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Acute Ethanol Administration Rapidly Increases Phosphorylation of Conventional Protein Kinase C in Specific Mammalian Brain Regions in Vivo

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Abstract

Background—Protein kinase C (PKC) is a family of isoenzymes that regulate a variety of functions in the central nervous system including neurotransmitter release, ion channel activity, and cell differentiation. Growing evidence suggests that specific isoforms of PKC influence a variety of behavioral, biochemical, and physiological effects of ethanol in mammals. The purpose of this study was to determine whether acute ethanol exposure alters phosphorylation of conventional PKC isoforms at a threonine 674 (p-cPKC) site in the hydrophobic domain of the kinase, which is required for its catalytic activity.

Methods—Male rats were administered a dose range of ethanol (0, 0.5, 1, or 2 g/kg, intragastric) and brain tissue was removed 10 minutes later for evaluation of changes in p-cPKC expression using immunohistochemistry and Western blot methods.

Results—Immunohistochemical data show that the highest dose of ethanol (2 g/kg) rapidly increases p-cPKC immunoreactivity specifically in the nucleus accumbens (core and shell), lateral septum, and hippocampus (CA3 and dentate gyrus). Western blot analysis further showed that ethanol (2 g/kg) increased p-cPKC expression in the P2 membrane fraction of tissue from the nucleus accumbens and hippocampus. Although p-cPKC was expressed in numerous other brain regions, including the caudate nucleus, amygdala, and cortex, no changes were observed in response to acute ethanol. Total PKCγ immunoreactivity was surveyed throughout the brain and showed no change following acute ethanol injection.

Conclusions—These results suggest that ethanol rapidly promotes phosphorylation of cPKC in limbic brain regions, which may underlie effects of acute ethanol on the nervous system and behavior.

Keywords

Ethanol; Conventional Protein Kinase C; PKC; PKCγ; Phosphorylation

Protein Kinase C (PKC) is a group of Ca^{2+} and phospholipid-dependent serine/threonine kinases that are involved in a variety of cell signaling systems (Nishizuka, 1984). The PKC family of enzymes is divided into subgroups based on the structural and functional properties of the kinase regulatory domain: *conventional* (α, βI, βII, and γ), *novel* (δ, ε, η, and θ), *atypical* (λ and ς), and the recently described (μ and ν) isoforms (Casabona, 1997; Dempsey

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et al., 2000; Newton, 2003; Nishizuka, 1984). The various isoforms of PKC also show distinct patterns of tissue expression and cellular localization (Akita, 2002; Saito et al., 1988), which suggests specificity of function. Indeed, specific members of the PKC family regulate a variety of neurobiological functions including ion channel activity, neurotransmitter release, receptor desensitization, and cell differentiation (Martelli et al., 2006; Tanaka and Nishizuka, 1994).

In addition to these more general functions, specific isoforms of PKC have been shown to regulate behavioral, biochemical, and physiological responses to ethanol (Hodge et al., 1999). Growing evidence from gene knockout mice indicates that PKC*γ*, a member of the conventional PKC (cPKC) subgroup, mediates both acute and chronic effects of ethanol. For example, PKC*γ* null mice show decreased sensitivity to the acute hypnotic effects of ethanol but display normal responses to other sedative/hypnotic compounds such as pentobarbital (Harris et al., 1995). PKC*γ* null mice also fail to develop tolerance to ethanol hypnosis after 10 days of ethanol liquid diet exposure (Bowers et al., 1999). Ethanol-induced enhancement of *γ*-amino butyric acid $(GABA)_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) from hippocampal CA1 pyramidal neurons (Proctor et al., 2003) and stimulation of $GABA_A$ receptor-mediated Cl-flux from cortical membranes (Harris et al., 1995) are blunted in PKC*γ* null mice. In agreement with these data showing loss of ethanol sensitivity, PKC*γ* null mice voluntarily consume significantly more ethanol than wild-type controls (Bowers and Wehner, 2001). Ethanol also alters association of PKC*γ* with $GABA_A$ receptors in the cerebral cortex (Kumar et al., 2002) and mediates the effects of ethanol withdrawal on *N*-methyl-D-aspartate receptor currents in the spinal cord (Li et al., 2005). Moreover, ethanol prevents translocation of the *β*II isoform of PKC, another member cPKC subgroup (Ron et al., 2000). Together, these findings suggest that conventional cPKC isoforms may regulate response to ethanol.

Members of the cPKC subfamily are activated by Ca^{2+} and diacylglycerol (DAG) in the presence of phosphatidylserine (Nishizuka, 1995). Ethanol has been shown to activate phospholipase C, which initiates intracellular signaling responses including formation of inositol-1,4, 5-trisphosphate, the release of intracellular Ca^{2+}, and formation of DAG, which leads to stimulation of cPKC (Hoek and Rubin, 1990). Evidence also indicates that ethanol (100 mM) induces translocation of PKC*γ* from the nucleus to the cytoplasm in neonatal spinal cord motor neurons (Li et al., 2005). However, to be catalytically active, cPKC isoforms also require a series of phosphorylations (Newton, 2003). For PKC*γ*, the activation loop threonine (T514) of the kinase is phosphorylated first by phosphoinositide-dependent kinase-1 (PDK1), which then enables an apparent autophosphorylation on T655 in the turn loop, and subsequent phosphorylation at a T674 site in the hydrophobic loop of the c-terminus (Parekh et al., 2000).

