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Binding site and interlobe interactions of the ionotropic glutamate receptor GluK3 ligand binding domain revealed by high resolution crystal structure in complex with (*S*)-glutamate

Raminta Venskutonytė [a,b], Karla Frydenvang [a], Michael Gajhede [a], Lennart Bunch [a], Darryl S. Pickering [b], Jette S. Kastrup [a,*]

[a] Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
[b] Department of Pharmacology and Pharmacotherapy, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

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ABSTRACT

Ionotropic glutamate receptors (iGluRs) are involved in excitatory signal transmission throughout the central nervous system and their malfunction is associated with various health disorders. GluK3 is a subunit of iGluRs, belonging to the subfamily of kainate receptors (GluK1–5). Several crystal structures of GluK1 and GluK2 ligand binding domains have been determined in complex with agonists and antagonists. However, little is known about the molecular mechanisms underlying GluK3 ligand binding properties and no compounds displaying reasonable selectivity towards GluK3 are available today. Here, we present the first X-ray crystal structure of the ligand binding domain of GluK3 in complex with glutamate, determined to 1.6 Å resolution. The structure reveals a conserved glutamate binding mode, characteristic for iGluRs, and a water molecule network in the glutamate binding site similar to that seen in GluK1. In GluK3, a slightly lower degree of domain closure around glutamate is observed compared to most other kainate receptor structures with glutamate. The volume of the GluK3 glutamate binding cavity was found to be of intermediate size between those of GluK1 and GluK2. The residues in GluK3 contributing to the subfamily differences in the binding sites are primarily: Thr520, Ala691, Asn722, Leu736 and Thr742. The GluK3 ligand binding domain seems to be less stabilized through interlobe interactions than GluK1 and this may contribute to the faster desensitization kinetics of GluK3.

1. Introduction

Ionotropic glutamate receptors (iGluRs) are tetrameric ligand gated ion channels, widely spread throughout the central nervous system where they play a crucial role in fast excitatory signal transmission. iGluRs are divided into three subfamilies on the basis of ligand selectivity and protein sequence identity: 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), N-methyl-D-aspartic acid (NMDA) and kainate receptors (KARs). GluK3 belongs to the kainate receptor subfamily (GluK1–5) (Traynelis et al., 2010). It has been shown that GluK1–3 may form functional homomeric or heteromeric receptors whereas GluK4 and GluK5 only form functional receptors in combination with other KAR subunits (Alt et al., 2004; Cui and Mayer, 1999). KARs have been linked to various neurological conditions including pain, epilepsy and migraine. Further, KARs differ from the remaining iGluRs as they mostly have a modulatory function in synaptic transmission, a feature that makes them an attractive therapeutic target (Contractor et al., 2011; Jane et al., 2009).

GluK3 is differentially expressed in various brain regions; however, the physiological function of this subunit is not clear. On a gene level, *GRIK3* was associated with recurrent major depressive disorder (Schiffer and Heinemann, 2007), thus making it a potential drug target. Furthermore, evidence exist that GluK3 functions as a presynaptic receptor in MF-CA3 synapses as a heteromer together with GluK2, where it facilitates synaptic transmission. It was shown that long term potentiation is impaired in GluK3−/− mice (Pinheiro et al., 2007). Interestingly, GluK3 displays a very low potency to glutamate compared to other iGluRs, with EC_{50} values being around 10 mM, which suggests a specific physiological function (Perrais et al., 2009; Pinheiro et al., 2007; Schiffer et al., 1997).

iGluRs consist of an intracellular carboxy-terminal domain (CTD), a transmembrane domain (TMD), an extracellular ligand

Abbreviations: AMPA, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; ATD, amino-terminal domain; CTD, carboxy-terminal domain; iGluRs, ionotropic glutamate receptors; IPTG, isopropyl-β-D-thiogalactopyranoside; KARs, kainate receptors; LBD, ligand binding domain; NMDA, *N*-methyl-D-aspartic acid; TMD, transmembrane domain.

* Corresponding author. Fax: +45 3533 6001.
E-mail address: jsk@farma.ku.dk (J.S. Kastrup).

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binding domain (LBD) and an extracellular amino-terminal domain (ATD). One X-ray structure of a full length iGluR (AMPA subtype) is available today (Sobolevsky et al., 2009); however, most of the structural studies of iGluRs were made on isolated soluble domains. Within the kainate receptor subfamily numerous structures of the GluK1 LBD and several structures of the GluK2 LBD have been solved in complex with agonists: among others glutamate (Mayer, 2005), kainate (Plested et al., 2008), domoic acid (Hald et al., 2007), dysiherbaine (Frydenvang et al., 2009) and antagonists, such as ATPO (Hald et al., 2007) and compounds of UBP series (Alushin et al., 2010; Mayer et al., 2006). The structural information has provided key insights into biostructural mechanisms underlying receptor activation (Hald et al., 2007; Mayer, 2005) and continuously aids the design of new potential iGluR ligands (Larsen and Bunch, 2010; Larsen et al., 2010). Although there are no fully GluK3-selective ligands, many GluK1-selective compounds were shown to bind to GluK3 with a lower affinity, but not to GluK2 (Dargan et al., 2009; Jane et al., 2009). Structural information on GluK3 is therefore important to fully understand differences governing ligand selectivity within these three KAR subunits. Here, we report the X-ray structure of the ligand binding domain of GluK3 in complex with its endogenous ligand glutamate as well as a detailed comparison to the GluK1 and GluK2 LBD:glutamate complexes. This insight into the structural differences in the glutamate binding sites of GluK1, GluK2 and GluK3 should facilitate the design of GluK3 selective compounds.

