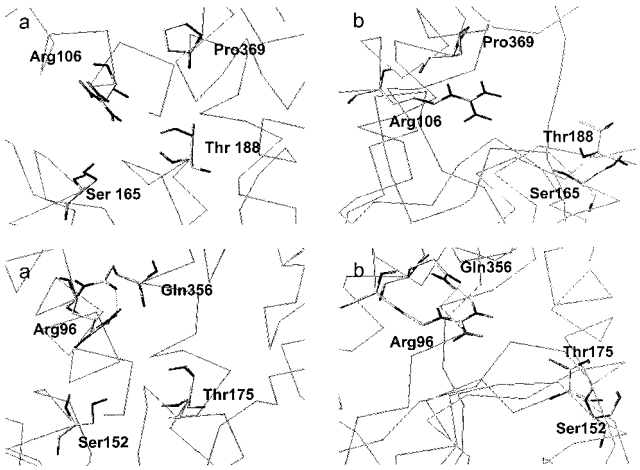


Figure 7. Two different views of the different orientations of the arginine residue involved in glutamate binding in mGlu1 (Top, Arg106), and mGlu5 (Bottom, Arg96).

mGlu1, and Ser152 and Thr175 by similarity for mGlu5).[14] Among the possible dispositions, the one with lower energy was stored and minimized (Tripos force field, after having added all the hydrogens) only in an interesting region defined in a sphere of 6 Å around the ligand position by using the "minimize subset" option of Sybyl. Since in this study we were interested in possible orientation of ligands inside the active sites rather than in accurate estimation of the energetic of binding, explicit desolvation effects were not taken into account.The conjugate gradient method was used for minimization of docked compounds and a gradient of 0.5 kcal/mol·A^2 was chosen as convergence criteria. Atomic charges were calculated using Gasteiger−Huckel dictionary. A distance-dependent dielectric function was used for these minimization procedures.

Electrostatic potential surface analysis was performed by measuring the electrostatic potential between a probe charge (+1) and the surface of the protein and by displaying the potential over the surface by color code. A physiological pH was assumed where the acidic and basic side chains of amino acids localized on the surface of the proteins are in their ionized states.

Conformational searches were performed by using GRID search module of Sybyl by setting 1000 interactions of Conjugate gradient and a dielectric constant of 80. The torsional bonds under study were selected as the Cα-Car1. The range of the analysis was 0−355° with a torsional increment of 5°. For the conformational study of the AIDA (**2**), the two possible cyclopentane conformation were analyzed. All the minima conformations were further geometry optimized by using the Tripos force field and the Conjugate gradient method. The minimization was performed until a gradient of 0.05 kcal/mol·A^2 was reached. Atomic charges were retrieved from Gasteiger−Huckel dictionary. A dielectric constant of 80 was used for these minimization procedures.

All computations were carried out on a SGI O2 R5500 workstation using Sybyl 6.3 molecular modeling software package.

Results

Three-Dimensional Models of mGlu1 and mGlu5. On the basis of the sequence homology with PBPs (Figure 1), the 3D-models of ATD of mGlu1 and mGlu5 were obtained (Figure 2). Thus, the mGlu1 and mGlu5's ATD are characterized by an ellipsoid shape, with two lobes constituted by α-helices alternated with *β*-sheets connected by a hinge region.

The amino acids directly involved in glutamate binding, namely, Ser 165 (Ser152 in mGlu5) and Thr188 (Thr175 in mGlu5), are localized in the carboxy terminus of ATDs, which keeps the characteristics of LIVBP's binding lobe[15b] and are disposed on the surface of one lobe (binding lobe) directly faced toward the cleft. The goodness of the obtained folding was evaluated through Verify 3D (Figure 3) and the geometry was checked with ProCheck.

Because of the new alignment strategy that we have used (see Method section), the three-dimensional model of mGlu1's ATD is different from that previously reported by O'Hara et al.[14] and that reported by us.[13] In particular, Arg 335, which was situated in the hinge region in the old model and proposed to be involved in agonist binding, is now localized on the surface of the binding lobe and cannot participate in ligand binding. However, as discussed below, the general validity of the old model is confirmed, either in terms of proposed binding mode of agonists or in term of hypothesized molecular determinants for signal transduction.

