Ethanol Activation of Protein Kinase A Regulates GABA_A α 1 Receptor Function and Trafficking in Cultured Cerebral Cortical Neurons

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ABSTRACT

Ethanol exposure produces alterations in GABAergic signaling that are associated with dependence and withdrawal. Previously, we demonstrated that ethanol-induced protein kinase C (PKC) γ signaling selectively contributes to changes in GABA_A α 1 synaptic receptor activity and surface expression. Here, we demonstrate that protein kinase A (PKA) exerts opposing effects on GABA_A receptor adaptations during brief ethanol exposure. Cerebral cortical neurons from day 0–1 rat pups were tested after 18 days in culture. Receptor trafficking was assessed by Western blot analysis, and functional changes were measured using whole-cell patch-clamp recordings of evoked and miniature inhibitory postsynaptic current (mIPSC) responses. One-hour ethanol exposure increased membrane-associated PKC and PKA, but steady-state GABA_A α 1 subunit levels were maintained.

Activation of PKA by Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine alone increased GABA_A α 1 subunit surface expression and zolpidem potentiation of GABA responses, whereas coexposure of ethanol with the PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine decreased α 1 subunit expression and zolpidem responses. Exposure to the PKC inhibitor calphostin-C with ethanol mimicked the effect of direct PKA activation. The effects of PKA modulation on mIPSC decay τ were consistent with its effects on GABA currents evoked in the presence of zolpidem. Overall, the results suggest that PKA acts in opposition to PKC on α 1-containing GABA_A receptors, mediating the GABAergic effects of ethanol exposure, and may provide an important target for the treatment of alcohol dependence/withdrawal.

Introduction

Ethanol elicits a number of adaptations in GABAergic signaling following both acute and chronic exposure. Evidence suggests that acute ethanol intoxication increases overall GABAergic inhibition, whereas chronic ethanol leads to deficits in GABA functioning [reviewed in Kumar et al. (2004, 2009)]. Numerous studies suggest that the major changes associated with GABAergic deficits following chronic ethanol exposure include a decrease in surface expression of GABA_A receptor α 1 subunits in both the cerebral cortex (Devaud et al., 1997; Kumar et al., 2003) and hippocampus (Cagetti et al., 2003; Liang et al., 2004, 2006, 2007). Chronic ethanol exposure produces a withdrawal syndrome upon discontinuation of ethanol that is mimicked, in part, by genetic deletion of α 1 subunits in mice that were never exposed to ethanol.

This suggests that the loss of GABA_A α 1 receptor surface expression is involved in tremor and enhanced seizure susceptibility associated with ethanol dependence (Kralic et al., 2002, 2005). Additionally, similar alterations in GABA_A α 1 subunit levels are found in other disorders, including models of epilepsy and benzodiazepine tolerance (Chen et al., 1999; Zeng and Tietz, 1999; Gonzalez and Brooks-Kayal, 2011). Therefore, a better understanding of GABA_A receptor regulation following ethanol exposure may provide both valuable insights into the general mechanism of GABA_A receptor regulation as well as potential therapeutic targets for pathologies linked to these disorders.

Recently, we and others have shown that GABA_A receptor adaptations to ethanol are recapitulated in vitro following a brief ethanol exposure in cultured cortical and hippocampal neurons (Kumar et al., 2010; Shen et al., 2011; Werner et al., 2011). The effect of ethanol is dependent upon the selective activation of protein kinase C (PKC) γ to induce the internalization of GABA_A α 1 subunits (Kumar et al., 2010), consistent with the ability of ethanol to activate PKC γ and reduce GABA_A α 1 subunit surface expression in vivo. However, there

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ABBREVIATIONS: ANOVA, analysis of variance; CalC, calphostin-C; mIPSC, miniature inhibitory postsynaptic current; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; Rp-cAMP, Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine; Sp-cAMP, Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine.

is a mismatch between the time-dependent effects of ethanol on PKC γ and GABA $_A$ $\alpha 1$ receptors, suggesting the involvement of other mechanisms (Kumar et al., 2010).

