Discrimination between Agonists and Antagonists by the α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid-selective Glutamate Receptor

A MUTATION ANALYSIS OF THE LIGAND-BINDING DOMAIN OF GluR-D SUBUNIT

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The crystal structures of the ligand-binding core of the agonist complexes of the glutamate receptor-B (GluR-B) subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective glutamate receptor indicate that the distal anionic group of agonist molecules are stabilized by interactions with an N-terminal region of an α-helix (helix F) in the lobe 2 (“domain 2,” Armstrong, N., and Gouaux, E. (2000) Neuron 28, 165–181) of the two-lobed ligand-binding domain. We used site-directed mutagenesis to further analyze the role of this region in the recognition of both agonists and antagonists by the AMPA receptor. Wild-type and mutated versions of the ligand-binding domain of GluR-D were expressed in insect cells as secreted soluble polypeptides and subjected to binding assays using [3H]AMPA, an agonist, and [3H]Ro 48-8587 (9-imidazol-1-yl-8-nitro-2,3,5,6-tetrahydro[1,2,4]triazolo[1,5-c]quinazoline-2,5-dione), a high affinity AMPA receptor antagonist, as radioligands. Single alanine substitutions at residues Leu-672 and Thr-677 severely affected the affinities for all agonists, as seen in ligand competition assays, whereas similar mutations at residues Asp-673, Ser-674, Gly-675, Ser-676, and Lys-678 selectively affected the binding affinities of one or two of the agonists. In striking contrast, the binding affinities of [3H]Ro 48-8587 and of another competitive antagonist, 6,7-dinitroquinoxaline-2,3-dione, were not affected by any of these alanine mutations, suggesting the absence of critical side-chain interactions. Together with ligand docking experiments, our results indicate a selective engagement of the side chains of the helix F region in agonist binding, and suggest that conformational changes involving this region may play a critical role in receptor activation.

The ligand-binding site of ionotropic glutamate receptors is composed of two extracellular segments, S1 and S2, homologous to bacterial amino acid-binding proteins (1–3). Recent determination of the crystal structure of an S1S2 fusion protein of the Glu-B1 (GluR2) α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit as complexed with ligands (4, 5) provided the first atomic resolution view into a neurotransmitter-binding pocket and confirmed the earlier predictions of a close structural and functional similarity to the bacterial proteins (1–3, 6–9). The agonists kainate, glutamate, and AMPA are engaged in multiple polar and van der Waals contacts that stabilize a closed state of the two-lobed binding domain (4, 5). The charged α-aminocarboxylate group of glutamate and AMPA and the pyrrolidine carboxyl and imino groups of kainate are accommodated by hydrogen bonds and ion pair interactions with the oppositely charged side chains of Arg-485 and Glu-705, respectively. The distal negatively charged group of the agonists, the carboxylmethyl group of kainate, the γ-carboxylate of glutamate, or the isoxazole ring hydroxyl of AMPA, make close polar contacts with the base of an α-helix (“helix F”) in the lobe 2, but the exact hydrogen bonding patterns differ between agonists (4, 5). In contrast to the agonist complexes, the ligand-free apo form and one antagonist complex (6,7-dinitroquinoxaline-2,3-dione (DNQX)) of GluR-B S1S2 assume a more open state, suggesting that agonist activity is based on their ability to induce a slight closure of the lobes (5).

Although the stereochemical features of the agonist-receptor interaction revealed by the crystal structure are largely supported by mutagenesis data, not much is yet known on how the receptor discriminates between agonists and antagonists or on how structurally different antagonists interact with the receptor. In particular, it is currently unclear to what extent the contacts antagonists make with the receptor overlap those made by agonists. Although previous mutagenesis work has identified amino acid residues that affect antagonist affinities in the AMPA receptor, for example Lys-471 and Tyr-472 in GluR-D (9), no mutations that would selectively affect only agonists or antagonists have been reported. As a step toward understanding the structural basis of discrimination between agonists and antagonists by AMPA receptors, we have analyzed the ligand binding properties of the mutated GluR-D ligand-binding domain constructs by using [3H]AMPA, an agonist, and [3H]Ro 48-8587, a high affinity AMPA receptor an-

