

Ethanol Regulation of Synaptic GABA_A α 4 Receptors Is Prevented by Protein Kinase A Activation

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Received November 3, 2015; accepted January 28, 2016

ABSTRACT

Ethanol alters GABA_A receptor trafficking and function through activation of protein kinases, and these changes may underlie ethanol dependence and withdrawal. In this study, we used subsynaptic fraction techniques and patch-clamp electrophysiology to investigate the biochemical and functional effects of protein kinase A (PKA) and protein kinase C (PKC) activation by ethanol on synaptic GABA_A α 4 receptors, a key target of ethanol-induced changes. Rat cerebral cortical neurons were grown for 18 days in vitro and exposed to ethanol and/or kinase modulators for 4 hours, a paradigm that recapitulates GABAergic changes found after chronic ethanol exposure in vivo. PKA activation by forskolin or rolipram during ethanol exposure prevented increases in P2 fraction α 4 subunit abundance, whereas inhibiting PKA had no effect. Similarly, in the synaptic fraction, activation of PKA by

rolipram in the presence of ethanol prevented the increase in synaptic α 4 subunit abundance, whereas inhibiting PKA in the presence of ethanol was ineffective. Conversely, PKC inhibition in the presence of ethanol prevented the ethanol-induced increases in synaptic α 4 subunit abundance. Finally, we found that either activating PKA or inhibiting PKC in the presence of ethanol prevented the ethanol-induced decrease in GABA miniature inhibitory postsynaptic current decay τ_1 , whereas inhibiting PKA had no effect. We conclude that PKA and PKC have opposing effects in the regulation of synaptic α 4 receptors, with PKA activation negatively modulating, and PKC activation positively modulating, synaptic α 4 subunit abundance and function. These results suggest potential targets for restoring normal GABAergic functioning in the treatment of alcohol use disorders.

Introduction

Ethanol causes adaptations in GABA_A receptors that are associated with alcohol dependence and withdrawal (Kumar et al., 2009). GABA_A receptors are ligand-gated chloride channels mediating the majority of inhibitory neurotransmission in the brain through both phasic and tonic currents (Farrant and Nusser, 2005). These channels consist of five subunits typically composed of two α subunits (1–6), two β subunits (1–3), and a γ (1–2) or δ subunit (Tretter and Moss, 2008). Two important targets of ethanol regulation in particular are the synaptic α 4 β γ 2 and extrasynaptic α 4 β δ GABA_A receptors mediating a portion of phasic and tonic inhibition, respectively (Olsen and Sieghart, 2009). The extrasynaptic α 4 receptors have been the subject of much study, owing to their responses to relatively low doses of ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004); however, surprisingly little is known about the regulation of synaptic α 4 receptors.

Although early studies showed that ethanol regulates the abundance of α 4 subunit mRNA and protein expression

(Devaud et al., 1996, 1997; Papadeas et al., 2001; Cagetti et al., 2003), the question of whether this regulation reflected synaptic or extrasynaptic receptor adaptations depended on subsequent functional studies of synaptic α 4 receptor kinetics. Synaptic α 4 receptors are upregulated after both acute (Liang et al., 2007) and chronic (Liang et al., 2006; Werner et al., 2011) ethanol exposure in the rat hippocampus and cortex. More recently, we demonstrated ethanol regulation of synaptic α 4 subunits in C57BL/6J mice after an acute injection of ethanol, using subcellular fractionation to isolate synaptic versus subsynaptic receptors (Carlson et al., 2014). Isolating the precise physiologic and behavioral ramifications of these changes in synaptic α 4 receptors is difficult because there are no selective pharmacological agents targeting these receptors. The α 4 β γ 2 receptors are benzodiazepine insensitive, although they show relatively high affinity for the GABA inverse agonist Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate) (Knoflach et al., 1996). However, Ro15-4513 also antagonizes effects of ethanol on α 4 β δ receptors (Hancher et al., 2006) that likely mediate effects of ethanol on tonic inhibition.

Recent studies from our laboratory have uncovered that kinase activation by ethanol plays a major role in GABA_A receptor regulation, and that protein kinase A (PKA) and protein kinase C (PKC) may have opposing effects on GABAergic trafficking and function. PKA activity positively

This research was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grants P60-AA11605 and T32-AA007573] and the University of North Carolina Bowles Center for Alcohol Studies.

dx.doi.org/10.1124/jpet.115.230417.

ABBREVIATIONS: ANOVA, analysis of variance; CalC, calphostin C; mIPSC, miniature inhibitory postsynaptic current; PdBu, phorbol-12,13-dibutyrate; PKA, protein kinase A; PKC, protein kinase C; Ro15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate; Rp-cAMP, Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine.