Ethanol has long been known to directly activate protein kinase A (PKA) (Dohrman et al., 1996). In vivo studies implicate PKA as playing a role in the behavioral effects of ethanol (Thiele et al., 2000; Pandey et al., 2003; Lai et al., 2007). Additionally, PKA has been shown to directly phosphorylate and modulate GABA $_A$ receptor activity (Ives et al., 2002; Brandon et al., 2003). Recently, we found that ethanol dose- and time-dependently increased PKA membrane levels in rat cerebral cortex, which corresponded to increased GABA $_A$ $\alpha 1$ subunit expression (Kumar et al., 2012). The precise physiological consequences of ethanol regulation of GABAergic inhibition via PKA, however, are unclear. As recent studies from our laboratory suggest that both PKC and PKA activation may influence GABA $_A$ receptor activity during ethanol exposure, we investigated the dualistic role of PKA and PKC in modulating the actions of ethanol on GABA $_A$ receptor $\alpha 1$ subunit expression and function in cortical cultured neurons.

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. Cultured cerebral cortical neurons were isolated as described elsewhere (Kumar et al., 2010). Briefly, 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 CO $_2$ -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, while cells used for electrophysiology were plated onto poly-D-lysine-coated cover slips in 12-well plates. Cells were maintained in a 5% CO $_2$ 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 at least 17 days before conducting experiments, as 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 chamber within the incubator. 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 vapor chamber with a beaker containing water. Cells were exposed to ethanol for various times (0–60 minutes). To examine PKA involvement, the PKA activator Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Sp-cAMP) (70 μ M) and the PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Rp-cAMP) (50 μ M) were added to the cell media. These doses were chosen based on previous studies (Zhang and Pandey, 2003). To examine PKC involvement, the PKC inhibitor calphostin-C (CalC) (0.3 μ M in 0.1% dimethyl sulfoxide, final concentrations) was used as previously described (Kumar et al., 2010).

P2 Fractionation and Biotinylation. After experiments, the reactions were stopped by placing the flasks on ice. Cells were washed with cold phosphate-buffered saline (PBS), 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. The final pellet was resuspended in PBS. Protein concentrations for the isolated P2 fraction were made using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Biotinylation experiments were performed as previously described (Kumar et al., 2010). Briefly, cells were washed twice with ice-cold PBS, followed by addition of sulfo-succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate diluted in PBS, and incubated for 30 minutes at 4°C. Unbound biotin was inactivated using a quenching solution. Cells were scraped and spun three times at 500g in Tris-buffered saline. Biotin-labeled (surface) and flow-through (cytosolic) proteins were separated using NeutrAvidin slurry (Thermo Fisher Scientific). Biotinylated proteins were eluted from the beads by incubation for 60 minutes at 22°C in Laemmli sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer. Samples were then subjected to gel electrophoresis and Western blotting.

Western Blot Analysis. GABA $_A$ receptor $\alpha 1$ subunits, PKA subunits, and PKC isoforms were analyzed by Western blotting as described elsewhere (Kumar et al., 2010). Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using Novex Tris-Glycine (8–16%) gels and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). Membranes were probed with GABA $_A$ receptor $\alpha 1$ subunit (Millipore, Billerica, MA) or PKA RII α , RII β , or PKC γ (BD Biosciences, Franklin Lakes, NJ) antibodies. Blots were then exposed to an antibody for β -actin for normalization. Proteins were detected with enhanced chemiluminescence (GE Healthcare, Amersham, UK). Membranes were exposed to film under nonsaturating conditions. Densitometric analysis was conducted using NIH Image 1.57 (Bethesda, MD). Comparisons were made within blots and expressed as % averaged control values. Statistics were conducted using analysis of variance (ANOVA) or Student's *t* test.