The abbreviations used are: GluR, glutamate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; Ro 48-8587, 9-imidazol-1-yl-8-nitro-2,3,5,6-tetrahydro[1,2,4]triazolo[1,5-c]quinazoline-2,5-dione.
agonist (10), as radioligands. The results from our ligand binding and molecular docking experiments indicate a selective engagement of the helix F region in agonist binding, suggesting that conformational transitions involving this region are involved in receptor activation.

EXPERIMENTAL PROCEDURES

Chemicals—[^1]H]AMPA (specific activity, 60 Ci/mmol) was obtained from PerkinElmer Life Sciences. [^1]H]Ro 48-8587 (specific activity, 44 Ci/mmol) was obtained from Amersham Biosciences. Unlabeled RS-AMPA and kainate were obtained from Sigma. Unlabeled Ro 48-8587 is a kind gift from Drs René Wyler and Vincent Mutel (Hoffmann-La Roche).

Site-directed Mutagenesis—The residues from Leu-672 to Lys-678 in the ligand-binding domain of GluR-D (flip isoform; see Ref. 11) were individually replaced by alanine residues by using a PCR-assisted mutagenesis approach. The plasmid pK503-4 (3) encoding the wild-type GluR-D ligand-binding domain (S1S2) carrying an N-terminal FLAG tag was used as a template. The mutations were generated by using a common 5'-primer (5'GGGGGGGCTATAGGTTATTCGGTTCA-GCTATAATTATGAGGATTATTTGCAGG-3') and the following muta-
tion-specific 3'-primers: 5'-GGGGGGGCTATAGGTTATTCGGTTCA-GCTATAATTATGAGGATTATTTGCAGG-3' (L672A), 5'-GGGGGGGC-TATAGGTTATTCGGTTCA-GCTATAATTATGAGGATTATTTGCAGG-3' (D673A), 5'-GGGGGGGCTATAGGTTATTCGGTTCA-GCTATAATTATGAGGATTATTTGCAGG-3' (D674A), 5'-GGGGGGGCTATAGGTTATTCGGTTCA-GCTATAATTATGAGGATTATTTGCAGG-3' (T677A), and 5'-GGGGGGGCTATAGGTTATTCGGTTCA-GCTATAATTATGAGGATTATTTGCAGG-3' (T678A). The PCR products were digested with NcoI and EcoRI and ligated into a similarly treated pK503-4 vector. The presence of all of the designed mutations was confirmed by DNA sequencing.

Expression in Insect Cells—Recombinant baculoviruses were generated by using the Bac-to-Bac system (Invitrogen) and used to infect Trichoplusia ni (High Five, Invitrogen) cells growing in T-25 culture flasks as described previously (9). The cells and culture media were harvested 3–4 days after infection.

Radioligand Binding—The culture supernatants were extensively dialyzed against 20 mM Tris-HCl, pH 7.2, 2.5 mM CaCl2, 100 mM KSCN (AMPA binding buffer; see Ref. 12), or 50 mM Tris-HCl, pH 7.0 (Ro 48-8587 binding buffer; see Ref. 10) to remove endogenous glutamate. Dialyzed samples (20–100 μl) were equilibrated with [^1]H]AMPA (5 nM) or [^1]H]Ro 48-8587 (1 nM) in the respective binding buffers for 1 h on ice in a total volume of 0.5 ml. The bound and free radioactivity were separated by filtration through a 0.2-μm filter. The free radioligand concentration was determined by liquid scintillation counting in OptiPhase (Wallac). Apparent dissociation constants (Kd) were calculated using the program LIGAND.

