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A Novel Selective Allosteric Modulator Potentiates the Activity of Native Metabotropic Glutamate Receptor Subtype 5 in Rat Forebrain

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ABSTRACT

We found that *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA), is a potent and selective positive allosteric modulator of the metabotropic glutamate receptor subtype 5 (mGluR5). CPPHA alone had no agonist activity and acted as a selective positive allosteric modulator of human and rat mGluR5. CPPHA potentiated threshold responses to glutamate in fluorometric Ca^{2+} assays 7- to 8-fold with EC_{50} values in the 400 to 800 nM range, and at 10 μM shifted mGluR5 agonist concentration-response curves to glutamate, quisqualate, and (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG) 4- to 7-fold to the left. The only effect of CPPHA on other mGluRs was weak inhibition of mGluR4 and 8. Neither CPPHA nor the previously described 3,3′-difluorobenzaldazine (DFB) affected [^{3}H]quisqualate binding to mGluR5, but although DFB partially competed for [^{3}H]3-methoxy-5-(2-pyridinylethynyl)pyridine binding, CPPHA had no effect on the binding of this 2-methyl-6-(phenylethynyl)-pyridine analog to mGluR5. Although the binding sites for the two classes of allosteric modulators seem to be different, these different allosteric sites can modulate functionally and mechanistically similar allosteric effects. In electrophysiological studies of brain slice preparations, it had been previously shown that activation of mGluR5 receptors by agonists increased *N*-methyl-D-aspartate (NMDA) receptor currents in the CA1 region of hippocampal slices. We found that CPPHA (10 μM) potentiated NMDA receptor currents in hippocampal slices induced by threshold levels of DHPG, whereas having no effect on these currents by itself. Similarly, 10 μM CPPHA also potentiated mGluR5-mediated DHPG-induced depolarization of rat subthalamic nucleus neurons. These results demonstrate that allosteric potentiation of mGluR5 increases the effect of threshold agonist concentrations in native systems.

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors (GPCRs) that bind glutamate to modulate neurotransmitter release or postsynaptic excitatory neurotransmission, and hence they modulate the strength of synaptic transmission. The mGluRs are members of GPCR family C and possess a large extracellular agonist binding domain in the amino-terminal portion of the receptor. This agonist binding domain distinguishes family C from the other GPCR families in which the agonist binding sites are associated with the seven-strand transmembrane spanning region or with the extracellular loops that connect the strands of this region. Thus, in the mGluRs, interaction of the agonist with the transmembrane domains is thought to be indirect (O'Hara et al., 1993; for reviews, see Conn and Pin, 1997; Bockaert and Pin, 1999).

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; DFB, 3,3′-difluorobenzaldazine; CPPHA, *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide; LC/MS, liquid chromatography/mass spectrometry; TFA, trifluoroacetic acid; CHO, Chinese hamster ovary; methoxy-PEPy, 3-methoxy-5-(2-pyridinylethynyl)pyridine; STN, subthalamic nucleus; ACSF, artificial cerebrospinal fluid; NMDA, *N*-methyl-D-aspartate; DHPG, (*R*,*S*)-3,5-dihydroxyphenylglycine; FLIPR, fluorometric imaging plate reader; 4C3HPG, 4-carboxy,3-hydroxyphenylglcyine; PPI, prepulse inhibition; QX314, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide; SIB-1893, 2-methyl-6-(phenylethynyl) pyridine hydrochloride; PHCCC, *N*-phenyl-7-(hydroxylimino)cyclopropa[*b*]chromen-1*a*-carboxyamide; RO 67-7476, 2-(4-fluorophenyl)-1-[(4-methylphenyl)sulfonyl]pyrrolidine.

Eight mGluRs have been identified and cloned and have been assigned to three groups based on structural similarity, primary coupling to intracellular signaling pathways, and pharmacology. Group I (mGluR1 and mGluR5) mGluRs are coupled through Gαq/11 to increases in inositol phosphate metabolism and resultant increases in intracellular Ca^{2+}. Group I mGluRs are mostly located postsynaptically and have a modulatory effect on postsynaptic signaling. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) mGluRs are coupled through Gαi/o to decreased cAMP synthesis. Group II and group III mGluRs are mostly located presynaptically and modulate (inhibit) neurotransmitter release. The group II and group III mGluRs can be distinguished functionally by group-selective pharmacological tools (for review, see Conn and Pin, 1997).

The group I receptor mGluR5 may play a role in a number of disease states, including anxiety (Spooren et al., 2000; Tatarczynska et al., 2001), pain (Salt and Binns, 2000; Bhave et al., 2001), addiction to cocaine (Chiamulera et al., 2001), and schizophrenia (for review, see Chavez-Noriega et al., 2002). We are interested in studying the function of mGluR5 and initiated an effort to discover and develop novel pharmacological tools specific for this receptor. Although a number of mGluR agonists and antagonists have previously been discovered using traditional approaches, these compounds are mostly analogs of glutamate, quisqualate, or phenylglycine (for review, see Schoepp et al., 1999) that bind to the agonist binding site (orthosteric site) in the amino-terminal domain of the receptor. Although group-selective agonists and antagonists were obtained with these approaches, it has been much more challenging to develop compounds that are subtype-selective, probably because the structure of the orthosteric agonist binding site is well conserved within an mGluR group. Recently, it has become possible to use functional assays to search for compounds that interact with receptors at allosteric sites that are separate from the orthosteric site. For the mGluRs, the first compounds clearly shown to interact with a allosteric sites were 7-(hydroxylimino) cyclopropa[*b*]chromen-1*a*-carboxamide ethyl ester (mGluR1-selective; Litschig et al., 1999) and 2-methyl-6-(phenylethynyl)-pyridine (MPEP, mGluR5-selective; Gasparini et al., 1999). These compounds do not bind to the orthosteric binding sites of their respective receptors, but rather they act as negative allosteric modulators, binding to sites in the seven-strand transmembrane spanning domain of their cognate receptors to exert their inhibitory effects. Several other examples of negative allosteric modulators of mGluRs have since been reported. Subsequently, positive allosteric modulators were reported for mGluR1 (Knoflach et al., 2001), mGluR2 (Schaffhauser et al., 2003), and mGluR4 (Marino et al., 2003), and we have recently reported 3,3′ difluorobenzaldazine (DFB) as a selective positive allosteric modulator of mGluR5 (O'Brien et al., 2003). We have continued with this approach of using functional assays to identify and characterize allosteric modulators, and now report *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl] phenyl}-2-hydroxybenzamide (CPPHA), a novel mGluR5-selective positive allosteric modulator of a different structural class from DFB.

Materials and Methods

Compounds

A synthetic scheme for CPPHA is shown in Fig. 1.

2-{[(4-Chloro-2-methylphenyl)amino]carbonyl}phenyl acetate (**3**) was synthesized as follows: to a stirred solution of 4-chloro-2-methylaniline, **1** (10.0 g, 0.071 mol) in toluene was added (24.7 ml, 0.014 mol) *N*,*N*-diisopropylethylamine, followed by slow addition of acetylsalicyloyl chloride **2**. The mixture was stirred until complete by thin layer chromatography. Reaction was filtered and dried under vacuum to afford 9.4 g of **3**. Analytical LC/MS: (CH_3CN/H_2O/1% TFA, 4-min gradient), 88% pure, M + 1 peak *m*/*e* 304.