Electrophysiology. Whole-cell voltage clamp recordings were used to assess evoked currents and miniature inhibitory postsynaptic currents (mIPSCs). Electrodes were pulled using a PP-830 (Narishige, Tokyo, Japan) and fire-polished to a resistance of 2–3 M Ω . Intracellular solution contained 150 mM KCl, 3.1 mM MgCl $_2$, 15 mM HEPES, 5 mM KATP, 5 mM EGTA, and 15 mM phosphocreatine, adjusted to pH 7.4 with KOH, while extracellular solution contained 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl $_2$, 1 mM MgCl $_2$, 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, Bristol, UK), and tetrodotoxin (1 μ M) (Sigma-Aldrich). Membrane potential was held at –60 mV, and currents were recorded with an Axopatch ID (Axon Instruments, Union City, CA) amplifier. Data were collected using Clampex software (Axon Instruments). Drugs were diluted in the extracellular solution and applied using a U-tube apparatus. Zolpidem (100 nM) was coapplied with GABA (1 or 10 μ M) to observe potentiation. Potentiation by zolpidem is expressed as the current in response to (GABA + zolpidem/GABA alone $\times$ 100). Dose-response curves were generated from GABA-evoked responses at 1, 3, 10, 30, 100 μ M, and 1 mM GABA. Current amplitudes were normalized to the averaged control current response to 1 mM GABA and fit using GraphPad Prism software (GraphPad Software, La Jolla, CA) with the sigmoidal dose-response equation:

$$Y = \min + (\max - \min) / [1 + 10^{(\text{LogEC}_{50} - X)}]$$

mIPSCs were analyzed using the Mini-Analysis 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 and amplitude were 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 to negate artifacts due to event summation. 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. Numerical data are presented as mean $\pm$ S.E.M. Statistics were conducted using ANOVA or Student's *t* test.

Results

PKA Activation by Ethanol Modulates GABA_A Receptor Trafficking and Activity. Cultured cerebral cortical neurons were exposed to ethanol (50 mM) for 1 hour to test for its effects on membrane expression of GABA_A α 1 subunit and protein kinase levels (Fig. 1). Whereas 4-hour ethanol exposure produces a decrease in GABA_A α 1 levels (Kumar et al., 2010), no effects on this subunit are observed at the 1-hour time point (Fig. 1A). However, 1-hour ethanol exposure produced a significant increase in the abundance of both PKC γ ($78.1\% \pm 21.0\%$; $n = 7$; $P < 0.05$, Student's *t* test) (Fig. 1B) and the PKA regulatory subunits RII α ($35.5\% \pm 12.7\%$; $n = 6$; $P < 0.05$, Student's *t* test) (Fig. 1C) and RII β ($36.4\% \pm 11.1\%$; $n = 6$; $P < 0.05$, Student's *t* test) (Fig. 1D) in the P2 fraction. Table 1 provides a comparison of the GABA_A α 1 and protein kinase trafficking changes induced by 1-hour and 4-hour ethanol exposure in cerebral cortical neurons. Whereas GABA_A α 1 membrane levels are unchanged at 1 hour and decreased following 4-hour ethanol, PKC γ levels are elevated at both time points. Conversely, whereas PKA RII α

and RII β are elevated at the 1-hour time point, PKA levels return to baseline after 4-hour ethanol (Table 1).

To determine the effects of ethanol that are mediated by PKA, GABA_A receptor subunit levels were assessed after either direct activation of PKA or ethanol exposure in the presence of a PKA inhibitor. Additionally, whole-cell patch-clamp recordings were used to measure functional changes in GABA_A receptor electrophysiological responses. GABA (1–1000 μ M) responses were first determined, and then zolpidem (100 nM) enhancement of GABA (10 μ M) responses was used as a pharmacological indicator for altered synaptic GABA_A α 1-containing receptor activity (Liang et al., 2004; Kumar et al., 2010). Currents evoked by GABA alone were compared with currents evoked by coapplication of GABA + zolpidem to measure percent potentiation.

