Pharmacological Characterization of cGMP Regulation by the Biarylpropylsulfonamide Class of Positive, Allosteric Modulators of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors

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ABSTRACT

The biarylpropylsulfonamide class of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) potentiators represented by N-2-(4-(4-cyanophenol)phenol)propyl-2-propanesulfonamide (LY404187) and (R)-4-[1-fluoro-1-methyl-2-(propane-2-sulfonylamo)-ethyl]-biphenyl-4-carboxylic acid methylamide (LY503430) are positive, allosteric AMPA receptor activators, which enhance AMPA receptor-mediated neurotransmission by reducing desensitization of the ion channel. Although these compounds have efficacy in in vivo rodent models of cognition, depression, and Parkinson’s disease, little is known about biochemical pathways activated by these agents. Given the well established regulation of the nitric oxide/cGMP pathway by excitatory neurotransmission, the current study characterized AMPA receptor potentiator-mediated cGMP response in mouse cerebellum. Acute treatment by both LY404187 and LY503430 [2.0, 5.0, or 10 mg/kg subcutaneously (s.c.)] elevated basal cerebellar cGMP levels in a dose-dependent manner. Pretreatment with the noncompetitive, allosteric AMPA receptor activators, which enhance AMPA receptor-mediated neurotransmission by reducing desensitization of the ion channel. Although these compounds have efficacy in in vivo rodent models of cognition, depression, and Parkinson’s disease, little is known about biochemical pathways activated by these agents. Given the well established regulation of the nitric oxide/cGMP pathway by excitatory neurotransmission, the current study characterized AMPA receptor potentiator-mediated cGMP response in mouse cerebellum. Acute treatment by both LY404187 and LY503430 [2.0, 5.0, or 10 mg/kg subcutaneously (s.c.)] elevated basal cerebellar cGMP levels in a dose-dependent manner. Pretreatment with the noncompetitive, allosteric AMPA receptor-selective antagonist 7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine-7-carboxamide, 5-(4-amino-phenyl)-8,9-dihydro-N,8-dimethyl-monohydrochloride-(9CI) (GYKI 53655) [3.0 mg/kg intraperitoneally (i.p.)], completely blocked the effect of LY404187, demonstrating that activation of AMPA receptors induces cGMP levels. Interestingly, pretreatment with the N-methyl-D-aspartate (NMDA) open channel blocker dizocilpine (0.3 and 1.0 mg/kg i.p.) also abolished the AMPA receptor potentiator-mediated cGMP accumulation, indicating that activation of AMPA receptors leads to NMDA receptor-mediated transmission involved in cGMP regulation. Pharmacological augmentation of the endogenous glutamate tone via the alkaloid harmaline (20–60 mg/kg i.p.) synergized with AMPA potentiator activity and provided further direct evidence of in vivo allosteric activation of AMPA receptors by LY404187. The synergism between harmaline and LY404187 was specific, since cGMP accumulation induced by foot-shock stress was not augmented by the AMPA receptor potentiator. Taken together, these data indicate that the cGMP system may play an important role in pharmacological efficacy of the biarylpropylsulfonamide class of AMPA receptor potentiators.

Glutamate is the major excitatory neurotransmitter in the brain acting at ionotropic and metabotropic receptors. Glutamate controls fast synaptic transmission and also plays a key role in synaptic plasticity (Parsons et al., 2002). Ionotropic glutamate receptors are ligand-gated ion channels that mediate the majority of fast synaptic transmission in the brain. There are three classes of ionotropic glutamate receptors: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainite receptors (Hollmann and Heinemann, 1994). Of these, AMPA receptors have received significant attention as a result of their dominant role in direct excitatory neurotransmission and neuromodulation contributing to pre- and postsynaptic plasticity phenomena. Indeed, the wide range of AMPA receptor pharmacology seems to contribute to the therapeutic potential of AMPA receptor activators for a variety of central nervous system disorders, such as major depression, schizophrenia, and addiction.