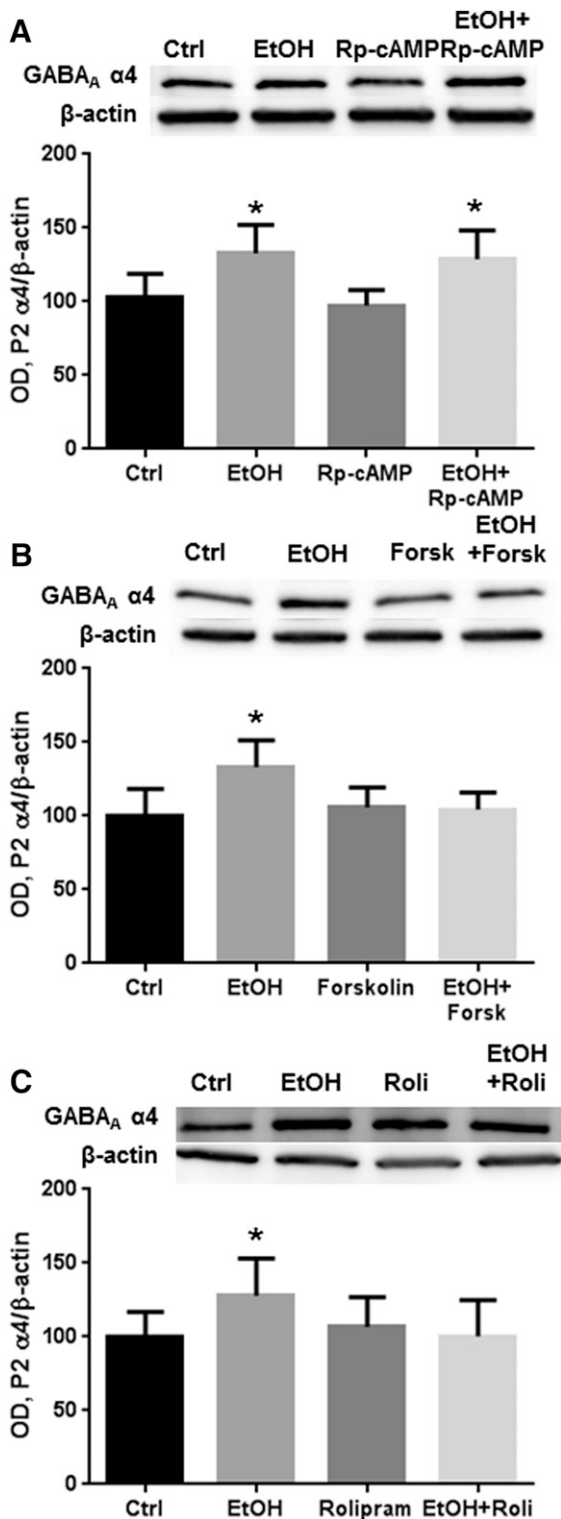


Fig. 1. PKA activation prevents ethanol-induced increases in P2 fraction GABA_A α 4 subunits. Cortical neurons were exposed to vehicle, ethanol (50 mM), Rp-cAMP (50 μ M), forskolin (10 μ M), and/or rolipram (10 μ M) for 4 hours, followed by P2 fractionation and western blot analysis. (A) Exposure to ethanol for 4 hours increased P2 fraction levels of the GABA_A α 4 subunit, which was not affected by coexposure with Rp-cAMP. (B and C) Exposure to the adenylyl cyclase activator forskolin (B) or the phosphodiesterase inhibitor rolipram (C) with ethanol prevented the increase in P2 fraction levels of α 4 subunits induced by ethanol. * $P < 0.05$ (one-way ANOVA, Bonferroni post-test, $n = 6-8$ per group). Ctrl, control; EtOH, ethanol; Forsk, forskolin; OD, optical density; Roli, rolipram.

regulates synaptic GABA_A α 1 subunit abundance and function in cortical neurons, whereas PKC γ activity negatively regulates these receptors (Kumar et al., 2010; Carlson et al., 2013). PKA activation also positively regulates extrasynaptic GABA_A α 4 and δ subunits in cortical neurons, whereas PKC is not involved in regulating these receptors in this brain region (Carlson et al., 2016). Finally, PKC γ activation by ethanol causes an increase in GABA_A α 4 subunits, although it is unclear whether this effect is specific to the synaptic population of receptors (Werner et al., 2011). It is also unclear what role PKA activity may play in regulating this subset of α 4 receptors.

This study elucidates the role of PKA and PKC in ethanol regulation of synaptic α 4-containing GABA_A receptors in cerebral cortical cultured neurons. We used a 4-hour ethanol exposure paradigm that recapitulates many of the GABAergic adaptations observed after chronic ethanol exposure in vivo (Devaud et al., 1997; Kumar et al., 2003). We measured changes in subunit abundance using a subcellular fractionation technique that enriches synaptic proteins combined with western blot analysis, whereas functional changes were measured using whole-cell patch-clamp analysis of GABA miniature inhibitory postsynaptic currents (mIPSCs).

