In vitro pharmacological characterization of novel isoxazolopyridone derivatives as allostERIC metabotropic glutamate receptor 7 (mGluR7) antagonists

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Nonstandard abbreviations:
5-HT, 5-hydroxytryptamine; AMN082, N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride; CHO, Chinese hamster ovary; CHO-rat mGluR7, Chinese hamster ovary cells expressing rat mGluR7; CHO-rat mGluR7/Gα15, Chinese hamster ovary cells co-expressing rat mGluR7 with Gα15; CHO-human mGluR7/Gα15, Chinese hamster ovary cells co-expressing human mGluR7 with Gα15; CNS, central nervous system; CPPG, (RS)-α-cyclopropyl-4-phosphonophenylglycine, dhfr, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FLIPR,
fluorometric imaging plate reader; FTIDC, 4-[1-(2-fluoropyridin-3-yl)-5-methyl-1H-1,2,3-triazol-4-yl]-N-isopropyl-N-methyl-3,6-dihydropyridine-1(2H)-carboxamide; hCT, human calcitonin; IBMX, 3-isobutyl-1-methyl-xantine; L-AP4, L-(-)-2-amino-4-phosphonobutyric acid; L-CCG-I, (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine; LY341495, (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; MDIP, 5-methyl-3,6-diphenylisoxazo[4,5-c]pyridine-4(5H)-one; mGluR, metabotropic glutamate receptor; MMPIP, 6-(4-methoxyphenyl)-5-methly-3-pyridine-4-ylisoxazo[4,5-c]pyridine-4(5H)-one; MPEP, 2-methyl-6-(phenylethynyl)pyridine; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTX, pertussis toxin; RFU, relative fluorescence units; RT-PCR; reverse transcription-polymerase chain reaction.

Recommended section assignment: Neuropharmacology
ABSTRACT

Novel isoxazolopyridone derivatives which are mGluR7 antagonists were discovered and pharmacologically characterized. 5-Methyl-3,6-diphenylisoxazolo[4,5-c]pyridine-4(5H)-one (MDIP) was identified by random screening, and 6-(4-methoxyphenyl)-5-methly-3-pyridine-4-ylisoxazolo[4,5-c]pyridine-4(5H)-one (MMPIP) was produced by chemical modification of MDIP. MDIP and MMPIP inhibited L-AP4-induced intracellular Ca\(^{2+}\) mobilization in CHO cells co-expressing rat mGluR7 with \(G_{\alpha 15}\) (IC\(_{50}\) = 20 and 26 nM). The maximum response in agonist concentration-response curves was reduced in the presence of MMPIP and its antagonism is reversible. MMPIP did not displace \(^{3}H\)LY341495 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 (IC\(_{50}\) = 99 and 220 nM). In CHO cells co-expressing human mGluR7 with \(G_{\alpha 15}\), MDIP and MMPIP also inhibited the L-AP4-induced cAMP response. The maximum degree of inhibition by MMPIP was higher than that by MDIP in a cAMP assay. MMPIP was able to antagonize an allosteric agonist, the 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 PTX-sensitive and mGluR7-dependent. MMPIP at concentrations of at least 1 \(\mu\)M 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 CNS functions.
Introduction

Metabotropic glutamate receptors (mGluRs) belong to a family of G-protein-coupled receptors believed to contribute to the modulation of neuronal excitability and neurotransmitter release. Eight mGluR subtypes (mGluR1-mGluR8) have been cloned and classified into three groups based on sequence homology, pharmacological profile and signal transduction pathway. mGluR1 and mGluR5 belong to group I mGluRs, and 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 (CNS) (Okamoto et al., 1994; Saugstad et al., 1994; Kinoshita et al., 1998; Kosinski et al., 1999). Interestingly, mGluR7 is primary localized on presynaptic terminals where it is thought to regulate neurotransmitter release, and is highly concentrated at specific neuronal terminals (Shigemoto et al., 1996). Based on its specific presynaptic localization and much lower L-glutamate affinity compared to other mGluR subtypes, mGluR7 appears 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; Holscher et al., 2004; Holscher et al., 2005; Callaertd-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 in order 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). On the other hand, high-throughput functional assays for detecting Ca$^{2+}$ mobilization have led to the identification of subtype-selective ligands in Gq-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$\alpha_{15}$ and G$\alpha_{16}$ are known to allow Gi-coupled receptors to couple to phospholipase C, resulting in Ca$^{2+}$ mobilization in response to agonist stimulation (Offermanns and Simon, 1995). In addition, mGluR7 is known to efficiently couple with G$\alpha_{15}$, but not with G$\alpha_{16}$ (Parmentier et al., 1998). In order to identify mGluR7-selective ligands, we generated CHO cells stably co-expressing mGluR7 with G$\alpha_{15}$, and screened chemical libraries using the cells and FLIPR. Since mGluR7 appears to be coupled to Gi rather than to G$\alpha_{15}$ in neurons (Wright and Schoepp, 1996), their activities were further confirmed by Gi-coupled cAMP response. Evaluation of mGluR7 ligands on mGluR7-mediated cAMP response could be a physiologically relevant assay since mGluR7 is known to regulate L-glutamate release through modulation of cAMP levels in cerebrocortical nerve terminals (Millan et al., 2002).