ABBREVIATIONS: NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR, glutamate receptor; LY404187, N-2-(4-(4-cyanophenol)phenol)propyl-2-propanesulfonamide; LY503430, (R)-4-[1-fluoro-1-methyl-2-(propane-2-sulfonylamo)-ethyl]-biphenyl-4-carboxylic acid methylamide; GYKI 53655, 7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine-7-carboxamide, 5-(4-amino-phenyl)-8,9-dihydro-N,8-dimethyl-monohydrochloride-(9CI); FS, foot-shock; ANOVA, analysis of variance; s.c., subcutaneous; i.p., intraperitoneal; MK-801, dizocilpine maleate.
depression, anxiety disorders, cognition, schizophrenia, and Parkinson's disease (Danyasz, 2002; O’Neill et al., 2004; Black, 2005; Alt et al., 2006).

AMPA receptors are cation-selective tetrameric complexes composed of homomeric and heteromeric combinations of subunits termed GluR1, GluR2, GluR3, or GluR4 (Hollmann and Heinemann, 1994). Like other ligand-gated ion channels, AMPA receptors possess multiple allosteric modulatory sites. The positive allosteric modulators do not possess direct agonist activity at the channel but markedly potentiate the effects of the endogenous transmitter glutamate or exogenously applied AMPA, by reducing the desensitization (e.g., cyclothiazide) and/or desensitization (e.g., aniracetam) of the agonist/channel complex (Yamada, 1998). These data suggest that multiple allosteric sites regulate the function of AMPA receptors. This property has been taken advantage of by several research groups who have generated a number of small molecule chemical classes of positive AMPA receptor modulators termed AMPA receptor potentiators or AMPA-kines (Arai et al., 1994; Bleakman and Lodge, 1998; Borges and Dingledine, 1998; Arai et al., 2002; Danyasz, 2002; Lynch, 2004; O’Neill et al., 2004). Among these, a new class of biarylpopylsulfonylamides, represented by LY404187 and LY503430 act as potent and selective AMPA receptor potentiators (Ornstein et al., 2000, Miu et al., 2001; Quirk and Nisenbaum, 2002; Murray et al., 2003). These compounds augment AMPA-mediated neurotransmission by reducing desensitization of the channel and have been shown to have efficacy in a variety of cell-based and in vivo models indicative of potential therapeutic efficacy in cognitive disorders, major depression, and Parkinson’s disease (O’Neill et al., 2004).

Despite the well characterized pharmacology of the biarylpopylsulfonylamide AMPA receptor potentiators on behavioral endpoints in several rodent models, little is known about the downstream biochemical signaling pathways activated by these agents. The primary objective of the present set of studies was to characterize the activation of synthesis of cGMP in response to the AMPA receptor potentiators. The focus on cGMP stems from its well documented coupling to the cGMP response induced by the AMPA receptor potentiator. To investigate the role of the NMDA receptor potentiators, mice were pretreated with the noncompetitive, selective NMDA open channel blocker dizocilpine (0.1 or 1.0 mg/kg i.p.) for 30 min, followed by AMPA receptor antagonist mediation. To assess the effects of endogenous glutamate tone on AMPA receptor potentiator-mediated cGMP accumulation, mice were treated with harmaline (10–60 mg/kg i.p.), an alkaloid that releases glutamate from the climbing fibers, 10 min before AMPA receptor antagonist treatment. As a means to establish the specificity of interactions between the glutamate system and AMPA receptor potentiators, we undertook a nonpharmacologic approach by evaluating whether footshock (FS) stress, a paradigm known to induce cerebellar cGMP response, modulates allosteric AMPA receptor antagonist-mediated cGMP accumulation. Mice were euthanized at 30 min to 1.0 h after the treatment with the AMPA receptor potentiator (as specified in figure legends) using a beam of microwave radiation (microwave fixation system model GA5013; Gerling Applied Engineering, Modesto, CA) focused on the skull for 0.5 s at high power setting. This method was used to preserve tissue cGMP content. All studies were carried out between 9:00 AM and 12:00 PM to reduce the effects of diurnal fluctuations.

