The Structure of (−)-Kaitocephalin Bound to the Ligand Binding Domain of the (S)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA)/Glutamate Receptor, GluA2*

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Background: The natural product (−)-kaitocephalin is a potential scaffold for development of subtype-specific glutamate receptor antagonists.

Results: The crystal structure of (−)-kaitocephalin bound to an AMPA receptor ligand binding domain was determined.

Conclusion: The orientation of (−)-kaitocephalin in the binding site can explain the subtype selectivity of the parent compound.

Significance: Comparisons with the structure of other glutamate receptors provides avenues for development of new subtype-specific antagonists.

Glutamate receptors mediate the majority of excitatory synaptic transmission in the central nervous system, and excessive stimulation of these receptors is involved in a variety of neurological disorders and neuronal damage from stroke. The development of new subtype-specific antagonists would be of considerable therapeutic interest. Natural products can provide important new lead compounds for drug discovery. The only natural product known to inhibit glutamate receptors competitively is (−)-kaitocephalin, which was isolated from the fungus Eupenicillium shearii and found to protect CNS neurons from excitotoxicity. Previous work has shown that it is a potent antagonist of some subtypes of glutamate receptors (AMPA and NMDA, but not kainate). The structure of kaitocephalin bound to the ligand binding domain of the AMPA receptor subtype, GluA2, is reported here. The structure suggests how kaitocephalin can be used as a scaffold to develop more selective and high affinity antagonists for glutamate receptors.

Activation by glutamate of AMPA (GluA1–4),2 kainate (GluK1–5), and NMDA (GluN1–3) receptors mediates the majority of excitatory synaptic transmission in the central nervous system. The divergent functional roles of the ionotropic glutamate receptor (iGluR) subfamilies arise from the molecular diversity of each subtype. In particular, AMPA receptors (GluA1–GluA4) mediate most of the fast synaptic transmission in the brain, whereas NMDA receptors (made up of GluN1, GluN2A–D, and GluN3 subunits) activate more slowly and have a high Ca²⁺ permeability and voltage-sensitive block by magnesium (1). AMPA and NMDA receptors control the synaptic plasticity underlying memory formation and learning (2–5), and AMPA channels contribute to excitotoxic cell death in neurodegenerative diseases, epilepsy, and hypoxic ischemia (5–8). Therefore, development of specific antagonists to modulate AMPA and NMDA receptor activity is of considerable interest (9).

Glutamate receptors are tetrameric, membrane-bound proteins, with each subunit consisting of two extracellular domains, a membrane-associated domain (three transmembrane helices and one reentrant loop), and a variable C-terminal domain (10). One of the two extracellular domains, the ligand binding domain (LBD), is made up of a portion of the extracellular N-terminal region and a region between two transmembrane helices (Fig. 1). The structure of the nearly full-length AMPA receptor, GluA2, has been determined (11), and structures of the extracellular domains of all three subtypes have been determined (12–17). Structures of the LBD of AMPA receptors show that full agonists bind in the cleft between two lobes, and after binding to Lobe 1, the lobes close to allow intercalation of NMDA receptor; ATPO, (S)-2-amino-3-[5-tert-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNOX, 6,7-dinitroquinoxaline-2,3-dione; iGluR, ionotropic glutamate receptor; KCP, (−)-kaitocephalin; LBD, extracellular ligand binding domain of GluA2; NS1209, (5S)-8-methyl-5-(4-(N,N-dimethylsulfamoyl)pheryl)-6,7,8,9-tetrahydro-1H-pyrrolo[3,2-h]isoquinoline-2,3-dione-3-O(4-hydroxybutyrate-2-y1)oxime; PDB, Protein Data Bank; UBP277, (5S)-3-(4-carboxyethyl)willardline; UBP282, (5S)-3-(4-carboxybenzyl)willardline.
action with Lobe 2, leading to the activation of the ion channel. In general, antagonists are larger molecules than agonists, and although interactions can be made with both lobes, the lobes remain largely open (although a rare transition to the closed lobe state is possible for some antagonists 18).

