

Title Page

**The mGluR5 antagonist MPEP decreases ethanol consumption via a
PKC ϵ -dependent mechanism**

M. Foster Olive, Andrew J. Mcgeehan, Jennifer R. Kinder, Thomas McMahon,
Clyde W. Hodge, Patricia H. Janak, Robert O. Messing

Ernest Gallo Clinic & Research Center, Department of Neurology, University of California at San
Francisco, Emeryville, CA 94608 USA (MFO, AJM, JRK, TM, PHJ, ROM)

Bowles Center for Alcohol Studies, Departments of Psychiatry and Pharmacology, University of
North Carolina at Chapel Hill, Chapel Hill, NC 27599 USA (CWH)

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Corresponding author:

M. Foster Olive, Ph.D.
Ernest Gallo Clinic and Research Center
Department of Neurology
University of California at San Francisco
5858 Horton Street, Suite 200
Emeryville, CA 94608
Phone: (510) 985-3922
Fax: (510) 985-3101
E-mail: folive@itsa.ucsf.edu

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Abbreviations: mGluR5, type 5 metabotropic glutamate receptor; PKC ϵ , protein kinase C epsilon; PI3K, phosphatidylinositol-3 kinase; PDK1, phospholipids-dependent kinase 1; CHPG, (RS)-2-chloro-5-hydroxyphenylglycine; MPEP, 6-methyl-2-(phenylethynyl)pyridine; aCSF, artificial cerebrospinal fluid

Abstract

Glutamatergic neurotransmission plays a critical role in addictive behaviors, and recent evidence indicates that genetic or pharmacological inactivation of the type 5 metabotropic glutamate receptor (mGluR5) reduces the self-administration of cocaine, nicotine and alcohol. Since mGluR5 is coupled to activation of protein kinase C (PKC), and targeted deletion of the epsilon isoform (PKC ϵ) in mice reduces ethanol self-administration, we investigated whether there is a functional link between mGluR5 and PKC ϵ . Here we show that acute administration of the mGluR5 agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) to mice increases phosphorylation of PKC ϵ in its activation loop (T566) as well as in its C-terminal region (S729). Increases in phosphoPKC ϵ are dependent not only on mGluR5 stimulation, but also on phosphatidylinositol-3 kinase (PI3K). In addition, the selective mGluR5 antagonist 6-methyl-2-(phenylethynyl)pyridine (MPEP) reduced basal levels of phosphorylation of PKC ϵ at S729. We also show that MPEP dose-dependently reduced ethanol consumption in wildtype but not in PKC ϵ null mice, suggesting that PKC ϵ is an important signaling target for modulation of ethanol consumption by mGluR5 antagonists. Radioligand binding experiments using [3 H]MPEP revealed that these genotypic differences in response to MPEP were not a result of altered mGluR5 levels or binding in PKC ϵ null mice. Our data indicate that mGluR5 is coupled to PKC ϵ via a PI3K-dependent pathway, and that PKC ϵ is required for the ability of the mGluR5 antagonist MPEP to reduce ethanol consumption.

Introduction

Glutamatergic neurotransmission plays an important role in the behavioral and neurochemical effects of drugs of abuse as well as in drug self-administration. Although most studies have examined the role of ionotropic glutamate receptor subtypes (Kalivas, 2004; Tzschentke and Schmidt, 2003), there is increasing evidence that metabotropic glutamate receptors (mGluRs) also play an important role in drug-related behaviors (Kenny and Markou, 2004; Olive, in press). For example, mice lacking the type 5 mGluR (mGluR5) show dramatically reduced levels of cocaine self-administration and cocaine-induced hyperactivity (Chiamulera et al., 2001). In addition, studies using selective mGluR5 antagonists have confirmed a role for mGluR5 in cocaine-related behaviors such as conditioned place preference (Mcgeehan and Olive, 2003), hyperlocomotion (Mcgeehan et al., 2004) and self-administration (Chiamulera et al., 2001; Kenny et al., 2003b; Tessari et al., 2004). Self-administration of nicotine (Kenny et al., 2003b; Paterson et al., 2003; Tessari et al., 2004) and ethanol (Backstrom et al., 2004; Sharko et al., 2002) are also reduced by mGluR5 antagonists. Finally, blockade of mGluR5 increases threshold current levels for intracranial self-stimulation (Harrison et al., 2002; Kenny et al., 2003a), suggesting that these receptors are centrally involved in primary reward mechanisms.