Tissue Dissection and cGMP Radioimmunoassay. After microwaving, a small piece (10–20 mg) of cerebellar cortex was quickly removed from the skull, tissue weight was recorded, and the tissue was homogenized in 2 ml of 1.0% perchloric acid (Sigma-Aldrich). Tissue homogenate was kept on ice for 30 min and then placed in a boiling water bath for 5 min followed by centrifugation at 11,700g for 20 min. One milliliter of supernatant was then acetylated with 40 μl of triethylamine (Sigma-Aldrich) and 20 μl of acetic anhydride (Sigma-Aldrich), vortexed, and centrifuged at 13,000g for 20 min at 4°C. Acetylated mouse cerebellum samples were stored at 4°C until cGMP analysis by radioimmunoassay. Cerebellar cGMP content in the acetylated samples was determined using the cGMP 125I Flash Plate radioimmunoassay (PerkinElmer Life and Analytical Sciences, Boston, MA) on duplicate samples from each animal as per manufacturer’s instructions.
Foot-Shock Stress. For mouse foot-shock stress studies, the Habitest Modular Test System was used (Coulbourn Instruments, Allentown, PA). Mice were subjected to either a 0.5- or 1.0-mA foot-shock for 10 or 30 s in the Habitest Operant cage equipped with a modular shock floor using an automated remote timer. As with drug treatments, all foot-shock studies were carried out between 9:00 AM and 12:00 PM to reduce the effects of diurnal fluctuations.

Data Presentation and Statistical Analysis. For each animal, cGMP levels were normalized to wet tissue weight and used to compute group averages and S.E.M. All data were analyzed on the log scale to correct problems with heterogeneity of variance. However, the figures represent data in raw picromoles per gram. For Figs. 1, 4, and 5A, statistical analyses were performed using ANOVA followed by Dunnett’s method to compare each treatment group to the vehicle group. For Figs. 2 and 3, statistical analyses were performed using ANOVA followed by multiple comparisons using Bonferroni’s method to compare selected treatment groups to each other. For Fig. 5B, statistical analysis was performed using ANOVA followed by Fisher’s least significant difference method to test for treatment differences from FS alone.

Results

Acute Effects of LY404187 and LY503430 on Basal Cerebellar cGMP Concentration. A single, acute, subcutaneous administration of LY404187 or LY503430 (2 or 5 mg/kg) for 30 min dose dependently increased cGMP levels in the cerebellar cortex (Fig. 1). Maximal induction in cGMP accumulation by LY404187 was 2.5× over the vehicle-treated control group and evident already by 5 mg/kg, since a higher dose (10 mg/kg) had effects similar to 5 mg/kg (Fig. 2). To determine whether another biarylpropylsulfonamide AMPA receptor potentiatior also augments cerebellar cGMP levels, acute effects of LY503430 were investigated under the same treatment conditions. Further shown in Fig. 1, LY503430 also dose dependently and significantly elevated cerebellar cGMP content, with a maximal induction of 2.7× over the vehicle-treated control group; ##, p < 0.01 versus the corresponding LY404187 group.

Effects of the Noncompetitive AMPA Receptor Antagonist GYKI 53655 on AMPA Receptor Potentiator-Mediated cGMP Accumulation. To establish the role of AMPA receptors directly, we investigated the effects of the noncompetitive AMPA receptor antagonist GYKI 53655 on LY404187-induced cGMP accumulation. Mice were treated with a single acute injection of the allosteric AMPA receptor antagonist GYKI 53655 (3 mg/kg i.p.) 5 min before LY404187 (5 or 10 mg/kg s.c.) and euthanized 30 min later for cGMP determination. Each column represents mean ± S.E.M. of cGMP content normalized by wet tissue weight for each treatment group (n = 6/group). Augmentation in cerebellar cGMP content induced by both doses of LY404187 was prevented by pretreatment with the AMPA receptor antagonist GYKI 53655, ANOVA, p < 0.0001; **, p < 0.01 versus the vehicle control group. For Figs. 2 and 3, statistical analyses were performed using ANOVA, followed by Dunnett’s method to compare each treatment group to the vehicle group; ##, p < 0.01 versus the corresponding LY404187 group.

