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Antagonist Pharmacology of Metabotropic Glutamate Receptors Coupled to Phospholipase D Activation in Adult Rat Hippocampus: Focus on (2*R*,1′*S*,2′*R*,3′*S*)-2-(2′-Carboxy-3′-phenylcyclopropyl)glycine Versus 3,5-Dihydroxyphenylglycine

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ABSTRACT

Metabotropic glutamate (mGlu) receptors coupled to phospholipase D (PLD) appear to be distinct from any known mGlu receptor subtype linked to phospholipase C or adenylyl cyclase. The availability of antagonists is necessary for understanding the role of these receptors in the central nervous system, but selective ligands have not yet been identified. In a previous report, we observed that 3,5-dihydroxyphenylglycine (3,5-DHPG) inhibits the PLD response induced by (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate in adult rat hippocampal slices. We now show that the antagonist action of 3,5-DHPG (IC_{50} = 70 μM) was noncompetitive in nature and nonselective, because the drug was also able to reduce PLD activation elicited by 100 μM norepinephrine and 1 mM histamine. In the search for a selective and more potent antagonist, we examined the effects of sixteen stereoisomers of 2-(2′-carboxy-3′-phenylcyclopropyl)glycine (PCCG) on the PLD-specific transphosphatidylation reaction resulting in the formation of [^{3}H]phosphatidylethanol. The (2*R*,1′*S*,2′*R*,3′*S*)-PCCG stereoisomer (PCCG-13) antagonized the formation of [^{3}H]phosphatidylethanol induced by 100 μM (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate in a dose-dependent manner and with a much lower IC_{50} value (25 nM) compared with 3,5-DHPG. In addition, increasing concentrations of PCCG-13 were able to shift to the right the agonist dose-response curve but had no effect when tested on other receptors coupled to PLD. The potent, selective, and competitive antagonist PCCG-13 may represent an important tool for elucidating the role of PLD-coupled mGlu receptors in adult hippocampus.

Glutamate receptors of the ionotropic (iGlu) and metabotropic (mGlu) types are known to mediate the excitatory and potentially neurotoxic effects of glutamate in the central nervous system. iGlu receptors are ligand-gated ion channels, whereas mGlu receptors are coupled to a variety of effector systems through GTP-binding proteins (Conn et al., 1994). Based on sequence homology, agonist pharmacology, and coupling to second-messenger systems, cloned mGlu receptors have been subdivided into three groups (for a review, see Conn and Pin, 1997). Group I receptors (mGlu 1 and mGlu 5, and their splice variants) are coupled to activation of PLC in a number of heterologous expression systems, whereas group II (mGlu 2 and mGlu 3) and group III (mGlu 4, mGlu 6, mGlu 7, and mGlu 8) receptors are both negatively coupled to adenylyl cyclase. It has been demonstrated that the nonselective mGlu receptor agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate [(1*S*,3*R*)-ACPD] is also able to stimulate PLD activity in neonate and adult hippocampal slices (Boss and Conn, 1992; Holler et al., 1993). Recent studies suggest that the glutamatergic activation of phospholipase D (PLD) in immature tissue is indirectly promoted by group I mGlu receptors via protein kinase C (PKC) activation (Klein et al., 1997, 1998), whereas in adult hippocampus, the pharmacology of PLD-coupled mGlu receptors does not appear to correspond to the profile of known subtypes coupled to

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ABBREVIATIONS: (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate; 3,5-DHPG, 3,5-dihydroxyphenylglycine; iGlu, ionotropic glutamate; IP, inositol phosphate; L-CCG-I, L(2*S*,1′*S*,2′*S*)-(carboxycyclopropyl)glycine; (+)-MCPG, (+)-α-methyl-4-carboxyphenylglycine; mGlu, metabotropic glutamate; PCCG, 2-(2′-carboxy-3′-phenylcyclopropyl)glycine; PEt, phosphatidylethanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

PLC or adenylyl cyclase (Boss et al., 1994; Pellegrini-Giampietro et al., 1996a).

PLD is the key enzyme in a signal transduction pathway that hydrolyzes phosphatidylcholine and leads to the formation of the second messengers phosphatidic acid and diacylglycerol (for reviews, see Klein et al., 1995; Morris et al., 1996; Exton, 1997). A number of neurotransmitter receptors, including muscarinic, α_1-adrenergic, histamine H_1, and mGlu receptors, are known to be coupled to both PLC and PLD: their agonists may therefore induce the formation of diacylglycerol by directly stimulating either phosphoinositide or phosphatidylcholine hydrolysis. Because phorbol esters activate PLD, it has also been suggested that PLD activation may be secondary to phosphoinositide hydrolysis via diacylglycerol formation and PKC activation. Compared with PLC-produced diacylglycerol, the formation of diacylglycerol through direct stimulation of the PLD pathway is expected to be 1) slower in time course, 2) more abundant because the substrate phosphatidylcholine is more abundant than phosphatidylinositol in membranes, and 3) long lasting because PKC activated by diacylglycerol desensitizes mGlu receptors coupled to PLC (Catania et al., 1991) but further stimulates PLD. The mutual interaction between PKC and PLD has been described as a "positive feedback loop" by Löffelholz (1989), and it has been proposed as an important mechanism for the generation of increasing amounts of second messengers and prolonged activation of PKC in response to receptor stimulation (Nishizuka, 1998).

To understand the role of mGlu receptors linked to PLD activation in the central nervous system, the development of selective antagonists is crucial. In a recent report (Pellegrini-Giampietro et al., 1996a), we showed that (+)-MCPG (a competitive antagonist of group I and II mGlu receptors) displays mixed agonist/antagonist effects on PLD-coupled mGlu receptors in adult rat hippocampal slices, whereas 3,5-dihydroxyphenylglycine (3,5-DHPG), which is known to be a selective agonist of group I mGlu receptors coupled to PLC activation, was able to antagonize the stimulatory effects of (1*S*,3*R*)-ACPD when tested on PLD activity. We now examine in further detail the pharmacological profile of 3,5-DHPG on PLD-coupled mGlu receptors.

In addition, we focused our attention on (carboxycyclopropyl)glycines, a valuable source of potent and selective ligands for numerous members of the glutamate receptor family, including mGlu receptors. Considering that the introduction of a hydrophobic moiety, such as a phenyl ring, in position 3′ of 2-(2′-carboxycyclopropyl)glycine could be useful for mapping the presence of unexplored areas in the recognition site of members of the glutamate receptor family, a complete stereolibrary of 16 2-(2′-carboxy-3′-phenylcyclopropyl)glycines (PCCGs) was recently synthesized and tested for activity on known mGlu receptors (Pellicciari et al., 1996). In the present study, we investigated whether any of the PCCG isomers lacking activity on known mGlu receptors could selectively antagonize the activation of PLD induced by mGlu receptor agonists. We report on the identification of the (2*R*,1′*S*,2′*R*,3′*S*)-PCCG stereoisomer (PCCG-13) as a selective, potent and competitive antagonist of PLD-coupled mGlu receptors in the adult rat hippocampus.