Ligand Docking—AUTODOCK 3.0 (16) is a semirigid docking program that considers the whole ligand molecule in the conformational search at the binding site, and it is possible to choose the torsion angles of the ligand that are allowed to rotate, whereas bond angles and bond length are kept fixed. The overall interaction energy between chemical species is estimated by considering both Lennard-Jones atom-atom potentials and electrostatic effects, summed for the individual interactions between atoms. Partial charges for the receptor model were calculated using the AMBER force field (17, 18) in SYBYL. For ligands, the semiempirical molecular orbital method PM3 (19) from the MOPAC package within SYBYL was used. This semiempirical method considers only the valence-shell electrons explicitly. The interaction of a probe corresponding to any type of atom in the ligand) with a receptor model was calculated, using the program AUTOGRID in the AUTODOCK package, at grid position 0.25 Å apart in a 20 × 20 × 20 Å box centered at the binding site. For each ligand, 10 separate docking simulations were performed.

Receptor Mapping Studies—Atomic and chemical group affinity maps in the binding site were calculated with GRID 19 (20). This program evaluates interaction energies between various test probes and the target protein molecule, predicting where a particular chemical probe interacts most favorably within the receptor-binding site. For the calculation, a cubic box (15 × 15 × 15) Å3 was centered in the binding site, and residue side-chain flexibility was allowed.

Other Methods—SDS-PAGE and Western blotting were performed as described previously (3, 9). Protein concentrations were measured by using the bicinchoninic acid assay (Pierce) as described by the manufacturer.

RESULTS

The N-terminal end of an α-helix (helix F) and the preceding residues in the S2 segment of the ligand-binding domain of the AMPA receptor form a major region of contact for the distal anionic group of agonists (subsites D, E, and F in the crystal structure of GluR-B S1S2-agonist complexes; see Ref. 5). In the present study, we have used site-directed mutagenesis and direct binding experiments with radiolabeled agonist and antagonist compounds to further examine the role of this region in ligand recognition. Mutated GluR-D S1S2 constructs having an alanine substituting individually for residues from Leu-672 to Lys-678 were expressed in recombiant baculovirus-infected models using SYBYL. To optimize the intramolecular interactions in the receptor models, hydrogen atoms were minimized (keeping the rest of the model rigid) using AMBER charges.

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High Five cells and appeared as a 40–42-kDa anti-FLAG immunoreactive species in the culture supernatants (Fig. 1). Comparison of secreted and cell-associated immunoreactivities revealed no major differences in the expression levels or in the relative amount of secreted protein, suggesting that the mutations do not cause any gross misfolding of the respective S1S2 proteins (Fig. 1B). The relative amounts of the 42-kDa- and 40-kDa- secreted species, probably representing two differently glycosylated forms, were variable but did not show any consistent differences between the mutant and the wild-type polypeptide.

To separately address the agonist and antagonist binding properties of the mutated S1S2 proteins, we used [\(^{3}H\)]AMPA, a high affinity agonist, and [\(^{3}H\)]Ro 48-8587, a recently described high affinity competitive AMPA receptor antagonist (10), as radioligands. First, the ability of the S1S2 mutants to bind [\(^{3}H\)]AMPA was determined in a qualitative binding assay using a single 100 nm concentration, which is saturating for the wild-type S1S2. Clear binding activity was observed for the mutants D673A, S674A, S676A, and K678A, whereas no binding above the nonspecific background measured in the presence of 1 mM glutamate was observed for the mutants L672A, G675A, and T677A (Fig. 2). The [\(^{3}H\)]AMPA binding properties of the four active mutants were analyzed in more detail by using competition experiments with unlabeled AMPA and L-glutamate. The apparent affinities for AMPA (D673A, S676A, and Lys-678 had only minor effects (Tables I and II). Generally, we found these findings to be in good agreement with a reported value for glutamate as 97 \(\mu M\); 78-fold decrease in affinity) and T677A (\(K_f\) 4.8 mM; 3840-fold decrease). Interestingly, the G675A mutant, which did not bind [\(^{3}H\)]AMPA, displayed a similar \(K_f\) value for glutamate as the wild-type S1S2 (Table II). Kainate inhibited [\(^{3}H\)]Ro 48-8587 binding to all S1S2 proteins at micromolar concentrations. Significantly reduced affinities were observed with the L672A (26-fold) and T677A (13-fold) mutants, whereas somewhat smaller affinity decreases were seen with the G675A and K678A mutants (Table II).

