Ethanol Regulation of D_1 Dopamine Receptor Signaling is Mediated by Protein Kinase C in an Isozyme-Specific Manner

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Ethanol consumption potentiates dopaminergic signaling that is partially mediated by the D_1 dopamine receptor; however, the mechanism(s) underlying ethanol-dependent modulation of D_1 signaling is unclear. We now show that ethanol treatment of D_1 receptor-expressing cells decreases D_1 receptor phosphorylation and concurrently potentiates dopamine-stimulated cAMP accumulation. Protein kinase C (PKC) inhibitors mimic the effects of ethanol on D_1 receptor phosphorylation and dopamine-stimulated cAMP levels in a manner that is non-additive with ethanol treatment. Ethanol was also found to modulate specific PKC activities as demonstrated using *in vitro* kinase assays where ethanol treatment attenuated the activities of lipid-stimulated $PKC\gamma$ and $PKC\delta$ in membrane fractions, but did not affect the activities of $PKC\alpha$, $PKC\beta_1$, or $PKC\varepsilon$. Importantly, ethanol treatment potentiated D_1 receptor-mediated DARPP-32 phosphorylation in rat striatal slices, supporting the notion that ethanol enhances D_1 receptor signaling *in vivo*. These findings suggest that ethanol inhibits the activities of specific PKC isozymes, resulting in decreased D_1 receptor phosphorylation and enhanced dopaminergic signaling.
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INTRODUCTION

Dopaminergic signaling in the brain is the target of many drugs of abuse, including alcohol. Changes in dopaminergic signaling within the mesolimbic network contribute to the rewarding and reinforcing effects of alcohol abuse (Samson *et al*, 1992). A major component of the mesolimbic dopaminergic system is the nucleus accumbens (NAc) that receives dopaminergic innervations from the ventral tegmental area (VTA). Among the many effects of ethanol (EtOH) consumption is the potentiation of dopamine (DA) release from the VTA dopaminergic neurons and the elevation of DA levels in the NAc (Weiss *et al*, 1993; Brodie *et al*, 1999). During withdrawal, in the absence of EtOH, the spontaneous activity of the VTA dopaminergic neurons is reduced (Shen and Chiodo, 1993; Shen, 2003). Given the well-accepted role of DA in addiction and reward pathways, it is not surprising that DA receptors have been implicated in alcohol-related behaviors.

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DA receptors are members of the G-protein-coupled receptor superfamily and five distinct subtypes have been cloned and pharmacologically characterized (D_1–D_5). Although gene deletion and pharmacological studies have implicated both D_1 and D_2 receptors in mediating dopaminergic responses to EtOH administration (El-Ghundi *et al*, 1998; Phillips *et al*, 1998; Eiler *et al*, 2003; Inoue *et al*, 2007), the D_1 subtype appears to be particularly important with respect to the rewarding properties of EtOH and motivation for its consumption. For instance, administration of the D_1 receptor antagonists SCH23390 or ecopipam decreases EtOH consumption in mice, whereas administration of the D_1 agonist SKF81297 with EtOH facilitates alcohol-related behaviors (El-Ghundi *et al*, 1998; D'Souza *et al*, 2003; Price and Middaugh, 2004). DARPP-32, the downstream target of D_1 receptor signaling, has also been implicated in EtOH-mediated signaling. At the cellular level, EtOH treatment increases phosphorylation of DARPP-32 and is reported to regulate NMDA receptor activity in the NAc (Maldve *et al*, 2002). Moreover, in mice lacking DARPP-32, EtOH-dependent reinforcement is absent (Maldve *et al*, 2002). Despite a growing body of evidence implicating D_1 receptor function in EtOH-mediated behaviors, nothing is known about the mechanisms by which EtOH regulates D_1 receptor signaling.

In the present study, we show that EtOH treatment of cells expressing the D_1 receptor diminishes its phosphorylation

state, resulting in enhanced DA stimulation of intracellular cyclic AMP (cAMP) levels. These effects of EtOH appear to be mediated through cellular inhibition of specific protein kinase C (PKC) isozymes that constitutively phosphorylate the D$_1$ receptor and dampen its signaling potential. We additionally show that EtOH treatment potentiates D$_1$ receptor-stimulated DARPP-32 phosphorylation in rat striatal slices, thus supporting the notion that EtOH enhances D$_1$ receptor-signaling pathways in vivo. These results reveal a new mechanism of action for how EtOH consumption potentiates dopaminergic neurotransmission and activates reward and reinforcement pathways in the brain.

MATERIALS AND METHODS

Materials

HEK293-tsa201 (HEK293T) cells were a gift from Dr V Ramakrishnan. FLAG-PKCε and HA-PKCβ1 were gifts from Dr A Toker. HA-PKCδ and 3XFLAG-PKCγ were gifts from Dr JF Mushinski (Mischak et al, 1993) and Dr A Newton, respectively. GFP-PKCα was a gift from Dr M Akbar. The CnA* construct was a kind gift from Dr S Williams. The G-protein-coupled receptor kinase 2 (GRK2) and GRK3 constructs were a kind gift from Dr JL Benovic. Okadaic acid and cantharidin were purchased from Sigma (St Louis, MO). Rottlerin and GF109203X were purchased from Tocris (Ellisville, MI). Gö6983 and the PKCβ inhibitor 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione were purchased from Calbiochem (San Diego, CA). FK-506 was purchased from LC Laboratories (Woburn, MA).

Cell Culture and Transfection

HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 µg/ml streptomycin, and 50 U/ml gentamicin at 37°C in 5% CO$_2$. HEK293T cells were seeded in 150-mm culture dishes and transfected 24 h later by calcium phosphate precipitation (Clontech, Mountain View, CA). Each plate was transfected with FLAG-tagged rat D$_1$ receptor DNA (Monsma et al, 1990) or cotransfected with vector, CnA*, GRK2, GRK3, or PKC DNA constructs.

Radioligand-Binding Assays

Transfected HEK293T cells were incubated in the presence or absence of EtOH, harvested in Ca^{2+}/Mg^{2+}-free EBSS supplemented with 5 mM EDTA, and centrifuged at 200g for 10 min. Cells were lysed in a dounce homogenizer in 5 mM Tris-HCl, pH 7.4 (at 4°C), and 5 mM MgCl$_2$. The lysate was centrifuged at 20 000g for 30 min and the pellet resuspended in 50 mM Tris-HCl (crude membrane fraction). A portion of the membrane suspension was quantitated using BCA protein assay kit (Pierce, Rockford, IL). The membrane fraction (100 µl) was added to tubes in triplicate containing [^{3}H]-SCH23390 (Perkin Elmer, Boston, MA) at a range of concentrations. Non-specific binding was determined in the presence of (+)-butaclamol (3 µM) (Sigma). Assay tubes were incubated at room temperature for 1.5 h and then terminated by rapid filtration through GF/C filters pre-treated with 0.6% polyethyleneimine. Bound radioactivity was quantitated by liquid scintillation counting.

cAMP Assay

Transfected HEK293T cells were seeded into 24-well plates coated with poly-D-lysine. Duplicate wells were exposed to dopamine dilutions that were prepared in 20 mM HEPES buffered DMEM supplemented with 200 µM sodium meta-bisulfite and 30 µM RO-20-1724 (a phosphodiesterase inhibitor) (Sigma). Basal activity was determined in the absence of dopamine. The plates were incubated at 37°C for 15 min. The reaction was terminated by removing the medium and adding 3% perchloric acid to each well for 30 min on ice. Each reaction was neutralized by adding 15% KHCO$_3$. cAMP accumulation was measured using the [^{3}H]-cAMP assay kit from DPC® (Los Angeles, CA).

