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The role of Arg^{78} in the metabotropic glutamate receptor $mGlu_1$ for agonist binding and selectivity

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Abstract

The metabotropic glutamate receptors belong to family C of the G-protein coupled receptor superfamily. These receptors all possess large extracellular amino terminal domains, where agonist binding takes place. We have previously constructed a molecular model of the amino terminal domain of the $mGlu_1$ receptor based on a weak amino acid sequence similarity with a family of bacterial periplasmic binding proteins (PBPs). The residues Ser^{165} and Thr^{188} were demonstrated to be involved in agonist binding to the receptor. Here, we report that mutation of Arg^{78} in the $mGlu_{1b}$ receptor to leucine or glutamate completely knocks out [^{3}H]quisqualic acid binding to the receptor. The constructed mutants, R78L and R78E, have also been characterized in a inositol phosphate assay. Here, the potency of (*S*)-glutamic acid and (*S*)-quisqualic acid was reduced 1000- and 100-fold, respectively, on R78L compared to the wild type (WT) receptor. (*S*)-Quisqualic acid was as potent on mutant R78E as it was on R78L, whereas (*S*)-glutamic acid was unable to activate R78E significantly at concentrations up to 10 mM. In conclusion, Arg^{78} appears to be essential for agonist binding to the $mGlu_1$ receptor, most likely, through the formation of an ionic bond between its positively charged side chain and the distal acid group of the agonists. Furthermore, the different impact of the two mutations on (*S*)-glutamic acid and (*S*)-quisqualic acid potencies strongly indicates that while Arg^{78} appears to be a common site of interaction for the agonists, the Group I subtype selectivity of (*S*)-quisqualic acid is probably determined by other residues in the amino terminal domain.

Keywords: $mGlu_1$ receptor, Arg^{78}; Agonist binding; Glutamic acid; Quisqualic acid; Mutagenesis

1. Introduction

The metabotropic glutamate receptors are members of family C of the G-protein coupled receptor superfamily (Nakanishi, 1994; Conn and Pin, 1997; Pin et al., 1999). The family also includes two γ-aminobutyric acid receptors type B ($GABA_B$) (Möhler and Fritschy, 1999), the Ca^{2+}-sensing receptor (Brown, 1999) and a family of putative pheromone receptors (Tirindelli et al., 1999). Eight metabotropic glutamate receptor subtypes have so far been identified, and some of these subtypes have numerous C-terminal splice variants. The eight subtypes have classically been divided into three subgroups based on their amino acid sequence similarity, pharmacological profile and signal transduction mechanism (Conn and Pin, 1997). Group I consists of the $mGlu_1$ and $mGlu_5$ receptors, Group II consists of the $mGlu_2$ and $mGlu_3$ receptors, and Group III of the remaining four subtypes.

The metabotropic glutamate receptors and the other family C receptors are characterized by their large extracellular, amino terminal domains, which can be up to 600 amino acids long. Numerous experiments have established that the binding site of the endogenous agonist resides in this region of the family C receptor (Takahishi et al., 1993; O'Hara et al., 1993; Tones et al., 1995; Okamoto et al., 1998; Han and Hampson, 1999; Bräuner-Osborne et al., 1996b; Malitschek et al., 1999). The amino terminal domains display weak amino acid sequence similarities with a family of bacterial periplasmic binding proteins (PBPs) (O'Hara et al., 1993). Crystal structures of PBPs have

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revealed that they consist of two globular domains ('lobes') connected by a hinge region (Quiocho and Ledvina, 1996). The ligands for these proteins bind to residues in the cleft between these lobes, and the existence of at least two conformations of the PBP has been postulated: an 'open' non-ligand bound and a 'closed' ligand-bound conformation (Quiocho and Ledvina, 1996).