In additional experiments, the ability of unlabeled AMPA to displace [\(^{3}H\)]Ro 48-8587 binding to the “AMPA-negative” S1S2 mutants was determined. L672A, G675A, and T677A mutant S1S2 polypeptides were tested. In all three cases, severe, −2000-fold (L672A), −300-fold (G675A), and −11,000-fold decreases in the binding affinity were observed (Table II), consistent with the failure to see any [\(^{3}H\)]AMPA binding activity in the filtration assay. Thus, mutations in the helix F region seem to selectively affect agonist affinities in an agonist-specific manner, with little or no effect on the binding of two different competitive antagonists. This striking behavior is illustrated in Fig. 3, which shows the inhibition of [\(^{3}H\)]Ro 48-8587 binding to L672A, G675A, and T677A by unlabeled agonist and antagonist compounds. These findings suggest that in contrast to AMPA receptor agonists, neither DNQX nor Ro 48-8587 does interact with the side chains of residues 672–678.

TABLE I

| Construct | \(K_f\) AMPA | \(K_f\) L-Glu |
|-----------|-------------|-------------|
| S1S2      | 24.8 ± 3.65 (n=3) | 0.279 ± 0.365 (n=3) |
| L672A     | NB          | 1.807 ± 0.369*(n=3) |
| D673A     | 65.1 ± 18.8* (n=4) | ND          |
| S674A     | 32.6 ± 3.78*(n=4) | ND          |
| G675A     | NB          | 0.596 ± 0.181*(n=4) |
| S676A     | 92.6 ± 24.1**(n=4) | ND          |
| T677A     | 103 ± 20.8**(n=3) | 0.840 ± 0.124**(n=3) |

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| T677A     | 103 ± 20.8**(n=3) | 0.840 ± 0.124**(n=3) |

Molecular modeling was used to interpret the ligand binding data in terms of the three-dimensional structure of the receptor. Four models of GluR-D S1S2, differing in the degree of domain closure, were built on the basis of the GluR-B S1S2 structures with bound l-glutamate, AMPA, kainate, and DNQX (5). The sequence identity between GluR-D and GluR-B over the S1S2 region is 89.6% with only one difference near the binding site: a phenylalanine residue (Phe-724) in GluR-D replaces a tyrosine residue in GluR-B. AMPA, L-glutamate, and kainate were automatically docked to the respective GluR-D S1S2 model structures (Fig. 4, A–C). The modeled conformations of these ligands are close to those derived from the corresponding crystal structures: the root mean squared deviations over all ligand atoms between the GluR-D model and the structures of GluR-B S1S2 complexes with AMPA, L-glutamate, and kainate were 0.51, 0.64, and 0.60, respectively.

Alanine substitutions at three positions, residues Leu-672, Gly-675, and Thr-677, resulted in clear changes in agonist binding, whereas similar mutations of Asp-673, Ser-674, Ser-676, and Lys-678 had only minor effects (Tables I and II). Generally, we found these findings to be in good agreement...
with the model. Three (Asp-673, Ser-674, and Lys-678) of the four residues that show no gross alterations when mutated do not have any contacts with the agonists. The side chains of Asp-673, Ser-674, and Lys-678 point away from the binding cavity, but a salt bridge with a local stabilizing effect may form between Asp-673 and Lys-678. Such an indirect role is in agreement with the observed, relatively minor affinity changes when these residues were mutated. The main chain amide nitrogens of Ser-676 and Thr-677 carry a partial positive charge due to their location at the N terminus of the helix F dipole and are hydrogen-bonded to the distal anionic group of the agonists. The side chain of Ser-676, however, does not have a direct contact with any of the agonists. Instead, its hydroxyl group is linked via a water bridge (Fig. 4D, w2) to Glu-727 in GluR-D, perhaps explaining the modest decreases in the binding affinities of agonists caused by the S676A mutation (Tables I and II).

