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Selective agonist binding of (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and 2*S*-(2α,3β,4β)-2-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid (kainate) receptors: a molecular modeling study

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Abstract

Molecular models were constructed, using the published X-ray structure of rat glutamate receptor 2 (GluR2), for the ligand-binding domains of the human (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA)- and kainate-selective *ionotropic* glutamate receptors (iGluRs): GluR1–7 and KA1–2. Based on the analysis of the known X-ray structures of GluR2 in complex with glutamate, kainate, and AMPA, we have constructed binding motifs (relative positioning of a ligand in the binding site and the physicochemical interactions that take place) for selected agonist ligands and found explanations for ligand-binding selectivity to *homomeric* receptors among the different iGluRs. Even a single sequence difference can explain significant differences in ligand-binding affinities between two receptors. In total, there are seven residues surrounding the binding cavity that affect agonist selectivity: in GluR2, these residues are Pro478, Thr480, Leu650, Ser654, Thr686, Tyr702, and Met708. Each of these seven positions has been shown, or is predicted, to influence the presence of one or more water molecules that, when present, may form bridging hydrogen bonds between particular ligands and receptors. By using this knowledge it should be possible to design new selective agonist ligands with high affinity for any AMPA/kainate receptor.

Keywords: Ionotropic glutamate receptors; Agonist binding; Structural modeling; Structure–activity relationship

Ionotropic glutamate receptors (iGluRs) have been divided into three subclasses based on their binding affinities for agonist ligands: *N*-methyl-D-aspartic acid (NMDA) receptors, AMPA receptors (GluR1–4), and kainic acid (KA) receptors (GluR5–7 and KA1–2). Glutamatergic neurotransmission mediated by iGluRs plays an important pharmacological role in many processes such as memory, learning, and synaptic plasticity, as well as in neuronal degeneration associated with cerebral ischaemia, Parkinson's disease and Alzheimer's disease [1–3]. Consequently, iGluRs have been intensively studied in order to understand their normal functions and roles in disease processes, as well as to develop ligands that can discriminate among different subunits. The pharmacology of AMPA and KA receptor ligands has been recently reviewed [4,5].

In AMPA, KA, and NMDA receptors and representatives of periplasmic amino acid binding proteins, two sequence positions, corresponding to Arg485 and Glu705 in GluR2, have been shown to play key roles in ligand binding where they respectively form ionic

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Abbreviations: iGluR, *ionotropic* glutamate receptor; GLU, (*S*)-glutamic acid or (*S*)-2-amino-pentanedioic acid; AMPA, (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; KA, kainic acid: (2*S*-(2α,3β,4β))-2-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid; ACPA, (*S*)-2-amino-3-(3-carboxy-5-methylisoxazol-4-yl)propionic acid; Br-HIBO, (*S*)-2-amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid; Domoic acid, (2*S*-(2α,3β,4β))-2-carboxymethyl-4*t*-((5Θ)-carboxy-1-methyl-hexa-1,3*t*-dien-*t*-yl)pyrrolidine-2*r*-carboxylic acid; ATPA, (*S*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-isoxazol-4-yl)propionic acid; Willardiine, (*S*)-2-amino-3-(5-R-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)propionic acid (R = H, F, Cl, Br, I); SYM2081, (2*S*,4*R*)-4-methylglutamic acid or (2*S*,4*R*)-2-amino-4-methyl-pentanedioic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione.

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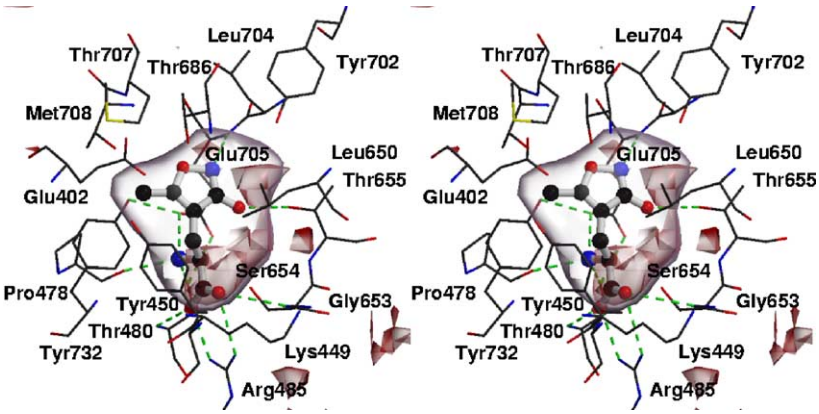

Fig. 1. Key interactions (hydrogen bonds, green dotted lines) at the binding site of the GluR2-AMPA crystal structure (in stereo). The binding cavity is shown as a transparent surface. GRID [38] maps for a COO^- probe at an energy contour of −10 kcal/mol (transparent pink) indicate regions of the binding cavity where negatively charged functional groups from a ligand can be placed. The figure was produced using BODIL, MOLSCRIPT [63], and Raster3D [64]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interactions with the charged α-carboxylate group and α-amino group of bound ligands (see Fig. 1). For example, in the AMPA receptor GluR4, Arg507 is essential for ligand binding, and even conservative mutations at this position have been shown to abolish agonist binding [6,7]. Mutations that neutralize the negative charge of glutamate lead to the complete loss of agonist binding, whereas the conservative mutation Glu705Asp causes agonist-selective changes in the binding affinity [6,8]. A wide range of other mutants have been made to different AMPA and KA receptor subunits and used to study their ligand-binding properties, receptor activation and receptor desensitization [3,6–18].

The first three-dimensional structure of an iGluR was solved for the rat GluR2 subunit (AMPA receptor) with the bound partial agonist kainate [19], followed by the crystal structures of the apo-form and complexes with the agonists AMPA and L-glutamate and with one antagonist DNQX [20]. Additional structures of GluR2 have now been reported where mutations have been made to the dimer interface affecting receptor desensitization [21], a Tyr702Phe mutant in complex with Br-HIBO and ACPA [12], as well as structures of GluR2 with bound Br-HIBO, ACPA, and with (*S*)-2-amino-3-(3-hydroxy-5-(2-methyl-2*H*-tetrazol-5-yl)4-isoxazolyl)propionic acid (2-Me-Tet-AMPA) [12], (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (Des-Me-AMPA) [22], quisqualate [23], and the antagonist ATPO [24]. No high-resolution structural information is currently available for any kainate- or NMDA-selective iGluR subunit. Nevertheless, due to the relatively high sequence similarity among the iGluRs, all of the AMPA and KA receptor ligand-binding domain structures can be modeled with reasonable accuracy. (At the time of this study, the following structures of GluR2 had not been published: 5-F-, 5-Br-, and 5-I-willardiine [25], (*S*)-ATPA [26], the Leu650Thr mutant with bound AMPA and KA [18], and the structure of the NR1 subunit of the NMDA receptor [27].)