The purpose of the present study was to determine whether acute ethanol exposure alters the phosphorylation state of cPKC in the mammalian brain. To accomplish this goal, male Long–Evans rats were administered either an acute dose of ethanol or a corresponding volume of water via intragastric (IG) gavage. Animals were killed after 10 minutes and the brains were prepared for analysis of phosphorylated PKC*γ* (phospho T674; p-PKC*γ*) expression by immunohistochemistry or Western blot methods. The T674 site was chosen for analysis because phosphorylation of the hydrophobic loop is required for PKC*γ* to be fully active (Newton, 2003). It should be noted that peptide competition assays have shown that the p-PKC*γ* antibody also labels the other cPKC isoforms. For this reason, here we refer to the antibody as phosphorylation of cPKC (p-cPKC). Total PKC*γ* immunoreactivity was also analyzed by immunohistochemistry throughout the brain and by Western blot analysis in the nucleus accumbens to determine whether acute ethanol produced any changes in the abundance of PKCγ.

METHODS

Animals

Male Long–Evans rats (Harlan, Indianapolis, IN) were housed 2 per cage in standard Plexiglas cages (17.8*W*×29.2*L*×12.7*H* cm) with food and water always available. Rats were 58 to 64 days of age and weighed an average of 335.76 ± 3.51 g (SEM) at the time of testing. The colony room was maintained on a 12:12 light–dark cycle (lights on at 6:00 AM) at 22°C. All procedures were approved by the Institutional Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals (1996).

Acute Ethanol Administration

Rats were allowed approximately 2 weeks to acclimate to the laboratory. For the following 2 weeks, rats were handled and weighed daily. To habituate the rats to the IG gavage procedure, rats received water (IG) administration daily for 5 days before the start of the experiment. On the day of the experiment, rats (n=6 per dose) used for the immunohistochemistry analysis received ethanol (0, 0.5, 1.0, or 2 g/kg, IG), and after 10 minutes were deeply anesthetized with pentobarbital, and brain tissue was prepared as described below. Similarly, rats used for the Western blot analysis received ethanol (0 or 2 g/kg, IG) and were decapitated 10 minutes following drug administration (n=23 or 24 per dose, respectively).

Immunohistochemical Analysis

Rats were transcardially perfused with 100 mM phosphate buffer, followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were postfixed overnight, rinsed 4 times with phosphate-buffered saline (PBS) over a 6-hour period, and stored at 4 °C. Coronal vibratome sections (40 μm) were collected and stored in a cryoprotectant at 20 °C until processed for immunohistochemistry. Endogenous peroxidase was blocked by incubating the free-floating sections for 10 minutes in 0.6% H_2O_2, followed by a citra buffer antigen retrieval step performed at 70 °C for 30 minutes (Antigen Retrieval Citra, BioGenex, San Ramon, CA). Sections were blocked in PBS/0.1% Triton-X/4% horse serum for 30 minutes and incubated at +4 °C overnight in primary polyclonal antibody to p-PKCγ (T674) 1:200 or PKCγ 1:1,200 (Abcam, Cambridge, MA). The sections were incubated in the appropriate biotinylated secondary antibody for 1 hour. Avidin–biotin–peroxidase complex (ABC elite kit, Vector Labs, Burlingame, CA) was applied for 1 hour and immunoreactivity was detected with nickel-enhanced diaminobenzidine as a chromagen. Sections were counterstained with toluidine blue, mounted, dried, and coverslipped with Cytoseal.

The immunoreactivity of PKCγ and p-cPKC was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita model, QImaging, Burnaby, BC) that was interfaced to a desktop computer (Dell, Round Rock, TX). Image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN) was used to quantify pixel density in brain regions of interest. Pixel density measurements were divided by the area of the region and expressed as pixels/mm^2. Data were analyzed within the brain region statistically by 1-way ANOVA followed by the Tukey multiple comparison procedure (GraphPad Software, San Diego, CA).

Western Blot Analysis

Following ethanol injection, the brains were rapidly removed and dissected over ice using a brain matrix. The dissected brain regions from individual rats were then rapidly frozen and placed in a −80 °C freezer for storage before homogenization. Tissue corresponding to the nucleus accumbens, caudate-putamen, hippocampus, or amygdala from 4 individual animals that received the same drug condition were pooled and homogenized. Homogenization buffer

consisted of 147 mM NaCl; 2 mM$Na_2HPO_4 \cdot 7H_2O$; 8 mM$NaH_2PO_4 \cdot H_2O$; and 0.32 M sucrose with Phosphatase Inhibitor Cocktail I (Sigma-Aldrich, St. Louis, MO), pH 7.4, with 10 M NaOH. Pooled tissue was placed in 10 mL of ice-cold homogenization buffer and homogenized for 2 periods of 10 seconds using an Omni 2000 hand-held electric homogenizer. The homogenate underwent low-speed centrifugation at 1,000×*g* for 15 minutes at 4 °C, followed by centrifugation of the supernatant at 12,000×*g* for 30 minutes at 4 °C. The pellet was resuspended in ice-cold homogenization buffer and centrifuged at 12,000×*g* for an additional 30 minutes at 4 °C to yield a washed crude membrane pellet (P2). The pellet was resuspended in homogenization buffer and stored in 400 μL aliquots at −80 °C.