Exposure to the PKA activator Sp-cAMP (50 μ M) for 1 hour produced a significant increase in GABA_A α 1 subunits relative to control values in the P2 fraction ($58.4\% \pm 13.7\%$; $n = 7$; $P < 0.05$, Student's *t* test) (Fig. 2A), with a corresponding increase in surface biotinylated protein ($50.48\% \pm 18.45\%$; $n = 5$; $P < 0.05$, Student's *t* test) (Fig. 2B) and decrease in the cytosolic fraction ($54.03\% \pm 10.74\%$; $n = 5$; $P < 0.05$, Student's *t* test) (Fig. 2C). Sp-cAMP exposure had no effect on the EC₅₀ or amplitude of GABA-evoked responses (Fig. 2D) or whole-cell GABA-evoked current amplitude at the dose used to test zolpidem enhancement of GABA responses (Fig. 2E). Sp-cAMP exposure did, however, increase zolpidem potentiation of GABA responses by $78.1\% \pm 9.4\%$ ($n = 6$ per group; Student's *t* test, $P < 0.05$) (Fig. 2, F and G) compared with control cells. Currents evoked over the course of 1 hour during Sp-cAMP

Fig. 1. One-hour ethanol (EtOH) (50 mM) alters expression of protein kinase subunits in the P2 fraction of cultured cortical neurons. Cortical neurons were exposed to EtOH (50 mM for 60 minutes), followed by preparation of P2 fractions. Western blot analysis of P2 fractions of cortical neurons revealed that P2 fraction levels of GABA_A α 1 were unchanged (A), whereas PKC γ subunit levels were increased by $78.1\% \pm 25.2\%$ (B), PKA RII α subunit levels were increased by $35.5\% \pm 12.7\%$ (C), and PKA RII β subunit levels were increased by $36.4\% \pm 11.1\%$ (D) following ethanol exposure. Graphs show the mean $\pm$ S.E.M. of percent control optical density (OD) values normalized to β -actin levels ($n = 4$ – 7 per group). * $P < 0.05$ compared with vehicle (Student's *t* test).

TABLE 1

Comparison of effects of ethanol at 1 and 4 hours on P2 fraction protein levels

Data for 4-hour time point levels of GABA_A α 1 and PKC γ are from Kumar et al. (2010).

Protein	1-H Ethanol	4-H Ethanol
GABA _A α 1	109.4 $\pm$ 9.6%	$\downarrow$ 59.8 $\pm$ 15.0%*
PKC γ	$\uparrow$ 178.1 $\pm$ 21.0%*	$\uparrow$ 152.3 $\pm$ 19.0%*
PKA RII α	$\uparrow$ 135.5 $\pm$ 12.7%*	97.1 $\pm$ 5.3%
PKA RII β	$\uparrow$ 136.4 $\pm$ 11.1%*	100.7 $\pm$ 4.2%

* P < 0.05 compared with controls, Student's t test.

exposure revealed that direct PKA activation resulted in a rapid increase in potentiation by zolpidem that was sustained over the course of the hour (n = 4; repeated-measures ANOVA, f = 22.03, P < 0.05, significantly increased at t = 12–60 minutes, Bonferroni post-test, P < 0.05), whereas current amplitude was stable for currents evoked under control conditions (Fig. 3).

Although ethanol alone did not significantly alter GABA_A α 1 levels after 1 hour, coexposure of ethanol and the PKA inhibitor Rp-cAMP decreased GABA_A α 1 membrane levels by 33% (n = 4; one-way ANOVA, f = 12.42, P < 0.05, Newman-Keuls post-test, P < 0.05) (Fig. 4A). Ethanol, ethanol + Rp-cAMP, or Rp-cAMP exposure had no effect on the GABA dose response (Fig. 4B) or GABA-evoked current amplitude (Fig. 4C). Reduced membrane levels of α 1 subunits corresponded to reduced zolpidem potentiation of GABA responses in ethanol + Rp-cAMP-exposed cells (n = 6–11; one-way ANOVA, f = 4.031, P < 0.05, Newman-Keuls post-test, P < 0.05) (Fig. 4, D and E). Rp-cAMP exposure alone had no effect on GABA_A α 1 subunit levels or zolpidem potentiation.