Here, we have constructed molecular models for the binding sites formed by the S1 and S2 segments of the non-NMDA-type GluR subunits, GluR1–4, GluR5–7, and KA1–2, primarily using the published X-ray structures of rat GluR2 [19,20]. Based on the analysis of the known GluR2 structures with different agonist ligands we have constructed binding motifs (relative positioning of a ligand in the ligand-binding site and the physicochemical interactions that take place) for selected agonist ligands and found plausible explanations for ligand-binding selectivity among the different iGluRs. An analysis of this kind should yield information that would facilitate the design of new subclass- and subunit-selective ligands for studies of the roles of iGluRs in both normal and dysfunctional nervous systems as well as for the development of novel ligands with potential therapeutic roles.

1. Materials and methods

1.1. Structural modeling

The three-dimensional structures of the GluR2 S1S2 construct complexed with kainate (KA; PDB access code: 1gr2; [19]), AMPA (PDB access code: 1ftm) and (*S*)-glutamic acid (GLU; PDB access code: 1ftj; [20]) were obtained from the Protein Data Bank [28]. The sequence alignment of human GluR1–4 (AMPA-receptors), GluR5–7, and KA1–2 (KA-receptors) was made using MALIGN [29] in the BODIL Modeling Environment[1] using a structure-based sequence comparison matrix [30]. MALIGN constructs a multiple-sequence alignment from pairwise

[1] Lehtonen JV, Still D-J, Rantanen V-V, Ekholm J, Björklund D, Iftikhar Z, Huhtala M, Jussila A, Jaakkola J, Pentikäinen OT, Nyrönen T, Salminen TA, Gyllenberg M, Johnson MS. BODIL: a molecular modeling environment for structure-function analysis and drug discovery. Unpublished observations. Available: http://www.abo.fi/fak/mnf/bkf/research/johnson/bodil.html.

alignments according to a tree relating the sequences being matched.

The program HOMODGE in BODIL was used to construct three-dimensional model structures by keeping the side-chain conformations of all identical residues fixed and by maintaining the corresponding torsion angles of similar residues in the alignment. HOMODGE employs a ''minimum change'' or ''minimum damage'' approach to modeling and performs as well as, and sometime better than restraint-based methods over a wide range of protein similarity.[2] Here, the sequence identity over S1 and S2 varies from approximately 90% (among AMPA receptors) to about 50% (between GluR2 and KA receptors) and it is even higher for residues directly involved in ligand binding, thus experience dictates that high-quality models should be obtained. With the exception of differences in domain closure observed for GluR2 with different agonist ligands and changes in the backbone structure at positions 674–675 when GluR2 is complexed with either Br-HIBO or quisqualate, the main-chain conformation of GluR2 is affected little by the binding of different ligands. Since the main chain appears to be largely unaffected by the ligand that is bound, we focused our attention on the side-chain conformations, such that for each bound ligand the intramolecular and intermolecular interactions were optimized by using the amino acid side-chain rotamer library [31] incorporated within BODIL. (In support of this modeling approach, since the submission of this work for publication the structures of complexes of GluR2 with bound (*S*)-ATPA [26] and with bromo- and iodo-willardiine [25] have been reported. In each case, we have correctly predicted the mode of binding to GluR2, including the need for the rearrangement of the Met708 side-chain conformation.)

1.2. Ligand minimization

Ligands were built with the program SYBYL 6.7 (Tripos Inc.). Each ligand structure was energy minimized prior to docking to the receptor models using the MMFF94s force field in Sybyl and conjugate gradient method until the energy gradient was less than 0.05 kcal/mol. Hybridization and protonization of the ligands were confirmed by examining similar (sub)structures obtained from the Cambridge Structural Data Bank (CSD System Documentation, Cambridge Crystallographic Data Centre, 1992) [32]; published literature was used as well (e.g. protonization state of AMPA [33]).

1.3. Ligand docking

AUTODOCK 3.0 [34] was used to dock agonist ligands into the different AMPA/KA receptors. The partial charges of atoms of the receptor model were calculated using the AMBER force field [35,36] in SYBYL; and, for ligands, the MMFF94 force field [37] was used. However, in most cases the docking modes produced by AUTODOCK were unreasonable and resulted in docked conformations different to those seen in the published X-ray structures for rat GluR2. This is mainly a consequence of the different number of water molecules that could participate in ligand binding in the different receptors and for different ligands. Consequently, we docked all ligands interactively using the X-ray structure of rat GluR2 in complex with ligands as the best guide to the likely binding modes for the human ionotropic glutamate receptors. To make the best possible assignments of the docking modes, for ligands not represented by crystal structures, we used our knowledge of the existing crystal structure complexes, similarities in the ligand structures, and the sequence differences among receptor subunits. GRID [38] maps (see below) made for different atom types seen in the ligands were used to guide the docking modes. After reasonable docking modes were achieved, the ligand structures were energy minimized within each binding site model using the MMFF94s force field and the Powell method.

1.4. Chemical interactions

Independently, the essential chemical interactions within the GluR2 binding site were mapped using the program GRID version 20 [38]. GRID calculates energies of interaction between a probe and the receptor. In the calculation, the probe that mimics a chemical group is placed at different positions throughout the binding site, and the receptor side chains are allowed to move in order to minimize the interaction energy between the probe and the receptor (using the side-chain flexibility option in GRID). The GRID maps were then compared with the docked ligands.

2. Results and discussion

2.1. Ligand binding and selectivity

Although radioligand-binding data for ionotropic glutamate receptors are available, detailed structural explanations for data across all AMPA and kainate subtypes have so far not been reported. Results have generally been limited to a small subset of receptors: for example, ATPA binding to GluR1 and GluR5 [16]; Br-HIBO binding to GluR1 and GluR3 [11]; binding of halogenated willardiines to GluR1 [10] (a very recent study [39] has primarily examined the effects of stereoisomers on binding to AMPA and kainate subtypes); other very recent studies have modeled the interactions of the GluR5-selective agonist (*R*,*S*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[-*d*]isoxazole-4-yl)propionic acid [40] and used ''low-mode'' docking calculations to explain kainoid selectivity

[2] Settimo L, Vainio M, Pentikainen OT, Lehtonen JV, Hoffrén A-M, Johnson MS, submitted for publication.

at AMPA receptors [41]. In the current study, we have considered agonist ligand binding across the set of AMPA and kainate receptors. Thus, we have (i) interpreted published ligand-binding data on recombinant iGluR subunits expressed as *homomeric* receptors, obtained, as far as possible in the same laboratory in order to minimize errors in the comparison of the data (ligand-binding data are tabulated for 23 selected ligands in Table 1 and the structures of these 23 ligands are shown in Fig. 6). These ligands were chosen because of their receptor selectivity; glutamate (GLU) is the natural ligand of the AMPA and KA receptors. Due to methodological differences, ligand-binding data may show differences between laboratories as illustrated by the two sets of binding data for GLU (Table 1). We have (ii) used the known rat GluR2 crystal structures to model the ligand-binding site of each of the human AMPA (GluR1–4) and kainate (GluR5–7, KA1–2) receptors (amino acids in the rat and human GluR2 ligand-binding site are 100% identical); (iii) docked the selected 23 agonist ligands to the models; and (iv) explained the role of the key amino acids at the ligand-binding site, boxed in Fig. 2, in order to understand the subtype selectivity of the different ligands.