Using the screening strategy mentioned above, we have identified isoazolopyridone derivatives as mGluR7 antagonists. Based on descriptions in the patent
application (Nakamura et al., 2002), several isozazolopyridone 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 isozazolopyridone derivatives. 5-Methyl-3,6-diphenylisoxazolo[4,5-c]pyridine-4(5H)-one (MDIP) was identified as a novel mGluR7 antagonist by random high-throughput functional screening, whereas 6-(4-methoxyphenyl)-5-methly-3-pyridine-4-ylisoxazolo[4,5-c]pyridine-4(5H)-one (MMPIP) was obtained by subsequent chemical modification of MDIP. These isoxazolopyridone derivatives were pharmacologically characterized using recombinant rat and human mGluR7-expressing cells.
Materials and Methods

Materials

5-Methyl-3,6-diphenylisoxazolo[4,5-c]pyridine-4(5H)-one (MDIP, Fig. 1A) was originally identified as a mGluR7 ligand from an in-house chemical library.

6-(4-Methoxyphenyl)-5-methly-3-pyridine-4-ylisoxazolo[4,5-c]pyridine-4(5H)-one (MMPIP, Fig. 1B) was synthesized in-house. L-(-)-2-amino-4-phosphonobutyric acid (L-AP4),

(2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I),

2-methyl-6-(phenylethynyl)pyridine (MPEP),

(RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG),

(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (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-methyl-xantine (IBMX), pertussis toxin (PTX) and human calcitonin (thyrocalcitonin) were purchased from Sigma-Aldrich (St. Louis, MO). AMN082 (Fig. 1C) was purchased from Ascent Scientific (North Somerset, UK). FTIDC was synthesized in-house (Suzuki et al., 2007).

L-proline and forskolin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dialyzed fetal bovine serum, culture media and other reagents used for cell culture were purchased from Invitrogen (Carlsbad, CA). All other reagents used were 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 transfected with rat mGluR7 cDNA cloned into pIRESNeo (Clontech, Palo Alto, CA) and selected in medium (Dulbecco’s modified Eagle’s medium with 10% dialyzed fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin and 1% proline supplemented with 500 µg/ml geneticin (Invitrogen). The stable cell lines were isolated and 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 Gα15 cDNA (Aurora Biosciences) cloned into pIREShyg (Clontech) and selected in medium supplemented with 500 µg/ml hygromycin B (Invitrogen) and 500 µg/ml geneticin. The stable cell lines were isolated and selected by their abilities to elicit Ca$^{2+}$ mobilization following L-AP4 addition (CHO-rat mGluR7/Gα15). CHO cells stably co-expressing human mGluR7 with Gα15 (CHO-human mGluR7/Gα15) and expressing human mGluR5 were obtained as described previously by O’Brien et al. (2004). CHO-dhfr− cells stably expressing human mGluR1a were obtained as described previously by Ohashi et al. (2002). CHO-dhfr− cells stably expressing rat mGluR3 and rat mGluR4 were kindly donated by Dr. S. Nakanishi. CHO-dhfr− cells stably co-expressing human mGluR2 with Gα16 and CHO K1 cells stably expressing human mGluR8 were obtained as described previously by Suzuki et al. (2007).

Intracellular Ca$^{2+}$ mobilization

Intracellular Ca$^{2+}$ mobilization was measured according to the method described by Suzuki et al. (2007). Briefly, CHO cells co-expressing rat mGluR7 with Gα15 were seeded at 5 x 10^4 cells/well in a 96-well black well/clear bottom plate (PerkinElmer Life...
and Analytical Sciences, Boston, MA) and 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 probenecid) containing 1% dialyzed fetal bovine serum for 1 hr at 37°C with 5% CO₂ in a humidified atmosphere. The extracellular dye was removed and Ca²⁺ flux was measured using a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). The cells were preincubated with a test compound for 5 min to evaluate its agonistic activity. After preincubation, antagonistic activity was evaluated for 3 min after addition of an agonist. The final concentration of L-AP4 was 500 µM in the antagonist assay for rat mGluR7. In the antagonist assay for human mGluR1a, human mGluR5 and human mGluR2, the final concentration of L-glutamate was 10 µM. CHO cells expressing human mGluR5 were seeded at 7.5 x 10⁴ cells/well and loaded with 4 µM Fluo-4. In order to access the reversibility of the antagonism by MMPIP, Fluo-3-loaded CHO cells co-expressing rat mGluR7 with Gα15 were preincubated for 5 min in the absence or presence of MMPIP, and then washed 3 times with 250 µl of assay buffer before agonist stimulation. An agonist (0.5 mM L-AP4) was added 5 min after washout in the FLIPR.

