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A NOVEL DUALISTIC PROFILE OF AN ALLOSTERIC AMPA RECEPTOR MODULATOR IDENTIFIED THROUGH STUDIES ON RECOMBINANT RECEPTORS, MOUSE HIPPOCAMPAL SYNAPSES AND CRYSTAL STRUCTURES

G. B. CHRISTIANSEN,[a] B. HARBAK,[b] S. E. HEDE,[c,d] A. H. GOULIAEV,[d†] L. OLSEN,[b] K. FRYDENVANG,[b] J. EGEBJERG,[c,e] J. S. KASTRUP[b] AND M. M. HOLM[a,c*]

[a] *Department of Biomedicine, Aarhus University, DK-8000 Aarhus C, Denmark*

[b] *Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Jagtvej 162,, DK-2100 Copenhagen, Denmark*

[c] *Department of Molecular Biology and Genetics, Aarhus University, DK-8000 Aarhus C, Denmark*

[d] *NeuroSearch A/S, Pederstrupvej 93, DK-2750 Ballerup, Denmark*

[e] *H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark*

Abstract—Positive allosteric modulators (PAMs) of 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid (AMPA) receptors receive increasing interest as therapeutic drugs and have long served as important experimental tools in the study of the molecular mechanisms underlying glutamate-mediated neurotransmission. The aim of this study was to investigate functional and structural aspects of a novel analog of the AMPA receptor PAM cyclothiazide (CTZ) on recombinant and native glutamate receptors. We expressed rat GluA4flip and flop in *Xenopus* oocytes and characterized NS1376 and CTZ under two-electrode voltage-clamp. The dose–response analyses revealed dual effects of NS1376. The modulator induced 30-fold and 42-fold reductions in glutamate potency and increased the glutamate efficacy by 3.2-fold and 5.3-fold at GluA4flip and GluA4flop, respectively. Rapid application of glutamate to excised outside-out patches showed that NS1376 markedly attenuated desensitization, supporting the increased efficacy observed in the oocytes. Furthermore, when applied to acutely isolated mouse brain slices, NS1376 reduced the field excitatory postsynaptic potentials (fEPSPs) in the hippocampus to 51.6 ± 4.3% of baseline, likely as a consequence of reduced glutamate potency. However, the modulator displayed no effects on a sub-maximal long-term potentiation (LTP) protocol. We confirmed that CTZ increases presynaptic transmitter release, a property which was not shared by NS1376. Finally, we obtained detailed molecular information through X-ray structures, docking and molecular dynamics, which revealed that NS1376 interacts at the dimer interface of the ligand-binding domain in a manner overall similar to CTZ. NS1376 reveals that minor structural changes in CTZ can result in an altered modulatory profile, both enhancing agonist efficacy while markedly reducing agonist potency. These unique properties add new aspects to the complexity of allosteric modulations in neuronal systems. © 2015 Published by Elsevier Ltd. on behalf of IBRO.

Key words: allosteric modulator, hippocampus, synaptic plasticity, AMPA receptor, crystal structure, outside-out patch.

INTRODUCTION

Ionotropic glutamate receptors in the brain are essential for excitatory neurotransmission, key players in synaptic plasticity and display important therapeutic potentials (Collingridge et al., 2004; Traynelis et al., 2010). The 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid (AMPA) receptors are members of the glutamate receptor family and are divided into GluA1, GluA2, GluA3 and GluA4, with further specializations arising through a range of post-transcriptional and post-translational modifications (Traynelis et al., 2010). In glutamatergic synapses of the brain additional complexity of native AMPA receptor complexes occurs by coassembly with auxiliary proteins creating large assemblies with altered functional properties (Schwenk et al., 2012; Howe, 2015).

In a simplified situation, upon agonist binding AMPA receptors undergo a conformational change which opens a central ion channel. In the continued presence of an agonist, further rearrangements of the complex may result in channel desensitization. The functionality of the AMPA receptors can be altered by allosteric modulators interacting at sites distinct from the agonist

*Correspondence to: M. M. Holm, Department of Biomedicine, Aarhus University, Ole Worms Allé, Building 1160, Room 122, DK-8000 Aarhus C, Denmark. Tel: +45-8716-7715.
E-mail address: mmh@biomed.au.dk (M. M. Holm).
URL: http://www.biomed.au.dk/mmh (M. M. Holm).
† Present address: Nuevolution A/S, Roennegade 8, DK-2100 Copenhagen, Denmark.

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; BDNF, brain-derived neurotrophic factor; CA1, *cornu ammonis* area 1; CTZ, cyclothiazide; DEPC, diethylpyrocarbonate; DMSO, dimethyl sulfoxide; fEPSP, field excitatory postsynaptic potential; GluA4i, glutamate receptor 4 of the flip splice variant; GluA4o, glutamate receptor 4 of the flop splice variant; LBD, ligand-binding domain; LTP, long-term potentiation; MD, molecular dynamics; molA, molecule A; NS1376, 3,4-dihydro-3-cyclohexyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide-7-sulfonamide; PAM, positive allosteric modulator; PPF, paired-pulse facilitation; RMSD, root mean square deviation.

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binding site, thereby either enhancing or reducing the activity of the agonist (Neubig et al., 2003). Thus allosteric modulators represent valuable pharmacological tools to control AMPA receptor activity in the brain. Recent extensive structural studies of full-length glutamate receptors have consolidated that these receptors are highly dynamic complexes which can be preferentially stabilized in a range of states depending on interacting antagonists, agonists and allosteric modulators (Sobolevsky et al., 2009; Durr et al., 2014; Meyerson et al., 2014). For further details on gating of the glutamate receptors, please consult recent reviews by Dawe et al. (2015) and Sobolevsky (2015).