Agonist Binding Sites of mGlu1 and mGlu5. Comparison with Ligand-Based Pharmacophore Models. Site-directed mutagenesis experiments have identified Ser165 and Thr188 as directly involved in L-Glu binding in mGlu1 subtypes.[14] These two amino acids align with Ser79 and Thr102 in LIVBP, two residues that crystallographic studies have demonstrated to be involved in the binding of the α-amino acidic moiety of LIVBP substrates.[15a] In the homology model of mGlu1, Ser165 and Thr188 are located in the carboxy-terminal lobe of ATD and are exposed to the cleft. Conserved residues in mGlu5 (Ser 152 and Thr 175) show the same localization. The major difference between substrates of LIVBP and mGlu receptor agonists is the presence, in the latter molecules, of a distal acidic function, that is indispensable for activity. The distal acidic function must have a suitable counterpart in the binding site. Arg106, conserved as Arg96 in mGlu5, may serve at this aim. Indeed, Arg106 (Arg96) is localized in the same region where the hydrophobic residues in LIVBP make up the substrate's side chain specificity pocket. It should be mentioned that the 3D translation of the original alignment proposed by O'Hara is characterized by the presence of Arg78 in the same spatial position of Arg106 in our model. Arg78 is fully conserved in all the mGlu receptor family and may, therefore, be considered as having a potential functional role. The construction of the 3D model based on that alignment, however, gives rise to the insertion of large loops in the core of the C-terminal lobe that can hardly be modeled (Figure 4). Whereas the mechanism of ligand recognition that we propose below is not affected by the replacement of Arg78 with Arg106, it should be reminded that Arg78, in view of its highly conserved nature, may be as well a suitable target for site-directed mutagenesis experiments.

Docking experiments of L-Glu (**10**) into mGlu1 and mGlu5 binding sites supports the involvement of the arginine residues in agonist recognition (Figure 5).

Indeed, the α-amino acidic moiety of L-Glu (**10**) binds, through hydrogen bonds, Ser165 (Ser152) and Thr188, (Thr175) while its distal carboxylate interacts with Arg106 (Arg96) through salt bridge formation. There is no significant difference in the binding mode between mGlu1 and mGlu5, i.e., L-Glu (**10**) adopts an identical conformation at both receptor subtypes. In a conceptu-