[^3H]LY341495 binding

CHO cells expressing rat mGluR7 were seeded at 3 x 10⁵ cells/well in a 24-well plate and cultured overnight. The culture medium was then removed and the cells were incubated in 250 µl of culture medium containing 100 nM[^3H]LY341495 in the presence or absence of test compounds at 37°C with 5% CO₂ in a humidified atmosphere. After 1 hr, the cells were washed three times with ice-cold PBS and solubilized in 2M NaOH. Radioactivity was measured using Tricarb2500 (PerkinElmer Life and Analytical Sciences) after addition of ULTIMA GOLD XR (PerkinElmer Life and Analytical Sciences). Nonspecific binding was defined as binding in the presence of 100 µM
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 x 10^4 cells/well in a 96-well clear bottom plate and cultured overnight. The culture medium was then replaced with Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2 mM CaCl_2, 1 mM MgCl_2, 3.6 mM NaHCO_3, 5.6 mM glucose, 10 mM Hepes, pH 7.4) containing 1 mM 3-isobutyl-1-methyl-xanthine (IBMX), and the cells were incubated for 20 min at 37°C with 5% CO_2 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 (EIA) system (GE Healthcare, Piscataway, NJ). CHO cells stably co-expressing human mGluR7 with G_α15 were seeded at 4 x 10^4 cells/well in a 96-well clear bottom plate and 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 mM and 1 mM, respectively, whereas in the antagonist assay for mGluR3, mGluR4 and mGluR8, the final concentrations of L-glutamate were 100 µM, 70 µM and 10 µM, respectively. In order to evaluate the effects of compounds on cAMP response in pertussis toxin (PTX)-treated CHO cells expressing rat mGluR7, the cells were incubated with 100 ng/ml PTX for 24 hr before the cAMP assay.

Knockdown of mGluR7 using siRNA

Four siRNA 21-mers matching the human mGluR7 sequence (referred to as LY341495.
mGluR7 siRNA) were purchased in a pooled form (siGENOME SMARTpool, M-005622-00) from Dharmacon (Lafayette, CO). A 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 co-expressing human mGluR7 with Gα15 were seeded at \(2.5 \times 10^5\) cells in a 25 cm\(^2\) flask and 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) according to the manufacturer’s instructions. After 2 days, the cells were seeded at \(4 \times 10^4\) cells in a 96-well white well/clear bottom plate (PerkinElmer Life and Analytical Sciences) and 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, 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 \(\mu\)g total RNA was performed in a total volume of 25 \(\mu\)l using random hexamers and a TaqMan Reverse Transcription reagent kit (Applied Biosystems) according to the manufacturer's protocol. The resultant cDNA sample (25 \(\mu\)l) was diluted with 75 \(\mu\)l of nuclease-free water. Quantitative real-time PCR was performed in a total volume of 25 \(\mu\)l containing 12.5 \(\mu\)l of TaqMan universal PCR MasterMix, 1.25 \(\mu\)l of TaqMan probe/primers mixture and 3 \(\mu\)l of the diluted cDNA templates using the ABI Prism 7700 cycler (Applied Biosystems). The cycling profile was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C, as recommended by the manufacturer. Relative quantification of the real-time PCR results was performed using the comparative Ct method with GAPDH as the internal control, according to the manufacturer's protocol (Applied Biosystems User Bulletin #2, ABI Prism 7700 Sequence Detection system).
Data Analyses and Statistics

Data analyses were performed using Prism (version 4.03) from GraphPad Software (San Diego, CA). Concentration-response curves for Ca^{2+} mobilization and cAMP accumulation were fitted using nonlinear regression analysis. In order to determine the potency of a non-competitive antagonist, $K_B$ value was calculated from the equation: $K_B = [B]/(\text{slope} - 1)$, 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 towards recombinant mGlur7

In CHO cells co-expressing rat mGlur7 with G\(\alpha_{15}\) (CHO-rat mGlur7/G\(\alpha_{15}\)), the mGlur7 agonists L-AP4 and L-CCG-I were able to increase intracellular Ca\(^{2+}\) concentrations, with EC\(_{50}\) values of 140 ± 18 µM (n=5) and 70 ± 6.4 µM (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 (RFU) in CHO-rat mGlur7/G\(\alpha_{15}\) were 16000 ± 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/G\(\alpha_{15}\), and MMPIP was obtained by successive chemical modification of MDIP. In CHO-rat mGlur7/G\(\alpha_{15}\), MDIP and MMPIP inhibited 0.5 mM L-AP4-induced Ca\(^{2+}\) mobilization with IC\(_{50}\) values of 20 ± 2.4 nM and 26 ± 3.4 nM (n=8), respectively (Fig. 2B). These compounds did not show any agonist activity in CHO-rat mGlur7/G\(\alpha_{15}\). In order to analyze the mode of action of these isoxazolopyridone derivatives, the effect of MMPIP on agonist concentration-response curves of intracellular Ca\(^{2+}\) mobilization was evaluated in CHO-rat mGlur7/G\(\alpha_{15}\). Agonist concentration-response curves for L-AP4 and L-CCG-I-induced increases in intracellular Ca\(^{2+}\) concentrations were obtained in the presence or absence of MMPIP. The maximum responses of L-AP4 and L-CCG-I were reduced in the presence of MMPIP (Fig. 3A and 3B), 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 Ca\(^{2+}\) responses was analyzed by a model of non-competitive antagonism (Kenakin, 1997). In order 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. 1A and 1B). Equally effective concentrations were calculated from curves in the absence or presence
of 100 nM MMPIP shown in Fig. 3A and in the absence or presence 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 $24 \pm 3.4$ and $30 \pm 2.7$ nM, respectively. Schild plot analysis of the antagonism produced by CPPG yielded a $pA_2$ of $4.7 \pm 0.053$ and a slope factor of $0.91 \pm 0.055$. (Supplemental Fig. 1C). The reversibility of the antagonism by MMPIP was determined by comparing Ca$^{2+}$ responses to 0.5 mM L-AP4 in CHO-rat mGluR7/αG15, with and without washout procedure. MMPIP (0.01 – 1 µM) dose-dependently inhibited L-AP4-induced Ca$^{2+}$ response. The agonist-induced responses were recovered within 5 min after washout of MMPIP (Fig 3D).