Fig. 1. Effects of LY404187 and LY503430 on basal cerebellar cGMP content. A single injection of LY404187 (2 or 5 mg/kg), LY503430 (2 or 5 mg/kg), or vehicle was administered subcutaneously to C57BL/6j mice for 30 min followed by euthanasia by focal microwaving for determination of cerebellar cGMP content. Each AMPA receptor potentiatior dose dependently induced cerebellar cGMP content with comparable potencies and efficacies. ANOVA, p = 0.0052; *, p < 0.05 versus the vehicle control group.

Fig. 2. Effect of the noncompetitive AMPA receptor antagonist GYKI 53655 on AMPA receptor potentiator-induced cGMP accumulation. Mice were treated with a single acute injection of the allosteric AMPA receptor antagonist GYKI 53655 (3 mg/kg i.p.) 5 min before LY404187 (5 or 10 mg/kg s.c.) and euthanized 30 min later for cGMP determination. Each column represents mean ± S.E.M. of cGMP content normalized by wet tissue weight for each treatment group (n = 6/group). Augmentation in cerebellar cGMP content induced by both doses of LY404187 was prevented by pretreatment with the AMPA receptor antagonist GYKI 53655, ANOVA, p < 0.0001; **, p < 0.01 versus the vehicle control group.

Fig. 3. Effects of the noncompetitive NMDA receptor antagonist dizocilpine on AMPA receptor potentiator-mediated cGMP accumulation. Mice were treated with a single acute injection of the NMDA receptor antagonist dizocilpine (0.3 or 1.0 mg/kg i.p.), 30 min before LY404187 (5 mg/kg s.c.) and euthanized 30 min later for cGMP determination. Each column represents mean ± S.E.M. of cGMP content normalized by wet tissue weight for each treatment group (n = 6/group). The NMDA antagonist by itself reduced cerebellar cGMP content at 1 mg/kg and abolished the response to AMPA receptor potentiation at both doses. ANOVA, p < 0.0001; *, p < 0.05 and **, p < 0.01 versus the corresponding vehicle control group. For Figs. 2 and 3, statistical analyses were performed using ANOVA, followed by Dunnett’s method to compare each treatment group to the vehicle group; ##, p < 0.01 versus the corresponding LY404187 group.

AMPA receptors directly, we investigated the effects of the noncompetitive AMPA receptor antagonist GYKI 53655 on LY404187-induced cGMP content (Fig. 2). Treatment with GYKI 53655 (3.0 mg/kg i.p.) by itself tended to reduce cerebellar cGMP levels, but the effect did not reach statistical significance. Treatment with LY404187 (5 or 10 mg/kg s.c.) for 30 min augmented cerebellar cGMP concentration to approximately 2.8-fold of the vehicle control level. Pretreatment with GYKI 53655 5 min before LY404187 administration abolished the increase in cerebellar cGMP observed with the latter compound.

Effects of Noncompetitive NMDA Receptor Antagonist Dizocilpine on AMPA Receptor Potentiator-Mediated cGMP Accumulation. To determine whether the AMPA receptor-mediated cerebellar cGMP response involves downstream participation of the NMDA receptor, we assessed the effects of dizocilpine, a noncompetitive, NMDA open channel blocker. As seen before, LY404187 (5 mg/kg s.c.; 30 min) significantly augmented cGMP concentrations to 200% of vehicle control. A 30-min pretreatment with dizocilpine at either 0.3 or 1.0 mg/kg i.p. completely blocked LY404187-induced cerebellar cGMP accumulation (Fig. 3).

For Fig. 5B, statistical analysis was performed using ANOVA followed by Dunnett’s method to compare each treatment group to the vehicle group; ##, p < 0.01 versus the corresponding LY404187 group.

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Notably, dizocilpine by itself reduced basal cerebellar cGMP levels with a statistically significant effect evident at 1 mg/kg i.p. However, this reduction in cGMP levels was no longer evident in mice that received dizocilpine plus LY404187.

