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A Family of Highly Selective Allosteric Modulators of the Metabotropic Glutamate Receptor Subtype 5

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ABSTRACT

We have identified a family of highly selective allosteric modulators of the group I metabotropic glutamate receptor subtype 5 (mGluR5). This family of closely related analogs exerts a spectrum of effects, ranging from positive to negative allosteric modulation, and includes compounds that do not themselves modulate mGluR5 agonist activity but rather prevent other family members from exerting their modulatory effects. 3,3′-Difluorobenzaldazine (DFB) has no agonist activity, but it acts as a selective positive allosteric modulator of human and rat mGluR5. DFB potentiates threshold responses to glutamate, quisqualate, and 3,5-dihydroxyphenylglycine in fluorometric Ca^{2+} assays 3- to 6-fold, with EC_{50} values in the 2 to 5 μM range, and at 10 to 100 μM, it shifts mGluR5 agonist concentration-response curves approximately 2-fold to the left. The analog 3,3′-dimethoxybenzaldazine (DMeOB) acts as a negative modulator of mGluR5 agonist activity, with an IC_{50} of 3 μM in fluorometric Ca^{2+} assays, whereas the analog 3,3′-dichlorobenzaldazine (DCB) does not exert any apparent modulatory effect on mGluR5 activity. However, DCB seems to act as an allosteric ligand with neutral cooperativity, preventing the positive allosteric modulation of mGluRs by DFB as well as the negative modulatory effect of DMeOB. None of these analogs affects binding of [^{3}H]quisqualate to the orthosteric (glutamate) site, but they do inhibit [^{3}H]3-methoxy-5-(2-pyridinylethynyl)pyridine binding to the site for 2-methyl-6-(phenylethynyl)-pyridine, a previously identified negative allosteric modulator. With the use of these compounds, we provide evidence that allosteric sites on GPCRs can respond to closely related ligands with a range of pharmacological activities from positive to negative modulation as well as to neutral competition of this modulation.

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that bind glutamate to exert a modulatory influence on neuronal excitability and synaptic transmission in the central nervous system. The eight known members of the mGluR subfamily have been divided into three groups on the basis of their sequence identity, pharmacology, and preferred signal transduction mechanism. Group I mGluRs (mGluRs 1 and 5) are primarily localized postsynaptically where they modulate ion channel activity and neuronal excitability. The group I mGluRs are coupled to $G_{\alpha q}$ and its associated effectors, such as phospholipase C. Groups II (mGluRs 2 and 3) and III (mGluRs 4, 6, 7, and 8) are primarily located presynaptically and regulate the release of neurotransmitters, including glutamate. Group II and III mGluRs are coupled to $G_{\alpha i}$ and its associated effectors, such as adenylate cyclase. These latter two groups are distinguished from each other by their pharmacology; selective agonists and antagonists have been identified for each group (Conn and Pin, 1997). All mGluR subtypes possess a large (~560 amino acids) extracellular amino-terminal domain that contains the glutamate agonist binding site. Thus, in the mGluRs, interaction of the agonist with the transmembrane domains is believed to be indirect (O'Hara et al., 1993; Conn and Pin, 1997; Bockaert and Pin, 1999).

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; DFB, 3,3′-difluorobenzaldazine; DCB, 3,3′-dichlorobenzaldazine; DMeOB, 3,3′-dimethoxybenzaldazine; methoxy-PEPy, 3-methoxy-5-(2-pyridinylethynyl)pyridine; DHPG, 3,5-dihydroxy-phenylglycine; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; GPCR, G protein-coupled receptor; NPS 2390, 2-quinoxaline-carboxamide-*N*-adamantan-1-yl; SIB-1757, 6-methyl-2-(phenylazo)-3-pyridinol; SIB-1893, (*E*)-2-methyl-6-(2-phenylethenyl)pyridine; CPCCOEt, 7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester; PHCCC, *N*-phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide; BAY 36-7620, (3a*S*,6a*S*)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopenta[*c*]furan-1-on; EM-TBPC, 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[*d*]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile; R214127, 1-(3,4-dihydro-2H-pyrano[2,3-*b*]quinolin-7-yl)-2-phenyl-1-ethanone; LCMS, liquid chromatography mass spectrometry; TFA, trifluoroacetic acid; ELSD, evaporative light-scattering detection; HRMS, high-resolution mass spectrometry; CHO, Chinese hamster ovary; FLIPR, fluorometric imaging plate reader; DMSO, dimethyl sulfoxide; KRB, Krebs' bicarbonate buffer; PI, phosphatidyl inositol.

It has been suggested that the group I receptor mGluR5 may play a role in a number of central nervous system disease states, including pain (Salt and Binns, 2000; Bhave et al., 2001), anxiety (Spooren et al., 2000; Tatarczynska et al., 2001), addiction to cocaine (Chiamulera et al., 2001), and schizophrenia (Chavez-Noriega et al., 2002). We are interested in mGluR5 function and initiated an effort to identify and develop novel pharmacological tools to study the role of mGluR5 in normal and pathological states. Most of the currently available agonists and antagonists used to study mGluR function have been developed as structural analogs of glutamate, quisqualate, or phenylglycine (Schoepp et al., 1999) and act through binding at or near the orthosteric agonist site in the amino-terminal domain. Although it has been possible to develop group-selective agonists and antagonists using this approach, it has been considerably more challenging to obtain potent, agonist-site compounds that are subtype-selective. A novel alternative approach has been to identify compounds that act through allosteric mechanisms, modulating the receptor by binding to sites different from the highly conserved orthosteric binding site. Receptor modulation by binding to an allosteric site may afford very high subtype selectivity, assuming there would be less pressure to conserve such a site within a subfamily of receptors. NPS 2390 (van Wagenen et al., 1998) and SIB-1757 and SIB-1893 (Varney et al., 1999) were the earliest compounds reported as noncompetitive antagonists of mGluR1 and mGluR5, respectively. The potent, noncompetitive group I antagonists MPEP (mGluR5-selective) (Gasparini et al., 1999) and CPCCOEt (mGluR1-selective) (Litschig et al., 1999) were the first reported examples of negative allosteric modulators of group I mGluRs. These compounds inhibit the agonist activation of group I mGluRs in functional assays but do not affect agonist affinity. They have been shown to exert their effects by binding to the transmembrane region of the receptors (Pagano et al., 2000). Additional examples of negative allosteric modulators of group I mGluRs include PHCCC (Annoura et al., 1996), a structural analog of CPCCOEt, and BAY 36-7620 (mGluR1-selective) (Carroll et al., 2001); methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine, [^{3}H]3-methoxy-5-(2-pyridinylethynyl)pyridine (methoxy-PEPy), and 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]-pyridine (mGluR5-selective) (Cosford et al., 2003a,b); EM-TBPC (rat mGluR1-selective (Malherbe et al., 2003); and R214127 (mGluR1-selective) (Lavreysen et al., 2003). Recently, Knoflach et al. (2001) reported examples of potent, positive allosteric modulators of mGluR1a. These compounds also were shown to bind to the transmembrane regions of the receptors.

We have identified a family of selective allosteric modulators of mGluR5. Unlike previously identified allosteric regulators of GPCRs, this family of closely related analogs exerts a spectrum of effects, ranging from positive to negative allosteric modulation, and includes compounds that do not themselves modulate mGluR5 agonist activity but rather prevent other family members from exerting their modulatory effects. These findings expand our understanding of allosteric sites on GPCRs and suggest that small molecules can interact with allosteric sites with a range of intrinsic activities in a manner analogous to what is seen at traditional neurotransmitter binding sites.

