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The importance of two specific domains in ligand binding to the AMPA/kainate glutamate receptors GluR2 and GluR6

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Abstract Chimeric receptor subunits of the AMPA receptor subunit GluR2 and the kainate receptor subunit GluR6 were constructed and stably expressed in baby hamster kidney cells. By using Ca^{2+} imaging and radioligand binding, we demonstrated that substitution of a specific domain showing homology to a bacterial leucine–isoleucine–valine binding protein (LIVBP) had no effect on the affinities of the tested agonists, but decreased the affinities of the antagonists CNQX, DNQX, and NBQX. On the other hand, when the first of two domains showing homology to a bacterial glutamine binding protein (QBP) in GluR2 was substituted with the corresponding region from GluR6, the affinity of AMPA decreased sevenfold and the affinity of kainate increased fourfold, indicating the importance of this domain in binding of these agonists. In contrast to this, the affinities of quisqualate and domoate, two other agonists, were unchanged, indicating that a region located C-terminal to the QBP domain is also involved in agonist binding.

Key words: Glutamate receptor; AMPA; Kainate; Ligand binding site

1. Introduction

L-Glutamate (L-Glu) is the most prevalent excitatory neurotransmitter in the central nervous system and is believed to play a key role in the mediation of fast synaptic transmission. Furthermore, L-Glu is probably involved in memory and learning, as well as in several neurological diseases [1,2].

Receptors for L-Glu are classified into ionotropic and metabotropic receptors. Based on pharmacology and electrophysiology, the ionotropic receptors have been further subdivided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptors [3]. At present, four AMPA receptor subunits, GluR1–4 [4–6], and five kainate receptor subunits, GluR5–7 [7–9] and KA-1/KA-2 [10,11], have been cloned. In heterologous systems, GluR1–4 form functional homomeric channels with high affinity for AMPA and quisqualate, and low affinity for kainate and domoate. In contrast, homomeric receptors of GluR5–7 and KA-1/KA-2 show high affinity for kainate and domoate, and very low affinity for AMPA. Several edited variants of the receptor subunits have been identified. Most important is the Q586R editing of GluR2 [12], rendering the receptor impermeable to Ca^{2+} [13].

The first predicted topology model for the AMPA/KA receptor subunits suggested the presence of a long extracellular N-terminus followed by four transmembrane domains (TMD1–4), and a short extracellular C-terminus [4]. However, recently this model has been brought into question [14–16] as experiments have indicated that the C-terminus is intracellular [17,18], and that at least part of the loop between TMD3 and TMD4 is extracellular. This is in disagreement with the four TMD model which places the loop between TMD3 and TMD4 intracellularly. At present, both a five and a three TMD model have been proposed [16,18,19].

Two bacterial binding proteins, a glutamine binding protein (QBP) [20] and a leucine–isoleucine–valine binding protein (LIVBP) [21], have been aligned to the ionotropic GluRs [22,23]. LIVBP shows homology with the AMPA/KA receptor subunits in a domain between residues 1 and 400, whereas QBP aligns to two domains, one located between residues 400 and 500, and another located between the original TMD3 and TMD4 (residues ~720–760). Previous studies, where point mutations were introduced in the first QBP domain, have suggested that this domain is involved in agonist binding [24,25]. This hypothesis is supported by the observation that the two QBP domains, but not the LIVBP domain, are also found in two kainate binding proteins from frog and chick brain [26,27].

In order to localize domains important for ligand binding, we have generated four chimeric receptor subunits from GluR2 and GluR6, exchanging either the LIVBP or the first QBP. The homomeric receptors were stably expressed in baby hamster kidney (BHK) cells, and functionality and ligand binding properties were investigated. Parts of this study (pharmacology and function of GluR6 and GluR2(Q)) have previously been presented in [28–30].