2.2. Modeling of the ligand-binding sites: AMPA receptors (GluR1–4)

A three-dimensional model structure representing the GluR3 and GluR4 ligand-binding domain (AMPA receptor) was constructed based on the 1.7 Å resolution structure of rat glutamate receptor 2 in complex with the agonist AMPA (GluR2; PDB code: 1ftm; [20]). GluR4 shares with GluR2 88% sequence identity and there is only one sequence difference in the vicinity of the binding site (Fig. 2): Tyr702Phe (GluR2 → GluR4; GluR2 crystal structure numbering used throughout, see Fig. 2). Around the agonist binding site, GluR1 is identical in sequence to GluR2, and GluR4 is identical in sequence to GluR3. Furthermore, amino acids at the agonist binding site are identical in rat and human AMPA receptor subunits.

Table 1
Published binding data for selected agonists to different *homomeric* AMPA and KA receptors

Compound	AMPA receptors		Kainate receptors			
	GluR1/GluR2	GluR3/GluR4	GluR5	GluR6	GluR7	KA1/KA2
AMPA	103 ± 13/107 ± 16[a,b]	103[b,c]/155 ± 10[a,b]	2144 ± 416[a,b]	>100000[b]	>100000[b]	nd/>100000[b]
ATPA	10000/12000[b,c]	9000/9500[b,c]	4.3 ± 1.1[b]	>100000[b]	40000[b,c]	nd/50000[b,c]
ACPA	45 ± 5/15 ± 7[d]	28 ± 6/20 ± 3[d]	330 ± 30[d]	nd	nd	nd/nd
Br-HIBO	69 ± 11[e]/nd	8640 ± 1900[e]/nd	nd	nd	nd	nd/nd
KA	7449 ± 2018/12221 ± 2744[a,b,f,g]	1800[b,c]/1714 ± 170[a,b,f,g]	177 ± 22[a,b,f,g]	32 ± 13[b,f,g]	10 ± 2[b,f]	nd/8 ± 2[b,f]
Domoic acid	nd/12[h]	nd/nd	2[h]	59[h]	12[h]	40/275[h]
GLU	1362 ± 257/940 ± 93[a,f,g]	nd/868 ± 219[a,f,g]	701 ± 46[a,f,g]	1106 ± 159[f,g]	789 ± 83[f]	nd/750 ± 77[f]
	nd/346[g]	nd/nd	290[h]	3100[h]	1100[h]	200/480[h]
SYM2081	>10000/>10000[g]	nd/>10000[g]	3[g]	10[g]	nd	nd/nd
5-R-Willardiine						
H	386 ± 92/898 ± 86[a]	nd/8850 ± 923[a]	28900 ± 7350[a]	nd	nd	nd/nd
F	14.7 ± 1.3/25.1 ± 5.2[a]	nd/305 ± 107[a]	1820 ± 149[a]	nd	nd	nd/nd
Cl	65 ± 11/53.1 ± 4.4[a]	nd/451 ± 65[a]	57.1 ± 23.5[a]	nd	nd	nd/nd
Br	92 ± 25/101 ± 19[a]	nd/457 ± 49[a]	9.1 ± 2.4[a]	nd	nd	nd/nd
I	163 ± 14/176 ± 29[a]	nd/972 ± 155[a]	0.24 ± 0.06[a]	nd	nd	nd/nd
(2*S*,4*R*,*E*)-2-Amino-4-(3-R-prop-2-enyl)pentanedioic acid						
Ph (*E*)	nd/nd	nd/nd	44[f]	1580[f]	nd	nd/nd
Ph (*Z*)	nd/nd	nd/nd	157[f]	339[f]	nd	nd/nd
4-OMe-Ph (*E*)	nd/nd	nd/nd	284[f]	22340[f]	nd	nd/nd
4-F-Ph (*E*)	nd/nd	nd/nd	78[f]	559[f]	nd	nd/nd
4-Cl-Ph (*E*)	>10000/>10000[f]	nd/>10000[f]	8[f]	3859[f]	423[f]	nd/>10000[f]
4-Br-Ph (*E*)	nd/nd	nd/nd	30[f]	7216[f]	nd	nd/nd
4-Me-Ph (*E*)	nd/nd	nd/nd	172[f]	4375[f]	nd	nd/nd
4-CO2H-Ph (*E*)	nd/nd	nd/nd	3590[f]	27280[f]	nd	nd/nd
2-Naphthyl (*E*)	>10000/>10000[f,g]	nd/>10000[f,g]	14[f,g]	13723[f,g]	617[f]	nd/>10000[f]
1-Naphthyl (*E*)	nd/nd	nd/nd	7[f]	4366[f]	nd	nd/nd

All K_i values in nanomolar; nd: no available data.
[a] Data from ref. [48].
[b] Data from ref. [68].
[c] Extrapolated from the graphs of ref. [68].
[d] Data from ref. [70].
[e] Data from ref. [69].
[f] Data from ref. [53].
[g] Data from ref. [67].
[h] Data from ref. [47].