2-({[2-(Bromomethyl)-4-chlorophenyl]amino}carbonyl)phenyl acetate (**4**) was synthesized as follows: **3** (9.4 g, 0.031 mol) was immediately taken up in a solution of CCl_4 with recrystallized *N*-bromosuccinimide (5.5 g, 0.031 mol) and benzoyl peroxide (Bz_2O_2) (0.75 g, 3.10 mmol). The reaction was heated at 90°C, along with a light source, until complete by thin layer chromatography. Upon completion, the solvent was reduced by two-thirds and filtered through a small plug of silica gel, yielding 10.5 g of **4**. Analytical LC/MS: (CH_3CN/H_2O/1% TFA, 4-min gradient), 85% pure, M + 1 peak *m*/*e* 384.

CPPHA (**6**) was synthesized as follows: compound **4** (6.0 g, 0.015 mol) was dissolved in 50 ml of dimethylformamide. Then, pthalimide **5** (3.31g, 0.023 mol), K_2CO_3 (6.2 g, 0.045 mol), and a catalytic amount of KI were added and allowed to stir at 50°C overnight. Upon completion the reaction was diluted with ethyl acetate and then washed with brine (6 × 25 ml) to afford 2.9 g of **6** in a crude mixture, which was then purified by normal phase chromatography. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.83 (S, 2H), 7.05 (m, 2H), 7.36 (dd, J = 2.4 Hz, 8.6 Hz, 2H), 7.50 (dt, J = 1.5 Hz, 8.5Hz, 1H), 7.59 (d, J = 2.4Hz, 1H), 7.75 ppm (m, 3H), 7.91 (m, 2H), 8.18 (d, J = 7.2 Hz, 2H); 10.17 (s, 1H), 12.27 (s, 1H) Analytical LC/MS: single peak (214 nm) at 3.633 min (CH_3CN/H_2O/1% TFA, 4-min gradient), high-resolution mass spectrometry calculated for $C_{22}H_{15}N_2O_4Cl$ (M + H), 407.0799; found 407.0793 (M + H).

The structure of DFB is shown in Fig. 1. Synthesis of DFB was detailed in O'Brien et al. (2003).

Stable Cell Lines

CHO cells were transfected with cloned human mGluR5 (human mGluR5 CHO cells) as follows: pCMV-T7-hmGluR5 (Daggett et al., 1995) was digested with HpaI and EcoRI (New England Biolabs, Beverly, MA) and the isolated human mGluR5 fragment was subcloned into pIRESpuro2 (BD Biosciences Clontech, Palo Alto, CA). Stable cell lines were established after transfection of CHONFAT-β-lactamase cells with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and drug selection with 10 μg/ml puromycin (BD Biosciences Clontech). Clonal cell lines were generated by limited dilution. Cells were grown in Dulbecco's modified Eagle's medium (11960; Invitrogen) containing 10% dialyzed fetal bovine serum (26400; Invitrogen), 2 mM L-glutamine (25030; Invitrogen), 100 units/ml penicillin/streptomycin (15070; Invitrogen), nonessential amino acids (11120; Invitrogen), 25 mM HEPES (15630; Invitrogen), 55 μM β-mercaptoethanol (21985; Invitrogen), and 10 μg/ml puromycin (8052-2; BD Biosciences Clontech). Positive expression was determined by measuring Ca^{2+} flux using a $FLIPR_{384}$, fluorometric imaging plate reader (Molecular Devices Corp., Sunnyvale, CA). Cloned rat mGluR5a receptors were transfected, and the resulting cells (rat mGluR5 CHO cells) were grown in the same manner. Cell lines expressing mGluR1b, 4, 7, and 8 were developed that were compatible with Ca^{2+}-sensitive fluorescence assays. Cells expressing mGluR4 were coexpressed with the chimeric G protein $G_{\alpha qi5}$ (Conklin et al., 1993), and cells expressing mGluR7 and mGluR8 were coexpressed with the promiscuous G protein $G_{\alpha 15}$.

Fluorometric Imaging Plate Reader

Methods used for Ca^{2+} flux measurements using $FLIPR_{384}$, fluorometric imaging plate reader (Molecular Devices Corp.) have been

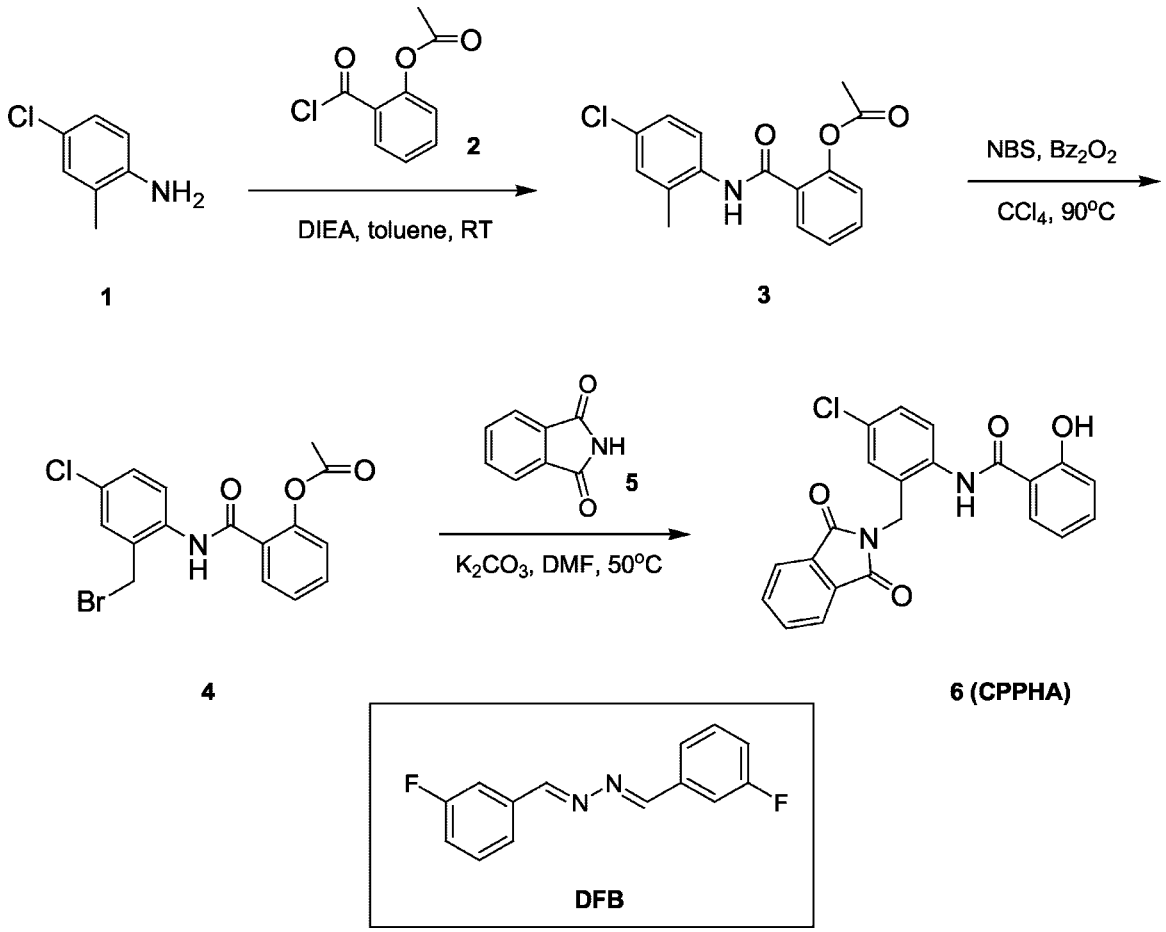

Fig. 1. Synthetic scheme for CPPHA. The structure of DFB is shown in an inset.

detailed previously (O'Brien et al., 2003). The peak of the calcium response was used to construct agonist concentration-response curves.

