In Vitro Pharmacological Characterization of Novel Isoxazolopyridone Derivatives as Allosteric Metabotropic Glutamate Receptor 7 Antagonists

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ABSTRACT

Novel isoxazolopyridone derivatives that are metabotropic glutamate receptor (mGluR) 7 antagonists were discovered and pharmacologically characterized. 5-Methyl-3,6-diphenylisoxazolo[4,5-c]pyridin-4(5H)-one (MDIP) was identified by random screening, and 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one (MMPIP) was produced by chemical modification of MDIP. MDIP and MMPIP inhibited L-AP4-induced intracellular Ca2+ mobilization in Chinese hamster ovary (CHO) cells coexpressing rat mGluR7 with Gα15. (IC50 = 20 and 26 nM). The maximal response in agonist concentration-response curves was reduced in the presence of MMPIP, and its antagonism is reversible. MMPIP did not displace [3H]L-CCG-I, a metabotropic glutamate receptor 5 (mGluR5) antagonist, bound to mGluR7. These results suggested that these isoxazolopyridone derivatives are allosteric antagonists. In CHO cells expressing rat mGluR7, MDIP and MMPIP inhibited L-AP4-induced inhibition of forskolin-stimulated cAMP accumulation (IC50 = 99 and 220 nM).

Metabotropic glutamate receptors (mGluRs) belong to a family of G protein-coupled receptors thought to contribute to the modulation of neuronal excitability and neurotransmitter release. Eight mGluR subtypes (mGluR1–mGluR8) have been cloned, and they have been classified into three groups based on sequence homology, pharmacological profile, and signal transduction pathway. mGluR1 and mGluR5 belong to group I mGluRs, and they are coupled to phospholipase C and subsequent intracellular calcium release via Gq protein. mGluR2 and mGluR3 belong to group II mGluRs, whereas mGluR4, mGluR6, mGluR7, and mGluR8 belong to group III mGluRs. The subtypes of group II and group III mGluRs are negatively coupled to adenylate cyclase via Gi protein (Conn and Pin, 1997).

mGluR7 is widely expressed in the central nervous system

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; CNS, central nervous system; AMN082, N,N′-dibenzhydryl-ethane-1,2-diamine dihydrochloride; hCT, human calcitonin; CHO, Chinese hamster ovary; CHO-rat mGluR7, Chinese hamster ovary cells expressing rat mGluR7; CHO-rat mGluR7/Gα15, Chinese hamster ovary cells coexpressing rat mGluR7 with Gα15; CHO-human mGluR7/Gα15, Chinese hamster ovary cells coexpressing human mGluR7 with Gα15; FLIPR, fluorometric imaging plate reader; MDIP, 5-methyl-3,6-diphenylisoxazolo[4,5-c]pyridin-4(5H)-one; MMPIP, 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one; L-AP4, L-AP4-induced cAMP response. In CHO cells coexpressing human mGluR7 with Gα15, MDIP and MMPIP also inhibited the L-AP4-induced cAMP response. The maximal degree of inhibition by MMPIP was higher than that by MDIP in a cAMP assay. MMPIP was able to antagonize an allosteric agonist, the N,N′-dibenzhydryl-ethane-1,2-diamine dihydrochloride (AMN082)-induced inhibition of cAMP accumulation.

In the absence of these agonists, MMPIP caused a further increase in forskolin-stimulated cAMP levels in CHO cells expressing mGluR7, whereas a competitive antagonist, LY341495, did not. This result indicates that MMPIP has an inverse agonistic activity. The intrinsic activity of MMPIP was pertussis toxin-sensitive and mGluR7-dependent. MMPIP at concentrations of at least 1 μM had no significant effect on mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, and mGluR8. MMPIP is the first allosteric mGluR7-selective antagonist that could potentially be useful as a pharmacological tool for elucidating the roles of mGluR7 on central nervous system functions.
mGluR7 is primary localized on presynaptic terminals where it is thought to regulate neurotransmitter release, and it is highly concentrated at specific neuronal terminals (Shigemoto et al., 1996). Based on its specific presynaptic localization and much lower L-glutamate affinity compared with other mGluR subtypes, mGluR7 seems to function as a low-pass filter, inhibiting synapses from firing above a certain frequency (Shigemoto et al., 1996). mGluR7 knockout mice showed reduced levels of anxiety, increased susceptibility to convulsants, and impaired working memory (Masugi et al., 1999; Sansig et al., 2001, Bough et al., 2004; Hölscher et al., 2004, 2005; Callaerts-Vegh et al., 2006; Mitsukawa et al., 2006).

However, the interpretation of phenotype analyses using genetically manipulated mGluR7 knockout mice might be limited by gene compensation, developmental effects, and variance among strains. Therefore, the pharmacological manipulation of mGluR7 by agonists and antagonists is useful to explore the physiological and pathophysiological roles of mGluR7. Recently, AMN082 was identified as the first mGluR7-selective allosteric agonist, and activation of mGluR7 with AMN082 was shown to modulate plasma stress hormone concentrations (Mitsukawa et al., 2005). Therefore, mGluR7 antagonists may be useful for treating conditions involving chronic stress, such as depression and anxiety disorders (Conn and Niswender, 2006). However, no mGluR7-selective antagonist has been discovered to date.