Through their enhancement of AMPA receptor activity, positive allosteric modulators (PAMs) promote long-term potentiation (LTP) (Arai and Lynch, 1992; Arai et al., 2004) and increase expression of brain-derived neurotrophic factor (BDNF) in e.g. hippocampus (Lauterborn et al., 2000) opening up for therapeutic applications in disorders with impaired neurotrophin signaling, such as Alzheimer's and depression (Nagahara and Tuszynski, 2011). During the past years PAMs have received considerable interest as therapeutic drugs (Pirotte et al., 2013) prompting more mechanistic research to optimize future applications in health and disease.

One of the pioneering PAMs was the benzothiadiazide cyclothiazide (CTZ, Fig. 1) which potently reduced glutamate receptor desensitization (Yamada and Tang, 1993). Crystallographic analyses revealed that the modulator stabilized the dimeric GluA2 ligand-binding domain (LBD) through interactions at the dimer interface (Sun et al., 2002). Several CTZ derivatives have been structurally and functionally characterized (Hald et al., 2009). Patch-clamp analyses combined with high-resolution crystal structures of CTZ and analogs like NS5206 and NS5217 (without the NH at the 4-position) demonstrated the NH hydrogen bond donor in the CTZ 4-position to be a key determinant for effective inhibition of the desensitization (Hald et al., 2009). The norbornenyl group in the 3-position of CTZ displays an almost perfect fit into the allosteric binding site and replacing this group with a smaller cyclopentyl ring results in increased flexibility of this part of the modulator (Hald et al., 2009). In this study we report the modulatory effects of 3,4-dihydro-3-cyclohexyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide-7-sulfonamide (NS1376), which contains an intermediate sized functional group in the 3-position while keeping the NH group in the 4-position (Fig. 1).

Accumulating evidence indicates that allosteric glutamate receptor modulators act in more complex ways, which also depend on the biological system. For example, it has been shown that GYKI 52466, although interacting at a site distinct from CTZ, can display both negative and positive modulatory actions. GYKI 52466 belongs to a group of 2,3-benzodiazepines showing potent noncompetitive antagonism of AMPA and kainate-evoked currents from cultured hippocampal neurons (Donevan and Rogawski, 1993). On the other hand, low concentrations of GYKI 52466 have been reported to show positive modulatory effects on the steady-state current recorded from outside-out patches

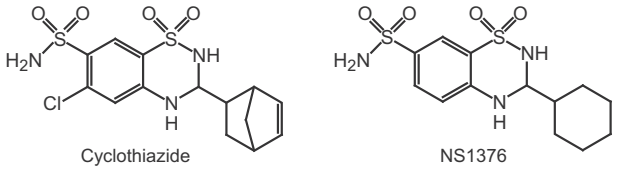

Fig. 1. Chemical structures of the modulators. Structural formulas of the two allosteric modulators, cyclothiazide (CTZ) and NS1376, employed in the present study.

isolated from *cornu ammonis* area 1 (CA1) pyramidal neurons, in addition to an inhibiting action at the peak current (Arai, 2001). Further complexity arises through the modulator-receptor interactions as exemplified in a report of crystal structures of the PAM piracetam in complex with GluA2-LBD revealing as much as three binding sites on each receptor subunit, each with low occupation (Ahmed and Oswald, 2010).

In order to further characterize the functional and structural characteristics of AMPA receptor modulators we here studied the analog NS1376 (Fig. 1) and compared it to the lead compound CTZ. Structurally, the chloride atom of CTZ was replaced by hydrogen and the norbornenyl moiety was replaced by a cyclohexyl ring in NS1376. The modulators were analyzed on recombinant AMPA receptors expressed in *Xenopus* oocytes and in hippocampal synapses in acute brain sections prepared from mice. To map the molecular interactions in detail, structural information was obtained through crystallization, docking and molecular dynamics. Interestingly, we revealed that despite our finding that NS1376 interacts in a manner overall similar to CTZ, it presents a novel dualistic mechanism of action; increasing agonist efficacy while markedly reducing agonist potency.

EXPERIMENTAL PROCEDURES

Recombinant DNA and preparation of cRNA

The wild-type glutamate receptors (glutamate receptor 4 of the flip splice variant (GluA4i) and glutamate receptor 4 of the flop splice variant (GluA4o)) cDNA were subcloned into the vector pGEMHE-3z (Liman et al., 1992). Point mutations in GluA4i and GluA4o were constructed by QuickChange Site-Directed Mutagenesis (Stratagene) according to the manufacturer's instruction and confirmed by sequencing. The plasmid DNA was linearized by Nhe1, before *in vitro* transcription with T7 polymerase. cRNA transcripts were extracted with phenol/chloroform and purified by precipitation in ammonium acetate and ethanol. The cRNA was dissolved in diethylpyrocarbonate (DEPC)-water. In some cases, we used the mMESSAGE mMACHINE T7 kit (Ambion).

Xenopus laevis oocyte electrophysiology

Oocytes were prepared as previously described (Nielsen et al., 2003). Recordings were performed after 3–10 days using a two-electrode voltage-clamp amplifier (Warner OC-725C, Warner Instruments, Inc., Hamden, CT, USA)

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