Experimental Procedures

Materials. PCCG-13 was synthesized as described previously (Pellicciari et al., 1996). In a previous preliminary report (Pellegrini-Giampietro et al., 1996b), the active compound was erroneously indicated as PCCG-16, which differs from PCCG-13 in the stereochemistry at carbon 2. (1*S*,3*R*)-ACPD, 3,5-DHPG, and quisqualate were purchased from Tocris Cookson (Bristol, UK). Norepinephrine, histamine, and A23187 were from Sigma Chimica (Milan, Italy). The phosphatidylethanol (PEt) standard was from Avanti Polar Lipids (Pelham, AL). [1,2,3-^{3}H]Glycerol (30–60 Ci/mmol) and *myo*-[2-^{3}H(N)]inositol (10–25 Ci/mmol) were purchased from Du Pont/NEN (Milan, Italy). Dowex AG-1-X 8 anion exchange resin (100–200 mesh) was from Sigma Chimica, and precoated silica gel 60A (LK6D) plates were from Whatman.

Tissue Preparation. Adult (180–200 g) Wistar rats (Nossan strain; Milan) were used. After decapitation, brains were rapidly removed, and the hippocampi dissected and placed into chilled Krebs-bicarbonate buffer (122 mM NaCl, 3.1 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.3 mM $CaCl_2$, and 10 mM glucose) gassed with 95% O_2/5% CO_2. Hippocampal slices (350 μm thick) were prepared using a McIlwain tissue chopper and then placed in gassed Krebs-bicarbonate solution for 1 h at 37°C before use. For some experiments, thoracic aortas were removed after decapitation, and aorta rings were prepared as described by Jones et al. (1993).

Determination of Agonist-Induced PLD Activity. PLD activity was determined as described previously (Pellegrini-Giampietro et al., 1996a) by making use of the transphosphatidylation reaction between phosphatidylcholine and primary alcohols specifically catalyzed by PLD. Thus, in the presence of exogenously added ethanol, PLD preferentially transfers the alcohol rather than water to the phosphatidyl moiety of phosphatidylcholine, producing PEt in place of phosphatidic acid. Briefly, membrane phospholipids were labeled by incubating hippocampal slices or aorta rings with [^{3}H]glycerol (final concentration, 60 μCi/ml) for 2 h at 37°C. Slices or rings were then rinsed and transferred to test tubes (two slices or four rings per test tube) containing 500 μl of drug-containing buffer gently stirred at 37°C by bubbling 95% O_2/5% CO_2. Antagonists were applied for 10 min before adding the agonists together with 170 mM ethanol, and the reaction was then carried out for 1 additional hour. Previous experiments in hippocampal slices had shown that a steady-state level of PEt formation was reached within 30 min and was stable for at least 2 h. All experiments were run in triplicate; two control sets of triplicate samples were always included in which 1) only Krebs-bicarbonate buffer (background) and 2) only buffer plus 170 mM ethanol (basal PLD activity) were present.

The reaction was stopped by adding 2 ml of ice-cold chloroform/methanol/HCl (100:200:2). The phases were then separated by adding 0.65 ml of chloroform and 0.65 ml of water and, after sonication (30 min), by low-speed centrifugation. Aliquots (1 ml) of the lipid phase were dried under a stream of N_2, resuspended in 70 ml of chloroform, and spotted onto precoated silica gel 60A plates together with aliquots of a PEt standard solution. [^{3}H]PEt was separated from major phospholipids by thin-layer chromatography using the upper phase of the solvent system ethyl acetate/2,2,4-trimethyl pentane/acetic acid/water (12:5:1:10). Spots were visualized with iodine vapor, and [^{3}H]PEt was identified by comparison with the PEt standard. The region corresponding to [^{3}H]PEt was scraped off, and radioactivity was counted by liquid scintillation spectrometry. The formation of [^{3}H]PEt for each individual sample was expressed as the percentage of radioactivity incorporated into the total lipids present in the organic phase. Because PEt was formed only in the presence of ethanol, the amount of label comigrating with [^{3}H]PEt in ethanol-free controls was considered as background and subtracted from the mean of each ethanol-containing triplet. Radioactivity present in ethanol-free (background) samples never exceeded 10% of the radioactivity present in ethanol-containing samples. After sub-

tracting the background, basal [^{3}H]PEt formation, expressed as [^{3}H]PEt/[^{3}H]total lipids $\times$ 10^4, was consistently about 12.0 $\pm$ 0.8. Data are expressed as percentages of incorporation of label into [^{3}H]PEt occurring under agonist-free (basal) conditions.

Determination of Agonist-Induced PLC Activity. Agonist-induced phosphoinositide hydrolysis was assayed essentially as described previously (Pellegrini-Giampietro et al., 1996a). Briefly, hippocampal slices incubated for 2 h at 37°C with [^{3}H]inositol (final concentration, 20 μCi/ml) were rinsed and then transferred to test tubes (two slices each) with 500 μl of drug-containing buffer gently stirred at 37°C in the presence of 10 mM LiCl by bubbling 95% O_2/5% CO_2. Antagonists were applied for 10 min before adding the agonists, which were then allowed to react for an additional 15 min. All experiments were run in triplicate. The reaction was stopped by adding 1.88 ml of ice-cold chloroform/methanol (1:2). The phases were separated by adding 0.65 ml of chloroform and 0.65 ml of water and, after brief sonication, by low-speed centrifugation. The upper phase, containing the water-soluble [^{3}H]inositol phosphates (IPs) (inositol monophosphate, inositol-1,4-bisphosphate, and inositol-1,4,5-trisphosphate), was transferred to Dowex AG 1-X 8 (formate form, 100–200 mesh) anion exchange resin columns. After washing with water and eluting [^{3}H]glycerophosphorylinositol with 16 ml of 5 mM sodium tetraborate, 60 mM ammonium formate, the three [^{3}H]IPs were eluted together with 16 ml of 1 M ammonium formate, 0.1 M formic acid. The dpm of the corresponding fractions was determined by liquid scintillation spectrometry, and the radioactivity present in [^{3}H]IPs was normalized to radioactivity incorporated into [^{3}H]glycerophosphorylinositol. Data are expressed as percentages of incorporation of label into [^{3}H]IPs occurring under agonist-free (basal) conditions.