$[^{3}H]LY341495$ binding assays, carried out to evaluate if MMPIP binds to the L-glutamate binding site of mGluR7, showed that it did not displace $[^{3}H]LY341495$ bound to CHO-rat mGluR7 (Fig. 3E). In contrast to MMPIP, both LY341495 and CPPG displaced $[^{3}H]LY341495$ 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 $86 \pm 12$ µM (n=12) and $170 \pm 76$ µM (n=4) in CHO-rat mGluR7 and CHO-human mGluR7/Go15, respectively (Fig. 4A and 4B). In CHO-rat mGluR7, MDIP and MMPIP dose-dependently antagonized L-AP4-induced inhibition of cAMP accumulation with $IC_{50}$ values of $99 \pm 25$ nM (n=6) and $220 \pm 23$ nM (n=5), respectively (Fig. 4C). MDIP and MMPIP also antagonized L-AP4-induced inhibition of cAMP accumulation with $IC_{50}$ values of $140 \pm 18$ nM (n=6) and $610 \pm 130$ nM (n=5), respectively, in CHO-human mGluR7/Go15 (Fig. 4D). The orthosteric mGluR antagonist LY341495 antagonized L-AP4-induced inhibition of cAMP accumulation in CHO-rat mGluR7 and CHO-human mGluR7/Go15 with $IC_{50}$
values of 1600 ± 360 nM (n=3) and 2300 ± 1300 nM (n=3), respectively. Since the maximum degree of inhibition by MMPIP was higher than that by MDIP as shown in Fig. 4C and 4D, MMPIP was selected for further pharmacological characterization.

An allosteric mGluR7 agonist, AMN082 (Fig. 1C) inhibited forskolin-stimulated cAMP accumulation with an EC\textsubscript{50} value of 95 ± 36 nM (n=4) in CHO-human mGluR7/G\textalpha15 (Fig. 5A) while AMN082 up to 10 \mu M did not induce intracellular Ca\textsuperscript{2+} mobilization (Supplemental Fig. 2). MMPIP dose-dependently antagonized AMN082-induced inhibition of cAMP accumulation in CHO-human mGluR7/G\textalpha15 (Fig. 5B). In contrast, even at 1 mM, an orthosteric mGluR antagonist, CPPG, did not antagonize AMN082-induced inhibition of cAMP accumulation in CHO-human mGluR7/G\textalpha15, while L-AP4-induced inhibition of cAMP accumulation was antagonized by CPPG.

**Intrinsic activity of MMPIP towards mGluR7**

In addition to antagonism against agonist-induced responses, MMPIP increased forskolin-stimulated cAMP accumulation in CHO cells expressing mGluR7 in the absence of the agonist. MMPIP increased forskolin-induced cAMP accumulation with EC\textsubscript{50} values of 97 ± 15 nM (n=7) and 260 ± 77 nM (n=4) in CHO-rat mGluR7 (Fig. 6A) and CHO-human mGluR7/G\textalpha15 (Fig. 6B), respectively. On the other hand, LY341495 did not increase forskolin-induced cAMP accumulation in either cell type (Fig. 6A and 6B). In CHO-rat mGluR7 treated with 100 ng/ml pertussis toxin (PTX), L-AP4 did not inhibit forskolin-stimulated cAMP accumulation, and MMPIP did not increase forskolin-stimulated cAMP accumulation in the absence of agonist. Differences in the activities of these compounds acting on control and PTX-treated cells were statistically significant (Fig. 6C).

**Effect of mGluR7 knockdown on intrinsic activity of MMPIP**
In order to confirm the intrinsic activity of MMPIP on mGluR7, its activity was evaluated in CHO-human mGluR7/Gα15 transfected with mGluR7 siRNA. mGluR7 siRNA and negative control siRNA were transfected into CHO-human mGluR7/Gα15. Three days after transfection, mGluR7 mRNA levels were measured using quantitative real-time RT-PCR (Fig. 7A). In CHO-human mGluR7/Gα15 transfected with mGluR7 siRNA (mGluR7 knockdown cells), mGluR7 mRNA levels were decreased to 34 ± 6.7% (n=4, % of non-transfected cells), while mGluR7 mRNA levels in CHO-human mGluR7/Gα15 transfected with negative control siRNA (control mGluR7 cells) were unaffected (102 ± 21%, n=4, % of non-transfected 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 µM AMN082 inhibited forskolin-stimulated cAMP accumulation to 37 ± 9.2% (n=4) (% of forskolin-stimulated cAMP accumulation). In contrast, cAMP levels in the presence of 1 µM AMN083 were 78 ± 12% (n=4) in mGluR7 knockdown cells (Fig. 7B). MMPIP increased forskolin-stimulated cAMP accumulation in the mGluR7 control cells, while 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). In order to confirm the specificity of knockdown by mGluR7 siRNA, the effects of 5-HT and human calcitonin (hCT) on cAMP accumulation were evaluated in mGluR7 control cells and knockdown cells endogenously expressing Gi-coupled 5-HT receptors and Gs-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.