Frozen aliquots of P2 membrane fractions were thawed over ice, diluted 1:1 with Tris-glycine sodium dodecyl sulfate (SDS) sample buffer, and boiled at 95 to 100 °C for 7 minutes. Proteins were separated on 8 to 16% Tris-glycine gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a running buffer consisting of 25 mM Tris-base, 192 mM glycine with 1% SDS, pH 8.6, with 10 M NaOH. Proteins were transferred onto polyvinylidene fluoride membranes using a transfer buffer consisting of 12mM Tris-base, 96mM glycine with 20% methanol, pH 8.3, with 10 M NaOH. Membranes were blocked overnight at 4 °C using Odyssey blocking bluffer (Li-Cor Biosciences, Lincoln, NE) and then probed with polyclonal antiphospho T674 PKCγ, or total PKCγ, rabbit antibody (Abcam) (1:500 dilution). Membranes were concurrently probed with monoclonal antiactin mouse antibodies (Chemicon, Temecula, CA) (1:2,000) as a loading control.

Following incubation with anti-rabbit IgG conjugated to Alexa 680 (Invitrogen, Carlsbad, CA) (1:5,000 dilution) and anti-mouse IgG conjugated to immunoreactivity dye 800 (Rockland Immunochemicals, Gilbertsville, PA) (1:10,000 dilution), the membranes were scanned in 2 different channels using the Odyssey IR imaging system (Li-Cor Biosciences). Band intensities were quantified using the Odyssey software. All samples were run in quadruplicate and band intensities were normalized to vehicle controls. The mean ethanol and vehicle band intensities of samples derived from the nucleus accumbens, caudate-putamen, hippocampus, and amygdala were compared by a *t*-test, with significance at $p<0.05$.

Antibody Specificity

The specificity of phospho-PKCγ (p-cPKC) antibody was confirmed by dephosphorylating proteins in a cortical membrane fraction (P2) by incubating the P2 fraction with protein phosphatase enzyme (λ-PPase, 40,000 U, New England Biolabs, Ipswich, MA) for 90 minutes (30 °C). Both control and de-phosphorylated proteins were subjected to SDS-PAGE analysis and probed with p-PKCγ or PKCγ (Abcam, Cambridge, MA). As p-PKCγ and PKCγ bands are approximately of the same size (~80 kDa), membranes were stripped using Restore™ (Western blotting stripping buffer, Pierce Biotechnology, Rockford, IL) and reprobed with the PKCγ antibody.

Anatomical Coordinates

Anatomical coordinates used for analysis were according to the atlas of Paxinos and Watson (1998). Analysis of PKCγ and p-cPKC (based on the nonspecific p-PKCγ antibody) immunoreactivity was conducted in coronal sections encompassing mesocorticolimbic brain regions that have been shown to regulate the behavioral effects of ethanol including the nucleus accumbens, prefrontal cortex, hippocampus, and amygdala (Hodge et al., 1992, 1996; Hodge and Cox, 1998). Analyses of the nucleus accumbens and proximal brain regions were in sections ranging from 1.0 to 1.7 mm anterior to Bregma. The hippocampus, amygdala, and proximal regions were evaluated in coronal sections ranging from −2.56 to −3.3 mm posterior to Bregma.

RESULTS

Antibody Specificity

The specificity of the p-PKC*γ* and PKC*γ* antibodies was evaluated by Western blot analysis. As shown in Fig. 1A and 1B, both antibodies detected a single band at the appropriate molecular weight, demonstrating that there were no cross-reacting bands labeled by the antibody in the cortical membrane (P2) fraction. Phospho-specificity of the p-PKC*γ* (p-cPKC) antibody was confirmed by dephosphorylating proteins in cortical membrane fraction (P2) via preincubation with protein phosphatase enzyme (Fig. 1A).

Distribution of PKC*γ* and p-cPKC in Rat Brain

Phosphorylation of cPKC—Immunoreactivity of p-cPKC was detected in numerous brain regions (Table 1). The highest density of p-cPKC immunoreactivity was observed in the indusium griseum, basolateral amygdala, and CA3 regions of the hippocampus. Moderate and low levels of immunolabeling were observed in most other structures including the prefrontal cortex, nucleus accumbens, amygdala, and thalamus. The cytological pattern of p-cPKC immunoreactivity suggested diverse cellular localization with intense immunoreactivity in the nucleus or membrane of cells as well as a more diffuse pattern (Table 1). Membrane-like immunoreactivity was observed throughout the cortex, the basolateral amygdala, regions of the hippocampus, and other structures. By contrast, the cytological pattern of p-cPKC immunoreactivity appeared to be nuclear in portions of the striatum including the nucleus accumbens.

PKC-γ—PKC-*γ* immunoreactivity was detected in the same brain regions that expressed p-cPKC. Nuclei showing the highest density of immunoreactivity included the prefrontal cortex, nucleus accumbens core, lateral septum, amygdala, subnuclei of the hippocampus, and several thalamic nuclei (Table 1). The nucleus accumbens shell, dorsal caudate, dentate gyrus of the hippocampus, and other brain regions were classified as showing a medium density of PKC*γ* immunoreactivity. Low levels of immunoreactivity were detected in motor, somatosensory, and other cortical regions. In general, PKC*γ* was expressed in numerous cells and fibers in most of the brain regions listed in Table 1. The cytological pattern of PKC*γ* immunoreactivity suggested a diverse localization with intense immunoreactivity in the nucleus or membrane of cells as well as a more diffuse pattern in a variety of brain regions (Table 1).