Data and Statistical Analysis. Agonist dose-response and antagonist inhibition curves were analyzed by nonlinear regression, and EC_{50} and IC_{50} values were calculated with the Prism software package (GraphPAD Software, San Diego, CA). The PCCG-13 dissociation constant (K_B) was estimated from the results of the (1*S*,3*R*)-ACPD dose-response curve and the PCCG-13 inhibition curve in the presence of a fixed concentration (A_f) of (1*S*,3*R*)-ACPD, applying the "null" method and the equation $K_B = IC_{50}'/([A_f]/EC_{50}' - 1)$, where EC_{50}' and IC_{50}' are equiactive concentrations derived from agonist and antagonist dose-response curves, respectively, constrained to have the same maximum (Lazareno and Birdsall, 1993). The slope and the pA_2 value of the Schild plot of PCCG-13 were calculated by linear regression analysis with the Prism software package. Statistical significance of differences between results was evaluated by performing ANOVA followed by Tukey's *w* test for multiple comparisons.

Results

3,5-DHPG Is a Noncompetitive and Nonselective Antagonist of PLD-Coupled mGlu Receptors. In a previous study, we showed that the selective group I mGlu receptor agonist 3,5-DHPG fails to activate PLD in adult rat hippocampal slices but rather reduces the stimulation of PLD induced by (1*S*,3*R*)-ACPD (Pellegrini-Giampietro et al., 1996a). To better define the pharmacological behavior of 3,5-DHPG on PLD-coupled mGlu receptors, we first compared the agonist effects of (1*S*,3*R*)-ACPD, quisqualate, and 3,5-DHPG on PLC and PLD activities. Consistent with previous findings (Schoepp and Johnson, 1988; Schoepp et al., 1994), quisqualate, (1*S*,3*R*)-ACPD, and 3,5-DHPG induced a dose-dependent accumulation of [^{3}H]IPs in adult rat hippocampal slices (Fig. 1A), with EC_{50} values of 15 $\pm$ 3, 50 $\pm$ 2, and 65 $\pm$ 2 μM, respectively. The maximal PLC response elicited by 3,5-DHPG was approximately four times the basal value but only about half the maximal response induced by quisqualate and (1*S*,3*R*)-ACPD. The latter two compounds acted as agonists also when tested on PLD activity (Fig. 1B). As previously reported (Pellegrini-Giampietro et al., 1996a), the maximal PLD response induced by quisqualate and (1*S*,3*R*)-ACPD was approximately twice the basal value, but quisqualate displayed a much lower EC_{50} value (80 $\pm$ 3 nM) than (1*S*,3*R*)-ACPD (30 $\pm$ 2 μM). On the contrary, 3,5-DHPG was unable to stimulate the formation of [^{3}H]PEt in adult rat hippocampal slices up to the concentration of 300 μM (Fig. 1B).

Preincubation of adult hippocampal slices with 3,5-DHPG dose-dependently inhibited the formation of [^{3}H]PEt induced by 100 μM (1*S*,3*R*)-ACPD (Fig. 2A). The antagonism provided by 3,5-DHPG was complete at 1 mM, and the IC_{50}

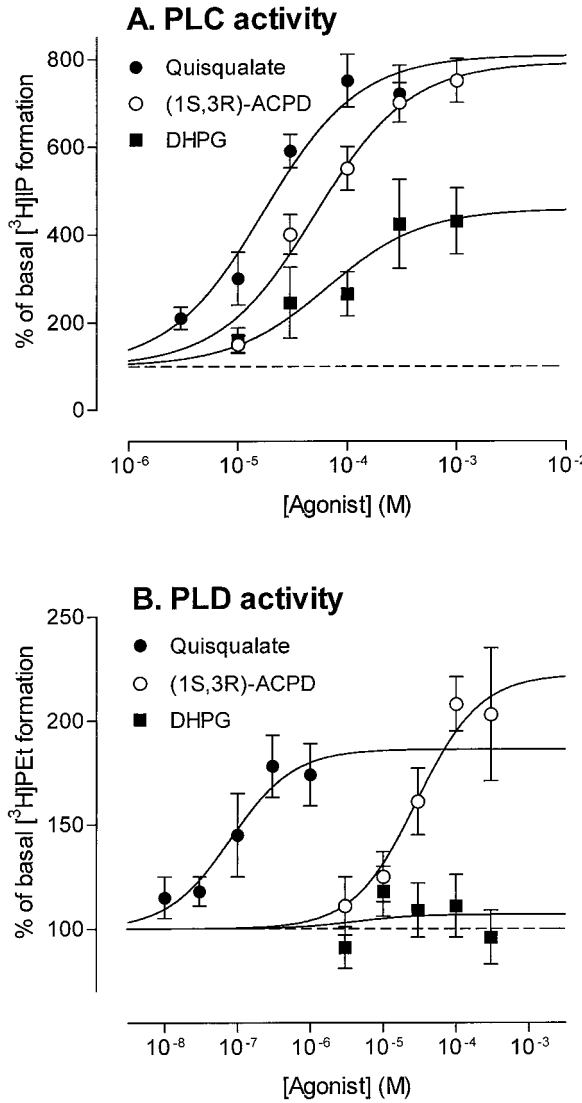


Fig. 1. 3,5-DHPG is a partial agonist of mGlu receptors coupled to phosphoinositide hydrolysis but has no agonist activity on mGlu receptors coupled to PLD in adult rat hippocampus. A, slices were labeled with [^{3}H]inositol, washed, and incubated for 15 min at 37°C in the presence of increasing concentrations of quisqualate, (1*S*,3*R*)-ACPD, or 3,5-DHPG. B, slices were labeled with [^{3}H]glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of increasing concentrations of quisqualate, (1*S*,3*R*)-ACPD, or 3,5-DHPG. PLC (A) and PLD (B) activity are expressed as percentage of basal (agonist-free) incorporation of label into [^{3}H]IPs or [^{3}H]PEt, respectively. Results represent the mean $\pm$ S.E.M. of at least four experiments run in triplicate.

value for this effect was 70 ± 3 μM. When the (1*S*,3*R*)-ACPD-evoked hydrolysis of phosphoinositides was measured, preincubation with 3,5-DHPG did not result in any additive or antagonist effect (Fig. 2A). Because receptor-mediated activation of PLD may be subject to desensitization (for a review, see Klein et al., 1995), hippocampal slices were also incubated with 100 μM 3,5-DHPG for different periods of time (1, 5, 10, 15, 30, and 60 min). The resulting formation of [^{3}H]PEt, however, was always negligible (data not shown), suggesting that the antagonist effect observed after the preincubation of adult hippocampal slices with 3,5-DHPG is not mediated by PLD-coupled mGlu receptors that desensitize after a rapid and transient response. In addition, 100 μM 3,5-DHPG did not modify the kinetics of 100 μM (1*S*,3*R*)-ACPD-evoked [^{3}H]PEt formation in adult hippocampal slices (Fig. 3)

