Effects of the Allosteric Antagonist 1-(4-Chlorophenyl)-3-[3-(6-pyrrolidin-1-ylpyridin-2-yl)phenyl]urea (PSNCBAM-1) on CB1 Receptor Modulation in the Cerebellum

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ABSTRACT

1-(4-Chlorophenyl)-3-[3-(6-pyrrolidin-1-ylpyridin-2-yl)phenyl] urea (PSNCBAM-1) has recently been described as a cannabinoid CB1 receptor allosteric antagonist associated with hypophagic effects in vivo; however, PSNCBAM-1 effects on CB1 ligand-mediated modulation of neuronal excitability remain unknown. Here, we investigate PSNCBAM-1 actions on CB1 receptor-stimulated miniature inhibitory postsynaptic currents (mIPSCs) in cerebellar membranes and on CB1 ligand modulation of presynaptic CB1 receptors at inhibitory interneuron-Purkinje cell synapses in the cerebellum using whole-cell electrophysiology. PSNCBAM-1 caused noncompetitive antagonism in [35S]GTP binding studies, with higher potency against the CB receptor agonist (–)-cis-3-[2-hydroxy-4-(1,1-dimethyl heptyl)phenyl]-trans-4-[3-hydroxypropoxy]cyclohexanol (CP55940) than for R(+)-[2,3-dihydro-5-methyl-3-[morpholino]methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-1-[naphthalenyl]methyl) methane mesylate [WIN55,212-2 (WIN55)]. In electrophysiological studies, WIN55 and CP55940 reduced miniature inhibitory postsynaptic currents (mIPSCs) frequency but not amplitude. PSNCBAM-1 application alone had no effect on mIPSCs; however, PSNCBAM-1 pretreatment revealed agonist-dependent functional antagonism, abolishing CP55940-induced reductions in mIPSC frequency but having no clear effect on WIN55 actions. The CB1 antagonist/inverse agonist N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) increased mIPSC frequency beyond control; this effect was reversed by PSNCBAM-1. PSNCBAM-1 pretreatment also attenuated AM251 effects. Thus, PSNCBAM-1 reduced CB1 receptor ligand functional efficacy in the cerebellum. The differential effect of PSNCBAM-1 on CP55940 versus WIN55 actions in [35S]GTP binding and electrophysiological studies and the attenuation of AM251 effects are consistent with the ligand-dependence associated with allosteric modulation. These data provide the first description of functional PSNCBAM-1 allosteric antagonist effects on neuronal excitability in the mammalian central nervous system (CNS). PSNCBAM-1 allosteric antagonism may provide viable therapeutic alternatives to orthosteric CB1 antagonists/inverse agonists in the treatment of CNS disease.

Introduction

Numerous G protein-coupled receptors (GPCRs) contain allosteric binding sites for endogenous and synthetic ligands that are topographically different from the orthosteric site (Christopoulos and Kenakin, 2002; Christopoulos, 2002; May and Christopoulos, 2003). On the basis of the ternary complex model, ligands at such allosteric sites mediate their cooperative effects on orthosteric affinity by altering rates of orthosteric ligand association and/or dissociation from the receptor (Ehlert, 1988; Christopoulos and Kenakin, 2002; May et al., 2004, 2007). Various therapeutic advantages of allosteric over orthosteric modulation have been suggested based on the differences between their underlying mecha-
nalms (May and Christopoulos, 2003). Price et al. (2005) identified three Organon compounds—Org 27569 (5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)-ethyl]-amide), Org 27759 (3-ethyl-5-fluoro-1H-indole-2-carboxylic acid [2-94-dimethylamino-phenyl]-ethyl]-amide), and Org 29647 (5-3-ethyl-1H-indole-2-carboxylic acid (1-benzyl-pyrrolidin-3-yl)-amide, 2-enedioc acid salt)—as allosteric modulators at CB1 receptors, thereby revealing a CB1 allosteric binding site that could hold potential as an alternative target for therapeutic modulation. Horswill et al. (2007) have described the actions of a novel CB1 allosteric antagonist, 1-(4-chlorophenyl)-3-[3-(6-pyrrolidin-1-ylpyridin-2-yl)phenyl]urea (PSNCBAM-1). The Organon compounds and PSNCBAM-1 all showed ligand-dependent effects in that they increased agonist (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexan (CP55940) equilibrium binding but decreased antagonist rimonabant (SR141716A) binding. Interestingly, and so far unique to CB1 receptors, allosteric modulation of orthosteric efficacy occurs in a direction opposite to the effects on orthosteric binding affinity in these studies; thus, CB1 allosteric antagonists produced noncompetitive functional antagonism of CB1 agonist effects (Ross, 2007a,b). PSNCBAM-1 was also suggested to exhibit agonist-dependent actions on the basis that antagonism of human CB1 stimulation by different agonists exhibited a wide range of potencies in a yeast reporter assay (Horswill et al., 2007). Finally, CB1 allosteric antagonism was proposed to be of functional relevance, as PSNCBAM-1 was also shown to possess hypogagic activity in vivo (Horswill et al., 2007). It has yet to be determined whether CB1 allosteric antagonists influence functional CB1-mediated modulation of neuronal excitability in the CNS. CB1 receptors are prominently expressed on presynaptic terminals within the CNS, in which they are intimately involved in the dynamic modulation of transmitter release. Therefore, we have investigated PSNCBAM-1 modulation of the effects of exogenous ligands on synaptic transmission at the well characterized inhibitory pathway between molecular layer interneurons (INs) and Purkinje cells (PCs). We and others have demonstrated that CB1 receptor activation at IN-PC synapses reduces inhibitory neurotransmission (Llano et al., 1991; Takahashi and Linden, 2000; Diana et al., 2002; Diana and Marty, 2003; Kawamura et al., 2006; Yamashiki et al., 2006; Kelm et al., 2008; Ma et al., 2008). We have also previously combined patch-clamp and multi electrode array electrophysiological recording techniques to show that CB1 receptor ligand effects on inhibitory neurotransmission at IN-PC synapses functionally modulate cerebellar network activity (Ma et al., 2008). In particular, CB1 receptor antagonist action at IN-PC synapses is consistent with block of endocannabinoid tone to increase GABA release and reduce overall excitability in the cerebellum (Stephens, 2009). The recent clinical withdrawal of rimonabant, an agent shown to function as an inverse agonist at CB1 receptors, has been linked to an inhibition of constitutive CB1 activity, which can occur even in the absence of endocannabinoid release (Jones, 2008). Such potentially unfavorable complications could be avoided by the use of neutral antagonists, which will only block the action of endocannabinoids released “on demand.” However, a further attractive option is the development of allosteric CB1 receptor antagonists to offer alternative therapeutic potential to orthosteric antagonists/inverse agonists (Ross, 2007a,b). Moreover, because PC axons represent the sole efferent system from the cerebellar cortex, the present study provides an opportunity to determine whether CB1 allosteric antagonists have the potential to functionally modulate the output of the cerebellum.

