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Pharmacological and structural characterization of conformationally restricted (*S*)-glutamate analogues at ionotropic glutamate receptors

Lina Juknaitė [a,1], Raminta Venskutonytė [a,1], Zeinab Assaf [b], Sophie Faure [b], Thierry Gefflaut [b], David J. Aitken [c], Birgitte Nielsen [a], Michael Gajhede [a], Jette S. Kastrup [a], Lennart Bunch [a,*], Karla Frydenvang [a,*], Darryl S. Pickering [a,*]

[a] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
[b] Institut de Chimie de Clermont-Ferrand, Clermont Université, Université Blaise Pascal, BP 10448, F-63000 Clermont-Ferrand and CNRS, UMR 6296, ICCF, 63177 Aubière, France
[c] Laboratoire de Synthèse Organique et Méthodologie, ICMMO, UMR 8182-CNRS, Université Paris-Sud 11, 15 rue Georges Clémenceau, F-91405 Orsay Cedex, France

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ABSTRACT

Conformationally restricted glutamate analogues have been pharmacologically characterized at AMPA and kainate receptors and the crystal structures have been solved of the ligand (2*S*,1′*R*,2′*S*)-2-(2′-carboxycyclobutyl)glycine (CBG-IV) in complex with the ligand binding domains of the AMPA receptor GluA2 and the kainate receptor GluK3. These structures show that CBG-IV interacts with the binding pocket in the same way as (*S*)-glutamate. The binding affinities reveal that CBG-IV has high affinity at the AMPA and kainate receptor subtypes. Appreciable binding affinity of CBG-IV was not observed at NMDA receptors, where the introduction of the carbocyclic ring is expected to lead to a steric clash with binding site residues. CBG-IV was demonstrated to be an agonist at both GluA2 and the kainate receptor GluK1. CBG-IV showed high affinity binding to GluK1 compared to GluA2, GluK2 and GluK3, which exhibited lower affinity for CBG-IV. The structure of GluA2 LBD and GluK3 LBD in complex with CBG-IV revealed similar binding site interactions to those of (*S*)-glutamate. No major conformational rearrangements compared to the (*S*)-glutamate bound conformation were found in GluK3 in order to accommodate CBG-IV, in contrast with GluA2 where a shift in lobe D2 binding site residues occurs, leading to an increased binding cavity volume compared to the (*S*)-glutamate bound structure.

1. Introduction

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate fast excitatory neurotransmission in the mammalian central nervous system (CNS) and are activated by the endogenous ligand (*S*)-glutamate (Glu). Glu signalling is essential for normal neurodevelopment and synaptic plasticity associated with learning and memory. On the other hand, overstimulation can cause degeneration of neurons, which may result in neuropathological processes such as ischemia and diseases like Alzheimer's, Parkinson's and Huntington's diseases (Bowie, 2008).

On the basis of pharmacological studies and protein sequence similarity, the iGluRs are divided into three subfamilies: (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionate (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors. The AMPA receptor subunits GluA1-4 and the KA receptor subunits GluK1–3 can form both homomeric and heteromeric receptors within their families, whereas GluK4 and GluK5 form functional receptors only when co-expressed with GluK1–3 (Traynelis et al., 2010).

Activation of AMPA and KA receptors is initiated by binding of the neurotransmitter Glu or another agonist to the extracellular ligand binding domain (LBD) of iGluRs. This binding promotes conformational changes of the receptor complex, leading to opening of the transmembrane ion channel pore (Mayer, 2011; Sun et al., 2002). The LBD consists of two lobes (D1 and D2), which form a clamshell-like structure with ligands binding between the two lobes (Armstrong and Gouaux, 2000). When an agonist is bound to the receptor, the two lobes will close around the agonist. This domain closure leads to opening of the ion channel, and thereby to signal transmission. When an antagonist is bound between the two lobes, the LBD remains in an open conformation and the receptor is kept in the resting state with the ion channel closed.