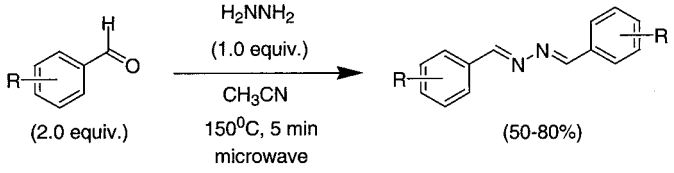


Fig. 1. Generalized synthesis scheme for benzaldazine analogs.

Materials and Methods

Compounds

A generalized synthetic scheme for the compounds reported in this article is shown in Fig. 1.

Representative Synthesis (3,3-Difluorobenzaldazine). Into a 5-ml Smithsynthesizer reaction vial (Personal Chemistry, Foxboro, MA) with a stir bar was placed 3-fluorobenzaldehyde (530 μl, 5 mmol, 2.0 equivalents) in 2.5 ml of acetonitrile. Then hydrazine (80 μl, 2.5 mmol, 1.0 equivalents) was added, and the vial was capped. The vial was then heated in the Smithsynthesizer reaction cavity at 150°C for 5 min. After 5 min, the reaction vial was rapidly cooled to 40°C. Upon removal from the reactor cavity, a bright yellow precipitate was collected by filtration and washed with water. After drying overnight in a vacuum oven at 50°C, 378 mg (62%) of the title compound was obtained as a bright yellow powder. Analytical LCMS indicated a single peak (3.82 min, CH_3CN/H_2O/0.1% TFA, 4-min gradient) that was >98% pure by UV (214 nm) and 100% pure by ELSD analysis. ^{1}H NMR (300 MHz, $CDCl_3$) findings were the following: δ 8.61 (s, 2H), 7.59 (m, 4H), 7.43 (m, 2H), and 7.16 (m, 2H); HRMS calculated for $C_{14}H_{10}F_2N_2$ (M+H), was 245.0885; found 245.0867 (M+H).

Analytical Data for Other Substituted Benzaldazines. For 2,2-difluorobenzaldazine, we obtained 408 mg (67%) of the title compound as a bright yellow powder. Analytical LCMS indicated a single peak (3.92 min, CH_3CN/H_2O/0.1% TFA, 4-min gradient) that was >98% pure by UV (214 nm) and 100% pure by ELSD analysis. ^{1}H NMR (300 MHz, $CDCl_3$) findings were the following: δ 8.93 (s, 2H), 8.13 (*td*, J = 1.8, 7.35 Hz, 2H), 7.44 (m, 2H), 7.22 (m, 2H), and 7.13 (m, 2H); HRMS calculated for $C_{14}H_{10}F_2N_2$ (M+H) was 245.0885, and we found 245.0865 (M+H). For 3,3-dimethoxybenzaldazine, we obtained 536 mg (80%) of the title compound as a bright yellow flocculent solid. Analytical LCMS indicated a single peak (3.65 min, CH_3CN/H_2O/0.1% TFA, 4-min gradient) that was >98% pure by UV (214 nm) and 100% pure by ELSD analysis. ^{1}H NMR (300 MHz,

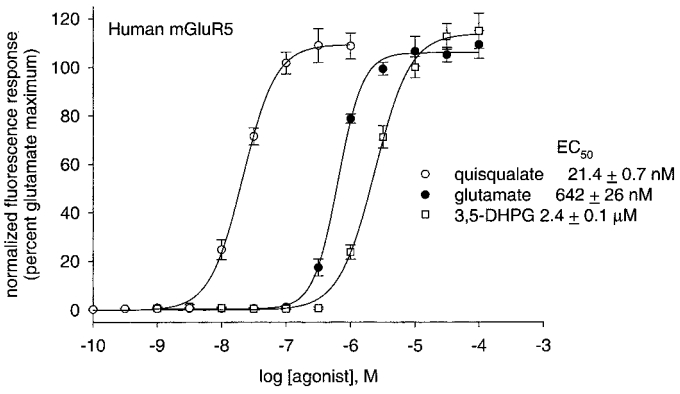


Fig. 2. Quisqualate, glutamate, and DHPG activate Ca^{2+} transients in human mGluR5-expressing CHO cells. Human mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. Agonists were added after 10 s of baseline determination. Data were normalized to a glutamate (10 μM) control maximum. Concentration-response curves were generated from the mean data of five experiments. Error bars represent S.E.M. Similar results were obtained for rat mGluR5 CHO cells. EC_{50} values for these cells are shown here and are summarized in Table 3.

$CDCl_3$) findings were the following: δ 8.63 (s, 2H), 7.44 (*d*, J = 2.7 Hz, 2H), 7.37 (s, 2H), 7.35 (m, 2H), 7.1 (m, 2H), and 3.88 (s, 6H); HRMS calculated for $C_{16}H_{16}N_2O_2$ (M+H) was 269.1285, and we found 269.1259 (M+H). For 3,3-dichlorobenzaldazine, we obtained 346 mg (50%) of the title compound as a bright yellow solid. Analytical LCMS indicated a single peak (4.1 min, CH_3CN/H_2O/0.1% TFA, 4-min gradient) that was >98% pure by UV (214 nm) and 100% pure by ELSD analysis. ^{1}H NMR (300 MHz, $CDCl_3$) findings were the following: δ 8.59 (s, 2H), 7.87 (m, 2H), 7.68 (*dt*, J = 1.5, 7.2Hz, 2H), and 7.42 (m, 4H); HRMS calculated for $C_{14}H_{10}Cl_2N_2$ (M+H) was 277.0294, and we found 277.0267 (M+H).

Cells

Cloned human mGluR5a receptors were transfected into CHO cells (human mGluR5 CHO cells) using a cytomegalovirus-based promoter to drive expression. Cells were grown in Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, nonessential amino acids, 25 mM HEPES, 55 μM β-mercaptoethanol (all listed materials were from Invitrogen, Carlsbad, CA), and 10 μg/ml puromycin (BD Biosciences Clontech, Palo Alto, CA). Cloned rat mGluR5a receptors were transfected, and the resulting cells (rat mGluR5 CHO cells) were grown in the same manner. Group III mGluRs were expressed in cell lines in the presence of chimeric or promiscuous G proteins to make them compatible with assays measuring increases in intracellular Ca^{2+} (mGluR4, mGluR7, and mGluR8 were coexpressed with $G_{\alpha qi5}$, $G_{\alpha 15}$, and $G_{\alpha 15}$, respectively).

Fluorometric Imaging Plate Reader

CHO cells expressing mGluR5 receptors (mGluR5 CHO cells) were plated in clear-bottomed, poly-D-lysine–coated 384-well plates (BD Biosciences, Franklin Lakes, NJ) in glutamate/glutamine-free medium using a Multidrop 384 cell dispenser (Thermo Electron Corporation, Waltham, MA). The plated cells were grown overnight at 37°C in the presence of 6% CO_2. The next day, the cells were washed with 3 × 100 μl of assay buffer (Hanks' balanced salt solution; Invitrogen) containing 20 mM HEPES (Invitrogen), 2.5 mM probenecid (Sigma Chemical Co., St. Louis, MO), and 0.1% bovine serum albumin (Sigma) using an EMBLA cell washer (Skatron, Lier, Norway). The cells were incubated with 1 μM Fluo-4AM (Molecular Probes, Eugene, OR) for 1 h at 37°C and 6% CO_2. The extracellular dye was removed by washing as described above. Ca^{2+} flux was measured using $FLIPR_{384}$ fluorometric imaging plate reader (Molecular Devices Corp., Sunnyvale, CA). Compounds were serially diluted in 100% DMSO and then diluted in assay buffer to a 3× stock at 2% DMSO. This stock was then applied to the cells for a final DMSO concentration of 0.67%. For potency determination, the cells were preincubated with various concentrations of compound for 5 min and then stimulated for 3 min with either an EC_{20} or EC_{50} concentration of agonist (i.e., glutamate) for potentiation measurements or inhibition measurements, respectively.