Extensive efforts to identify subtype-selective mGluR ligands by competitive binding assays have been unsuccessful, probably due to the fact that the amino acid sequences of L-glutamate binding sites are highly conserved among mGluR subtypes (Kunishima et al., 2000). In contrast, high-throughput functional assays for detecting Ca\(^{2+}\)/H\(_{11001}\) mobilization have led to the identification of subtype-selective ligands in G\(_{i}\)-coupled mGluR1 and mGluR5 (Varney et al., 1999; Suzuki et al., 2007). However, it is difficult to directly apply this type of high-throughput functional assay to the identification of subtype-selective ligands for Gi-coupled group II or group III mGluR subtypes. Promiscuous G proteins such as G\(_{a15}\) and G\(_{a16}\) are known to allow Gi-coupled receptors to couple to phospholipase C, resulting in Ca\(^{2+}\)/H\(_{11001}\) mobilization in response to agonist stimulation (Offermanns and Simon, 1995). In addition, mGluR7 is known to efficiently couple with G\(_{a15}\), but not with G\(_{a16}\) (Parmentier et al., 1998). To identify mGluR7-selective ligands, we generated CHO cells stably coexpressing mGluR7 with G\(_{a15}\), and we screened chemical libraries using the cells and fluorometric imaging plate reader (FLIPR). Because mGluR7 seems to be coupled to G\(_{i}\) rather than to G\(_{a15}\) in neurons (Wright and Schoepp, 1996), their activities were further confirmed by G\(_{i}\)-coupled cAMP response. Evaluation of mGluR7 ligands on mGluR7-mediated cAMP response could be a physiologically relevant assay, because mGluR7 is known to regulate L-glutamate release through modulation of cAMP levels in cerebrocortical nerve terminals (Millán et al., 2002).

Using the screening strategy mentioned above, we have identified isooxazolopyridone derivatives as mGluR7 antagonists. Based on descriptions in the patent application (Nakamura et al., 2002), several isooxazolopyridone derivatives have been synthesized, and preliminary pharmacological characterization of the compounds has been reported (Niswender et al., 2006). In the present study, we describe the comprehensive in vitro pharmacological characterization of two isooxazolopyridone derivatives. 5-Methyl-3,6-diphenylisoxazolo[4,5-c]pyridin-4(5H)-one (MDIP) was identified as a novel mGluR7 antagonist by random high-throughput functional screening, whereas 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one (MMPIP) was obtained by subsequent chemical modification of MDIP. These isooxazolopyridone derivatives were pharmacologically characterized using recombinant rat and human mGluR7-expressing cells.

Materials and Methods

MDIP (Fig. 1A) was originally identified as a mGluR7 ligand from an in-house chemical library. MMPIP (Fig. 1B) was synthesized...
in-house. L(-)-2-amino-4-phosphonobutyric acid (l-AP4), (2S,1'S,2'S)-2-carboxycyclopolyglycine (l-CCG-I), 2-methyl-6-(phenylethynyl)pyridine (MPEP), (R,S)-α-cyclopenty1-4-phosphony
ephosphoglycerine (CPPG), LY341495, and [3H]LY341495 (36.5 Ci/mmol) were purchased from Tocris Cookson Inc. (Bristol, UK). l-Glutamate, 5-hydroxytryptamine (5-HT), 3-isobutyl-1-methylxanthine (IBMX), pertussis toxin (PTX), and human calciitonin (hCT; thyrocalcitonin) were purchased from Sigma-Aldrich (St. Louis, MO). AMN082 (Fig. 1C) was purchased from Ascent Scientific (North Somerset, UK). PTxDC was synthesized in-house (Suzuki et al., 2007). l-Proline and foskolin were purchased from Wako Pure Chemicals (Osaka, Japan). Dialyzed fetal bovine serum, culture media, and other reagents used for cell culture were purchased from Invitrogen (Carlsbad, CA). All other reagents were used of molecular or analytical grade, where appropriate.

Methods

Stable Cell Lines. Rat mGluR7 cDNA was kindly donated by Dr. S. Nakanishi (Osaka Bioscience Institute, Osaka, Japan). CHO-NFAT-bla cells from Aurora Biosciences (San Diego, CA) were transfectected with rat mGluR7 cDNA cloned into pIREShyg (Clontech), and they were selected in medium [Dulbecco’s modified Eagle’s medium with 10% dialyzed fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 1% proline supplemented with 500 μg/ml Geneticin (G-418; Invitrogen). The stable cell lines were isolated, and they were selected by their abilities to inhibit forskolin-stimulated cAMP accumulation following l-AP4 addition. CHO-NFAT-bla cells expressing rat mGluR7 (CHO-rat mGluR7) were transfected with G015 cDNA (Aurora Biosciences) cloned into pIREShyg (Clontech), and they were selected in medium supplemented with 500 μg/ml hygromycin B (Invitrogen) and 500 μg/ml G-418. The stable cell lines were isolated, and they were selected by their abilities to elicit Ca2+ mobilization following l-AP4 addition (CHO-rat mGluR7/G015). CHO cells stably coexpressing human mGluR7 with G015 (CHO-human mGluR7/G015) and expressing human mGluR5 were obtained as described previously by O’Brien et al. (2004). CHO-dhfr- cells stably expressing human mGluR1a were described previously by Ohashi et al. (2002). CHO-dhfr<sup>−</sup> cells stably expressing rat mGluR3 and rat mGluR4 were kindly donated by Dr. S. Nakanishi. CHO-dhfr<sup>−</sup> cells stably coexpressing human mGluR2 with G015 and CHO-K1 cells stably expressing human mGluR8 were obtained as described previously by Suzuki et al. (2007).