In Situ Phosphorylation Assay

These assays were performed as described previously (Rankin et al, 2006). Briefly, transfected HEK293T cells were seeded into six-well plates coated with poly-D-lysine. A portion of the transfection was retained in a 100-mm dish for radioligand-binding assay to quantitate the expression of D$_1$ receptor expression. Forty-eight hours post transfection, medium from each well was replaced with phosphate-free medium supplemented with 10% FBS, 50 U/ml penicillin, and 10 µg/ml gentamicin, and incubated for 1 h. The medium was replaced with 1 ml phosphate-free DMEM containing 106 µCi/ml [^{32}P]-orthophosphoric acid (Perkin Elmer) for 50 min. EtOH was added to appropriate wells and incubated for a further 10 min. Subsequently, specific wells were challenged with basal medium ± EtOH or media containing 10 µM dopamine ± EtOH for 10 min. The cells were placed on ice, rinsed with ice-cold EBSS, and lysed with solubilization buffer (50 mM HEPES, pH 7.4, 40 mM sodium pyrophosphate, 50 mM NaF, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM EDTA) supplemented with MiniComplete™ protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 1 h at 4°C. Cell lysates were cleared by centrifugation and protein concentration were quantitated using BCA protein assay kit. D$_1$ receptor expression was determined by radioligand-binding assays using cells seeded in the 100-mm dish. Equal amounts of D$_1$ receptor for each condition were incubated with anti-FLAG-M2 agarose gel (Sigma) overnight, washed three times, and resolved using 4–12% Bis–Tris NuPage gels using MOPS buffer (Invitrogen, Carlsbad, CA). Dried gels were subjected to autoradiography.

PKC Activity

HEK293T cells expressing tagged PKC isozymes were seeded into duplicate culture dishes. One plate was used for EtOH pretreatment (100 mM/10 min). Cells were harvested in phosphate-buffered saline (PBS), centrifuged at 200g for 10 min, and the pellet was homogenized in 1 ml homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 30 mM NaF, and 20 mM sodium pyrophosphate) supplemented with MiniComplete protease

inhibitor cocktail. The samples were centrifuged at 20 000g for 30 min at 4°C and the supernatant was collected as the cytosolic fraction. The pellet was resuspended and homogenized with 1 ml homogenization buffer containing 1% Triton X-100 and supplemented with MiniComplete protease inhibitor cocktail. The homogenate was centrifuged for 5 min at 9000g and the supernatant used as the membrane fraction. For FLAG-tagged or HA-tagged PKC constructs, each fraction was transferred to tubes containing 50 μl of equilibrated anti-FLAG M2-gel or anti-HA agarose gel (Sigma), respectively, and rotated for 60 min at 4°C. For the GFP-tagged PKC construct, fractions were incubated with a rabbit polyclonal antibody to GFP (Novus Biologicals, Littleton, CO) and rotated for 60 min at 4°C, followed by incubation with 50 μl equilibrated protein-G agarose for a further 60 min at 4°C. Samples were centrifuged at 9000g for 1 min at 4°C and the pellets were washed three times with PBS. Each pellet was resuspended with 70 μl PBS. Kinase assays were performed using a PKC assay kit (Upstate, Lake Placid, NY) according to the manufacturer's instructions. The kinase activity in 10 μl of each immunoprecipitated fraction was assayed by measuring the transfer of $^{32}P_i$ from $[\gamma\text{-}^{32}P]ATP$ to a specific substrate peptide (Upstate). PKC activity was measured in an assay containing assay dilution buffer II (ADBII end concentration–3.33 mM MOPS (pH 7.2), 4.2 mM β-glycerol phosphate, 0.17 mM sodium orthovanadate, 0.17 mM dithiothreitol, and 0.12 mM CaCl$_2$), 83 μM PKC substrate peptide, 0.3 μM PKA inhibitor peptide, 3.3 μM CaMK inhibitor, and PKC lipid activators (80 ng/μl phosphatidyl serine (PS), and 8 ng/μl diacylglycerol (DAG)). EtOH was added from a concentrated stock solution. Maximal PKC activity was achieved in the presence of PS and DAG. Basal activity was measured in the presence of 0.5 mM EGTA instead of PS and DAG. Non-specific activity was determined in the absence of substrate peptide. Each condition was performed in duplicate. Kinase reactions were initiated by adding 10 μCi $[\gamma\text{-}^{32}P]ATP$ in Mg^{2+}/ATP cocktail, and were incubated for 10 min at 30°C. The reactions were terminated by transferring 25 μl of the mixture to P81 filter papers, followed by washing three times with 0.75% phosphoric acid and once with acetone. The amount of phosphorylated peptide was determined by scintillation counting.

Immunohistochemistry

Fresh frozen rat brains were sectioned at 10 μm in the coronal plane, and three sections were mounted onto glass microscope slides. After drying at room temperature until the sections were opaque, slides were treated at the same temperature as follows: control, incubated in PBS (30 min); agonist–control, incubated in PBS (15 min) then SKF 81297 (1 μM, 15 min); ethanol–control, incubated in 25 mM EtOH (15 min) then PBS (15 min); ethanol–agonist, incubated in 25 mM EtOH (15 min) then SKF 81297 (1 μM, 15 min). Slides were dipped in PBS, fixed in 4% paraformaldehyde buffered in PBS for 5 min, followed by a 5-min PBS rinse. Sections were incubated in primary phospho-DARPP-32 antisera (diluted 1:100; Chemicon Inc, Temecula CA) overnight in a humidified chamber at 4°C. Slides were rinsed in PBS then exposed to fluorescently labeled secondary antisera (Invitrogen Molecular Probes, Carlsbad, CA) for 1.5 h in a humidified chamber at 4°C. After a final PBS rinse, sections were examined immediately using epifluorescence. Image acquisition and analysis was as described previously (Ariano et al, 2005). Briefly, image exposure durations were based on the control sections, and were taken from this treatment set. The other three treatments used these settings, and again, a minimum of six images was obtained. Fluorescence intensities were determined using the histogram function of Adobe Photoshop (San Jose, CA) and compared with the control condition, which was arbitrarily set to 100. Experiments were performed three times.

Data Analysis

For phosphorylation assays, the relative intensities of bands on the autoradiographs were determined by scanning and analyzing the bands using LabWorks 4.0 (UVP Inc., Upland, CA). Figures depict representative graphs or autoradiographs for each experimental condition. All experiments were performed at least three independent times. Where shown, data are presented as the mean ± SEM. Comparisons of all data to the control were performed using analysis of variance (one-way ANOVA) and Dunnett's post hoc test. Comparisons between selected data pairs were analyzed using ANOVA followed by the Bonferroni post test, or in some cases by Student's t-test. All statistical analyses were performed with a level of significance established at $p < 0.05$. Statistical analyses were conducted using GraphPad Prism 4 (GraphPad Prism Inc., San Diego, CA) software.