(−)-Kaitocephalin (KCP; Fig. 2) is the only known naturally occurring glutamate receptor competitive antagonist. This compound was isolated from the fungus, *Eupenicillium shearii*, and found to protect the chick primary telencephalic and rat hippocampal neurons from kainate excitotoxicity (19). Its total synthesis was subsequently reported by five groups (20–25). KCP displays a differential affinity for different subtypes of glutamate receptor (NMDA > AMPA > kainate (26)) and is of interest as a scaffold for rational drug design (27). We have solved the three-dimensional structure of the LBD of the AMPA receptor subunit, GluA2, bound to KCP. Analysis of the structure demonstrates the orientation of the natural product in the binding site and the important interactions within the binding cleft. Using this information, models were built of KCP in the binding site of a kainate receptor subunit (GluK2) and two NMDA receptor subunits (GluN1 and GluN2A). The models predict the structural basis for the subtype specificity of KCP.

**EXPERIMENTAL PROCEDURES**

**Materials**—KCP was synthesized as described previously (20). The GluA2 LBD construct was obtained from Professor Eric Gouaux (Vollum Institute). The construct consists of residues Asn392–Lys506 and Pro632–Ser775 of the full rat GluA2-flop subunit (28) with a GA segment at the N terminus and a GT linker connecting Lys506 and Pro632 (13).

**Protein Preparation and Purification of LBD**—pET-22b(+) plasmids containing the coding region for GluA2 were transformed in *Escherichia coli* (Origami B (DE3)) and grown at 37 °C in LB medium supplemented with antibiotics (ampicillin and kanamycin) to an A600 of 0.9 to 1.0. Cultures were cooled to 20 °C for 20 min, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. Cultures were then allowed to grow at 20 °C for an additional 20 h. The bacteria were then pelleted, and the LBD was purified using a nickel-nitritotriacetic acid column. The His6 tag was cleaved with thrombin, and the protein was further purified with a sizing column (Superose 12, XK 26/100) followed by an HT-SP-ion exchange-Sepharose column (Amersham Biosciences). Glutamate (1 mM) was maintained in all buffers throughout purification. The protein was then concentrated and stored in 20 mM sodium acetate, 1 mM sodium azide, and 10 mM glutamate at pH 5.5. To exchange the protein to KCP, the protein was concentrated to 50 μl and resuspended in 600 μl of 20 mM acetate buffer. This was repeated seven times. The protein was then concentrated again to 50 μl and resuspended in 600 μl of 2 mM iodosulfamide in acetate buffer. This was repeated two times. This was followed by seven more exchanges with acetate buffer. Finally, two successive dilution and concentration steps were performed with 1 mM KCP, in this case, diluting to 300 μl. Exchange to iodosulfamide was used to ensure that glutamate was removed and replaced with a lower affinity compound. This facilitated the exchange to KCP, of which only a small quantity was available. The final protein concentration was ~0.3 mM.

**Crystallization**—Crystals were grown at 4 °C using the hanging drop technique. The drops contained a 1:1 (v/v) ratio of protein solution to reservoir solution (16–18% PEG 8000, 0.1 M ammonium sulfate, 50 mM sodium phosphate, pH 6.0) and equilibrated against a reservoir solution containing 16–18% PEG 8000, 0.1 M ammonium sulfate, 50 mM sodium phosphate, pH 6.0.
TABLE 1
Data collection and refinement statistics (molecular replacement)

| Parameters | KCP bound to the LBD of GluA2 (4GXS) |
|------------|-------------------------------------|
| Space group | P2₁,2,2 |
| Data collection | |
| No. reflections | 257,994 |
| I/σ(I) | 14.3 (4.12) |
| Completeness (%) | 99.9 (100.0) |
| Redundancy | 6.2 (5.1) |