Intracellular Ca2+ Mobilization. Intracellular Ca2+ mobilization was measured according to the method described by Suzuki et al. (2007). In brief, CHO cells coexpressing rat mGluR7 with G015 were seeded at 5 × 10<sup>5</sup> cells/well in a 96-well black-well/clear-bottomed plate (PerkinElmer Life and Analytical Sciences, Boston, MA), and they were cultured overnight. The cells were then incubated with 4 μM Fluo-3 in assay buffer (Hanks’ balanced salt solution containing 20 mM HEPES and 2.5 mM proceneb) containing 1% dialyzed fetal bovine serum for 1 h at 37°C with 5% CO2 in a humidified atmosphere. The extracellular dye was removed, and the cell culture was washed three times with ice-cold phosphate-buffered saline and solubilized in 2 M NaOH. Radioactivity was measured using TritonX-2500 (PerkinElmer Life and Analytical Sciences) after addition of Ultima Gold XR (PerkinElmer Life and Analytical Sciences). Non-specific binding was defined as binding in the presence of 100 μM LY341495.

Intracellular cAMP Measurements. Intracellular cAMP was measured by a modification of the method of Tanabe et al. (1992). CHO cells expressing rat mGluR7, rat mGluR3, rat mGluR4, and human mGluR8 were seeded at 5 × 10<sup>4</sup> cells/well in a 96-well clear-bottomed plate, and they were cultured overnight. The culture medium was then replaced with Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 3.6 mM NaHCO<sub>3</sub>, 5.6 mM glucose, and 10 mM HEPES, pH 7.4) containing 1 mM IBMX, and the cells were incubated for 20 min at 37°C with 5% CO2 in a humidified atmosphere. The cells were then incubated with Locke’s buffer containing 1 mM IBMX and 10 μM forskolin in the presence or absence of test compounds for an additional 20 min. The amount of intracellular cAMP was determined using the cAMP enzyme immunoassay Biotrak (enzyme immunoassay) system (GE Healthcare, Piscataway, NJ). CHO cells stably coexpressing human mGluR7 with G015 were seeded at 4 × 10<sup>4</sup> cells/well in a 96-well clear-bottomed plate, and they were cultured overnight. The final concentrations of forskolin and IBMX were 3 μM and 0.5 mM, respectively. Intracellular cAMP levels were determined using an AlphaScreen CAMP Assay kit (PerkinElmer Life and Analytical Sciences) according to the manufacturer’s instructions. In the antagonist assay for rat and human mGluR7, the final concentrations of l-AP4 were 0.5 and 1 mM, respectively, whereas in the antagonist assay for mGluR3, mGluR4, and mGluR8, the final concentrations of l-glutamate were 100, 70, and 10 μM, respectively. To evaluate the effects of compounds on cAMP response in PTX-treated CHO cells expressing rat mGluR7, the cells were incubated with 100 ng/ml PTX for 24 h before the cAMP assay.

Knockdown of mGluR7 Using siRNA. Four siRNA 21-mers matching the human mGluR7 sequence (referred to as mGluR7 siRNA) were purchased in a pooled form (siGENOME SMARTpool, M-005622-00) from Dharmacon RNA Technologies (Lafayette, CO). An siRNA 21-mer matching the luciferase GL2 sequence was used as a negative control siRNA (referred to as control siRNA) (Elbashir et al., 2002). CHO cells coexpressing human mGluR7 with G015 were seeded at 2.5 × 10<sup>5</sup> cells in a 25-cm<sup>2</sup> flask, and they were cultured overnight in the culture medium described above, but without antibiotics. siRNA was transfected into cells at a final concentration of 25 nM using DharmaFECT 4 (Dharmacon RNA Technologies) according to the manufacturer’s instructions. After 2 days, the cells were seeded at 4 × 10<sup>4</sup> cells in a 96-well white-well/clear-bottomed plate (PerkinElmer Life and Analytical Sciences), and they were cultured overnight. Intracellular cAMP levels were measured as described above.

Total RNA was extracted from the cells 3 days after transfection using an RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany). TaqMan Gene Expression Assays (Assay ID, Hs00356067_m1) and TaqMan Rodent GAPDH Control Reagents (Applied Biosystems, Foster City, CA) were used to quantify mRNA expression of mGluR7 and GAPDH, respectively. Reverse transcription of 0.5 μg of total RNA was performed in a total volume of 25 μl using random hexamers and a TaqMan Reverse Transcription Reagent kit (Applied Biosystems) according to the manufacturer’s protocol. The resultant cDNA sample (25 μl) was diluted with 75 μl of nucleash-free water. Quantitative real-time PCR was performed in a total volume of 25 μl containing 12.5 μl of TaqMan Universal PCR Master Mix, 1.25 μl of
The response to 3 mML-AP4 (Fig. 2A). Relative fluorescence agonist activity in CHO-rat mGluR7/G respectively (Fig. 2B). These compounds did not show any statistically significant.