2. Materials and methods

2.1. Construction of mutant receptor subunits

2.1.1. Chimeras. The first GlnH domain was defined as amino acid K393–Q508 in GluR2, and amino acid R400–N515 in GluR6 [22]. The LIV domain was defined as amino acid V1–I365 in GluR2 and amino acid T1–I371 in GluR6 (both amino acids are included and the signal peptides are excluded). The chimeric cDNAs were generated by conventional polymerase chain reaction (PCR) methods using *Pfu* polymerase (Stratagene, La Jolla, CA) or Ampli*Taq* DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT) for 25 cycles of 1 min at 94°C, 2 min at 40°C, and 2 min at 72°C. The final PCR constructs were sequenced by the method of Sanger et al. [31]. All chimeric constructs were incorporated into Zem219b [32], which utilizes the constitutively active mouse metallothionein promoter for expression, and the dihydrofolate reductase gene for selection of stable transformants.

2.1.2. GluR2(Q). A ~700 bp fragment was generated from three PCR reactions using GluR2(R) cDNA as template. One of the primers

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; NMDA, *N*-methyl-D-aspartate; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; DNQX, 6,7-dinitro-quinoxaline-2,3-dione; NBQX, 6-nitro-7-sulfamoyl-benzo[f]quin-oxaline-2,3-dione; QBP, glutamine binding protein; LIVBP, leucine-isoleucine-valine binding protein.

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hybridized to a region containing bp G1757 (excluding the signal peptide sequence), however, the primer contained a mismatch in the position corresponding to bp 1757 (creating a G→A substitution), resulting in the R586Q mutation. The fragment was digested with *Spe*I/*Bsp*EI (New England BioLabs) and assembled with the rest of GluR2. The GluR2(Q) cDNA was inserted into Zem219b, and the PCR fragment was sequenced.

2.2. Expression system and generation of cell lines

For transfection, the DNA was purified using CsCl centrifugation. The plasmids were transfected into BHK570 cells by Lipofectin (BRL), and colonies were isolated as described previously [30]. The selected clones were screened for receptor expression by Western blotting. The cell lines were grown in standard high-glucose Dulbecco's modified Eagle's medium with Glutamax (Gibco-BRL), with the addition of 10% fetal calf serum and 5 μM methotrexate.

2.3. [^{3}H]Kainate binding

Confluent cells were washed twice with phosphate-buffered saline at 25°C, followed by incubation for 30 min with 10 ml of 10 mM EDTA. The cells were scraped off, and membrane preparation and radioligand binding was carried out as described in Tygesen et al. [30]. The final protein concentration in the assay was 0.05–0.2 mg/ml (GluR6, GluR6/LIVBP, GluR2). Non-specific binding was defined as [^{3}H]kainate bound in the presence of 1 mM L-Glu. [^{3}H]Kainate (58 Ci/mmol) was purchased from NEN-Dupont.

2.4. [^{3}H]AMPA binding

Confluent cells were scraped off, and membrane preparation and radioligand binding was performed as described in Tygesen et al. [30]. The final protein concentration in the assay was 0.05–0.1 mg/ml (GluR2/LIVBPGluR6), 0.3 mg/ml (GluR2(Q)), or 0.6 mg/ml (GluR2/QBPGluR6), depending on the level of receptor expression. Non-specific binding was defined as [^{3}H]AMPA bound in the presence of 1 mM L-Glu. [^{3}H]AMPA (46.1 Ci/mmol) was purchased from NEN-Dupont.

All binding data were analyzed with EBDA-SCARFIT (Elsevier-Biosoft, Cambridge, UK). Data are expressed as mean ± S.D.

2.5. Western blot analysis

Samples were subjected to SDS-PAGE (4–20% gradient gels) and transferred to a nitrocellulose membrane by electroblotting (200 mA, 2 h). The blots were blocked for 30 min with 3% non-fat dry milk in Tris-buffered saline (TBS) at room temperature followed by incubation for 2 h at room temperature with affinity-purified GluR6- or GluR2-specific anti-peptide antibody (GluR6, amino acids 862–877; GluR2, amino acids 843 862; kindly supplied by Dr. Craig Blackstone) [33,34] diluted 1:200 in 1.5% non-fat dry milk in TBS. Following several washes in TBS, 0.1% Tween 20, the blot was incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Dako P217) diluted 1:2000 in 1.5% non-fat dry milk in TBS. After washes, immunoreactive protein was visualized with ECL detection reagent (Amersham).

2.6. Protein determination

Protein contents were determined by the Bradford (Bio-Rad) assay using gamma-globulin as a standard.