RESULTS

### Ethanol Treatment Modulates D$_1$ Receptor Phosphorylation and Signaling

EtOH-dependent modulation of DA-stimulated cAMP levels was examined in HEK293T cells expressing the FLAG-tagged rat D$_1$ receptor. Importantly, HEK293T cells provide a suitable cellular environment to examine D$_1$ receptor signaling, desensitization, and trafficking (Rankin et al, 2006). Treatment of cells with EtOH (100 mM) for 15 min significantly potentiated maximal DA-stimulated cAMP levels by ∼30%, without altering agonist potency (Figure 1a). Potentiation of cAMP accumulation was observed with EtOH concentrations as low as 10 mM (data not shown); however, we routinely used 100 mM to obtain maximally consistent results. We also examined the effect of longer EtOH treatment periods (up to 60 min) and observed a similar potentiation of DA-stimulated cAMP levels (data not shown). Notably, EtOH-dependent potentiation of cAMP production required a pretreatment period, as direct addition of EtOH to the cAMP assay did not potentiate cAMP levels (Figure 1b). To establish if the EtOH effect was due to an increase in D$_1$ receptor-binding activity, membranes prepared from either control cells or cells pretreated with EtOH were assayed using the D$_1$-specific radioligand [^{3}H]SCH23390. EtOH treatment did not significantly alter D$_1$ receptor levels (Figure 1c), suggesting
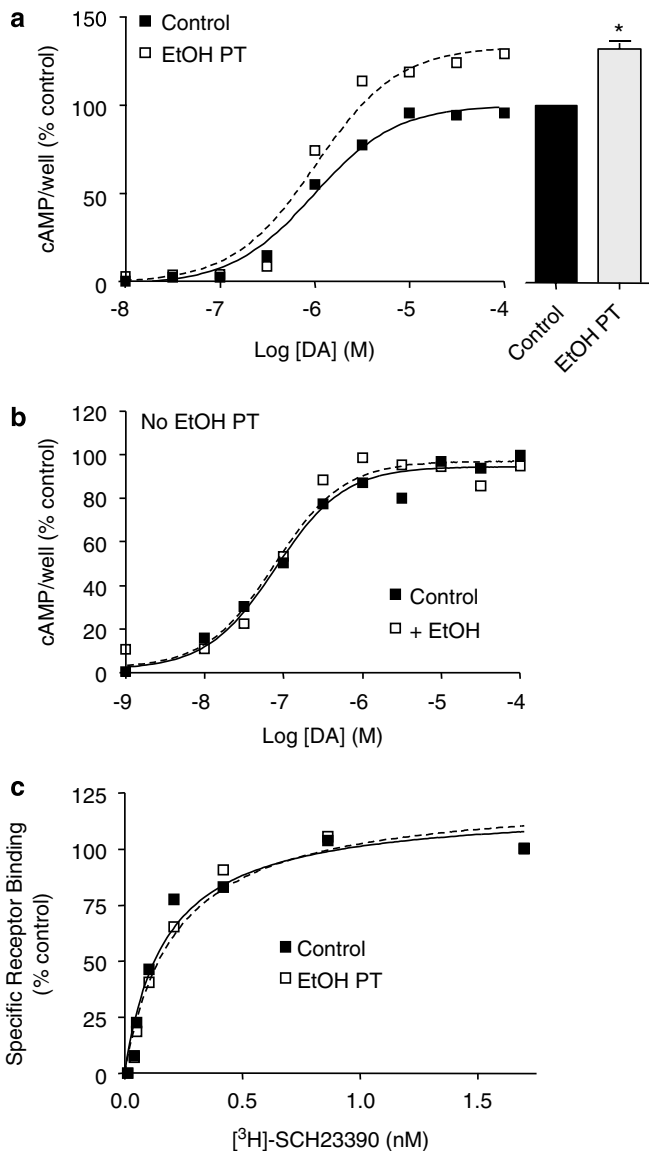
Figure 1 EtOH pretreatment potentiates D_1 receptor-mediated cAMP accumulation. (a) HEK293T cells expressing the rat D_1 receptor were pretreated with EtOH (100 mM) for 15 min prior to cAMP assay. For those cells pretreated with EtOH (EtOH PT), EtOH was also maintained in the assay buffer. Control cells were never exposed to EtOH. EtOH pretreatment E_{max}, 132 ± 3% of control. Average EC_{50} values ± SEM for control and EtOH pretreatment are 473 ± 200 and 446 ± 200 nM, respectively. (b) EtOH-dependent cAMP potentiation is not observed without pretreatment. EtOH (100 mM) was added to the cells with the addition of DA. (c) EtOH pretreatment does not significantly alter the expression of the D_1 receptor. Radioligand-binding experiments were performed on membranes prepared from control cells or cells pretreated with 100 mM EtOH for 15 min. All data are normalized as the percentage of control and are representative of 3–4 independent experiments (*$p < 0.05$, paired Student's t-test).

that EtOH increases the coupling of the D_1 receptor to its G-protein.

To more definitely determine the mechanism responsible for EtOH-dependent modulation of D_1 receptor signaling, we examined the effect of EtOH on D_1 receptor phosphorylation, as this post-translational modification is known to regulate D_1 receptor function primarily in a negative
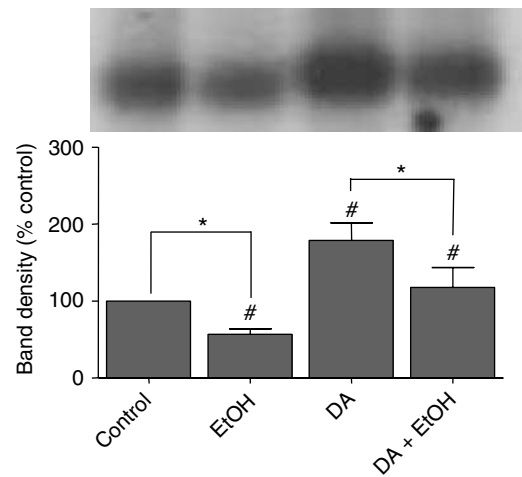
Figure 2 EtOH pretreatment reduces phosphorylation of the D_1 receptor. *In situ* phosphorylation assays were performed on HEK293T cells expressing the D_1 receptor. Cells were pretreated with EtOH (100 mM) or media for 10 min prior to a further 10 min incubation with either media (control), dopamine (DA, 10 μM), EtOH (100 mM), or DA + EtOH as indicated. Control and cells stimulated with only DA were never exposed to EtOH. (Top) Autoradiogram of D_1 receptor immunoprecipitates from a representative *in situ* phosphorylation assay; (bottom) band density for each condition. The data are normalized as the percentage of control and are representative of four independent experiments (*$p < 0.05$, ANOVA followed by Bonferroni pairwise comparisons; #$p < 0.05$ compared with the control, ANOVA followed by Dunnett's *post hoc* test).

manner (Tiberi *et al*, 1996; Jiang and Sibley, 1999; Gardner *et al*, 2001; Mason *et al*, 2002; Kim *et al*, 2004; Rankin *et al*, 2006). Cells expressing the D_1 receptor were metabolically labeled with $H_3[^{32}P]PO_2$ and either pretreated with EtOH for 10 min or never exposed to EtOH (control cells), followed by a 10-min treatment with either media, EtOH, DA, or DA + EtOH. The D_1 receptor was then immunoprecipitated and subjected to autoradiography. Under basal conditions, EtOH treatment decreased phosphorylation of the D_1 receptor by 43 ± 6% (Figure 2). EtOH also decreased agonist-stimulated D_1 receptor phosphorylation by a similar magnitude, suggesting that the EtOH-dependent decrement of receptor phosphorylation was primarily due to a decrease in basal phosphorylation. On the basis of these data, we hypothesized that EtOH decreases basal D_1 receptor phosphorylation, with a concomitant enhancement of signaling, by either activating a protein phosphatase or inhibiting a protein kinase that constitutively phosphorylates the receptor.

Serine/Threonine Phosphatases, GRKs, and EtOH-Dependent Potentiation of D_1 Receptor Signaling

Accordingly, if EtOH was activating a protein phosphatase, then treatment of cells with protein phosphatase inhibitors should abolish EtOH-dependent potentiation of cAMP accumulation and decrement of basal D_1 receptor phosphorylation. As the D_1 receptor is known to be phosphorylated exclusively on serine and threonine residues (Tiberi *et al*, 1996; Gardner *et al*, 2001; Mason *et al*, 2002; Kim *et al*, 2004), the repertoire of serine/threonine phosphatase expression in HEK293T cells was determined by western blot analysis (Supplementary Figure S1). Protein

phosphatases PP1, PP2A, PP2B, and PP4–6 were found to be expressed in these cells, whereas no signal was observed for PP2C. The effects of three phosphatase inhibitors were subsequently examined. Cantharidin inhibits protein phosphatases PP1 and PP2A, while FK-506 is specific for PP2B (calcineurin) and okadaic acid (OA) is a broad-spectrum phosphatase inhibitor that targets PP1, PP2A, and PP4–6. Cells were pretreated with the phosphatase inhibitors cantharidin, FK-506, or OA for 45 min. EtOH was added to samples 15 min prior to the end of the 45-min incubation period, followed by cAMP assay. In the absence of EtOH, pretreatment with either cantharidin or OA significantly decreased maximal cAMP accumulation when compared with the control. However, EtOH-dependent potentiation of DA-stimulated cAMP levels was not abolished (Figure 3a). Treatment of cells with FK-506 alone did not significantly alter DA-stimulated cAMP accumulation. However, EtOH treatment still significantly potentiated cAMP accumulation in cells pretreated with FK-506 (Figure 3a).