Inhibition of PKC in the Presence of Ethanol Mimics Direct PKA Activation. As ethanol is known to activate PKC and inhibition of PKC prevents the GABAergic changes seen after 4-hour ethanol exposure (Kumar et al., 2010; Werner et al., 2011), P2 fractions levels of GABA_A subunits

Fig. 2. PKA activator Sp-cAMP increases GABA_A α 1 subunit expression and zolpidem potentiation in cultured cortical neurons. Cortical neurons were exposed to vehicle or Sp-cAMP for 60 minutes, followed by preparation of cellular fractions for Western blot analysis or patch-clamp recording. Activation of PKA increased GABA_A receptor α 1 subunit expression in the P2 fraction (58.4% $\pm$ 13.7%) (A) and in the biotinylated surface protein fraction (50.5% $\pm$ 18.5%) (B) compared with vehicle controls. There was a corresponding decrease in α 1 subunit levels in the cytosolic fraction following Sp-cAMP (54.0% $\pm$ 10.7%) (C). There was no effect of Sp-cAMP exposure on GABA dose response (control EC₅₀ = 15.2 μ M; Sp-cAMP EC₅₀ = 17.5 μ M) (D) or whole-cell current amplitude (E) in response to 10 μ M GABA. Sp-cAMP exposure significantly increased zolpidem potentiation relative to controls (78.1% $\pm$ 9.4%) (F). (G) Representative GABA-evoked (10 μ M) current traces for control and Sp-cAMP exposure with and without 100 nM zolpidem. Data are presented as mean $\pm$ S.E.M. Representative blots are shown. * P < 0.05, Student's t test; n = 6–18 per group.

Fig. 3. PKA activation produces rapid increases in zolpidem potentiation of GABA responses. Whole-cell currents were evoked by application of GABA (1 μ M) and zolpidem (100 nM) at 2-minute intervals over the course of 1 hour with vehicle or Sp-cAMP (50 μ M) exposure. (A) Currents evoked in Sp-cAMP-exposed cells were increased from 12–60 minutes (repeated-measures ANOVA, $f = 3.648$, $P < 0.05$, Bonferroni post-test), whereas control (Ctrl) cells were unaffected. Data are presented as mean $\pm$ S.E.M.; $n = 3$ –4. (B) Representative whole-cell current traces elicited by GABA and zolpidem before ($t = 0$) and after ($t = 60$ minutes) exposure.

were measured following 1-hour ethanol exposure in the presence of the PKC inhibitor CalC (0.3 μ M). Coexposure of ethanol and CalC uncovered effects not normally observed at 1 hour. GABA α 1 membrane levels were increased 56% ($n = 6$; one-way ANOVA, $f = 11.91$, $P < 0.05$, Newman-Keuls post-test, $P < 0.05$) (Fig. 5A).

The regulation of GABA α receptor α 1 subunit expression in the P2 fraction was corroborated by the effects of ethanol plus kinase inhibitors on GABA-evoked currents (compare Figs. 3 and 5, B and C). Ethanol (50 mM) was applied via the extracellular solution at the start of the time course, while CalC or Rp-cAMP was in the patch pipette. Currents evoked by GABA and zolpidem over the course of 1 hour were compared with currents averaged for 10 minutes prior to drug exposure. Current amplitudes were stable prior to application of ethanol and kinase inhibitors (Fig. 5). Current amplitude was not significantly altered in the presence of ethanol alone (Fig. 5, B and C). Coapplication of ethanol + CalC, however, rapidly increased the current amplitude produced by GABA + zolpidem, mimicking the results of direct PKA activation shown above ($n = 3$; repeated-measures ANOVA, $f = 3.863$, significantly increased at $t = 14$ –60 minutes, Bonferroni post-test, $P < 0.05$). Conversely, coapplication of ethanol + Rp-cAMP resulted in a rapid decrease in current amplitude that was also sustained for the duration of 1 hour ($n = 3$; repeated-measures ANOVA, $f = 11.10$, significantly decreased at $t = 8$ –60 minutes, Bonferroni post-test, $P < 0.05$).