Radioligand Binding Assays

Methods used for binding studies using the agonist binding site radioligand [^{3}H]quisqualate and the MPEP analog radioligand [^{3}H]3-methoxy-5-(2-pyridinylethynyl)pyridine (methoxy-PEPy) have been detailed previously (O'Brien et al., 2003).

Electrophysiology

Slice Preparation. All harvesting of animal tissues was carried out using protocols approved by the West Point Institutional Animal Care and Use Committee in accordance with the provisions of the ILAR Guide for the Care and Use of Laboratory Animals. All patch-clamp experiments on CA1 pyramidal cells were performed on slices from 20- to 30-day-old Sprague-Dawley rats (Taconic Farms, Germantown, NY). Subthalamic nucleus neuron recordings were performed on slices from 15- to 20-day-old Sprague-Dawley rats. After decapitation, brains were rapidly removed and submerged in an ice-cold choline chloride buffer: 126 mM choline chloride, 2.5 mM KCl, 8 mM $MgSO_4$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 26 mM $NaHCO_3$, equilibrated with 95% O_2, 5% CO_2 (Cooper and Stanford, 2001). Parasagittal slices (300 μm in thickness) were obtained using a Vibraslicer (WPI, Sarasota, FL). Slices were transferred to a holding chamber containing normal ACSF: 124 mM, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 20 mM glucose, and 26 mM $NaHCO_3$, equilibrated with 95% O_2, 5% CO_2 at room temperature. In all experiments, 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenic acid were included in the choline chloride buffer and holding chamber to increase slice viability.

Electrophysiological Recordings. Whole cell patch-clamp recordings from CA1 pyramidal neurons were obtained using the "blind" patch technique. Whole cell patch-clamp recordings from subthalamic nucleus (STN) neurons were obtained under visual control as described previously (Marino et al., 2001). Neurons were visualized using a differential interference contrast microscope and an infrared video system. During recordings, slices were maintained fully submerged on the stage of a brain slice chamber at 34°C and perfused continuously with equilibrated ACSF (2–3 ml/min). Patch electrodes were pulled from borosilicate glass on a two-stage vertical patch pipette puller. For recordings in CA1, pipettes were filled with 140 mM cesium methane sulfonate, 16 mM HEPES, 10 mM NaCl, 2 mM EGTA, 0.2 mM NaGTP, and 2 mM MgATP, pH adjusted to 7.5 with 1 N CsOH. In all experiments performed in CA1, 1 mM QX314 (Tocris Cookson Inc., Ballwin, MO) was added to the internal pipette solution. Electrode resistance was 5 to 8 MΩ. For recordings of STN neurons, pipettes were filled with 120 mM potassium gluconate, 10 mM KCl, 10 mM NaCl, 10 mM EGTA, 10 mM HEPES, 1 mM $CaCl_2$, 2 mM Mg-ATP, and 0.5 mM Na-GTP, pH adjusted to 7.4 with 0.5 N KOH. Electrode resistance was 3 to 5 MΩ. All recordings were performed using a HEKA EPC10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). For measurement of NMDA-evoked currents, NMDA (100 μM; Tocris Cookson Inc.) was applied directly to the postsynaptic cell for 0.5 to 2.0 s with a modification of the U-tube fast application system (Alagarsamy et al., 1999). NMDA-evoked currents were recorded at a holding potential of −60 mV and tetrodotoxin (1 μM; Sigma-Aldrich, St. Louis, MO) was present in the bath to block synaptic transmission. Percentage of potentiation was

defined by using the ratio of maximum current during 3,5-dihydroxyphenylglycine (DHPG) application (average of three trials during maximal drug effect) to average current amplitude of three trials immediately preceding drug application. All data are expressed as mean ± S.E.M.

Drugs and Drug Application. Drugs were made into 10 mM stock solutions and diluted to the desired concentration in ACSF immediately before bath application. All experiments were performed in 1% dimethyl sulfoxide. (*R*,*S*)-3,5-DHPG 10 mM stock was made fresh in water every day. CPPHA 10 mM stock was made in dimethyl sulfoxide and aliquoted and stored at −20°C. CPPHA was applied 10 to 15 min before application of (*R*,*S*)-3,5-DHPG. (*R*,*S*)-3,5-DHPG, NMDA, and QX314 were obtained from Tocris Cookson Inc.. All other compounds were obtained from Sigma-Aldrich.

Mathematical Modeling. Data were compared with the model of Hall (2000) using Prism 4.0 (GraphPad Software Inc., San Diego, CA). This model includes three equilibrium constants: one each for agonist and modulator binding to the receptor (*K* and *M*, respectively) and a third for the equilibrium between active and inactive states of the receptor (L). In addition, four allosteric constants are included for agonist intrinsic efficacy (α), modulator intrinsic efficacy (β), binding cooperativity between agonist and modulator (γ), and activation cooperativity between agonist and modulator (δ). The Hall equations were modified so that the values of equilibrium constants, cooperativity parameters, and concentrations were entered as logarithms (Christopoulos, 1998). Thus, Hall's eq. 3 (Hall, 2000) was modified to the following:

$$\text{fractional occupancy} = \frac{10^{A}}{\dfrac{1 + (10^{L}) + \{(10^{M})(10^{B})[1 + (10^{\beta})(10^{L})]\}}{(10^{K})[1 + ((10^{\alpha})(10^{L})) + \{(10^{\gamma})(10^{M})(10^{B})[1 + ((10^{\alpha})(10^{\beta})(10^{\delta})(10^{L}))]\}} + 10^{A}}$$

Hall's eq. 10, which gives fractional activation of the receptor, was modified to the following:

$$\text{fractional activation} = \frac{(10^{L})\{1 + [(10^{\alpha})(10^{K})(10^{A})] + \{[(10^{\beta})(10^{M})(10^{B})][1 + (10^{\alpha})(10^{\gamma})(10^{\delta})(10^{K})(10^{A})]\}}{1 + (10^{L}) + [(10^{M})(10^{B})(1 + (10^{\beta})(10^{L}))] + [(10^{K})(10^{A})(1 + (10^{\alpha})(10^{L}) + [(10^{\gamma})(10^{M})(10^{B})(1 + \{(10^{\alpha})(10^{\beta})(10^{\gamma})(10^{L})\}}$$

Nonlinearity of stimulus-response coupling was accounted for by transforming fractional activation with a general logistic function (eq. 4.5; Kenakin, 1997):

$$\text{response} = \frac{(\text{fractional activation})^{n}}{(\text{fractional activation})^{n} + K_{E}}$$

The resulting equation was fit to the FLIPR functional data.