Activation of group I mGluRs, which include mGluR1 and mGluR5, stimulates the activity of phospholipase C (PLC) thereby generating diacylglycerol (DAG), which in turn can activate PKC (Hermans and Challiss, 2001). Nine different PKC genes have been identified, seven of which encode isozymes activated by DAG. It is currently not known which PKC isozyme(s) are activated by mGluR5. One candidate is PKC ϵ which, like mGluR5, is enriched in regions of the brain involved in drug self-administration and addiction, including the frontal cortex, striatum, nucleus accumbens and hippocampus (Minami et al., 2001; Saito et al., 1993). Moreover, there is considerable anatomical overlap in the expression of mGluR5 and PKC ϵ in the brain (Minami et al.,

2001; Romano et al., 1995; Saito et al., 1993; Shigemoto et al., 1993), suggesting that this PKC may be a downstream signaling target of mGluR5. Interestingly, our research has shown that similar to pharmacological blockade of mGluR5, genetic deletion of PKC ϵ reduces ethanol consumption in mice (Choi et al., 2002; Hodge et al., 1999; Olive et al., 2000). Here, we sought to establish biochemical link between mGluR5 and PKC ϵ , and to determine if PKC ϵ is necessary for the ability of mGluR5 antagonists to reduce ethanol consumption.

Materials and Methods

Animals. Male mice (age 2-4 months) were used for all experiments and were housed in temperature and humidity controlled Plexiglas cages (BioZone, Fort Mill, SC) under a 12:12 light-dark cycle with lights-on at 0600 h. Food and water were available *ad libitum* except where noted. Male wildtype C57BL/6J x 129SvJae littermates (F1 or F2 generation) were used for all studies except ethanol consumption experiments (see below). All procedures were conducted in accordance with institutional IACUC procedures and the Society for Neuroscience's Policy on the Use of Animals in Neuroscience Research.

Drugs and Reagents. (RS)-2-chloro-5-hydroxyphenylglycine (CHPG, Tocris, Ellisville, MO) was dissolved in 110 mM NaOH, and LY294002 (Calbiochem, San Diego, CA) was suspended in DMSO prior to dilution in artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Holliston, MA) for intracerebroventricular (i.c.v.) administration. 6-methyl-2-(phenylethynyl)pyridine (MPEP, Sigma-Aldrich, St. Louis, MO) was dissolved in physiological saline prior to intraperitoneal (i.p.) administration. All other chemical reagents were obtained from Fisher Scientific (Santa Clara, CA) or Sigma-Aldrich.

Intracerebroventricular Cannula Implantation and Drug Treatments. For experiments involving administration of CHPG, mice were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (7 mg/kg i.p.) and placed in a stereotaxic frame equipped with a mouse adapter (Harvard Apparatus). A plastic guide cannula (26 ga OD, Plastics One, Roanoke, VA) aimed at the lateral ventricle was unilaterally implanted using the following coordinates with bregma and skull surface as reference points (Franklin and Paxinos, 2001): posterior -0.3 mm, lateral +1.0 mm, ventral -2.0 mm. The

cannula was secured with skull screws and dental cement, and mice were allowed at least 5 d of post-surgical recovery prior to experiments.

A 33-ga OD microinjection needle (Plastics One) that extended 1 mm beyond the ventral tip of the guide cannula was used for i.c.v. drug administration. Microinjection needles were connected to gas-tight Hamilton syringes via FEP tubing (CMA/Microdialysis, North Chelmsford, MA). Syringes were driven by a Harvard syringe pump at a flow rate of 1 $\mu\text{l}/\text{min}$ for a total infusion of 1 μl of fluid. The microinjection needle was left in place for an additional 1 min following each infusion to allow for diffusion of the drug into the ventricle.