Here, we show that PSNCBAM-1 causes an agonist-dependent functional antagonism of CB1 receptor-mediated modulation of inhibitory neurotransmission at IN-PC synapses, consistent with the demonstration of noncompetitive antagonism and agonist-dependent potency in [35S]GTPγS binding studies in isolated cerebellar membranes. We extend data to PSNCBAM-1 effects to actions on the CB1 receptor agonist/inverse agonist N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-multipyrarazole-3-carboxamide (AM251). The reduction in CB1 receptor ligand-mediated functional efficacy in the cerebellum also extends previous studies demonstrating a unique response profile for CB1 allosteric antagonists to actions on neuronal excitability.

Materials and Methods

[35S]GTPγS Binding Assays

Cerebellar membranes were prepared from C57BL/6 mice, and [35S]GTPγS binding experiments were performed as described previously (Horswill et al., 2007; Dennis et al., 2008). In brief, 10 μg of membranes per reaction was incubated with 10 μM GDP and 0.1 nM [35S]GTPγS in assay buffer (20 mM HEPES, 3 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, 100 mM 8-cyclopentyl-1,3-dipropylxanthine, and 1 mg/ml bovine serum albumin, pH 7.4). 8-Cyclopentyl-1,3-dipropylxanthine, an adenosine A1 receptor antagonist, was included in the buffer to reduce non-CB1 receptor-mediated [35S]GTPγS binding. Reactions were performed in duplicate or triplicate in 96-well, round-bottomed plates in a final volume of 200 μl; nonspecific binding was determined using 10 μM unlabeled GTPγS. Antagonists were preincubated with membranes for 10 min at room temperature before adding agonists. After addition of agonists, plates were incubated for a further 15 min at room temperature (22–24°C) before adding [35S]GTPγS. Plates were then incubated at 30°C for a further 45 min. Reactions were terminated by rapid filtration onto GF/B filter mats, which were subsequently washed five times with 300 μl/well of 50 mM Tris-HCl buffer, pH 7.4. Filter mats were dried, and scintillation reagent was added before counting radioactivity using a PerkinElmer scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). PSNCBAM-1 effects were tested against a range of CB agonist concentrations. Dose-titration effects were also tested against a fixed agonist concentrations, as allowed by the noncompetitive nature of antagonism; thus, CP55940 (150 nM) or R(+)-[2,3-dihydro-5-methyl-3-[(mor- pholiny)ethyl]-pyrrolo[1,2,3,-d e]-1,4-benzoxazinyl]-[1-naphthalenyl]methanone mesylate [WIN55,212-2 (WIN55)] (0.5 μM) was used to stimulate [35S]GTPγS binding (to approximately 165% of basal) in the presence of a range of PSNCBAM-1 concentrations. Binding data were analyzed using Prism 5 (GraphPad Software Inc., San Diego, CA). Unpaired Student’s t tests were used to compare different agonist treatment values and pIC50 values.
Electrophysiology