Families of theoretical curves were developed in GraphPad Prism 4.0 to determine the effect of systematically changing the various parameters in this model and to determine reasonable ranges of values that would fit the binding and FLIPR functional data. Initial values for the agonist-receptor and allosteric modulator-receptor binding association constants (K and M, respectively) were taken from the agonist and potentiator EC_{50} values. The initial value for agonist intrinsic efficacy α was taken as 100, the Hill slope value of 2 for the agonist concentration-response curves was used for the slope factor n in the general logistic equation, and the fitting parameter K_E in this equation (inversely proportional to the efficiency of stimulus-response coupling) was adjusted to match the maxima and minima of the FLIPR concentration-response curves for agonist alone. Because the modulators did not show agonist activity (see below), the initial value β for intrinsic activity of the modulator was set at 1. Because the modulators did not affect agonist binding (see below), the initial value γ for binding cooperativity was set at 1. The initial value for the equilibrium constant for isomerization between active and inactive states of the receptor was taken to be low (0.0003) because the receptor is not constitutively active. The initial value for the activation cooperativity constant δ was set greater than 1 but less than 10, because positive modulation was observed. It is important to remember that the concentrations of agonists and modulators, the equilibrium constants and cooperativity constants are all entered as the logarithms of the values given above. Finally, the model was fit to the experimental data using ranges of values we found to be reasonable from the theoretical curves. The agonist and modulator affinity constants as well as the equilibrium constant for isomerization between active and inactive receptors were shared for all data sets in a given study and were fit globally.

Results

FLIPR Assays. In $FLIPR_{384}$ assays, both human and rat mGluR5 CHO cells exhibited concentration-dependent increases in Fluo-4 fluorescence in response to quisqualate, glutamate, and DHPG as shown in O'Brien et al. (2003). These compounds seemed to act as full agonists, with potencies consistent with previously reported EC_{50} values. This assay was used to screen compounds for their ability to increase the response of human mGluR5 CHO cells to a low concentration of glutamate (300 nM) without eliciting a response by themselves. The mGluR5 allosteric potentiator DFB, reported by O'Brien et al. (2003), was first identified with this assay (Fig. 1). We now report that CPPHA, a compound from a different structural class (Fig. 1), was also found to be active as an mGluR5 potentiator.

Like DFB, CPPHA caused concentration-dependent potentiation of the response of human mGluR5 CHO cells to agonists in this assay. For example, the maximal potentiation of the response to 8 nM quisqualate was approximately 4.0-fold

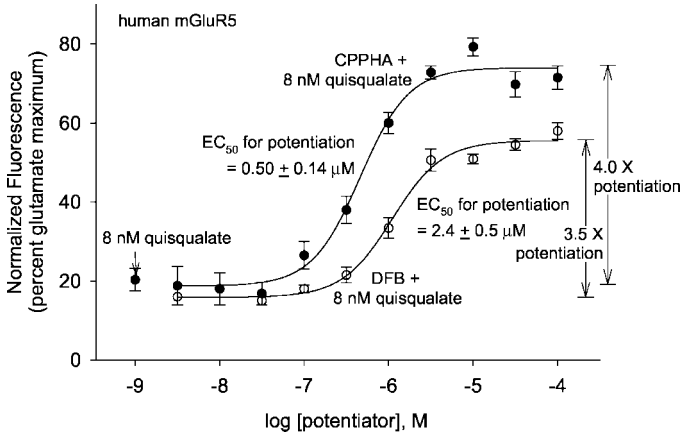

Fig. 2. DFB and CPPHA potentiate mGluR5 activation by quisqualate. Human or rat mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. A range of concentrations of DFB or CPPHA were added to cells (human mGluR5 CHO cells in this figure) after 10 s of baseline determination. Five minutes later, a fixed concentration (approximate EC_{10} concentration) of agonist (8 nM quisqualate in this figure) was added, and the Ca^{2+} response was measured by $FLIPR_{384}$. The fluorescence response was normalized to the response to glutamate control in each plate. Concentration-response curves were generated from mean data of six experiments. Error bars are S.E.M. Results for glutamate, 3,5-DHPG, and quisqualate on both human and rat mGluR5 CHO cells are summarized in Table 1. Fold potentiation was calculated from the maxima and minima determined by nonlinear curve fitting of the mean data.

TABLE 1
CPPHA and DFB potentiation of human and rat mGluR5 activation by glutamate, 3,5-DHPG, and quisqualate

Agonist	CPPHA EC_{50} for Potentiation	Maximum Potentiation, CPPHA[a]	DFB EC_{50} for Potentiation	Maximum Potentiation, DFB[a]
	μM		μM	
Human mGluR5a CHO Cells				
300 nM Glutamate	0.393 ± 0.037 (11)	6.8-fold	2.3 ± 0.6 (7)	4.2-fold
1 μM 3,5-DHPG	0.316 ± 0.045 (4)	3.2-fold	2.5 ± 0.6 (11)	3.4-fold
8 nM Quisqualate	0.500 ± 0.145 (4)	4.0-fold	2.4 ± 0.5 (8)	3.5-fold
Rat mGluR5 CHO Cells				
300 nM Glutamate	0.81 ± 0.19 (4)	7.9-fold	7.2 ± 1.9 (6)	6.1-fold
1 μM 3,5-DHPG	0.634 ± 0.071 (4)	5.4-fold	4.2 ± 1.1 (6)	4.9-fold
8 nM Quisqualate	1.16 ± 0.38 (4)	7.6-fold	4.4 ± 1.2 (5)	6.7-fold

[a] Ratio of the maximum to the minimum response determined by nonlinear curve fitting of mean data (see Fig. 2).

for CPPHA, with an EC_{50} value for potentiation of 0.50 ± 0.14 μM (n = 4) (Fig. 2). By comparison, DFB caused a 3.5-fold potentiation with an EC_{50} value for potentiation of 2.4 ± 0.5 μM (n = 8) for this concentration of quisqualate. Similar to the results previously obtained with DFB (O'Brien et al., 2003), CPPHA caused potentiation of the responses of both human mGluR5 and rat mGluR5 CHO cells to glutamate, quisqualate, and 3,5-DHPG (Table 1). Like DFB, potencies were comparable for potentiation of these agonists as well as across species. DFB and CPPHA were both found to potentiate the glutamate response of human embryonic kidney 293 cells expressing human mGluR5 (data not shown). CPPHA alone (100 μM) did not cause a response on either human or rat mGluR5 CHO (or human embryonic kidney 293) cells in this assay, indicating that, like DFB, CPPHA did not act as an agonist.

Reversibility of CPPHA was determined by comparing the FLIPR response of human mGluR5 CHO cells to 300 nM glutamate in the presence of a range of concentrations of CPPHA, with and without a washout step. The response to CPPHA was virtually abolished after washing [without washout, CPPHA EC_{50} = 0.32 + 0.05 μM (n = 3); with washout, CPPHA EC_{50} >100 μM], indicating CPPHA was reversible (data not shown).

In other functional studies, CPPHA did not potentiate the responses of other mGluRs or endogenous receptors. Besides mGluR5 potentiation, the only activities observed for CPPHA were weak negative modulation of the responses of human mGluR4 and rat mGluR8 to glutamate in the FLIPR assay (Fig. 3). Further testing of CPPHA against a panel of 175 receptors, transporters, ion channels, and enzymes (MDS Panlabs, Bothell, WA) indicated submicromolar activity in the following: phosphodiesterase 6 (IC_{50} of ~0.5 μM), opiate δ (K_i = 352 nM), opiate κ (K_i = 474 nM), and at the human *ether-a-go-go*-related gene potassium channel (K_i = 813 nM).