**Interactions between Endogenous Glutamate Tone on LY404187-Induced cGMP Accumulation.** Since the allosteric AMPA receptor potentiator LY404187 by itself does not activate AMPA channels but facilitates the activation by the endogenous ligand glutamate, we investigated the cGMP response to LY404187 under the condition of augmented glutamate tone induced by harmaline. In concurrence with published reports (Wood, 1991), harmaline dose dependently increased cGMP levels in the cerebellum, with maximal induction of approximately 390% of control reached at 60 mg/kg i.p. (Fig. 4A). Pretreatment with harmaline (20 and 60 mg/kg i.p.) for 15 min before a subthreshold dose LY404187 (2 mg/kg) resulted in a synergistic response between the two drugs as evident by approximately a 4.5-fold increase in the cerebellar cGMP content over vehicle control (Fig. 4B).

**Assessment of Interactions between LY404187-Induced and Foot-Shock Stress-Induced cGMP Accumulation.** Mice were treated with LY404187 30 min before a brief, acute foot-shock stressor that is known to increase glutamate release in the brain. Figure 5A demonstrates that foot-shock stress induces cerebellar cGMP levels in an intensity- and duration-dependent manner. Pretreatment with subthreshold doses of LY404187 (0.5 or 2 mg/kg s.c.), which alone do not induce cerebellar cGMP, did not modify the cGMP response induced by the foot-shock stress (1.0 mA × 10 s) (Fig. 5B).

Discussion

The studies detailed here establish that the biarylpropylsulfonamide class of AMPA receptor potentiators, represented by LY404187 and LY503430, augment basal cerebellar cGMP accumulation in a dose-dependent manner. Although representative data are shown here, the overall results were replicated in additional independent experiments. The two AMPA receptor potentiators tested are members of a structurally novel class of highly potent biarylpropylsulfonamides that have been shown to positively modulate AMPA receptor activity in vitro and show central nervous system activity in vivo (Ornstein et al., 2000; Miu et al., 2001). These compounds show in vivo efficacy in rodent models of cognition (O’Neill et al., 2004), mood disorders (Alt et al., 2005, 2006) and Parkinson’s disease (Murray et al., 2003; O’Neill et al., 2004). The current results demonstrate that a common pharmacological effect of the two compounds is the dose-dependent induction in basal cGMP levels in the mouse cerebellum. These data clearly indicate that AMPA receptor potentiator treatment by itself is sufficient to induce basal cerebellar cGMP. To our knowledge, this is the first report of in vivo activation of tissue cGMP levels by positive allosteric modulators of the AMPA receptors and suggests that under basal conditions, the tonic activity of the AMPA...
aspartate, or harmaline-induced accumulation of cGMP in the cerebellum (Llinas and Volkind, 1973; Batini et al., 1979). Indeed, harmaline is an alkaloid that preferentially enhances synaptic activity of climbing fibers originating in the inferior olive (Lamarre and Mercier, 1971; Llinas and Volkind, 1973; Batini et al., 1979). Indeed, harmaline-induced accumulation of cGMP in the cerebellum has been shown to be dependent on an intact olivary-cerebellar climbing fiber projection system (Biggio and Guidotti, 1976). These fibers use excitatory amino acids (glutamate, aspartate, or N-acetyl-aspartyl glutamate) as the neurotransmitter (Wiklund et al., 1982; Renno et al., 1997). Thus, the data presented here indicate that activation of climbing fibers leads to activation of AMPA receptors by an endogenously released excitatory neurotransmitter, whose efficacy is potentiated by LY404187. These data are consistent with the positive allosteric property of LY404187, which augments the effects of glutamate and AMPA by reducing the deactivation of AMPA channels (Gates et al., 2001; O’Neill et al., 2004). We conclude that cerebellar cGMP offers a pharmacodynamic marker of in vivo allosteric potentiation pharmacology of this class of AMPA receptor potentiators.

Although harmaline synergized with LY404187, no interaction was seen between acute foot shock stress-induced elevation in cGMP and the AMPA receptor potentiator. This is surprising since stress is thought to increase excitatory neurotransmission. It is possible that the stress-induced excitatory neurotransmission affects cGMP levels primarily via non-AMPA glutamate receptors, likely the NMDA receptor or metabotropic glutamate receptors. Alternatively, the stress-induced elevation in cerebellar cGMP levels may be dependent on nonexcitatory neurotransmission such as dopaminergic or GABAergic pathway (Dinnendahl and Gumulka, 1977). Regardless, the data shown here establish the specificity of the pharmacological responses induced by AMPA receptor potentiators.