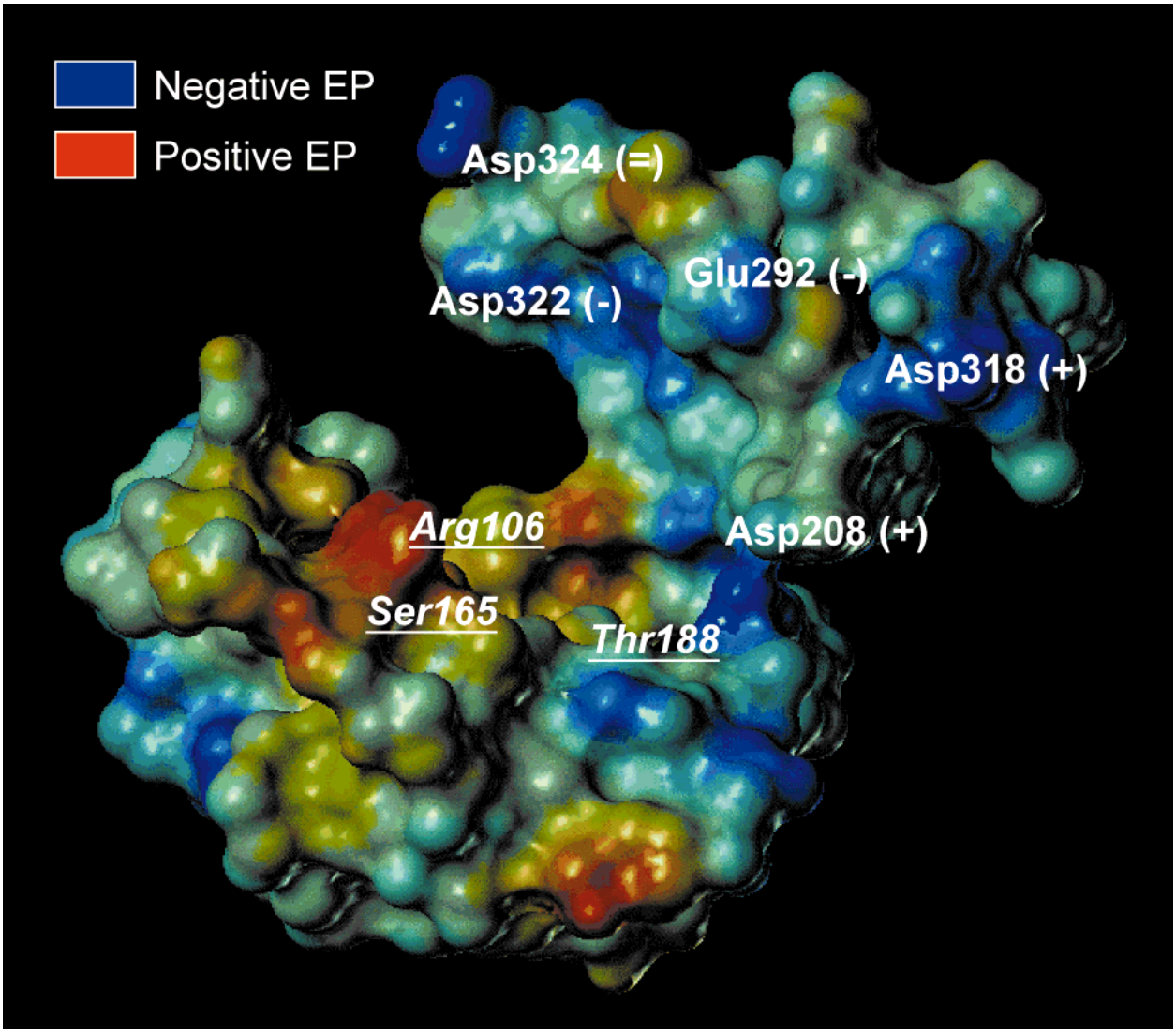


Figure 8. Electrostatic potential analysis on the mGlu1 surface of binding cavity. A (+) indicates acidic residues conserved in group I receptors and LIVBP; (−) indicates acidic residues conserved in group I receptors; (=) indicates acidic residues presents only in mGluR1. Key residues involved in glutamate binding are also indicated.

ally different approach, we have previously derived a ligand-based group I pharmacophore,[27] highlighting conformational and steric features responsible for agonist activity. Briefly, the structures used for that study were the most potent group I agonists, i.e., quisqualic acid (**11**, Chart 2), ibotenic acid (**12**), (1*S*, 3*R*)-ACPD (**13**), 3,5-DHPG (**9**), and ABHxD-1 (**14**).

The obtained pharmacophore models were featured by two donor sites along the *syn* lone-pairs of distal carboxy group, a positive charge on the amino group and two negative charges on the carboxylate group of amino acidic moiety, disposed in a nearly all-extended conformation. Moreover, excluded volume studies have indicated the presence of two forbidden regions in agreement with those proposed by Kozikowski et al.[28] The first one, called region A, is localized near the agonist amino acidic moiety, the second one, called region B, is localized on the upper face of ABHxD-I (**14**). The direct comparison of the pharmacophore model, obtained through a ligand-based approach, and the binding site obtained through a homology modeling approach should disclose the inherent robustness of the two models. Thus, the group I pharmacophore model was inserted into the binding sites of both mGlu1 and mGlu5 (Figure 6) and was shown to fit well the proposed key residues of binding sites, (Ser165, Thr188, Arg106 for mGlu1, and Ser152, Thr175, Arg96 for mGlu5) i.e., those indicated by experimental mutagenesis and our docking experiments as directly involved in the agonist recognition process. Moreover, when projected into the active site of both mGlu1 and mGlu5, region A clearly overlaps with the van der Waals radii of receptors residues, while region B does not intersect the active site residues and is localized in the cleft. This observation suggests that the open-bound state of the receptors is insufficient to explain steric requirements for agonist selectivity.

Both homology and pharmacophore modeling were thus unable to disclose structural features that may allow to discriminate between mGlu1 and mGlu5. A closer inspection of the active sites of mGlu1 and mGlu5, as obtained by homology modeling, points out an extremely high degree of amino acid conservation. More in detail, if a sphere of radius 10 Å centered on the active site residues Ser165 (Ser152) and Thr188 (Thr175) is considered, only one nonconserved residue is found,

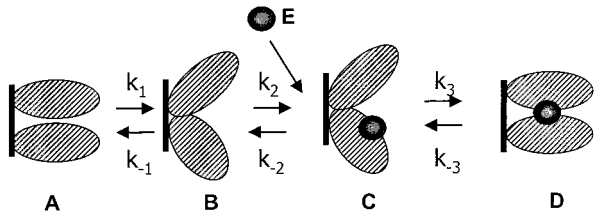


Figure 9. Functional equilibrium state of ATDs of metabotropic glutamate receptors. A: Closed form. B: Open form. C: Open ligand bound form. D: Close ligand bound form. E: Agonists.

namely a glutamine (Gln356) in mGlu5 instead of a proline (Pro 369) in mGlu1.