Preparation of Acute Cerebellar Brain Slices. Three- to five-week-old male C57BL/6 mice were used for all experiments, which followed methods outlined previously (Ma et al., 2008). All work was conducted in accordance with UK Home Office regulations [Animals (Scientific Procedures) Act 1986] and every effort was made to minimize any discomfort experienced by animals. Mice were killed by cervical dislocation and decapitated. The brain was rapidly removed and submerged in ice-cold sucrose-based aCSF solution. The cerebellum was then hemisected along the midline and a hemisphere glued to the stage of a Vibratome (R and L Slaughter, Upminster, UK) that was used to prepare 300-μm parasagittal cerebellar slices. After sectioning, each slice was immediately moved to a holding chamber containing standard aCSF, which consisted of 124 mM NaCl, 3 mM KCl, 26 mM NaHCO3, 2.5 mM NaH2PO4, 2 mM MgSO4, 2 mM CaCl2, and 10 mM d-glucose, maintained at pH 7.3 by bubbling with 95% O2/5% CO2. Slices were kept at 37°C for at least 30 min and then superfused with carboxygenated standard aCSF. Temperature and superfusion with carboxygenated standard aCSF immediately before application.

Whole-Cell Patch-Clamp Recording. Individual cerebellar brain slices were placed in a recording chamber maintained at room temperature and superfused with carboxygenated standard aCSF. PCs were identified using an IR-DIC upright Olympus BX50WI microscope (Olympus, Tokyo, Japan) with a 60×, 0.9 numerical aperture water immersion lens. Whole-cell patch-clamp recordings were made from PCs at a holding potential of −70 mV using an EPC-9 amplifier (HEKA, Lambrecht/Pfalz, Germany) controlled by Pulse software (HEKA) using a Macintosh G4 computer (Apple Computer, Cupertino, CA). Electrodes (3–7 MΩ) were made from PCs at a holding potential of −70 mV using an EPC-9 amplifier (HEKA, Lambrecht/Pfalz, Germany) controlled by Pulse software (HEKA) using a Macintosh G4 computer (Apple Computer, Cupertino, CA). Electrodes (3–7 MΩ) were filled with borosilicate glass (Harvard Apparatus, Kent, United Kingdom) filled with an intracellular solution containing 140 mM CsCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM EGTA, 4 mM MgATP, 0.4 mM NaGTP, and 10 mM HEPES, pH 7.3. For whole-cell recording, slow capacitance was compensated, and series resistance was typically monitored at 15 to 20 MΩ with 70 to 90% compensation. Data were sampled at 5 kHz and filtered at one-third of the sampling frequency. Spontaneous inhibitory postsynaptic currents were first recorded for a minimum of 15 min to ensure recording stability. To isolate mIPSCs, slices were treated with tetrodotoxin (TTX, 1 μM) and NBQX (2.5 mM). In all experiments, ligands were bath-applied for a minimum of 20 min to achieve maximal steady-state effects, and a minimum 150-s recording was used as raw data for event detection. Solvent was present at a maximum final concentration of 0.1% (v/v); solvent, applied alone at equivalent experimental concentrations, has no effect on synaptic responses in this preparation (Bardo et al., 2002).

Data and Statistical Analysis

Data were initially exported using Pulsefit (HEKA) and then imported to Axograph 4.0 software for event detection (http://axographx.com/index.html). All electrophysiology data were analyzed using Prism 4 or Axograph 4.0 software. Cumulative frequency plots were constructed for interval intervals using 5-ms bins. In all cases, because amplitude distributions were skewed and did not pass the D’Agostino and Pearson omnibus normality test, differences between the means of the medians were used for parametric testing. Cumulative frequency plots were analyzed using the Kolmogorov-Smirnov test. Paired Student’s t tests were used to compare control and first treatment values. Comparison of the means from multiple treatment groups was performed using repeated measurement one-way ANOVA tests. Tukey’s HSD tests were performed when a repeated-measurement one-way ANOVA yielded significant differences. Differences were considered significant if p < 0.05. All data are expressed as means ± S.E.M.; n refers to the number of replicants used.

Pharmacology

The following agents were used: AM251, CP55540, NBQX (disodium salt), WIN55 (all from Tocris Cookson, Bristol, UK); (R)-baclofen (Ascent Scientific, Bristol, UK); and TTX-citrate (Alomone Labs, Jerusalem, Israel). PSNCBAM-1 was kindly provided by Prosidion Limited (Oxford, United Kingdom). TTX-citrate was dissolved in distilled water, and NBQX, WIN55, PSNCBAM-1, and AM251 were dissolved in dimethyl sulfoxide. All drugs were made up as 1000× stock solutions except for AM251 (made up as 5000× stock) and stored at −20°C. Drug stock solutions were diluted in carboxygenated standard aCSF immediately before application.