Materials and Methods

Cultured Cerebral Cortical Neurons. All experiments were conducted in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee at the University of North Carolina. Mixed-sex rat pups from Sprague-Dawley breeding pairs (Harlan, Indianapolis, IN) were decapitated on postnatal day 0–1. Brains were rapidly dissected and the cerebral cortices were isolated. Cortical halves were minced into fine pieces and tissue was incubated in CO₂-independent media containing papain (50 U/ml; Worthington, Lakewood, NJ) and L-cysteine and DNase (both from Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Tissue pieces were gently washed, followed by gentle trituration in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% horse serum, penicillin-streptomycin, and DNase. Cells used for biochemistry were plated onto poly(D-lysine)-coated flasks, whereas cells used for electrophysiology were plated onto poly(D-lysine)-coated coverslips in 12-well plates. Cells were maintained in a 5% CO₂ humidified incubator. After day 3, cells were fed with serum-free medium containing B27 and penicillin-streptomycin (10,000 U/ml; final concentration 50 U per flask). Media were changed twice per week with no more than one-third of the media being removed during exchanges. For all experiments, penicillin-streptomycin was removed from cultures on day 14 to prevent interactions with GABA_A receptors. Cultures were maintained for 18 days before conducting experiments, because prior studies determined that GABA_A receptor expression was stable between 15 and 19 days in vitro.

Ethanol and Drug Exposure. Cultured cells were exposed to 50 mM ethanol and placed in a plastic vapor chamber within the incubator. This concentration was chosen because it produces changes in GABAergic inhibition consistent with in vivo models (Devaud et al., 1997; Kumar et al., 2003). A beaker of water with 50 mM ethanol was used to maintain stable ethanol concentrations in the chamber. Control cells were exposed to an equivalent amount of water and placed in a plastic vapor chamber with a beaker containing water. Cells were exposed to ethanol for 4 hours. To examine PKA involvement, the PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphate triethylamine (Rp-cAMP; 50 μ M), the adenylyl cyclase activator forskolin (10 μ M; Tocris Bioscience, Minneapolis, MN), or the phosphodiesterase inhibitor rolipram (10 μ M; Sigma-Aldrich) was added to the cell media. To examine PKC involvement, the PKC inhibitor calphostin C (CalC; 0.3 μ M in 0.1% dimethylsulfoxide, final