This lack of conservative substitution may, however, be relevant for achieving subtype selectivity. In particular, Gln356 interacts with the key residue Arg96 in mGlu5 while Pro369 does not with Arg106 in mGlu1, and this causes a different orientation of the Arg side chain in the two subtypes (Figure 7). The effects on receptor selectivity of this mutation will be discussed below.

Molecular Determinants for Domain Closure in Group I mGlu Receptor Subtypes. The central assumption underlined by the homology of the ATD of mGlu receptors with PBPs is that the transductional event is initiated by a collapse of the two lobes upon agonist binding. A electrostatic potential analysis allowed us to speculate on the driving force responsible for the domain closure (Figure 8).

A high-negative electrostatic potential surface exists on the complementary lobe facing the other one in which is localized the agonist binding site. In particular, a cluster of acidic residues (mGlu1: Asp208, Glu292, Asp318, Asp322, Asp324), among which Asp208 and Asp318 are conserved in mGlu5 and LIVBP, contribute to the negative potential surface. On the contrary, a region of positive electrostatic potential is localized on the binding lobe upon agonist binding. Thus, electrostatic complementarity between the two lobes must be seen as the prevalent driving force for domain closure. We can imagine a dynamic situation where the two lobes are shielded by water molecules, localized into the cleft, so that the open form is the more stable one. Binding of glutamate or an agonist transiently destroys the water shell. The positively charged amino group of agonists will serve as molecular probes that recognizes the negatively charged complementary lobe and breaks down the electrostatic equilibrium of the two lobes leading to their collapse and to the formation of closed, agonist-bound, form of the ATD. This hypothesis is in agreement with the functional mechanism of bacterial periplasmic proteins.[29] Indeed, PBPs exist in an equilibrium between four forms: a closed empty state, an open empty state, a open substrate-bound state, a closed substrate-bound state. We can assume a similar equilibrium to be operative in the case of metabotropic glutamate receptors, i.e., an equilibrium between a closed empty form of the receptor, an open unbound form, an open agonist-bound form and, finally, the closed agonist bound form that is responsible of the signal transduction (Figure 9).

Thus, agonists first bind the receptor in its unbound open form, then the ATD reorganizes into the more stable closed ligated form of the receptor that activates the transduction process, possibly by interacting with extracellular loop(s). In agreement with the above observations, agonist selectivity should be explained not only through an open agonist-bound model of the receptor, but also it would be necessary a closed model of the receptor. The availability of the open form only of LIVBP as template for homology modeling has so far prevented us to explicitly consider the closed form, and this is a limitation in the applicability of 3D-model in discussing all the aspects relative to agonist binding and selectivity. Nevertheless, we are currently developing a model for the closed form of ATD of mGluR1, based on structural similarities with sequence-unrelated proteins.[32] The result will be communicated in the due course.

Antagonist Binding Sites of mGlu1 and mGlu5. We have previously proposed that group I antagonists, and carboxyphenylglycines (**1**, **2**, **6**, **7**, Chart 1) in particular, are characterized by a peculiar mode of binding that involves the simultaneous interaction with both the binding and the complementary lobes of ATD.[13] This hypothesis is herewith confirmed and is in strict agreement with the proposed mechanism of domain closure gathered by agonists. Indeed, mGlu1 and mGlu5 antagonists interact with Ser165 (Ser152) and Thr188 (Thr175) through the α-amino acidic moiety and with Lys341 (Lys328), localized into the complementary lobe, through the distal carboxylate. Because of the model

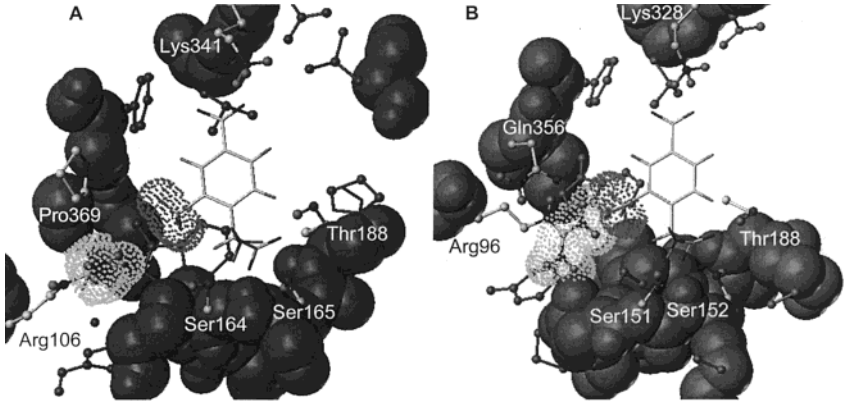


Figure 10. Docking of LY-367385 (**7**) into mGlu1 (A) and mGlu5 (B) binding sites. Dot surfaces show the van der Waals contacts between the ligand's methyl moiety and the key residues Arg106 and Arg96.