Based on the crystal structure of one of these PBPs, the leucine/isoleucine/valine binding protein (LIVBP), we have previously proposed a molecular model of the tertiary structure of the 'open' amino terminal domain of the $mGlu_1$ receptor (O'Hara et al., 1993). Recently, the crystal structure of the leucine binding protein (LBP) has been applied as a template for a model of the amino terminal domain of the $GABA_{B1}$ receptor (Galvez et al., 1999). In the $mGlu_1$ receptor, Ser^{165} and Thr^{188}, located in the cleft between the two lobes of the amino terminal domain, have been shown to take part in the binding of the agonists (*S*)-glutamic acid and (*S*)-quisqualic acid (O'Hara et al., 1993). The two residues correspond to the Ser^{79} and Thr^{102} residues in LIVBP. Based on the protein–ligand interactions derived from the crystal structure of LIVBP (Sack et al., 1989), Ser^{165} and Thr^{188} in the $mGlu_1$ receptor are believed to form hydrogen bonds to the α-carboxylate and the α-amino groups of the glutamatergic agonists, respectively. In concordance with this, residues corresponding to Ser^{79} and Thr^{102} in LIVBP have been demonstrated to be equally important for agonist binding to the $mGlu_4$ and $GABA_{B1}$ receptors (Hampson et al., 1999; Galvez et al., 1999), and in the Ca^{2+}-sensing receptor, mutations of the corresponding Ser^{147} and Ser^{170} residues greatly reduce the ability of the receptor to become activated (Bräuner-Osborne et al., 1999b).

In contrast to the detailed knowledge of the interactions between LIVBP and the 'amino acid backbone' of its ligands, information of the binding pattern of the side chains of the amino acids is more sparse (Sack et al., 1989), as is the case with the metabotropic glutamate receptors. However, in a recent publication, a conserved arginine residue in the amino terminal domain of all the metabotropic glutamate receptors was suggested as a possible contributor to agonist binding (Pin et al., 1999). This arginine is not conserved in the PBPs upon which the amino terminal domains of the $mGlu_1$ and $GABA_{B1}$ receptors have been modeled (Fig. 1). In a recent study, Hampson et al. (1999) have shown that the arginine residue in the $mGlu_4$ receptor, Arg^{78}, is in fact involved in the binding of the Group III selective agonist (*S*)-2-amino-4-phosphonobutyric acid ((*S*)-AP4). The authors concluded that the residue most likely serves as a ligand for the distal phosphono acid group of the agonist. However, the importance of the residue for (*S*)-glutamic acid binding was not addressed. Furthermore, no explanation for the differences in selectivities between (*S*)-AP4 and the endogenous agonist was offered. Finally, the importance of the Arg^{78} mutation for receptor functionality was not investigated.

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LIVBP    13  ---GPVA-QYGDQEFTGAEQAVADINA 35
LBP      13  ---GPIA-QWGIMEFNGAEQAIKDINA 35
mGluR1   65  RKCGEIREQYGIQRVEAMFHTLDKINA 91
mGluR2   48  EECGPVNEHRGIQRLEAMLFALDRINR 74
mGluR3   55  EECGRINEDRGIQRLEAMLFAIDEINK 81
mGluR4   65  KACGELKKEKGIHRLEAMLFALDRINN 91
mGluR5   55  RKCGAVREQYGIQRVEAMLHTLERINS 81
mGluR6   52  RACGALKKEQGVHRLEAMLYALDRVNA 75
mGluR7   65  VPCGDIKRENGIHRLEAMLYALDQINS 91
mGluR8   62  VPCGELKKEKGIHRLEAMLYAIDQINK 88
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Fig. 1. A sequence alignment of two bacterial PBPs and the metabotropic glutamate receptors. Alignment of part of LIVBP and LBP with part of the amino terminal domains of the rat $mGlu_1$, $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_5$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors. Arg^{78} in the $mGlu_1$ receptor and the corresponding amino acids in the other sequences are indicated in bold. LIVBP and LBP from *Escherichia coli*.

In the present study, we have investigated the importance of the arginine residue in the $mGlu_1$ receptor, also termed Arg^{78}, for the agonist binding to and activation of the receptor.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma (St. Louis, MO). Culture media, serum, antibiotics and buffers for cell culture were obtained from Life Technologies (Paisley, UK). (*S*)-Quisqualic acid was purchased from Tocris (Bristol, UK). [^{3}H]Quisqualic acid was purchased from Amersham (Buckinghamshire, UK). The pSI vector was obtained from Promega (Madison, WI). The tsA cells were a generous gift from Dr. Penelope S.V. Jones (University of California, San Diego, CA).