Replacement of Leu-672 by alanine resulted in a total loss of [3H]AMPA binding and dramatically lowered the affinities of glutamate and kainate when measured in the [3H]Ro 48-8587 competition binding assay (Table II). In the GluR-B S1S2 structures with bound agonist, the corresponding residue, Leu-650, is found in two different conformations: one present in the AMPA and glutamate complexes and the other one observed in the kainate complex. We suggest that the latter conformation is likely to be relevant for Leu-672 in GluR-D in all three agonist complexes. This conformation of Leu-672 will ideally pack against the aromatic ring of Phe-727 (which substitutes for Tyr-702 in GluR-B), resulting in slightly better contact with the agonists.

**Table II**

| Construct | $K_d$ [3H]Ro 48-8587 (nM) | $K_i$ AMPA (µM) | $K_i$ l-Glu (µM) | $K_i$ kainate (µM) | $K_i$ DNQX (µM) |
|-----------|--------------------------|---------------|---------------|----------------|---------------|
| S1S2      | 15.6 ± 6.56              | 0.027 ± 0.005 | 1.25 ± 0.827  | 3.72 ± 1.07    | 0.200 ± 0.091 |
| L672A     | 20.1 ± 5.89              | 47.4 ± 6.42   | 97.3 ± 14.0** | 95.2 ± 29.9** | 0.136 ± 0.037 |
| D673A     | 9.53 ± 2.31              | ND            | 2.55 ± 1.49   | 1.63 ± 0.20*   | 0.282 ± 0.146 |
| S674A     | 23.8 ± 3.45              | ND            | 1.02 ± 0.07   | 2.36 ± 0.338   | 0.218 ± 0.033 |
| G675A     | 18.4 ± 6.92              | 5.98 ± 2.98   | 1.33 ± 0.404  | 22.5 ± 8.21**  | 0.214 ± 0.163 |
| S676A     | 26.1 ± 9.98              | ND            | 3.49 ± 1.07** | 4.49 ± 1.57    | 0.323**       |
| T677A     | 13.8 ± 1.45              | 230*          | 4800 ± 1400***| 49.2 ± 6.22*** | 0.112 ± 0.029 |
| K678A     | 14.6 ± 0.473             | ND            | 2.14 ± 0.441  | 9.05 ± 2.64**  | 0.169 ± 0.017 |

* The value is from a single experiment.

Fig. 3. [3H]Ro 48-8587 binding properties of wild-type and mutated GluR-D S1S2 constructs. Binding of [3H]Ro 48-8587 to GluR-D S1S2 (○) and three S1S2 mutants that do not have [3H]AMPA binding activity, L672A (■), G675A (●), and T677A (□) was determined in the presence of increasing concentrations of unlabeled agonists (l-glutamate and kainate) or antagonists (DNQX and Ro 48-8587) as indicated. The displacement curves represent a best fit to a one-site model obtained by nonlinear curve fitting.
hydrophobic parts of the agonist ligands (Figs. 4, A–C, and 5). In the case of AMPA, the total loss of binding with the L672A mutation is in agreement with extensive hydrophobic contacts between leucine and both the methylene group and the aromatic/hydrophobic part of the isoxazol ring of AMPA (Fig. 4A).

Furthermore, the considerable extra space created by the alanine substitution is not easily accommodated by side-chain rearrangements and would provide a less than ideal location for a water molecule. Similarly, the impaired binding affinity of L-glutamate for the L672A mutant is consistent with a total or partial loss of hydrophobic contacts with the two methylene groups of the agonist. Interestingly, the bulky isopropenyl group of kainate is predicted to interact favorably with an alanine side chain at position 672, in agreement with the observed moderate (26-fold) decrease in binding affinity with L672A (Table II).