Results

Previous studies have identified PSNCBAM-1 as an allosteric antagonist at recombinant human CB1 receptors (Horswill et al., 2007). We first sought to extend studies to CB1 receptors in native murine cerebellar membranes (Dennis et al., 2008). The CB receptor agonist CP55940 stimulated [35S]GTPγS binding to a maximum of 172.0 ± 7.3% of basal (n = 4) with a pEC50 of 8.04 ± 0.03 (Fig. 1A). The CB receptor agonist WIN55 also stimulated [35S]GTPγS binding, but with a significantly higher maximum effect (236.5 ± 6.2% of basal, n = 4, p < 0.01, Student’s t test) and a significantly less potent pEC50 of 6.22 ± 0.10 (p < 0.01, Student’s t test) than seen for CP55940 (Fig. 1B). PSNCBAM-1 behaved noncompetitively against both CB receptor agonists, producing a substantial reduction in maximal [35S]GTPγS stimulation and only weakly affecting agonist potency. Agonist-dependent effects were also seen; for example, 1 μM PSNCBAM-1 was sufficient to almost fully inhibit CP55940 stimulation; by contrast, 10 μM PSNCBAM-1 was unable to fully inhibit the WIN55-induced response. We further investigated this phenomenon by determining PSNCBAM-1 potency for inhibition against a single agonist concentration that stimulated a similar amount of GTPγS binding. Although these experiments are dependent on the agonist concentration chosen, PSNCBAM-1 displayed significantly greater potency against CP55940 than WIN55 (pIC50 of 7.32 ± 0.20 and 6.50 ± 0.15, respectively; p < 0.05, Student’s t test). Overall, these data confirm PSNCBAM-1 noncompetitive, agonist-dependent actions in [35S]GTPγS binding assays.

Effects of PSNCBAM-1 on CB Receptor Agonist Modulation of Inhibitory Neurotransmission. Having demonstrated that PSNCBAM-1 has differential agonist-dependent actions in [35S]GTPγS binding assays in isolated cerebellar membranes, we next examined the potential functional effects of PSNCBAM-1 on modulation of synaptic transmission at IN-PC synapses in C57BL/6 mice. We first confirmed that WIN55 exerted a comparable agonistic effect to that demonstrated previously in TO strain mice (Ma et al., 2008). Bath application of WIN55 (5 μM) significantly decreased mean mIPSC frequency (73 ± 6%, n = 8, p < 0.01, Student’s t test; Fig. 2A), there was no accompanying change in mean mIPSC amplitude (n = 8; p = 0.52, Student’s t test). CP55940 (5 μM) also significantly decreased mean mIPSC frequency (64 ± 6%, n = 6, p < 0.01, Student’s t test; Fig. 2B), again, with no effect
on amplitude distribution \((n = 6; p = 0.99, \text{Student's } t \text{ test})\). In these and all subsequent experiments, no statistically significant changes in mean mIPSC amplitude were seen, and these effects are not further discussed. Overall, these data are consistent with a reduction in presynaptic GABA release without accompanying postsynaptic changes and also confirm CB\(_1\) agonist effects at IN-PC synapses (Ma et al., 2008) to be independent of the mouse strain used.

To examine the effects of PSNCBAM-1 upon CB\(_1\) agonist-mediated modulation of inhibitory transmission in the cerebellum, WIN55 or CP55940 was applied in the presence of PSNCBAM-1 (Fig. 3). Pretreatment with 10 \(\mu M\) PSNCBAM-1 had no significant effect on WIN55-induced inhibition of synaptic transmission, such that 5 \(\mu M\) WIN55 still caused a significant decrease in mean mIPSC frequency \((76 \pm 4\% , n = 7, p < 0.01, \text{Student's } t \text{ test}; \text{Fig. } 3\text{A})\). By marked contrast, PSNCBAM-1 abolished the inhibitory effect of 5 \(\mu M\) CP55940 on mean mIPSC frequency \((97 \pm 11\% , n = 5, p = 0.83, \text{Student's } t \text{ test}; \text{Fig. } 3\text{B})\). Together, these data demonstrate PSNCBAM-1 can cause CB receptor functional antagonism at IN-PC synapses and reflect an agonist-dependent effect.

**Effects of PSNCBAM-1 on CB\(_1\) Receptor Antagonist Modulation of Inhibitory Neurotransmission.** In our previous study, we reported that the synthetic CB\(_1\) antagonist/inverse agonist AM251 increased inhibitory neurotransmission at IN-PC synapses in TO strain mice, as demonstrated by an increase in mean mIPSC frequency beyond control levels (Ma et al., 2008). This increase can be generally attributable to the removal of endocannabinergic tone by a CB\(_1\) antagonist action or the blockade of constitutive activity by an inverse agonist action. Therefore, we next compared the actions of PSNCBAM-1 with those of AM251 at IN-PC synapses in C57BL/6 mice. PSNCBAM-1 \((10 \mu M)\) had no significant effect on mean mIPSC frequency \((99 \pm 6\% , n = 12, p = 0.80, \text{Student's } t \text{ test}; \text{Fig. } 4\text{A})\). By contrast, 2 \(\mu M\) AM251 caused a significant increase in mean mIPSC frequency \((117 \pm 4\% , n = 7, p < 0.01, \text{Student's } t \text{ test}; \text{Fig. } 4\text{B})\).

These data suggest that PSNCBAM-1 lacks the CB\(_1\) neutral antagonist or inverse agonist properties associated with AM251 in this system.