Abbreviations: AMPA, (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionate; CBG-IV, (2*S*,1′*R*,2′*S*)-2-(2′-carboxycyclobutyl)glycine; CNS, central nervous system; iGluRs, ionotropic glutamate receptors; KA, kainate; LBD, ligand binding domain; NMDA, *N*-methyl-D-aspartate; Glu, (*S*)-glutamate; TEVC, two electrode voltage clamp.

* Corresponding authors. Fax: +45 3533 6001.
E-mail addresses: lebu@farma.ku.dk (L. Bunch), kf@farma.ku.dk (K. Frydenvang), picker@farma.ku.dk (D.S. Pickering).

[1] These authors contributed equally to this work and are joint first authors.

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In order to study the physiological roles of different AMPA and KA receptor subtypes in the CNS, the discovery of subtype selective ligands is essential. While Glu is a flexible molecule, it is well-accepted that it binds in a partly folded conformation when activating the iGluRs (Armstrong and Gouaux, 2000; Mayer, 2005; Bunch and Krogsgaard-Larsen, 2009; Vogensen et al., 2011). Introduction of substituents or conformational restriction so as to favour a folded conformation of Glu has long been a key strategy to obtain selective iGluR agonists. Based on this strategy a number of potent Glu analogues have been reported (Vogensen et al., 2011). However, a perfect conformational mimic is no guarantee for activity and an unexpected lack of affinity at the iGluRs has been reported for some compounds (Bunch et al., 2003).

Here, we present a pharmacological and structural study of the four stereoisomers of 2-(2′-carboxycyclobutyl)glycine (CBG-I to IV), a conformationally restricted Glu analogue which contains a fused 3,4-ring system (Fig. 1) (Faure et al., 2006). The design and numbering of these analogues are based upon previously published conformationally restricted cyclopropyl Glu analogues (Kawai et al., 1992). Binding affinities are presented for the AMPA receptor subunit GluA2 and the KA receptor subunits GluK1–3, together with the crystal structures of the LBD of GluA2 and GluK3 with the highest affinity analogue CBG-IV.

2. Material and methods

2.1. Binding pharmacology

Radioligand binding assays were performed as previously described at native and recombinant rat iGluRs: GluA2$(R)_o$, GluK1$(Q)_{1b}$, GluK2$(VCR)_a$, GluK3$_a$ (Clausen et al., 2009) and at the purified soluble proteins (GluA2 and GluK3 LBDs) (Nielsen et al., 2005; Frydenvang et al., 2009). (*RS*)-[^{3}H]-AMPA, (45.8 Ci/mmol, PerkinElmer, Waltham, MA) was used as the radioligand for GluA2$(R)_o$, K_d = 16.8 nM and GluA2 LBD, K_d = 12.8 nM. [^{3}H]-SYM2081, (40 Ci/mmol, ARC, St. Louis. MO) was used as the radiolabel for GluK1–3 and GluK3 LBD. GluK1$(Q)_{1b}$, K_d = 0.66 nM; GluK2$(VCR)_a$, K_d = 17 nM; GluK3$_a$, K_d = 5.7 nM; GluK3 LBD, K_d = 63 nM.

2.2. Functional pharmacology

TEVC electrophysiology in oocytes was performed as previously described (Nielsen et al., 2005; Venskutonytė et al., 2011a).

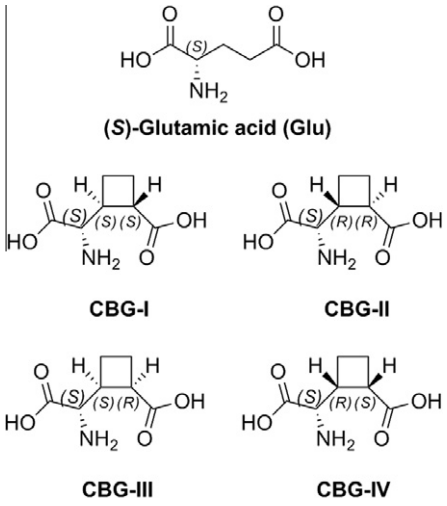

Fig.1. Chemical structures of (*S*)-glutamate (Glu) and conformationally restricted cyclobutyl (CBG) analogues.