The effects of the alanine substitution at residue Gly-675 were remarkably specific for AMPA binding: despite the total loss of [3H]AMPA binding, the affinities of glutamate and kainate, as measured in the [3H]Ro 48-8587 competition assay, were either
unchanged or slightly decreased, respectively. A close inspection of the water-mediated hydrogen-bonding network in the crystal structures of the three different GluR-B S1S2-agonist complexes reveals that although most water molecules in the binding site occupy essentially identical positions, one water molecule (Fig. 4D, w1) is found near Gly-653 (equivalent to Gly-675 in GluR-D) only in the AMPA complex. This water molecule plays a key role in agonist binding, forming hydrogen bonds with the oxygen atom at the 3-position of the isoxazole ring, shown to be unprotonated (21), to the α-carboxylate of AMPA, and to the main chain NH group of Thr-655 (Thr-677 in GluR-D). The distance between the α-carbon of Gly-653 to the oxygen atom of w1 is 3.3 Å, whereas with the glycine-to-alanine mutation, the distance between the methyl carbon in the alanine side chain and w1 would be only about 2.0 Å (Fig. 4D). Accordingly, the methyl group would displace the water molecule, and in the absence of any alternative mechanism to restabilize the interactions with AMPA, a severe decrease in binding affinity would ensue. Such an effect is not expected for kainate and glutamate, as the respective complexes do not harbor this specific water molecule, and modest torsional rotations of these agonist molecules should easily accommodate the methyl group of alanine without any undue steric interference.

The side-chain OH group of Thr-677 directly interacts with agonists, forming hydrogen bonds with the negatively charged oxygen attached to the isoxazole ring of AMPA and with the distal carboxylate groups of L-glutamate and kainate. This essential role in agonist binding is consistent with the loss of [3H]AMPA binding and the 4000-fold reduction in L-glutamate binding measured for the T677A mutant (Tables I and II). With kainate, only an ~13-fold decrease in affinity was measured in the [3H]Ro 48-8587 competition assay, indicating that hydrogen bond interactions and favorable electrostatic attraction with the main chain amides at residues 676 and 677 provide sufficient stabilization for the distal carboxylate group of the rigid kainate molecule. The loss of one hydrogen bond concomitant with the alanine mutation is much less well tolerated by L-glutamate and AMPA. Glutamate has fewer interactions overall with the receptor than either kainate or AMPA, whereas AMPA has only one negatively charged oxygen atom binding to the N terminus of helix F, and with this mutation, it will entirely lose a key interaction with the receptor.

In complete contrast to the agonists, the alanine substitutions at residues 672–678 did not have any significant effects on the binding affinities of the two antagonists, [3H]Ro 48-8587 or DNQX. The docked conformation of DNQX in the GluR-D model is expected to be very similar to the conformation present in the corresponding GluR-B S1S2 crystal structure (root mean squared deviation of 0.88 Å; Fig. 6), and no close contacts between DNQX and the helix F region are seen. The proposed docked conformation for Ro 48-8587 largely
superimposes with that of DNQX (Fig. 6). In particular, the two-ring systems of the antagonist molecules occupy similar positions and share several hydrogen-bonding interactions with the receptor: in both DNQX and Ro 48-8587, one nitro group interacts with Tyr-754, one carbonyl group interacts with Arg-507, and one amicd nitrogen donates a hydrogen to the carbonyl oxygen of Pro-500. The two vicinal carbonyl groups in DNQX that interact with Arg-507 are replaced in Ro 48-8587 by one carbonyl group and an amicd nitrogen in the triazolyl ring substituent. The hydrogen atom attached to this nitrogen can easily shift to the adjacent carbonyl oxygen, creating a partial negative charge, and therefore, the carbonyl/amicd nitrogen region of Ro 48-8587 is able to interact with Arg-507, much like the two carbonyl groups in DNQX. The imidazolyl ring of Ro 48-8587 fits well into a cavity generated by the binding site residues Thr-708, Leu-726, and Met-730, and a hydrogen bond can form between one ring nitrogen and the side-chain hydroxyl group of Thr-708. These favorable interactions may contribute to the high binding affinity of Ro 48-8587. Importantly, no interactions are seen between Ro 48-8587 and the helix F region.