To determine the mode of action of 3,5-DHPG at PLD-coupled mGlu receptors, dose-response curves for PLD activation by (1*S*,3*R*)-ACPD were performed in the absence and presence of 3,5-DHPG (Fig. 2B). 3,5-DHPG (100 and 300 μM) induced a clear nonsurmountable shift of the agonist dose-response curve, indicating that the antagonism at PLD-coupled mGlu receptors was noncompetitive. When tested on other receptor types that are known to be linked to PLD activation, 100 μM 3,5-DHPG significantly reduced the formation of [^{3}H]PEt induced by 100 μM norepinephrine or 1 mM histamine (Fig. 4A). However, there was no antagonist effect on the formation of [^{3}H]PEt when PLD was stimulated by the calcium ionophore A23187 or the direct G protein activator aluminum fluoride (10 μM $AlCl_3$ plus 10 mM NaF; see Holler et al., 1994) (Fig. 4A), suggesting that the block of agonist-induced [^{3}H]PEt formation observed with 3,5-DHPG occurs at the receptor level and not downhill along the PLD pathway. Because 3,5-DHPG stimulates phosphoinositide hydrolysis per se acting at group I mGlu receptors, the stimulatory effects of 3,5-DHPG and norepinephrine were additive when assayed together in hippocampal slices (Fig. 4B). On the contrary, when tested in a tissue not expressing group I mGlu receptors, such as the rat aorta, 3,5-DHPG significantly inhibited the formation of [^{3}H]PEt evoked by norepinephrine (Fig. 4C), further substantiating a possible interaction at the PLD-coupled norepinephrine receptor level for these two drugs.

PCCG-13 Is a Selective, Potent, and Competitive Antagonist of PLD-Coupled mGlu Receptors. In the search for a selective antagonist of PLD-coupled mGlu receptors, we tested a number of isomers of a recently characterized stereolibrary of PCCGs (Pellicciari et al., 1996) for their effects on the PLD-specific transphosphatidylation reaction stimulated by (1*S*,3*R*)-ACPD. Among the PCCGs that were reported to be inactive on a number of known iGlu or mGlu receptors, the PCCG-13 stereoisomer (Fig. 5) appeared to be the most in-

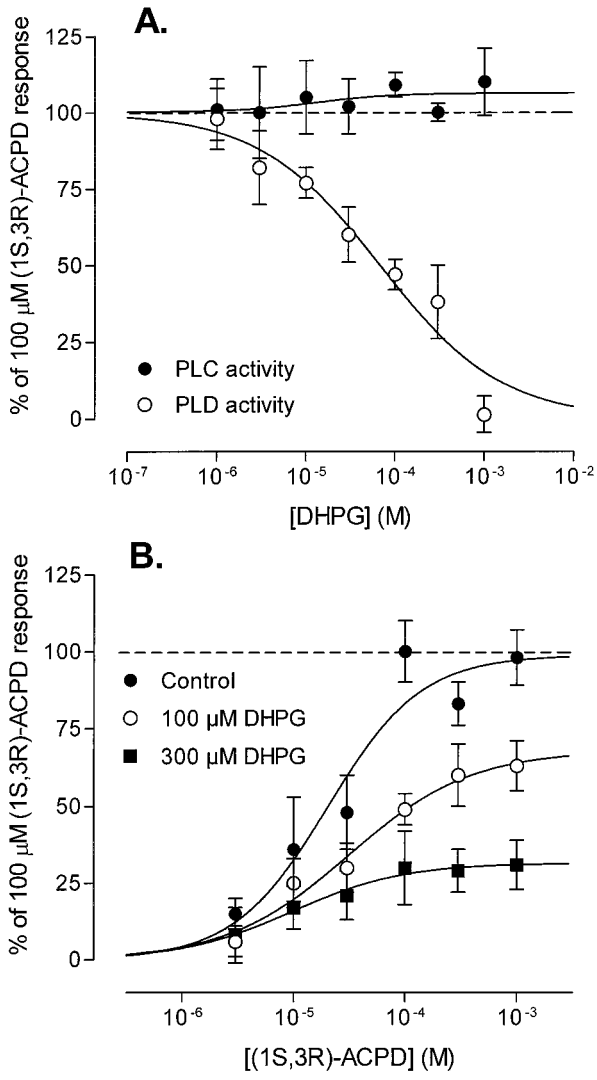


Fig. 2. 3,5-DHPG is a noncompetitive antagonist of mGlu receptors coupled to PLD in adult rat hippocampus. Slices were labeled and incubated as indicated in Fig. 1 in the presence of 100 μM (A) or increasing concentrations (B) of (1*S*,3*R*)-ACPD. 3,5-DHPG, at the indicated concentrations, was applied 10 min before (1*S*,3*R*)-ACPD. Results were normalized to a reference response of (1*S*,3*R*)-ACPD (100 μM) and are the mean ± S.E.M. of at least six experiments run in triplicate.

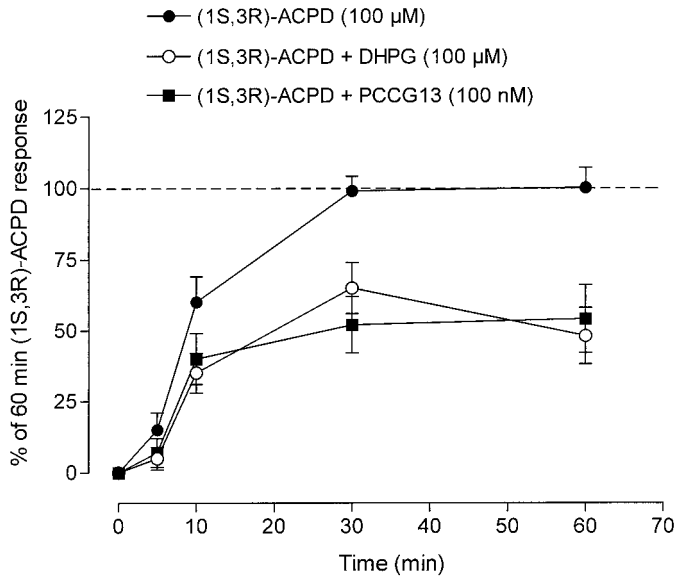


Fig. 3. The temporal pattern of (1*S*,3*R*)-ACPD-evoked [^{3}H]PEt formation in adult hippocampus is not altered by the antagonists 3,5-DHPG and PCCG-13. Slices were labeled and incubated as indicated in Fig. 1B in the presence of 100 μM (1*S*,3*R*)-ACPD for various periods of time. 3,5-DHPG (100 μM) and PCCG-13 (100 nM) were applied 10 min before (1*S*,3*R*)-ACPD. Results were normalized to a reference response of 100 μM (1*S*,3*R*)-ACPD (60 min) and are the mean ± S.E.M. of at least four experiments run in triplicate.