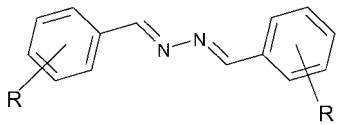
Reversibility of modulators was determined by adding a range of concentrations of modulator to half of a plate of Fluo-4–loaded mGluR5 CHO cells (with vehicle added to the other half), allowing it to incubate for 5 min, washing with 100 μl of assay buffer 10 times, allowing the plate to incubate for 15 min at 37°C, and washing another five times. Then the same range of concentrations of modulator was added to the vehicle-treated half of the plate, and vehicle was added to the half originally treated with modulator and allowed to incubate for 5 min; then agonist was added in the FLIPR. The response of the cells to agonist on the two halves of the plate were compared to determine the effect of washing on the modulatory effect. A rightward shift of the concentration-dependent modulatory effect after washing indicated reversibility of the modulator. In the case of compounds with neutral cooperativity, the assay was done as above, except positive or negative modulators were added after all wash steps to both halves of the plate in addition to the vehicle on the treated half and the compound with neutral cooperativity on the untreated half of the plate. The plate was allowed to incubate for 5 min, and agonist was added in the FLIPR. A rightward shift in the effect of the neutrally cooperative compound in blocking the effect of either the positive or negative allosteric modulator indicated reversibility of the neutrally cooperative compound.

Phosphoinositide Hydrolysis

Cells. CHO mGluR5 cells were loaded in their culture flasks overnight with *myo*-[^{3}H]inositol, suspended, and pooled. The radiotracer was removed, and glutamate-pyruvate transaminase, pyridoxal phosphate, and pyruvate were added to the buffer to reduce endogenous extracellular glutamate, and LiCl was added to inhibit the last step in the pathway of inositol trisphosphate breakdown. The cells were distributed to polypropylene culture tubes, and test compounds (serially diluted in 100% DMSO, then diluted in assay buffer to a 20× stock at 1% DMSO, then added to assay for a final DMSO concentration of 0.05%) or vehicle were added to each tube, followed by quisqualate, an mGluR5 agonist that is not affected by glutamate-pyruvate transaminase. The reaction was allowed to proceed for 45 min and was stopped by the addition of perchloric acid to each tube. The perchloric acid was neutralized by the addition of KOH, and the contents of each tube were diluted and applied to Dowex 1 × 8 columns (Dow Chemical Company, Midland, MI). The columns were washed with water and then with 60 mM ammonium formate and 5 mM sodium tetraborate. The columns were eluted with 1 M ammonium formate and 0.1 M formic acid. Scintillation cocktail was added to the eluates, and the amount of radiolabel for each sample was determine by scintillation counting.

Rat Hippocampal Slices. Rat pups (postnatal day 9) were decapitated, and their brains were removed and placed in ice-cold,

TABLE 1
Structures and activities of benzaldazine analogs



	R	Allosteric Activity	Potency
			μM
3,3′-Difluorobenzaldazine	3,3′-F	Positive	2.6 ± 0.4
2,2′-Difluorobenzaldazine	2,2′-F	Positive	14 ± 12
4,4′-Difluorobenzaldazine	4,4′-F	Negative	>100
3,3′-Dimethoxybenzaldazine	3,3′-OCH_3	Negative	3.0 ± 0.4
4,4′-Dimethoxybenzaldazine	4,4′-OCH_3	Negative	>100
3,3′-Dichlorobenzaldazine	3,3′-Cl	Neutral	7.6 ± 1.9 vs. DFB
		Neutral	17 ± 16 vs. DMeOB
3,3′-Dihydroxybenzaldazine	3,3′-OH	Neutral	>50

oxygenated Krebs' bicarbonate buffer (KRB). The hippocampus was dissected on ice and sliced with use of a McIlwain tissue chopper (Campden Instruments Ltd., Loughborough, UK). Slices were placed in fresh ice-cold oxygenated KRB and dispersed, and then they were placed in a 37°C water bath with gentle shaking. Note that decapitation, dissection, and slicing were carried out to this point separately for each animal to minimize the time between hippocampal dissection and slice preparation. After the slices from the last animal had incubated for 30 min, the slices were pooled in a conical-bottomed centrifuge tube and allowed to settle by gravity. The supernatant was aspirated off, and warmed oxygenated KRB was added to wash the slices. This was repeated two to three times. Finally, the excess KRB was removed from the gravity-packed slices. Slices were distributed to capped tubes containing 1 μCi *myo*-[^{3}H]inositol and test compounds (diluted as a 50× stock in 100% DMSO and then added to assay for a final DMSO concentration of 2%), concluding each addition by oxygenating and then capping the tubes. Slices in the tubes were incubated at 37°C for 2 h. Then LiCl (to a final concentration of 10 mM) and KRB or test compound (i.e., DFB) was added to each tube, each of which was gassed and capped. The tubes were incubated at 37°C for 15 min, and then agonist was added to the tubes, which were again gassed and capped. After 20 min, chloroform/methanol (1:2) was added to each tube, and the tubes were vortexed for 1 min and then stood at room temperature for 15 min. HCl and chloroform were added to each tube and mixed by vortexing. Tubes were centrifuged to separate the phases, and an aliquot of the aqueous (upper) phase was applied to a Dowex 1 × 8 formate column. The column was washed with 5 mM inositol and then 5 mM sodium borate and 60 mM ammonium formate. The sample was eluted with 200 mM ammonium formate and 100 mM formic acid into scintillation vials. Scintillation cocktail (ReadySafe; Beckman Coulter, Fullerton, CA) was added to each vial, and radioactivity was determined by scintillation counting.

Radioligand Binding Assays

The orthosteric agonist binding site radioligand [^{3}H]quisqualate and the allosteric inhibitor MPEP analog radioligand [^{3}H]methoxy-PEPy (Cosford et al., 2003) were used to evaluate the interaction of DFB, DMeOB, and DCB with mGluR5. Membranes were prepared from CHO mGluR5 cells or rat cerebral cortex. Aliquots of these membranes were added to tubes containing test compound (serially diluted in 100% DMSO, then diluted in assay buffer to a 4× stock at 1% DMSO; stock was then added to assay for a final DMSO concentration of 0.25%) or vehicle and either [^{3}H]quisqualate (25 nM final concentration in 20 mM HEPES, 2 mM $CaCl_2$ and $MgCl_2$, pH 7.2) or [^{3}H]methoxy-PEPy (1 or 2 nM final concentration in 50 mM Tris/0.9% NaCl, pH 7.4). The tubes were incubated for 60 min at room temperature with shaking, and the membrane-bound ligand was separated from the free ligand by filtration onto glass-fiber filters presoaked with 20 mM HEPES, 2 mM $CaCl_2$ and $MgCl_2$, pH 7.2, for the [^{3}H]quisqualate or with 0.2% polyethyleneimine for the [^{3}H]methoxy-PEPy. Filters were punched out into vials, Aquassure (PerkinElmer Life Sciences, Boston, MA) was added, and membrane-bound radioactivity was determined by scintillation counting of the filters. Nondisplaceable binding was estimated using 1 mM glutamate for the [^{3}H]quisqualate assay and 1 μM MPEP for the [^{3}H]methoxy-PEPy assay.