2.7. Imaging analysis

The imaging analysis was performed essentially as decribed in Tygesen et al. [30]. In some experiments (GluR6, GluR6/LIVGluR2, GluR6/QBPGluR2), the extracellular solution contained *N*-methyl-D-glucamine (NMG) instead of NaCl. Eventually the cells were incubated with 1 mg/ml concanavalin A (Con A) prior to analysis in order to inhibit receptor desensitization [8,35].

3. Results

3.1. Western blotting

Membrane preparations of rat brain, wild-type BHK cells, and BHK cells transfected with the native and mutant receptor cDNAs were subjected to SDS-PAGE and to immunoblot analysis using GluR6- or GluR2-specific antibodies. A protein of approximately 100 kDa was detected in rat brain, and in all transfected cell lines, whereas no band was detected in the wild-type cell line (Fig. 1). The size of the bands is in accordance with the expected molecular weight, and also with data reported by others [19,33,34]. In one of the cell lines (GluR2(Q); Fig. 1B) a doublet band was detected, and in the GluR6 cell line, faint bands of higher molecular weight were detected. In the cell line expressing the chimera GluR6/QBPGluR2, a lower molecular weight band was also detected.

3.2. Radioligand binding studies

We investigated the binding of [^{3}H]kainate and [^{3}H]AMPA to membranes of cells expressing the native and mutant receptors. For receptor characterization, inhibition studies were performed in which binding of [^{3}H]kainate and [^{3}H]AMPA competed against the non-NMDA agonists AMPA, kainate, quisqualate, domoate, and L-Glu, and the non-NMDA antagonists CNQX, DNQX, and NBQX. The results from the radioligand binding experiments are summarized in Table 1.

As reported previously [30], binding of [^{3}H]kainate to GluR6 was saturable and best fitted to a single-site model with a K_d

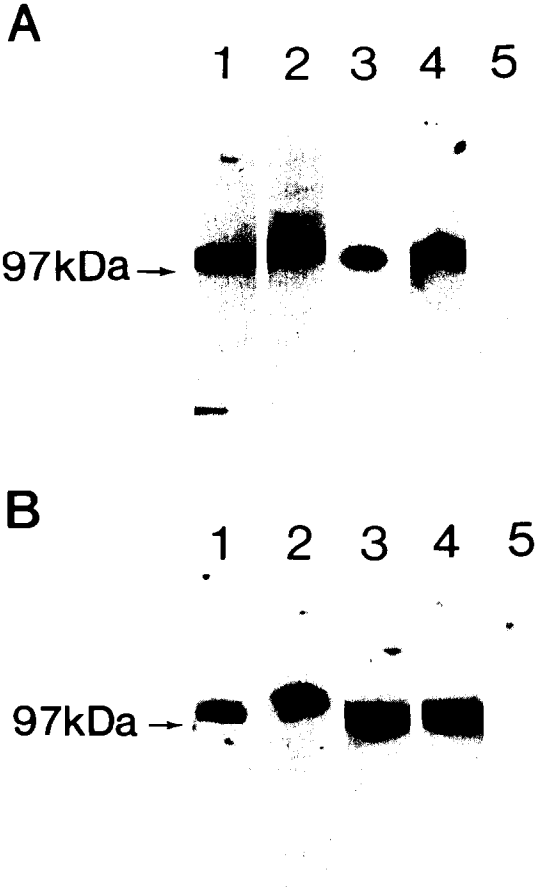

Fig. 1. Western blots of membrane preparations from baby hamster kidney cells transfected with native and mutant glutamate receptors, using GluR6 (A) or GluR2 (B) specific antibodies recognizing the C-terminus. The amount of protein loaded is noted in brackets. (A) lane 1, GluR6/QBPGluR2 (45 μg); lane 2, GluR6 (5 μg); lane 3, GluR6/LIVBPGluR2 (5 μg); lane 4, rat brain (20 μg); lane 5, wild-type cell line (20 μg). (B) Lane 1, GluR2/QBPGluR6 (90 μg); lane 2, GluR2/LIVBPGluR6 (5 μg); lane 3, GluR2(Q) (5 μg); lane 4, rat brain (20 μg); lane 5, wild-type cell line (20 μg).

of 12.9 ± 2.4 nM (n = 4). B_{max} was followed for several months and was determined to be approximately 3 pmol/mg protein. [^{3}H]Kainate was most potently displaced by domoate (K_i = 5.1 ± 3.0 nM, n = 4), whereas AMPA was unable to inhibit the binding ($K_i \gg 15$ μM) (Table 1). No specific [^{3}H]AMPA binding to the GluR6 receptors could be detected.