To confirm these data, next we activated CB receptors with WIN55 before the application of either AM251 or PSNCBAM-1 (Fig. 5). In the first of these experiments, 5 \(\mu M\) WIN55 caused a significant reduction in mean mIPSC frequency \((74 \pm 4\% , p < 0.01, \text{Student's } t \text{ test})\) that was reversed to beyond control level by subsequent application of 2 \(\mu M\) AM251 \((115 \pm 7\% , n = 7, p < 0.001, \text{repeated-measurement one-way ANOVA followed by a Tukey's HSD test}; \text{Fig. } 5\text{A})\). By contrast, in a separate set of experiments, the 5 \(\mu M\) WIN55-induced significant reduction in mean mIPSC fre-

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**Fig. 1.** Effects of PSNCBAM-1 on \([\gamma^3\text{S}]\text{GTP}\gamma\text{S}\) binding in mouse cerebellar membranes. Effects of PSNCBAM-1 on log concentration-response curves for CP55940 (A) and WIN55 (B) on the percentage of basal stimulation of \([\gamma^3\text{S}]\text{GTP}\gamma\text{S}\) binding (both mean \(\pm\) S.E.M. of four experiments). C, dose–titration effect of PSNCBAM-1 on either CP55940 \((150 \text{ nM})\) or WIN55 \((0.5 \mu M)\) stimulated \([\gamma^3\text{S}]\text{GTP}\gamma\text{S}\) binding in mouse cerebellar membranes. Data points are mean \(\pm\) S.E.M. of three experiments. PSNCBAM-1 had agonist-dependent potency differences between CP55940 (\(pIC_{50} = 7.32 \pm 0.20\)) versus WIN55 (\(pIC_{50} = 6.50 \pm 0.15\)); \(p < 0.05, \text{Student's } t \text{ test}).
frequency (74 ± 6%, p < 0.01, Student’s t test) was not significantly affected by 10 μM PSNCBAM-1 (71 ± 8%, n = 8, p > 0.05, repeated-measurement one-way ANOVA followed by a Tukey’s HSD test; Fig. 5B). Subsequent application of AM251 was still able to reverse WIN55 effects (mean mIPSC frequency increased to 97 ± 6%, n = 8; p < 0.01, repeated-measurement one-way ANOVA followed by a Tukey’s HSD test; Fig. 5B). In this experiment, mean mIPSC frequency in the presence of AM251 did not exceed control levels, suggesting that PSNCBAM-1 pretreatment can affect AM251 actions; therefore, we next examined the effects of PSNCBAM-1 application after AM251 (Fig. 6). In this set of experiments, 2 μM AM251 caused a significant increase in mean mIPSC frequency (117 ± 4%, n = 7; p < 0.01, repeated-measurement one-way ANOVA followed by a Tukey’s HSD test), which, in turn, was significantly reduced by subsequent application of 10 μM PSNCBAM-1 (104 ± 3%, n = 7; p < 0.01, repeated-measurement one-way ANOVA followed by a Tukey’s HSD test; Fig. 6). These data demonstrate that PSNCBAM-1 had actions on a CB1 antagonist/inverse agonist in addition to the agonist-dependent modulation described above.

Finally, to test for potential actions at other Gαi/o-coupled GPCRs that modulate inhibitory transmission at IN-PC synapses, we investigated PSNCBAM-1 effects on GABAR receptor-mediated inhibition (Harvey and Stephens, 2004). Bath application of the GABAR agonist baclofen (20 μM) significantly decreased mean mIPSC frequency (78 ± 3%, n = 4, p < 0.01, Student’s t test; Fig. 7A). Pretreatment with 10 μM PSNCBAM-1 had no significant effect on baclofen-induced inhibition of synaptic transmission, such that 20 μM baclofen still caused a significant decrease in mean mIPSC frequency (72 ± 4%, n = 5, p < 0.01, Student’s t test; Fig. 7B). Taken together, these electrophysiological data suggest that PSNCBAM-1 displays CB1 receptor ligand-dependent actions consistent with functional allosteric antagonism at cerebellar IN-PC synapses.

Discussion

Recent studies have identified a series of Organon compounds and the structurally similar PSNCBAM-1 as CB1 receptor allosteric antagonists (Price et al., 2005; Horswill et al., 2007). Here, we demonstrate PSNCBAM-1 func-

![Fig. 2. Effects of CB agonists on inhibitory neurotransmission at IN-PC synapses. Effect of 5 μM WIN55 (n = 8) (A) and 5 μM CP55940 (n = 6) (B) on mIPSCs; example raw data traces (top), summary bar graph showing effects on normalized mIPSC frequency (middle), and cumulative frequency curves for effects on interevent interval (bottom). Both WIN55 and CP55940 significantly reduced normalized mIPSC frequency * , p < 0.01 versus control (CTL), Student’s t test) and caused a significant rightward shift of the CTL interevent interval curve (both p < 0.0001 in individual cells, Kolmogorov-Smirnov test).](https://molpharm.aspetjournals.org/content/early/2017/08/02/mol.0b3.18002643.full.html)
tional CB₁ antagonism and agonist-dependent effects on [³⁵S]GTPγS binding in native cerebellar membranes and reveal that these effects extend to CB₁ ligand-dependent modulation of inhibitory neurotransmission in the mammalian CNS.