Results

FLIPR Assays. In $FLIPR_{384}$ assays, mGluR5 CHO cells exhibited concentration-dependent increases in Fluo-4 fluorescence in response to quisqualate, glutamate, and 3,5-dihydroxy-phenylglycine (DHPG) (Fig. 2). These compounds seemed to act as full agonists, with potencies consistent with EC_{50} values reported previously. Compounds were screened in this assay 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. DFB was identified as active in this assay (Table 1).

DFB caused a concentration-dependent potentiation of the response of human mGluR5 CHO cells to 300 nM glutamate in this assay. The maximal potentiation at this concentration of glutamate was approximately 3.1-fold, with an EC_{50} value for potentiation of 2.6 ± 0.4 μM (n = 6, Fig. 3). As shown in Table 2, DFB caused similar potentiation of the responses of both human mGluR5 and rat mGluR5 CHO cells to glutamate, quisqualate, and DHPG with comparable potencies. DFB also potentiated the glutamate response of human embryonic kidney 293 cells expressing human mGluR5 (data not shown). DFB alone (100 μM) caused no response on either human or rat mGluR5 CHO (or human embryonic kidney 293) cells in this assay, indicating that the compound did not act as an agonist.

Reversibility of DFB was determined by comparing the FLIPR response of mGluR5 CHO cells to 300 nM glutamate in the presence of a range of concentrations of DFB, with and without a washout step. In this series of experiments, the concentration-response curve of DFB was shifted >15-fold to the right after washing (without washout, DFB EC_{50} = 5.8 ± 2.0 μM (n = 3); with washout, DFB EC_{50} > 100 μM), indicating that DFB was reversible.

In other functional studies, DFB did not potentiate the activity of mGluR1, -2, -3, -4, -7, or -8. Although some antagonism (>30%) of mGluR4 and -8 occurred at 100 μM DFB, none was observed at 30 μM. Thus, in FLIPR assays, the effects of DFB (100 μM) as a percentage of the control response were the following: mGluR1b, 80 ± 10% at 50 μM glutamate (~EC_{20} concentration); human mGluR4, 57 ± 17% and 71 ± 12% at 2 μM and 30 μM glutamate, respectively (~EC_{10} and ~EC_{70} concentrations, respectively); human mGluR7, 75 ± 8% at 1 mM glutamate; and rat mGluR8, 25 ±

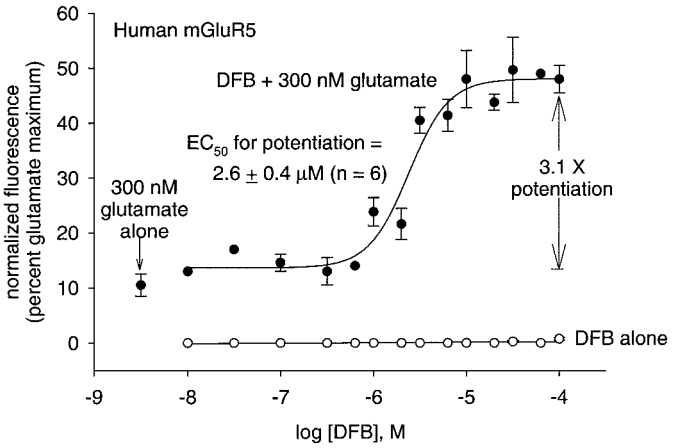


Fig. 3. DFB potentiates mGluR5 activation by glutamate. mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. A range of concentrations of DFB were added to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

1% and 59 ± 15% at 400 nM and 10 μM glutamate, respectively (~EC_{10} and ~EC_{70} concentrations, respectively). DFB was also tested for any modulatory effect on the glutamate-stimulated increases in guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding to membranes prepared from human mGluR2 and human mGluR3-expressing cells. The effects of DFB as a percentage of control response were the following: mGluR2, 100% and 81 ± 7% at 1 and 100 μM glutamate, respectively (~EC_5 and ~EC_{90} concentrations, respectively); and mGluR3, 100% at both 100 and 500 nM glutamate, respectively (~EC_5 and ~EC_{90} concentrations, respectively). DFB (100 μM) also had no effect on responses to agonists of endogenous $G_{\alpha q}$-coupled receptors (e.g., 200 nM ATP, 200 nM UTP, and 5 nM thrombin at purinergic or thrombin receptors) in the parental cell line (data not shown).

Increasing concentrations of DFB caused a parallel, leftward shift of mGluR5 CHO cell glutamate concentration-response curves with no increase in maximal response (Fig. 4). The maximum change in EC_{50} was 2.2-fold. Similar shifts in agonist concentration-response curves were observed for quisqualate and DHPG in both human and rat mGluR5 CHO cells, again with no increase in the maximal response to the agonists (Table 3).

Phosphatidyl Inositol Hydrolysis. To assess the effect of DFB in other functional assays, phosphatidyl inositol hydrolysis assays were performed in mGluR5 CHO cells and in rat brain slices. In the presence of LiCl, human mGluR5 CHO cells exhibited a concentration-dependent, quisqualate-stimulated accumulation of inositol phosphates with an EC_{50} of 49.1 ± 5.1 nM (n = 3) and an E_{max} approximately 4.5-fold over basal activity. DFB (10 μM) had no effect by itself on basal activity in this assay, but it shifted the concentration-response curve to quisqualate to the left (EC_{50} = 27.1 ± 2.6 nM, n = 3, with no increase to maximal response) (Fig. 5), which is consistent with the FLIPR fluorescence data. Results obtained for DHPG were similar to those for the agonist (DHPG, EC_{50} = 10.3 ± 3.1 μM, n = 3; DHPG + 10 μM DFB, EC_{50} = 4.4 ± 1.8 μM, n = 3). In addition, similar results were obtained with rat mGluR5 CHO cells with these two agonists (quisqualate, EC_{50} = 86.1 ± 14.8 nM, n = 3; quisqualate + 10 μM DFB, EC_{50} = 28.3 ± 8.8 nM, n = 3; DHPG, EC_{50} = 7.9 ± 1.8 μM, n = 4; DHPG + 10 μM DFB, EC_{50} = 3.0 ± 0.4 μM, n = 3).

In hippocampal slices from young (postnatal day 9) rats, quisqualate also stimulated phosphatidyl inositol (PI) hydrolysis in a concentration-dependent manner (EC_{50} = 0.92 ± 0.14 μM, n = 3) (Fig. 6). This quisqualate-stimulated PI hydrolysis was inhibited approximately 80% by MPEP (10 μM), an mGluR5-selective negative allosteric modulator, suggesting that quisqualate was activating mGluR5 (data not shown). DFB (100 μM) shifted the quisqualate concentration-response curve to the left in this assay (EC_{50} = 0.42 ± 0.10 μM, n = 3) (Fig. 6), which is consistent with the data obtained in the PI hydrolysis assays with the cloned receptors expressed in CHO cells.

