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In vitro and *in vivo* characterization of MPEP, an allosteric modulator of the metabotropic glutamate receptor subtype 5: Review article

R. Kuhn[1], **A. Pagano**[1], **N. Stoehr**[1], **I. Vranesic**[1], **P. J. Flor**[1], **K. Lingenhöhl**[1], **W. Spooren**[1], **C. Gentsch**[1], **A. Vassout**[1], **A. Pilc**[2], and **F. Gasparini**[1]

[1] Nervous System Research, Novartis, Pharma AG, Basel, Switzerland
[2] Institute of Pharmacology, Polish Academy of Science, Krakow, Poland

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Summary. There is a need to identify subtype-specific ligands for mGlu receptors to elucidate the potential of these receptors for the treatment of nervous system disorders. To date, most mGlu receptor antagonists are amino acid-like compounds acting as competitive antagonists at the glutamate binding site located in the large extracellular N-terminal domain.

We have characterized novel subtype-selective $mGlu_5$ receptor antagonists which are structurally unrelated to competitive mGlu receptor ligands. Using a series of chimeric receptors and point mutations we demonstrate that these antagonists act as inverse agonists with a novel allosteric binding site in the seven-transmembrane domain. Recent studies in animal models implicate $mGlu_5$ receptors as a potentially important therapeutic target particularly for the treatment of pain and anxiety.

Keywords: Group I metabotropic glutamate receptors – MPEP – SIB-1757 – Anxiety – Pain

Introduction

L-glutamate is the main excitatory neurotransmitter in the mammalian brain and acts through two heterogeneous families of receptors: ionotropic and metabotropic glutamate (mGlu) receptors. Ionotropic glutamate receptors are ligand gated channel whereas metabotropic receptors are G-protein coupled receptors linked to second messenger pathways. To date, eight subtypes of mGlu receptors have been cloned and classified into three groups on the basis of sequence similarities and pharmacological properties. When expressed in heterologous systems, group I mGlu receptors ($mGlu_1$ and $mGlu_5$) activate phospholipase C via $G\alpha_{q/11}$ and a subsequent release of calcium from internal stores. Group II mGlu receptors ($mGlu_2$ and $mGlu_3$) and group III receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$) inhibit adenylate cyclase via $G\alpha_{i/o}$ following exposure of cells to forskolin or activation of intrinsic $G\alpha_s$-coupled receptors. Many of these receptors exist as various isoforms with different intracellular carboxy-termini generated by alternative splicing. Pharmacological and immunocytochemical studies have indicated that individual mGlu receptor subtypes and splice variants exhibit distinct expression patterns, subcellular localizations and physiological properties (reviewed in Conn and Pin, 1997).

The $mGlu_5$ receptor protein has been localized primarily postsynaptically at glutamatergic synapses. $MGlu_5$ immunoreactivity is found abundantly throughout cortex, the hippocampus, particularly in the CA1, CA3 and dentate gyrus, and the caudate-putamen and nucleus accumbens. Moderate expression is found in the output structures of the basal ganglia such as the pallidal complex, subthalamic nucleus and the substantia nigra (Shigemoto et al., 1993; Romano et al., 1995; Shigemoto and Mozuno, 2000). As these brain areas are thought to be involved in emotional and motivational processes the $mGlu_5$ receptor has been considered as a potential drug target for treatment of psychiatric and neurological disorders. $mGlu_5$ mRNA, protein and functional responses have also been found in peripheral endings of primary nociceptive afferents (Walker et al., 2001a; Bhave et al., 2001), small diameter dorsal root ganglia (Valerio et al., 1997) and superficial dorsal horn neurons (lamina I and II) (Vidnyanszki et al., 1994; Valerio et al., 1997; Jia et al., 1999; Berthele et al., 1999; Alvarez et al., 2000) as well

as cortex and thalamus (Romano et al., 1996; Neto et al., 2000) suggesting an involvement in the modulation of nociceptive information.