PKA Activation by Ethanol Regulates Zolpidem Modulation of mIPSC Decay τ_2 . As ethanol exposure has been shown to alter mIPSC decay kinetics, which is related to changes in GABA α receptor subunit expression (Liang et al., 2006; Fleming et al., 2009; Werner et al., 2011), recordings of mIPSCs were made to further investigate the functional consequences of ethanol activation of PKA on synaptic GABA α receptor signaling. Although there appeared to be a slight shift in the mIPSC decay kinetics suggestive of an altered synaptic GABA α receptor expression profile, there was not a significant direct effect of 1 hour ethanol and/or PKA modulation on GABA mIPSC kinetics (Fig. 6A; Table 2). To further investigate the potential for receptor subtype-specific effects on mIPSCs, recordings were also made in the presence of 100 nM zolpidem. Under control conditions zolpidem increased decay τ_2 as expected (comparison of control with and without zolpidem, $P < 0.05$, Student's t test). Further, decay τ_2 in the presence of zolpidem (Fig. 6B; Table 2) was decreased after ethanol + Rp-cAMP exposure ($24.7\% \pm 3.2\%$; $n = 6$) and increased after Sp-cAMP exposure ($68.8\% \pm 9.0\%$; $n = 6$) relative to controls ($46.1\% \pm 3.7\%$; $n = 8$; one-way ANOVA, $f = 9.673$, $P < 0.05$, Newman-Keuls post-test, $P < 0.05$ for both comparisons). No other mIPSC characteristics were altered in the presence of zolpidem following modulation of PKA activity (Table 2).

Discussion

The present results demonstrate that ethanol activation of PKA plays an active role in GABA α receptor trafficking and function. PKA activation results in an increase in GABA α α 1 subunit expression in P2 and biotinylated fractions of surface receptors in cultured cortical neurons. The finding that these alterations corresponded to functional changes in zolpidem enhancement of GABA responses corroborates this conclusion. Additionally, our results highly implicate oppositional roles of PKA and PKC in GABA α receptor regulation. Ethanol is known to activate both PKA and PKC pathways, and our past results coupled with the present studies indicate that such changes in activity are accompanied by increases in PKA and PKC abundance in the P2 fraction. We demonstrate that inhibiting the activity of either kinase pathway in the presence of ethanol results in GABA α α 1 subunit alterations that are dependent on the sum of the activity of these protein kinase pathways. Specifically, inhibition of PKC in the presence of ethanol results in increased GABA α α 1 surface expression, similar to that observed following exposure to PKA activators alone. Furthermore, a decrease in GABA α receptor α 1 subunit levels similar to that found in models of ethanol dependence in vivo (Devaud et al., 1997; Cagetti et al., 2003; Liang et al., 2004) or 4-hour ethanol exposure in vitro (Kumar et al., 2010; Werner et al., 2011) could be observed after 1-hour ethanol exposure in the presence of a PKA antagonist.

The results suggest that ethanol activates at least two opposing pathways in the cerebral cortex: PKA and PKC. Whereas PKC γ activation results in decreased GABA α receptor α 1 subunit (Kumar et al., 2010), PKA appears to counteract these changes. Such opposition is consistent with previous studies that have found an apparent antagonistic role of PKA and PKC working in other areas of the central nervous system (Vaello et al., 1994; Brandon et al., 2000; Grey and Chang,

Fig. 4. PKA inhibitor Rp-cAMP decreases GABA_A $\alpha 1$ expression and zolpidem potentiation in the presence of ethanol. Neurons were exposed to vehicle, ethanol (EtOH) (50 mM), EtOH with Rp-cAMP (50 μ M), or Rp-cAMP alone for 60 minutes, followed by preparation of P2 fractions for Western blot analysis or patch-clamp recording. EtOH or Rp-cAMP exposure alone did not alter GABA_A receptor $\alpha 1$ subunit expression; however, EtOH exposure in the presence of the PKA inhibitor Rp-cAMP decreased GABA_A receptor $\alpha 1$ subunit expression by 33% (one-way ANOVA, $f = 12.42$, $P < 0.05$, Newman-Keuls post-test) (A). There was no effect of drug exposure on GABA dose response (not all dose-response curves shown for visual simplicity; EtOH + Rp-cAMP $EC_{50} = 16.4$ μ M) (B) or whole-cell current amplitude (C) in response to 10 μ M GABA. (D) EtOH + Rp-cAMP decreased zolpidem potentiation (one-way ANOVA, $f = 4.031$, $P < 0.05$, Newman-Keuls post-test) of GABA-evoked currents compared with vehicle control, whereas EtOH or Rp-cAMP had no effect. (E) Representative GABA-evoked (10 μ M) current traces for control and EtOH + Rp-cAMP exposure with and without 100 nM zolpidem. Data are presented as mean $\pm$ S.E.M. Optical density (OD) values normalized to β -actin. Representative blots are shown. * $P < 0.05$, one-way ANOVA, Newman-Keuls post-test; $n = 3$ –21 per group.