DISCUSSION

We introduced alanine substitutions into the ligand-binding domain (S1S2) of the AMPA receptor subunit GluR-D in an area that corresponds to a loop (Leu-672–Ser-674) and the N-terminal end of helix F (Gly-675–Lys-678) in the ligand-binding core of GluR-B (4, 5). Due to the cumulative dipole moment of helix F (residues 675–684), which produces a positively charged N terminus, this region forms an excellent interaction site for the negatively charged functional groups of the agonists. Both the invariant α-carboxylates and the distal anionic groups of the agonists, i.e. the γ-carboxylate of glutamate, the carboxylmethyl group of kainate, and the hydroxyl group in the isoxazol ring of AMPA whose proton is nearly dissociated at pH 7 (21), interact with this region in GluR-B S1S2 (5). Consistent with this, our results showed ligand-specific decreases in agonist affinities by alanine mutations at residues 672–678. Interestingly, none of the mutations in this area had any significant effect on the binding affinities of two antagonists, Ro 48-8587 and DNQX, indicating that these antagonist molecules do not have specific side-chain interactions with this region.

Three mutations had particularly striking effects on agonist affinities, which could all be interpreted in the light of the ligand docking models. For leucine 672, the decrease in AMPA and glutamate binding seems to be due to the loss of favorable hydrophobic packing contacts. Interestingly, this residue has been implicated previously as a selective determinant of the nondesensitizing nature of kainate-induced responses (5, 22). The steric clash between the leucine side chain and the isopropenyl group of kainate may prevent the closure of the binding domain to the extent seen with AMPA and glutamate in the respective GluR-B S1S2 complexes (5). Although a more complete closure should be possible with alanine at this position, it does not appear to lead to a better overall stabilization of the closed complex, as suggested by the reduced binding affinity of kainate to the L672A mutant.

The G675A mutation results in a relatively selective loss of AMPA binding with only a modest effect on kainate and no effect on glutamate. A water molecule that is uniquely present in the GluR-B S1S2-AMPA complex (5), and participating in agonist binding, would be expelled by the methylene side chain of alanine, leading to the elimination of an important water-mediated interaction. In contrast, the mutation T677A strongly affected the binding of all three agonists. The side chain of this threonine has direct hydrogen bonding interactions with the agonists, and noticeable effects on agonist affinities by mutations at the corresponding residue have been reported previously (6, 23).

Our results are generally consistent with the analysis of the crystal structures of the GluR-B S1S2-ligand complexes (4, 5), and thereby, provide strong support for the relevance of these structures as models of receptor-ligand interaction in the AMPA receptor. Although the crystallographic data clearly support an agonist-induced domain closure as the mechanism that allosterically couples ligand binding to channel opening (5), currently there is still no generally accepted and comprehensive structural explanation for the unique ability of agonist compounds to activate the receptor. For example, small-angle x-ray scattering data on GluR-D S1S2 in the glutamate-occupied and free state are not consistent with any larger cleft closure induced by the agonist (25). Relatively smaller, local conformational changes induced by the agonist have been proposed as an alternative explanation for receptor activation (26, 27). Irrespective of the model, however, our findings indicate that agonists selectively recruit side chains of the helix F region to stabilize the ligand-receptor complex, whereas antagonists do not necessarily interact with this region at all. In the native receptor, both the C terminal of S1 and the N terminus of S2, which flank the M1–M3 channel domain in the native receptor channel, are located in lobe 2 and are in the vicinity of the helix F. Therefore, even small conformational changes in this region may, in principle, exert an influence on the channel gating mechanism.

In conclusion, alanine substitutions at residues preceding and including the N terminus of helix F in the ligand-binding domain of the GluR-D AMPA receptor drastically affect agonist binding with little or no affect on two different antagonists. Analysis of the likely docking modes of AMPA receptor ligands in the binding site provides plausible explanations for the striking and agonist-selective effects of the mutations.

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