**PSNCBAM-1 Displays Agonist-Dependent Potency in [³⁵S]GTPγS Binding Assays.** PSNCBAM-1 caused a noncompetitive antagonism of CB receptor agonist-stimulated [³⁵S]GTPγS binding in cerebellar membranes. Overall, PSNCBAM-1 achieved only a partial inhibition of WIN55-induced responses, with PSNCBAM-1 displaying higher potency for inhibition of CP55940 versus WIN55 stimulation when tested against a range of agonist concentration and in dose-titration experiments against a fixed agonist concentration. A similar differentiation was seen in human embryonic kidney 293 membranes expressing the human CB₁ receptor, in which PSNCBAM-1 also displayed higher potency for inhibition of agonist-stimulated [³⁵S]GTPγS binding by CP55940 than by WIN55 (Supplemental Fig. 1). Thus, PSNCBAM-1 displays agonist-dependent potency at mouse CB₁ receptors in native membranes and recombinant human CB₁ receptors. A caveat to these data is that WIN55 may stimulate a non-CB₁-mediated component of [³⁵S]GTPγS binding (Breivogel et al., 2001). In the present study, WIN55 displayed a higher efficacy than CP55940 in stimulation of [³⁵S]GTPγS binding in cerebellar membranes, as reported in some previous studies (Breivogel et al., 2003; Childers, 2006). However, we have shown that WIN55-stimulated [³⁵S]GTPγS binding in cerebellar membranes is potently blocked by the CB₁ antagonists AM251 and Δ⁹-tetrahydrocannabinin (Δ⁹-THCV) (Dennis et al., 2008), and that WIN55 inhibition at IN-PC synapses is prevented by Δ⁹-THCV (Ma et al., 2008), arguing against WIN55 causing functional CB₁-independent effects here. With regard to alternative receptor targets, it is of interest that CP55940, but not WIN55, is a ligand at GPR55, a lysophosphatidyllysinoi receptor, with some sensitivity to cannabinoids (Ross, 2009). However, in our hands, the GPR55 agonist 5-methyl-4-[(1R,6R)-3-methyl-6-(1-methyl-ethenyl)-2-cyclohexen-1-yl]-1,3-benzenediol (O-1602) lacked effects on [³⁵S]GTPγS binding in cerebellar membranes (I. Smith, B. J. Whalley, and G. J. Stephens, unpublished data). Moreover, it is currently unknown whether PSNCBAM-1 acts at GPR55. In the future, it will be useful to test the effects of CB₁ allosteric antagonists in cnr1(−/−) and GPR55(−/−) animals. Overall, PSNCBAM-1 displays both noncompetitive antagonism and agonist-dependent potency in [³⁵S]GTPγS binding studies, characteristics associated with allosteric mechanisms (Conn et al., 2009).

**C57BL/6 Mouse IN-PC Synapses Possess Functional CB₁ Receptors.** Activation of CB₁ receptors at IN-PC synapses attenuates inhibitory neurotransmission, resulting in consequential synaptic disinhibition of PC output (Ma et al.,

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**Fig. 3.** Effect of pretreatment with PSNCBAM-1 on CB agonists. Effect of 10 μM PSNCBAM-1 on 5 μM WIN55 (n = 7) (A) and 5 μM CP55940 (n = 5) (B) actions on mIPSCs; example raw data traces (top), summary bar graph showing effects on normalized mIPSC frequency (middle), cumulative frequency curves for effects on interevent interval (bottom). PSNCBAM-1 had agonist-dependent potency differences, blocking CP55940 effects, but lacking action on WIN55 effects, such that the WIN55-mediated significant reduction in normalized mIPSC frequency [*p < 0.01 versus control (CTL), Student’s t test] and significant rightward shift of the CTL interevent interval curve (p < 0.0001 in individual cells, Kolmogorov-Smirnov test) were sustained.
2008). Here, both WIN55 and CP55940 caused a significant decrease in mIPSC frequency but produced no change in mIPSC amplitude, consistent with CB1 agonist-mediated presynaptic inhibition of GABA release. We chose to record mIPSCs not only to isolate presynaptic events but also to negate any potentially confounding WIN55-mediated effect on Ca\(^{2+}\) channels, which has been proposed to underlie WIN55 CB1 receptor-independent effects on synaptic transmission (Németh et al., 2008) and postsynaptic PC output (Fisyunov et al., 2006). Because miniature, action potential-independent events at IN-PC synapses occur independently of Ca\(^{2+}\) influx into the axon terminal via voltage-dependent Ca\(^{2+}\) channels (Harvey and Stephens, 2004; Stephens, 2009), it is unlikely that such a mechanism could account for the observed WIN55-induced reduction in inhibitory transmission. Consequently, we propose that the CB1 pathway-mediated responses seen here occur downstream of Ca\(^{2+}\) entry, involves the release machinery, and results in the inhibition of exocytosis of GABA-containing vesicles. We also demonstrate that AM251 increases inhibitory neurotransmission. Together, these results confirm functional presynaptic CB1 receptors at IN-PC synapses in C57BL/6 mice, thereby validating the preparation for the study of functional PSNCBAM-1 effects.