teresting compound. In adult rat hippocampal slices, preincubation with increasing doses of PCCG-13 inhibited the formation of [^{3}H]PEt induced by 100 μM (1*S*,3*R*)-ACPD in a dose-dependent manner, whereas the drug had no effect on the agonist-evoked stimulation of PLC-coupled mGlu receptors (Fig. 6A). When tested alone, PCCG-13 did not stimulate PLC activity (data not shown). The IC_{50} value for the antagonist effect of PCCG-13 on PLD-coupled mGlu receptors was 25 $\pm$ 2 nM, and the K_B value estimated from agonist and inhibition curves with the "null method" (see *Experimental Procedures*) was 11.0 $\pm$ 1.2 nM. PCCG-13, however, was unable to completely inhibit the effect of 100 μM (1*S*,3*R*)-ACPD: the maximal degree of antagonism, observed at 1 μM PCCG-13, was approximately 80% (Fig. 6A). Like 3,5-DHPG, PCCG-13 (100 nM) failed to alter the temporal pattern of (1*S*,3*R*)-ACPD-induced PLD activation in adult hippocampus slices (Fig. 3).

We then performed dose-response curves of PLD-coupled mGluR agonists in the absence and presence of PCCG-13 (0.1, 0.3, and 1 μM). The PCCG derivative clearly produced a parallel rightward shift in the dose-response curve obtained by stimulating the formation of [^{3}H]PEt with quisqualate (Fig. 6B). The inhibition of quisqualate-induced PLD activation was surmounted by increasing the concentration of the agonist. The Schild plot analysis of PCCG-13 revealed a pA_2 value of 7.05 (r = 0.991) with a slope of 0.89 $\pm$ 0.12, suggesting a competitive fashion of antagonism for this compound. When (1*S*,3*R*)-ACPD, at 100 or 300 μM, was used as an

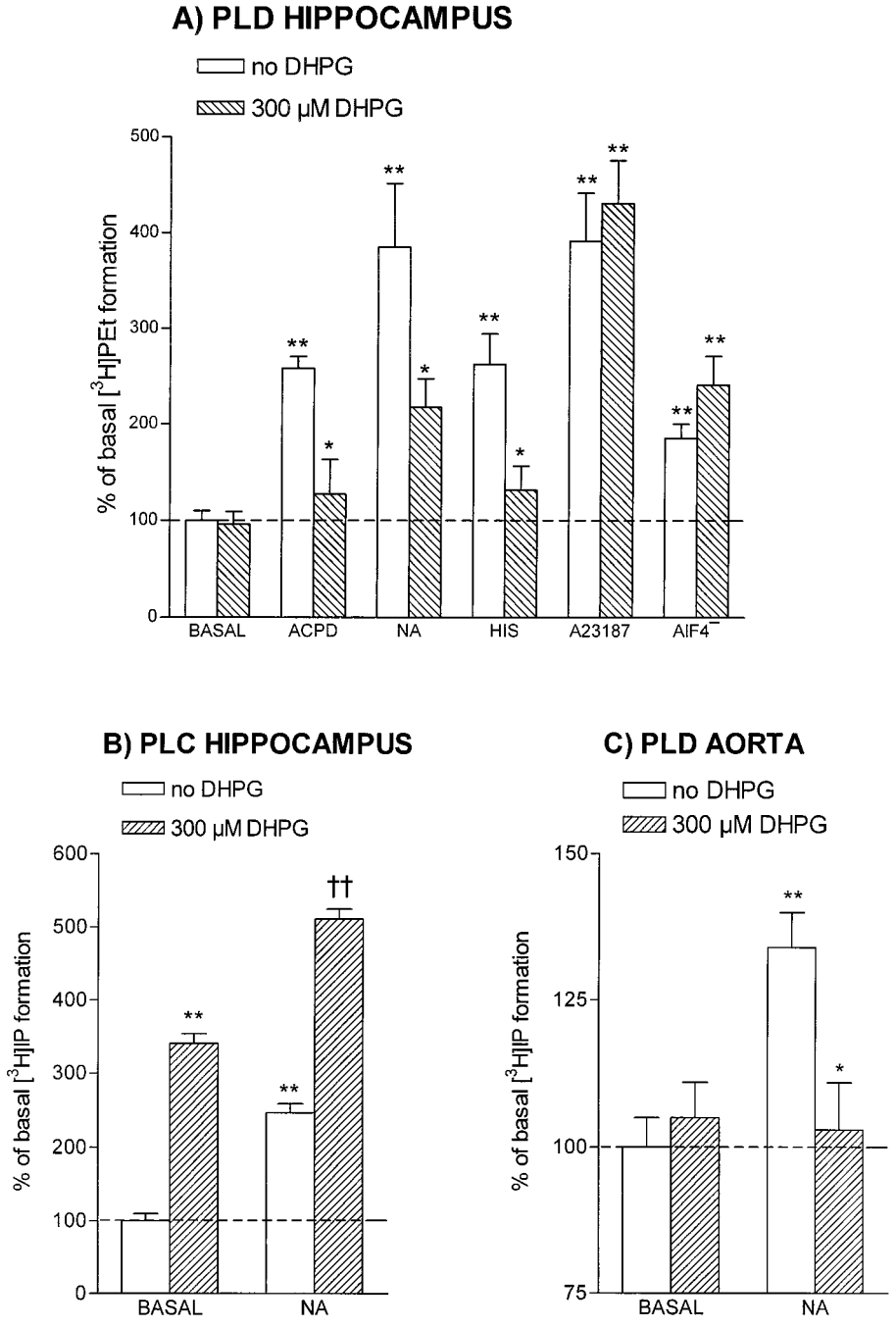
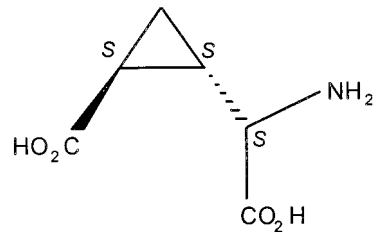