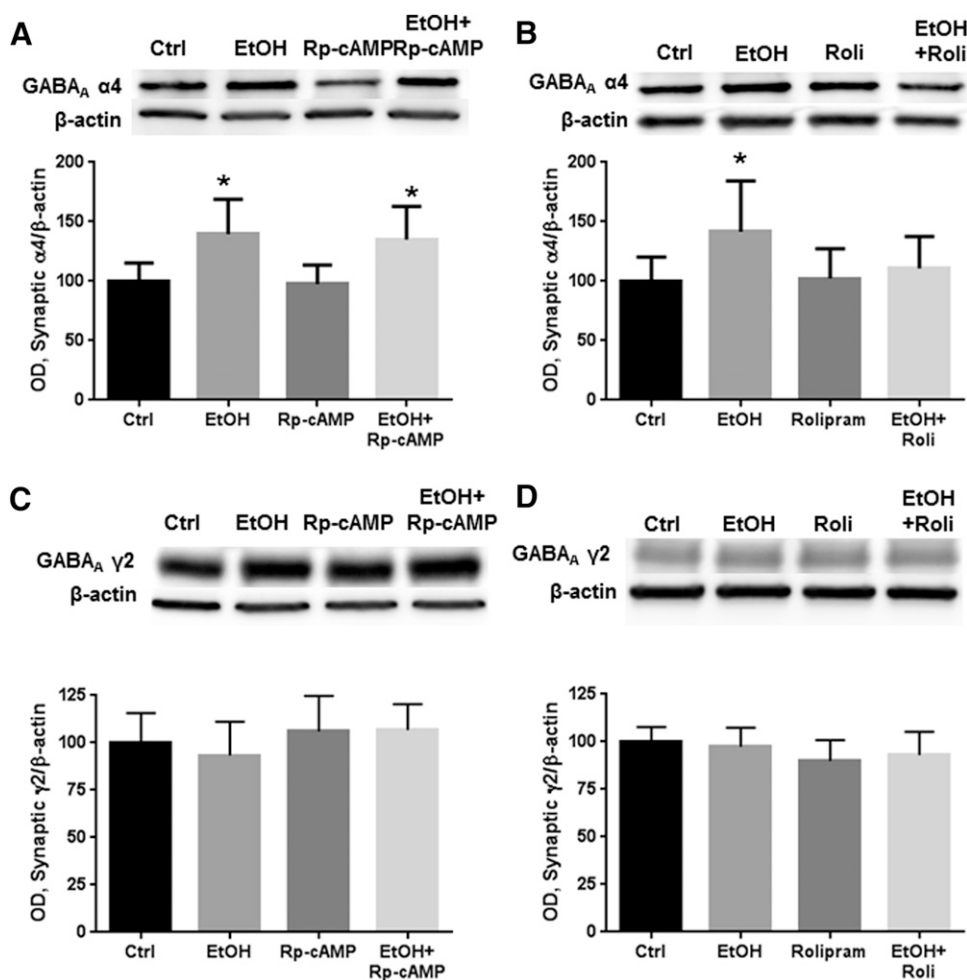


Fig. 2. PKA activation prevents the increased abundance of synaptic $\alpha 4$ receptors induced by ethanol. Cortical neurons were exposed to vehicle, ethanol (50 mM), Rp-cAMP (50 μ M), and/or rolipram (10 μ M) for 4 hours, followed by synaptic fractionation and western blot analysis. (A) Ethanol decreased synaptic GABA_A $\alpha 4$ abundance, an effect that was not altered by coexposure with Rp-cAMP. (B) Coexposure with rolipram prevented ethanol-induced increases in synaptic $\alpha 4$ abundance. (C and D) GABA_A $\gamma 2$ abundance was not altered by exposure to ethanol or either Rp-cAMP (C) or rolipram (D). * $P < 0.05$ (one-way ANOVA, Bonferroni post-test, $n = 6-10$). Ctrl, control; EtOH, ethanol; OD, optical density; Roli, rolipram.

concentrations; Sigma-Aldrich) or the activator phorbol-12,13-dibutyrate (0.1 μ M; Sigma-Aldrich) was added to the cell media. The concentrations of PKA and PKC modulators were chosen based on previous studies (Zhang and Pandey, 2003; Carlson et al., 2013).

Subcellular Fractionation. After experiments, the reactions were stopped by placing the flasks on ice. Cells were washed with cold phosphate-buffered saline, scraped, centrifuged at 1000g for 18 minutes, and stored at -80°C until fractionation. Cell pellets were homogenized in 0.32 M sucrose and centrifuged at 1000g for 10 minutes. The supernatant was then centrifuged twice for 30 minutes at 12,000g to yield the P2 fraction pellet. For experiments examining the synaptic fraction, the P2 fraction was further purified into the synaptic fraction according to the methods of Goebel-Goody et al. (2009) as previously described (Carlson et al., 2014, 2016). The fractions were separated by 30-minute incubation in 0.5% Triton-X, followed by two centrifugations at 32,000g for 30 minutes. The resulting pellet was resuspended to yield the synaptic fraction. Protein concentrations for isolated P2 fraction or synaptic fractions were calculated using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were then subjected to gel electrophoresis and western blot analysis.

Western Blot Analysis. GABA_A receptor $\alpha 4$ subunit abundance was analyzed by western blot. Protein samples were subjected to SDS-PAGE using Novex Tris-Glycine (8%–16%) gels and were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Membranes were probed with GABA_A receptor $\alpha 4$ antibody (1:500 dilution; Abcam, Cambridge, MA), anti-GABA_A δ (1:750 dilution; Novus, St. Louis, MO), or anti-GABA_A $\gamma 2$ (1:1000 dilution; Novus), followed by β -actin antibody (1:3000 dilution; Millipore, Billerica, MA) for normalization. Proteins were detected with

enhanced chemiluminescence (GE Healthcare, Amersham, UK). Membranes were imaged using a LAS-4000 (GE Healthcare), and densitometric analysis was conducted using GE ImageQuant software. Comparisons were made within blots and expressed as a percentage of averaged control values.

Electrophysiology. Whole-cell voltage clamp recordings were used to assess mIPSCs. Electrodes were pulled using a PP-830 device (Narishige, Tokyo, Japan) and were fire polished to a resistance of 2 to 3 M Ω . Intracellular solution contained 150 mM KCl, 3.1 mM MgCl₂, 15 mM HEPES, 5 mM KATP, 5 mM EGTA, and 15 mM phosphocreatine, adjusted to pH 7.4 with KOH. Extracellular solution contained 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM sucrose, and 10 mM glucose, adjusted to pH 7.4 with NaOH. For mIPSC recordings, the external solution also contained 6-cyno-7-nitroquinoxaline-2,3-dione (10 μ M; Sigma-Aldrich), D-2-amino-5-phosphonopentanoic acid (40 μ M; Tocris Bioscience), and tetrodotoxin (1 μ M; Sigma-Aldrich). Membrane potential was held at -60 mV and currents were recorded with an Axopatch ID amplifier (Axon Instruments, Union City, CA). Data were collected using Clampex software (Axon Instruments). mIPSCs were analyzed using miniAnalysis software (version 5.6.4; Synaptosoft, Decatur, GA). mIPSCs were recorded for a minimum of 3 minutes. Minimum threshold detection was set to 5 pA. Frequency was determined using automatic detection of each recording. To assess mIPSC kinetics, the recording trace was visually inspected and only events with a stable baseline, sharp rising phase, and single peak were used. Only recordings with a minimum of 25 events fitting these criteria were analyzed. Decay time constants were obtained by using a double exponential fit for the average of the mIPSCs in a single recording.