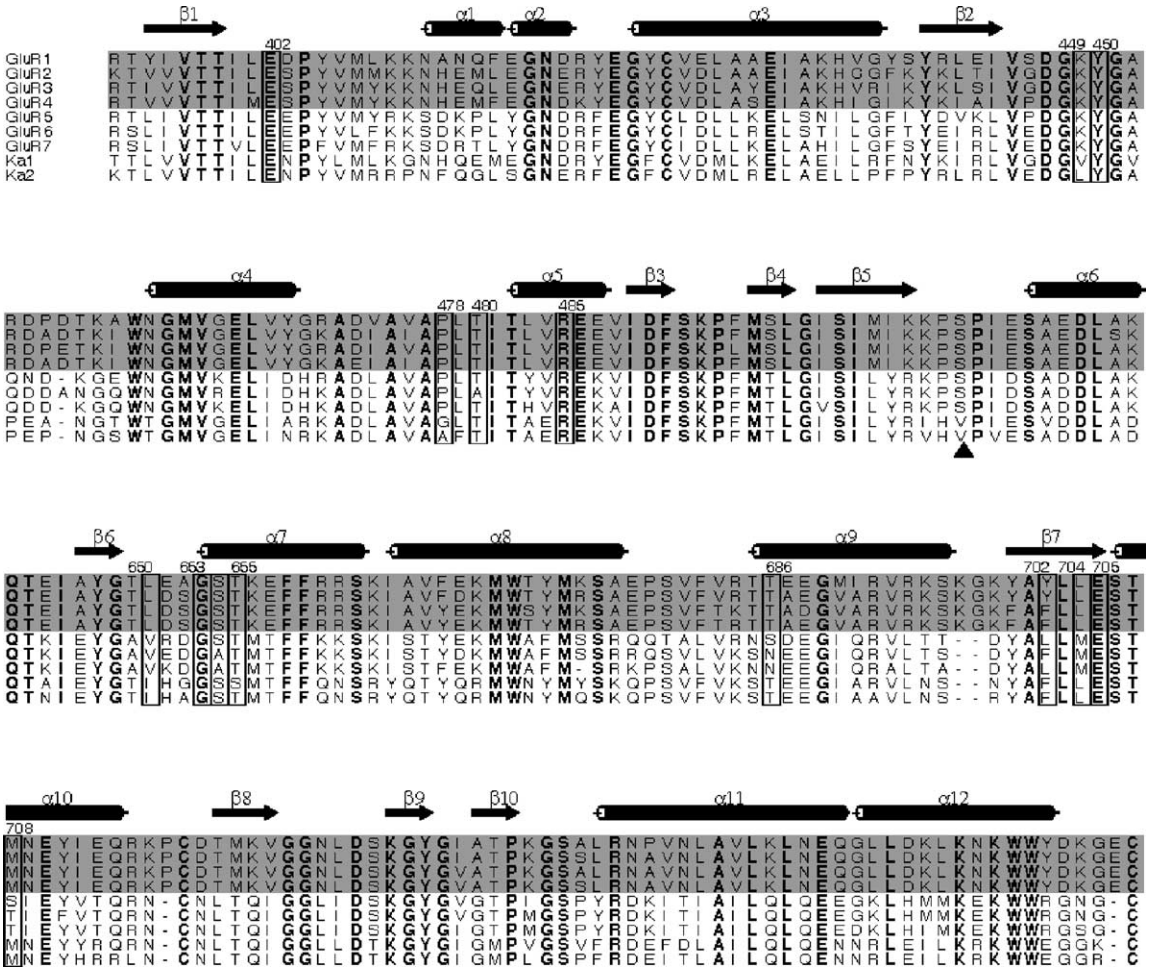

Fig. 2. Sequence alignment of AMPA (gray) and KA receptors with the S1–S2 construct of the GluR2 crystal structures (triangle indicates the S1–S2 junction). Residues within the ligand-binding pocket are boxed; conserved residues are shown in bold type; the secondary structure and residue numbering are from the GluR2 crystal structure, PDB code 1ftm. All AMPA sequences correspond to the ''flop'' variants [65] as does the GluR2 crystal structure. The SwissProt sequence of human GluR4 contains the error Asp710–Asn711, which has been corrected to Glu710–Tyr711. Position 743 in the AMPA receptors is subject to RNA editing whereby genome-encoded arginine in GluR2–4 may be replaced by glycine. The figure was prepared using ALSCRIPT [66].

2.3. Modeling of the ligand-binding sites: kainate receptors (GluR5–7 and KA1–2)

The model structure for each human KA receptor ligand-binding domain was constructed in a similar way as for the GluR3–4 ligand-binding domain. The sequence identity with the rat GluR2 crystal structure varies from 52% (GluR7) to 56% (KA1). In GluR5 there are, compared with rat GluR2, five sequence differences (Fig. 2) in the vicinity of the agonist ligand-binding site: Leu650Val (GluR2 → GluR5), Met708Ser, Thr686Ser, Leu704Met and Tyr702Leu. Among the GluR5–7 and KA1–2 receptors there are some additional sequence differences. GluR5 and GluR7 have three differences in the agonist ligand-binding site: Ser654Ala (GluR5 → GluR7), Ser686Asn, and Ser708Thr. GluR6 has phenylalanine at position 702 (as do GluR3–4), which in GluR5,7 is leucine; at position 480 GluR6 has alanine, whereas all other AMPA/KA receptors have threonine.

The ligand-binding sites of KA1–2 are nearly identical in sequence to the AMPA receptors (Fig. 2). With respect to GluR3–4, the only sequence differences at the ligand-binding site (Fig. 2) are: Pro478Gly/Ala (GluR4 → KA1/KA2), Leu650Ile, Lys449Val/Leu and, in addition, KA1 has serine instead of threonine at position 655.

2.4. Sequence differences at position 478: different ligand-binding affinities for KA1–2 in comparison with AMPA receptors

Both KA1–2 and AMPA receptors have methionine at position 708, which plays a crucial role in binding (discussed below), instead of the serine or threonine in GluR5–7. This similarity together with other identities between the ligand-binding residues in the GluR1–4 and KA1–2 subunits would make one expect similar ligand binding to KA1–2 as to AMPA receptors but, from the experimental data (Table 1), this is clearly not true. The differences in

binding affinities between KA1–2 and AMPA receptors, for all but the smallest of ligands (i.e. glutamate and SYM2081), can be partially attributed to the residue at position 478: GluR1–7 have a proline, while KA1 and KA2 have glycine and alanine, respectively. (Bacterial GluR0 [42] also has proline at this position, but the binding mode for GLU in the GluR0 complex structure is different from that in rat GluR2 due to numerous sequence differences).

In the published GluR2 structures, each of the bound agonist ligands forms a hydrogen bond between the α-amino group of the ligand and the main-chain oxygen atom of Pro478 (e.g. bound AMPA in Fig. 1); the main-chain carbonyl oxygen atom of residue 477 does not point towards the binding site. In contrast, glycine and alanine are found at position 478 in KA1 and KA2, respectively; glycine, alanine and serine are present in the bacterial periplasmic binding proteins [43–46]. If the main-chain carbonyl oxygen atom of residue 477 in KA1–2 were oriented as in the periplasmic binding proteins (although the Cα atom backbone, residues 477–479 in GluR2 (proline), closely superposes with the corresponding amino acids in the periplasmic binding proteins (glycine, alanine, serine) referenced above, the main-chain carbonyl oxygen atoms at position 477 do not), the carbonyl oxygen atom would point towards Tyr450, forcing the phenolic side chain to alter its conformation (Fig. 3). In turn, reorientation of Tyr450 could help to shield the isopropenyl group of KA from the surrounding solvent molecules leading to the higher affinity observed for KA to KA1–2 in comparison with other iGluRs (Table 1).

2.5. The sequence differences at positions 654 and 480: small effects on ligand binding

The natural ligand, GLU, binds to AMPA receptors, and to GluR5 and KA1–2 with approximately the same affinity (200–480 nM), but three to eight times weaker binding is observed for GluR6 (3100 nM) and GluR7 (1100 nM) [47] (Table 1). When we compared the residues that interact with GLU in the rat GluR2-GLU complex X-ray structure [20] with those in the model structures, only one residue at position 654 in helix F differs between the AMPA and the KA receptors (Fig. 2). Serine is found at sequence position 654 in GluR1–5 and KA1–2, while alanine is found in GluR6–7. The hydrogen-bonding network in GluR2, in which the hydroxyl group of serine interacts with GLU via a water molecule, would be disturbed when the hydrophobic side chain of alanine replaces serine at position 654, resulting in lowered affinity for GLU in GluR6–7. Mutation of serine to alanine in GluR4 resulted in reduced binding for AMPA (>3-fold), GLU (∼3-fold) and KA (<1.5-fold), as well as for two antagonists, DNQX (∼1.5-fold) and Ro 48-8587 (>1.5-fold) [15].