In contrast, GluR2(Q) bound [^{3}H]AMPA with high affinity. Scatchard analysis revealed a single binding site with K_d = 15.7 ± 4.5 nM (n = 2) [29]. B_{max} varied between 0.3 and 2.5 pmol/mg protein, probably as a result of channel opening induced by L-Glu present in the medium [30]. Quisqualate was the most potent inhibitor of [^{3}H]AMPA binding (K_i = 10.3 ± 2.1 nM, n = 2), and kainate was the least potent (K_i = 3054 ± 230 nM, n = 2) (Table 1).

The chimera GluR6/LIVBPGluR2 (Fig. 2) bound [^{3}H]kainate with K_d = 13.3 ± 1.8 nM and B_{max} = 3.0 ± 0.4 pmol/mg protein (n = 3). No specific [^{3}H]AMPA binding could be detected. The pharmacological agonist profile closely resembled the one obtained for GluR6, with high affinity for domoate (K_i = 4.0 ± 3.0 nM, n = 3) and very low affinity for AMPA ($K_i \gg 15$ μM) (Table 1).

The chimera GluR2/LIVBPGluR6 (Fig. 2) bound [^{3}H]AMPA with high affinity (K_d = 10.0 ± 3.7 nM, n = 4). B_{max} was 5.3 ± 1.8 pmol/mg protein (n = 4). No specific [^{3}H]kainate binding could be detected. The pharmacological agonist profile closely resembled the one obtained for GluR2(Q), with high affinity for quisqualate (K_i = 4.7 ± 3.6 nM, n = 3) and low affinity for kainate (K_i = 2111 ± 484 nM, n = 3) (Table 1).

The chimera GluR2/QBPGluR6 (Fig. 2) bound [^{3}H]AMPA with K_d = 107 ± 11 nM and B_{max} = 630 ± 254 fmol/mg protein (n = 4). Thus, the affinity of AMPA had decreased from 15.2 nM to 107 nM. Quisqualate was still the most potent displacer with a K_i = 11.7 ± 0.4 nM (n = 2), similar to the one found in GluR2(Q). Interestingly, the affinity of kainate had slightly increased (K_i = 716 ± 90 nM, n = 2) compared to GluR2(Q), indicating the importance of this domain for binding of kainate (Table 1). In accordance with the results obtained from inhibition of [^{3}H]AMPA binding, the chimera GluR2/QBPGluR6 only bound [^{3}H]kainate with low affinity.

Neither specific [^{3}H]AMPA nor [^{3}H]kainate binding could be detected in four different subclones of BHK cells transfected with the chimera GluR6/QBPGluR2.

3.3. Functional studies

The function of the native and mutant receptors was tested by Ca^{2+} imaging, taking Ca^{2+} influx as a measurement of functionality. The only mutant which could not be tested was chimera GluR2/QPBGluR6, as this mutant was generated from the GluR2(R) version, and therefore was not permeable to Ca^{2+}. All other native and mutant receptors were found to be functional.

As we have reported previously [30] 5 μM kainate (in NMG containing buffer) induced a sustained increase in the intracellular Ca^{2+} concentration in 75% (n = 58) of the GluR6 expressing BHK cells (Con A pretreated). The increase in intracellular Ca^{2+} concentration varied between 200 nM and 3 μM. When GluR2(Q) expressing cells were stimulated with 200 μM kainate (in high Na^+ buffer, no Con A pretreatment), a similar increase in intracellular Ca^{2+} concentration was observed in 74% of the cells (n = 65). 90% of the cells (n = 41) expressing the chimera GluR6/LIVBPGluR2 responded, when stimulated with 100 μM kainate (NMG buffer, Con A pretreatment). 81% of the cells (n = 32) expressing the chimera GluR2/LIVBPGluR6 responded to 500 μM kainate (high Na^+ buffer, no Con A pretreatment). The cells expressing the chimera GluR6/QBPGluR2 also responded to 500 μM kainate (47% responded, n = 15), however, the increase in intracellular Ca^{2+} was smaller (50–300 nM).