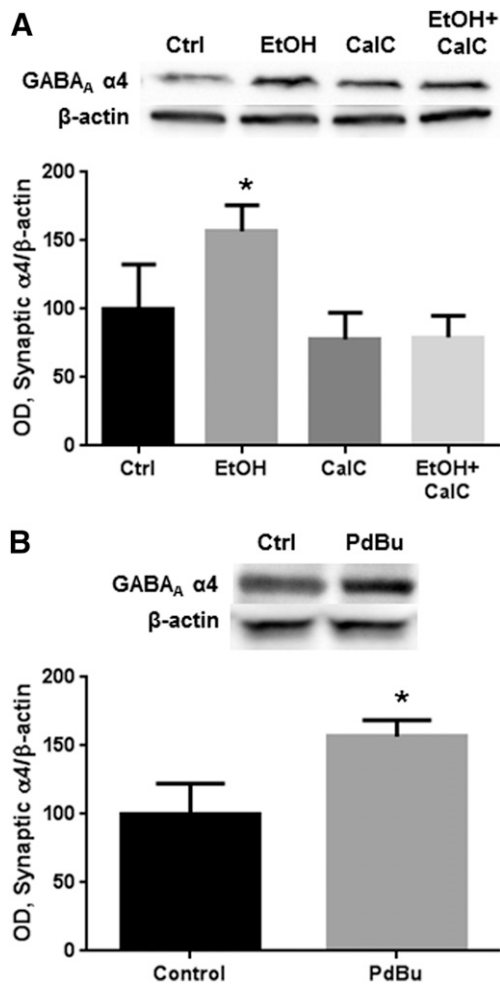


Fig. 3. PKC activation by ethanol increases GABA_A α 4 subunit levels in the synaptic fraction. Cortical neurons were exposed to vehicle, ethanol (50 mM), CalC (0.3 μ M), and/or PdBu (0.1 μ M) for 4 hours followed by synaptic fractionation and western blot analysis. (A) Exposure to ethanol for 4 hours increased synaptic fraction levels of the GABA_A α 4 subunit, which was prevented by coexposure with CalC. (B) Exposure to PdBu mimicked the effect of ethanol on synaptic GABA_A α 4 levels. * $P < 0.05$ (t test or one-way ANOVA, Bonferroni post-test, $n = 5$ to 6 per group). Ctrl, control; EtOH, ethanol; OD, optical density; PdBu, phorbol-12,13-dibutyrate; CalC, Calphostin C.

Statistical Analysis. Numerical data are presented as means \pm S.E.M. Analyses were conducted using analysis of variance (ANOVA) and the Bonferroni post-test or t test.

Results

Direct PKA Activation Prevents the Effects of 4-Hour Ethanol Exposure on Total GABA_A α 4 Abundance in the P2 Fraction. We first examined the effect of PKA modulation on GABA_A α 4 subunits during ethanol exposure. Exposure to ethanol for 4 hours increased GABA_A α 4 subunit levels in the P2 fraction of cerebral cortical neurons as expected based on previous studies (Fig. 1). Inhibition of PKA by Rp-cAMP did not affect ethanol-induced increases in α 4 abundance (Fig. 1A). Conversely, activation of PKA during ethanol exposure by either the adenylyl cyclase activator forskolin (10 μ M; Fig. 1B, $P < 0.01$, one-way ANOVA, $F = 6.330$, $P < 0.01$, Bonferroni post-test, $n = 6$ to 7 per group) or the phosphodiesterase inhibitor rolipram (10 μ M; Fig. 1C,

$P < 0.05$, one-way ANOVA, $F = 3.107$, $P < 0.05$, Bonferroni post-test, $n = 7$ to 8 per group) prevented the increase of α 4 subunit abundance. None of the PKA modulators alone had any effect on GABA_A α 4 subunit abundance.