The effect of phosphatase inhibitor treatment on D₁ receptor phosphorylation was also examined. Cells were pretreated for 45 min with the phosphatase inhibitors and EtOH was added to the samples 10 min prior to the end of the treatment period, followed by incubation with media ± EtOH for further 10 min. In agreement with the cAMP data shown in Figure 2a, pretreatment of cells with either cantharidin or OA increased basal D₁ receptor phosphorylation (Figure 3b). However, an EtOH-dependent decrease of D₁ receptor phosphorylation was still observed in cells pretreated with cantharidin, OA, or FK-506 (Figure 3b).

While the above results argue against EtOH modulation of phosphatase activity as a mechanism for enhancement of D₁ receptor signaling, we wished to further investigate the potential role of PP2B (calcineurin), as calcineurin has been reported to directly associate with the D₁ receptor (Adlersberg *et al*, 2004), although its role in receptor phosphorylation remains unclear. We thus employed an approach using cells cotransfected with the D₁ receptor and a constitutively active subunit of calcineurin (CnA*) (Chin *et al*, 1998). If the effect of EtOH involved activation of calcineurin, then the constitutively active subunit may mimic these effects. Cells coexpressing the D₁ receptor + CnA* or D₁ receptor + empty vector (control) were incubated with media or DA and D₁ receptor phosphorylation was assessed. As expected, treatment with DA increased D₁ receptor phosphorylation in control cells; however, coexpression with CnA* did not diminish basal or DA-stimulated D₁ receptor phosphorylation (Figure 3c). In fact, basal and DA-stimulated receptor phosphorylation was increased in cells coexpressing CnA* (Figure 3c). Taken together, these data further support the lack of calcineurin involvement in EtOH modulation of D₁ receptor signaling.

Our next approach was to investigate protein kinases known to phosphorylate and regulate the D₁ receptor. The best-characterized family of kinases in this regard are the GRKs, which primarily mediate agonist-induced phosphorylation and desensitization of the D₁ receptor (Tiberi *et al*, 1996; Rankin *et al*, 2006). On the basis of the *in situ* phosphorylation results (Figure 2); however, the EtOH-dependent decrease of D₁ receptor phosphorylation was not changed in cells treated with DA, suggesting that the effects of EtOH are agonist-independent and may not involve
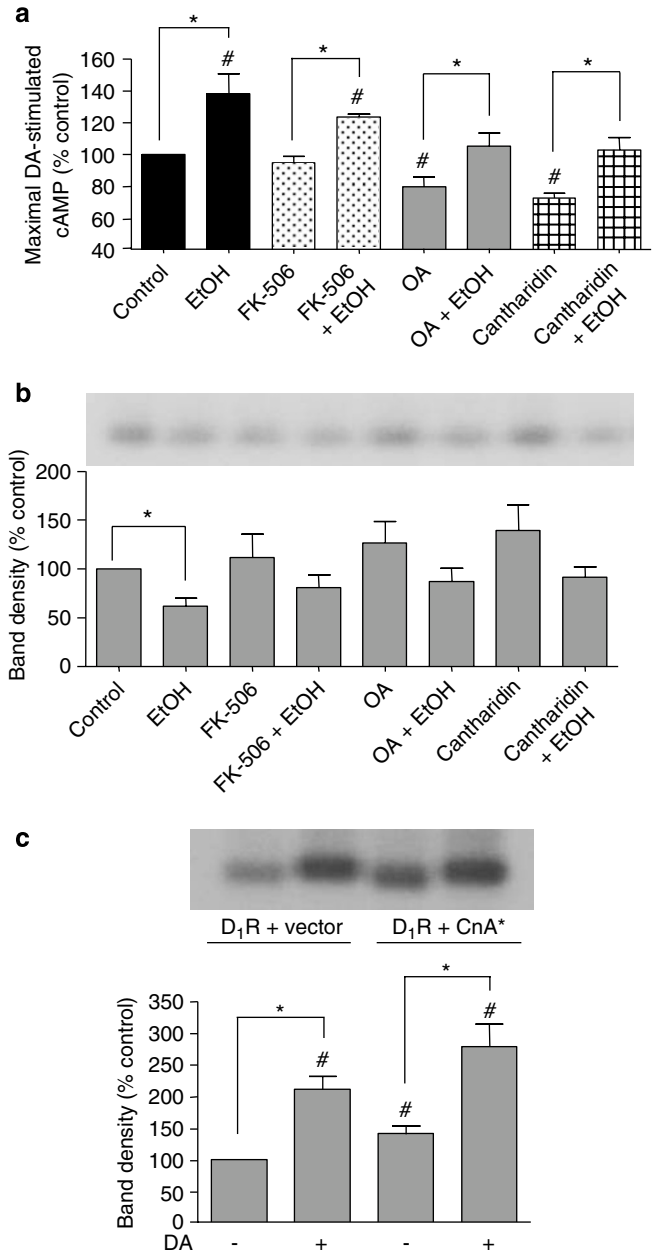
Figure 3 EtOH-dependent modulation of D₁ receptor signaling does not involve serine/threonine phosphatases. (a) HEK293T cells were pretreated with the phosphatase inhibitors cantharidin (10 μM), FK-506 (10 μM), or OA (1 μM) for 45 min. EtOH (100 mM) was then added to the indicated samples 15 min prior to the end of the 45-min incubation period, followed by addition of 10 μM DA to stimulate cAMP accumulation. (b) EtOH (100 mM) was added to the indicated samples 10 min prior to the end of the 45-min incubation period with individual phosphatase inhibitors, followed by assessment of basal receptor phosphorylation. (Top) Autoradiogram of D₁ receptor immunoprecipitates from a representative *in situ* phosphorylation assay; (bottom) band density for each condition. The data are normalized as the percentage of control and are representative of three independent experiments. (c) (Top) Autoradiogram of D₁ receptor immunoprecipitates isolated from cells coexpressing CnA* or empty vector. Cells were stimulated with either buffer (basal) or DA (10 μM) for 10 min. The autoradiogram is from a representative *in situ* phosphorylation assay; (bottom) band density for each condition. The data are normalized as the percentage of control for each individual experiment. The histograms represent mean ± SEM from three independent experiments (*p < 0.05, ANOVA followed by Bonferroni pairwise comparisons; #p < 0.05 compared with the control, ANOVA followed by Dunnett's *post hoc* test).
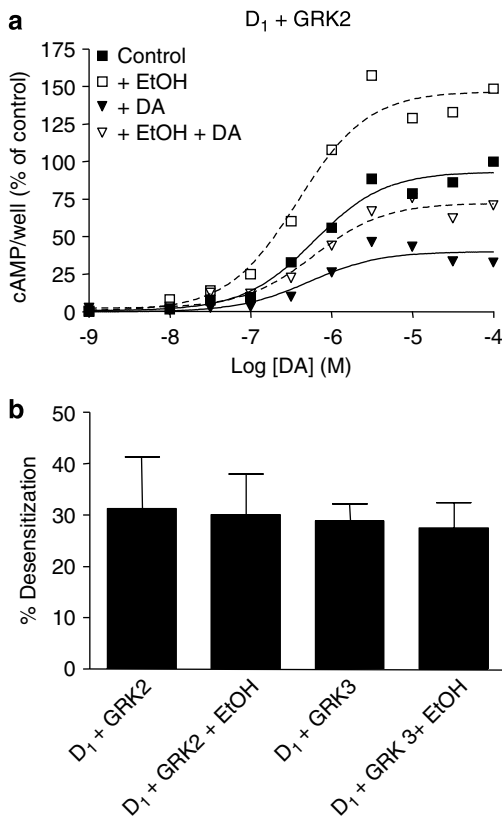
Figure 4 EtOH pretreatment does not alter GRK2- or GRK3-mediated D₁ receptor desensitization. (a) HEK293T cells cotransfected with the D₁ receptor and GRK2 were pretreated with DA (10 μM) ± EtOH (100 mM) for 60 min prior to stimulation with the indicated concentrations of DA. Control cells were never exposed to EtOH or pretreated with DA. Data are normalized as percentage of the E_{max} value of the control. The dose response curves shown are from a representative experiment, whereas the histogram in (b) shows the mean percentage of D₁ receptor desensitization ± SEM from three independent experiments. Percent desensitization for each condition was calculated by dividing the E_{max} of the desensitized cells (DA pretreatment) by the E_{max} of the corresponding control cells (no DA pretreatment), and then subtracting this ratio from 1 and converting to percentage.