Selectivity of MMPIP towards other mGluR subtypes
L-glutamate induced intracellular Ca\(^{2+}\) mobilization in CHO cells expressing mGluR1 or mGluR5, and in CHO cells co-expressing mGluR2 with G\(\alpha_{16}\) (mGluR2/G\(\alpha_{16}\)). FTIDC (an mGluR1 antagonist; 0.1 \(\mu\)M), 0.1 \(\mu\)M MPEP (an mGluR5 antagonist) and 1 \(\mu\)M 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 towards these mGluR subtypes (Fig. 8). In CHO cells expressing mGluR3, mGluR4 or mGluR8, L-glutamate inhibited forskolin-stimulated cAMP accumulation, while 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, neurotransmitter receptors, transporters and ion channels; these ion channels included ionotropic glutamate receptors (NMDA, AMPA and kainate) and other neurotransmitter receptors (dopamine, serotonin, acetylcholine, GABA, adrenalin and histamine) (MDS Pharma, Bothell, WA). The IC\(_{50}\) values of MMPIP were higher than 10 \(\mu\)M against all these targets except for 51% inhibition at 10 \(\mu\)M towards monoamine oxidase MAO-A (data not shown).
Discussion

CHO cells were constructed which co-express mGluR7 with promiscuous G protein (Gα15), and co-expression was shown to be coupled to intracellular Ca\(^{2+}\) mobilization. Using CHO-rat mGluR7/Gα15, the isoxazolopyridone derivatives MDIP and MMPIP were identified as mGluR7 antagonists. These isoxazolopyridone derivatives fully inhibited L-AP4-induced Ca\(^{2+}\) mobilization in CHO-rat mGluR7/Gα15. MMPIP caused the reduced maximum Ca\(^{2+}\) response to agonist in CHO-rat mGluR7/Gα15 whereas an orthosteric antagonist, CPPG did not reduce the maximum response.

Antagonism of MMPIP was analyzed using a model of non-competitive antagonism, indicating that MMPIP has a potent mGluR7 antagonist (K_B values = 24-30 nM). An insurmountable antagonism in the measurement of transient Ca\(^{2+}\) responses could be caused not only by an allosteric antagonist but also a slowly dissociating or irreversible orthosteric antagonist (Kenakin, 1997; Christopoulos et al., 1999). The agonist-induced Ca\(^{2+}\) mobilization in CHO-rat mGluR7/Gα15 was fully recovered within 5 min after washout of MMPIP, suggesting that MMPIP is a reversible antagonist with fast dissociation kinetics. \(^{[3]H}\)LY341495 is an orthosteric radioligand that binds to the L-glutamate binding site of mGluR7 (Wright et al., 2000); CPPG displaced \(^{[3]H}\)LY341495 bound to rat mGluR7 expressed in CHO, but not MMPIP. 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 support that MMPIP is an allosteric mGluR7 antagonist and 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, while Gα15 expression is normally limited to certain cells derived from the hematopoietic lineage (Offermanns and Simon, 1995). Thus, mGluR7 is not likely to couple with Gα15 in the CNS under physiological conditions. In heterologous expression systems, mGluR7 is negatively coupled with adenylate cyclase via Gi 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, L-AP4, inhibits forskolin-stimulated cAMP accumulation in neuronal cells. The inhibitory effect is biphasic, with the low-affinity component likely mediated by mGluR7 and the high-affinity component possibly mediated by mGluR4 (Wright and Schoepp, 1996). In the present study, MMPIP antagonized L-AP4-induced inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing mGluR7. L-AP4 is known to inhibit forskolin-stimulated cAMP levels and resultant L-glutamate release in cerebrocortical nerve terminals, presumably via mGluR7 (Millan et al., 2002). Therefore, the inhibitory activity of MMPIP shown in CHO cells expressing mGluR7 could be biologically relevant since the compound exhibits inhibitory activities not only in Gα15-coupled Ca2+ mobilization, but also in Gi-coupled cAMP response.

In the absence of an agonist, MMPIP caused a further increase in forskolin-stimulated cAMP levels in CHO cells expressing mGluR7. This is in contrast to the effect of an agonist such as L-AP4, suggesting that MMPIP exhibits inverse agonist activity and thus might inhibit the agonist-independent constitutive activity of mGluR7. An alternative interpretation could be that MMPIP inhibits activation of mGluR7 caused by either residual L-glutamate in the assay medium or endogenous L-glutamate released from cells. However, an orthosteric mGluR antagonist, LY341495, did not increase
forskolin-stimulated cAMP in CHO cells expressing mGluR7 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 mGluR7, and not from inhibition of mGluR7 activation due to L-glutamate contamination from the medium or cells. Treatment of CHO cells expressing mGluR7 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 Gi/o proteins, and that the compound does not directly activate endogenous adenylate cyclase in the cells. To date, the constitutive activity of mGluR7 has not been directly demonstrated under physiological conditions in the CNS. However, in the absence of an agonist, mGluR7 constitutively inhibited voltage-sensitive Ca\(^{2+}\) channels (VSCCs) via G\(\beta\gamma\) in cerebellar granule neurons transfected with mGluR7 cDNA (Bertaso et al., 2006). This mGluR7-dependent constitutive inhibition of VSCCs might be mediated via constitutive activity of mGluR7 and suggests that a constitutively-active mGluR7 might have physiological roles in the cerebellum. MMPIP could therefore be useful for revealing the functions of the agonist-independent activity of mGluR7.