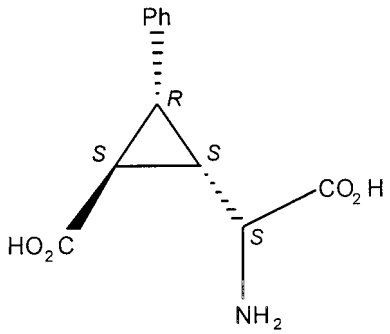
Fig. 4. 3,5-DHPG is a nonselective antagonist among PLD-coupled mGlu receptors in adult rat hippocampus and aorta. A, hippocampal slices were labeled and incubated as indicated in Fig. 1B in the presence of 100 μM (1*S*,3*R*)-ACPD, 100 μM norepinephrine (NA), 1 mM histamine (HIS), 100 μM A23187, or aluminum fluoride (AlF_4^-; 10 μM $AlCl_3$ plus 10 mM NaF). B, hippocampal slices were labeled and incubated as indicated in Fig. 1A in the presence of 100 μM norepinephrine. C, aorta rings were labeled with [^{3}H]glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of 100 μM norepinephrine. 3,5-DHPG (300 μM) was applied in all cases 10 min before the agonists. PLD and PLC activities are expressed as indicated in Fig. 1. Each column represents the mean $\pm$ S.E.M. of at least four experiments run in triplicate. $^{**}p < .01$ versus basal; $^{*}p < .05$ versus agonist alone; $^{\dagger\dagger}p < .01$ versus norepinephrine alone.

agonist, PCCG-13 was able to significantly reduce the PLD responses (see, for example, Fig. 6A). However, PCCG-13 did not inhibit the formation of [^{3}H]PEt evoked by lower (3–10 μM) concentrations of (1*S*,3*R*)-ACPD (data not illustrated).

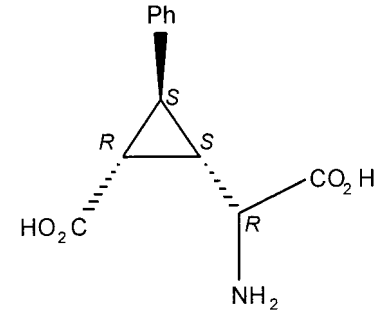
PCCG-13 (100 nM) was also able to antagonize the weak agonist effect displayed by (+)-MCPG on PLD-coupled mGlu receptors in adult hippocampus (see Pellegrini-Giampietro et al., 1996a), but when tested on other receptors coupled to PLD it failed to antagonize the formation of [^{3}H]PEt evoked by 100 μM norepinephrine or 1 mM histamine (Fig. 7). Moreover, (2*R*,1′*R*,2′*S*,3′*R*)-PCCG (PCCG-14), (2*S*,1′*R*,2′*S*,3′*R*)-PCCG (PCCG-15), and (2*S*,1′*S*,2′*R*,3′*S*)-PCCG (PCCG-16), the other three isomers derived by diastereoselective Strecker synthesis from the same racemic aldehyde precursor as PCCG-13 (Pellicciari et al., 1996), displayed no antagonist activity when tested (at 100 nM and 1 μM) on (1*S*,3*R*)-ACPD-evoked PLD activation in hippocampal slices (Fig. 8). When tested alone, PCCG-13 did not stimulate the formation of [^{3}H]PEt, whereas PCCG-15 was able to evoke a significant and dose-dependent PLD response.



L-CCG-I



PCCG-4



PCCG-13

Fig. 5. Chemical structures of L-(2*S*,1′*S*,2′*S*)-(carboxycyclopropyl)glycine (L-CCG-1), PCCG-4, and PCCG-13. PCCG-4 and PCCG-13 are member of a stereolibrary of 16 isomers (Pellicciari et al., 1996).

Discussion

Our results show that PCCG-13 is a selective, potent, and competitive antagonist of PLD-coupled mGlu receptors in adult rat hippocampus. PCCG-13 is one of the 16 members of a stereolibrary of PCCGs that was recently synthesized and tested for functional activity on iGlu and mGlu receptors, as

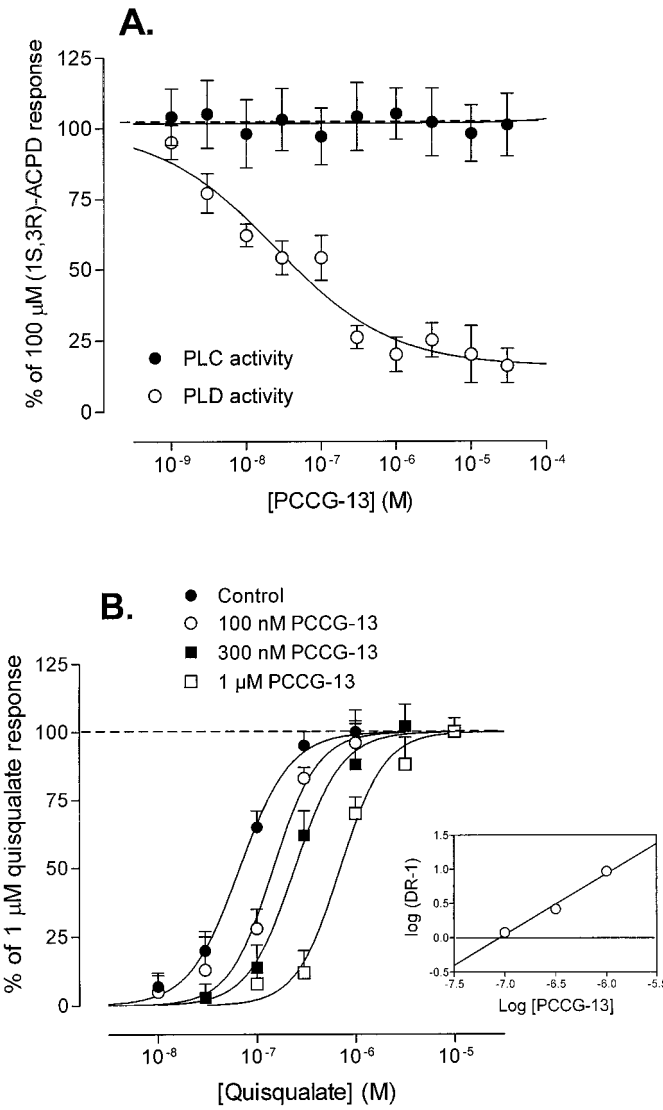


Fig. 6. PCCG-13 is a potent and competitive antagonist of mGlu receptors coupled to PLD in adult rat hippocampus. Slices were labeled and incubated as indicated in Fig. 1 in the presence of 100 μM (1*S*,3*R*)-ACPD (A) or increasing concentrations of quisqualate (B). PCCG-13, at the indicated concentrations, was applied 10 min before the agonists. Results were normalized to a reference response of (1*S*,3*R*)-ACPD (100 μM, A) or quisqualate (1 μM, B) and are the mean ± S.E.M. of at least six experiments run in triplicate. The inset in B shows the Schild plot obtained from the EC_{50} values calculated from B.