GRKs. Nonetheless, to further investigate possible GRK involvement, the effect of EtOH on GRK2- and GRK3-mediated D₁ receptor desensitization was examined. If EtOH inhibits GRK activity, then agonist-induced desensitization should be reduced. Our previous observations indicated that GRK2 and GRK3 primarily mediate agonist-induced desensitization of the D₁ receptor (Rankin et al, 2006). Cells coexpressing the D₁ receptor and GRK2 or GRK3 were pretreated with DA to promote agonist-induced desensitization in the absence or presence of EtOH (Figure 4). In the absence of EtOH, DA pretreatment promotes an ∼30% decrease (desensitization) in maximum DA-induced cAMP response. EtOH treatment alone potentiated DA-stimulated cAMP levels in cells coexpressing GRK2 (Figure 4a). However, EtOH treatment did not affect the ability of the D₁ receptor to undergo agonist-mediated desensitization in cells coexpressing GRK2 (Figure 4a and b). This was also true for cells coexpressing GRK3 (Figure 4b). Taken together, these results suggest that the effects of EtOH on D₁ receptor signaling are not mediated by inhibition of GRKs.

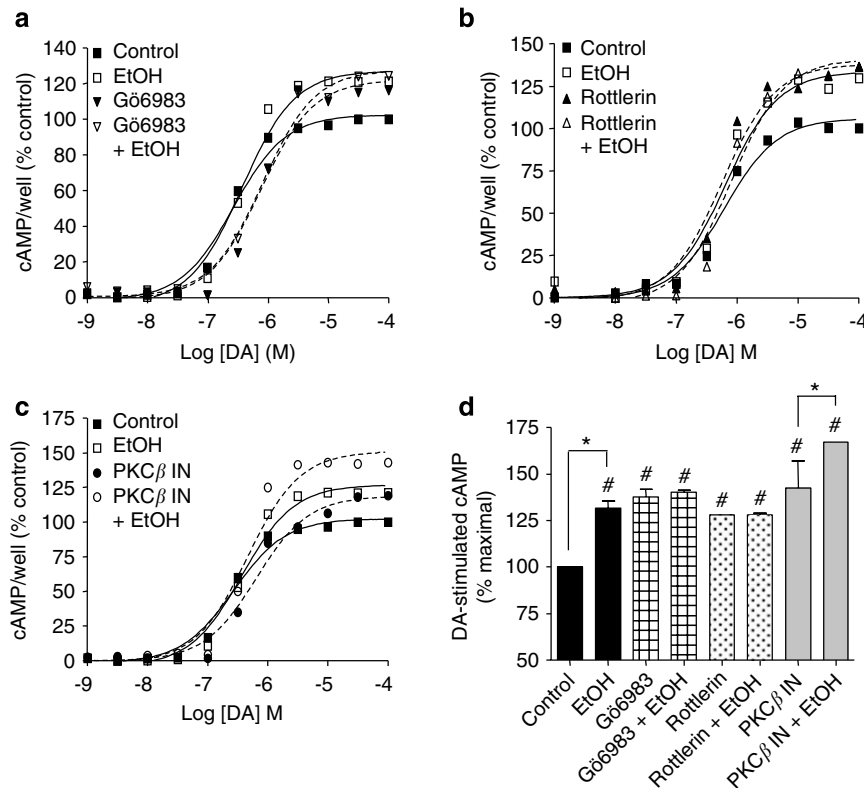## PKC Inhibitors Mimic the Effects of EtOH on D₁-Dependent Signaling

We previously published evidence that PKC may phosphorylate the D₁ receptor in an agonist-independent manner (Gardner et al, 2001). More recently, we have found that PKC phosphorylation of the D₁ receptor is constitutive in nature and primarily regulates the basal level of receptor phosphorylation (unpublished observations). As such, we were interested in examining the possibility that EtOH may inhibit the activity of PKC. As PKC phosphorylation of the D₁ receptor appears to be isozyme-specific (unpublished observations), we first examined the repertoire of PKC isozyme expression in HEK293T by western blot analysis (Supplementary Figure S1). PKCα, PKCβ₁, PKCδ, PKCε, PKCζ, PKCλ, PKCμ, and PKCν were found to be expressed in these cells at varying levels. In contrast, no signal was observed for PKCβ₂, PKCγ, and PKCθ.

Using an approach similar to that described for the phosphatase inhibitors, cells were pretreated with the PKC inhibitors Gö6983, rottlerin, or PKCβ inhibitor for 45 min. EtOH was added to samples 15 min prior to the end of the 45-min incubation period, followed by cAMP assay. The broad-spectrum PKC inhibitor, Gö6983, and the reported PKCδ-selective inhibitor, rottlerin (Gschwendt et al, 1994), mimicked the effects of EtOH treatment and potentiated D₁ receptor-stimulated cAMP levels (Figure 5a, b, and d). Importantly, EtOH treatment did not further potentiate cAMP accumulation beyond that observed with the PKC inhibitor (ie, the effects were non-additive). The PKCβ-specific inhibitor also potentiated DA-stimulated cAMP levels; however, the response was further increased when cells were pretreated with EtOH (Figure 5c and d). It should be noted that the effect of Gö6983 on D₁-stimulated signaling was not observed in a recent study by Jackson et al, where Gö6983 had no effect on D₁ receptor accumulation of cAMP. Surprisingly, PMA treatment potentiated D₁ receptor signaling (Jackson et al, 2005). Such discrepancies may be due to differences in experimental procedures and perhaps more importantly differences in the expression/activation profile of PKC isozymes.

We next examined the effect of PKC inhibitors on basal D₁ receptor phosphorylation. Cells were pretreated with the broad-spectrum PKC inhibitor GF109203X or rottlerin for 45 min. EtOH was added to samples 10 min prior to the end of the 45-min incubation period, followed by a 10-min incubation with media ± EtOH. Consistent with cAMP data, treatment of cells with GF109203X or rottlerin mimicked the effect of EtOH and significantly reduced basal D₁ receptor phosphorylation (Figure 6). Importantly, basal D₁ receptor phosphorylation was not further decreased in cells pretreated with EtOH + PKC inhibitor beyond that observed with either agent alone. These results suggest a role for specific PKC isozymes in the EtOH-dependent modulation of D₁ receptor signaling.