RNA interference using siRNA was used to confirm whether the intrinsic activity of MMPIP was dependent on mGluR7 expression. mGluR7 knockdown cells exhibited decreased agonistic activity of AMN082, suggesting that mGluR7 knockdown cells have lost mGluR7 receptor function. In addition, the intrinsic activity of MMPIP was significantly diminished in the same mGluR7 knockdown cells. These results indicate that the intrinsic activity of MMPIP is mediated via mGluR7. In order to confirm the specificity of mGluR7 knockdown, the effects of 5-HT and hCT on cAMP levels were compared between mGluR7 knockdown cells and mGluR7 control cells. CHO cells endogenously express the 5-HT\(_{1B}\) receptor and 5-HT inhibits forskolin-stimulated cAMP accumulation via Gi protein in CHO cells (Giles et al., 1996). In the present study, forskolin-stimulated cAMP accumulation was comparably inhibited by 5-HT in both
mGluR7 knockdown cells and mGluR7 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 mGluR7 knockdown cells as well as in mGluR7 control cells. These results indicate that the diminished intrinsic activity of MMPIP towards mGluR7 knockdown cells is due to specific mGluR7 knockdown, and not to non-specific effects of mGluR7 siRNA.

The results presented here show that both MDIP and MMPIP fully inhibit agonist-induced Ca^{2+} mobilization via the Gq pathway. On the other hand, the maximum degree of inhibition by MDIP was less than that of MMPIP in eliciting a cAMP response from Gi protein in both human and rat mGluR7. Although the exact reason for this difference is not presently clear, it might be related to the property of allosteric antagonists which 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 towards mGluR7-mediated Ca^{2+} mobilization and cAMP response might be explained by permissive antagonism, suggesting that isoxazolopyridone derivatives could exhibit signal-pathway dependent antagonistic activity. In addition, the antagonist potencies (IC_{50} values) of MMPIP and MDIP on cAMP response were less potent than those on Ca^{2+} 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 confirmations (R* and R**) that preferentially interact with different G proteins (G1 and G2, respectively). MMPIP and MDIP might have different affinities for two confirmations of mGluR7 coupling to G\alpha 15 and Gi/o. It may therefore be worth evaluating the activities of isoxazolopyridone derivatives towards other functional responses (Saugstad et al., 1996, Perroy et al., 2000 and Millan et al., 2002, 2003) in order to further understand their...
signal pathway-dependent activities.

Counter assays using CHO cells expressing other mGluR subtypes showed that MMPIP is selective for mGluR7. MMPIP did not exhibit agonistic or antagonistic activity towards Gq-coupled mGluR1 and mGluR5. Furthermore, MMPIP did not inhibit agonist-induced Ca\(^{2+}\) mobilization in CHO cells co-expressing mGluR2 with promiscuous G protein. These results further suggest that the inhibitory activity of MMPIP in CHO-rat mGluR7/G\(\alpha_{15}\) does not arise from non-selective inhibition of the signaling pathway via Gq proteins including promiscuous G protein. Furthermore, MMPIP at concentrations of at least 1 \(\mu\)M exhibited no significant effect on cAMP response mediated via mGluR3, mGluR4 or mGluR8.

The limitation of the present study was that the all results were obtained using recombinant systems, thus the mode of action of these compounds might be different in native tissues where expression level of mGluR7 might be different from those in the recombinant systems. It will be necessary to confirm their actions in native mGluR7 in future studies.

In conclusion, MMPIP is a potent mGluR7 antagonist against agonist-induced Gq-coupled Ca\(^{2+}\) mobilization and Gi-coupled cAMP pathway. The inhibitory mode is non-competitive and allosteric. In the absence of agonist, MMPIP showed PTX-sensitive and mGluR7-dependent intrinsic activities, suggesting inverse agonist activity. MMPIP showed no significant effect on other mGluR subtypes or on other molecules tested. This is the first detailed description of allosteric mGluR7-selective antagonists. It is expected that MMPIP will be a useful pharmacological tool for elucidating the role of mGluR7 on CNS functions.
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Footnotes

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**Legends for figures**

**Fig. 1.** Chemical structures of MDIP (A), MMPIP (B) and an allosteric mGluR7 agonist, AMN082 (C). MDIP; 5-methyl-3,6-diphenylisoxazolo[4,5-c]pyridine-4(5H)-one, 6-[5-(dimethylamino)pyridine-3-yl]-5-methyl-3-phenylisoxazolo[4,5-c]pyridine-4(5H)-one, MMPIP; 6-(4-methoxyphenyl)-5-methyl-3-pyridine-4-ylisoxazolo[4,5-c]pyridine-4(5H)-one, AMN082; N,N'-dibenzhydryl-ethane-1,2-diamine dihydrochloride.

