Neuropharmacology 60 (2011) 126–134

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Binding site and ligand flexibility revealed by high resolution crystal structures of GluK1 competitive antagonists

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ARTICLE INFO

Article history:
Received 2 April 2010
Accepted 8 June 2010

Keywords:
Glutamate receptors
Crystal structure
Conformational flexibility
Halogen bond

ABSTRACT

The availability of crystal structures for the ligand binding domains of ionotropic glutamate receptors, combined with their key role in synaptic function in the normal and diseased brain, offers a unique selection of targets for pharmaceutical research compared to other drug targets for which the atomic structure of the ligand binding site is not known. Currently only a few antagonist structures have been solved, and these reveal ligand specific conformational changes that hinder rational drug design. Here we report high resolution crystal structures for three kainate receptor GluK1 antagonist complexes which reveal new and unexpected modes of binding, highlighting the continued need for experimentally determined receptor–ligand complexes.

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1. Introduction

ööKainate receptors (KARs) are members of the ionotropic glutamate receptor (iGluR) family which also includes *N*-methyl-D-aspartate receptors (NMDARs) and (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid receptors (AMPARs). KARs are tetrameric assemblies of GluK1-5 subunits (Collingridge et al., 2009) and have been implicated in various functions in the central nervous system (Jane et al., 2009; Lerma, 2006; Pinheiro and Mulle, 2006). In addition, KARs and in particular those containing the GluK1 subunit have been implicated in a number of neurological conditions, such as chronic pain (Jones et al., 2006; Simmons et al., 1998), migraine (Weiss et al., 2006), epilepsy (Smolders et al., 2002) and neurodegeneration (O'Neill et al., 2000) as well as in psychiatric conditions, such as schizophrenia (Beneyto et al., 2007) and anxiety (Alt et al., 2007).

Subunit selective antagonists are required to investigate the functions of KARs in more detail and to identify new targets for drug discovery. The first KAR antagonists to be reported, particularly those based on the quinoxalinedione nucleus, also bind to AMPARs with high affinity and showed no KAR subtype selectivity (Jane et al., 2009). More recently, antagonists showing good selectivity for KARs versus AMPARs and selectivity for the GluK1 subunit within the KAR family have been reported. The most potent GluK1 selective antagonists reported to date include those based on the natural product willardiine, such as UBP310 (Dolman et al., 2007; Mayer et al., 2006) and UBP316 (Dargan et al., 2009; Dolman et al., 2007), and a structurally distinct set of ligands such as LY466195 based on the decahydroisoquinoline nucleus (Weiss et al., 2006).

A breakthrough in our understanding of the molecular interactions between agonists and iGluRs came with the publication of high resolution crystal structures of the ligand binding domains (LBDs) of AMPAR subunits, in particular GluA2 (Armstrong and Gouaux, 2000; Hogner et al., 2002; Jin et al., 2003; Sun et al., 2002); the NMDA receptor subunits GluN1 (Furukawa and Gouaux, 2003), GluN2A (Furukawa et al., 2005), GluN3A and GluN3B (Yao et al., 2008); and the KAR GluK1 and GluK2 subunits (Frydenvang et al., 2009; Hald et al., 2007; Mayer, 2005; Nanao et al., 2005; Naur et al., 2005). By contrast, due to the difficulty in crystallizing antagonist-LBD complexes there are far fewer crystal structures available. Indeed, only two antagonist-LBD structures have been reported for the NR1 NMDAR subunit (Furukawa and Gouaux, 2003; Inanobe et al., 2005), eight for the GluA2 AMPAR subunit (Ahmed et al., 2009; Armstrong and Gouaux, 2000; Cruz et al., 2008; Hogner et al., 2003; Kasper et al., 2006; Menuz et al., 2007; Sobolevsky et al., 2009), and four for the GluK1 KAR subunit (Dargan et al., 2009; Hald et al., 2007; Mayer et al., 2006). However, these structures have been limited in their usefulness for

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0028-3908/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.neuropharm.2010.06.002

drug design due to the conformational flexibility of iGluR LBDs and the dependence of the degree of domain closure on both the ligand and the iGluR subunit (Mayer, 2006). In addition, a complex network of water molecules within the LBDs of iGluRs plays a major role in receptor–ligand interactions, and this network is difficult to model without prior structural knowledge. Molecular dynamics studies on ligand–LBD complexes have made some progress in solving some of these problems (Arinaminpathy et al., 2006; Dolman et al., 2007; Lau and Roux, 2007; Postila et al., 2010), but are not yet sufficiently accurate that they can replace data obtained for experimentally determined complexes with novel ligands.