GluA2$(Q)_i$ and non-desensitizing mutants of GluK1$(Q)_{1b}$ and GluK3$_a$ (Weston et al., 2006) were expressed homomerically and responses of 1 mM CBG-IV compared to that of saturating concentrations of Glu (GluA2, 1 mM; GluK1, 10 mM; GluK3, 100 mM). Drugs were dissolved in recording buffer. For GluA2 recordings, 100 μM cyclothiazide was included in order to block receptor desensitization.

2.3. Crystallization

2.3.1. GluA2 LBD in complex with CBG-IV

The GluA2 LBD (GluR2-S1S2J; Armstrong and Gouaux, 2000) comprises a *N*-terminal Gly-Ala cloning remnant, amino-acid residues 413–527 from segment S1 of the membrane-bound receptor, a Gly-Thr linker and residues 653–796 from segment S2 (numbering with signal peptide). The protein was expressed and purified essentially as previously described (Ramanoudjame et al., 2006), except for trypsin digestion, which was used to remove the His-tag (buffer: 20 mM sodium acetate, 1 mM Glu, 10 mM EDTA, 5 mM (*RS*)-methionine, 10 mM NaCl, pH 5.5) followed by ion-exchange chromatography (HiTrap SP).

The GluA2 LBD in complex with CBG-IV was crystallized using the hanging drop vapour diffusion method at 7 °C. The drop contained 1 μL of complex solution (6.7 mg/mL GluA2 LBD and 11 mM CBG-IV in 10 mM HEPES, pH 7.0, 20 mM NaCl, 1 mM EDTA) and 1 μL of reservoir solution of 24.4% PEG4000, 0.3 M Li_2SO_4, 0.1 M phosphate–citrate, pH 4.5. The crystals appeared within a week and they were flash cooled with liquid nitrogen after immersion in the reservoir solution containing 20% glycerol.

2.3.2. GluK3 LBD in complex with CBG-IV

The soluble ligand binding domain of GluK3 (GluK3 LBD) was purified as previously described (Venskutonytė et al., 2011b). The protein contains three additional *N*-terminal amino acids: Gly, Pro and Gly, residues 432–546 of segment S1, a Gly-Thr linker and residues 669–806 of segment S2 of the GluK3 receptor (numbering with signal peptide). The protein was crystallized in complex with Glu using the hanging drop method at room temperature, with reservoir solution containing 2.2 M sodium/potassium phosphate, pH 8.5. The drop contained 1 μL of 8.8 mg/mL protein in 10 mM HEPES, pH 7.0, 20 mM NaCl, 1 mM Glu plus 1 μL of reservoir solution. Soaking for three weeks of the Glu:GluK3 LBD crystals with CBG-IV was performed by adding 0.5 μL of a 50 mM CBG-IV solution. The crystals were flash cooled with liquid nitrogen after immersion in the reservoir solution containing 20% glycerol.

2.4. X-ray structure determination

Data were collected at the I911–3 beamline (MAX-Lab, Lund, Sweden) and processed using XDS (Kabsch, 2010) and the CCP4 suite of programs (Collaborative Computational Project, No. 4, 1994). The structures were solved by molecular replacement using PHASER within CCP4. GluA2 LBD with (*S*)-ATPA (PDB-code 1NNP, molA (Lunn et al., 2003)) was used as a search model for the GluA2 complex, whereas GluK3 LBD with Glu (PDB-code 3S9E, molA (Venskutonytė et al., 2011b)) was used as a search model for the GluK3 complex. In each case, a clear solution was obtained. Subsequently, the amino acid residues of the GluA2 complex were automatically modelled into the electron density using ARP/wARP within CCP4, except for a few amino acids which were manually built using COOT (Emsley and Cowtan, 2004). The model was refined in PHENIX (Adams et al., 2010). For the GluK3 complex, the PHASER model was used directly for examination in COOT and refinement in PHENIX. Between each refinement step the structures were checked and edited in COOT. The ligand coordinates were created in Maestro (Maestro version 9.1, Schrödinger, LLC,