**Fig. 2.** Effects of MDIP and MMPIP on agonist-induced Ca\(^{2+}\) mobilization in CHO-rat mGluR7/\(\alpha\)G15. A, Concentration-response curves of L-AP4 (closed squares) and L-CCG-I (open squares) in CHO-rat mGluR7/\(\alpha\)G15. L-AP4 and L-CCG-I increased intracellular Ca\(^{2+}\) concentrations with EC\(_{50}\) values of 140 ± 18 µM (n=5) and 70 ± 6.4 µM (n=4). Relative fluorescence units (RFU) in CHO-rat mGluR7/G\(\alpha\)15 were 16000 ± 1200 (n=5) and 8500 ± 1400 (n=4) in the presence of 3 mM L-AP4 and L-CCG-I, respectively. Data are shown as a percentage of the response to 3 mM L-AP4. B, Concentration-response curves of MDIP (closed triangles) and MMPIP (open triangles) for 0.5 mM L-AP4-induced Ca\(^{2+}\) mobilization in CHO-rat mGluR7/G\(\alpha\)15. IC\(_{50}\) values of MDIP and MMPIP were 20 ± 2.4 nM and 26 ± 3.4 nM (n=8). Data are shown as a percentage of the response to 0.5 mM L-AP4. All data are the means ± S.E.M. from more than four individual experiments performed in duplicate.

**Fig. 3.** Mode of inhibitory action of MMPIP. Effects of MMPIP on concentration-response curves for L-AP4 (A) and L-CCG-I (B)-induced increases in intracellular Ca\(^{2+}\) mobilization. Concentration-response curves of agonists were obtained in the presence or absence of MMPIP in CHO-rat mGluR7/G\(\alpha\)15. Results are expressed as a percentage of the response to L-AP4 (3 mM) or L-CCG-I (3 mM). Effects of an orthosteric antagonist, CPPG on concentration-response curves for L-AP4-induced
increases in intracellular Ca\textsuperscript{2+} mobilization (C). Concentration-response curves of L-AP4 were obtained in the presence or absence of CPPG in CHO-rat mGluR7/G\alpha15. Results are expressed as a percentage of the response to L-AP4 (3 mM). Results are indicated as means \pm S.E.M. from three individual experiments performed in triplicate. Reversibility of MMPIP was determined by comparing Ca\textsuperscript{2+} responses to 0.5 mM L-AP4 in CHO-rat mGluR7/\alphaG15, with and without washout procedure (D). L-AP4-induced Ca\textsuperscript{2+} response was inhibited by preincubation with MMPIP (0.01 – 1 \muM) for 5 min without washout procedure. The agonist-induced response was fully recovered within 5 min after washing 3 times with assay buffer. Results are expressed as a percentage of the response to L-AP4 (0.5 mM) and are the means \pm S.E.M. from three individual experiments performed in duplicate. Displacement of [\textsuperscript{3}H]LY341495 bound to CHO-rat mGluR7 (E). Results are expressed as a percentage of the specific binding of [\textsuperscript{3}H]LY341495 (100 nM) and are the means \pm S.E.M. from three individual experiments performed in triplicate. Specific binding was obtained by calculating the difference between total binding and nonspecific binding. Nonspecific binding was determined using 100 \muM LY341495. The specific binding of [\textsuperscript{3}H]LY341495 in the presence of 1% DMSO to CHO-rat mGluR7 was 2100 \pm 100 (dpm, n=4). LY341495 and CPPG displaced [\textsuperscript{3}H]LY341495 bound to CHO-rat mGluR7 while MMPIP did not.

**Fig. 4.** Effects of MDIP and MMPIP on agonist-induced inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing mGluR7. L-AP4 inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner in CHO-rat mGluR7 (A) or CHO-human mGluR7/G\alpha15 (B). MDIP and MMPIP antagonized L-AP4-induced inhibitions of cAMP accumulation in CHO-rat mGluR7 (C) or CHO-human mGluR7/G\alpha15 (D). In the antagonist assay for rat and human mGluR7, the final concentrations of L-AP4 were 0.5 mM and 1 mM, respectively. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation and are the means
± S.E.M. from more than four individual experiments. Basal and forskolin-stimulated cAMP levels (pmol/well) were 0.36 ± 0.023 and 8.6 ± 1.1 (n=12), respectively, in CHO-rat mGluR7. Basal and forskolin-stimulated cAMP levels (pmol/well) were 0.23 ± 0.040 and 3.1 ± 0.37 (n=9), respectively, in CHO-human mGluR7/Gα15.

**Fig. 5.** Effect of MMPIP on an allosteric mGluR7 agonist, AMN082-induced inhibition of cAMP accumulation in CHO-human mGluR7/Gα15. **A**, AMN082 inhibited forskolin-stimulated cAMP accumulation with an EC₅₀ value of 95 ± 36 nM (n=4) in CHO-human mGluR7/Gα15. **B**, MMPIP (0.1 – 10 μM) dose-dependently antagonized AMN082 (1 μM)-induced inhibition of cAMP accumulation, while the orthosteric antagonist CPPG (CP) did not antagonize even at 1 mM. An orthosteric agonist, L-AP4 (1 mM), induced inhibition of cAMP accumulation and was antagonized by CPPG (CP) at 1 mM. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation and are the means ± S.E.M. from four or five individual experiments.

**Fig. 6.** Intrinsic activity of MMPIP towards mGluR7. In the absence of agonist, MMPIP increased forskolin-stimulated cAMP accumulation in CHO-rat mGluR7 (**A**) or CHO-human mGluR7/Gα15 (**B**). Effects of MMPIP on cAMP accumulation in CHO-rat mGluR7 treated with pertussis toxin (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 0.01-1 μM, respectively. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation and 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.