Novel allosteric $mGlu_5$ receptor antagonists

The first specific mGlu receptor agonists and antagonists identified, 1S,3R-ACPD and S-MCPG, were conformationally constraint glutamate analogs, which competitively interact at the glutamate-binding site located in the large extracellular N-terminal domain (O'Hara et al., 1993; Kunishima et al., 2000). These compounds have been very important tools for the early characterization of mGlu receptor mediated physiological responses, however, they lack mGlu receptor subtype-selectivity and have a low affinity: 1S,3R-ACPD is an agonist of group I and II mGlu receptors; MCPG is an antagonist of most mGlu receptor subtypes (for review, see Schoepp et al., 1999). In the following years, synthesis of novel ligands focussed on derivates of amino acids and phenylglycines, respectively. Despite significant improvement in potencies and selectivities for compounds acting act group II mGlu receptors, very few "selective" agonists and antagonists were identified for group I mGlu receptors. Currently, the most potent competitive group I mGlu receptor antagonist is LY393675, a non-selective antagonist with IC_{50}'s of 0.48 and 0.35 μM at $mGlu_5$ and $mGlu_1$ receptors, respectively (Baker et al., 1998). More recently, LY344595 was described as a $mGlu_5$ selective competitive antagonist with a selectivity factor of seven compared to the $mGlu_1$ receptor subtype. However, LY344595 (IC_{50} = 5.5 μM) has lower potency and also antagonises group II mGlu receptors in a similar potency range (Doherty et al., 2000). Despite these significant advances, highly potent and subtype-selective competitive mGlu receptor ligands have not been identified. Furthermore, amino acid derived mGlu receptor ligands have generally shown poor blood brain barrier penetration limiting their use in *in vivo* studies.

To overcome these hurdles we began in the middle of the 90s to search for non-amino acid-like antagonists that could modulate receptor activity possibly via new regulatory sites on the receptor protein. The first subtype selective $mGlu_5$ receptor antagonists reported from a collaborative effort of SIBIA Neurosciences Inc., and Novartis were SIB-1757 and SIB-1893 with IC_{50} values of 3.7 and 3.5 μM respectively, in the PI hydrolysis assay and at least 10-fold selectivity over other metabotropic or ionotropic glutamate receptor subtypes (Varney et al., 1999). Shortly after, we described 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a 100-fold more potent antagonist derived from chemical variation of SIB-1893 (Gasparini et al., 1999). At the human $mGlu_{5a}$ receptor expressed in recombinant cells, MPEP completely inhibited quisqualate-stimulated PI-hydrolysis with an IC_{50} value of 36 nM while having no significant agonist or antagonist activities at cells expressing other mGlu or iGlu receptor subtypes at concentrations up to 10 μM. In rat neonatal brain slices, MPEP inhibited DHPG-stimulated PI hydrolysis with a potency and selectivity similar to that observed on human mGlu receptors. Furthermore, in extracellular recordings in the CA1 area of the hippocampus, microiontophoretic application of DHPG or (1S,3R)-ACPD induced neuronal firing that was completely inhibited by co-administration of MPEP either iontophoretically or intravenously. Excitation induced by AMPA was not affected. Similarly, iontophoretic MPEP reduced responses of thalamic neurones to the $mGlu_5$ receptor agonist CHPG selectively compared to NMDA (Salt et al., 1999) suggesting that MPEP is a selective $mGlu_5$ receptor antagonist *in vivo*.