Separate experimental groups of mice were treated as follows: saline or MPEP (20 mg/kg i.p.) 15 min prior to the administration of CHPG (3 $\mu\text{g}/\mu\text{l}$ i.c.v.) or its corresponding vehicle (dilute NaOH); LY294002 (30 $\mu\text{g}/\mu\text{l}$ i.c.v.) or its corresponding vehicle (dilute DMSO) 15 min prior to the administration of CHPG (3 $\mu\text{g}/\mu\text{l}$ i.c.v.); and saline or MPEP (20 mg/kg i.p.). Fifteen minutes following the final drug treatment, mice were euthanized and brains removed and placed into ice-cold phosphate buffered saline (PBS, pH=7.2) for dissection of striatal tissue.

Striatal Homogenate Preparation and Immunoblot Analysis. Striatal tissue was placed in 10 volumes of homogenization buffer (pH 7.5) consisting of 20 mM Tris, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM phenylmethylsulfonylfluoride, and a 1:100 dilution of a protease inhibitor cocktail (Sigma-Aldrich) and a serine/threonine phosphatase inhibitor cocktail (Sigma-Aldrich). Tissue was homogenized with 10-12 strokes of a motorized homogenizer at 5000 rpm and centrifuged at 10,000 g at 4°C for 10 min. The supernatant was then stored at -70°C until Western analysis.

For Western analysis, samples were split into two equal aliquots (20 μg of protein each) and subjected to SDS-PAGE using separate 10% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins

were transferred to nitrocellulose membranes, preblocked with PBS containing 0.1% (v/v) Tween 20 and either 5% (w/v) bovine serum albumin (for phosphoPKC) or 5% (w/v) nonfat dried milk powder (for total PKC) for 1 h prior to overnight incubation with primary antibodies. We used the following rabbit polyclonal antibodies: anti-phosphoPKC ϵ T566 (Parekh et al., 1999); 1:5000 dilution), anti-phosphoPKC ϵ S729 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000 dilution), anti-phosphoPKC α T638/PKC β T641 (Cell Signaling Technology, Beverly, MA; 1:1000 dilution), anti-PKC ϵ (Choi et al., 2002); 1:5000 dilution), and anti-PKC β II (Santa Cruz Biotechnology, 1:500 dilution). Membranes were then washed and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antisera (Jackson ImmunoResearch, West Grove, PA, 1:10,000 dilution) for 90 min, and immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ). Protein bands corresponding to a molecular weight of approximately 90 kDa were visualized on a Storm 860 Phosphoimager (Amersham Biosciences) at 100 μ m resolution using ImageQuant software (Amersham Biosciences). The optical density of the phosphoPKC band was divided by that of the corresponding total PKC band to yield a phosphoPKC/total PKC ratio.

Radioligand Binding. Cerebral cortex and striatum tissue from naïve wildtype and PKC ϵ null mice (Hodge et al., 1999; Khasar et al., 1999) were homogenized in 10 volumes of ice-cold assay buffer consisting of 50 mM Tris-HCl, 0.9% (w/v) NaCl, pH=7.5, and the homogenate was centrifuged at 39,800 x g for 10 min at 4°C. The pellet was re-suspended in assay buffer immediately prior to use. Receptor binding in crude membrane fractions was determined using [3 H]MPEP (46.85 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) over a concentration range of 0.075–10 nM, and non-specific binding was determined using 10 μ M MPEP. Binding studies were performed using 300 μ g protein in a 1 ml reaction volume. After incubation for 1 h at room temperature tissues were

rapidly filtered over Whatman GF/C filters, and radioactivity remaining on the filters was detected by liquid scintillation counting. Each binding experiment (n=4 animals) was performed with 3-5 replicate experiments for each genotype and brain region.

Ethanol consumption. For these studies, C57BL/6J x 129/SvJae PKC ϵ heterozygous null mice (F2 generation; see Hodge et al., 1999; Khasar et al., 1999) were backcrossed onto a C57BL/6J background for 10 generations, and mice heterozygous for the PKC ϵ null mutation were subsequently mated with female 129/SvJae PKC ϵ heterozygous mice to generate F1 hybrid wildtype and PKC ϵ null mice for study. Mice were housed individually in cages equipped with two bottle grommets at one end, and allowed simultaneous access for 16 h to one 50 ml bottle containing 10% v/v ethanol and another containing tap water, beginning at 1700 h. On the following morning at 0900 h, the two bottles were removed from the cages and the amount of each fluid consumed was recorded. Animals did not have access to either fluid from 0900 to 1700 h. These 16-h two-bottle choice sessions were conducted 4 sequential days per week. For the remaining 3 days, animals were allowed 24-h access to both solutions. At least two weeks of baseline consumption was established prior to administration of any pharmacological agents. Mice were administered saline or MPEP (3, 10 or 20 mg/kg i.p.) in a volume of 10 ml/kg immediately prior to 16 h access sessions. All animals received saline or doses of MPEP in a balanced and randomized order, with a maximum of two injections per week. Ethanol consumption was defined as g/kg/16 h, ethanol preference was defined as (ml ethanol consumed per 16 h/total ml consumed per 16 h) x 100, and water consumption was defined as ml/16 h.