We have previously reported that the ligand binding domains are hyper-extended in GluK1 complexes with antagonists and that consequently the key ligand binding residue Glu723 exists in two conformations, one of which plays no direct role in antagonist binding (Mayer et al., 2006). This is unusual as this residue plays a critical role in stabilizing GluK1 and GluK2 agonist complexes (Mayer, 2005) and also GluA2 agonist and antagonist complexes (Armstrong and Gouaux, 2000; Hogner et al., 2003).

Here, we report crystal structures of the GluK1 LBD with three high affinity antagonists UBP315, UBP318 and the structurally unrelated compound LY466195. These structures reveal a much wider variation in ligand–receptor interactions and ligand binding domain closure than found in our previous studies with the GluK1 antagonists UBP302, UBP310 and UBP316 (Dargan et al., 2009; Mayer et al., 2006). For instance, the 4-bromo group on the thiophene ring of UBP315 forms an unusual interaction with the carboxylate group of Glu426, producing a 5° greater degree of domain closure compared to the UBP302, UBP310, UBP316 and UBP318 complexes. In addition, LY466195 forms interactions with residues in the LBD of GluK1 that do not occur in the willardiine-based antagonist structures. Linked to these changes the network of ordered solvent molecules in the GluK1 binding pocket undergoes ligand specific rearrangements. These variations in the structures of antagonist-GluK1 complexes underline the necessity of obtaining X-ray crystal structures of the LBDs of iGluRs in complex with structurally unrelated antagonists to inform the drug design process.

2. Methods

The GluK1 ligand binding domain (LBD) was expressed as a soluble protein in *E. coli* and purified by affinity and ion exchange chromatography with no modifications from the previously reported protocol (Mayer, 2005). The construct consisted of residues N416 – K529, preceded by an 18 amino acid peptide encoding an IMAC His tag and thrombin site, and was linked via a GT dipeptide to residues P652 – E791; the affinity tag was removed by proteolysis prior to ligand binding studies and crystallization. For ligand binding assays apo protein was prepared by exhaustive dialysis of the purified GluK1 LBD with 5 buffer changes over a period of three days, with a total volume exchange of $>10^{10}$. Displacement assays were performed using 15 nM ^{3}[H]-glutamate as reported previously (Mayer, 2005), and the data fit with a single binding site isotherm for a competitive interaction,

$$\text{Bound} = \text{Bmax}/(1 + [\text{Antag}]/(K_i + K_i * [\text{Glu}]/K_{dGlu}))$$

where K_i is the dissociation constant for the cold ligand, with the previously measured value of 57 nM used as the dissociation constant for glutamate (K_{dGlu}).

Crystals were grown using the hanging drop technique at a temperature of 20 °C, typically with a 1 to 1 dilution of protein with reservoir. To prepare antagonist complexes the protein was dialyzed against a 2× crystallization buffer containing 40 mM NaCl, 20 mM HEPES pH 7.0, 2 mM EDTA and 10–20 μM ligand, with up to four buffer changes for a total volume exchange of $>10^{7}$; the protein was then diluted by 50% with 10 mM ligand dissolved in water adjusted to pH 7.0 with NaOH, and then concentrated between 5 and 10 mg/ml. Seeding was required to obtain diffraction quality crystals. Cryopreservation was achieved by rapid serial transfers to mother liquor supplemented with increasing amounts of glycerol to a maximum concentration of 18–20%, followed by flash cooling in liquid N_2. The reservoir solution contained 100 mM Tris pH 8.5, 18–21% PEG 1 K (UBP315 and UBP318), or 250 mM $(NH_4)_2$citrate pH 5.35 and 20% PEG 3350 (LY466195).