2.2. Subcloning and site-directed mutagenesis

The $mGlu_{1b}$ receptor was subcloned from the ZEM228 vector to the pSI vector as described previously (Bräuner-Osborne et al., 1999a). Mutations were made using the Quickchange mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA), and the mutant receptors were sequenced on an ABI 310 using Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer, UK).

2.3. Cell culture

tsA cells (a transformed human embryonic kidney (HEK) 293 cell line (Chahine et al., 1994)) were maintained at 37°C in a humidified 5% CO_2 incubator in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% calf serum.

2.4. [^{3}H]Quisqualic acid binding assays

2×10^6 cells were split into a 15 cm tissue culture plate and transfected with 10 μg plasmid the following day using Superfect as a DNA carrier according to the protocol by the manufacturer (Qiagen, Hilden, Germany). The day after transfection, the medium was changed. The following day, the cells were suspended in ice-cold 20 mM HEPES-NaOH/10 mM EDTA (pH 7.4) and centrifuged at 50.000 $\times g$ at 4°C for 15 min. The pellet was homogenized (using a polytron for 5 s) in ice-cold 20 mM HEPES-NaOH/0.1 mM EDTA (pH 7.4) and centrifuged at 50.000 $\times g$ at 4°C for 15 min. This step was performed twice. Then, the membranes were homogenized in the assay buffer, ice-cold 20 mM HEPES-NaOH/2 mM $MgCl_2$/2 mM $CaCl_2$ (pH 7.4), and centrifuged at 50.000 $\times g$ at 4°C for 15 min. The membranes were resuspended in assay buffer, and 50–150 μg of membrane fractions were incubated for 1 h on ice with 100 nM of [^{3}H]quisqualic acid in a total volume of 250 μl. Non-specific binding was determined in the presence of 1 mM (*S*)-glutamic acid. 5-ml ice cold assay buffer was added, and the mixture was aspirated onto a GF/B filter (Whatman Paper, Gaithersburg, MD). After being washed twice with 5 ml ice-cold assay buffer, the filters were dried and counted in a scintillation counter. All binding experiments were performed in duplicate twice.

2.5. Inositol phosphate assays

1×10^6 cells were split into a 10-cm tissue culture plate and transfected with 5 μg plasmid the following day using Superfect as a DNA carrier according to the protocol by the manufacturer (Qiagen, Hilden, Germany). The day after transfection, the cells were split into a poly-D-lysine coated 24-well tissue culture plate in inositol-free DMEM, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% dialysed fetal calf serum and 1 μCi/ml *myo*-[2-^{3}H]inositol (Amersham). 16–24 h later, the cells were washed with Hanks' balanced saline solution (HBSS), and incubated at 37°C for 20 min in HBSS, supplemented with 0.9 mM $CaCl_2$ and 1.05 mM $MgCl_2$. The buffer was removed and the cells were incubated for 10 min in HBSS supplemented with 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$ and 10 mM LiCl. The buffer was removed and the cells were incubated for 40 min in HBSS supplemented with 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$, 10 mM LiCl and various concentrations of (*S*)-glutamic acid or (*S*)-quisqualic acid. The reactions were stopped by exchanging the buffer with 500 μl ice-cold 20 mM formic acid and the separation of total [^{3}H]inositol phosphates was carried out by ion-exchange chromatography as previously described (Nanevicz et al., 1996). All inositol phosphate experiments were performed in triplicate, and the results are given as mean ± S.E. of at least three independent experiments.

2.6. Data analysis

Data from the inositol phosphate assays were fitted to the simple mass equation: $R = R_{max}/(1 + (EC_{50}/[A])^n) + R_{basal}$ where [A] is the concentration of agonist, n is the Hill coefficient and R is the response. Curves were generated by non-weighted least-squares fits using the program KaleidaGraph 3.08 (Synergy Software, Reading, PA).

3. Results

In the binding experiments, we used a concentration of 100 nM [^{3}H]quisqualic acid. This concentration is fourfold higher than the K_d value obtained in a recent study of the $mGlu_{1a}$ receptor transiently expressed in HEK 293 cells (Mutel et al., 1999), and fivefold higher than the concentration used in a study of ligand inhibition of [^{3}H]quisqualic acid binding to the $mGlu_{1a}$ receptor in CHO cells (Okamoto et al., 1998). We obtained a significant degree of specific binding of the radioligand to the wild type (WT) $mGlu_{1b}$ receptor (Fig. 2). However, when Arg^{78} was mutated to a leucine or a glutamate residue (mutant R78L and R78E, respectively), [^{3}H]quisqualic acid binding to the receptor was completely eliminated (Fig. 2). The level of non-specific binding in cells transfected with WT, R78L and R78E was similar in all experiments.