Effect of Acute Ethanol on p-cPKC Immunoreactivity

Nucleus Accumbens, Lateral Septum, and Caudate Nucleus—Acute ethanol (0.5–2 g/kg, IG) induced a rapid and dose-dependent increase in p-cPKC immunoreactivity in the nucleus accumbens and lateral septum. Both the nucleus accumbens core [$F(3, 23)=5.9$, $p=0.005$] and shell [$F(3, 23)=3.4$, $p=0.04$] showed significant increases in p-cPKC immunoreactivity 10 minutes after acute ethanol treatment that were solely attributable to the highest dose of ethanol (2 g/kg) tested (Fig. 2A). The increase in immunoreactivity in the nucleus accumbens shell was associated with a significant increase in the number of p-cPKC-positive cells. Under vehicle conditions, the mean ± SEM number of p-cPKC-positive cells in the nucleus accumbens shell was $27.8 \pm 7.5/mm^2$ and this was significantly increased by almost 10-fold to $246.7 \pm 75/mm^2$ after ethanol (2.0 g/kg) administration [$t(10)=2.9$, $p<0.05$]. Similarly, acute ethanol significantly increased the number of p-cPKC-positive cells in the nucleus accumbens core from 39.7 ± 6.4 to $208.4 \pm 71/mm^2$ [$t(10)=2.6$, $p<0.05$]. In the lateral septum, only 2 g/kg ethanol significantly increased p-cPKC immunoreactivity [$F(3, 23)=4.2$, $p=0.02$] but there was a trend toward an increase following 1 g/kg ethanol. The ethanol-induced increase in p-cPKC immunoreactivity in the lateral septum was not associated with a significant increase in the number of c-PKC-positive cells, which is reflective of an increase in diffuse staining in this brain region. Representative photomicrographs illustrating the cytological

pattern of p-cPKC immunoreactivity in the lateral septum and nucleus accumbens following ethanol (0 or 2 g/kg) are shown in Fig. 2B. No effect of acute ethanol injection on p-cPKC immunoreactivity was observed in a variety of other proximal nuclei including the caudate nucleus (Fig. 2A), prelimbic cortex, and piriform cortex (data not shown). Total PKCγ immunoreactivity was unchanged in all brain regions (data not shown).

Hippocampus and Amygdala—The effects of acute ethanol (0.5–2.0 g/kg) administration on p-cPKC immunoreactivity in the hippocampus and amygdala were dose and brain region specific. In the granule cell layer of the dentate gyrus of the hippocampus, acute ethanol produced a significant increase in p-cPKC immunoreactivity following ethanol [$F(3, 23)=8.1$, $p=0.001$]. Similarly, acute ethanol increased p-cPKC immunoreactivity in the CA3 subregion of the hippocampus [$F(3, 23)=3.3$, $p=0.04$]. In both cases, the significant increase was due to the effects of the highest dose of ethanol (Fig. 3A). Other subregions of the hippocampus, including CA1, showed no significant change in p-cPKC immunoreactivity in response to ethanol. There was also no significant change in the number of p-cPKC-positive cells in any subregion of the hippocampus reflecting the mostly diffuse cytological pattern of immunoreactivity (Fig. 3B). Acute ethanol produced no change in p-cPKC immunoreactivity in a number of nuclei proximal to the hippocampus including the thalamus, rhinal cortex, and piriform cortex (data not shown). There was also no change in the central and basolateral nuclei of the amygdala (Fig. 3A), although there was a trend in the central amygdala (Fig. 3A and 3B). In addition, no changes were observed in total PKCγ immunoreactivity in any brain region, suggesting that the ethanol-induced changes in p-cPKC were not related to changes in the abundance of conventional PKC.

Effect of Ethanol on Membrane Expression of p-cPKC

To determine whether the ethanol-induced increases in p-cPKC immunoreactivity were associated with changes in the cellular distribution of the kinase, P2 membrane fractions were prepared from nucleus accumbens, caudate nucleus, hippocampus, and amygdala tissue that was dissected 10 minutes following ethanol (0 or 2 g/kg, IG). Western blot analysis showed that acute ethanol (2 g/kg, IG) significantly increased p-cPKC in P2 membrane fractions in the nucleus accumbens and hippocampus (Fig. 4A and 4B). No significant changes in membrane expression of p-cPKC were observed in the caudate nucleus or amygdala, which corresponds to the lack of change in these regions seen with immunohistochemistry. Moreover, there was no significant change in total PKCγ membrane expression in the nucleus accumbens (101.2% of water control) or hippocampus (103% of water control) following acute ethanol, suggesting that the effects were specific to phosphorylation and not related to changes in the abundance or cellular location of conventional PKCγ.

DISCUSSION

Phosphorylation is a general mechanism that regulates the function of protein kinases (Krebs et al., 1959). The activity and stability of cPKC isoforms are modulated by a series of phosphorylations at 3 conserved sites: the activation loop, the turn motif, and the hydrophobic motif (Newton, 2003). Phosphorylation of the hydrophobic site is thought to be important for kinase stability (Edwards and Newton, 1997) and function as it provides a docking site for PDK-1 (Gao et al., 2001). Ethanol can increase intracellular Ca^{2+} and DAG (Hoek and Rubin, 1990), which then leads to cPKC association with cell membranes where it can phosphorylate target proteins and modulate cell signaling.

The present study was conducted to determine whether acute ethanol exposure alters phosphorylation of cPKC in vivo. We found that acute IG administration of ethanol produces a rapid dose-dependent increase in cPKC phosphorylation in specific limbic brain regions.

Ethanol (2 g/kg) administration increased p-cPKC immunoreactivity in the lateral septum, nucleus accumbens (core and shell), and hippocampus (dentate gyrus and CA3) 10 minutes after administration. Numerous other brain regions including the cortex, thalamus, striatum, and amygdala showed no change in p-cPKC immunoreactivity. Interestingly, Western blot analysis also showed that p-cPKC was increased in the P2 membrane fraction in the nucleus accumbens and hippocampus. These results suggest that acute ethanol increases cPKC activity in a brain region-dependent manner.