Structure-Activity Relationships. In exploration of the structure-activity relationships of DFB, we found that minor alterations in the structure afforded a variety of pharmacological profiles (Table 1). Changing the position of the fluoro groups from 3,3′ to 2,2′ decreased the potency of the potentiation of the FLIPR Ca^{2+} response caused by the compound by approximately 5-fold (EC_{50} = 14 ± 12 μM) without affecting the maximal potentiation at this concentration of glutamate (Fig. 7). Replacement of the 3,3′ fluoro groups on DFB with 3,3′ methoxy groups (DMeOB) changed the pharmacology from potentiation of the glutamate response to negative

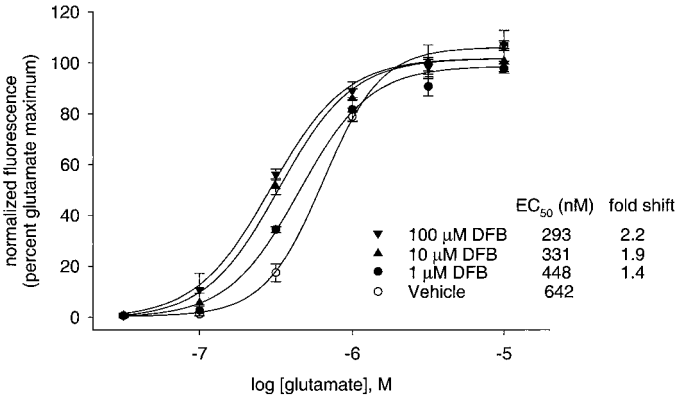


Fig. 4. DFB potentiation of response to glutamate is manifested as increased mGluR5 agonist sensitivity. mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$.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

TABLE 2

DFB potentiation of human and rat mGluR5 activation by glutamate, DHPG, and quisqualate

Maximum potentiation is the ratio of the maximum to the minimum response determined by nonlinear curve fitting of mean data (see Fig. 3). Data are expressed as mean ± S.E.M. (*n*)

[Agonist]	EC_{50} for Potentiation	Maximum Potentiation
	μM	*-fold*
Human mGluR5a CHO cells		
300 nM Glutamate	2.6 ± 0.4 (6)	3.1
1 μM DHPG	2.0 ± 0.5 (6)	3.4
8 nM Quisqualate	2.3 ± 1.2 (6)	3.7
Rat mGluR5 CHO cells		
300 nM Glutamate	5.3 ± 1.4 (5)	4.1
1 μM DHPG	3.6 ± 1.4 (5)	6.2
8 nM Quisqualate	3.6 ± 1.1 (4)	3.7

TABLE 3

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

EC_{50} change at 100 μM DFB was calculated from the EC_{50} values determined by nonlinear curve fitting of the mean data (see Fig. 4) Data are expressed as mean ± S.E.M. (*n*)

Agonist	Control Agonist EC_{50}	EC_{50} Change at 100 μM DFB
		-fold
Human mGluR5a CHO cells		
Glutamate	642 ± 30 nM (5)	2.2
DHPG	2.36 ± 0.17 μM (5)	2.3
Quisqualate	21.4 ± 0.7 nM (5)	2.2
Rat mGluR5 CHO cells		
Glutamate	659 ± 48 nM (5)	1.9
DHPG	2.07 ± 0.12 μM (5)	2.0
Quisqualate	22.0 ± 0.9 nM (5)	1.7

modulation with an IC_{50} of 3.0 ± 0.4 μM) (Fig. 8). These mGluR5 CHO cells exhibited no constitutive activity in FLIPR assays (mGluR5 receptor density was 240 fmol/mg), and it was therefore not possible to distinguish between antagonist-like or inverse agonist activity for DMeOB. The inhibitory effects of this compound are noncompetitive-like, because increasing concentrations of compound not only shift the concentration-response curves of glutamate and quisqualate to the right, but they also reduce the maximal response to agonist similar to the behavior reported for MPEP (Fig. 9). Reversibility of DMeOB was determined by comparing the FLIPR response of mGluR5 CHO cells to 600 nM glutamate in the presence of a range of concentrations of DMeOB, with and without a washout step. The concentration-response curve of DMeOB under these conditions was shifted >15-fold to the right after washing, indicating that DMeOB was reversible (data not shown).

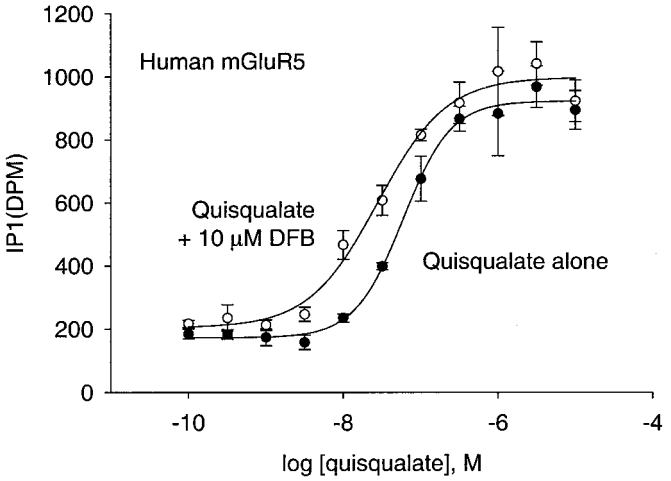


Fig. 5. DFB potentiates quisqualate-stimulated PI hydrolysis in human mGluR5 CHO cells. Phosphatidyl inositol hydrolysis was measured in human mGluR5 CHO cells as outlined under *Materials and Methods*. DFB (10 μM) had no effect by itself on basal activity. A representative experiment is shown. Error bars represent S.D. Similar results were obtained with rat mGluR5 CHO cells with these two agonists.

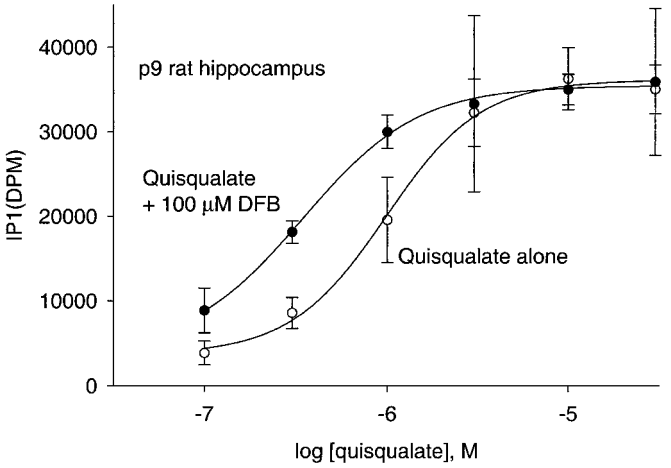


Fig. 6. DFB potentiates quisqualate-stimulated PI hydrolysis in rat postnatal day 9 (p9) hippocampal slices. Phosphatidyl inositol hydrolysis was measured in rat postnatal day 9 hippocampal slices as described under *Materials and Methods*. A representative experiment is shown. Error bars represent S.D.

Substitution of the 3,3′-fluoro groups of DFB with 3,3′-chloro groups resulted in a compound (DCB) that acted to prevent the allosteric modulatory effects of other compounds in this structural class. For example, although DCB had no effect on the response of mGluR5 to glutamate alone, it reduced the ability of DFB to potentiate the FLIPR Ca^{2+} response of mGluR5 to 300 nM glutamate (Fig. 10A). In Fig. 10A, the upper and lower curves correspond to the responses of 600 and 300 nM glutamate, respectively, in the presence of increasing concentrations of DCB. At concentrations as high as 100 μM, DCB has very little effect on the FLIPR Ca^{2+}

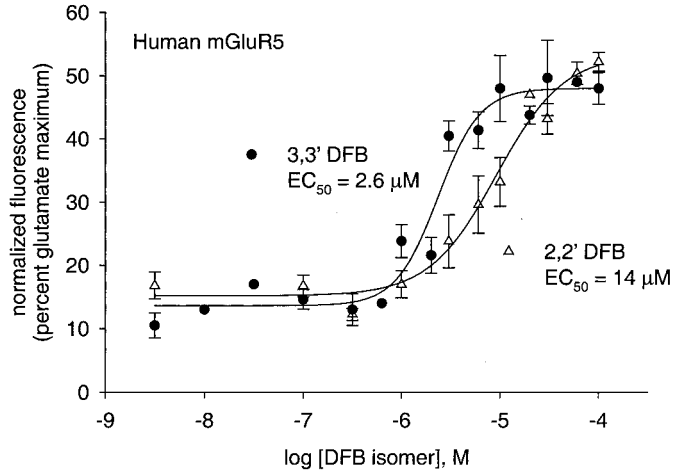


Fig. 7. Positional isomers of DFB exhibit different potencies of potentiation. Human mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. A range of concentrations of 2,2′-DFB and 3,3′-DFB were added to the cells after 10 s of baseline determination. Five minutes later, a fixed concentration (~EC_{10} concentration) of glutamate was added, and the Ca^{2+} response was measured with the use of $FLIPR_{384}$. Concentration-response curves were generated from the mean data of five experiments. Error bars represent S.E.M.

