Crystal Structures of Potent Dimeric Positive Allosteric Modulators at the Ligand-Binding Domain of the GluA2 Receptor

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Supporting Information

ABSTRACT: The ionotropic glutamate receptor GluA2 is considered to be an attractive target for positive allosteric modulation for the development of pharmacological tools or cognitive enhancers. Here, we report a detailed structural characterization of two recently reported dimeric positive allosteric modulators, TDPAM01 and TDPAM02, with nanomolar potency at GluA2. Using X-ray crystallography, TDPAM01 and TDPAM02 were crystallized in the ligand-binding domain of the GluA2 flop isoform as well as in the flip-like mutant N775S and the preformed dimer L504Y-N775S. In all structures, one modulator molecule binds at the dimer interface with two characteristic hydrogen bonds being formed from the modulator to Pro515. Whereas the GluA2 dimers and modulator binding mode are similar when crystallized in the presence of L-glutamate, the shape of the binding site differs when no L-glutamate is present. TDPAM02 has no effect on domain closure in both apo and L-glutamate bound GluA2 dimers compared to structures without modulator.

KEYWORDS: Dimeric positive allosteric modulator, GluA2 ligand-binding domain, glutamate bound structure, apo structure, X-ray crystallography

I onotropic glutamate receptors (iGluRs) comprise a family of ligand-gated ion channels. They are located in the central nervous system where they engage in fast excitatory synaptic transmission triggered by binding of the neurotransmitter L-glutamate. One class of iGluRs is the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.¹ The AMPA receptors consist of four different subunits, GluA1–4, that combine to form homotetrameric or heterotetrameric functional receptors.² Each subunit is composed of the extracellular N-terminal domain, the ligand-binding domain (LBD) that binds L-glutamate, the transmembrane domain that forms the ion channel, and a cytosolic C-terminal domain.¹ The LBD layer in the tetrameric receptor forms a dimer-of-dimers arrangement.³ Each LBD is composed of two lobes, D1 and D2, that move upon binding of L-glutamate and close around the ligand.⁴

Previous studies have reported a decrease of the AMPA receptor signaling in brain disorders, e.g., attention deficit hyperactivity disorder (ADHD), Alzheimer's disease, or schizophrenia.^{5–7} Therefore, the AMPA receptors seem to be an interesting target for development of medicines for treatment of such diseases.

Positive allosteric modulators (PAMs) are important drug candidates or valuable pharmacological tool compounds for studying receptor function. The PAMs bind at the LBD dimer

interface and thereby stabilize the receptor in the activated form and delay either deactivation of the receptor or its entrance into a desensitized state where the channel is closed despite L-glutamate is still bound.^{8–10}

The GluA2 receptor is the most studied of the AMPA receptors. The receptor exists in two splice variants, i.e., the flip and flop isoforms.¹¹ Of the nine residues differing in the flip/flop cassette, the residue located at position 775 in the LBD has attracted attention.¹² This residue corresponds to Asn in the flop variant and Ser in the flip variant. PAMs bind in the vicinity of Asn/Ser775 and have been shown to affect the two splice variants differentially.^{13,14}

The ligand-binding domain of GluA2 (GluA2-LBD) was first crystallized in 1998,¹⁵ and since then, numerous structures have been reported.¹⁴ GluA2-LBD is predominantly monomeric in solution with a K_d of dimerization of 6 mM that decreases to 1.2 μ M in the presence of the modulator cyclothiazide.¹⁶ Introduction of a L504Y mutation at the dimer

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interface has been shown to dramatically strengthen the dimer, resulting in a K_d of dimerization of 30 nM without altering the PAM binding cavity.¹⁶ By introducing the L504Y mutation into GluA2-LBD, we have previously studied modulator binding to a preformed dimer.^{17–20}

Recently, we synthesized two highly potent dimeric PAMs of the AMPA receptors, TDPAM01 and TDPAM02 (Figure 1).²⁰

Figure 1. Structures of positive allosteric modulators.

Using a calcium flux experiment on HEK293 cells expressing GluA2 flop (Q), the potency of TDPAM01 and TDPAM02

was determined to 13.4 and 1.4 nM, respectively. TDPAM01 was successfully cocrystallized with the GluA2-LBD flop, and based on the structure we predicted that TDPAM02 would bind in a similar manner to that of TDPAM01. Here, we provide direct evidence that this prediction was correct. Crystallization of TDPAM02 turned out to be challenging, requiring DMSO to be present during crystallization. In total five structures of GluA2-LBD were determined, which allowed us to explain the 10-fold better potency of TDPAM02 over TDPAM01 and showed that TDPAM01 and TDPAM02 can bind to both the GluA2 flop and flip variants as well as to a preformed dimer of GluA2. In addition, we show that TDPAM02 can bind to both the L-glutamate bound and unbound (apo) structures.