2011). The data further suggest that these kinases are under time-dependent regulation by ethanol. Although PKA and PKC are both active at 1 hour of ethanol exposure, no overall change in GABA_A $\alpha 1$ subunit expression is observed. By 4 hours, however, PKA activity has returned to baseline while PKC remains active, and trafficking of GABA_A $\alpha 1$ receptors is observed (Kumar et al., 2010; Werner et al., 2011). An increase in PKA translocation to the P2 synaptosomal region is consistent with ethanol-induced activation of PKA observed in other cellular systems (Diamond and Gordon, 1997). Although the exact role of increased PKA subunits in the synaptosomal region is unclear, the effects of direct activation by Sp-cAMP, or inactivation by Rp-cAMP in the presence of

ethanol, would suggest that ethanol is increasing overall catalytic activity by PKA. Future studies might investigate the specific phosphorylation states regulating trafficking of $\alpha 1$ subunits by the oppositional PKA and PKC activation by ethanol.

The present results extend several recent studies characterizing altered GABA_A regulation following ethanol exposure. These alterations include a shift in the normal synaptic and extrasynaptic GABA_A receptor trafficking. Studies in the hippocampus have found a decrease in synaptic $\alpha 1$ and extrasynaptic $\alpha 4\delta$ receptors, and a corresponding increase in synaptic $\alpha 4$ GABA_A receptors, following both acute (Liang et al., 2007) and chronic (Cagetti et al., 2003; Liang et al., 2004)

Fig. 5. PKA and PKC activation by ethanol (EtOH) produces opposing effects on GABA_A $\alpha 1$ expression and zolpidem potentiation of GABA responses. (A) Neurons were exposed to vehicle, EtOH (50 mM), or EtOH with CalC (0.3 μ M) for 60 minutes, followed by preparation of P2 fractions and Western blot analysis. EtOH or CalC exposure alone did not alter GABA_A receptor $\alpha 1$ subunit expression; however, EtOH exposure in the presence of the PKC inhibitor CalC increased GABA_A receptor $\alpha 1$ subunit expression by 56% (one-way ANOVA, $f = 11.91$, $P < 0.05$, Newman-Keuls post-hoc test). (B and C) Whole-cell currents were evoked by application of GABA (1 μ M) and zolpidem (100 nM) at 2-minute intervals over the course of 1 hour during exposure to EtOH (50 mM), EtOH and Rp-cAMP (50 μ M), or EtOH and CalC (3 μ M). Currents were stable for the 10-minute period prior to bath application of EtOH and during diffusion of CalC or Rp-cAMP via the recording pipette. Currents evoked by EtOH + CalC were significantly increased from 14–60 minutes (repeated-measures ANOVA, $f = 3.863$, $P < 0.05$, Bonferroni post-test). Currents evoked by EtOH + Rp-cAMP were significantly decreased at 8–60 minutes (repeated-measures ANOVA, $f = 11.10$, $P < 0.01$, Bonferroni post-test). (C) Representative whole-cell current traces elicited by GABA and zolpidem before ($t = 0$) and after ($t = 60$ minutes) EtOH and drug exposure. Data are presented as mean $\pm$ S.E.M.; $n = 3$ –6 per group.

ethanol exposure, thereby producing an overall decrease in GABAergic inhibition and increasing central nervous system hyperexcitability. The present results suggest that these processes are conserved in the cerebral cortex as well (Kumar

et al., 2010; Werner et al., 2011). The lack of an effect of brief (1-hour) ethanol exposure on GABA_A $\alpha 1$ receptors in the present study is consistent with prior reports in cerebral cortex and in cultured cortical neurons (Kumar et al., 2003,