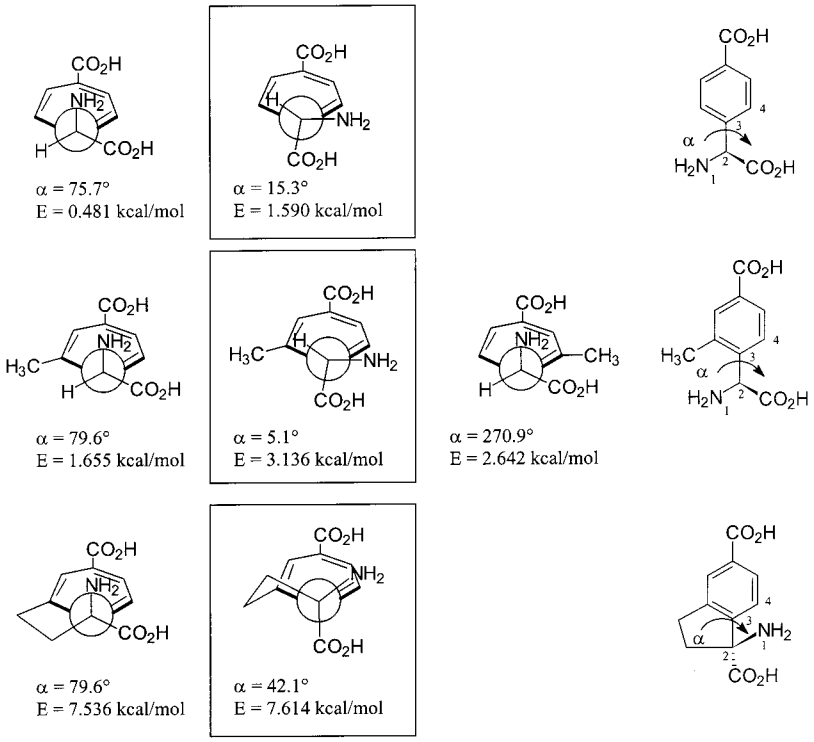


Figure 11. Conformational analysis of 4-CPG (**1**), AIDA (**2**), and LY-367385 (**7**). The proposed bioactive conformation are highlighted.

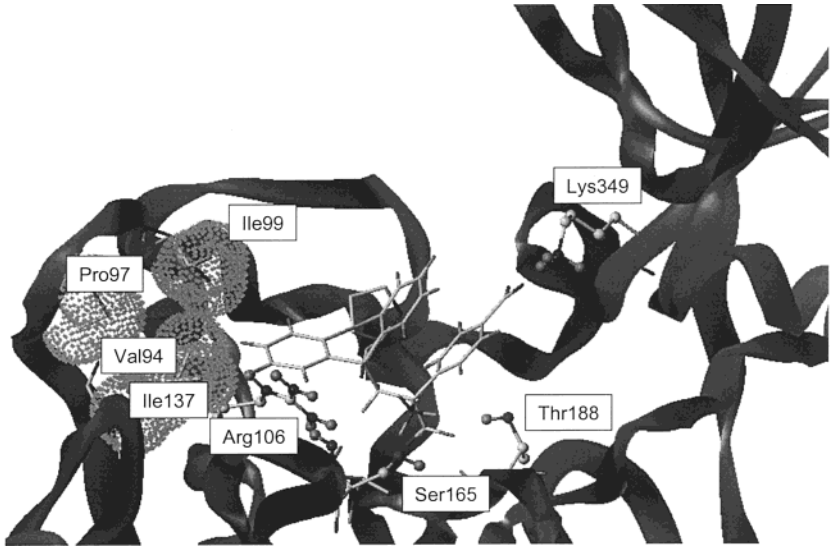


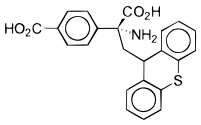
Figure 12. Docking of LY-367366 (**14**) into mGlu1 binding site. Dot surfaces show the hydrophobic pocket defined by Val94, Pro97, Ile99, and Ile137 (mGlu 1 numbering).