Refinement

| Resolution (Å) | 50–1.97 |
| Root mean square deviations | |
| Bond lengths (Å) | 0.010 |
| Bond angles (º) | 1.312 |
| B-factors | |
| Protein | 35.9 |
| Ligand/ion | 44.1 |
| Water | 43.2 |

| Values in parentheses are for highest resolution shell. |

M sodium cacodylate, 0.1–0.15 M zinc acetate, pH 6.5). Data were collected at the Cornell High Energy Synchrotron Source beam line A1 (wavelength, 0.987 Å; temperature, 100 K) using a Quantum-210 Area Detector Systems charge-coupled device detector. Data sets were indexed and scaled with HKL-3000 (29). Structures were solved with molecular replacement using Phenix (30). Refinement was performed with Phenix (30), and Coot 0.6.1 (31) was used for model building. Lobe orientation was determined as described by Gill et al. (32) relative to the A protomer of the GluA2, LBD bound to glutamate (Protein Data Bank (PDB) code 3DP6 (12)). The Ramachandran plot indicated that 94% of the residues were in the most favored regions, 6% in the additional allowed regions, and 0% were in the generously allowed or disallowed regions.

Models of GluA6, GluN1, and GluN2A—To assess the potential differences in affinity of different glutamate receptor subtypes, KCP was modeled into the binding sites of GluA2 (PDB code 1TT1), GluN1, and GluN2A (PDB 2A5T). This was done by aligning separately Lobe 1 and Lobe 2 of each of the structures with the KCP-GluA2 structure. Small deviations from standard bond lengths and angles in the linker regions were corrected using Coot (31). This provided a structure for each of the three glutamate receptor subtypes with a lobe orientation identical to the KCP-GluA2 structure. The energy was then minimized using Amber12 (33) with explicit water.

RESULTS

Structure of GluA2 LBD Bound to KCP—The complex of the GluA2 LBD with KCP crystallizes in the P₂₁,2,2 space group with two copies of the protein in the asymmetric unit. The structure was solved to 1.97 Å using molecular replacement with the DNQX-bound structure of GluA2 LBD (PDB code 1FTL, with DNQX removed (13)) as the search model (Table 1). KCP could be easily modeled into the binding site using Coot (31) followed by further refinement in Phenix (30). The density for the dichlorohydroxybenzoyl moiety was somewhat weaker than for the remainder of the molecule (Fig. 3c), suggesting that this portion of KCP may not interact strongly with the protein.

As noted elsewhere (27), the KCP backbone contains two moieties that can mimic glutamate. Either the C-2 nitrogen or the nitrogen on the pyrrolidine ring (similar to that of kainate) could serve the function of the glutamate α-amine (Fig. 2). The C-1 carboxyl would serve the function of the α-carboxylate of glutamate in the former case, and the C-18 carboxyl in the latter case. The density clearly shows that the C-2 nitrogen and carboxyl make most of the same contacts as glutamate; that is, the C-1 carboxyl interacts with the side chain guanidinium of Arg485 and the backbone amide of Thr480, and the C-2 amine interacts with the backbone carboxyl of Pro678, the side chain carboxyl of Glu705, and the side chain hydroxy of Thr480 (Fig. 3).

In the glutamate-bound structure (PDB codes 1FTJ and 3DP6), the backbone amide of Ser654 interacts with the α-carboxylate, but in the case of the KCP-bound structure, the amide of Ser654 interacts with the C-18 carboxyl. This is a consequence of the difference in orientation between the two lobes, with a greater degree of lobe closure in the case of the glutamate structures (see below). The C-18 carboxyl also interacts with the amide and side chain hydroxy of Thr485 through a water molecule. The C-3 hydroxy of KCP hydrogen bonds with the side chain hydroxy of Ser654, and the C-17 carboxyl of KCP interacts with the backbone amides of both Tyr450 and Gly451. The side chain of Tyr450 forms a hydrophobic surface for the pyrrolidine ring and C-2.