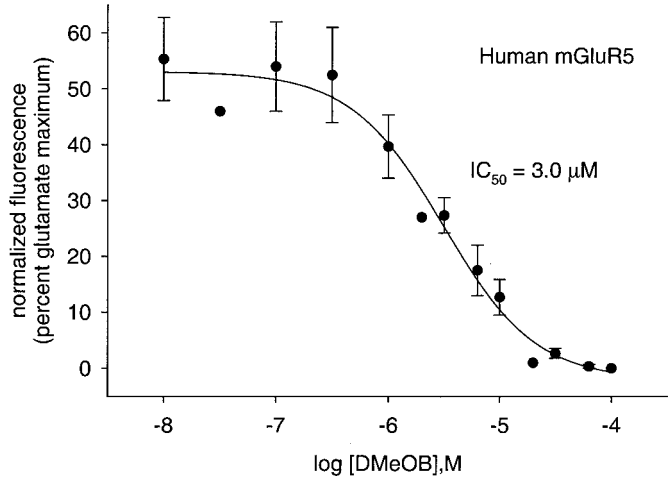


Fig. 8. DMeOB causes the inhibition of glutamate response. Human mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. A range of concentrations of DMeOB was added to the cells after 10 s of baseline determination. Five minutes later, a fixed concentration (~EC_{10} concentration) of glutamate was added, and the Ca^{2+} response was measured with the use of $FLIPR_{384}$. Concentration-response curves were generated from the mean data of three experiments. Error bars represent S.E.M.

response of mGluR5 to glutamate alone. In the presence of 6 μM DFB, the response of mGluR5 to 300 nM glutamate is potentiated in this system to approximate the response to 600 nM glutamate alone. The presence of DCB in this system attenuates the potentiation conferred by DFB in a concentration-dependent manner (IC_{50} = 7.6 ± 1.9 μM).

In a similar manner, DCB relieved the inhibitory effect of 8 μM DMeOB on the FLIPR Ca^{2+} response of mGluR5 to 600 nM glutamate (IC_{50} = 17 ± 16 μM, Fig. 10B) and blocked approximately 50% of the inhibition of this system by 30 nM MPEP (IC_{50} = 3.0 ± 0.9 μM, data not shown). Increasing fixed concentrations of DCB shifted the MPEP inhibition curves to the right in a parallel manner (Fig. 11).

Reversibility of DCB was determined by comparing the FLIPR response of mGluR5 CHO cells to 300 nM glutamate in the presence of 6 μM DFB or to 600 nM glutamate in the presence of 8 μM DMeOB in the presence of a range of concentrations of DCB, with and without a washout step. The DFB or DMeOB were added after the washout step. The concentration-response curve of DCB was shifted 3- to 4-fold to the right after washing, indicating that DCB was reversible (data not shown).

As was the case with DFB, none of the analogs reported here interacted with human mGluR1b, -2, -3, or -7 or with endogenous purinergic or thrombin receptors present in the parental cell lines (data not shown). However, DMeOB was found to act as an inhibitor of mGluR4 (IC_{50} of approximately 35 μM) and mGluR8 (IC_{50} of approximately 50 μM) activation. Also similar to findings with DFB, all analogs had the same pharmacological profile on rat mGluR5a (data not shown).

Several other close structural analogs exhibited activity as negative modulators (4,4′-difluorobenzaldazine and 4,4′-dimethoxybenzaldazine) or in preventing allosteric modulation by other analogous compounds (3,3′-dihydroxybenzaldazine) in ways similar to those of DMeOB and DCB, respectively. However, the potency of these compounds in the FLIPR assay was weak (>50–100 μM), and they were not characterized further (Table 1).

Neither DFB, DMeOB, nor DCB (each at 100 μM) had any effect on the binding of [^{3}H]quisqualate (25 nM, K_d = 110 nM)) to membranes prepared from CHO mGluR5 cells (Fig. 12), suggesting that this series of analogs did not bind at the orthosteric agonist binding site. Nondisplaceable [^{3}H]quisqualate binding was estimated in the presence of 1 mM

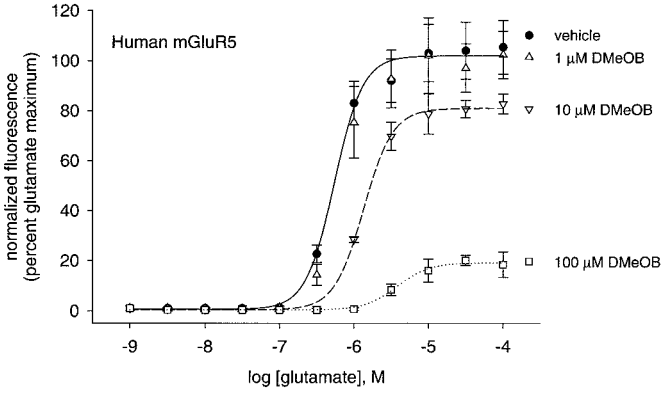


Fig. 9. DMeOB seems to be a noncompetitive antagonist. mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. Several fixed concentrations of DMeOB were added to cells (human mGluR5 CHO cells) after 10 s of baseline determination. Five minutes later, a range of concentrations of agonist (glutamate) was added. The concentration-response curves are shifted to the right with increasing concentrations of DMeOB (EC_{50} for glutamate = 0.55 μM for vehicle control, 0.64 μM with 1 μM DMeOB, 1.36 μM with 10 μM DMeOB, and 3.84 μM with 100 μM DMeOB), and the maximum response to glutamate is reduced at the highest concentrations of DMeOB. Concentration-response curves were generated from the mean data of three experiments. Error bars represent S.E.M.