We previously showed that TDPAM01 could be cocrystallized with GluA2-LBD flop by mixing a suspension of TDPAM01 in aqueous buffer containing 10% DMSO. However, this strategy showed not to be applicable for crystallization of TDPAM02, probably due to its very low solubility in water. Therefore, different methods and buffers were tested. Well-defined electron density for TDPAM02 was observed when TDPAM02 was dissolved in 100% DMSO and then added to the protein solution, resulting in a protein–TDPAM02 suspension containing 20% DMSO. Furthermore, the receptor was crystallized at room temperature. We suggest that the high concentration of DMSO helps keeping sufficient amount of modulator in solution to fully occupy the GluA2-LBD binding site.

Figure 2. Dimeric structures of GluA2-LBD in complex with TDPAM02. (a) Left: side-view of the GluA2-LBD flip dimer in cartoon representation (salmon for chain A; beige for chain B), showing that TDPAM02 (purple spheres) binds in the lower part of the dimer interface. L-Glutamate is shown as orange spheres and Leu504 and Ser775 as yellow spheres. Right: a 90° horizontally rotated view (top view). (b) Left: side-view of the apo GluA2-LBD flop dimer (dark cyan for chain A; light cyan for chain B). Leu504 and Asn775 are shown as yellow spheres. Right: a 90° horizontally rotated view (top-view). White arrow: D1–D2 domain closure.

To establish the binding mode of TDPAM02, we crystallized the modulator in complex with the ligand-binding domain of the GluA2 flip-like N775S (GluA2-LBD flip) and L-glutamate. Diffraction data were collected to a resolution of 1.6 Å (Table S1). The complex crystallized as a dimer with L-glutamate in the two orthosteric binding sites and one molecule of TDPAM02 at the dimer interface (Figure 2a). Unambiguous electron density was observed for both L-glutamate and TDPAM02 (Figure S1c). L-Glutamate induces a domain closure of 20.4° (chain A) and 21.0° (chain B) in the presence of TDPAM02, being similar to the domain closure introduced by L-glutamate without modulator present.¹⁴

TDPAM02 binds in a region comprising residues Ile502, Lys514, Pro515, Phe516, Met517, Ser518, Ser750, Lys751, Gly752, Leu772, Ser775, and Leu780 of both subunits (within 4 Å of TDPAM02; numbering of residues with signal peptide) (Figure 3). Each benzothiadiazide (BTD) moiety of

Figure 3. Zoom on the TDPAM02 binding site in the GluA2-LBD flip dimer. The receptor is shown in salmon (chain A) and beige (chain B) cartoon representation. Residues within 4 Å of TDPAM02 (purple sticks) are shown as lines (residues discussed in main text are shown as sticks) and water molecules as red spheres. Direct hydrogen bonds of TDPAM02 with P515 are shown as stippled black lines. In addition, water-mediated contacts between TDPAM02 and protein residues are shown as red stippled lines and selected water-mediated contacts between receptor subunits as stippled yellow lines.

TDPAM02 makes a direct hydrogen bond from the sulfonamide nitrogen atom to the backbone oxygen atom of Pro515 in each subunit (Figure 3). Furthermore, one of the sulfonamide oxygen atoms forms a water-mediated contact to Ile502 of chain B. The side chain of Ser775 being part of the flip/flop cassette forms a water-mediated contact to Ser750 in the other subunit (Figure 3) but only forms indirect contacts to the modulator through two water molecules. Ser518 is

present in two side-chain conformations and forms water-mediated contacts to Ser750 in the other subunit (Figure 3).

The *N*-cyclopropyl group in TDPAM02 is within van der Waals distance from the backbone atoms of Phe516 and Met517 (Figure 3). The cyclopropyl ring forms stacking interactions with the peptide backbone of Phe516 and Met517. Also, a network of water molecules is within van der Waals distance from the cyclopropyl group (Figure 3).

For the first time, we have crystallized a BTD-containing modulator with GluA2-LBD in the apo state. GluA2-LBD flop crystallized with TDPAM02 as a dimer with an expanded orthosteric binding cleft compared to the GluA2-LBD flip structure with TDPAM02 and L-glutamate (Figure 2). The conformation of apo GluA2-LBD flop cocrystallized with TDPAM02 is very similar to the previously reported apo structure of GluA2-LBD flop without modulator (PDB code 1FTO).⁴

Unambiguous electron density for one molecule of TDPAM02 was seen at the dimer interface (Figure S1a). In the apo form, TDPAM02 binds in the same manner at the PAM binding site and essentially forms the same hydrogen bonding interactions as seen for the L-glutamate bound form (Figure 4a). However, differences are seen, one of which is an additional water-mediated contact from one of the sulfonamide groups in TDPAM02 to the side-chain of Asn775 (Figure 4a,b). Furthermore, Asn775 forms both a direct and a water-mediated contact to the backbone of Ser750 in the other subunit, thereby stabilizing the dimer (Figure 4a,b). In GluA2-LBD flop with TDPAM01 this water-mediated hydrogen bond to one of the sulfonamide oxygen atoms was also observed.²⁰ In addition, the shape of the PAM binding site differs between the apo and L-glutamate bound structures. While keeping the tight contact from the cyclopropyl group of TDPAM02 to the peptide bond of Met516 and Phe517 (3.2–3.5 Å), the other GluA2 subunit moves approximately 0.5 Å further away from the cyclopropyl group in the apo structure (Figure 4a,c). However, the PAM binding site in the apo structure seems to be more narrow in the region where a network of water molecules was observed to surround the cyclopropyl group in the L-glutamate bound structure (Figures 3 and 4c).