**Fig. 7.** Effect of mGluR7 knockdown on intrinsic activity of MMPIP. **A**, Knockdown
effect of siRNA against mGluR7 on expression of mGluR7 mRNA. In CHO-human mGluR7/Gα15 transfected with siRNA against human mGluR7 (mGluR7 siRNA), the expression level of mGluR7 mRNA was decreased to 34 ± 6.7% (n=4, % of non-transfected cells). Control siRNA (siRNA against luciferase GL2) did not show significant knockdown of mGluR7 mRNA (102 ± 21%, n=4, % of non-transfected cells).

B. Effect of AMN083, 5-HT and hCT on cAMP accumulation in CHO-human mGluR7/Gα15 transfected with mGluR7 siRNA (mGluR7 knockdown cells) or control siRNA (mGluR7 control cells). C. Effect of MMPIP on forskolin-stimulated cAMP accumulation in mGluR7 knockdown cells and mGluR7 control cells. The final concentrations of MMPIP were 0.1, 1 and 10 µM. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation and are the means ± S.E.M. from more than three individual experiments. Basal cAMP levels (pmol/well) in mGluR7 knockdown cells and mGluR7 control cells were 0.24 ± 0.086 (n=4) and 0.24 ± 0.036 (n=4), respectively. Forskolin-stimulated cAMP levels (pmol/well) in mGluR7 knockdown cells and mGluR7 control cells were 5.4 ± 0.44 (n=4) and 7.4 ± 1.1 (n=4), respectively. Statistical analyses were conducted with Student's t-test. *P< 0.05 versus control.

**Fig. 8.** Selectivity of MMPIP towards mGluR1, mGluR5 and mGluR2. MMPIP (1 and 10 µM) was tested to investigate its effect on intracellular Ca^{2+} concentrations mediated via mGluR1 (A), mGluR5 (B) and mGluR2 with Gα16 (C). In the antagonist assay, the final concentration of L-glutamate was 10 µM. FTIDC (FT; 0.1 µM), 0.1 µM MPEP (MP) and 1 µM LY341495 (LY) were used as positive control antagonists for mGluR1, mGluR5 and mGluR2, respectively. Data are expressed as a percentage of 10 µM L-glutamate response and the means ± S.E.M. from more than three individual experiments performed in duplicate.

**Fig. 9.** Selectivity of MMPIP towards 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 µM, 70 µM and 10 µM, respectively. LY341495 (LY) was used as a positive control antagonist. The final concentration of LY341495 was 10 µM, 100 µM and 10 µM for mGluR3, mGluR4 and mGluR8, respectively. Data are expressed as a percentage of forskolin-stimulated cAMP accumulation and the means ± S.E.M. from more than three individual experiments performed in duplicate. Basal cAMP levels (pmol/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 (pmol/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.
Fig. 1

A, MDIP

B, MMPIP

C, AMN082

2 HCl
Fig. 2

A

Log [agonist] (M)

Ca$^{2+}$ mobilization (% of 3 mM L-AP4 response)

L-AP4
L-CCG-I

B

Log [compound] (M)

Ca$^{2+}$ mobilization (% of 0.5 mM L-AP4 response)

MDIP
MMPIP
Fig. 3

A

- Ca^{2+} mobilization
- (% of 3 mM L-AP4 response)
- Log [L-AP4] (M)
- Control
- 10 nM MMPIP
- 100 nM MMPIP
- 1000 nM MMPIP

B

- Ca^{2+} mobilization
- (% of 3 mM L-CCG-I response)
- Log [L-CCG-I] (M)
- Control
- 3 nM MMPIP
- 30 nM MMPIP
- 300 nM MMPIP

C

- Ca^{2+} mobilization
- (% of 3 mM L-AP4 response)
- Log [L-AP4] (M)
- Control
- 10 µM CPPG
- 30 µM CPPG
- 100 µM CPPG

D

- Ca^{2+} mobilization
- (% of 0.5 mM L-AP4 response)
- Log [MMPIP] (µM)
- No wash
- Wash

E

- [3H]LY341495 binding
- (% of specific binding)
- Log [compound] (M)
- LY341495
- MMPIP
- CPPG
Fig. 4

A

rat mGluR7

cAMP level (% of forskolin response)

Log [L-AP4] (M)

B

human mGluR7

cAMP level (% of forskolin response)

Log [L-AP4] (M)

C

MDIP

MMPIP

cAMP level (% of forskolin response)

Log [compound] (M)

D

MDIP

MMPIP

cAMP level (% of forskolin response)

Log [compound] (M)
Fig. 5

A

CAMP level (% of forskolin response) vs. Log [AMN082] (M).

B

CAMP level (% of forskolin response) vs. MMPIP (µM) with AMN082 and L-AP4.
Fig. 7

A  

Control siRNA  

mGluR7 siRNA  

mGluR7 expression  

(% of non-transfected cells)  

B  

Control siRNA  

mGluR7 siRNA  

cAMP level  

(% of forskolin response)  

0  

AMN  

5-HT  

CT  

C  

Control siRNA  

mGluR7 siRNA  

cAMP level  

(% of forskolin response)  

0  

0.1  

1  

10  

MMPIP (µM)
**Fig. 9**

**A**

Assessment of mGluR3 activity with L-glutamate and MMPIP (µM) in terms of cAMP levels (% of forskolin response).

**B**

Assessment of mGluR4 activity with L-glutamate and MMPIP (µM) in terms of cAMP levels (% of forskolin response).

**C**

Assessment of mGluR8 activity with L-glutamate and MMPIP (µM) in terms of cAMP levels (% of forskolin response).