Increasing concentrations of CPPHA caused a parallel, leftward shift of mGluR5 CHO cell quisqualate concentration-response curves with no increase in maximal response, similar to the results obtained with DFB (Fig. 4; O'Brien et al., 2003). The change in EC_{50} in the presence of 10 μM CPPHA was 4.8-fold. The potentiation of a fixed concentration of agonist seems to be maximal at this concentration of CPPHA (Fig. 2). Similar shifts in agonist concentration-responses were observed for glutamate and 3,5-DHPG in both human and rat mGluR5 CHO cells, again with no increase in the maximal response to the agonists (Table 2). The studies with CPPHA and DFB were performed separately, accounting for the slight difference in agonist EC_{50} values.

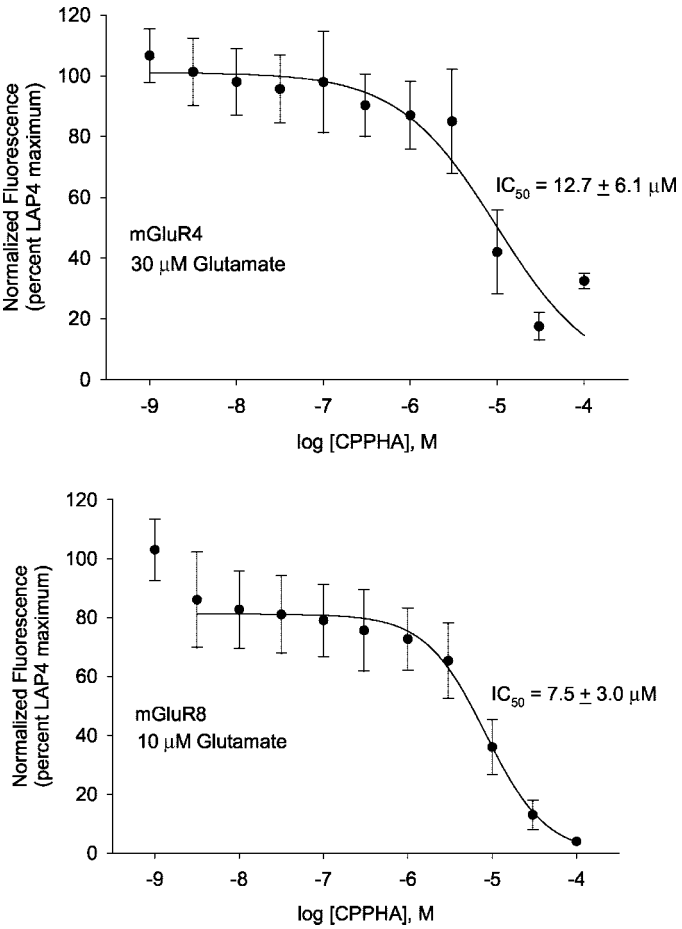

Fig. 3. CPPHA inhibits mGluR4 and mGluR8 activation by glutamate. Human mGluR4 and rat mGluR8 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. A range of concentrations of CPPHA were added to cells after 10 s of baseline determination. Five minutes later, a fixed concentration (approximate EC_{70} concentration) of agonist (30 μM glutamate for mGluR4, 10 μM glutamate for mGluR8) was added, and the Ca^{2+} response was measured by $FLIPR_{384}$. The fluorescence response was normalized to the response to an L-AP4 control in each plate. Concentration-response curves were generated from mean data of three experiments. Error bars are S.E.M.

Both CPPHA and DFB revealed 4-carboxy,3-hydroxyphenylglcyine (4C3HPG) to be a partial agonist of mGluR5 in FLIPR assays. Indeed, 4C3HPG has no apparent agonist effect on its own at concentrations up to 10 mM, but rather antagonizes mGluR5 responses to glutamate. However, in

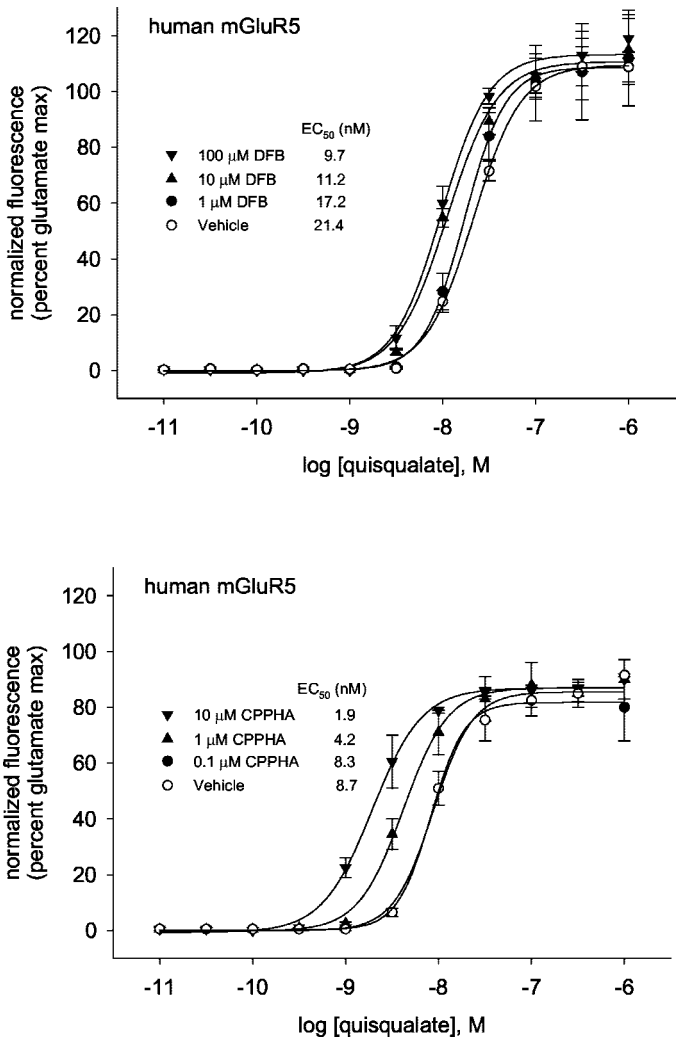

Fig. 4. DFB and CPPHA potentiation of response to quisqualate is manifested as increased mGluR5 agonist sensitivity. Human or rat mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. Several fixed concentrations of DFB (top) or CPPHA (bottom) were added to cells (human mGluR5 CHO cells in this figure) after 10 s of baseline determination. Five minutes later, a range of concentrations of agonist (quisqualate in this figure) was added. The fluorescence response was normalized to the response to glutamate control in each plate. The leftward shift of the quisqualate concentration-response curves seems to approach a maximum at the highest concentrations of DFB or CPPHA. Concentration-response curves were generated from mean data of five experiments. Error bars are S.E.M. Results for glutamate, 3,5-DHPG, and quisqualate on both human and rat mGluR5 CHO cells are summarized in Table 2, calculated from the EC_{50} values determined by nonlinear curve fitting of the mean data.

the presence of CPPHA (10 μM) or DFB (100 μM), 4C3HPG acts as a partial agonist of mGluR5 with EC_{50} values (and apparent maximal effects) of 140 μM (80% of glutamate maximum) and 870 μM (~50% of glutamate maximum) respectively (data not shown).