4. Discussion

BHK570 cells have previously been demonstrated to provide a convinient system for stable expression of AMPA/kainate glutamate receptors. In this system, the AMPA/kainate receptors have been studied by radioligand binding, electrophysiology, and Ca^{2+} imaging [28–30,36,37]. Interestingly, the pharmacological profiles of these stably expressed receptors resemble the ones reported from transient systems [5,9,36,38,39]. Further, the pharmacological profile of GluR2(Q) (Table 1) is similar to that previously reported for GluR2(R) [5,36,39], thus, the R586Q point mutation does not affect the ligand binding affinities of GluR2.

The pharmacological profile of the chimera GluR2/LIVBPGluR6 resembles GluR2, with unchanged (or slightly increased) affinities for all agonists. Similarily, the agonist affin-

Table 1
Binding affinities (K_i or K_d, nM) of various agonists and antagonists for GluR2(Q), GluR6, and chimeras hereof

Compound	[a]GluR2(Q)	[b]GluR6	[a]GluR2/QBPGluR6	[a]GluR2/LIVBPGluR6	[b]GluR6/LIVBPGluR2
AMPA	15.7 ± 4.5	>15,000	107 ± 11	10.0 ± 3.7	>15,000
Kainate	3054 ± 230	12.9 ± 2.4	716 ± 90	2111 ± 484	13.3 ± 1.8
Quisqualate	10.3 ± 2.1	203 ± 114	11.7 ± 0.4	4.7 ± 3.6	193 ± 16
L-Glu	477 ± 91	355 ± 74	1344 ± 67	203 ± 69	565 ± 141
Domoate	2598 ± 230	5.1 ± 3.0	3305 ± 840	1691 ± 503	4.0 ± 3.0
CNQX	184 ± 98	528 ± 65	1273 ± 507	810 ± 172	1442 ± 316
DNQX	454 ± 55	351 ± 130	–	310 ± 30	922 ± 287
NBQX	394 ± 229	866 ± 118	–	153 ± 105	7499 ± 3848

Values represent mean ± S.D. The K_d and K_i values are calculated from 2–4 saturation or displacement experiments performed in duplicate. The concentrations of [^{3}H]AMPA were: 18 nM (GluR2(Q)), 25 nM (GluR2/QBPGluR6), 10 nM (GluR2/LIVBPGluR6). The concentrations of [^{3}H]kainate were 10 nM. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NBQX, 6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione.

[a] Measured by [^{3}H]AMPA binding. Affinity of AMPA calculated from Scatchard analysis using [^{3}H]AMPA.

[b] Measured by [^{3}H]kainate binding. Affinity of kainate calculated from Scatchard analysis using [^{3}H]kainate.

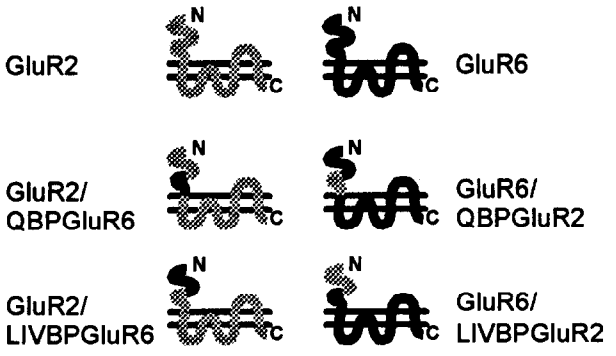

Fig. 2. Schematic presentation of the native and chimeric receptors inserted in the cell membrane. A three transmembrane topology model [14–16] is assumed. The N-terminus is extracellular, the C-terminus intracellular.

ities for chimera GluR6/LIVBPGluR2 resemble the affinities for GluR6. As both the rank order of affinities and the K_i values for five different agonists thus were unchanged, and as both receptors were functional when stimulated with kainate, this strongly indicates that the LIVBP domain does not play a role in agonist binding.