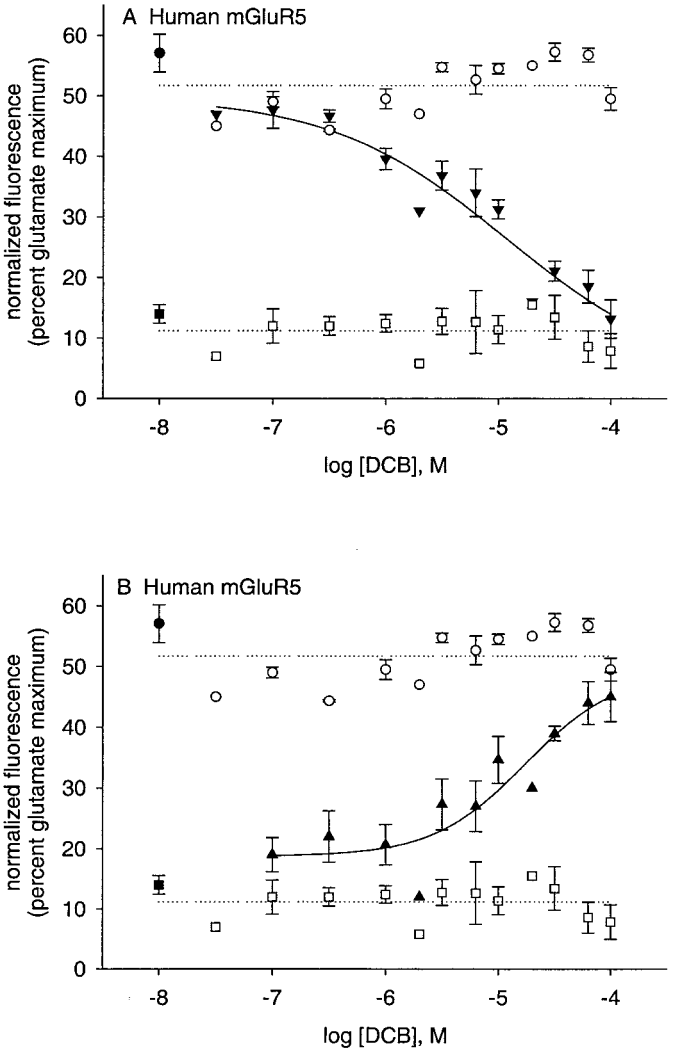


Fig. 10. DCB reduces allosteric modulation by DFB and DMeOB. mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. A, a fixed concentration of DFB (6 μM) and a range of concentrations of DCB were added to the cells after 10 s of baseline determination. Five minutes later, a fixed concentration (300 nM) of glutamate was added, and the Ca^{2+} response was measured by $FLIPR_{384}$. Squares represent the response to 300 nM glutamate (■, glutamate alone; □, glutamate in the presence of increasing concentrations of DCB). Circles represent the response to 600 nM glutamate (●, glutamate alone; ○, glutamate in the presence of increasing concentrations of DCB). ▼, the response of 300 nM glutamate potentiated by the presence of 6 μM DFB. B, a fixed concentration of DMeOB (8 μM) and a range of concentrations of DCB were added to the cells after 10 s of baseline determination. Five minutes later, a fixed concentration (600 nM) of glutamate was added, and the Ca^{2+} response was measured by $FLIPR_{384}$. The symbols represent the same responses described in A, except that ▲ represents the response of 600 nM glutamate inhibited by the presence of 8 μM DMeOB. Concentration-response curves were generated from the mean data of four experiments. Error bars represent S.E.M.

glutamate. All three benzaldazine analogs inhibited binding of the MPEP analog [^{3}H]methoxy-PEPy (2 nM) (Cosford et al., 2003) to these membranes (Fig. 13). DFB partially inhibited [^{3}H]methoxy-PEPy binding [51% inhibition, IC_{50} = 8.5 ± 4.4 μM (n = 3)], whereas DCB and DMeOB inhibited the binding more fully (75% and 92% inhibition, IC_{50} = 5.8 ± 2.6 μM (n = 3) and 13.8 ± 0.6 μM (n = 3), respectively). Saturation-binding studies using [^{3}H]methoxy-PEPy suggest that the presence of these analogs did not affect B_{max} but did increase K_d (Fig. 13B) (control, B_{max} = 143 fmol/mg protein, K_d = 4.7 ± 0.4 nM; in the presence of 15 μM DFB, B_{max} = 139 fmol/mg protein, K_d = 9.9 ± 1.1 nM; in the presence of 6 μM DMeOB, B_{max} = 141 fmol/mg protein, K_d = 11.5 ± 2.0 nM). Under these conditions, MPEP was found to inhibit [^{3}H]methoxy-PEPy binding fully. MPEP (1 μM) was used to estimate nondisplaceable binding (9% of total binding). Similar results were obtained using rat cortical membranes in place of the human mGluR5-expressing CHO cell membranes.

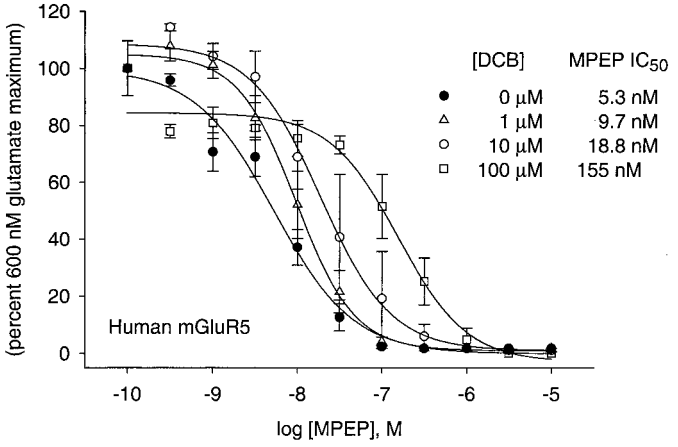


Fig. 11. DCB reduces allosteric modulation by MPEP. mGluR5 CHO cells were plated in clear-bottomed 384-well plates in glutamate/glutamine-free medium, loaded the next day with the calcium-sensitive fluorescent dye Fluo-4, and placed in $FLIPR_{384}$. Several fixed concentrations of DFB and a range of concentrations of MPEP were added to the cells after 10 s of baseline determination. Five minutes later, 600 nM glutamate was added, and the Ca^{2+} response was measured by $FLIPR_{384}$. Concentration-response curves were generated from the mean data of three experiments. Data are plotted as a percentage of the maximum response to 600 nM glutamate. Error bars represent S.E.M.

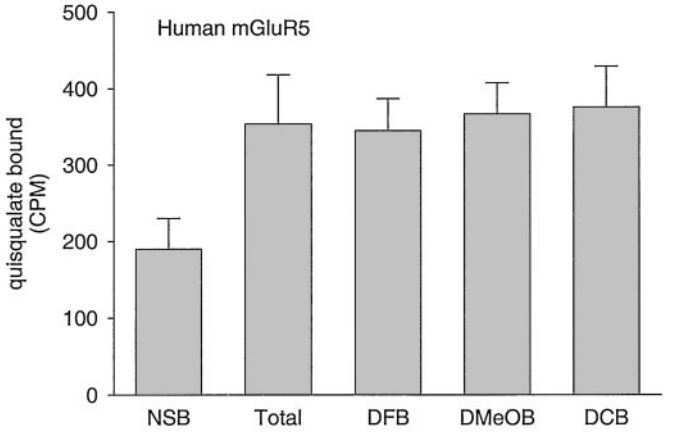


Fig. 12. Benzaldazine analogs have no effect on [^{3}H]quisqualate binding to human mGluR5 CHO cell membranes. The assay conditions were the following: membranes prepared from human mGluR5 CHO cells were incubated with [^{3}H]quisqualate (25 nM final concentration in 20 mM HEPES, 2 mM $CaCl_2$ and $MgCl_2$, pH 7.2) in the presence of 100 μM compound or vehicle for 60 min at room temperature. Samples were filtered onto glass fiber filters. Nondisplaceable binding (NSB) was estimated with the use of 1 mM glutamate. A representative experiment is shown. Error bars represent S.D.

Discussion

Potent and selective pharmacological tools are important in the characterization of GPCR function, and in the case of mGluRs, potent orthosteric agonists and antagonists have been discovered (Conn and Pin, 1997; Schoepp et al., 1999). With the advent of high-throughput functional assays, it has been possible to expand the search for pharmacological tools to include compounds that act on receptors at allosteric sites rather than at the historically targeted orthosteric sites. For the mGluRs, MPEP and CPCCOEt were the first compounds that were clearly shown to be negative allosteric modulators (selective for mGluR5 and mGluR1, respectively). Recently, positive allosteric modulators selective for mGluR1b also have been identified (Knoflach et al., 2001). In a continuation of this approach of using functional assays to discover allosteric modulators, DFB was identified as a novel positive allosteric modulator (i.e., potentiator) selective for mGluR5.