Data and Statistics

Data analyses were performed using Prism, version 4.03, from GraphPad Software Inc. (San Diego, CA). Concentration-response curves for Ca2+ mobilization and cAMP accumulation were fitted using nonlinear regression analysis. To determine the potency of a noncompetitive antagonist, the \( K_B \) value was calculated from the equation \( K_B = (B_{\text{max}} \times \text{slope}) \), where \( B \) is the concentration of antagonist (B), and slope is that of a double-reciprocal plot of equieffective concentrations of agonist (A) in the absence (1/\( A \)) and presence (1/\( A' \)) of antagonist (Kenakin, 1997). Competition binding experiments were analyzed using nonlinear regression analysis. Student's \( t \) test was used to analyze data obtained from studies with PTX and siRNA. A probability level of < 0.05 was considered statistically significant.

**Results**

**Activities of Isoxazolopyridone Derivatives toward Recombinant mGluR7.** In CHO cells coexpressing rat mGluR7 with Go15 (CHO-rat mGluR7/Go15), the mGluR7 agonists L-AP4 and L-CCG-I were able to increase intracellular Ca2+ concentrations, with \( EC_{50} \) values of 140 ± 18 \( \mu M \) \((n = 5)\) and 70 ± 6.4 \( \mu M \) \((n = 4)\), respectively. The maximum response of L-CCG-I was 53 ± 8.7% \((n = 4)\), a percentage of the response to 3 mM L-AP4 (Fig. 2A). Relative fluorescence units in CHO-rat mGluR7/Go15 were 16,000 ± 1200 \((n = 5)\) and 8500 ± 1400 \((n = 4)\) in the presence of 3 mM L-AP4 and 3 mM L-CCG-I, respectively. MDIP was identified from an in-house chemical library by random screening with CHO-rat mGluR7/Go15, and MMPIP was obtained by successive chemical modification of MDIP. In CHO-rat mGluR7/Go15, MDIP and MMPIP inhibited 0.5 mM L-AP4-induced Ca2+ mobilization, with \( IC_{50} \) values of 20 ± 2.4 and 26 ± 3.4 \( nM \) \((n = 8)\), respectively (Fig. 2B). These compounds did not show any agonist activity in CHO-rat mGluR7/Go15. To analyze the mode of action of these isoxazolopyridone derivatives, the effect of MMPIP on agonist concentration-response curves of intracellular Ca2+ mobilization was evaluated in CHO-rat mGluR7/Go15. Agonist concentration-response curves for L-AP4 and L-CCG-I-induced increases in intracellular Ca2+ concentrations were obtained in the presence or absence of MMPIP. The maximal responses of L-AP4 and L-CCG-I were reduced in the presence of MMPIP (Fig. 3, A and B), whereas an orthosteric antagonist, CPPG, caused a parallel rightward shift in the L-AP4 concentration-response curves with no effect on maximum response (Fig. 3C). The antagonism of MMPIP on L-AP4- and L-CCG-I-induced Ca2+ responses was analyzed by a model of noncompetitive antagonism (Kenakin, 1997). To estimate the \( K_B \) values of MMPIP, we used a double-reciprocal plot of equally effective concentrations of L-AP4 or L-CCG-I (A) in the absence (1/\( A \)) and presence (1/\( A' \)) of MMPIP (Supplemental Fig. 1, A and B). Equally effective concentrations were calculated from curves in the absence or presence of 100 nM MMPIP shown in Fig. 3A and in the presence or absence of 30 nM MMPIP shown in Fig. 3B, respectively. The \( K_B \) values of MMPIP calculated from the double-reciprocal plots of L-AP4 and L-CCG-I were 24 ± 3.4 and 30 ± 2.7 nM, respectively. Schild plot analysis of the antagonism produced by CPPG yielded a \( pA_2 \) of 4.7 ± 0.053 and a slope factor of 0.91 ± 0.055 (Supplemental Fig. 1C).

The reversibility of the antagonism by MMPIP was determined by comparing Ca2+ responses to 0.5 mM L-AP4 in CHO-rat mGluR7/Go15 with and without washout procedure. MMPIP (0.01–1 \( \mu M \)) dose-dependently inhibited L-AP4-induced Ca2+ response. The agonist-induced responses were recovered within 5 min after washout of MMPIP (Fig. 3D). [3H]LY341495 binding assays, carried out to evaluate whether MMPIP binds to the l-glutamate binding site of mGluR7, showed that it did not displace [3H]LY341495 bound to CHO-rat mGluR7 (Fig. 3E). In contrast to MMPIP, both LY341495 and CPPG displaced [3H]LY341495 bound to CHO-rat mGluR7.