Emerging evidence indicates that cPKC isoforms regulate the specific behavioral, biochemical, and physiological effects of ethanol (Bowers and Wehner, 2001; Proctor et al., 2003; Ron et al., 2000) that are consistent with the anatomical specificity observed in this study. For example, mice carrying a null mutation for PKCγ are less sensitive to the acute hypnotic effects of ethanol compared with the wild-type mice (Harris et al., 1995). Although it is not clear which brain region(s) regulate this effect, mice that were genetically selected for differential sensitivity to ethanol hypnosis (short-sleep and long-sleep mice) exhibit differential functional effects of ethanol in the hippocampus (Hanania et al., 2000). Here, we show that acute ethanol increases cPKC phosphorylation in the hippocampus. When taken together with evidence showing that PKCγ null mice show no ethanol-induced enhancement of hippocampal IPSCs (Proctor et al., 2003), these data suggest that cPKC activity in the hippocampus may mediate sensitivity to acute ethanol.

Other evidence indicates that PKCγ null mice consume more ethanol than wild-type control mice in a 2-bottle choice test (Bowers and Wehner, 2001). Although the neurobiological systems that regulate ethanol self-administration remain to be fully characterized, it is widely accepted that the nucleus accumbens plays a role in this behavior (e.g., Hodge et al., 1995, 1992; Rassnick et al., 1992; Weiss et al., 1993). We previously showed that PKCε null mice self-administer less ethanol than wild-type controls and show no increase in dopamine levels in the nucleus accumbens after acute ethanol treatment (Olive et al., 2000), suggesting that PKC modulation of dopamine in this brain region may regulate ethanol self-administration. In the present study, we show that ethanol increases the overall density of p-cPKC immunoreactivity, and the number of p-cPKC-positive cells, in the nucleus accumbens core and shell. We also obtained evidence showing that acute ethanol increased p-cPKC protein levels in the P2 membrane fraction in the nucleus accumbens. Similarly, previous work has shown that chronic exposure to an ethanol-containing liquid diet is associated with an increase in the abundance of cPKC isoforms (PKCα and PKCγ) in cell membranes within the larger limbic forebrain (Narita et al., 2001). Together, these findings suggest that activation of cPKC in the nucleus accumbens by ethanol may be a functional part of the central nervous system response to ethanol that inhibits, or terminates, ethanol self-administration. It would be of interest to know if PKCγ null mice show increased dopamine release in the nucleus accumbens after acute ethanol, which would be the inverse of PKCε null mice and correlate with differential alcohol intake between these lines of PKC mutant mice (e.g., Bowers and Wehner, 2001; Hodge et al., 1999).

An important finding of this study is that ethanol increased membrane-bound protein levels of the active (e.g., phosphorylated) form of cPKC in the nucleus accumbens and hippocampus. Although previous work has shown that ethanol can alter the cellular location of PKC isoforms (e.g., Gordon et al., 2001; Ron et al., 2000), these data are the first to show ethanol-induced increases in the mature, or phosphorylated, form of cPKC at the cell membrane. This finding is particularly important in view of evidence showing that chronic ethanol consumption decreases the association of PKCγ with $GABA_A$ α1 receptor subunits in vivo (Kumar et al., 2002). Together, these data suggest that acute ethanol may increase, whereas chronic ethanol may decrease, PKCγ activity at the most abundant form of $GABA_A$ receptors in the mammalian brain regions, which are known to regulate GABAergic properties of ethanol (Hodge et al.,

1995; Hodge and Cox, 1998). This may partly explain why PKCγ null mice are insensitive to acute ethanol (Harris et al., 1995) and show decreased tolerance to chronic exposure (Bowers et al., 1999). Additional work is needed to determine whether cPKC isoforms are required for ethanol-induced changes in $GABA_A$ receptor function.

Another potentially significant aspect of the present results is the rapid effect of ethanol on cPKC phosphorylation. We focused on a single 10 minutes time point after a single ethanol exposure. Ethanol levels are known to peak in the blood and brain between 10 and 20 minutes after administration (Ferraro et al., 1990, 1991; Quertemont et al., 2003), which corresponds well with the time course of the acute locomotor-activating and sedative/hypnotic effects of the drug (Hodge et al., 1999). Thus, the rapid induction of p-cPKC phosphorylation by ethanol suggests that altered activity of this class of PKC isoforms may play a role in the immediate effects of acute ethanol on brain and behavioral function. Interestingly, however, the effects of ethanol in the present study were dose dependent, with changes in cPKC phosphorylation observed only after the highest dose (2 g/kg) was tested. This suggests that some of the low-dose acute effects of ethanol, such as anxiolysis, may not be mediated by cPKC activity (but see Bowers et al., 2001).

Overall, the results of this study contribute to growing evidence regarding the complex, and differential, role of specific PKC isoforms in the biobehavioral effects of ethanol. For example, work with gene knockout mice has shown that PKCγ and PKCε may differentially regulate acute ethanol sensitivity and self-administration. As mentioned above, PKCγ null mice are less sensitive than wild-type controls to the acute hypnotic effects of ethanol (Harris et al., 1995) and, accordingly, consume more ethanol in a home-cage preference test (Bowers and Wehner, 2001). This is consistent with the present results showing that activation (i.e., increased phosphorylation) of cPKC is a response to acute ethanol, which, if removed by gene knockout, would be predicted to blunt acute response to ethanol. By contrast, PKCε null mice are more sensitive to acute ethanol and show reduced self-administration (Besheer et al., 2006; Hodge et al., 1999). This suggests that acute ethanol might inhibit PKCε activity, or its functional effects, which would lead to enhanced behavioral response. Additional work is needed to address this question and to determine the effects of acute, and chronic, ethanol on the activity of specific PKC isoforms in vivo.