DFB was found to potentiate the activation of human and rat mGluR5 receptors by glutamate, quisqualate, and DHPG, although it was not itself an agonist. The potentiation seems to be specific: it was observed for mGluR5 activation but not for the activation of mGluR1, -2, -3, -4, -7, or -8 or for endogenous purinergic or thrombin receptors. Potentiation of mGluR5 by DFB was observed in several functional assays in cloned cells as well as tissue slices, and it is therefore unlikely to be an artifact of the expression system.

Studies to determine the structure-activity relationships of DFB indicated that slight structural changes had profound effects on the pharmacological function of the resulting compounds without altering selectivity for receptor subtype or

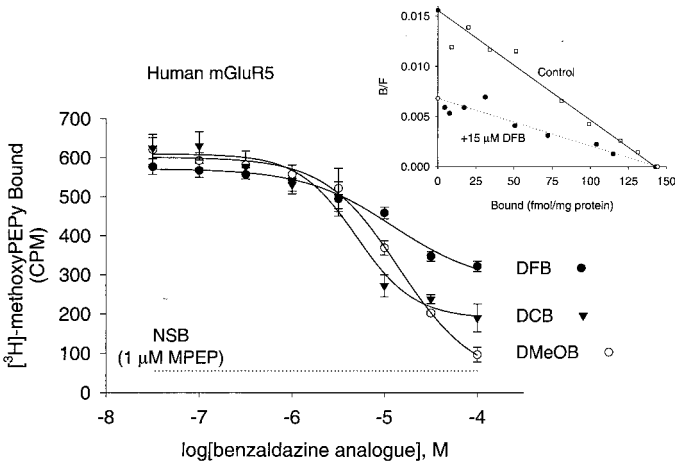


Fig. 13. Benzaldazine analogs seem to interact with the MPEP binding site. The assay conditions were the following: membranes prepared from human mGluR5 CHO cells were incubated with the radiolabeled MPEP analog [^{3}H]3-methoxy-5-(2-pyridinylethynyl)pyridine (1 or 2 nM final concentration in 50 mM Tris and 0.9% NaCl, pH 7.4), for 60 min at room temperature in the presence of varying concentrations of benzaldazine analogs. Samples were then filtered onto glass fiber filters. Nondisplaceable binding (NSB) was estimated with 1 μM MPEP. A representative competition experiment is shown. Error bars show S.D. Inset, a representative saturation experiment is shown in the presence and absence of 15 μM DFB.

species. Replacement of the fluoro groups with methoxy groups to give DMeOB changed the activity from potentiation by the original compound to antagonism by the resulting compound. On the other hand, substitution of chloro groups for the original fluoro groups to give DCB resulted in a compound that is neither an agonist, an inhibitor, nor an allosteric potentiator. This otherwise pharmacologically "silent" compound was found to interact with mGluR5 to prevent the pharmacological actions of DFB and DMeOB. Thus, the compounds in this structural series exhibit a range of pharmacological activities including positive allosteric modulation, negative allosteric modulation, and neutral modulation (i.e., preventing positive or negative modulation). We have not found a compound in this series that exhibits agonist-like effects.

The very close structural homology between these compounds and the ability of DCB to block completely both potentiation by DFB as well as antagonism by DMeOB is consistent with these compounds binding to the same site on mGluR5. Binding studies are consistent with the interaction of these analogs with the MPEP binding site. They inhibited the binding of the MPEP analog [^{3}H]methoxy-PEPy with IC_{50} values in a potency range similar to that observed for their functional effects. Saturation binding studies with [^{3}H]methoxy-PEPy were consistent with competitive inhibition, because B_{max} was not changed but K_d was increased. Although inhibition of [^{3}H]methoxy-PEPy binding by some of these compounds was not complete, this may have been caused by poor solubility in the longer binding assays, because data in other assays indicated a full effect of these compounds at 100 μM. Solubility may also play a role in the slight reduction in the maximal response to glutamate in the presence of 100 μM DCB (Fig. 11). Most of these data are consistent with MPEP and the benzaldazine analogs binding to the same site, although the lack of complete inhibition of [^{3}H]methoxy-PEPy binding is not consistent with this conclusion. Identification of the amino-acid interactions that are important for the various pharmacologies of these compounds will help clarify this and is currently being pursued with the construction of chimeric and mutated mGluR5 receptors. The lack of species selectivity between human and rat suggests that the binding site for these compounds is conserved between these species and further indicates that they may be suitable for studying the function of mGluR5 in both primates as well as rodents.

We have compared the data obtained for these allosteric modulators with published models of allosterism. One of the simplest models (Ehlert, 1988) describes the action of positive allosteric modulators through an increase in the intrinsic efficacy of agonists and/or an increase in affinity of the agonist for its binding site. The potency and solubility of these compounds did not permit us to make full use of the pharmacological null methods described in this article. However, the lack of effect of these compounds on [^{3}H]quisqualate binding suggests that they do not affect the affinity of agonists. Furthermore, 4-carboxy,3-hydroxyphenylglycine, which has no apparent mGluR5 agonist effects on its own and antagonizes mGluR5 responses to glutamate, is revealed as a partial agonist in the presence of 100 μM DFB (data not shown). These results suggest that the benzaldazine analogs may act by affecting the intrinsic efficacy of agonists.

Recently, Parmentier et al. (2002) proposed a model of family 3 GPCR function that considers the possibility of variable coupling of the equilibrium between the open and closed conformations of the agonist binding region and the equilibrium between the active and inactive conformations of the effector region. In this model, allosteric modulators could affect the coupling between these equilibria and/or the active/inactive conformational equilibrium of the effector region. Our results indicate that DFB did not increase the basal activity of the receptor (it did not exhibit apparent agonist activity), which is not consistent with its action applied through altering the equilibrium between active and inactive conformations of the effector region. Parmentier et al. (2002) also indicated that the maximum effect and affinity of agonists would increase in the presence of a positive allosteric modulator. As noted above, we did not observe any effect of DFB on [^{3}H]quisqualate binding, nor did we observe an increase in the maximal effect of glutamate, DHPG, or quisqualate in the presence of DFB. At present, we do not have an explanation for this discrepancy between our results with DFB and the results predicted for a positive allosteric modulator by this model. The alternative mode of action proposed by this model, involving alteration of the tightness of coupling between the agonist binding and effector regions of the receptor, may be consistent with the alteration in agonist intrinsic efficacy suggested by the Ehlert model.

Although receptors by definition exert their actions through allosteric mechanisms, it is only recently that interest in allosteric modulation of GPCR action by small molecules has progressed to active searches for pharmacological tools acting through these mechanisms. The series of closely related analogs described in this article was identified from an active search for such compounds, and they are members of a new class of pharmacological tools that includes the first reported example of an mGluR5-selective positive allosteric modulator, as well as an mGluR5-selective negative allosteric modulator, and a pharmacologically "silent" allosteric ligand with neutral cooperativity. The wide range in pharmacological profiles induced by subtle structural changes was unexpected, and therefore this set of tools will probably prove useful in studying the function of mGluR5. We believe that these compounds represent a rare example of a series of allosteric modulators of GPCRs that range in pharmacological activity from positive to negative modulation as well as neutral prevention of this modulation.

Acknowledgments

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