EtOH Attenuates Lipid-Activated PKCδ and PKCγ in Membrane Fractions

On the basis of our data, we hypothesized that EtOH reduces D₁ receptor phosphorylation and enhances signaling by decreasing the activity of specific PKC isozymes.

Figure 5 PKC inhibitors mimic the effects of EtOH on DA-dependent cAMP accumulation and receptor phosphorylation. HEK293T cells were pretreated with PKC inhibitors, (a) Gö6983 (10 μM), (b) rottlerin (10 μM), or (c) PKCβ inhibitor (PKCβ IN, 10 μM), for 45 min. EtOH was added to select samples 15 min prior to the end of the 45-min incubation period, followed by cAMP assay. The data are normalized as the percentage of control. The dose response curves shown are from a representative experiment, whereas the histogram in (d) shows the mean E$_{max}$ values ± SEM from three independent experiments (*$p < 0.05$, ANOVA followed by Bonferroni pairwise comparisons; #$p < 0.05$ compared with the control, ANOVA followed by Dunnett's *post hoc* test).

To address this, selected PKC isozymes were examined for EtOH-dependent changes in kinase activity.

Cells were transfected with one of the following tagged PKC isozymes, HA-PKCδ, FLAG-PKCγ, HA-PKCβ, FLAG-PKCε, or GFP-PKCα. These isozymes were selected on the basis of their known expression in HEK293T cells (with the exception of PKCγ) (Supplementary Figure S1) and/or preliminary observations that they mediate D$_1$ receptor phosphorylation. In these experiments, cells were either pretreated with EtOH or media for 10 min, followed by lysis and fractionation. For each condition, the PKC isozyme was immunoprecipitated from either the cytosolic fraction or a solubilized membrane preparation using an antibody to the appropriate tag and its activity measured using an *in vitro* kinase assay.

Three treatments were examined: (1) no pretreatment (cells were never exposed to EtOH prior to cell fractionation or during the kinase assay); (2) EtOH in assay (cells were never exposed to EtOH prior to cell fractionation but EtOH was included in the kinase assay); and (3) EtOH pretreatment (cells were pretreated with EtOH prior to cell fractionation and EtOH was included in the kinase assay). For each treatment, kinase activity was measured under basal (absence of the endogenous lipid activators, PS, and DAG) or lipid-activated conditions. Significantly, EtOH decreased lipid-dependent kinase activity of PKCδ and PKCγ, but only when they were isolated from the membrane

fraction (Figure 7). Moreover, adding EtOH directly to the kinase assay appeared to be sufficient to decrease kinase activities of either PKCγ or PKCδ (Figure 7). In contrast, EtOH did not significantly alter the *in vitro* kinase activities of PKCβ_1, PKCε, or PKCα isolated from the membrane fractions (Supplementary Figure S2).

EtOH Pretreatment and DARPP-32 Phosphorylation in Rat Striatum

To determine if the EtOH-dependent potentiation of D$_1$ receptor signaling observed in the HEK293T cells is physiologically relevant in the brain, we examined EtOH-dependent modulation of D$_1$ receptor-stimulated DARPP-32 phosphorylation in slices prepared from rat striatum. Importantly, DARPP-32 is converted to a potent inhibitor of PP1 upon phosphorylation by PKA in response to an elevation of cellular cAMP levels. Fresh frozen striatal sections were treated with PBS (control), EtOH, SKF81297, or pretreated with EtOH, followed by SKF81297 treatment. Slices were incubated with an antibody specific for the PKA-dependent phospho-Thr[34] site of DARPP-32. As expected, treatment with the D$_1$-selective agonist, SKF81297, increased the phosphorylation of DARPP-32 (Figure 8a and c). Elevation of phospho-DARPP-32 immunoreactivity is notably apparent in the cell bodies as well as in the surrounding neuropil (Figure 8a and c). Treatment with
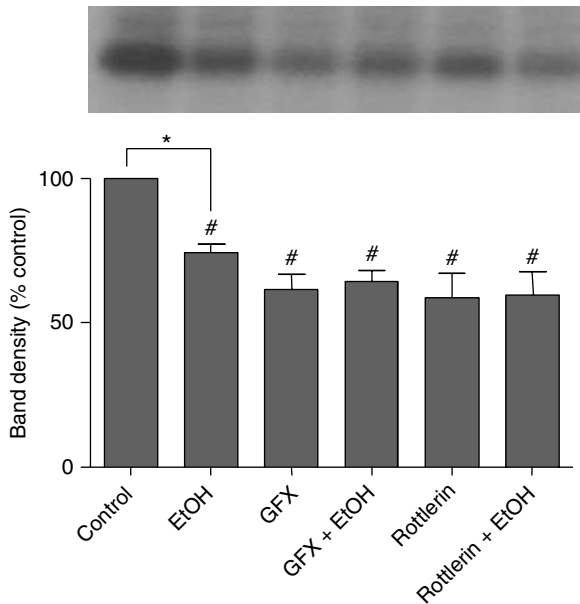
Figure 6 PKC inhibitors mimic the effects of EtOH and reduce basal D_1 receptor phosphorylation. HEK293T cells were pretreated with GFX109203X (GFX, 500 nM) or rottlerin (10 μM) for 45 min. EtOH was added to the indicated samples 10 min prior to the end of the 45 min incubation period. (Top) Autoradiogram of D_1 receptor immunoprecipitates from a representative *in situ* phosphorylation assay; (bottom) band density for each condition. The data are normalized as the percentage of control for each individual experiment. The histogram represents the mean ± SEM from nine independent experiments (*$p < 0.05$, ANOVA followed by Bonferroni pairwise comparisons; #$p < 0.05$ compared with the control, ANOVA followed by Dunnett's *post hoc* test).

EtOH alone also increased the level of DARPP-32 phosphorylation by a small, but significant extent (Figure 8a and c) in agreement with previous reports (Maldve *et al*, 2002; Donohue *et al*, 2005). Importantly, consistent with our findings with HEK293T cells, EtOH pretreatment synergistically potentiated SKF81297-stimulated DARPP-32 phosphorylation in comparison with the agonist treatment alone (Figure 8a–c). EtOH potentiation of agonist treatment is especially noticeable at higher magnification (Figure 8b). These results confirm the notion that EtOH exposure potentiates D_1 receptor signaling pathways in the brain.

DISCUSSION

Our current investigation illuminates a novel mechanism for how acute EtOH exposure can potentiate dopaminergic signaling mediated through the D_1 receptor subtype. This G-protein-coupled receptor is known to be phosphorylated by multiple protein kinases, including GRKs (Tiberi *et al*, 1996; Rankin *et al*, 2006), PKA (Jiang and Sibley, 1999; Mason *et al*, 2002), and PKC (Gardner *et al*, 2001). Phosphorylation by these various kinases negatively modulates receptor signaling and/or expression at the cell surface. Recently, we have determined that PKC constitutively phosphorylates the D_1 receptor in an isozyme-specific manner (unpublished observations). As shown in the present study, inhibition of this constitutive PKC phosphorylation potentiates agonist-stimulated D_1 receptor

signaling. Notably, EtOH exposure was found to diminish D_1 receptor phosphorylation and potentiate receptor signaling through inhibition of constitutive PKC phosphorylation. This effect appears to occur in an isozyme-specific manner in that only membrane-associated PKCδ or PKCγ activities were found to be inhibited by EtOH treatment.

On the basis of recent experimentation, we have found that the D_1 receptor is phosphorylated by multiple PKC isozymes, although the corresponding phosphorylation sites on the receptor have not been completely elucidated as of yet. We hypothesize that EtOH inhibits only a subset of the PKC isozymes engaged in D_1 receptor phosphorylation and/or diminishes phosphorylation on the subset of receptor residues selectively targeted by PKCδ or PKCγ. These PKCδ or PKCγ phosphorylation sites may be distinct from, or partially overlap with other PKC phosphorylation sites on the receptor, such as those for PKCβ. Complete mapping (in progress) of all the PKC phosphorylation sites on the D_1 receptor, and their isozyme specificity, should shed light on these issues.