In conclusion, the results of this study show that acute IG administration produces a rapid and dose-dependent increase in cPKC phosphorylation in the mammalian brain. Conventional PKC immunoreactivity was detected throughout the brain, but ethanol-induced changes were restricted to subregions of the septum, ventral striatum, and hippocampus. Increased immunoreactivity in the nucleus accumbens and hippocampus was associated with an increase in membrane-bound p-cPKC. It will be both interesting and important to determine whether these ethanol-induced changes in p-cPKC alter its association with specific neurotransmitter receptors, or other membrane targets, that regulate acute response to ethanol.

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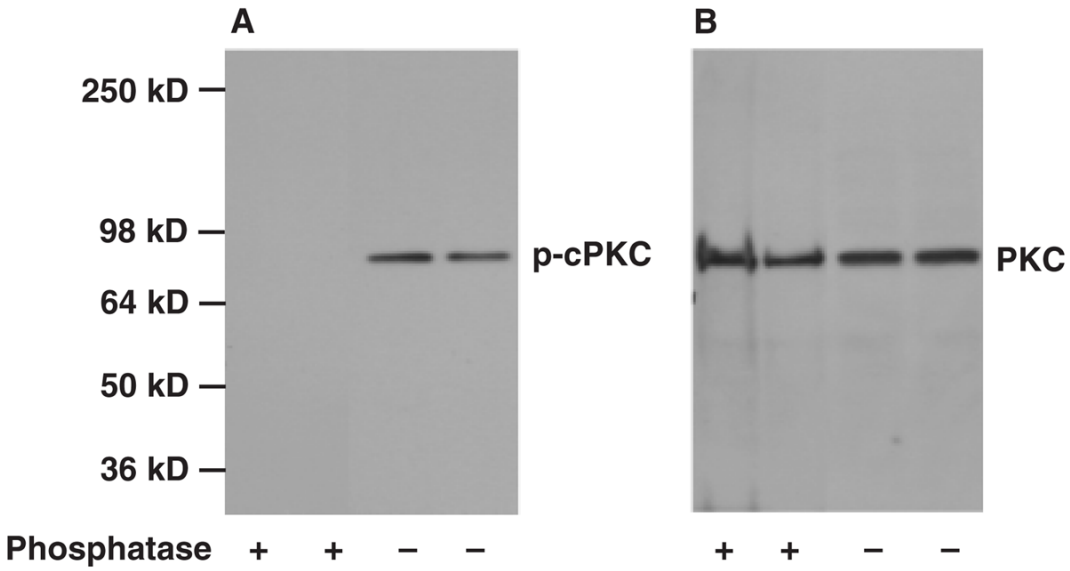

Fig. 1.

Antibody specificity. (**Panel A**) Representative Western blot showing phospho-specificity of the p-PKC*γ* (noted as p-cPKC) antibody. Single bands of approximately 84 kDa were detected in the control cortical P2 fraction (lanes 3 and 4) but no bands were apparent in dephosphorylated protein (lanes 1 and 2). (**Panel B**) After probing with p-PKC*γ*, membrane was stripped and reprobed with the PKC*γ* antibody, which also detected a single band at approximately 80 kDa that was not altered by dephosphorylation. p-PKC*γ*, phosphorylated protein kinase C-*γ*; p-cPKC, phosphorylation of conventional PKC.