Statistical Analyses. Data from immunoblot and radioligand binding experiments were analyzed by one-way analysis of variance (ANOVA) followed by Neuman-Keuls or Dunn's multiple

comparisons tests, or by a Kruskal-Wallis one-way ANOVA on ranks when appropriate. Data from ethanol consumption studies were analyzed by two-way ANOVA with one factor repetition (treatment) followed by Holm-Sidak multiple comparisons tests. $P < 0.05$ was considered statistically significant for all tests.

Results

PhosphoPKC experiments

We used phosphospecific antibodies to detect phosphorylation of PKC ϵ at T566 and S729, since phosphorylation of this kinase at these two sites is considered to reflect an increase in the activatable pool of PKC ϵ (Akita, 2002; Cenni et al., 2002; Parekh et al., 1999; Parekh et al., 2000). The activation loop of PKC ϵ is phosphorylated at T566 by phospholipid-dependent kinase 1 (PDK1), which in turn is activated by lipid products of phosphatidylinositol-3 kinase (PI3K) (Cenni et al., 2002; Parekh et al., 2000). Once PKC ϵ is phosphorylated at T566, it can be then be phosphorylated in the C-terminal at S729, either by autophosphorylation or by heterologous kinases (see Discussion).

Administration of the mGluR5 agonist CHPG to wildtype mice (Fig. 1a) increased the abundance of striatal phosphoPKC ϵ (S729) immunoreactivity relative to total PKC ϵ immunoreactivity ($F_{(2,14)} = 4.087$, $p < 0.05$). This was blocked by systemic pretreatment with MPEP. Surprisingly, CHPG also increased phosphorylation of PKC ϵ at T566 ($H_{(2)} = 8.681$, $p < 0.05$), and this also was also blocked by systemic MPEP pretreatment (Fig. 1b). The increase in phosphoPKC ϵ (T566) induced by CHPG ($F_{(2,21)} = 3.901$, $p < 0.05$) was also inhibited by pretreatment with the PI3K inhibitor LY294002 (Fig. 1c). In contrast, CHPG did not modify the ratio of phosphoPKC α/β II (T638/641) to total PKC β II immunoreactivity (Fig. 1d, $F_{(2,9)} = 0.081$, $p > 0.05$). Additional experiments revealed that a single peripheral administration of MPEP (20 mg/kg i.p.), but not saline, reduced basal levels of phosphoPKC ϵ (S729) relative to total PKC ϵ immunoreactivity (Fig. 1e, $F_{(1,12)} = 5.063$, $p < 0.05$).

Ethanol consumption

As we previously reported (Hodge et al., 1999; Olive et al., 2000), baseline ethanol consumption (Fig. 2a) was significantly higher in wildtype mice compared with PKC ϵ null mice ($H_{(1)} = 9.694$, $p < 0.005$). Similarly, baseline ethanol preference (Fig. 2b) was significantly higher in wildtype as compared to PKC ϵ null mice ($H_{(1)} = 7.467$, $p < 0.01$). Administration of MPEP dose-dependently reduced ethanol consumption in wildtypes, but not in PKC ϵ null mice. Analysis of ethanol consumption in both genotypes revealed a significant main effect of genotype ($F_{(1,63)} = 4.630$, $p < 0.05$), MPEP treatment ($F_{(3,63)} = 3.726$, $p < 0.05$) and a genotype x treatment interaction ($F_{(3,63)} = 3.854$, $p < 0.05$). Similarly, with regards to ethanol preference, there was a main effect of MPEP treatment ($F_{(3,63)} = 3.002$, $p < 0.05$) and a genotype x treatment interaction ($F_{(3,63)} = 4.841$, $p < 0.005$), with a trend towards a significant main effect of genotype on ethanol preference ($F_{(1,63)} = 3.208$, $p = 0.08$). No effect of genotype ($F_{(1,58)} = 1.510$, $p > 0.05$) or MPEP treatment ($F_{(3,58)} = 0.903$, $p > 0.05$) on water intake was observed, nor was there a genotype x treatment interaction ($F_{(3,58)} = 0.368$, $p > 0.05$) (see Fig. 2c).