In the GluR2 crystal structures, the side-chain hydroxyl group of threonine at position 480 accepts a hydrogen bond from the α-amino group of the ligand. In the kainate receptor GluR6, threonine is replaced by alanine whose methyl side chain cannot accept a hydrogen bond. Consequently, those ligands that have an α-amino group should bind to GluR6 worse than to the other GluRs. For most agonist ligands this is not a critical interaction, but for GLU, which overall has fewer interactions with receptor in comparison with most other ligands, a lower binding affinity would be expected. As discussed above, the presence of alanine at position 654 in helix F would itself lead to a lowering of the binding affinity of GLU for GluR6. Nonetheless, GluR7, which also contains alanine at position 654 but in common with the other receptors has threonine at position 480, can be used to study the effect of alanine at position 480 in GluR6. Indeed, the affinity of GLU for GluR6 is lowered by a factor of 3 in comparison to the affinity of GLU for GluR7 (Table 1). This sequence difference should not affect the binding of KA and its derivatives, since they have only two hydrogen atoms at the nitrogen equivalent to the α-amino group of GLU.

2.6. Selective ligand binding to GluR5 due to sequence differences at positions 708 and 702

The Met708-site is formed from hydrophobic side chains (Met708 and Pro478) and polar groups (the side-chain carboxylate group of Glu402; the side-chain OH groups of Tyr450, Thr707 and Tyr732; and the main-chain oxygen atom of Pro478). The polar residues form a network of conserved hydrogen bonds (Fig. 4) that fixes the side-chain orientations in that region (and no hydrogen bond donors are available for ligand binding). Due to the lack of hydrogen bond donors, this region is unsuitable for fully/partially negatively charged atoms on a ligand. Thr707 is hydrogen bonded to Tyr732, which is in turn hydrogen bonded to Glu705; these residues are also present in the other AMPA and KA receptor subunits.

Met708, present in GluR1–4 and KA1–2, has a highly flexible side chain and is the only residue whose side chain changes position in the different X-ray structures solved for rat GluR2 (e.g., the isoxazole ring of AMPA forces methionine to be positioned further away from the center of the binding site than is seen for GLU and KA). In GluR5–7 methionine is replaced by serine/threonine, thus changing both the size and the polarity of the binding site (the serine side chain is ∼50 $Å^3$ smaller than the methionine side chain). Ligands having bulkier groups, such as ATPA and 5-I-willardiine, would be able to take advantage of this additional space in GluR5–7.

In the ATPA [26] and (*S*)-2-amino-3-(3-hydroxy-5-(2-methyl-2*H*-tetrazol-5-yl)4-isoxazolyl)propionic acid (2-Me-Tet-AMPA) [12] GluR2 complex structures, at the Met708-site, a water molecule donates hydrogen bonds to the hydroxyl groups of Tyr405 and Tyr732 and accepts a hydrogen bond from the hydroxyl group Thr707. Nielsen *et al.* [16] have modeled the GluR5 structure and predicted

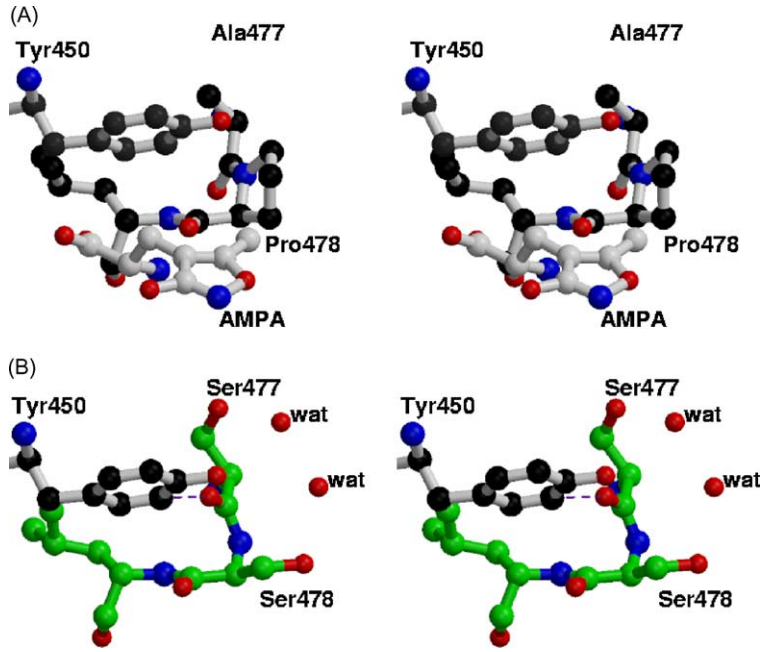

Fig. 3. Sequence differences at position 478 may alter the position of the main-chain oxygen atom of position 477, affecting the conformation of Tyr450 and thus the shape of the ligand-binding pocket. A backbone-constraining proline residue is found in GluR1–7, while glycine and alanine are found in KA1 and KA2. (A) Structure, in stereo, near Pro478 in the GluR2 structure (PDB code: 1ftm; AMPA is shown with white carbon atoms). (B) Like KA1–2, a nonproline residue, serine, is found at the equivalent position in the bacterial periplasmic lysine-arginine-ornithine binding protein (LAO; PDB code: 1lst; green carbon atoms); in LAO the main-chain oxygen atom of the previous residue points in the opposite direction of that in GluR2. If KA1–2 have a similar main-chain conformation as in LAO, then Tyr450 (black carbon atoms) positioned as in the GluR2 structure would clash (dotted line) with the main-chain oxygen atom at position 477; thus Ty450 in KA1–2 would need to alter its conformation. Two water molecules (wat, red balls) in LAO are not found in the GluR2 structure. The figure was produced using BODIL, MOLSCRIPT [63], and Raster3D [64]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that a water molecule could be bound to the serine located at position 708. This is very likely and would explain some of the ligand-binding specificity of certain ligands: we also suggest that this water molecule accepts a hydrogen bond from the hydroxyl group of serine and that the side-chain oxygen atom of serine then points towards the center of the ligand-binding cavity. Consequently, GluR5 would not be able to bind favorably any ligand where a hydrogen bond acceptor points towards Ser708 (e.g. the oxygen atom of the isoxazole ring of AMPA and ACPA).