**Effects of Isoxazolopyridone Derivatives on Agonist-Induced Inhibition of Forskolin-Stimulated cAMP Accumulation in CHO Cells Expressing mGluR7.** The effects of isoxazolopyridone derivatives on cAMP accumulation were evaluated using CHO-rat mGluR7 and CHO-human mGluR7/Go15. L-AP4 inhibited forskolin-stimulated cAMP accumulation with \( EC_{50} \) values of 86 ± 12 \( \mu M \) \((n = 12)\) and 170 ± 76 \( \mu M \) \((n = 4)\) in CHO-rat mGluR7 and CHO-human.
Characterization of Novel Allosteric mGluR7 Antagonists

Intrinsic Activity of MMPIP toward mGluR7. An allosteric mGluR7 agonist, AMN082 (Fig. 1C), inhibited forskolin-stimulated cAMP accumulation, with an EC50 value of 95 ± 36 nM (n = 4) in CHO-human mGluR7/Ga15 (Fig. 5A), whereas AMN082 up to 10 μM did not induce intracellular Ca2+ mobilization (Supplemental Fig. 2). MMPIP dose-dependently antagonized AMN082-induced inhibition of cAMP accumulation in CHO-human mGluR7/Ga15, with IC50 values of 1600 ± 360 nM (n = 3) and 2300 ± 1300 nM (n = 3), respectively. Because the maximal degree of inhibition by MMPIP was higher than that by MDIP, as shown in Fig. 4, C and D, MMPIP was selected for further pharmacological characterization.

mGluR7/Ga15, respectively (Fig. 4, A and B). In CHO-rat mGluR7, MDIP and MMPIP dose-dependently antagonized L-AP4-induced inhibition of cAMP accumulation with IC50 values of 99 ± 25 nM (n = 6) and 220 ± 23 nM (n = 5), respectively (Fig. 4C). MDIP and MMPIP also antagonized L-AP4-induced inhibition of cAMP accumulation with IC50 values of 140 ± 18 nM (n = 6) and 610 ± 130 nM (n = 5), respectively, in CHO-human mGluR7/Ga15 (Fig. 4D). The orthosteric mGluR antagonist LY341495 antagonized L-AP4-induced inhibition of cAMP accumulation in CHO-rat mGluR7 and CHO-human mGluR7/Ga15, with IC50 values of 1400 ± 360 nM (n = 3) and 2300 ± 1300 nM (n = 3), respectively. Because the maximal degree of inhibition by MMPIP was higher than that by MDIP, as shown in Fig. 4, C and D, MMPIP was selected for further pharmacological characterization.

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tion with EC\textsubscript{50} values of 97 ± 36 nM (n = 4) in CHO-human mGluR7/G\textalpha\textsubscript{15}. AMN082 inhibited forskolin-stimulated cAMP accumulation with an EC\textsubscript{50} value of 95 ± 36 nM (n = 4) in CHO-human mGluR7/G\textalpha\textsubscript{15}. AMN082 (0.1–10 \mu M) dose-dependently antagonized 1 \mu M AMN082-induced inhibition of cAMP accumulation, whereas the orthosteric agonist CPPG (CP) did not antagonize even at 1 mM. An orthosteric agonist, L-AP4, at 1 mM, induced inhibition of cAMP accumulation, and it was antagonized by CPPG at 1 mM. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation, and they are the means ± S.E.M. from four or five individual experiments.

**Fig. 5.** Effect of MMPIP on allosteric mGluR7 agonist AMN082-induced inhibition of cAMP accumulation in CHO-human mGluR7/G\textalpha\textsubscript{15}. A, AMN082 inhibited forskolin-stimulated cAMP accumulation with an EC\textsubscript{50} value of 97 ± 36 nM (n = 4) in CHO-human mGluR7/G\textalpha\textsubscript{15}. B, MMPIP (0.1–10 \mu M) dose-dependently antagonized 1 \mu M AMN082-induced inhibition of cAMP accumulation, whereas the orthosteric agonist CPPG (CP) did not antagonize even at 1 mM. An orthosteric agonist, L-AP4, at 1 mM, induced inhibition of cAMP accumulation, and it was antagonized by CPPG at 1 mM. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation, and they are the means ± S.E.M. from four or five individual experiments.

**Effect of mGluR7 Knockdown on Intrinsic Activity of MMPIP.** To confirm the intrinsic activity of MMPIP on mGluR7, its activity was evaluated in CHO-human mGluR7/G\textalpha\textsubscript{15} transfected with mGluR7 siRNA. mGluR7 siRNA and negative control siRNA were transfected into CHO-human mGluR7/G\textalpha\textsubscript{15}. Three days after transfection, mGluR7 mRNA levels were measured using quantitative real-time reverse transcription-PCR (Fig. 7A). In CHO-human mGluR7/G\textalpha\textsubscript{15} transfected with mGluR7 siRNA (mGluR7 knockdown cells), mGluR7 mRNA levels were decreased to 34 ± 6.7% (n = 4; percentage of nontransfected cells), whereas mGluR7 mRNA levels in CHO-human mGluR7/G\textalpha\textsubscript{15} transfected with negative control siRNA (control mGluR7 cells) were unaffected (102 ± 21%; n = 4; percentage of nontransfected cells). The difference in mGluR7 mRNA levels between mGluR7 knockdown cells and the control cells was statistically significant (P < 0.05). In mGluR7 control cells, 1 \mu M AMN082 inhibited forskolin-stimulated cAMP accumulation to 37 ± 9.2% (n = 4) (percentage of forskolin-stimulated cAMP accumulation).