To establish the receptor contingency of the cGMP response induced by AMPA receptor potentiators, we assessed the effects of the noncompetitive AMPA receptor antagonist GYKI 53655 and NMDA receptor antagonist dizocilpine. At the dose used, GYKI 53655 tended to decrease cerebellar cGMP levels, but the effect did not reach statistical significance. However, pretreatment with the AMPA antagonist abolished the cGMP accumulation induced by LY404187, thereby demonstrating the role of AMPA receptors in the cGMP response. Interestingly, the NMDA antagonist significantly reduced basal cGMP concentration, suggesting that tonic activity via the NMDA receptor may regulate cGMP levels in the cerebellum. Of further interest, dizocilpine at a dose that by itself does not significantly affect basal cGMP levels blocked LY404187-induced elevation in tissue cGMP content. These data indicate that although the effects of LY404187 on cGMP accumulation are contingent upon AMPA receptor activation (since AMPA receptor antagonist blocked the activity of LY404187), the NMDA receptor seems to participate in the effect (since the noncompetitive NMDA receptor antagonist also blocked the response to LY404187). Since LY404187 does not have any affinity for the NMDA receptors (Gates et al., 2001), it is plausible that in the presence of the AMPA potentiator, AMPA receptor activation by endogenous glutamate causes sufficient depolarization of cells to remove the voltage-dependent blockade of the NMDA receptor by Mg$^{2+}$ ions and thereby enables NMDA-dependent cGMP accumulation. This is consistent with previous work demonstrating that AMPA receptor potentiators LY392098 and LY404187 can activate AMPA- and NMDA-evoked firing in vivo in a manner that is sensitive to blockade by GYKI 53655 (Vandergriff et al., 2001). Also in agreement with this, the potentiation of AMPA-induced elevation in extracellular cGMP levels by the allosteric AMPA receptor potentiator cyclothiazide is also abrogated by pretreatment with dizocilpine (Fedele and Raiteri, 1999). Colocalization of NMDA and AMPA receptors in cerebellar granular and Purkinje cells provides the ability for this interaction between AMPA and NMDA channels to regulate cGMP levels. Such functional relationships between colocalized ionotropic GluRs are well documented, and, in particular, stimulation of AMPA sites seems to play a permissive role on the activation of NMDA receptors in the presence of otherwise inhibiting Mg$^{2+}$ concentrations (Dese et al., 1992; Raiteri et al., 1992).

The physiological or therapeutic relevance of cGMP accumulation by the AMPA receptor potentiators is unclear. There is ample evidence demonstrating that ionotropic glutamate receptor-mediated neurotransmission induces the synthesis of nitric oxide via neuronal or endothelial NO synthase (Bredt and Snyder, 1990). NO diffuses between cells and activates soluble guanylate cyclase to promote the synthesis of cGMP, which in turn activates protein kinase G- or cyclic nucleotide-gated ion channels (Barnstable et al., 2004). Thus, one function of the cGMP response to AMPA receptor potentiators may be to amplify the GluR-mediated signaling. Interestingly, AMPA receptors themselves are one of the targets of protein kinase G-mediated phosphorylation, or direct regulation of the channel gating by cGMP, both of which can affect AMPA-mediated plasticity such as long-term depression (Lei et al., 2000). It would be interesting to investigate whether the cGMP response induced by the AMPA receptor potentiator reflects an autoregulatory phenomenon that controls AMPA-mediated neurotransmission in the cerebellum.

In conclusion, the data presented here provide strong evidence that basal, ex vivo cerebellar cGMP assessment is a good indicator of in vivo allosteric activation of AMPA receptors by the biarylpropylsulfonamide class of AMPA receptor potentiators. Most interestingly, using a pharmacological approach, we show that AMPA receptor activation leads to the recruitment of NMDA activity to elicit the cerebellar cGMP response. We hypothesize that regulation of the cGMP pathway is a critical pharmacological response of the AMPA receptor potentiators that may contribute to the efficacy of AMPA modulators in rodent models of cognition, depression, and neurodegeneration in Parkinson’s disease.

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