PKA Activation Prevents the Effects of 4-Hour Ethanol Exposure on Synaptic Fraction GABA_A α 4 Abundance. To resolve whether the effects of ethanol and PKA on GABA_A α 4 subunits in the P2 fraction represent synaptic receptor regulation, we expanded the study shown in Fig. 1 to determine whether these effects occur within the synaptic fraction purified by subcellular fractionation (Carlson et al., 2014, 2016; Bohnsack et al., 2016). Coexposure of Rp-cAMP with ethanol did not prevent ethanol-induced increases in synaptic GABA_A α 4 abundance (Fig. 2A, $P < 0.01$, one-way ANOVA, $F = 5.776$, $P < 0.05$, Bonferroni post-test, $n = 6$ –8 per group). Conversely, coexposure of rolipram with ethanol prevented increases in synaptic GABA_A α 4 abundance (Fig. 2B, $P < 0.05$, one-way ANOVA, $F = 3.107$, $P < 0.05$, Bonferroni post-test, $n = 7$

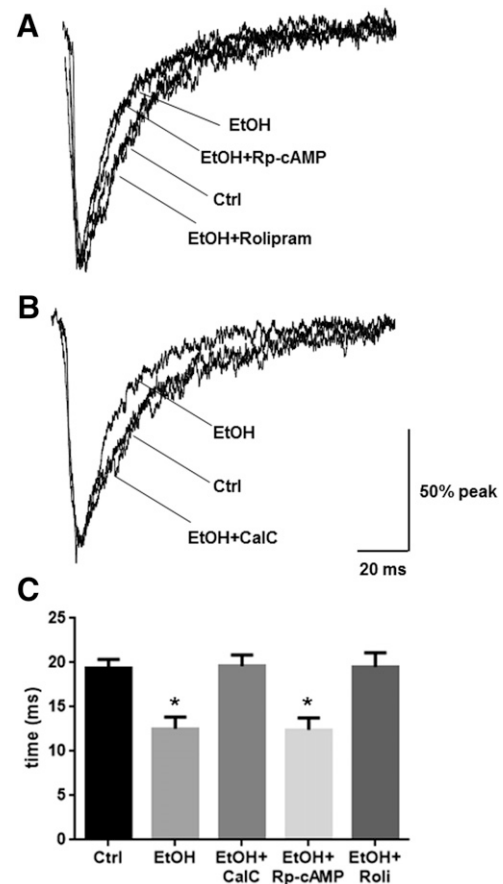


Fig. 4. PKA activation or PKC inhibition mitigate ethanol-induced alterations in mIPSC responses. Whole-cell patch-clamp recordings of cortical neurons were made in the presence of TTX (1 μ M), CNQX (10 μ M), and AP-5 (40 μ M) to isolate GABA mIPSCs after 4-hour exposure to ethanol and/or kinase modulatory drugs. (A) Decay time (decay τ_1) was significantly decreased by ethanol exposure, an affect that was not affected by coexposure of ethanol with Rp-cAMP; however, coexposure of ethanol with rolipram prevented this decrease. (B) Coexposure of ethanol with CalC also prevented the decrease in decay rate induced by ethanol alone. (C) Quantification of changes in decay τ_1 is shown. (* $P < 0.05$ compared with control, EtOH plus CalC, or EtOH plus rolipram groups; one-way ANOVA, Bonferroni post-test). Summarized mIPSC metrics are presented in Table 1. AP-5, D-2-amino-5-phosphopentanoic acid; CNQX, 6-cyno-7-nitroquinoxaline-2,3-dione; Ctrl, control; EtOH, ethanol; Roli, rolipram; TTX, tetrodotoxin.

TABLE 1
GABA mIPSC decay kinetics after exposure to PKA modulators and ethanol
Data are presented as means \pm S.E.M.

Measure	Control	Ethanol	Ethanol Plus CalC	Ethanol Plus Rp-cAMP	Ethanol Plus Rolipram
Rise time (ms)	2.4 \pm 1.0	3.1 \pm 1.4	2.8 \pm 0.9	1.9 \pm 1.0	2.6 \pm 0.6
Amplitude (pA)	22.1 \pm 1.2	23.5 \pm 1.0	22.8 \pm 1.9	21.6 \pm 0.9	22.5 \pm 1.1
Frequency (Hz)	1.9 \pm 0.4	1.5 \pm 0.8	2.1 \pm 0.6	1.8 \pm 0.4	2.0 \pm 0.5
Decay τ_1	19.4 \pm 1.0	12.5 \pm 1.3*	19.6 \pm 1.3	12.4 \pm 1.4*	19.5 \pm 1.6
Decay τ_2	30.1 \pm 2.3	32.1 \pm 2.6	31.2 \pm 2.5	32.0 \pm 1.9	31.6 \pm 1.4
Number	14	11	11	11	11

* $P < 0.05$ (compared with control, ethanol plus CalC, or ethanol plus rolipram groups, one-way ANOVA, Bonferroni post-test).

to 8 per group). No changes in GABA_A $\gamma 2$ subunit abundance were observed in response to ethanol or PKA modulators (Fig. 2, C and D). No δ subunits were detected in the synaptic fraction (data not shown), although δ subunit abundance is known to decrease after 4-hour ethanol exposure in the extrasynaptic fraction (Carlson et al., 2016).