In the functional experiments, the WT $mGlu_{1b}$ receptor displayed a concentration-dependent increase in inositol phosphate accumulation from three- to fourfold, when exposed to (*S*)-glutamic acid and (*S*)-quisqualic acid (Fig. 3A and B). Substitution of Arg^{78} with a leucine or a glutamate residue led to mutant receptors with markedly rightward shifted pharmacological profiles (Fig. 3A and B). The $Arg^{78} \rightarrow$ Leu mutation reduced the potency of (*S*)-glutamic acid and (*S*)-quisqualic acid with 1000- and 100-fold, respectively (Table 1). When the arginine was

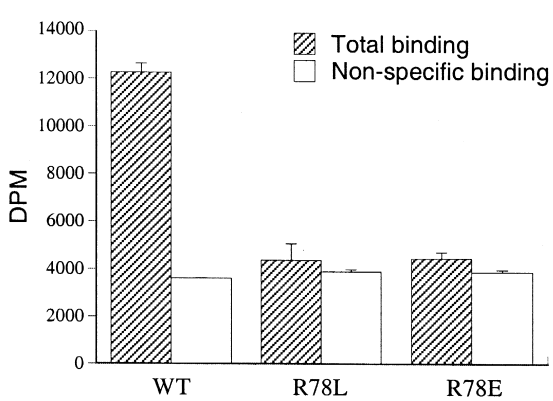

Fig. 2. [^{3}H]Quisqualic acid binding to tsA cells transfected with WT, R78L or R78E $mGlu_{1b}$ receptors. Fifty micrograms of membrane fractions prepared as described in Section 2 were incubated on ice for 1 h with 100 nM [^{3}H]quisqualic acid alone (total binding) or together with 1 mM (*S*)-glutamic acid (non-specific binding). Data are given as disintegration per minute (dis/min) and are mean ± S.D.

mutated to glutamate, the ability of the receptor to be stimulated by the (*S*)-glutamic acid was almost completely lost at concentrations up to 10 mM. In contrast, the potency of (*S*)-quisqualic acid was not significantly different when tested on R78E and R78L.

The observation that the mutants R78L and R78E were fully functional indicates that the mutations have not disrupted the protein folding or the expression of the mutant receptors in the cell membrane. The Hill coefficients of the two agonists on the mutants were significantly higher than those of the WT receptor (Table 1). Furthermore, the mutants displayed a considerably higher fold of stimulation than the WT receptor. The R_{max}/R_{basal} ratios of the mutants were two- to fourfold higher than those of the WT receptor. This phenomenon may, in part, be due to low endogenous levels of (*S*)-glutamic acid in the assay elevating the basal response of the WT receptor but not those of the less sensitive 'right-shifted' mutants.

Table 1
EC_{50} values and Hill coefficients (n_H) from agonist-induced inositol phosphate accumulation in tsA cells transfected with WT, R78L or R78E $mGlu_{1b}$ receptors

Receptor	(*S*)-glutamic acid		(*S*)-quisqualic acid	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
WT	3.7 ± 0.2	1.2 ± 0.2	0.3 ± 0.0	0.9 ± 0.0
R78L	2957 ± 23	2.5 ± 0.1	40.2 ± 9.4	2.0 ± 0.1
R78E	> 10.000[a]	–[b]	49.3 ± 4.0	2.3 ± 0.3

[a] 10 mM of (*S*)-glutamic acid induced a response < 10% of the maximal response obtained with (*S*)-quisqualic acid on the mutant.
[b] nd: not determinable.