Antagonists of AMPA receptors tend to be larger than agonists and bind in a manner that separates the two lobes. As shown in Table 2, the lobes of the LBD in the crystal are rotated open (relative to the A protomer of the glutamate-bound GluA2 LBD, PDB code 3DP6) by 13° ± 3° when KCP is bound. This about the same degree of lobe opening seen for crystals bound to CNQX (34) and DNQX (13). CNQX and DNQX are smaller molecules and bind deeper in the binding pocket. For this reason, full lobe closure is possible (18), and under some circumstances CNQX and DNQX can function as weak partial agonists (34–36). On the other hand, KCP is larger and can prevent the top portions of the binding cleft from closing, suggesting that it is unlikely to serve as even a weak partial agonist. Fig. 4 compares the binding of several antagonists to GluA2 LBD. In each case, antagonists interact with Arg485, and additional interactions vary widely. At the top of the cleft, KCP makes some of the same interactions as the antagonist, NS1209 (37). Carboxyl groups of both interact with the backbone amide of Gly451, and both interact with the side chain hydroxy of Ser654. Likewise, the phosphatase group of ATPO (38) interacts with the side chain hydroxy of Ser654 but also interacts with the backbone amide of Ser654. KCP uses a second group, the C-18 carboxyl, to interact with the amide of Ser654. The two other antagonists shown (UBP277 and UBP282 (39)) bind much deeper in the cleft and differ from CNQX and DNQX by making more extensive interactions with Lobe 2 (39).

Glutamate Receptor Subtype Specificity—KCP inhibits NMDA receptors with higher affinity than AMPA receptors, which in turn are inhibited with higher affinity than GluK2 (kainate) receptors (26). The IC₅₀ for rat brain cortex NMDA
receptors was 75 \pm 9 \text{nm}, for rat brain cortex AMPA receptors was 242 \pm 37 \text{nm}, for homomeric GluA3 receptors was 502 \pm 55 \text{nm}, and for homomeric GluK2 receptors was >100 \mu M (26). The binding pocket for GluK2 is slightly larger than that for GluA2 (15), but KCP binds in the superficial portion of the binding pocket, so the size of the pocket is unlikely to play a major role. More likely are specific differences in the points of contact of KCP with the binding domain, as indicated by molecular modeling. In particular, two important points of interaction on GluA2 are different in GluK2, which are likely to explain the difference in affinity (Fig. 5a). The position corresponding to Ser\textsuperscript{654} in GluA2 is an alanine in GluK2 (Ala\textsuperscript{660}). This would remove the H-bond with the C-3 hydroxy group of KCP. Thr\textsuperscript{480} in GluA2 is Ala\textsuperscript{487} in GluK2, which removes the H-bond with the C-2 amine of KCP. The loss of two hydrogen bonds could account for the \sim 3 \text{kcal/mol} difference in the inhibition of GluK2 relative to AMPA receptors.

For the NMDA receptor, KCP could potentially bind to either GluN1 or GluN2 or to both. We modeled both GluN1 (Fig. 5b) and GluN2A (Fig. 5c). As noted by Furukawa and Gouaux (14), the binding pocket of GluN1 is actually very similar to GluA2, with many of the same functional groups that contact glutamate in GluA2. However, GluN1 binds glycine and has extremely low affinity for glutamate. This is postulated to be due to Val\textsuperscript{609} (GluN1) in the place of Thr\textsuperscript{655} in GluA2 and

**TABLE 2**

Lobe orientation of GluA2 LBD bound to antagonists

The lobe opening of the GluA2 LBD bound to various antagonists or the apo state was measured relative to the A protomer of the structure of GluA2 LBD bound to glutamate (3DP6) as described by Gill et al. (32). The distance between the \beta-carbons of P632 on each monomer within a dimer is given. For comparison, the distance for the A protomer of 3DP6 is 40.6 Å. No value for NS1209 is given because it is a mixed agonist/antagonist dimer.