**PSNCBAM-1 Displays Agonist-Dependent Potency in Electrophysiological Studies.** PSNCBAM-1 pretreatment blocked CP55940-induced inhibition of synaptic transmission; by contrast, PSNCBAM-1 had no effect upon WIN55-induced inhibition. Thus, PSNCBAM-1 possesses an agonist-dependent functional antagonism at IN-PC synapses. This agonist-dependence is in agreement with our \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assays and data from yeast reporter assays by Horswill et al. (2007) that showed PSNCBAM-1 more potently antagonized the stimulation of CB1 receptors by CP55940 than by WIN55.

Here, PSNCBAM-1 had no effect on inhibitory neurotransmission when applied alone; by contrast, the CB1 antagonist/inverse agonist AM251 increased mIPSC frequency. We have shown previously that AM251, \(\Delta^9\)-THCV, and SR141716A all increase inhibitory neurotransmission at IN-PC synapses, an effect consistent with a blockade of endocannabinergic tone or abolition of constitutive CB1 activity (Ma et al., 2008). Moreover, pharmacological actions of PSNCBAM-1 exhibit assay-dependence, behaving as a noncompetitive antagonist in an in vivo feeding model and CB1 yeast reporter, GTP\gamma\text{S}, and cAMP assays but demonstrating partial inverse agonism against basal \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding (Horswill et al., 2007). Here, PSNCBAM-1 lacked the intrinsic activity associated with

![Fig. 4. Effect of PSNCBAM-1 and AM251 on inhibitory neurotransmission at IN-PC synapses. Effect of 10 μM PSNCBAM-1 (n = 12) (A) and 2 μM AM251 (n = 7) on mIPSCs (B); example raw data traces (top), summary bar graph showing effects on normalized mIPSC frequency (middle), and cumulative frequency curves for effects on interevent interval (bottom). PSNCBAM-1 lacked effects on mIPSCs; AM251 significantly increased mean normalized mIPSC frequency \(r, p < 0.01\) versus control (CTL), Student’s \(t\) test] and caused a significant leftward shift of the CTL interevent interval curve \(p < 0.0001\) in individual cells, Kolmogorov-Smirnov test.}
with CB₁ antagonists/inverse agonists at IN-PC synapses. We have proposed that AM251 acts to displace endocannabinoids at IN-PC synapses (Stephens, 2009), the lack of intrinsic PSNCBAM-1 effects further suggests that PSNCBAM-1 binds at a site distinct from that occupied by endocannabinoids. It is noteworthy that PSNCBAM-1 modulated the actions of AM251. Thus, PSNCBAM-1 attenuated the AM251-induced reversal of WIN55-induced inhibition and also reversed AM251-induced increases in inhibitory neurotransmission. These data are the first to describe PSNCBAM-1 functional effects on CB₁ antagonists/inverse agonists and suggest further mechanisms of CB₁ receptor allosteric modulation.

**Mechanisms of PSNCBAM-1 Allosteric Antagonism.** We demonstrate that PSNCBAM-1 has distinct effects on the actions of the CB agonist CP55940 versus WIN55 and also modulates the actions of the CB₁ antagonist/inverse agonist AM251. A parsimonious explanation of our data is that PSNCBAM-1 can bind to a CB₁ receptor allosteric site to exert a ligand-dependent antagonist action. Here, allosteric-orthosteric ligand interactions could also depend on the presence of different CB₁ orthosteric binding sites for distinct ligands. In this regard, molecular modeling studies have shown that CP55940 (a synthetic derivative of Δ⁹-tetrahydrocannabinol) and WIN55 (an aminoalkylindole) bind at distinct CB₁ binding sites (McAllister et al., 2003, 2004), in agreement with earlier mutagenesis studies (Song and Bonner, 1996). Thus, PSNCBAM-1 allosteric binding may selectively affect different CB₁ orthosteric sites. The phenomenon whereby certain allosteric agents can interact with one class of ligands, but not another, has been termed “probe-dependence” and is likely to be conserved across GPCR classes (Leach et al., 2007; May et al., 2007; Kenakin, 2008). In the classic ternary complex model for receptor allosteroism (Ehlert, 1988), binding to an allosteric site alters ligand affinity for the orthosteric site in either a positive, negative, or neutral direction. PSNCBAM-1 had positive cooperativity for agonist [³H]CP55940 binding but negative cooperativity for antagonist/inverse agonist [³H]SR141716A binding in competition experiments (Horswill et al., 2007). Comparable effects were reported for Organon CB₁ allosteric antagonists (Price et al., 2005). Thus, CB₁ allosteric antagonists exhibit ligand-dependent actions on orthosteric affinity (Ross, 2007a,b). Moreover, in a manner that is presently unique to CB₁ allosteric antagonists, changes in orthosteric efficacy are reported to occur in a direction opposite to those on orthosteric affinity, with these compounds causing noncompetitive antagonism of CB₁ receptor function. It may be hypothesized that interference with G proteins or G protein coupling may explain why CB₁ allosteric antagonists enhance CP55940 receptor binding but inhibit CP55940 functional effects. However, it has been reported previously that PSNCBAM-1 lacks effects on CB₂ receptors, the closest homolog to CB₁ (Horswill et al., 2007), and here we demonstrate that PSNCBAM-1 lacked modulatory effects on GABAergic receptor pathways at IN-PC synapses. Together with