In contrast, cAMP levels in the presence of 1 \mu M AMN083 were 78 ± 12% (n = 4) in mGluR7 knockdown cells (Fig. 7B). MMPIP increased forskolin-stimulated cAMP accumulation in the mGluR7 control cells, whereas the compound had little effect on mGluR7 knockdown cells (Fig. 7C). The differences in the activities of AMN082 and MMPIP between mGluR7 control cells and knockdown cells were statistically significant (P < 0.05). To confirm the specificity of knockdown by mGluR7 siRNA, the effects of 5-HT and hCT on cAMP accumulation were evaluated in mGluR7 control cells and knockdown cells endogenously expressing G\textsubscript{i}-coupled 5-HT receptors and G\textsubscript{\alpha}-coupled calcitonin receptors (Fig. 7B). 5-HT comparably inhibited forskolin-stimulated cAMP accumulation in both mGluR7 knockdown cells and mGluR7 control cells, whereas hCT increased cAMP accumulation with comparable efficacy in mGluR7 knockdown cells as well as the mGluR7 control cells.

**Fig. 6.** Intrinsic activity of MMPIP toward mGluR7. In the absence of agonist, MMPIP increased forskolin-stimulated cAMP accumulation in CHO-rat mGluR7 (A) or CHO-human mGluR7/G\textalpha\textsubscript{15} (B). Effects of MMPIP on cAMP accumulation in CHO-rat mGluR7 treated with PTX (C). PTX diminished L-AP4-induced inhibition of forskolin-stimulated cAMP and MMPIP-induced increase in forskolin-stimulated cAMP in CHO-rat mGluR7. The final concentrations of L-AP4 and MMPIP were 1 mM and 0.01 to 1 \mu M, respectively. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation, and they are the means ± S.E.M. from more than three individual experiments. Statistical analyses were conducted with Student’s t test. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus control.
Selectivity of MMPIP toward Other mGluR Subtypes. L-Glutamate induced intracellular Ca\(^{2+}\) mobilization in CHO cells expressing mGluR1 or mGluR5, and in CHO cells coexpressing mGluR2 with G\(_{\alpha_{16}}\) (mGluR2/G\(_{\alpha_{16}}\)). FTIDC (an mGluR1 antagonist; 0.1 μM), 0.1 μM MPEP (an mGluR5 antagonist), and 1 μM LY341495 (a group II/III mGluR antagonist) inhibited L-glutamate-induced increases in Ca\(^{2+}\) concentrations in CHO cells expressing mGluR1, mGluR5, and mGluR2/G\(_{\alpha_{16}}\), respectively. In contrast, MMPIP did not show either antagonistic or agonistic activity toward these mGluR subtypes (Fig. 8). In CHO cells expressing mGluR3, mGluR4, or mGluR8, L-glutamate inhibited forskolin-stimulated cAMP accumulation, whereas MMPIP did not. MMPIP also did not antagonize L-glutamate-induced inhibition of cAMP accumulation in CHO cells expressing mGluR3, mGluR4, or mGluR8, whereas LY341495 acted as an antagonist (Fig. 9). The selectivity of MMPIP was also tested against 168 target molecules, including enzymes, neu-
Discussion

CHO cells were constructed that coexpress mGluR7 with promiscuous G protein (G<sub>15</sub>), and coexpression was shown to be coupled to intracellular Ca<sup>2+</sup> mobilization. Using CHO-rat mGluR7/G<sub>15</sub>, the isoxazolopyridone derivatives MDIP and MMPIP were identified as mGluR7 antagonists. These isoxazolopyridone derivatives fully inhibited 1-AP4-induced Ca<sup>2+</sup> mobilization in CHO-rat mGluR7/G<sub>15</sub>. MMPIP caused the reduced maximal Ca<sup>2+</sup> response to agonist in CHO-rat mGluR7/G<sub>15</sub>, whereas an orthosteric antagonist, CPPG, did not reduce the maximum response. Antagonism of MMPIP was analyzed using a model of noncompetitive antagonism, indicating that MMPIP has a potent mGluR7 antagonist (<i>K<sub>i</sub></i> values = 24–30 nM). An insurmountable antagonism in the measurement of transient Ca<sup>2+</sup> responses could be caused not only by an allosteric antagonist but also by a slowly dissociating or irreversible orthosteric antagonist (Kenakin, 1997; Christopoulos et al., 1999). The agonist-induced Ca<sup>2+</sup> mobilization in CHO-rat mGluR7/G<sub>15</sub> was fully recovered within 5 min after washout of MMPIP, suggesting that MMPIP is a reversible antagonist with fast dissociation kinetics. [<sup>3</sup>H]LY341495 is an orthosteric radioligand that binds to the L-glutamate binding site of mGluR7 (Wright et al., 2000); CPPG displaced [<sup>3</sup>H]LY341495 bound to rat mGluR7 expressed in CHO, whereas MMPIP did not. These results suggested that the antagonism by MMPIP could be exerted via an allosteric mechanism. Chimeric and point-mutated receptors of mGluR7 will be useful for further characterization of an allosteric inhibitory mechanism by MMPIP.