2. Materials and methods

2.1. Expression and purification

The rat GluK3 LBD construct consisting of the S1 and S2 segments was synthesized at GenScript with optimized nucleotide sequence for *Escherichia coli* expression and cloned into the pOPINJ vector using InFusion cloning system (Berrow et al., 2009). The protein was expressed in the *E. coli* Origami 2 cell line (Novagen) as an *N*-terminal GST and His tagged fusion protein. The cleaved protein contains three additional *N*-terminal amino acids, Gly, Pro and Gly, GluK3 residues 432–546 (S1), a Gly-Thr linker and GluK3 residues 669–806 (S2).

The GluK3 LBD expression construct was transformed into bacterial cells, and 6 liters of Hyper Broth media (USBiological) containing 100 μg/ml ampicillin were inoculated with overnight cultures of the construct and cultivated at 37 °C at 200 rpm shaking until the OD_{600} reached ca. 1. Then cultures were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 20 °C overnight. *E. coli* cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM glutamate, 0.3 mg/ml lysozyme, 0.05 mg/ml DNAse, 1 mM $MgCl_2$, protease inhibitor) and applied to a cell disruptor at 1.4 bar. Resulting supernatant after centrifugation of the lysate was applied to an equilibrated (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM glutamate) GSTtrap 5 ml column (GE healthcare). 1 mg of 3C protease solution was applied to the column and it was incubated overnight at 4 °C. Cleaved protein was eluted from the column, concentrated and separated from the remaining impurities by gel filtration on a Superdex 75 column (GE healthcare) in 10 mM HEPES pH 7.0, 20 mM NaCl, 1 mM glutamate. Pure protein was concentrated to ~6 mg/ml and used for crystallization.

2.2. Crystallization and data collection

The GluK3 LBD containing 1 mM glutamate was screened for crystallization conditions at the EMBL HTX crystallization facility in Grenoble, France using the methods previously described (Dimasi et al., 2007). One condition that yielded crystals was reproduced manually by the hanging drop method. The drop contained 1 μl of ~6 mg/ml protein in HEPES pH 7.0, 20 mM NaCl and 1 mM glutamate and 1 μl of reservoir solution consisting of 1.8 M of sodium/potassium phosphate pH 8.2. Crystals were grown at room temperature and flash cooled with liquid nitrogen after immersion in reservoir solution containing 20% glycerol. The final data were collected at the BESSY II beamline (Berlin, Germany) with wavelength of 0.9184 Å using a MARMOSAIC 300 mm CCD detector. The CCP4 suite of programs was used for data processing (Collaborative Computational Project, No. 4, 1994).

2.3. Structure determination

The structure of GluK3 LBD was determined by molecular replacement using PHASER (McCoy et al., 2007). The GluK1 structure in complex with glutamate (pdb code 1YCJ, MolA, Naur et al., 2005) was used as a search model. ARP/wARP (Perrakis et al., 1999) was used to trace amino acid residues of the model. The structure was refined in PHENIX (Adams et al., 2002) and the model checked and edited in COOT (Emsley and Cowtan, 2004) between the refinements.

2.4. Structure analysis and figure preparation

The domain closure of the GluK3 LBD structure in comparison with the apo structure of GluA2 (pdb code 1FTO, MolA; Armstrong and Gouaux, 2000) was calculated using the DynDom server (Hayward and Lee, 2002). The cavity volume of GluK3 was calculated using VOIDOO (Kleywegt and Jones, 1994) as previously described (Mayer, 2005). The surface area of the dimer interface and protein–protein contacts within the dimer of GluK1–3 were analyzed using the PISA server (Krissinel and Henrick, 2007). PyMOL (The PyMOL Molecular Graphics System) was used to prepare the figures. The SSBOND program (Hazes and Dijkstra, 1988) was used to predict suitable mutation sites for introducing cysteines that would form disulfide bonds between protomers comprising the dimer.

2.5. PDB accession code

The atomic coordinates and structure factors of GluK3 LBD in complex with (*S*)-glutamate has been deposited at the Protein Data Bank with the accession code 3S9E.

2.6. Binding experiments

Affinities of glutamate and SYM 2081 at purified GluK3 LBD were determined by radioligand binding (displacement) assay using [^{3}H]-SYM 2081 (ARC, St. Louis, MO) as previously described (Frydenvang et al., 2009).

2.7. Mutagenesis

G772C/D777C mutations were introduced to wild type full length GluK3 in vector pGEMHE by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) with primers CATCGGCACGCCCATGTGCTCCCCCT-ACAGGTGCAAAATCACCATCGC and GCGATGGTGATTTTGCACCTG-TAGGGGGAGCACATGGGCGTGCCGATG; H523C and L784C mutations were introduced using the QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) with primers CCCCTGACCATCACCTGTGTCCGAGAGAAGGCC and GGGA-CAAAATCACGATCGCCATTTGCCAGCTGCAGGAGGAGG. Mutations were confirmed by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).