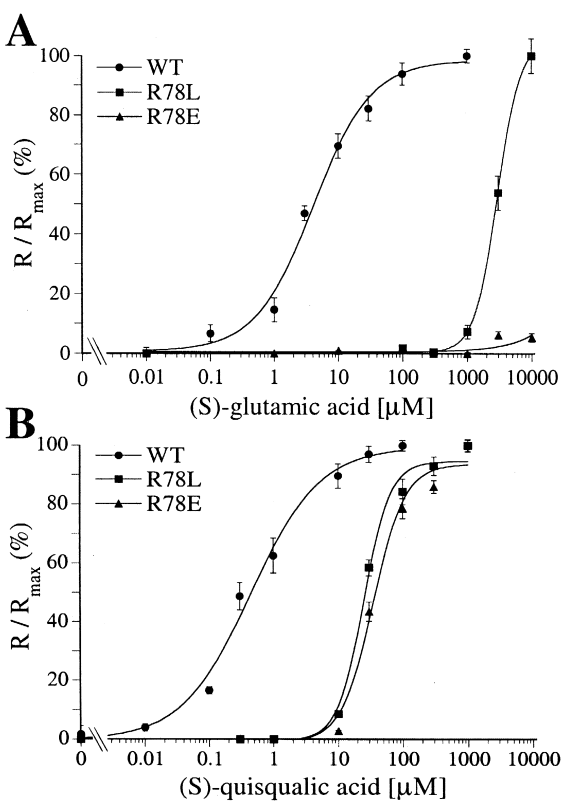

Fig. 3. Agonist-induced inositol phosphate accumulation in tsA cells transfected with WT, R78L or R78E $mGlu_{1b}$ receptors. Concentration–response curves for WT (closed circles), R78L (closed squares) and R78E (closed triangles) obtained with (*S*)-glutamic acid (A) and (*S*)-quisqualic acid (B). The inositol phosphate assays were performed as described in Section 2, and data are expressed as percentage of the maximal response, R_{max}. In the (*S*)-glutamic acid experiment, R_{basal} and R_{max} values (in dis/min mean ± S.D) were 6344 ± 302 and 21879 ± 356 for WT, 2786 ± 285 and 33562 ± 1780 for R78L, and 3621 ± 108 and 6022 ± 645 for R78E. In the (*S*)-quisqualic acid experiment, R_{basal} and R_{max} values (in dis/min mean ± S.D) were 6380 ± 445 and 21672 ± 265 for WT, 3279 ± 195 and 44875 ± 889 for R78L, and 3448 ± 211 and 49190 ± 857 for R78E. The R_{max} value for (*S*)-quisqualic acid on R78E was used in the calculation of the percentages for the (*S*)-glutamic acid curve on the mutant.

4. Discussion

Arginine residues are well known to be involved in the binding of carboxylate groups and analogous of these. Thus, they have been shown to participate in the agonist binding of ionotropic glutamate receptors, as well as the γ-aminobutyric acid receptor type A, $GABA_A$ (Armstrong et al., 1998; Lampinen et al., 1999; Laube et al., 1997; Westh-Hansen et al., 1999).

Here, we report that Arg^{78} is essential for (*S*)-glutamic acid and (*S*)-quisqualic acid binding to the $mGlu_1$ receptor, most likely, through an ionic interaction between the positively charged residue and the negatively charged distal group of the agonists. According to our model of the 'open' amino terminal domain of the receptor, Arg^{78} is located within a reasonable distance from Ser^{165} and Thr^{188}, which are also involved in the binding (O'Hara et al., 1993) (Fig. 4). Interestingly, the three residues are located in the same lobe of the amino terminal domain.

The involvement of Arg^{78} in agonist binding to the $mGlu_1$ receptor is in excellent agreement with a recent study of the importance of the corresponding arginine residue for [^{3}H]AP4 binding to the $mGlu_4$ receptor, a Group III metabotropic glutamate receptor (Hampson et al., 1999). Together, the two studies strongly suggest that the conserved arginine residue is involved in agonist binding to all metabotropic glutamate receptor subtypes.

In addition to the binding data demonstrating the essential role of Arg^{78} for agonist binding, the functional data reported in this study offer interesting information regarding $mGlu_1$ receptor agonist selectivity. The potency of (*S*)-glutamic acid is significantly more reduced than the potency of (*S*)-quisqualic acid, when Arg^{78} in the receptor is mutated to a leucine. Hence, although the residue is important for the binding of both agonists, it seems to be more crucial for (*S*)-glutamic acid activation than for (*S*)-quisqualic acid activation of the receptor. This hypothesis is further supported by the observed effects of the