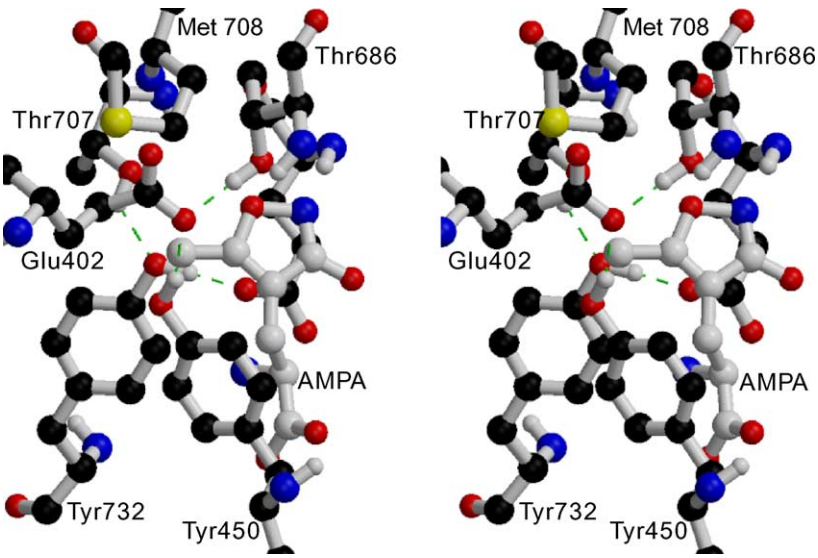

Fig. 4. The Met708-site (in stereo), where hydrogen bonding (green dotted lines) among side chain hydroxyl and carboxylate groups means there are no free hydrogen bond donor groups available for ligands. AMPA (white carbon atoms) bound to GluR2 is shown (PDB code: 1ftm); only polar hydrogens are shown. The figure was produced using BODIL, MOLSCRIPT [63], and Raster3D [64]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Although ATPA (Fig. 6) is an AMPA-analogue (where the *tert*-butyl group replaces the CH_3 group attached to the isoxazole ring), it is a selective ligand for GluR5 KA receptor (see Table 1). In GluR5, the small amino acid serine is found at position 708. In the AMPA receptors and in KA1–2, the presence of the larger methionine side chain makes it difficult to accommodate the *tert*-butyl group of ATPA. Threonine is found in GluR6–7 but ATPA binds only poorly: as with AMPA, the binding of ATPA would be obstructed by the asparagine at position 686 in GluR6–7 (see below). The recently published crystal structure of GluR2 in complex with (*S*)-ATPA [26] is in agreement with these proposals.

The Met708-site is particularly relevant to the willardiines and Br-HIBO. A set of willardiines (5-F-, 5-Cl-, 5-Br-, and 5-I-willardiine; Fig. 6) was docked to the model structures. The binding motif of the willardiines is very similar to the binding motif seen for AMPA in the X-ray structure of the rat GluR2 complex. In the willardiines, the carbonyl oxygen atom at ring-position 2 is equivalent to the distal negatively charged oxygen atom of AMPA. A halogen at ring-position 5 in the willardiines can interact with the Met708-site. When the effects of different halogens attached to position 5 of the willardiine ring are compared (Table 1), the order of the binding affinity in AMPA receptors is F > Cl > Br > I, which shows that a small substituent (fluorine) is better than a bulky one (iodine). Fluorine occupies approximately the same space as the methyl group in AMPA, whereas the much bigger iodine needs to go further away from the center of the binding site. This result, where ''size matters'', is in good agreement with the results for ATPA binding, but the differences in the binding affinities among the different halogens, i.e. the fluoro derivative is only 11 times better than the iodo derivative (Table 1), is not as large as one would expect based on size arguments alone: fluorine is also very electronegative and the Met708-site is filled with partially negatively charged oxygen atoms. Furthermore, the introduction of an electron-withdrawing group at position 5 might lead to a reduction in the pK_a of the ligand and consequently an increased affinity towards the AMPA receptor [39].

In contrast to GluR1–4, in GluR5 the small side chain of serine at position 708 would lead to a larger cavity accommodating bulkier groups (e.g. iodine). Iodine is less electronegative than the other halogens, thus it can locate itself between partially negatively charged oxygen atoms of the receptor: as a substituent of willardiine at the 5-position, iodine is 8000 times better than fluorine (Table 1; [48]). The conformation of the willardiines and the role of the halogen described here are in good agreement with studies on the binding of halogenated willardiines to GluR1 [10], the very recently released GluR2 structures with bound halogenated willardiines [25], and with the structure–activity studies for these ligands very recently reviewed by Johansen *et al.* [39].

Bromine in Br-HIBO would not be able reach as far as the iodine atom at the 5-position in 5-I-willardiine. In both GluR1 and GluR2, the bromine atom of Br-HIBO would fill the volume in this region better than any of the other halogens. Our docked conformation of Br-HIBO is in good agreement with the conformation seen in the recently published crystal structure of GluR2–Br-HIBO complex [12]. (Very recently, receptor affinity data have been reported for Cl-HIBO to GluR1–6 and the results are very similar to those obtained for Br-HIBO [49].)

2.7. Selective binding of some GLU derivatives to GluR5,7: sequence differences at positions 650 and 702

At position 650, leucine is present in the AMPA receptors, while valine, having a more rigid side chain, is found in GluR5–7. Because of this sequence difference, the (2*S*,4*R*)-isomer of 4-methyl glutamate, SYM2081, is one ligand that is selective for KA receptors [50]. Valine can interact favorably with the methyl group of SYM2081, while in AMPA receptors leucine would locate to the same space where the methyl group of SYM2081 would be optimally positioned. Similarly, the natural ligand dysiherbaine, similar in structure to glutamate but modified at the 4-position, binds four to six times better to KA receptors than to AMPA receptors [51]. Other methylglutamate compounds with the methyl group at the 2- or 3-position are inactive [52].

The role of the sequence difference Tyr702 → Phe702 between GluR1–2 and GluR3–4 has been discussed in several publications [8,15]. The importance of position 702 for ligand binding is supported by the crystal structures of GluR2 with the Tyr → Phe mutant [12], even though the residue at position 702 does not have a direct contact with the ligands bound in the wild type and mutant structures.

In the KA receptors, leucine is present at position 702 in GluR5,7 and phenylalanine is found in GluR6 and KA1–2. The leucine side chain, unlike bulky phenylalanine, would allow GluR5,7 to accommodate very bulky agonist ligands such as the 4-cinnamyl derivatives of GLU (Fig. 5). The attached 3-phenyl-prop-2-enyl group, or its modifications, plays a key role in the observed GluR5 selectivity (Table 1). Only GluR5 or GluR7 could accommodate this bulky hydrophobic part of these GLU derivatives (Fig. 5B and C). However, in the case of GluR7, the carbonyl oxygen atom of the Asn686 side chain would effectively reduce the ligand binding, both sterically and by having an unfavorable polar (receptor)—hydrophobic (ligand) contact (Fig. 5C).