AMN082 was recently identified as an mGluR7-selective agonist that activates mGluR7 via an allosteric site in the transmembrane domain (Mitsukawa et al., 2005). In the present study, MMPIP antagonized AMN082-induced inhibition of cAMP accumulation. In contrast, an orthosteric mGluR antagonist, CPPG, did not inhibit the effect of AMN082, consistent with previous observations (Mitsukawa et al., 2005). These results suggest that MMPIP is an allosteric mGluR7 antagonist, and they suggest that the binding regions of MMPIP might be shared with those of AMN082. However, further studies, such as a binding assay with radiolabeled MMPIP or AMN082, are necessary before this interpretation can be validated.

mGluR7 is expressed in many regions of the CNS, whereas G<sub>15</sub> expression is normally limited to certain cells derived from the hematopoietic lineage (Offermanns and Simon, 1995). Thus, mGluR7 is not likely to couple with G<sub>15</sub> in the CNS under physiological conditions. In heterologous expression systems, mGluR7 is negatively coupled with adenylate cyclase via G<sub>i</sub> protein, resulting in inhibition of forskolin-stimulated cAMP accumulation (Okamoto et al., 1994; Saugstad et al., 1994; Wu et al., 1998). In addition, a group III mGluR agonist, 1-AP4, inhibits forskolin-stimulated cAMP accumulation in neuronal cells. The inhibitory effect is biphase, with the low-affinity component probably mediated by mGluR7 and the high-affinity component possibly mediated by mGluR4 (Wright and Schoepp, 1996). In the present study, MMPIP antagonized 1-AP4-induced inhibition of forskolin-stimulated cAMP accumulation in Cho cells expressing mGluR7. 1-AP4 is known to inhibit forskolin-stimulated cAMP levels and resultant L-glutamate release in cerebrocortical nerve terminals, presumably via mGluR7 (Millán et al.,

Fig. 9. Selectivity of MMPIP toward mGluR3, mGluR4, and mGluR8. MMPIP (1 and 10 μM) was tested to investigate its effect on mGluR3 (A), mGluR4 (B), and mGluR8 (C). In the antagonist assay for mGluR3, mGluR4, and mGluR8, the final concentration of L-glutamate was 100, 70, and 10 μM, respectively. LY341495 (LY) was used as a positive control antagonist. The final concentration of LY341495 was 10, 100, and 10 μM for mGluR3, mGluR4, and mGluR8, respectively. Data are expressed as a percentage of forskolin-stimulated cAMP accumulation, and they are means ± S.E.M. from more than three individual experiments performed in duplicate. Basal cAMP levels (picomoles per well) in CHO cells expressing mGluR3, mGluR4, and mGluR8 were 0.17 ± 0.02 (n = 3), 0.43 ± 0.04 (n = 5), and 0.31 ± 0.041 (n = 4), respectively. Forskolin-stimulated cAMP levels (picomoles per well) in CHO cells expressing mGluR3, mGluR4, and mGluR8 were 5.5 ± 0.39 (n = 3), 15 ± 1.0 (n = 5), and 7.0 ± 0.99 (n = 4), respectively.

rotransmitter receptors, transporters, and ion channels; these included ionotropic glutamate receptors (N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainate) and other neurotransmitter receptors (dopamine, serotonin, acetylcholine, GABA, adrenalin, and histamine) (MDS Pharma, Bothell, WA). The IC<sub>50</sub> values of MMPIP were higher than 10 μM against all these targets, except for 51% inhibition at 10 μM toward monoamine oxidase-A (data not shown).
Therefore, the inhibitory activity of MMPIP shown in CHO cells expressing mGLR7 could be biologically relevant, because the compound exhibits inhibitory activities not only in Gαq-coupled Ca2+ mobilization but also in Gq-coupled cAMP response.

In the absence of an agonist, MMPIP caused a further increase in forskolin-stimulated cAMP levels in CHO cells expressing mGLR7. This is in contrast to the effect of an agonist such as L-AP4, suggesting that MMPIP exhibits inverse agonist activity and thus it might inhibit the agonist-independent constitutive activity of mGLR7. An alternative interpretation could be that MMPIP inhibits activation of mGLR7 caused by either residual L-glutamate in the assay medium or endogenous L-glutamate released from cells. However, an orthosteric mGLR antagonist, LY341495, did not increase forskolin-stimulated cAMP in CHO cells expressing mGLR7 in the absence of agonist. These results support the conclusion that the activity of MMPIP in the absence of agonist comes from inhibition of the constitutive activity of mGLR7, and not from inhibition of mGLR7 activation due to L-glutamate contamination from the medium or cells. Treatment of CHO cells expressing mGLR7 with PTX diminished the intrinsic activity of MMPIP in the absence of an agonist. This result suggests that the intrinsic activity of MMPIP is mediated via PTX-sensitive Gq proteins and that the compound does not directly activate endogenous adenylate cyclase in the cells. To date, the constitutive activity of mGLR7 has not been directly demonstrated under physiological conditions in the CNS. However, in the absence of an agonist, mGLR7 constitutively inhibited voltage-sensitive Ca2+ channels via Gq, in cerebellar granule neurons transfected with mGLR7 cDNA (Bertaso et al., 2006). This mGLR7-dependent constitutive inhibition of voltage-sensitive Ca2+ channels might be mediated via constitutive activity of mGLR7, and it suggests that a constitutively active mGLR7 might have physiological roles in the cerebellum. Therefore, MMPIP could be useful for revealing the functions of the agonist-independent activity of mGLR7.