Importantly, both PKCδ and PKCγ are known to be expressed in the brain, including the striatum and in particular the NAc (Tanaka and Saito, 1992; Merchenthaler *et al*, 1993); a region that is rich in D_1 receptor expression. Notably, the striatum is the site of action for many drugs of abuse. Differences between the dorsal striatum (a region that controls locomotion) and the ventral striatum (a region that is associated with emotion and motivational aspects of behavior) with regards to drug abuse have been reported. The dorsal striatum appears to be involved in the establishment of reward associated memories and habit formation (Vanderschuren *et al*, 2005; Volkow *et al*, 2006), whereas the major component of the ventral striatum, the NAc, is involved in the rewarding properties of many drugs of abuse such as EtOH (Carelli, 2002). In this regard, it is interesting to note that following acute administration, EtOH concentrations in the striatum rise to levels that are twofold greater than those observed in other brain regions (Chen *et al*, 2007).

Consistent with our present observations, it is interesting to note that mice lacking PKCγ have been found to consume more alcohol, display less behavioral inhibition, and are less sensitive to the anxiolytic effects of EtOH (Bowers *et al*, 2001; Bowers and Wehner, 2001). Although PKCγ is not expressed in the HEK293T cells, the overlapping expression of the D_1 receptor and PKCγ in the NAc suggests that EtOH-dependent modulation of D_1 receptor signaling by this PKC isozyme, as well as PKCδ, may indeed occur *in vivo*.

The ability of EtOH to directly regulate PKC activity has been investigated previously, but this work remains somewhat controversial. Modulation of PKC activity by EtOH appears to be dependent on assay conditions and the specific isozymes in question. For example, acute EtOH treatment has been found to inhibit PKC activity in membranes prepared from rat brain using protamine sulfate as both an activator and substrate (Slater *et al*, 1993). Similarly, EtOH reduced total PKC activity in membrane but not cytosolic fractions prepared from rat forebrains (Kruger *et al*, 1993). EtOH treatments were also found to reduce PKC activity in hippocampal homogenates (Davis *et al*, 1999). However, other studies showed no effect of EtOH on PKC activity, although some reports have
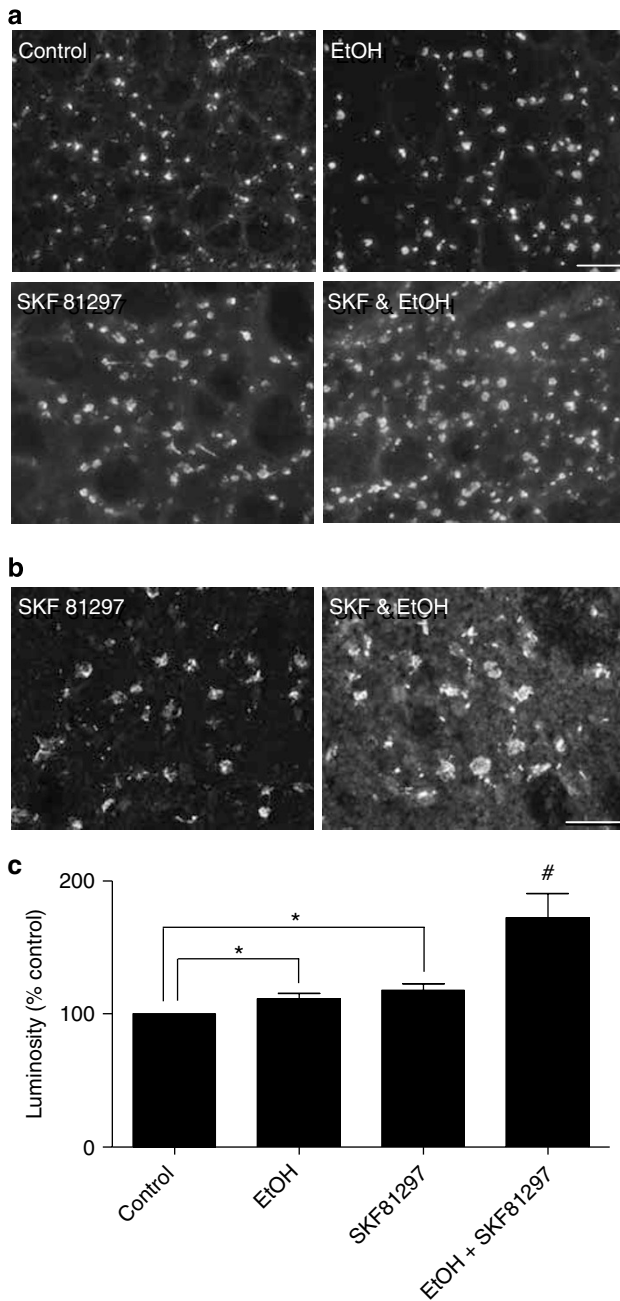
PKCδ

PKCγ
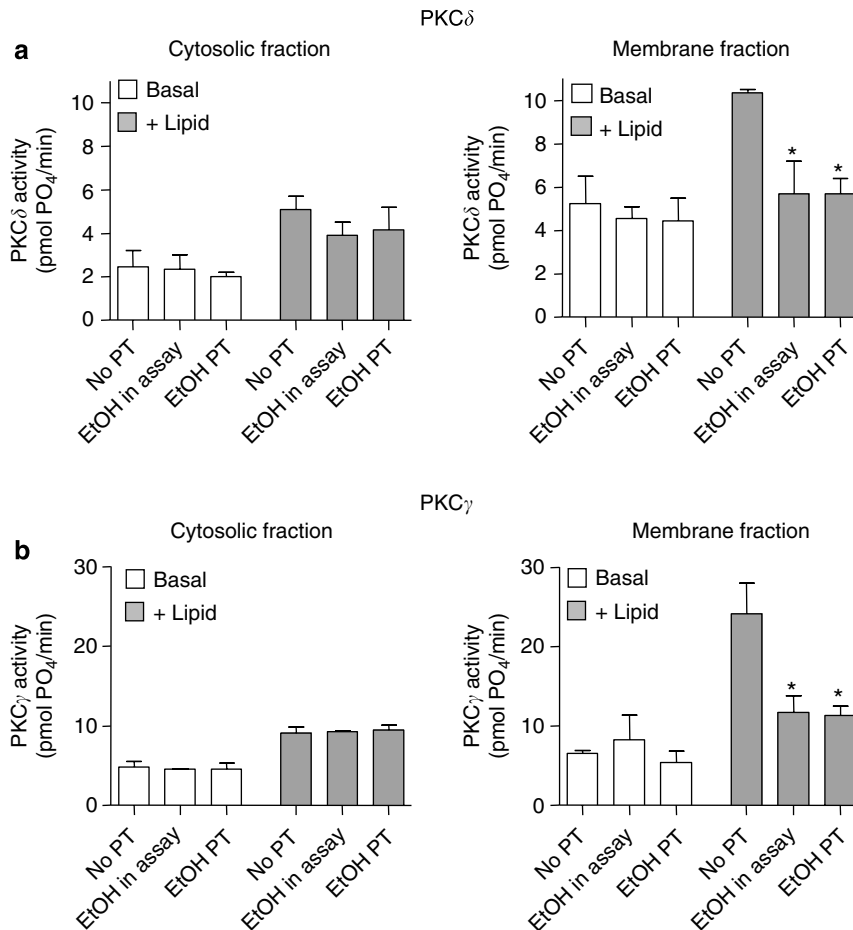
Figure 7 EtOH attenuates lipid-activated PKCδ and PKCγ activities in membrane fractions. Cells overexpressing either PKCδ or PKCγ were pretreated with media or EtOH (100 mM) for 10 min, followed by extraction and immunoprecipitation of the isozyme. The kinase activity of the isozyme-specific immunoprecipitates was directly assessed using an *in vitro* kinase assay. 'No pretreatment (PT)' samples were never exposed to EtOH both prior and during the kinase assay. 'EtOH in assay' samples were from the same cellular preparations as the 'no pretreatment' samples except that EtOH (100 mM) was included in the kinase assay. 'EtOH PT' samples were treated with 100 mM EtOH for 10 min prior to assay. For preparations that were pretreated with EtOH before cell fractionation, EtOH was maintained in the kinase assay. For each condition, kinase activity was measured under basal (no PS or DAG) or lipid-activated conditions. No specific phosphorylation was measured in cells transfected with empty tagged vector. Left panel, cytosolic fraction; right panel, membrane fraction. (a) PKCδ and (b) PKCγ. Data are presented as mean ± SEM of three independent experiments. Significantly different to 'no pretreatment' for lipid-activated conditions (*$p < 0.05$, ANOVA followed by Bonferroni pairwise comparisons).

described EtOH-dependent activation of PKC (Machu *et al*, 1991; Messing *et al*, 1991; Satoh *et al*, 2006).