PKC Activity Mediates Ethanol-Induced Increases in Synaptic GABA_A $\alpha 4$ Subunits. Although we previously found increases in P2 fraction levels of GABA_A $\alpha 4$ to be caused by PKC activity (Werner et al., 2011), we next used subcellular fractionation to confirm that these changes occur in the synaptic fraction. Ethanol increased synaptic GABA_A $\alpha 4$ levels, an effect that was prevented by inhibiting PKC with CalC (0.3 μ M; Fig. 3A, $P < 0.01$, one-way ANOVA, $F = 12.53$, $P < 0.01$, Bonferroni post-test, $n = 6$ per group). CalC alone had no effect on GABA_A $\alpha 4$ subunit abundance. Direct activation of PKC with the phorbol ester phorbol-12,13-dibutyrate mimicked the effect of ethanol in increasing synaptic GABA_A $\alpha 4$ abundance (Fig. 3B, $P < 0.01$, t test, $n = 5$ to 6 per group).

PKC Inhibition and PKA Activation Prevent Ethanol-Induced Changes in GABA mIPSCs. Finally, we investigated the ramifications of PKA and PKC activity on mIPSC kinetics during 4-hour ethanol exposure. We previously demonstrated that GABA mIPSC decay τ_1 is decreased after 4-hour ethanol exposure (Werner et al., 2011). Coexposure of ethanol with Rp-cAMP had no effect on the ethanol-induced decrease in GABA mIPSC decay τ_1 , whereas coexposure with rolipram prevented the decrease (Fig. 4, A and C, $P < 0.001$, one-way ANOVA, $F = 9.955$, $P < 0.001$, Bonferroni post-test, $n = 11$ –14 per group). Coexposure of ethanol with CalC prevented the decrease in GABA mIPSC decay τ_1 (Fig. 4, B and C, $P < 0.001$, one-way ANOVA, $F = 11.78$, $P < 0.001$, Bonferroni post-test, $n = 11$ –14 per group). There were no other effects on mIPSC kinetics (Table 1).

Discussion

In this study, we demonstrate that PKA and PKC have opposing effects on synaptic GABA_A $\alpha 4$ subunit abundance and function. We elucidate that PKC activation by ethanol

upregulates synaptic GABA_A $\alpha 4$ abundance to reduce the mIPSC decay rate, whereas maintaining PKA activation through phosphodiesterase inhibition prevents these effects. These data further characterize the regulation of a poorly understood GABA_A receptor that may be an important mediator of the chronic effects of ethanol, and these results enhance our understanding of kinase regulation of GABA_A receptors by ethanol (Table 2). Thus, these findings implicate potential therapeutic methods for restoring normal GABAergic functioning after chronic alcohol misuse.

We used a 4-hour in vitro ethanol exposure paradigm that recapitulates changes observed after chronic ethanol consumption and withdrawal (Devaud et al., 1997; Kumar et al., 2003; Carlson et al., 2013, 2016). It was not surprising that PKA inhibition during 4-hour ethanol exposure had no effect, because we previously found that PKA abundance (Carlson et al., 2013) and activity (Carlson et al., 2016) are not altered by ethanol exposure for 4 hours, despite the effects of ethanol at 1 hour. Nonetheless, it was possible that earlier PKA activation might produce downstream effects and this possibility was ruled out by testing the effect of PKA inhibition. Since PKA inhibition had no effect on synaptic GABA_A $\alpha 4$ receptor expression, it appears that constitutive PKA activity does not regulate synaptic GABA_A $\alpha 4$ receptors. The data suggest that PKA activation downregulates synaptic GABA_A $\alpha 4$ receptors, because either maintaining PKA activity through phosphodiesterase inhibition or activating PKA through adenylyl cyclase activation prevented ethanol-induced increases in GABA_A $\alpha 4$ subunits. Because GABA_A $\alpha 4\beta 2$ receptors are upregulated in other pathologic conditions, including seizure disorders (González and Brooks-Kayal, 2011) and progesterone withdrawal (Gulinello et al., 2002), PKA regulation of these receptors likely has broad implications deserving further exploration.

Our laboratory previously demonstrated that PKC activation by ethanol leads to an upregulation of the abundance of GABA_A $\alpha 4$ subunits in vitro after 4 hours, purportedly owing to an increase in the synaptic population of GABA_A $\alpha 4$ receptors (Werner et al., 2011). However, these studies did not isolate synaptic $\alpha 4$ receptors since δ subunits were

TABLE 2
Summary of findings on modulation of GABA_A $\alpha 1$ and $\alpha 4$ subunits by ethanol and kinases
Data are presented as means \pm S.E.M. Downward arrows indicate negative modulation, whereas upward arrows indicate positive modulation. The dash indicates no change.