The selectivity of GLU derivatives for GluR5 (Table 1) increases with the bulkiness of the group (methyl → 3-naphthyl-prop-2-enyl) attached to position 4 of L-glutamic acid (chirality as in (2*S*,4*R*)-methylglutamic acid). In agreement with these experimental observations, when we manually docked 3-phenyl-prop-2-enyl-glutamic

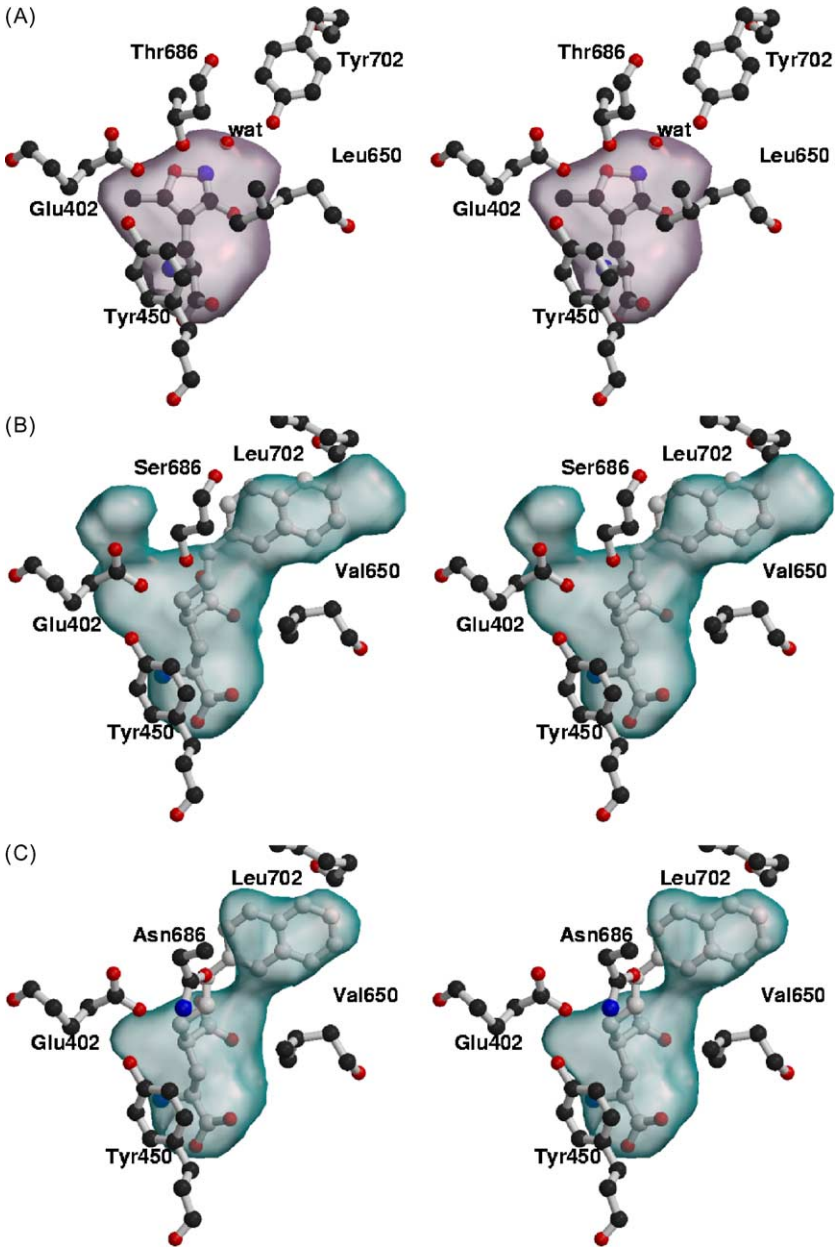

Fig. 5. Selectivity of cinnamyl compounds can be explained by sequences differences at positions 650, 686, and 702. (2*S*,4*R*,*E*)-2-Amino-4-(3-(2-naphthyl)prop-2-enyl)pentanedioic (LY339434) acid binds only poorly to GluR7 in comparison with GluR5 and not at all to the other AMPA and KA receptors. At position 650, leucine is present in the AMPA receptors while smaller, rigid valine is found in GluR5–7. With the exception of GluR5 and GluR7, in all other receptors Leu702 is replaced by a bulky side-chain—either tyrosine or phenylalanine—that prevents the ligand from binding, as illustrated by the differences in the binding cavity (in stereo) of panel (A). GluR2-AMPA structure (PDB code: 1ftm) (pink transparent surface) and the modeled binding cavities (cyan transparent surface) of panel (B), GluR5, and panel (C), GluR7, where docked LY339434 fills the cavity between Val650 and Leu702. The smaller side chain of Ser686 in GluR5 is replaced in GluR7 by the larger asparagine, which would lead to steric clashes and unfavorable interactions with the nearby CH group of the docked ligand, reflected by the differences in the observed affinities (see Table 1); the amide oxygen atom of Asn686 in GluR7 would be located close to the position of a water molecule (wat, red ball) seen in the GluR2-AMPA crystal structure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acid to the GluR5 model structure, the phenyl group partly fills the unoccupied space near Leu702, while the 3-(2-naphthyl)prop-2-enyl group fills the cavity fully (Fig. 5B). Completely hydrophobic or negatively charged groups at the *para*-position of the phenyl ring are not suitable because of the close proximity of the partially negatively charged main-chain carbonyl oxygen atom of Gly670. Pedregal *et al.* [53] did show, however, that a chlorine or bromine atom at the *para*-position is a reasonable choice, but fluorine is too electronegative and iodine is too bulky. We would predict that methylamine ($NH–CH_3$) attached at the *para*-position or the complete replacement of the phenyl ring with an indole ring system attached from ring-position 5 would donate one additional hydrogen bond to the main-chain oxygen atom of Gly648 and fill the cavity better than a phenyl group.

2.8. Differences between GluR6–7 and other AMPA and KA receptors at position 686: reduced binding affinity for several agonist ligands

When agonist ligands are bound to GluR2 in the X-ray structures, an important stabilizing interaction between the domains occurs: Glu402 in S1 accepts a hydrogen bond from the amino acid at position 686 in S2, otherwise there are few other direct interactions between S1 and S2 near the binding pocket. In all AMPA receptors, as well as in GluR5 and KA1–2, there is either threonine or serine at position 686 and the side-chain OH group can donate a hydrogen bond to Glu402. Asparagine is present at position 686 in GluR6 and GluR7 and the side-chain NH_2 group would serve to hydrogen bond to Glu402. As a result, the side-chain carbonyl oxygen atom of asparagine would point towards the ligand-binding site and interfere with the binding of several ligands. In Fig. 5C, the carbonyl oxygen atom from Asn686 (GluR7) occupies the same space as the water molecule seen in the crystal structure of GluR2-AMPA complex (Fig. 5A). Consequently, the partly negatively charged carbonyl oxygen atom of asparagine would be repulsed by contact with the negatively charged atoms in the isoxazole ring of AMPA.