Radioligand binding

To control for the possibility that differential effects of MPEP on ethanol consumption in wildtype and PKC ϵ null mice might result from genotypic differences in mGluR5 density or affinity, we performed radioligand binding studies using [^3H]MPEP in crude cortical and striatal membrane preparations from wildtype and PKC ϵ null mice. Saturation binding curves are shown in Figure 3. No genotypic differences in ligand binding density (B_{max}) were observed in the cortex (wildtype $B_{\text{max}} = 56.16 \pm 2.65$, PKC ϵ null $B_{\text{max}} = 66.96 \pm 11.84$, $F_{(1,5)} = 0.579$, $p > 0.05$) or striatum (wildtype $B_{\text{max}} = 44.29 \pm 11.34$, PKC ϵ null $B_{\text{max}} = 78.66 \pm 16.28$, $F_{(1,6)} = 2.190$, $p > 0.05$). Similarly, no genotypic differences in ligand affinity (K_D) were observed in the cortex (wildtype $K_D = 6.45 \pm$

0.48, PKC ϵ null $K_D = 6.32 \pm 1.42$, $F_{(1,5)} = 0.006$, $p > 0.05$) or striatum (wildtype $K_D = 4.60 \pm 0.35$,

PKC ϵ null $K_D = 5.04 \pm 0.50$, $F_{(1,6)} = 0.385$, $p > 0.05$).

Discussion

Our findings identify for the first time a novel mGluR5-PKC ϵ signaling pathway in the brain that regulates ethanol consumption. Using PKC ϵ null mice we found that the ability of the mGluR5 antagonist MPEP to reduce ethanol consumption was dependent on the presence of PKC ϵ . These alterations in ethanol consumption were not a result of altered mGluR5 number or binding in PKC ϵ null mutant mice, since no differences in radiolabeled MPEP binding to the cortex and striatum between wildtype and PKC ϵ null mice were observed. Furthermore, using a phospho-specific antibody against phosphorylated T566 of PKC ϵ and LY294002 (an inhibitor of PI3K), we found that activation of mGluR5 increases the activatable pool of PKC ϵ via PI3K and PDK1. Until now, regulated phosphorylation of PKC ϵ at T566 has only been demonstrated *in vitro* in NIH 3T3 cells stimulated with platelet derived growth factor (Cenni et al., 2002) or in HEK293 cells stimulated with serum following serum starvation (Parekh et al., 1999). Our results provide the first *in vivo* evidence for regulated PDK1 mediated phosphorylation of PKC ϵ through a G-protein-coupled neurotransmitter receptor (mGluR5). Moreover, our results also indicate that mGluR5 is coupled to PI3K in the striatum. These findings provide *in vivo* functional significance to the recent *in vitro* demonstration that mGluR5 can associate with PI3K via a PI3K enhancer (PIKE)/Homer protein complex (Rong et al., 2003).

We did not, however, examine the effects of LY294002 on basal levels of PKC ϵ phosphorylation, since it has previously been shown that inhibition of PDK1 function (through the utilization of kinase inactive mutants of PDK-1 rather than pharmacological inhibition of PI3K by LY294002) does not alter basal phosphorylation of PKC ϵ (Cenni et al., 2002). Thus, we predict that administration of LY294002 alone would not alter basal levels of PKC ϵ phosphorylation *in vivo*. Yet given recent evidence that PI3K contributes to behavioral plasticity associated with

repeated exposure to cocaine (Izzo et al., 2002), the role of this lipid kinase in drug and ethanol self-administration clearly merits further investigation.