Radioligand Binding. Neither DFB nor CPPHA (up to 100 μM) had any effect on the binding of [^{3}H]quisqualate (25 nM) to membranes prepared from CHO mGluR5 cells (data not shown), suggesting that these compounds did not bind at the agonist binding site. Nondisplaceable [^{3}H]quisqualate binding was estimated in the presence of 1 mM glutamate. We reported previously that DFB inhibited binding of the MPEP analog methoxy-PEPy (2 nM; Cosford et al., 2003) to these membranes (O'Brien et al., 2003), similar to the effect of MPEP (Fig. 5). Interestingly, CPPHA had no effect on binding of this radioligand (Fig. 5). Comparison of these two compounds in saturation binding studies using [^{3}H]methoxy-PEPy (Fig. 5, inset) suggest that the presence of DFB did not affect B_{max}, but increased K_d (control: B_{max} = 111 ± 8 fmol/mg protein, K_d = 5.1 ± 0.6 nM; in presence of 15 μM DFB: B_{max} = 104 ± 1 fmol/mg protein, K_d = 9.4 ± 1.4 nM), whereas the presence of CPPHA had no effect on either parameter (in presence of 15 μM CPPHA: B_{max} = 104 ± 9 fmol/mg protein, K_d = 5.1 ± 0.5 nM). MPEP (20 nM) by comparison, caused no change in B_{max} (118 ± 1 fmol/mg protein) but increased K_d (19.5 ± 1.9 nM).

Electrophysiology. Previous studies have shown that bath application of the group I mGluR-selective agonist DHPG potentiates NMDA receptor currents in hippocampal CA1 pyramidal cells in rat brain slices and that this potentiation is selectively mediated by activation of the group I mGluR-subtype mGluR5 (Mannaioni et al., 2001). We therefore were interested to test whether CPPHA could potentiate the effect of a low concentration of DHPG that by itself has little or no effect on NMDA receptor currents in this region.

We performed whole cell voltage-clamp recordings from CA1 pyramidal cells in rat brain slices. All recordings were performed at a holding potential of −60 mV in the presence of tetrodotoxin (TTX; 1 μM), to block synaptic transmission. Fast application of NMDA (0.5–1.5 s; 100 μM) directly to the recording site at 1-min intervals produced a stable inward current (30–100 pA) in CA1 pyramidal cells. Bath application of a low dose of DHPG alone (1 μM; 10 min) had no significant effect on the amplitude of NMDA-evoked currents (13.6 ± 6.8%; $n = 4$, $p > 0.05$; Fig. 6A$_1$, B, and C). Prior bath application (10–15 min) of the mGluR5 potentiator CPPHA (10 μM), which had no significant effect by itself (data not shown), induced a robust potentiation of NMDA-evoked currents by 1 μM DHPG (97.3 ± 22.0%; $n = 4$, $p < 0.05$; Fig. 6A$_2$, B, and D). The effect of 1 μM DHPG on NMDA-evoked currents in the presence of 10 μM CPPHA reached a maximum after approximately 5 min of bath application and returned almost back to baseline before the DHPG application was turned off (Fig. 6D).

Another brain region that shows a selective response to mGluR5 activation is the STN. Bath application of DHPG has been shown to induce a robust depolarization in STN neurons that is selectively mediated by activation of the group I mGluR subtype mGluR5 (Awad et al., 2000). This effect of DHPG therefore provided another physiological response induced by mGluR5 activation with which to test the effects of an mGluR5 potentiator. We performed whole cell current-clamp recordings from STN neurons in rat midbrain slices. All studies were performed in the presence of TTX (1 μM) to block action potential firing. As described previously (Awad et al., 2000), 2-min bath application of DHPG (100 μM) induced a robust depolarization in these cells (17.25 ± 2.1 mV; $n = 5$; data not shown). To test the effect of 10 μM CPPHA on this response, we chose a low concentration of DHPG (3 μM) that by itself had little or no excitatory effect on STN neurons (2.3 ± 0.8 mV; $n = 4$; Fig. 7, A and C). Bath application of 10 μM CPPHA for 10 to 15 min had no effect on membrane potential (data not shown). However, application of 3 μM DHPG in the presence of 10 μM CPPHA induced a

TABLE 2
Effect of CPPHA and DFB on FLIPR agonist concentration-response curves: human and rat mGluR5

Agonist	Control Agonist EC_{50}	EC_{50} Change at 10 μM CPPHA[a]	Control Agonist EC_{50}	EC_{50} Change at 100 μM DFB[a]
Human mGluR5a CHO Cells				
Glutamate	452 ± 63 nM (3)	7.0-fold	642 ± 30 nM (5)	2.2-fold
3,5-DHPG	1.26 ± 0.20 μM (3)	4.8-fold	2.36 ± 0.17 μM (5)	2.3-fold
Quisqualate	8.95 ± 0.7 nM (5)	4.8-fold	21.4 ± 0.7 nM (5)	2.2-fold
Rat mGluR5 CHO Cells				
Glutamate	766 ± 129 nM (5)	6.1-fold	659 ± 48 nM (5)	1.9-fold
3,5-DHPG	1.88 ± 0.11 μM (5)	4.7-fold	2.07 ± 0.12 μM (5)	2.0-fold
Quisqualate	15.3 ± 1.9 nM (5)	4.1-fold	22.0 ± 0.9 nM (5)	1.7-fold

[a] Calculated from the EC_{50} values determined by nonlinear curve fitting of the mean data (see Fig. 4).

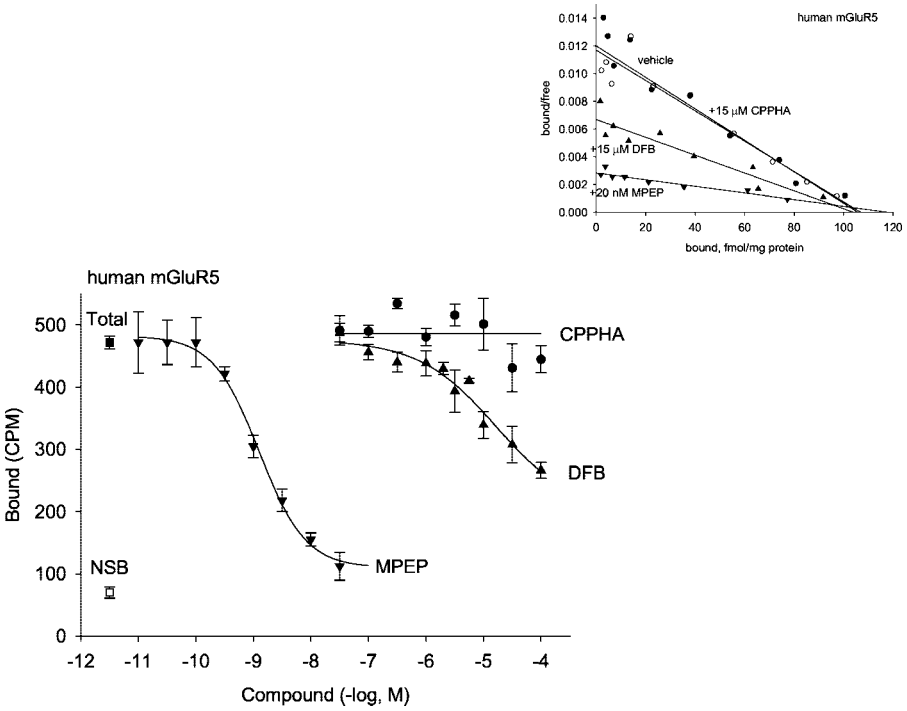

Fig. 5. Unlike DFB and MPEP, CPPHA does not affect [^{3}H]methoxyPEPy binding (inset, Scatchard plot). Membranes prepared from human mGluR5 CHO cells were incubated with the radiolabeled MPEP analog [^{3}H]3-methoxy-5-(2-pyridinylethynyl) pyridine (1 or 2 nM final in 50 mM Tris 0.9% NaCl, pH 7.4) for 60 min at room temperature in the presence of varying concentrations of allosteric modulators. Samples were then filtered onto glass fiber filters. Nondisplaceable binding was estimated with 1 μM MPEP. A representative competition experiment is shown. Error bars are standard deviation. Inset, representative saturation experiment is shown in the presence and absence of 15 μM DFB, 15 μM CPPHA, or 20 nM MPEP.