Subunit	Chronic Ethanol	PKC	PKA
GABA _A $\alpha 1$	↓ (Kumar et al., 2003)	↓ (Kumar et al., 2010)	↑ (Carlson et al., 2013)
Extrasynaptic GABA _A $\alpha 4$	↓ (Liang et al., 2006) ^a	— (Carlson et al., 2016)	↑ (Carlson et al., 2016)
Synaptic GABA _A $\alpha 4$	↑ (Liang et al., 2006) ^a	↑ (This study)	↓ (This study)

^aNote that these studies are in the hippocampus.

detected in the P2 fraction. Our current results, combined with recent findings demonstrating a lack of effect of PKC on extrasynaptic GABA_A α 4 δ receptors (Bohnsack et al., 2016; Carlson et al., 2016), support the hypothesis that PKC effects are specific for the synaptic GABA_A α 4 receptors in cortical neurons and that synaptic and extrasynaptic populations of receptors are subject to different methods of regulation. The similarity of ethanol effects in the synaptic fraction and P2 fraction suggests that the P2 fraction largely consists of synaptic components and that the subsynaptic fraction, although functionally relevant (Carlson et al., 2016), may not be present in sufficient quantities to confound these results.

Our results suggest that PKA and PKC play oppositional roles in synaptic GABA_A α 4 receptor regulation, similar to our previous findings on GABA_A α 1 receptor regulation (Carlson et al., 2013). These results are consistent with other studies demonstrating oppositional roles of PKC and PKA activity on GABA receptor functioning (Poisbeau et al., 1999; Brandon et al., 2000; Bohnsack et al., 2016). The observation that PKA activation decreases synaptic α 4 abundance could explain why we previously found no difference in whole-cell GABA-evoked current amplitude or GABA dose response after 1 hour of PKA activation, despite an increase in α 1 receptor abundance (Carlson et al., 2013). Similarly, the lack of change in GABA_A γ 2 subunit abundance observed in this study is likely attributable to opposing changes in α 4 β γ 2 vs. α 1 β γ 2 receptors (Kumar et al., 2010). The observation that kinase inhibition in the absence of ethanol had no effect on synaptic α 4 subunits suggests that these receptors do not undergo constitutive regulation by these pathways but are only altered after a physiologic insult such as high concentrations of ethanol. These findings are consistent with previous studies in which the PKA RII β subunit did not constitutively regulate α 4 subunits in vivo (Carlson et al., 2014).

The more rapid mIPSC decay constants after 4-hour ethanol exposure were consistent with previous findings from our laboratory (Werner et al., 2011). The synaptic GABA_A α 4 receptors display more rapid decay times than GABA_A α 1 receptors in recombinant systems (Whittemore et al., 1996; Brown et al., 2002) and α 4 knockout mice have longer decay times compared with wild-type mice (Chandra et al., 2006). Thus, the faster decay time after chronic ethanol exposure is consistent with a higher proportion of GABA_A α 4 receptors in the synaptic GABA receptor population; however, it will be important to confirm this conclusion in recombinant systems that can isolate synaptic α 4 receptors physiologically. These results mirror similar studies uncovering reduced decay times after chronic ethanol exposure in the hippocampus (Cagetti et al., 2003; Liang et al., 2006) and during withdrawal from the neuroactive steroid allopregnanolone (Hsu et al., 2003). These changes are correlated with a reduction in the anxiolytic and sleep-inducing effects of ethanol associated with withdrawal and dependence. Thus, the finding that PKC inhibition or PKA activation in the presence of ethanol prevented the faster GABA_A mIPSC decay time suggests two possible methods of preventing pathologic changes associated with ethanol dependence. Finally, the correspondence of the functional changes in mIPSCs with the change in α 4 subunit expression detected by synaptic fractionation technique further validates the viability of this method of isolating synaptic proteins.

Our results underscore the potential therapeutic relevance for phosphodiesterase inhibition using drugs such as rolipram. Recent studies in rodent models have demonstrated

decreased drinking behavior in animals given phosphodiesterase inhibitors (Hu et al., 2011; Wen et al., 2012; Blednov et al., 2014; Franklin et al., 2015). Together, these studies suggest that phosphodiesterase inhibition provides a promising target for the treatment of alcohol use disorders. Future in vivo studies examining effects of coadministration of ethanol and rolipram (or administration of rolipram after chronic ethanol) on GABAergic trafficking and GABA-related behavior would be a logical extension of these data.

This study expands our understanding of kinase signaling in modulating the GABAergic effects of ethanol. The data suggest that PKA activity may prevent alterations of GABAergic inhibition associated with chronic alcohol misuse, whereas PKC activity may facilitate them. These second messenger pathways could provide important targets for treatments to prevent or restore normal GABA_A receptor functioning associated with alcohol tolerance, dependence, and withdrawal.

Acknowledgments

The authors thank Todd K. O'Buckley, Raechel McKinley, and Danielle Morrow for technical assistance.

Authorship Contributions

Participated in research design: Carlson, Bohnsack, Morrow.

Conducted experiments: Carlson, Bohnsack.

Performed data analysis: Carlson, Bohnsack.

Wrote or contributed to the writing of the manuscript: Carlson, Bohnsack, Morrow.

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