Direct measurements of PKC ϵ activity were not performed in the present study. Rather, we quantified levels of phosphorylation of PKC ϵ using specific antisera against two phosphorylation sites on this enzyme, since phosphorylation of PKC isozymes is strongly correlated with increases in activity of these kinases, and appears to be essential for expression of maximal kinase activity (Mitchell et al., 1989; Newton, 2001; Parekh et al., 1999). Most PKC isoforms contain phosphorylation sites in the activation loop and in the C-terminal hydrophobic region, and it is thought that a sequential phosphorylation of these sites leads to activation of the enzyme (Newton, 1997; Newton, 2001). For example, it has been demonstrated that trans-phosphorylation of PKC β II in its activation loop (at T500) by PDK-1 renders it competent to autophosphorylate residues T641 and S660 (Dutil et al., 1994; Keranen et al., 1995). Once autophosphorylated, PKC β II remains catalytically competent, but inactive until it translocates to the membrane, where it binds lipid activators such as DAG. Since PKC ϵ contains similar phosphorylation sites in its activation loop (T566) and C-terminus (S710 and S729), it is likely that this PKC isoform undergoes a similar sequential activation sequence (Parekh et al., 2000). The activation loop of PKC ϵ is known to be phosphorylated by PDK1, which in turn is activated by lipid products of PI3K (Cenni et al., 2002; Parekh et al., 2000). PDK-1 mediated phosphorylation of the activation loop is a constitutive event in the maturation of conventional PKC isozymes (α , β and γ), but for other PKC isozymes is regulated by the activity of PI3K (Newton, 2001). PDK1-mediated phosphorylation of PKC ϵ at T566 then triggers sequential phosphorylation of S710 and S729. However, whether or not phosphorylation of S729 reflects autophosphorylation is a subject of debate, since some investigators have demonstrated a complete abolition of phosphorylation of PKC ϵ at S729 in the

presence of a PKC inhibitor (Parekh et al., 1999), whereas others found only partial reductions in phosphorylation at this site (Cenni et al., 2002). These latter findings suggest that under certain conditions, other kinases may participate in phosphorylation of PKC ϵ at S729. However, these same authors also showed evidence for autophosphorylation processes in PKC ϵ activation, as kinase inactive mutants of PKC ϵ failed to show phosphorylation of PKC ϵ at S729 (Cenni et al., 2002). Regardless of the mechanism, PKC ϵ phosphorylation at S729 has been used in several studies as a marker for changes in PKC ϵ activation (Bayer et al., 2003; Saitoh et al., 2001; Zhou et al., 2003).

In the present study we showed that activation of mGluR5 by CHPG stimulated phosphorylation of PKC ϵ at both T566 and S729, indicating that stimulation of mGluR5 increases the available pool of PKC ϵ that can become active. Our finding that MPEP alone reduced phosphorylation of PKC ϵ at S729 in the striatum indicates a role for mGluR5 in setting the basal level of activatable PKC ϵ in this brain region. Consistent with this result, it has been reported that mGluR5 exhibits constitutive activity, as these receptors undergo endocytosis in the absence of agonists in cultured hippocampal neurons (Fourgeaud et al., 2003).

Both mGluR5 and PKC ϵ are highly co-expressed in numerous regions of the adult rodent brain such as the cerebral cortex, olfactory bulb, striatum, nucleus accumbens, and hippocampus (Minami et al., 2001; Romano et al., 1995; Saito et al., 1993; Shigemoto et al., 1993). For the current study, we chose to examine the dorsal striatum, as this region contains very high levels of both proteins, is adjacent to the lateral ventricle making it easily accessible to i.c.v.-administered drugs, and plays an important role in the control of drug self-administration and persistent drug-related habits (Gerdeman et al., 2003; Wang et al., 2003; Winder et al., 2002). However, the striatum also contains significant levels of PKC β (Minami et al., 2001; Tanaka and Saito, 1992), and thus the possibility exists that mGluR5 in this region may also be coupled to this PKC isozyme.

Yet we found that CHPG did not modify the ratio of phosphoPKC α / β II (T638/641) to total PKC β II immunoreactivity, indicating that the effect of mGluR5 stimulation on PKC ϵ phosphorylation in the striatum is specific. Consistent with this, non-selective Group I mGluRs ligands have been reported to induce translocation of PKC ϵ but not PKC β in cortical synaptosomes (Pastorino et al., 2000), suggesting that mGluR5 coupling to PKC ϵ may be specific in other forebrain regions as well.