well as for glutamate uptake inhibition (Pellicciari et al., 1996). A number of PCCG stereoisomers are thus known to be active on known mGlu receptors, including (2*R*,1′*R*,2′*R*,3′*S*)-PCCG (PCCG-2), which is an mGlu 2 receptor agonist; (2*R*,1′*S*,2′*R*,3′*R*)-PCCG (PCCG-6), an mGlu 1 receptor antagonist; and (2*S*,1′*S*,2′*S*,3′*R*)-PCCG (PCCG-4, Fig. 4), which was recently demonstrated to be a potent and relatively selective antagonist of group II mGlu receptors (Thomsen et al., 1996; Cozzi et al., 1997). PCCG-13 has no effect on the hydrolysis of phosphoinositides or the formation of cAMP in cells expressing mGlu 1a, mGlu 2, or mGlu 4 receptors (Pellicciari et al., 1996) or in cells expressing mGlu 5a receptors (unpublished observations). Although PCCG-13 has not been tested directly on mGlu 3, mGlu 6, mGlu 7, and mGlu 8 receptors, which are all negatively coupled to adenylyl cyclase, our results in hippocampal slices confirm that PCCG-13 is inactive on mGlu receptors of the I group coupled to PLC and PKC activation. Moreover, PCCG-13 displays no inhibition of 1) the binding of selective ligands for *N*-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors to rat brain membranes (Pellicciari et al., 1996); 2) sodium-dependent and calcium/chloride-dependent glutamate uptake into rat cortical synaptosomes (Pellicciari et al., 1996); and 3) the PLD responses induced by norepinephrine or histamine (current report). Finally, our data with the closely related isomers PCCG-14, PCCG-15, and PCCG16 indicate that the inhibitory effect of PCCG-13 on PLD-coupled mGlu receptors is stereoselective.

PCCG-13 was able to selectively inhibit the PLD response induced by (1*S*,3*R*)-ACPD, quisqualate, and (+)-MCPG. The formation of [^{3}H]PEt induced by a quasimaximal concentration of (1*S*,3*R*)-ACPD was antagonized with remarkable potency (IC_{50} = 25 nM). However, PCCG-13 could not completely abolish the agonist-evoked stimulation of PLD, suggesting that under our experimental conditions, (1*S*,3*R*)-ACPD may stimulate the formation of [^{3}H]PEt by activating two different pathways: a PCCG-13-sensitive pathway responsible for 80% of the (1*S*,3*R*)-ACPD effect, and a PCCG-13-insensitive pathway responsible for 20% of the effect. Because PCCG-13 antagonized only the PLD responses evoked by relatively high (100–300 μM) concentrations of (1*S*,3*R*)-ACPD, we were unable to observe a reliable shift of the (1*S*,3*R*)-ACPD dose-response curve with PCCG-13. This peculiar antagonist behavior should be borne in mind when using PCCG-13 together with (1*S*,3*R*)-ACPD in functional studies investigating the role of PLD-coupled mGlu receptors in the central nervous system. When quisqualate was used as an agonist, PCCG-13 produced a parallel shift to the right in the dose-response curve. The inhibitory effects of 100 nM, 300 nM, and 1 μM PCCG-13 could be clearly surmounted by appropriately increasing the concentration of quisqualate up to 10 μM. Linear regression analysis of the corresponding Schild plot indicated that the the antagonism was competitive in nature. The different antagonist profile observed with the two agonists may be explained by the different potencies displayed by quisqualate (EC_{50} = 80 nM) and (1*S*,3*R*)-ACPD (EC_{50} = 30 μM) on PLD-coupled mGlu receptors or by the fact that quisqualate and (1*S*,3*R*)-ACPD, unlike PCCG-13, may also modify PLD activity through diverse and distinct mechanisms, including stimulation of AMPA receptors, opening of voltage-gated Ca^{2+} channels after depolarization, or inhibition of adenylyl cyclase via mGlu receptors of the II and III groups. The alternative possibility that (1*S*,3*R*)-ACPD and quisqualate may act at different sites or at different receptors cannot be ruled out at the present time and deserves further investigation.

3,5-DHPG stimulates phosphoinositide hydrolysis in functional systems expressing mGlu 1 and mGlu 5 receptors (Ito

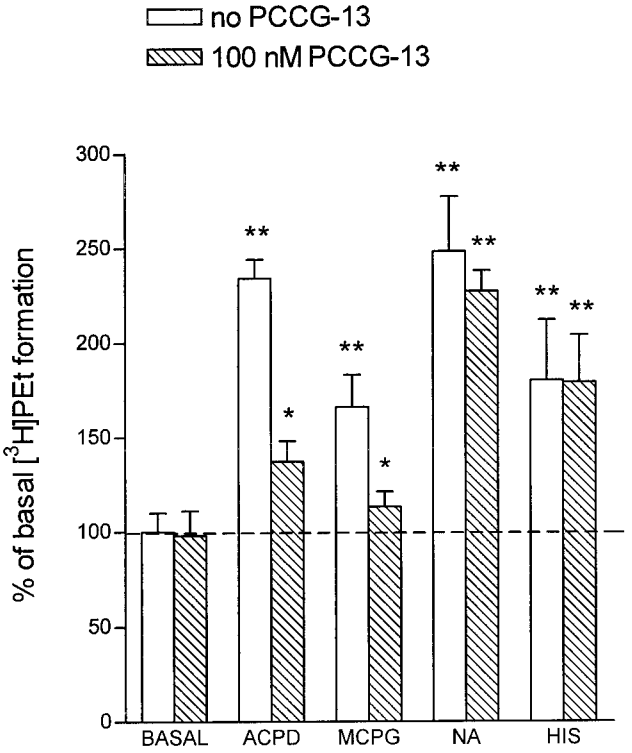


Fig. 7. PCCG-13 is a selective antagonist among mGlu receptors coupled to PLC in adult rat hippocampus. Slices were labeled and incubated as indicated in Fig. 1B in the presence of 100 μM (1*S*,3*R*)-ACPD, 1 mM (+)-MCPG, 100 μM norepinephrine (NA), or 1 mM histamine (HIS). PCCG-13 (100 nM) was applied 10 min before the agonists. PLD activity is expressed as indicated in Fig. 1. Each column represents the mean ± S.E.M. of at least four experiments run in triplicate. $^{**}p < .01$ versus basal; $^{*}p < .05$ versus (1*S*,3*R*)-ACPD alone.