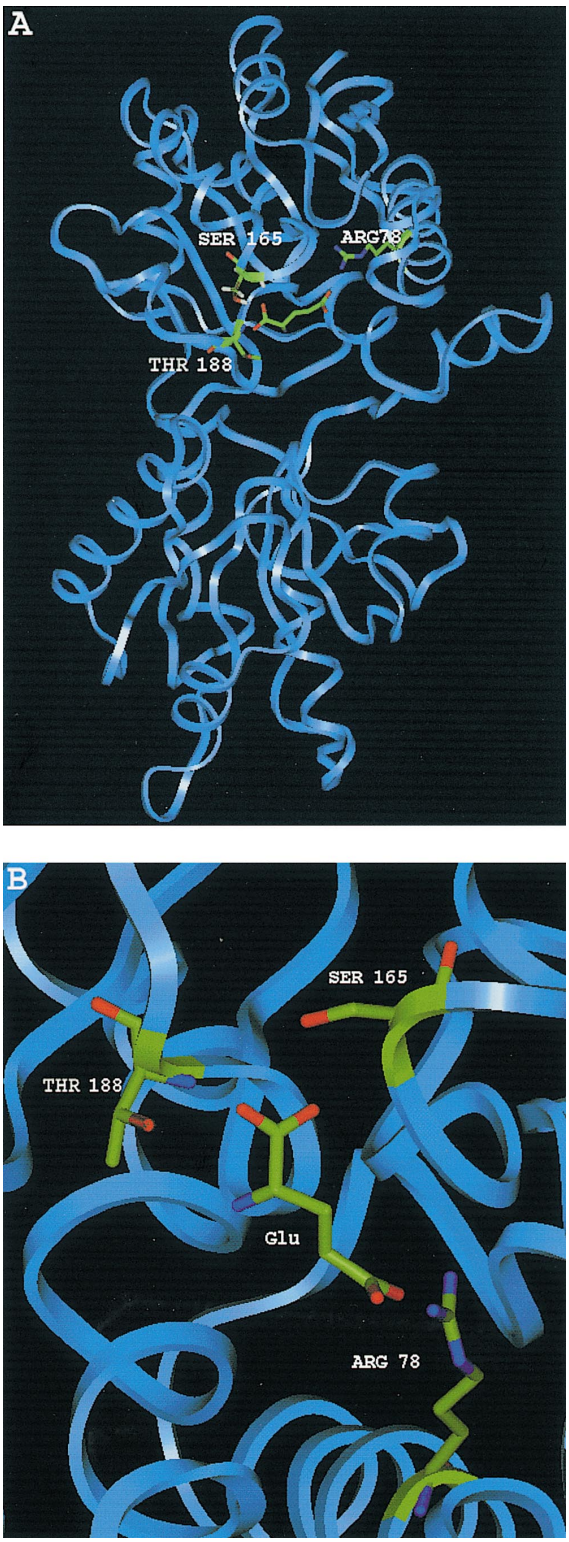

Fig. 4. Molecular model of the 'open' form of the amino terminal domain of the $mGlu_1$ receptor. Ser^{165}, Thr^{188} and Arg^{78} and docked (*S*)-glutamic acid are shown.

$Arg^{78} \rightarrow$ Glu mutation on the potencies of the two agonists. This mutation completely knocks out the (*S*)-glutamic acid activation of the receptor, whereas (*S*)-quisqualic acid retains its ability to activate the receptor to its maximal response. The repulsive forces between the distal carboxylate group of (*S*)-glutamic acid and the introduced glutamate residue in mutant R78E clearly represent a major obstacle to the binding of the agonist. In contrast, even though the removal of Arg^{78} significantly reduces (*S*)-quisqualic acid potency at the receptor, it does not seem to make a difference if it is substituted with a 'neutral' (R78L) or a negatively charged residue (R78E). In conclusion, (*S*)-glutamic acid appears to bind in a more 'simplistic' fashion to the receptor than (*S*)-quisqualic acid.

We have previously shown that a $Thr^{188} \rightarrow$ Ala mutation completely knocks out (*S*)-glutamic acid activation of the $mGlu_1$ receptor, whereas (*S*)-quisqualic acid is still able to bring the receptor to maximal response, albeit with a greatly reduced potency (O'Hara et al., 1993). Furthermore, it was shown that the contributions of Ser^{165} to (*S*)-glutamic acid and (*S*)-quisqualic acid binding are essentially the same. Hence, it is tempting to explain the differences in (*S*)-glutamic acid and (*S*)-quisqualic acid potencies on the T188A mutant by the binding component arising from their different side chains. The present data appear to support this proposal.