Consistent with the above prediction, the mutation Ser686Asn in GluR5 reduces the binding of AMPA [54] and 5-I-willardiine [55], suggesting that the polar atoms of the asparagine side chain do indeed adversely affect ligand binding. In addition to these two ligands, it is very likely that asparagine affects the binding of all ligands having a ring structure occupying the space equivalent to the isoxazole ring of AMPA (e.g. ATPA and ACPA, see Fig. 6). Furthermore, in the study of S1S2 chimeras formed between GluR4 and GluR6 [56], AMPA binding is specifically lost when residues 662–722 originated from GluR6, (thus, having asparagine at position 686). We predict, however, that Asn686 does interact with kainate in GluR6–7 and this notion is supported by the mutation Asn686Ser in GluR6, which decreases the affinity 5-fold towards kainate [54], and supporting Asn686 as the likely candidate for the residue in GluR6 that is irreversibly labeled by the photoactivatable analogue of kainate, DZKA: (2′*S*,3′*S*,4′*R*)-2′-carboxy-4′-(2-diazo-1-oxo-3,3,3-trifluoropropyl)-3′-pyrrolidinyl acetate [57].

2.9. Variation at position 650: kainate binding

In the GluR2 X-ray structure, the isopropenyl portion of the ligand KA occupies the space between Leu650 and Tyr450. The replacement of Leu650 by valine (valine is not as flexible as leucine and its conformation is unlikely to differ from that in our model) in GluR5–7 would make

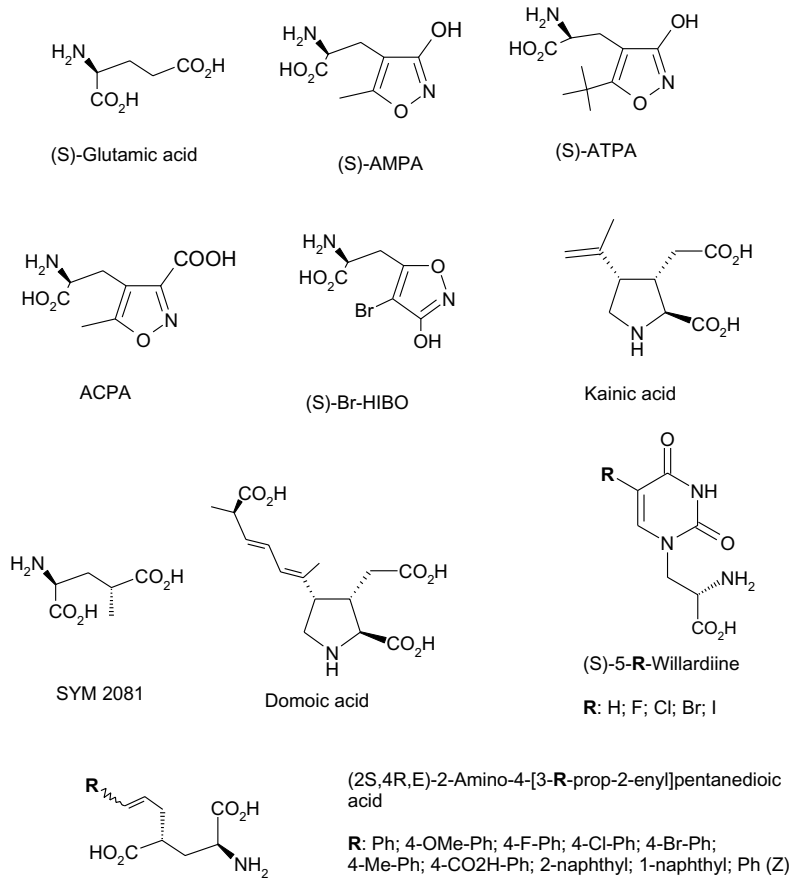

Fig. 6. Structures of the 23 agonist ligands considered in this study.

additional space available for ligands or lead to further domain closure such as that observed in the Leu650Thr mutant structure of GluR2 with bound KA [18]. Instead of having either leucine (GluR1–4) or valine (GluR5–7) at position 650, KA1–2 have isoleucine. Isoleucine at position 650 in KA1–2 is an ideal amino acid for binding KA because it can optimize the hydrophobic interactions both with Phe702 and bound KA, unlike either leucine (GluR1–4) or valine (GluR5–7). Nielsen and Liljefors [58] have used conformational analysis in aqueous solution to propose that the conformation of bound kainate is similar in the AMPA and KA receptors, suggesting that the observed affinity differences among the receptors primarily result from differences in the receptor–ligand interactions.

A variety of modifications to the isopropenyl group (C4-position) of KA have been introduced [5,39,59–61]. As seen, for example, in domoic acid (Fig. 6) and in 4-(2-methoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (MFPA)/4-(2-hydroxy)-2-carboxy-3-pyrrolidineacetic acid (HFPA) [59], despite having a large substituent the ligand remains a specific agonist for KA receptors. The stereochemistry is the same as in kainate and in each case the substituent can still favorably interact with Tyr450—both experimental [60,61] and theoretical [62] studies suggest that receptor interactions with the π-system of the C4 substituent of ligands plays an important role. Domoic acid binds with even higher affinity than KA to the KA receptors (Table 1). The third negatively charged group of the 2-methylvaleric acid moiety of domoic acid, which replaces the methyl group of the isopropenyl group in KA, should interact favorably with amino acids located outside of the binding pocket. For example, in GluR1–4 and GluR5–7, methylvaleric acid can interact with the positively charged side chain of Lys449. Indeed, the binding affinity of domoic acid is higher than that of KA for these receptors. In KA1/KA2, position 449 is occupied by valine/leucine residues that cannot interact with the acidic group of methylvaleric acid, consistent with the similar binding affinities of domoic acid and KA (Table 1).

3. Conclusions

The mechanism by which the sequence differences between AMPA and KA receptor subunits give rise to differences in ligand selectivity can be rationally explained in structural terms. Even a single sequence difference between two receptors can change the ligand-binding affinity dramatically. In total, there are seven residues surrounding the binding cavity that affect agonist selectivity either directly or via bridging water molecules: in GluR2, these residues are Pro478, Thr480, Leu650, Ser654, Thr686, Tyr702, and Met708. As described above, the similarity among AMPA receptor subunits coupled with the known structures of GluR2 in complex with numerous ligands allows us to suggest possible roles for bound water molecules based on the published crystal structures. In the KA receptors, however, the presence of several sequence differences makes it more difficult to locate potential sites for key water molecules within the binding site. Furthermore, several sequence differences can change the ability of a receptor to shield ligands from the surrounding solvent by opening channels for water molecules (Leu650Val/Ile, Tyr/Phe624Leu, Met708Ser) or by altering the degree of domain closure on ligand binding (Leu650Val/Ile). Taken together, computer-based analyses combined with published data on ligand binding to *homomeric* receptors provide a basis for understanding receptor-ligand selectivity and insight applicable to the design of novel selective ligands.

Acknowledgments

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