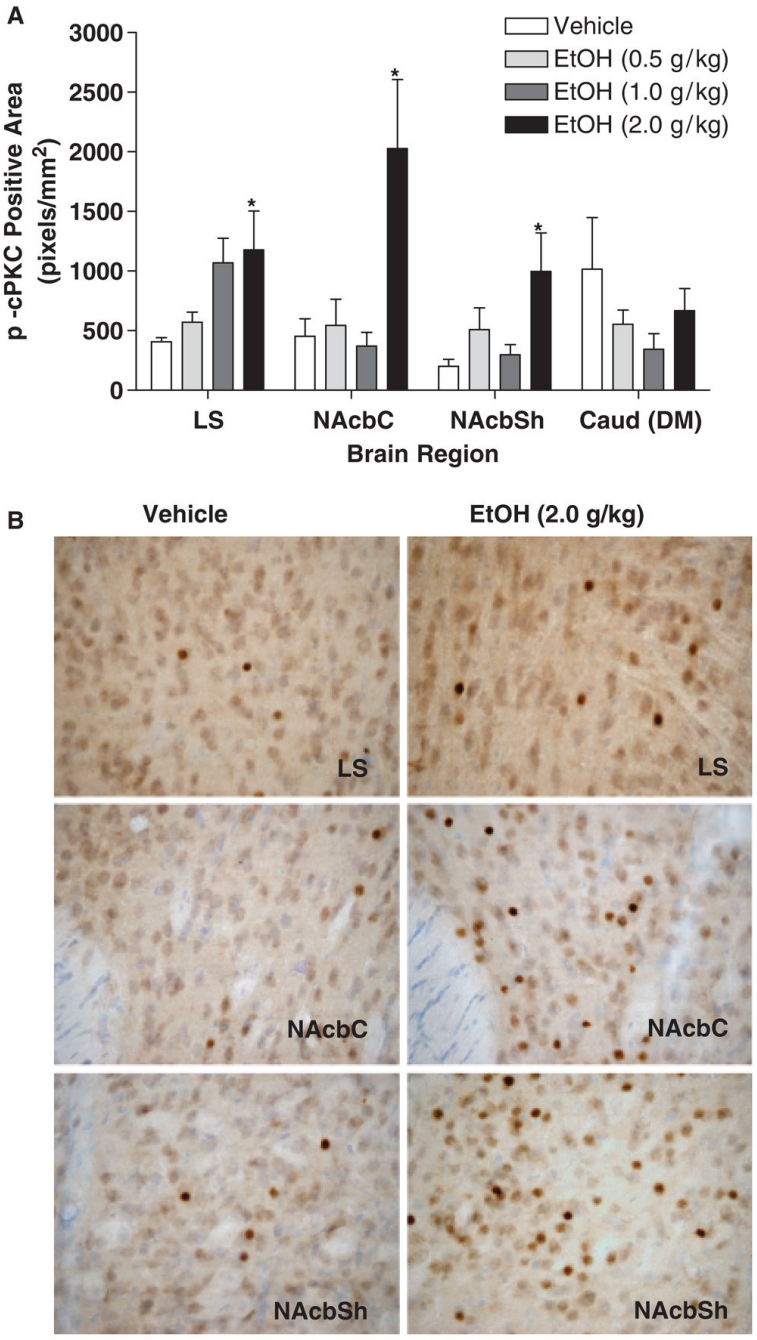

Fig. 2.

(**Panel A**) Mean (± SEM) immunoreactivity of the p-cPKC-positive area (pixels/mm2) in the lateral septum (LS), nucleus accumbens core (NAcbC), and shell (NAcbSh), and the caudate nucleus following treatment with vehicle, 0.5, 1.0, and 2.0 g/kg ethanol. *p<0.05—vehicle versus 2 g/kg ethanol. p-cPKC, phosphorylation of conventional protein kinase C. (**Panel B**) Representative photomicrographs of the cytological pattern of p-cPKC immunoreactivity in the LS, NAcbC, and NAcbSh following treatment with vehicle (left column) or ethanol 2 g/kg (right column).

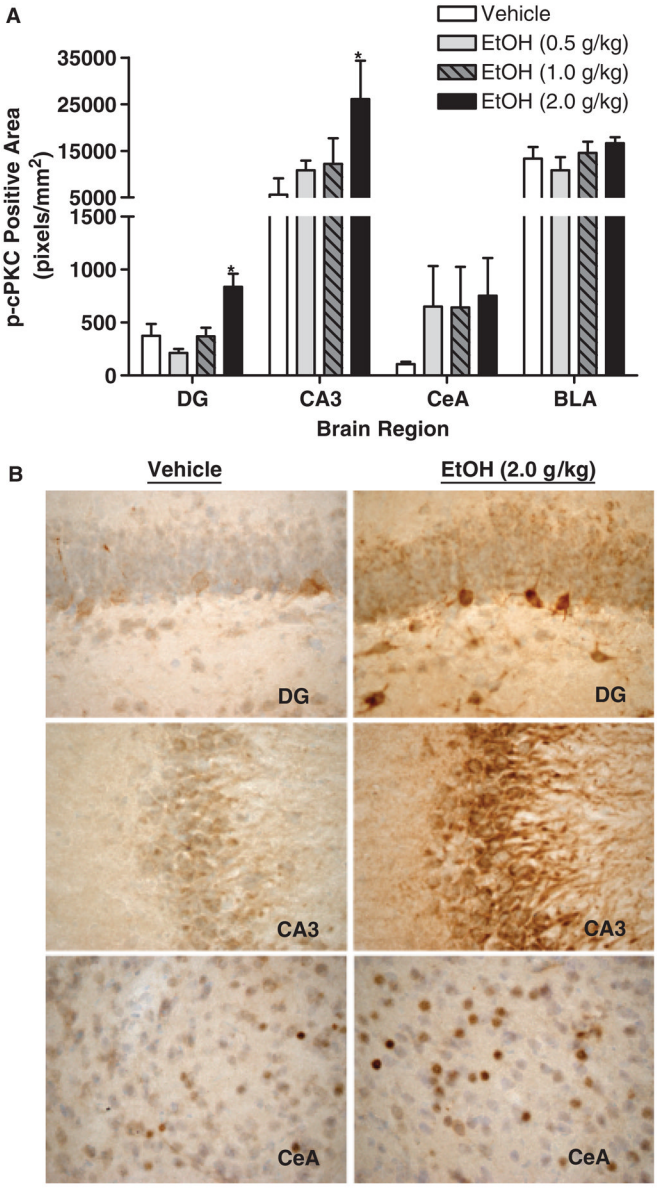

Fig. 3.

(**Panel A**) Mean (± SEM) immunoreactivity of the p-cPKC-positive area (pixels/mm^2) in the dentate gyrus (DG), the CA3 subregion of the hippocampus, and the central (CeA) and basolateral (BLA) nuclei of the amygdala following treatment with vehicle, 0.5, 1.0, and 2.0 g/kg ethanol. *p<0.05—vehicle versus 2 g/kg ethanol. p-cPKC, phosphorylation of conventional protein kinase C. (**Panel B**) Representative photomicrographs of the cytological pattern of p-cPKC immunoreactivity in the DG, the CA3 subregion of the hippocampus, and CeA following treatment with vehicle (left column) or ethanol 2 g/kg (right column).