A recent report has questioned the selectivity of MPEP (O'Leary et al., 2000). Faden and colleagues have shown that MPEP when applied at 20 and 200 μM inhibited NMDA receptor activity by 22.6% and 64.9% in electrophysiological recordings of cultured primary cortical neurons and have therefore concluded that some of the *in vitro* and *in vivo* effects observed with MPEP might be due to direct inhibition of NMDA receptors. This conclusion should be taken with some caution for several reasons. First, we have published previously that MPEP shows good selectivity *in vitro* over a concentration range of four magnitudes up to 10 μM. However, at concentrations higher than 10 μM non-specific effects were observed (a non significant 23% inhibition of NMDA-induced currents of hNMDAR1/R2A was obtained at 30 μM in electrophysiological recordings using Xenopus oocytes). Therefore, it is not surprising that testing of MPEP at a concentration of 200 μM (also above its limit of solubility in an aqueous solution) shows significant effects as documented by Faden and colleagues. Second, the interpretation that *in vivo* effects of MPEP are also at least in part mediated by inhibition of NMDA receptors, should be revised since additional experiments showed that the brain levels of MPEP at pharmacologically active doses following i.v., i.p. or

p.o administrations are far below the concentrations needed to inhibit NMDA receptors in vitro (F. Gasparini, manuscript in preparation). Furthermore, MPEP (up to 30 mg/kg i.p.) was inactive in inhibiting NMDA-induced convulsions in rats (A. Pilc, personal communication).

Characterization of the binding domain of allosteric $mGlu_5$ receptor antagonists

Concentration-response analysis of glutamate in the presence of different concentrations of MPEP indicated that MPEP acts in a non-competitive manner by decreasing the efficacy of glutamate-stimulated phosphoinositide hydrolysis without affecting the EC_{50} value of glutamate (Pagano et al., 2001). The non-competitive mode of inhibition suggested that MPEP and its close derivatives interacts at a different site than the glutamate binding site located in the large extracellular domain. To elucidate the site of action, we have generated a set of chimeric $mGlu_{1/5a}$ and $mGlu_{5/1b}$ receptors fused at the border between the large N-terminal extracellular domain and the first TM segment. These chimeric receptors were transiently expressed in COS cells and were shown to couple to PI turnover and a subsequent release of $[Ca^{2+}]_i$ from internal stores. Investigation of the inhibition by MPEP showed that the respective transmembrane domain of the $mGlu_5$ receptor is necessary to mediate the inhibitory activity (Pagano et al., 2001).

To identify the amino acid residues mediating the selective receptor/ligand interaction a series of chimeric receptors and receptor mutants with amino acid exchanges between $mGlu_1$ and $mGlu_5$ were constructed and studied using the novel $mGlu_5$ receptor radioligand [^{3}H]-M-MPEP (2-(3-Methoxyphenylethynyl)-6-methyl-pyridine), a close analog of MPEP with a K_D of 2 nM. Substitution of Ala810 in TMVII or Pro655 and Ser658 in TMIII in the $mGlu_5$ receptor sequence with the homologous residues of $hmGlu_1$ completely abolished [^{3}H]-M-MPEP binding. Likewise, introduction of these amino acid residues into the homologous positions of the $mGlu_1$ receptor conferred MPEP inhibition and high-affinity [^{3}H]-M-MPEP binding indicating that MPEP specifically interacts with non-conserved amino acid residues in TMIII and TMVII of $mGlu_5$ receptors.

It is currently not known how noncompetitive $mGlu_5$ receptor antagonists inhibit receptor activity but it can be assumed that receptor activation requires a series of conformational changes in the extracellular ligand binding domain which are transmitted to the 7TM domain. Binding of noncompetitive antagonists in the 7TM domain can specifically affect conformational changes of the 7TM domain and thus have no direct effect on the occupation of the agonist-binding site.