Data sets from single crystals were collected at APS beamline ID22 at 100 K using a MAR 300 CCD detector for the UBP318 and LY466195 complexes; for the UBP315 complex data was collected using a microfocus Cu-anode sealed X-ray tube with confocal optics (Rigaku Micromax 002) and a Mar345 image plate detector in an attempt to reduce radiation damage for Br atoms. Diffraction data was indexed, scaled and merged using HKL2000 (Otwinowski and Minor, 2001). For the LY466195 complex many crystals exhibited substantial merohedral twinning, but by screening diffraction data from multiple crystals using phenix.xtriage (Adams et al., 2010) we were able to select a crystal with a twin fraction of only 0.02% estimated by Maximum Likelihood, Britton alpha 0.015, and proceeded with standard refinement for untwinned data.

Structures for the UBP315 and UBP318 complexes were solved by Fourier difference techniques using the UBP310 complex dimer (PDB 2F34) striped of ligands, solvent, and alternative conformations as the initial model. For the LY466195 complex the structure was solved by molecular replacement with the program Phaser-1.3.1 (McCoy et al., 2007) using one monomer (2F34) as the search probe. The starting models for ligand structures were built in SYBYL 7.3 (Tripos Inc., St Louis, MO, USA) and optimized with the MMF94s force field using a dielectric constant of 78.5, and a library entry for refinement generated from these coordinates with REFMAC. Cycles of rebuilding and real space refinement in COOT (Emsley and Cowtan, 2004), alternated with cycles of restrained positional, individual B-factor, and TLS refinement using REFMAC5 (Winn et al., 2001), with TLS groups identified by TLSMD (Painter and Merritt, 2006) were performed until no interpretable features remained in Fo–Fc maps. Additional crystallographic calculations were performed using the CCP4 suite of programs (Collaborative computational project number 4, 1994). Data collection and refinement statistics are reported in Table 1.

Subsite maps were prepared according to the nomenclature reported previously for the GluK1 glutamate and UBP310 complexes (Mayer et al., 2006) and use the same numbering scheme for conserved water molecules. The subsite maps were assembled in Photoshop based on the output from LIGPLOT (Wallace et al., 1995) and show ligands as ball and stick figures in which torsion angles were manually adjusted compared to the conformation found in the crystal structure to bring the heterocyclic rings into approximately the same plane for ease of illustration. Electron density maps and crystal structures were illustrated using scripts written for PyMOL (DeLano, 2002). Coordinates and structure factors for the UBP315, UBP318 and LY466195 complexes have been deposited in the protein data bank with codes of 2QS1, 2QS2 and 2QS4 respectively.

3. Results

3.1. High affinity binding of UBP315, UBP318 and LY466195

In this study we characterize the interaction of three high affinity competitive antagonists (*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-4,5-dibromothiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione (UBP315), (*S*)-1-(2-amino-2-carboxyethyl)-5-bromo-3-(2-carboxythiophene-3-yl-methyl)pyrimidine-2,4-dione (UBP318), and (3*S*,4 *aR*,6*S*,8 +*aR*)-6-[[(2*S*)-2-carboxy-4,4-difluoro-1-pyrrolidinyl] methyl]decahydro-3-isoquinolinecarboxylic acid (LY466195), with the kainate receptor GluK1 ligand binding domain using X-ray crystallography and biochemical techniques. These ligands were chosen to investigate the structural effects of halogen substituents in two different positions in the willardiine series of antagonists (Dolman et al., 2007), and to explore how the binding of these ligands differs from that of a second class of competitive antagonist based on a decahydroisoquinoline backbone (Weiss et al., 2006).

We measured the affinity of UBP315, UBP318 and LY466195 for GluK1 using ^{3}[H]-glutamate displacement assays and the genetically isolated GluK1 ligand binding domain (LBD) expressed as a soluble protein, which was purified to homogeneity and exhaustively dialyzed against a ligand free buffer as described previously (Mayer, 2005). The results reveal that UBP315 and LY466195 bind to the GluK1 LBD with similar affinities, K_d 33 ± 4 nM and 38 ± 7 nM respectively, while UBP318, K_d 186 ± 23 nM ($n = 3$), binds with 5-fold lower affinity (Fig. 1). For UBP315 and LY466195 these results are consistent with prior studies on full length GluK1 for which K_{ds} of 10 ± 2 nM and 52 ± 22 nM were reported respectively, while for UBP318 we found a lower affinity than the value of 25 ± 2 nM reported in prior studies (Dolman et al., 2007; Weiss et al., 2006).