In a recent study, it has been suggested that (*S*)-glutamic acid binds to the $mGlu_1$ receptor in an 'extended', as well as in a 'folded' form, representing a high and a low affinity conformation, respectively (Jullian et al., 1999). Based on the structures of various 'rigid extended' and 'rigid folded' agonists, two different pharmacophore models of the receptor were constructed, and a wide range of glutamatergic agonists were tried fitted into these models. (*S*)-Quisqualic acid turned out to be the only agonist capable of fitting to all binding sites in both of these pharmacophore models. The authors suggested that the high affinity of the agonist for the $mGlu_1$ receptor could arise from its ability to mimick (*S*)-glutamic acid and bind in both conformations.

The present study may shed some light on this hypothesis. According to the hypothesis activation of the WT $mGlu_1$, receptor by both (*S*)-glutamic acid and (*S*)-quisqualic acid should primarily arise from binding of the extended forms of the agonists and only a marginal fraction of the total activation exerted by the agonists should be ascribed to binding of their folded forms. In mutant R78L, the removal of Arg^{78} has greatly reduced the affinity of both agonists, suggesting that the binding of the extended conformation of both agonists is eliminated or greatly reduced. For (*S*)-glutamic acid, this is reflected by a 1000-fold increased EC_{50} value, whereas the potency of (*S*)-quisqualic acid is 'only' 100-fold reduced. In mutant R78E, (*S*)-glutamic acid is no longer capable of activating the receptor, whereas the potency of (*S*)-quisqualic acid on mutant R78E is not significantly different from that on mutant R78L. Whether (*S*)-glutamic acid binds to the $mGlu_1$ receptor in a single conformation or in the two

conformations suggested by Jullian et al. (1999) is impossible to determine from this study. However, if there are indeed two receptor binding conformations of (*S*)-glutamic acid, substitution of the arginine with a glutamate residue clearly eliminates the ability of both conformations to activate the receptor. Furthermore, if (*S*)-quisqualic acid exerted its effect on the receptor by mimicking the two conformations of the endogenous agonist as the authors suggested based on the pharmacophore models (Jullian et al., 1999), one would expect the $Arg^{78} \rightarrow Glu$ mutation to knock out the ability of both (*S*)-glutamic acid and (*S*)-quisqualic acid to activate the receptor. This is clearly not the case.

Hence, we propose that (*S*)-quisqualic acid binding to the receptor is more 'complex' than that of (*S*)-glutamic acid. The presence of a distal 1,2,4-oxadiazol-3,5-dione group most likely enables the agonist to bind to additional residues in the receptor than those it shares with (*S*)-glutamic acid. Consequently, (*S*)-quisqualic acid is less dependent on the binding contributions from Arg^{78} than (*S*)-glutamic acid, even though the residue is still a major determinant for the overall binding of the agonist. These additional distal agonist–receptor interactions of (*S*)-quisqualic acid may very well be the reason for the significantly higher affinity for the $mGlu_1$ receptor displayed by the agonist in comparison with the endogenous agonist (Thomsen et al., 1993; Okamoto et al., 1998). It is also tempting to ascribe the agonist-dependent activity displayed by several competitive antagonists on the $mGlu_1$ and $mGlu_5$ receptors to these differences in nature and complexity of the receptor binding by various agonists (Brabet et al., 1995; Lin et al., 1997).

In conclusion, Arg^{78} in the $mGlu_1$ receptor appears to be essential for agonist binding to the receptor. The residue is conserved in all metabotropic glutamate receptors and is most likely binding the distal negatively charged side chains of the glutamatergic agonists. As it was to be expected, the amino acid clearly is not the only residue binding the side chains of the agonists. The high affinity and Group I selectivity of (*S*)-quisqualic acid is bound to arise from other side chain–receptor interactions than those of the endogenous agonist. In order to obtain information, which may assist in the design of agonists selective for the $mGlu_1$ receptor, it would be interesting to explore the receptor regions close to Arg^{78} for additional residues involved in (*S*)-quisqualic acid binding.

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