| Antagonist | PDB ID code | Lobe opening (°) | Pro\textsuperscript{632}–Pro\textsuperscript{632} | No. of protomers | Ref. |
|------------|-------------|------------------|-----------------|-----------------|-----|
| KCP        | 4GXS        | 13.3 \pm 2.3     | 31.6            | 2               | This paper |
| CNQX       | 3R7D        | 12.9 \pm 0.2     | 31.6 \pm 0.2    | 8               | 34  |
| DNQX       | 1FTL        | 14.6 \pm 0.7     | 33.2            | 2               | 13  |
| ATPO       | 1NOT        | 16.5 \pm 2.8     | 31.1 \pm 0.7    | 4               | 38  |
| UBP277     | 3H03        | 17.4 \pm 0.2     | 30.3            | 4               | 39  |
| NS1209     | 2CMO        | 19.4             | 30.3            | 1               | 37  |
| UBP282     | 3H06        | 23.3 \pm 1.9     | 26.0 \pm 1.1    | 8               | 39  |
| Apo        | 1FTO        | 17.1 \pm 1.6     | 30.8            | 2               | 13  |

FIGURE 4. **Comparison of the binding of KCP to the GluA2 LBD with the binding of other antagonists with GluA2.** Each antagonist is shown as a stick representation, and the protein to which it is bound is shown in the same color. Structures shown are: ATPO, PDB code 1NOT (38); NS1209, PDB code 2CMO (37); DNQX, PDB code 1FTL (13); UBP277, PDB code 3H03 (39); and UBP282, PDB code 3H06 (39).
Trp$^{731}$ (GluN1) in the place of Leu$^{704}$ (GluA2). The side chain of Thr$^{655}$ makes a water-mediated H-bond with KCP in GluA2. The C-18 carboxyl of KCP is in the same position as the γ-carboxyl of glutamate in GluA2. It is the steric clash of Trp$^{731}$ with the γ-carboxyl that seems to play an important role in preventing the binding of glutamate of GluN1. With KCP as a ligand, however, the lobes would be open to a large extent due to other interactions within the binding site, so that this steric clash would be less of an issue, similar to the structure of GluN1 bound to 5,7-dichlorokynurenic acid (14). In both GluN1 and GluN2A, the position corresponding to Glu$^{705}$ is an aspartate (Asp$^{732}$ in GluN1 and Asp$^{731}$ in GluN2A). In the model, it is not clear whether the shorter side chain would affect its interaction with the C-2 amine of KCP. Glu$^{657}$ of GluA2 corresponds to Ile$^{691}$ of GluN1. The hydrophobic isoleucine side chain may provide a hydrophobic surface for the face of the dichlorohydroxybenzoyl group of KCP. Finally, Glu$^{522}$ of GluN1 (Val$^{484}$ of GluN2A) is in an ideal position to form a H-bond with the hydroxy of the dichlorohydroxybenzoyl group of KCP. These two possible interactions could compensate for the loss of H-bond due to the presence of Val$^{689}$ (GluN1) in the place of Thr$^{655}$ (GluA2).

Likewise, GluN2A has all of the important determinants of binding found in GluA2. However, Trp$^{731}$ of GluN1 is Tyr$^{730}$ in GluN2A, decreasing the probability of a steric clash. Also, Glu$^{657}$ of GluA2 corresponds to Arg$^{692}$ of GluN2. The hydrophobic surface of the side chain of arginine could play a similar role as suggested above for Leu$^{691}$ of GluN1. Glu$^{517}$ of GluN2A is in the same position as Glu$^{522}$ of GluN1, perhaps forming an H-bond with the hydroxy of the dichlorohydroxybenzoyl moiety. Unlike GluN1, GluN2A has a threonine (Thr$^{690}$) in the same position as Thr$^{655}$ in GluA2, allowing the formation of the same H-bond network as seen in GluA2. Thus, all of the same interactions are possible in GluN2A as in GluA2; however, additional favorable interactions could be made with the dichlorohydroxybenzoyl moiety of KCP that could account for the 3–6-fold higher affinity.