robust depolarization of STN neurons (8.9 ± 2.2 mV; n = 6; Fig. 7, B and C) that in most cases led to the induction of membrane oscillations (Fig. 7B). Only cells that reached a plateau of membrane depolarization before oscillating were used and the maximum depolarization was measured at this plateau.

Together, these data show that CPPHA potentiates the effect of low concentrations of DHPG on NMDA-evoked currents in CA1 pyramidal cells and the direct excitatory effects of DHPG on STN neurons. Because it has been shown previously that both of these responses are mediated by activation of the group I mGluR-subtype mGluR5, these data indicate that CPPHA acts as a potentiator of native mGluR5 expressed in two different populations of neurons.

Discussion

Characterization of GPCR function depends on the identification and development of potent and selective pharmacological agents. These compounds are required as tools to elucidate details of GPCR activation and function as well as to understand the role of a given GPCR in complex systems. For the mGluR family of GPCRs, a number of potent orthosteric agonists and antagonists have been discovered and developed using traditional approaches (for reviews, see Conn and Pin, 1997; Schoepp et al., 1999). Recently, novel high-throughput functional assays have expanded the ability of researchers to identify and develop pharmacological tools to include compounds that act on receptors at novel allosteric sites rather than at the historically targeted orthosteric sites. This approach has resulted in the identification and characterization of MPEP and 7-(hydroxylimino) cyclopropa[*b*-]chromen-1*a*-carboxamide ethyl ester as the first negative allosteric mGluR modulators (selective for the group I receptors mGluR5 and mGluR1, respectively). More recently, positive allosteric modulators selective for mGluR1b also have been identified (Knoflach et al., 2001), and we have used functional assays to identify DFB as a novel positive allosteric modulator selective for mGluR5 (O'Brien et al., 2003). We now report discovery of CPPHA as a novel positive allosteric modulator selective for mGluR5.

Although CPPHA is in a different structural class from DFB, it is qualitatively similar to DFB in potentiating the activation of human and rat mGluR5 receptors in FLIPR assays by glutamate, quisqualate, and 3,5-DHPG; like DFB, CPPHA is not itself an agonist. Potentiation of mGluR5 by CPPHA was observed in several functional assays in cloned cells as well as tissue slices and is therefore unlikely to be an artifact of the expression system. As in the case of DFB, the

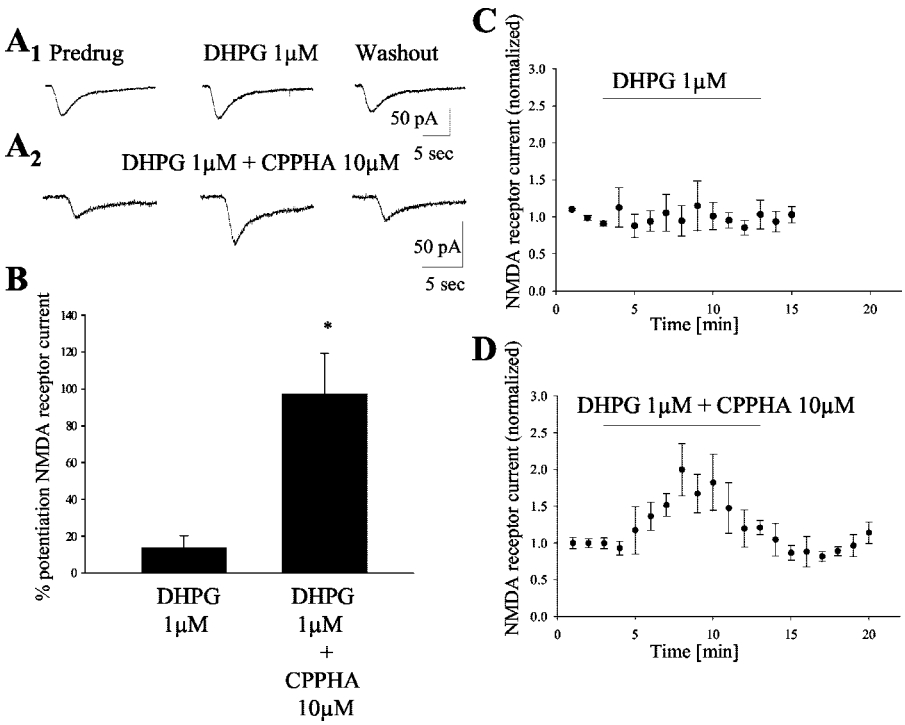

Fig. 6. CPPHA potentiates the effect of low, subthreshold doses of DHPG on NMDA-evoked currents in CA1 pyramidal cells. $A_{1,2}$, representative traces of NMDA-evoked currents before (predrug), during, and after (washout) application of 1 μM DHPG in the absence (A_1) and presence (A_2) of 10 μM CPPHA. B, bar graph showing the average maximum effect of 1 μM DHPG on NMDA-evoked currents in the absence and presence of 10 μM CPPHA. Each bar represents the mean ± S.E.M. of data from four cells; *, $p < 0.05$, Student's paired t test. C and D, average time courses of the effect of 1 μM DHPG on NMDA-evoked currents in the absence (C) and presence (D) of 10 μM CPPHA; each point represents the mean ± S.E.M. of data from four cells.

positive allosteric effect of CPPHA on mGluR activity seems to be specific: CPPHA potentiates mGluR5 activation, but not activation of mGluRs 1, 2, 3, 4, 7, or 8, or endogenous purinergic or thrombin receptors. CPPHA does have a negative allosteric effect on the group III receptors mGluR4 and mGluR8 with IC_{50} values 7- to 40-fold higher than the EC_{50} values for potentiation of mGluR5. Interestingly, the group I negative allosteric modulators SIB-1893, MPEP, and PHCCC have been shown to be positive allosteric modulators of the group III receptor mGluR4 (Maj et al., 2003; Marino et al., 2003; Mathiesen et al., 2003). Although these results suggest that there may be a functional reciprocity between allosteric modulators of group I and group III mGluRs, the binding site for MPEP (and presumably the structurally related SIB-1893) on mGluR5 is thought to be topologically distinct from the binding site for 7-(hydroxylimino) cyclopropa[*b*]chromen-1*a*-carboxamide ethyl ester (and presumably the structurally related PHCCC) on mGluR1 (Pagano et al., 2000). Elucidation and comparison of the binding sites for these allosteric modulators on group III as well as group I mGluRs may provide additional insight into their mechanism of action. Interestingly, despite the qualitative similarities between CPPHA and DFB, it is clear from the [^{3}H]methoxyPEPy binding data that these two allosteric potentiators do not interact with mGluR5 in the same manner. Although DFB competes with [^{3}H]methoxyPEPy binding to mGluR5, CPPHA does not affect the binding of this radioligand, indicating that its interaction with mGluR5 is different from that of DFB.