RNA interference using siRNA was used to confirm whether the intrinsic activity of MMPIP was dependent on mGLR7 expression. mGLR7 knockdown cells exhibited decreased agonistic activity of AMN082, suggesting that mGLR7 knockdown cells have lost mGLR7 receptor function. In addition, the intrinsic activity of MMPIP was significantly diminished in the same mGLR7 knockdown cells. These results indicate that the intrinsic activity of MMPIP is mediated via mGLR7. To confirm the specificity of mGLR7 knockdown, the effects of 5-HT and hCT on cAMP levels were compared between mGLR7 knockdown cells and mGLR7 control cells. CHO cells endogenously express the 5-HT1b receptor, and 5-HT inhibits forskolin-stimulated cAMP accumulation via Gq protein in CHO cells (Giles et al., 1996). In the present study, forskolin-stimulated cAMP accumulation was comparably inhibited by 5-HT in both mGLR7 knockdown cells and in mGLR7 control cells. hCT increased cAMP accumulation via activation of endogenous calcitonin receptors in CHO cells (George et al., 1997). In the present study, hCT increased cAMP accumulation in mGLR7 knockdown cells as well as in mGLR7 control cells. These results indicate that the diminished intrinsic activity of MMPIP toward mGLR7 knockdown cells is due to specific mGLR7 knockdown and not to nonspecific effects of mGLR7 siRNA.

The results presented here show that both MDIP and MMPIP fully inhibit agonist-induced Ca2+ mobilization via the Gq pathway. However, the maximum degree of inhibition by MDIP was less than that of MMPIP in eliciting a cAMP response from Gq protein in both human and rat mGLR7. Although the exact reason for this difference is not presently clear, it might be related to the property of allosteric antagonists that allow orthosteric agonist binding to the receptor. Allosteric antagonists might block specific signaling pathways while permitting other intracellular signaling, an idea recently proposed as “permissive antagonism” (Kenakin, 2005). The difference in maximum degree of inhibition toward mGLR7-mediated Ca2+ mobilization and cAMP response might be explained by permissive antagonism, suggesting that isoxazolopyridine derivatives could exhibit signal-pathway-dependent antagonistic activity. In addition, the antagonist potencies (IC50 values) of MMPIP and MDIP on cAMP response were less potent than those on Ca2+ mobilization. The permissive antagonism could also explain the difference between the two readouts. Alternatively, the discrepancy could be interpreted by a three-state receptor model (Leff et al., 1997). In the model, the receptor may be inactive (R) or can adopt two active conformations (R* and R**) that preferentially interact with different G proteins (Gq and Gq, respectively). MMPIP and MDIP might have different affinities for two conformations of mGLR7 coupling to Gα15 and Gq0. It may therefore be worth evaluating the affinities of isoxazolopyridine derivatives toward other functional responses (Saugstad et al., 1996, Perroy et al., 2000; Millán et al., 2002, 2003) to further understand their signal pathway-dependent activities.

Counter assays using CHO cells expressing other mGLR subtypes showed that MMPIP is selective for mGLR7. MMPIP did not exhibit agonistic or antagonistic activity toward Gq-coupled mGLR1 and mGLR5. Furthermore, MMPIP did not inhibit agonist-induced Ca2+ mobilization in CHO cells coexpressing mGLR2 with promiscuous G protein. These results further suggest that the inhibitory activity of MMPIP in CHO-rat mGLR7/Gα15 does not arise from nonspecific inhibition of the signaling pathway via Gq proteins, including promiscuous G protein. Furthermore, MMPIP at concentrations of at least 1 μM exhibited no significant effect on cAMP response mediated via mGLR3, mGLR4, or mGLR8.

The limitation of the present study was that all the results were obtained using recombinant systems; thus, the mode of action of these compounds might be different in native tissues where expression levels of mGLR7 could be lower than those in the recombinant systems. It will be necessary to confirm their actions in native mGLR7 in future studies.

In conclusion, MMPIP is a potent mGLR7 antagonist against agonist-induced Gq-coupled Ca2+ mobilization and Gq-coupled cAMP pathway. The inhibitory mode is noncompetitive and allosteric. In the absence of agonist, MMPIP showed PTX-sensitive and mGLR7-dependent intrinsic activities, suggesting inverse agonist activity. MMPIP showed no significant effect on other mGLR subtypes or on other molecules tested. This is the first detailed description of allosteric mGLR7-selective antagonists. It is expected that MMPIP will be a useful pharmacological tool for elucidating the role of mGLR7 on CNS functions.
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References