**Discussion**

The use of natural products as scaffolds for the development of specific inhibitors of receptors and enzymes has been a productive strategy (40, 41). KCP is the only known glutamate receptor competitive antagonist from a natural source (E. shearrii PF1191) and has been shown to have neuroprotective properties in a kainate model of excitotoxicity (19). It is of interest because of its inherent differential selectivity for glutamate receptor subtypes (26) and the possibility of using the scaffold to produce selective inhibitors of specific subtypes. We describe here the structure of KCP bound to the LBD of the AMPA receptor, GluA2, and propose possible interactions that give rise to the lower affinity of KCP for GluK2 and the higher affinity for NMDA receptors.

In their search for new small molecule modulators of iGlur activity, Vaswani et al. (27) reported the design, synthesis, and biological evaluation of four KCP analogs that retained the hydrophilic core structure but incorporated simple aromatic rings at C-7. However, their molecular modeling study found that the lowest energy structure of the parent 7-phenyl analog docked into GluA2 placed the pyrroline ring nitrogen in the orientation of the glutamate α-amino group, rotated ~180° relative to our structure of KCP bound to the LBD of GluA2. This would place the aryl group in a more buried location than the dichlorohydroxybenzoyl group of KCP. If the predicted orientation for this scaffold is correct, then aryl substitutions may well produce interesting changes in specificity and affinity. However, if it binds in a manner analogous to KCP, meta- and para-tolyl groups should have relatively minor effects on activity relative to the parent phenyl group. This seems to be the case because the sterically larger ortho-tolyl analog exhibited increased antagonist potency on a mixture of AMPA and kainate receptors activated by kainate. The affinity relative to KCP was not reported.

Of particular interest is the specificity of KCP for subtypes of glutamate receptors. Limon et al. (26) reported that GluK2 had at least 200-fold lower affinity for KCP than a mixture of AMPA receptors from the rat cerebral cortex or homomeric GluA3. The structure of the LBD of GluA3 is almost identical to that of GluA2 (12), so the comparison of the structure of KCP bound to GluA2 with the predicted binding of KCP to GluK2 should provide some insight into the difference in affinity. Although many of the same determinants of binding are present in both subtypes, GluK2 would lack two hydrogen bonds with KCP that are seen in GluA2. The difference in affinity would correspond to a free energy difference of about 3 kcal/mol at 25 °C, which could easily be accounted for by the loss of two H-bonds. The interaction with NMDA receptors is more difficult to analyze, particularly because KCP was found to have a mixed competitive
and noncompetitive inhibition (26) relative to NMDA as an agonist binding to the NR2 subunit. One possible reason for the noncompetitive inhibition is that KCP blocks both NR1 and NR2 subunits. Our models suggest that this is a plausible explanation. KCP can interact with essentially the same amino acids on both GluN1 and GluN2A. The major difference between the two subtypes is the position corresponding to Trp$^{731}$ on GluN1 and Tyr$^{730}$ on GluN2A. The tryptophan on GluN1 accommodates glycine but not glutamate when the lobes are in the closed position. Because of the smaller tyrosine side chain, glutamate can bind to GluN2A. However, when the lobes are forced open with a large antagonist, the relative bulk of W731 becomes less important as in the case of 5,7-dichlorokynurenic acid binding to GluN1 (14), making the binding of KCP to both GluN1 and GluN2A plausible. Thus, if KCP can bind to both sites, then it would behave as a competitive inhibitor of NMDA by virtue of its binding to GluN2 and a noncompetitive inhibitor of NMDA by virtue of its binding to GluN1. The higher affinity relative to GluA2 may arise from additional contacts with the dichlorohydroxybenzyl group. Potentially, the addition of substituents to the pyrrolidine ring could result in an analog that prevents binding to both GluN1 and GluN2A.

The natural product (−)-kaitocephalin binds to the GluA2 LBD in a unique manner that provides a basis for using this structure for further development of glutamate receptor antagonists. In particular, an analysis of key interactions in the KCP-GluA2 structure together with associated KCP-iGluR molecular modeling studies illustrates how this small molecule antagonist can have selectivity for AMPA and NMDA receptors over kainate receptors and provides some clues as to how to modulate the affinity of AMPA receptors over NMDA receptors.

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