We also crystallized TDPAM02 in complex with the preformed dimer of GluA2-L504Y-N775S (GluA2-LBD predimer) to investigate if TDPAM02 was capable of binding to this receptor form. As expected, GluA2-LBD predimer crystallized as a dimer, and the structure is very similar to the structure of GluA2-LBD flip (Figure S2). TDPAM02 binds in a similar way to the PAM binding site and the surrounding residues adopt similar conformations and contacts. Also, the region of the L504Y mutation is very similar to that of other GluA2-LBD predimer structures with PAMs.¹⁷

TDPAM01 has previously been crystallized in GluA2-LBD flop. Here, we also present the structures of GluA2-LBD flip (Figure 5) and GluA2-LBD predimer with TDPAM01 (Figure S2b). The structures are similar to those with TDPAM02. The potency of TDPAM02 was previously shown to be approximately 10 times greater than that of TDPAM01. We have previously investigated two monomeric PAMs containing an *N*-ethyl group (BPAM97) and *N*-cyclopropyl group (BPAM344: compound 3 in reference) (Figure 1).^{17,18} For these PAMs, we also observed an approximately 10-fold difference in potency in favor of the cyclopropyl containing PAM. The major structural factor increasing the binding affinity of BPAM344 was suggested to be the interaction

Figure 4. Zoom on the TDPAM02 binding site in the GluA2-LBD flop dimer. (a) The receptor is shown in dark cyan cartoon representation. Selected residues within 4 Å of TDPAM02 (purple) are shown as sticks. Direct hydrogen bonds of TDPAM02 with P515 are shown as stippled black lines. In addition, water-mediated contacts between TDPAM02 and protein residues are shown as red stippled lines and selected water-mediated contacts between receptor subunits as stippled yellow lines. The structure of the GluA2-LBD flop dimer (salmon) with TDPAM02 in light pink has been overlaid to illustrate similarities and differences. (b) Zoom on Asn775 (flop)/Ser775 (flip) protein–modulator and intersubunit interactions. Water molecules are colored according to the structures. (c) Zoom on the N-cyclopropyl group of TDPAM02 and its interactions. Phe516 and Met517 are shown in sticks representations. Water molecules are shown within 4.5 Å of the cyclopropyl group.

between the cyclopropyl group and the backbone amide of Phe516 and Met517. This stacking interaction might be the result of the π -character of the cyclopropyl ring. As TDPAM01 and TDPAM02 bind in a similar way as BPAM97 and BPAM344, respectively, we find that the difference in potency between TDPAM01 and TDPAM02 likewise can be attributed to the stacking interaction between the cyclopropyl group and the peptide bond in the GluA2 backbone.

In conclusion, we have shown that the two PAM binding sites at the GluA2-LBD dimer interface can host one molecule of the dimeric modulator TDPAM02, occupying the positions

Figure 5. Comparison of the binding mode of TDPAM01 (green) and TDPAM02 (purple) in the GluA2-LBD flop dimer. The receptor is colored with chain A in salmon and chain B in beige. Phe516 and Met517 are shown in sticks representation. Distances between the N-cyclopropyl group of TDPAM02 and the peptide bond between Phe516 and Met517 are shown as stippled black lines.

of two separated monomeric PAMs. TDPAM01 and TDPAM02 were capable of crystallizing with different variants of the GluA2-LBD: flop, flip-like, and flip-like preformed dimer as well as both the L-glutamate bound and L-glutamate unbound forms. Therefore, in the synapse the TDPAMs might bind to different states of the receptor and stabilize it in a compact conformation ready for activation by L-glutamate. This information may turn out to be valuable in future design and synthesis of PAMs for development of medicines for treatment of glutamate receptor related diseases in the central nervous system.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00369.

Materials and Methods; crystal data, data collection, and refinement statistics; omit electron density maps; structure of TDPAM01 and TDPAM02 in GluA2-LBD preformed dimer (PDF)

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K.V.H., M.M., K.F., and J.S.K. Manuscript: J.S.K. wrote the first draft of the manuscript, S.L., K.V.H., and M.M. prepared figures, and all authors contributed to and commented on the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BTB, benzothiadiazide; GluA2-LBD, ligand-binding domain of GluA2; GluA2-LBD flip, ligand-binding domain of the flip-like GluA2 N775S; GluA2-LBD predimer, preformed dimer of GluA2-L504Y-N775S; iGluRs, ionotropic glutamate receptors; LBD, ligand-binding domain; PAMs, positive allosteric modulators

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