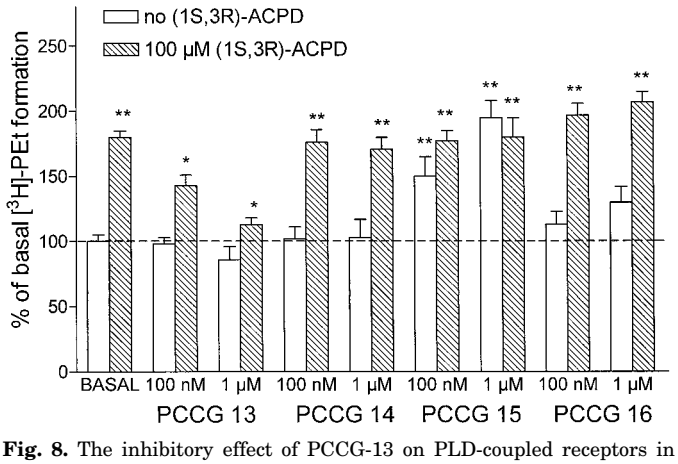


Fig. 8. The inhibitory effect of PCCG-13 on PLD-coupled receptors in adult rat hippocampus is stereoselective. Slices were labeled and incubated as indicated in Fig. 1B in the presence of PCCG-13, PCCG-14, PCCG-15, or PCCG-16 (at 100 nM and 1 μM). When PCCG derivatives were tested together with 100 μM (1*S*,3*R*)-ACPD, they were applied 10 min before the agonist. PLD activity is expressed as indicated in Fig. 1. Each column represents the mean ± S.E.M. of at least four experiments run in triplicate. $^{**}p < .01$ versus basal, no (1*S*,3*R*)-ACPD; $^{*}p < .05$ versus basal, 100 μM (1*S*,3*R*)-ACPD.

et al., 1992; Brabet et al., 1995) and is known to be a highly selective agonist for PLC-coupled mGlu receptors in the rat hippocampus (Schoepp et al., 1994). In a previous report, we demonstrated that 3,5-DHPG does not stimulate PLD but, quite unexpectedly, reduces the formation of [^{3}H]PEt induced by (1*S*,3*R*)-ACPD in adult rat hippocampal slices (Pellegrini-Giampietro et al., 1996a). The present study further confirms that 3,5-DHPG is an agonist of mGlu receptors linked to phosphoinositide hydrolysis but an antagonist of PLD-coupled mGlu receptors in adult hippocampus. The possibility that 3,5-DHPG might inhibit the formation of [^{3}H]PEt via activation of group I mGlu receptors appears unlikely because numerous reports (for a review, see Klein et al., 1995) have quite conclusively established that activation of PKC, the key product of the PLC transduction pathway, leads to stimulation of PLD in virtually all cell types studied. Rather, our findings with 3,5-DHPG are in line with previous reports showing that activation of PLC does not necessarily result in activation of PLD (Sarri et al., 1995) and that the PLC and PLD pathways can be independently activated in adult hippocampus (Holler et al., 1994; Pellegrini-Giampietro et al., 1996a). 3,5-DHPG was less potent (IC_{50} = 70 μM) than PCCG-13 in reducing the formation of [^{3}H]PEt evoked by 100 μM (1*S*,3*R*)-ACPD, but unlike PCCG-13, it completely inhibited the agonist-induced PLD response. Because (1*S*,3*R*)-ACPD promotes the release of glutamate (Herrero et al., 1992; Lombardi et al., 1996; Moroni et al., 1998) and glutamate, in turn, is known to stimulate the release of norepinephrine in the hippocampus (Jones et al., 1987; Vezzani et al., 1987; Pittaluga and Raiteri, 1992), 3,5-DHPG may be producing a complete inhibition of PLD activity because it is able, unlike PCCG-13, to prevent in a nonspecific manner the formation of [^{3}H]PEt induced by norepinephrine and histamine. Our results with a calcium ionophore and a direct G protein activator in hippocampal slices and the data obtained using aorta rings imply either receptor cross-talk or that 3,5-DHPG exerts its nonspecific antagonist effect at the receptor level. When agonist dose-response curves were performed in the presence of 3,5-DHPG, the reducing effect of the antagonist could not be surmounted by increasing the dose of (1*S*,3*R*)-ACPD. The nonspecific interaction between 3,5-DHPG and other receptors or, possibly, other sites on PLD-coupled mGlu receptors may contribute to the noncompetitive pattern of antagonism observed in our experiments.

In contrast with our results, Klein et al. (1997) have shown that 3,5-DHPG induces a full agonist PLD response in hippocampal slices from 8-day-old rats. It is therefore possible that, as recently postulated by these authors (Klein et al., 1998), glutamate-evoked stimulation of PLD occurs along different pathways in immature and adult hippocampus. In neonate tissue, PLD responses appears to be mediated by group I mGlu receptors via PLC and PKC activation, in a manner independent of extracellular Ca^{2+}. This "first-phase" PLD activation desensitizes within minutes and fades postnatally, disclosing a nondesensitizing "second phase" that is smaller but predominates in the adult. The second-phase PLD activation, observed in hippocampal slices from adult rats, appears to be PKC independent (Pellegrini-Giampietro et al., 1996a) but Ca^{2+} dependent (Sarri et al., 1995) and is mediated by mGlu receptors bearing characteristics of group I mGlu receptors (i.e., quisqualate is the most potent agonist) but also a number of unique pharmacological features, such as the selective activation by L-cystein sulfinic acid (Boss et al., 1994) and the antagonism by PCCG-13 and 3,5-DHPG (see also Pellegrini-Giampietro et al., 1996a). The distinct pharmacological profile of PLD-coupled mGlu receptors and other mGlu receptors described in rat brain (Thomsen et al., 1993; Scholz, 1994; Zheng et al., 1995; Mannaioni et al., 1996; Chung et al., 1997) strongly suggests that there may be still a number of novel subtypes that have not yet been cloned. Moreover, the stereochemical features of PCCG-13 indicate that PLD-coupled mGlu receptors may be different from known subtypes. Indeed, in contrast with other (carboxycyclopropyl)glycines, such as L-CCG-1 and PCCG-4 (see Fig. 4), that are known to interact with group II mGlu receptors in a fully extended disposition of the α-aminoacidic moiety and ω-carboxylate group (Costantino et al., 1993), PCCG-13 exhibits a *cis* configuration between the aminoacidic moiety and the distal acidic group. Even more surprisingly, PCCG-13 shows an unnatural *R* configuration at the carbon 2 aminoacidic center. Studies aimed at defining a pharmacophoric model for PLD-coupled mGlu receptors focused on the above structural features are under way.

In conclusion, 3,5-DHPG is a noncompetitive and nonselective antagonist of PLD responses evoked by (1*S*,3*R*)-ACPD in adult rat hippocampus. On the other hand, PCCG-13 is a potent, selective, and competitive antagonist of PLD-coupled mGlu receptors in the same tissue and thus should provide an excellent tool for unraveling the functional role of these receptors in the central nervous system.

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