The allosteric model of Parmentier et al. (2002) has been used to model family C GPCRs, treating the orthosteric and allosteric domains of the as separate, linked entities. This model is a more complex version of the model of Hall (2000). We found that the binding and functional data obtained for CPPHA and DFB could be nicely fit by the simpler model of allosterism developed by Hall (2000) if it is modified to take into account the nonlinearity of the stimulus-response relationship (see *Materials and Methods*). The modified Hall model accurately reflected both the lack of effect of CPPHA and DFB on binding of [^{3}H]quisqualate to mGluR5 as well as the agonist curve-shift data. Modeling of the binding and functional data suggested that the main effect of both CPPHA and DFB was to increase the activation cooperativity (δ) of the system, resulting in an increased fraction of activated, agonist-bound mGluR5 within the total population of receptors. The value of the activation cooperativity parameter δ that provided the best fits for the CPPHA data was about 5.2, whereas this value of δ for the best fits for the DFB data was less: about 1.9, consistent with the smaller curve shifts with this compound. Thus, the action of both modulators seems to be through the same parameter. This suggests that for a given receptor, multiple allosteric sites may modulate functionally (and mechanistically) similar allosteric effects.

Because CPPHA was more potent and efficacious than DFB, we were able to study its effects by electrophysiological techniques in rat brain slices. Earlier brain slice electrophysiological studies had found that NMDAR currents were increased by activation of mGluR5 by group I-selective agonists. The results presented above demonstrate that in the presence of CPPHA, NMDAR currents are increased at concentrations of mGluR5 agonists that would ordinarily be below the threshold of activation. Similarly, earlier studies had found that STN neurons were depolarized by activation of mGluR5 by group I-selective agonists. The results presented above indicate that in the presence of CPPHA, subthreshold concentrations of mGluR5 agonists cause depolarization of STN neurons. Thus, positive allosteric modulation of mGluR5 increases NMDAR currents in CA1 neurons and increases the depolarization of STN neurons evoked by low doses of mGluR5 agonists. This demonstrates that allosteric potentiation of mGluR5 can be observed by electrophysiological methods in native systems. Similar results have been reported for potentiation of mGluR1-mediated currents in rat

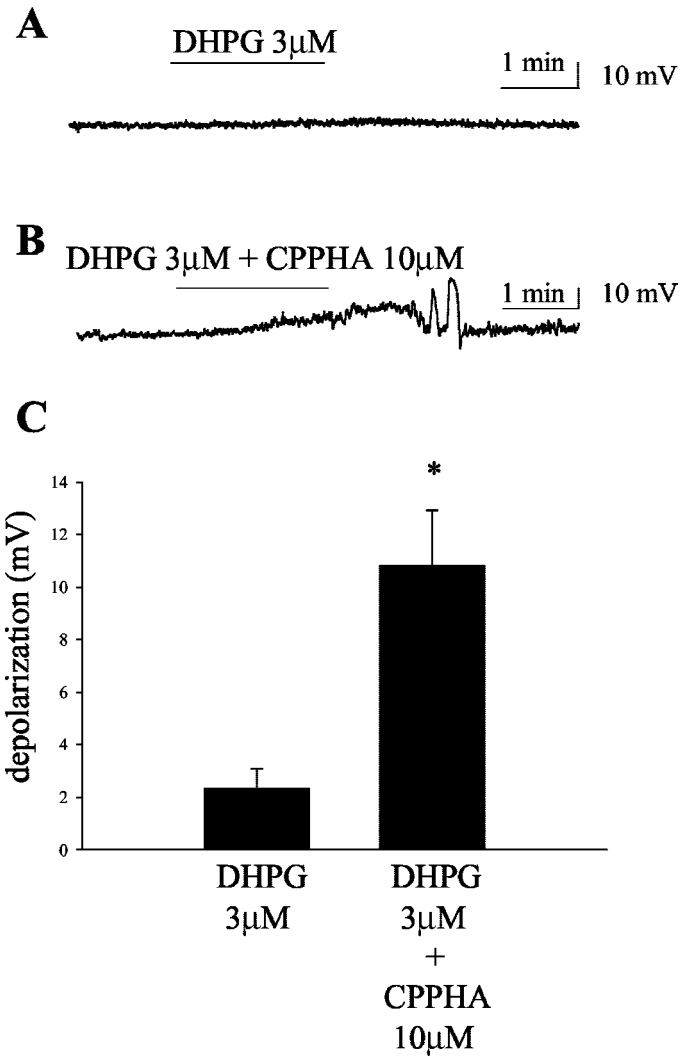

Fig. 7. CPPHA potentiates the effect of low doses of DHPG on the mGluR5-mediated depolarization of STN neurons. A and B, representative traces of the effect of 3 μM DHPG on membrane potential in STN neurons in the absence (A) and presence (B) of 10 μM CPPHA. Note that 3 μM DHPG had no or only a small effect on membrane potential by itself (A) but induced a large depolarization with membrane oscillations in the presence of 10 μM CPPHA (B). C, bar graph showing the average effect of 3 μM DHPG in the absence and presence of 10 μM CPPHA. Each bar represents the mean ± S.E.M. of data from four and six cells respectively; *, $p < 0.05$, Student's paired t test.

cerebellar slices by the rat-selective mGluR1 potentiator Ro 67-7476 (Knoflach et al., 2001).

The ability to potentiate NMDAR currents by potentiation of mGluR5 may have useful physiological implications. Because NMDAR-dependent long-term potentiation is thought to play a central role in cognition, the effect of mGluR5 potentiation on NMDARs could serve as a means of enhancing cognitive processes. Furthermore, use-dependent NMDAR antagonists lead to behaviors similar to schizophrenia in healthy volunteers, and exacerbate or lead to relapse of symptoms in patients suffering from schizophrenia. This has led to the hypothesis that hypofunction of NMDAR-mediated neuronal circuitry may be involved in the positive, negative, and cognitive symptoms of schizophrenia. Recently, Kinney et al. (2003) reported that the mGluR5 negative allosteric modulator MPEP potentiated both phencyclidine-induced motor activity and phencyclidine-induced disruption of prepulse inhibition (PPI, a model of sensorimotor gating) in rodents. Furthermore, mGluR5 knockout mice were found to display consistent deficits in PPI relative to wild-type controls. Finally, the mGluR5 agonist (*R*,*S*)-2-chloro-5-hydroxyphenylglycine was found to have no effect by itself on rodent PPI, but ameliorated amphetamine-induced disruption of PPI in these models. These results indicate that mGluR5 plays a modulatory role on rodent locomotor behavior and sensorimotor gating. An interesting question is whether a positive allosteric mGluR5 modulator can mimic the effects of (*R*,*S*)-2-chloro-5-hydroxyphenylglycine in these models. Although CPPHA does not have sufficient solubility to achieve pharmacologically active levels in vivo, it is likely that more potent, more soluble compounds will soon become available. These compounds will help determine whether potentiation of NMDAR by mGluR5 in neuronal circuitry relevant to schizophrenia or cognition might offer a novel approach for development of antipsychotic or cognition-enhancing drugs.

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