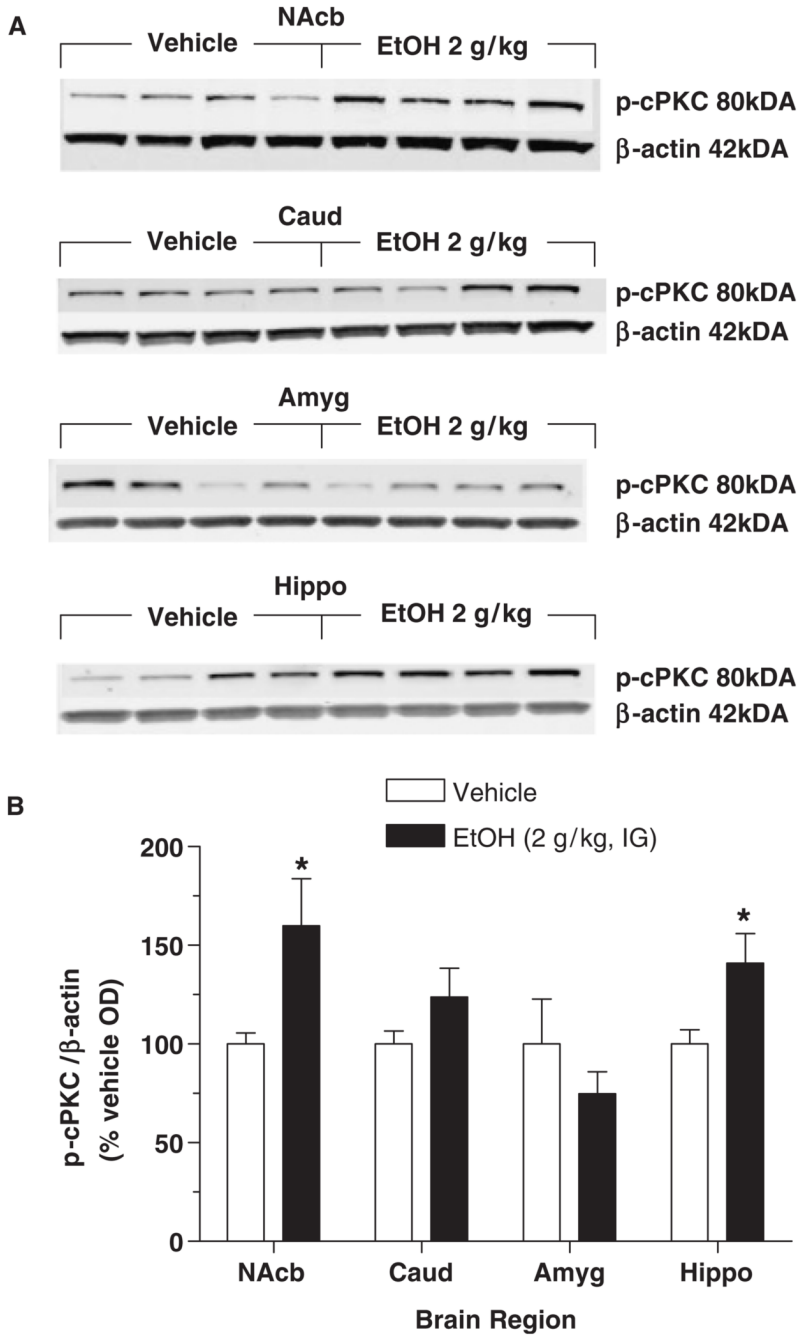

Fig. 4.

(**Panel A**) Mean (± SEM) phosphorylated cPKC relative to *β*-actin control in the P2 membrane fractions in the nucleus accumbens (NAcb), caudate nucleus (Caud), amygdala (Amyg), and hippocampus (Hippo) following treatment with vehicle or ethanol (2 g/kg). (**Panel B**) Immunoblots for phosphorylated cPKC and *β*-actin control from NAcb, Caud, Amyg, and Hippo following treatment with vehicle or ethanol (2 g/kg). **p*<0.05. cPKC, conventional protein kinase C.

Table 1

Evaluation of the Immunocytochemical Distribution of p-cPKC and PKCγ in Rat Brain

	p-cPKC (T674)		PKCγ	
Brain region	**IR[a]**	**Location[b]**	**IR[a]**	**Location[b]**
Cerebral cortex				
Prefrontal	++	m	++++	m
Motor (M1)	++	m	++	m
Somatosensory (S1)	+	m	+	m
Rhinal	++	m	++++	d
Piriform	++	m	++++	d
Olfactory tubercle	+	m	++	d
Nucleus accumbens				
Core	++	n	++++	m/d
Shell	++	n	+++	m/d
Caudate (dorsal medial)	+++	n	+++	m
Lateral septum	+++	n/d	++++	d
Indusium griseum	++++	m/d	++++	m
Amygdala				
Basolateral	+++	m	++++	m/d
Central	++	m	++++	m/d
Hippocampus				
CA1	+++	m	++++	m/d
CA3	+++	m	++++	m/d
Fissure	+++	d	++++	d
Dentate gyrus				
Granule/subgranule	++	m	+++	m
Polymorph layer	+	d	+++	d
Subiculum	+++	m/d	++++	m/d
Thalamus				
Dorsal	+++	m	++++	m
Reuniens	++	m	+++	m
Xiphoid	+	d	++++	m
Paraventricular	++	m	++++	m
Arcurate nucleus	+	m/d	+++	m/d

[a] Immunoreactivity (IR) was ranked as minimal (+), low (++), medium (+++), or high (++++).

[b] Cellular location was characterized as membrane (m), nucleus (n), or diffuse (d).

p-cPKC, Phosphorylation of conventional protein kinase C.