![Fig. 5. Effect of PSNCBAM-1 and AM251 on WIN55 actions.](image-url)

A. Effect of 5 µM WIN55 and subsequent application of 2 µM AM251 (n = 7) on mIPSCs; summary bar graph showing effects on normalized mIPSC frequency (top), and cumulative frequency curves for effects on interevent interval (bottom). WIN55 caused a reduction in normalized mIPSC frequency (*, p < 0.01, Student’s t test), which was reversed beyond control (CTL) levels by AM251 (**, p < 0.001, repeated-measurement one-way ANOVA followed by a Tukey’s HSD test). These data were reflected by significant shifts of the CTL interevent interval curve for each treatment (both p < 0.0001 in individual cells; Kolmogorov-Smirnov test). B. Effect of 5 µM WIN55 and subsequent applications of 10 µM PSNCBAM-1 and then 2 µM AM251 (n = 8) on mIPSCs; summary bar graph showing effects on normalized mIPSC frequency (top), and cumulative frequency curves for effects on interevent interval (bottom). WIN55 caused a reduction in normalized mIPSC frequency (*, p < 0.01, Student’s t test), which was not affected by PSNCBAM-1 but was increased by AM251 (*, p < 0.01, repeated-measurement one-way ANOVA followed by a Tukey’s HSD test). These data were reflected by significant shifts of the interevent interval curve for WIN55 and AM251 treatment (both p < 0.0001 in individual cells, Kolmogorov-Smirnov test) but a lack of effect of PSNCBAM-1.
the differential effects on CB1 ligands, these data suggest a lack of “nonselective” PSNCBAM-1 actions on G proteins or their coupling.

Overall, our data are consistent with PSNCBAM-1 binding to a CB1 receptor allosteric site, leading to a reduction of receptor function efficacy for both CP55940 and AM251. These data extend demonstrations of reduced receptor function by CB1 allosteric antagonists to functional assays of neuronal excitability and show that PSNCBAM-1 differentially affects CB1 ligand-mediated modulation of inhibitory neurotransmission.

Therapeutic Potential of CB1 Allosteric Antagonist. CB1 receptor activation by exogenous agents (e.g., Δ9-tetrahydrocannabinol) results in severe motor incoordination and associated cerebellar dysfunction (DeSanty and Dar, 2001; Patel and Hillard, 2001). Therefore, blockade of CB1 receptors could offer a viable therapeutic strategy in diseases such as cerebellar ataxia. A variety of therapeutic advantages of GPCR allosteric modulators have been described. First, GPCR allosteric binding sites show divergence (Christopoulos, 2002), which could be exploited to reduce side effects (Jensen and Spalding, 2004). Second, allosteric modulation...
lators may produce effects irrespective of dose, reducing the potential for toxicity (May and Christopoulos, 2003). For example, no adverse or toxic effects were seen after long-term PSNCBAM-1 administration in vivo (Horswill et al., 2007). Finally, allosteric antagonists can lack the intrinsic effects in the absence of an orthosteric ligand, allowing selective tuning of drug effects (Birdsall et al., 1996; Christopoulos and Kenakin, 2002). Here, PSNCBAM-1 lacked intrinsic activity at IN-PC synapses; by contrast, CB1 antagonists/inverse agonists increase inhibitory transmission (Ma et al., 2008). Intrinsic changes to neuronal excitability may be undesirable; for example, the withdrawal of rimonabant as an antiobesity agent was proposed to reflect constitutive CB1 activation as a result of inverse agonist actions (Jones, 2008). Thus, PSNCBAM-1 acting at an allosteric binding site has potential to reduce CNS side effects associated with orthosteric CB1 antagonist/inverse agonists.

Overall, we demonstrate PSNCBAM-1 actions consistent with noncompetitive, agonist-dependent allosteric antagonism of cerebellar CB1 receptors. These data extend PSNCBAM-1 functional effects to CB1 ligand modulation of inhibitory neurotransmission and suggest alternative pharmacological approaches to the therapeutic modulation of neuronal excitability in the CNS.

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Authorship Contributions
Participated in research design: Wang, Horswill, Whalley, and Stephens.
Conducted experiments: Wang and Horswill.
Contributed new reagents or analytic tools: Horswill.
Performed data analysis: Wang and Horswill.
Wrote or contributed to the writing of the manuscript: Wang, Horswill, Whalley, and Stephens.
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References

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