Effects of $mGlu_5$ receptor antagonists in nervous system disease models

Pain

The localization of $mGlu_5$ rceptor protein and mRNA in peripheral endings of nociceptive C-fibre afferents (Walker et al., 2001a; Bhave et al., 2001), dorsal root ganglia (Valerio et al., 1997), superficial dorsal horn neurones (Vidnyanszki et al., 1994; Valerio et al., 1997; Jia et al., 1999; Berthele et al., 1999; Alvarez et al., 2000), thalamus and cerebral cortex (Romano et al., 1996; Neto et al., 2000) suggested a role for $mGlu_5$ receptors in nociceptive processes. In models of inflammatory pain systemic administration of MPEP produced effective reversal of the hyperalgesia associated with inflammation, without affecting the normal behavioural responses to noxious stimulation in naïve rats (Walker et al., 2001b). Experiments involving different routes of administration of MPEP (intraplantar, intrathecal and intracerebroventricular) suggest that these effects are primarily mediated by $mGlu_5$ receptors expressed on the peripheral terminals of sensory neurons (Walker et al., 2001a). Furthermore, studies with SIB-1757 showed that blockade of peripheral $mGlu_5$ receptors can also reverse neuropathy-induced thermal hyperalgesia (Dogrul et al., 2000).

Taken together, it is apparent that $mGlu_5$ receptors represent a novel target for intervention in pain processes, and it is likely that $mGlu_5$ receptor antagonism may be particularly valuable in specific pain types, e.g. inflammatory pain. However, it should be remembered that other glutamate receptors, particularly $mGlu_1$ receptors, have also been implicated in peripheral and central pain processes (Hargett et al., 2000; Young et al., 1998).

Anxiety

Glutamate is known to play an essential role in fear, fear-related learning and memory. Given the expression of $mGlu_5$ receptors in limbic forebrain regions an

involvement of $mGlu_5$ receptors in motivational and emotional processes and accordingly in psychiatric conditions such as anxiety and depression was hypothesized and tested in animal models. In the so-called conditioned response tests anxiolytic-like activity of MPEP was observed in the Vogel test and the four-plate test (Tatarczynska et al., 2001; Klodzinska et al., 2000). MPEP (1–30 mg kg^{-1}) significantly increased the number of shocks accepted without affecting water intake or shock perception in the Vogel test and significantly increased the number of punished crossings albeit at higher dosages in the four plate test. In the Geller-Seifter test the effects of MPEP, up to a dose of 100 mg kg^{-1}, were inconclusive since treatment with MPEP tended to increase the number of punished responses as well as the number of received shocks, but neither effect reached statistical significance (Spooren et al., 2000). The acquisition of conditioned fear and at higher doses also the expression of fear was shown to be prevented by MPEP in the fear-potentiated startle test (Schultz et al., in press). Interestingly, acquisition but not expression of conditioned fear was also prevented by direct injection of MPEP into the lateral amygdala, directly linking $mGlu_5$ receptors in the amygdala to fear conditioning (Fendt et al., 2000).

MPEP was also broadly tested in so-called unconditioned response tests. MPEP showed significant anxiolytic-like activity in a model of social anxiety (social exploration test in rats, effective dose range 0.3–1.0 mg kg^{-1}) and novelty-induced anxiety (marble burying test in mice, effective dose range 1.5–30 mg kg^{-1}). Furthermore, MPEP was effective in an approach-avoidance conflict model (elevated plus maze in rats, effective dose range 0.1–30 mg kg^{-1}) and finally in a model of anticipatory anxiety (stress-induced hyperthermia in mice, effective dose range 1.5–30 mg kg^{-1}) (Spooren et al., 2000).

Together these findings indicate that MPEP has a very broad and potent anxiolytic-like activity in rodent models of anxiety. The novel mechanism and the potential absence of sedation and psychotomimetic effects as assessed by spontaneous locomotor activity and the prepulse inhibition paradigm, suggest that $mGlu_5$ receptor antagonists may indeed represent a new and safe approach to the treatment of anxiety.

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Authors' address: Dr. Rainer Kuhn, Nervous System Research, Novartis, Pharma AG, WKL-125.6.08, CH-4002 Basel, Switzerland, Fax +41 61 69 62809, E-mail: Rainer.kuhn@pharma.novartis.com