EtOH treatment has also been shown to promote the subcellular translocation of specific PKC isozymes. Chronic EtOH exposure (48 h) promotes translocation of PKCδ and PKCε between specific intracellular compartments (Gordon *et al*, 1997). The subcellular redistribution of PKCε but not PKCδ is also evident in cellular fractions prepared from cardiac myocytes (Miyamae *et al*, 1998). Moreover, acute EtOH administration differentially alters PKC expression in membrane and cytosolic fractions prepared from the rat cortex. Notably, EtOH treatment (60 min) decreases PKCγ expression in the membrane fraction (Kumar *et al*, 2006). Although not directly addressed in this study, our *in vitro* kinase assay results suggest that isozyme translocation is not responsible for the decrease of PKCδ/γ activities by EtOH. Notably, direct addition of EtOH to the *in vitro* kinase assays is sufficient to attenuate the kinase activities of PKCδ and PKCγ. Also treatment of cells with EtOH prior

to cell fractionation did not further decrease the kinase activities of PKCδ/γ immunoprecipitated from the membrane fraction as might be expected if EtOH promoted PKC translocation from the membrane to the cytosol.

The specific mechanism by which EtOH decreases the kinase activities of PKCδ and PKCγ is not completely clear. Notably, EtOH was found to only inhibit the activities of PKCδ or PKCγ when isolated from the membrane fraction—there was no effect of EtOH treatment on the activities of these enzymes when they were immunoprecipitated from the cytosolic fraction. Thus, the inhibitory effect of EtOH may be uniquely restricted to when the PKC isozyme is in association with plasma membrane components. Phosphorylation is a well-described mechanism for regulating PKC activity, with the general dogma being that threonine phosphorylation in the activation loop is required for conventional PKC activation (Parekh *et al*, 2000; Wilkie *et al*, 2007). Notably, the phosphorylation state of conventional PKCs is known to vary in a regulated manner based

on their subcellular compartmentalization (Gallegos et al, 2006). One might then speculate that EtOH diminishes the phosphorylation states and resulting activities of PKCδ and PKCγ only when they are in association with the plasma membrane.

Another intriguing possibility is that EtOH may target PKC isozyme-specific interacting proteins present in the membrane fraction. Indeed, a number of PKC isozyme-specific interacting proteins have been identified, which might regulate PKC activity (Poole et al, 2004). In this regard, EtOH has been shown to promote the uncoupling of the scaffolding protein, RACK1, from PKCβ_2 (Ron et al, 2000). Of note, in our study, any PKC-interacting protein co-immunoprecipitating with the PKC isozymes would be included in the in vitro kinase assays. In this scenario, association with a putative PKC-interacting protein may render the PKC isozyme susceptible to inhibition by EtOH (via regulating phosphorylation?) or, perhaps more interestingly, the PKC-interacting protein itself may be the target of EtOH. In this manner, the repertoire and location of isozyme-specific PKC-interacting proteins may prove to be critical determinants for EtOH-dependent modulation of PKCδ/γ activities. These various possibilities are currently under investigation.

Notably, additional effectors besides DARPP-32 that are downstream of the D$_1$ receptor are also likely to be modulated by the EtOH-dependent potentiation of D$_1$ receptor signaling. For example, the transcription factor CREB is phosphorylated and subsequently activated by kinases such as PKA, a downstream effector of D$_1$ receptor signaling. Indeed, increased adenylyl cyclase and PKA activity have been linked to increased ethanol consumption in mice (Wand et al, 2001). Moreover, many drugs of abuse, including EtOH, increase CREB phosphorylation in the striatum, a response that is postulated to promote EtOH self administration (Yang et al, 1998; Nestler, 2001; Newton and Messing, 2006). Equally conceivable is that other targets of PKC isozymes, not just the D$_1$ receptor, are regulated by EtOH. If so, this may partly explain the multifaceted effects of EtOH in vivo and potentially provide a platform for therapeutic intervention in treating alcoholism.

In summary, our model suggests that acute EtOH treatment decreases the enzymatic activities of PKCδ and/ or PKCγ in the plasma membrane, decreases basal D$_1$ receptor phosphorylation and potentiates DA-stimulated cAMP accumulation and downstream signaling pathways. We propose that the EtOH-dependent decrease of basal D$_1$ receptor phosphorylation is a consequence of reduced membrane PKC activity, specifically PKCδ in HEK293T cells and either PKCδ or PKCγ in the brain. This modification may stabilize the D$_1$ receptor in a conformation primed for efficient coupling to G-proteins (Gα_s/$_{olf}$), such that upon challenge with agonist, cAMP accumulation is potentiated. Increased phosphorylation of DARPP-32 on Thr^{34} is a clear downstream consequence of the potentiation by EtOH of D$_1$ receptor-stimulated cAMP production. Further, elevation of phospho-Thr^{34}-DARPP-32 levels will circumvent EtOH-dependent inhibition of the NMDA receptor by inhibiting the activity of PP1 and permitting increased phosphorylation of the NR1 subunit leading to enhanced channel activity (Maldve et al, 2002; Newton and Messing, 2006), a modification that contributes to synaptic plasticity and

Figure 8 EtOH pretreatment potentiates agonist-mediated phosphorylation of DARPP-32 in rat striatum. (a) Rat striatal slices were treated with PBS (control), EtOH (25 mM/15 min), SKF81297 (1 μM/15 min), or pretreated with EtOH (25 mM/15 min), followed by SKF81297 treatment (1 μM/15 min). Slices were incubated with an antibody that recognizes the phospho-Thr^{34} site of DARPP-32. (b) At higher magnification, EtOH pretreatment clearly increased SKF81297-stimulated phospho-DARPP-32 immunoreactivity in the cell bodies. (c) Histogram showing the percentage of phospho-DARPP-32 immunoreactivity compared with the control. The cumulative data indicate that EtOH pretreatment potentiated SKF81297-stimulated DARPP-32 phosphorylation by 172 ± 18.2% over control. In comparison, EtOH or SKF81297 treatment alone potentiated DARPP-32 phosphorylation by 112 ± 3.5 and 118 ± 4.8%, respectively. Data are normalized as the percentage of control and representative of three independent experiments (*p < 0.05, paired Student's t-test; #p < 0.05 compared with the control, ANOVA followed by Dunnett's post hoc test). Bar, 150 μm (a); bar, 60 μm (b).

EtOH tolerance (Lovinger, 2002). Therefore, the EtOH-dependent potentiation of D$_1$ receptor signaling and subsequent phosphorylation of Thr^{34}-DARPP-32 may contribute to the rewarding and reinforcing properties of alcohol.

DISCLOSURE/CONFLICT OF INTEREST

None.

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