The current study underlines the importance of PKC ϵ , a downstream signaling target of mGluR5, in the ability of the mGluR5 antagonist MPEP to reduce ethanol self-administration. Further studies are needed to determine if dysregulation of this signaling pathway contributes to disorders of excessive ethanol consumption such as alcohol abuse and alcoholism. While it has been demonstrated that mGluR5 also plays an important role in the reinforcing and behavioral effects of other drugs of abuse such as cocaine and nicotine (Kenny and Markou, 2004; Olive, in press), no studies to date have examined the role of PKC ϵ activity in modulation of cocaine and nicotine self-administration as well as behavioral and neural plasticity induced by these drugs. Such studies should provide a more detailed understanding of the cellular substrates underlying drug addiction. Interestingly, though, while we have shown that rewarding effects of morphine are not altered by co-administration of MPEP at doses up to 20 mg/kg in mice (Mcgeehan and Olive, 2003) (though higher doses may be required in other rodent species – see Popik and Wrobel, 2002), preliminary evidence suggests that morphine reward is enhanced in mice lacking PKC ϵ (Newton et al., 2004). Thus, more research is needed aimed at identifying G-protein coupled receptor other than mGluR5 that are coupled to PKC ϵ in brain regions known to be critically involved in the rewarding effects of morphine, such as the ventral tegmental area (McBride et al., 1999; Wise, 1989). In addition to drugs of abuse, this mGluR5-PKC ϵ signaling cascade may have relevance for other CNS pathologies, since both antagonism of mGluR5 as well as deletion of PKC ϵ produces parallel

reductions in anxiety and hyperalgesia (Conn, 2003; Hodge et al., 2002; Khasar et al., 1999; Kuhn et al., 2002; Spooren et al., 2003; Spooren et al., 2001).

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Footnotes

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Reprint requests to: M. Foster Olive, Ph.D., Ernest Gallo Clinic and Research Center, Department of Neurology, University of California at San Francisco, 5858 Horton Street, Suite 200, Emeryville, CA, 94608, USA, Phone: (510) 985-3922, Fax: (510) 985-3101, E-mail: folive@itsa.ucsf.edu

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Legends for Figures

Figure 1. The mGluR5 receptor is positively coupled to PKC ϵ via a pathway dependent on PI3K. Administration of the mGluR5 agonist CHPG significantly increased levels of active PKC ϵ as measured by the ratio of phosphoPKC ϵ (S729) (**a**) or phosphoPKC ϵ (T566) (**b**) immunoreactivities to total PKC ϵ immunoreactivity (* P <0.05 vs. saline i.p. followed by vehicle i.c.v.). These effects were significantly attenuated when animals were pretreated with MPEP ($\dagger P$ <0.05 vs. saline i.p. followed by CHPG i.c.v.). (**c**) The PI3K kinase inhibitor LY294002 significantly attenuated the ability of CHPG to increase levels of phosphoPKC ϵ (T566) relative to total PKC ϵ ($\dagger P$ <0.05 vs. vehicle prior to CHPG). (**d**) No effect of CHPG treatment was observed on levels of phosphoPKC α/β II (T638/641) relative to total PKC β II. (**e**) Systemic administration of MPEP (20 mg/kg) but not saline (0 mg/kg) resulted in a reduction in levels of phosphoPKC ϵ (S729) immunoreactivity relative to total PKC ϵ immunoreactivity (* P <0.05 vs. saline). Data shown represent mean \pm SEM of $n=3$ animals per condition and 4-8 immunoblot analyses.

Figure 2. PKC ϵ is required for the ability of the mGluR5 antagonist MPEP to reduce ethanol consumption (**a**) and preference (**b**). Saline (0 mg/kg) or MPEP (3, 10 or 20 mg/kg) were administered immediately prior to 16 h two-bottle access sessions. (**c**) Lack of effect of MPEP administration on water intake. * P <0.05 vs. saline-treated mice of the same genotype. $\dagger P$ <0.05 vs. wildtype at corresponding treatment. Values represent mean \pm SEM of $n=8-14$ animals per dose.

Figure 3. Saturation curves for [³H]MPEP binding to membranes from (a) cerebral cortex (b) or striatum of wildtype (●) and PKCε null (○) mice. Values represent mean ± SEM of n=3-5 replicate experiments per group (n=4 mice per experiment).