resolution and the uncertainty associated with the flexibility of the lysine side-chain, the involvment of Lys341 (Lys328) remains at a speculative level, but this result is in agreement with the collapse hypothesis of the transductional event. In fact, while agonist binding promotes conformational changes leading to domain closure through long-range Coulombic force between the positively charged amino group and the negative potential surface of the complementary lobe, antagonist binding freezes the ATD in an open form keeping the two lobes separated each from the other and shifting the equilibrium state toward an open, antagonist-bound form.

Docking studies on selective antagonists for individual group I subtypes turn out interesting results on structural motifs in group I ATDs, which may affect ligand selectivity.

LY-367385 (**7**) is a selective mGlu1 antagonist with no activity at mGlu5.[30] Docking of **7** into both ATD models may allow us to explain the mGlu1 selectivity. A key role could be played by the glutamine (Gln356) residue in mGlu5, which is not conserved in mGlu1 where it is substituted by a proline (Pro369). The substitution P → Q may produce significant alteration in the 3D-environment of the active sites. In particular, it may be proposed that Gln356 interacts with Arg96

Chart 3



15

LY-367366

in mGlu5 leading to a closer spatial grouping between Ser152, Thr175, and Arg96 than in mGlu1 and resulting in a forbidden steric overlap between the 2-methyl group of LY-367385 and Arg96, which is not present in mGlu1 (Figure 10). The same conclusions may be drawn to explain the mGlu1 selectivity of AIDA (**2**).

An alternative hypothesis that may explain the different activity at mGlu1 or mGlu5 of 4-CPG (**1**), AIDA (**2**), or LY-367385 (**7**) takes into account the possible different conformational profiles of the above derivatives. To test this possibility, a conformational analysis was carried out around the Cα-Car1 bond of carboxyphenylglycines. As reported in Figure 11, there is no significant differences in the conformational profile of carboxyphenylglycines that may account for the observed selectivity. Indeed, 4-CPG (**1**) and AIDA (**2**) are endowed with two low energy conformations (characterized by torsional angles $\alpha \approx 80°$ and $\alpha \approx 15°$, respectively), whereas LY-367385 (**7**) is endowed with three low energy conformation ($\alpha \approx 270°$). Also, in agreement with our docking studies, the conformation with the amino group staggered with the phenyl ring should be the active one, and this is owned by all the three derivatives. Thus, conformational properties cannot be seen as the only parameters responsible for subtype selectivity. It should be noted that the different torsional angle value of Cα-Car1 bond found between LY-367385 (**7**) and AIDA (**2**) (about 37° between their bioactive minima conformations) could explain the lack of potency of the latter.

LY-367366 (**15**, Chart 3)[31] is a very potent group I antagonist with no selectivity between mGlu1 and mGlu5. Docking of **15** into the active sites of both subtypes shows how this ligand adopts the same disposition as other "classical" CPGs. It is worth noting that the α-thioxantenic moiety undergoes a sort of hydrophobic collapse. In the binding site (Figure 12), the α-thioxantenic group adopts a disposition in which one of the two aromatic ring face-to-face interacts with the phenyl ring of the carboxyphenylglycine moiety, while the second one is forced toward a hydrophobic cluster formed by Val94, Pro97, Ile99, Ile137 residues (mGlu1 numbering). The same disposition is found on mGlu5 binding site, thus accounting for the lack of selectivity.

Conclusion

On the basis of the homology with members of thebacterial PBPs, 3D-models of the ATD of mGlu1 and mGlu5 have been derived. These constructs represent models of the open state of the receptors. Electrostatic potential analysis of the surfaces of the two lobes constituting the ATDs has allowed us to postulate a molecular mechanism leading to domain closure. Moreover, docking experiments of either mGlu1 or mGlu5 agonists and antagonists have allowed to propose amino acidic residues that are likely to be directly involved in the binding, to propose the mechanism by which agonist gathers the domain closure and to underlie the active site environment that may lead to mGlu1/mGlu5 selectivity.

The mechanistic conclusion of this work may have general validity and be applied to other members of the type C GPCR family. Our hypothesis on individual residues involved either in agonist/antagonist binding or in the mechanism of domain closure can be instrumental in designing specific site-directed mutagenesis experiments.

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