However, when the first QBP domain in GluR2 is exchanged with GluR6 (GluR2/QBPGluR6) the affinities of some of the agonists change. Most interesting is the sevenfold decrease in affinity of AMPA and the fourfold increase in affinity of kainate. This indicates that the first QBP domain is directly involved in binding of both AMPA and kainate. In contrast to this, the unchanged affinities of quisqualate and domoate indicate that the first QBP domain does not selectively contribute to the binding of these agonists (even though involvement of some residues can not be excluded, however, these residues cannot explain the difference in affinities of quisqualate and domoate for GluR2 and GluR6). Consequently, the binding of quisqualate and domoate directly involves residues located in the region C-terminal to the first QBP domain. The C-terminal region is probably also involved in binding of AMPA and kainate, as the affinity of AMPA further decreases from 107 nM in chimera GluR2/QBPGluR6 to >15 μM in chimera GluR6/LIVBPGluR2, and the affinity of kainate increases from 716 nM in chimera GluR2/QBPGluR6 to 13 nM in chimera GluR6/LIVBPGluR2. Chimera GluR2/QBPGluR6 could not be tested for functionality, as it was generated from the Ca^{2+} impermeable version of GluR2 (the R version). However, the increase in affinity of kainate and the unchanged affinities of quisqualate and domoate strongly indicates that the receptor has been properly processed.

When we turn to the antagonists, CNQX, DNQX, and NBQX, the affinities for chimera GluR2/LIVBPGluR6 and GluR6/LIVBPGluR2, decrease compared to GluR2(Q) and GluR6, respectively (Table 1). Along with the unchanged agonist affinities and the demonstrated functionality, this indicates that the LIVBP domain is involved in antagonist binding. The first QBP domain might also be involved, as the CNQX affinity decreases by a factor of seven in GluR2/QBPGluR6 compared to GluR2.

Our agonist studies are in accordance with previous reports [24,25]. Mishina et al. [24] and Uchino et al. [25] reported that introduction of a point mutation, K445E, in the first QBP domain in GluR1 led to a marked decrease in the apparant affinity for L-Glu, AMPA and quisqualate, suggesting that this amino acid is directly involved in agonist binding. Other mutations in the first QBP domain, namely E398K and D443K, also decreased the apparent agonist affinity [25]. Furthermore, recent experiments on the NMDA receptor subunit NMDA-R1 [40], indicate that amino acids located both in the first and second QBP domain are involved in binding of the co-agonist Gly to this receptor [41].

Further data supporting our results were very recently presented by Stern-Bach et al. [42], who generated a number of chimeras from GluR3 and GluR6. Functionality was studied in *Xenopus* oocytes, and the pharmacological agonist profiles for the native and mutant receptors were determined. They concluded that the N-terminal region (first 400 amino acids) is not involved in agonist binding, but that two regions containing the first and the second QBP domain are involved and that simultaneous exchange of both regions is needed to fully reverse the pharmacological profiles of GluR3 and GluR6. [^{3}H]AMPA and [^{3}H]kainate binding were also performed (to transfected HeLa cells). However, the results from their radioligand binding studies are far from conclusive, as specific [^{3}H]AMPA binding to the native GluR3 receptor could not be demonstrated. Furthermore, the determined affinities of [^{3}H]AMPA and [^{3}H]kainate for the mutant receptors (and for the native GluR6 receptor) are quite low, namely 100–300 nM. With affinities in that range a certain unreliability is introduced.

Altogether, we have demonstrated that a domain, located between amino acid 1 and 400 in GluR2 and GluR6, showing homology to the leucine–isoleucine–valine transporter protein, LIVBP, is important for binding of the antagonists CNQX, DNQX, and NBQX. The LIVBP domain seems not to be important for agonist binding. Another domain in GluR2 and GluR6, located between amino acids 400 and 500, which shows homology to a bacterial glutamine transporter protein, QBP, is directly involved in the binding of the agonists AMPA, kainate, and L-Glu. Also, a region located C-terminal to the first QBP domain is involved in binding of these three agonist, as well as in the binding of two other agonists, quisqualate and domoate. It is tempting to speculate that this C-terminal domain is the second QBP domain.

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