Fig. 6. Ethanol (EtOH) and PKA modulation of GABA_A mIPSC decay kinetics in the presence of zolpidem. Recordings were made in the presence of tetrodotoxin, 6-cyno-7-nitroquinoxaline-2,3-dione, and D-2-amino-5-phosphonopentanoic acid to pharmacologically isolate GABA mIPSCs. Representative averaged mIPSC traces, following exposure to vehicle (control) or drug and standardized for peak amplitude, are shown. (A) Representative control (no zolpidem) traces following control, EtOH, Sp-cAMP, or EtOH + Rp-cAMP exposure. (B) Representative traces in the presence of zolpidem (100 nM) following control, Sp-cAMP, or EtOH + Rp-cAMP exposure. Summarized data are shown in Table 2.

TABLE 2
GABA_A mIPSC kinetics following exposure to PKA modulators and ethanol

Measure	Control	Ethanol	Ethanol + Rp-cAMP	Sp-cAMP
Rise time (ms)	3.6 ± 1.5	1.2 ± 0.8	4.0 ± 1.5	1.3 ± 0.9
Decay 90–37% (ms)	14.4 ± 2.6	21.7 ± 1.6	14.9 ± 2.2	16.7 ± 2.2
Half-width (ms)	12.6 ± 2.5	20.1 ± 0.7	14.5 ± 3.2	16.4 ± 2.0
Amplitude (pA)	25.3 ± 0.8	23.4 ± 1.0	23.2 ± 1.1	21.8 ± 1.8
Frequency (Hz)	2.6 ± 0.9	1.6 ± 0.2	1.6 ± 0.5	1.5 ± 0.3
Decay τ_1 (ms)	13.2 ± 2.4	18.3 ± 2.0	15.0 ± 3.2	16.8 ± 1.7
Decay τ_2 (ms)	31.0 ± 6.4	36.6 ± 5.8	26.3 ± 2.5	45.6 ± 10
Decay τ_2 (ms) with zolpidem	46.1 ± 3.7**	46.2 ± 6.0	24.7 ± 3.2*	68.8 ± 9.0*
<i>n</i>	8	6	6	6

***P* < 0.05 compared with control decay τ_2 (without zolpidem), Student's *t* test; **P* < 0.05 compared with controls with zolpidem, one-way ANOVA, Newman-Keuls post-test.

2010); however, more rapid effects of ethanol have been observed in rat hippocampus (Liang et al., 2007), suggesting that the time course of ethanol regulation of GABA_A receptors may differ across brain regions. We have previously shown that ethanol activation of PKC differs in rat cortex and hippocampus (Kumar et al., 2006). Further studies are needed to determine if regional differences in the time course of ethanol regulation of GABA_A receptors are secondary to regional differences in ethanol activation of protein kinases.

Previous studies have shown that PKA and PKC activation have direct effects on GABA responses that were not observed in the present study (Kittler and Moss, 2003). This is likely due to the use of different model systems and the fact that we focused on the GABA_A $\alpha 1$ receptors in cerebral cortex that are sensitive to 100 nM zolpidem. To isolate effects on these receptors in cultured neurons, we used an IC₁₀ concentration of GABA. It is quite possible that effects of PKA or PKC inhibition may have been observed at higher concentrations of GABA; however, such an experiment would involve actions at additional receptor subtypes. Although effects of PKA on GABA_A receptor function have been previously observed (Brandon et al., 2003; Lilly et al., 2003), this is the first evidence that PKA specifically regulates trafficking and function of zolpidem-sensitive GABA_A $\alpha 1$ receptors. The observation that inhibition of PKA or PKC alone had no effect on $\alpha 1$ receptor abundance or function, however, suggests that these kinases do not constitutively regulate GABA_A $\alpha 1$ receptors. Rather, these pathways may only become active regulators of GABAergic inhibition following physiological insults such as ethanol exposure or processes associated with epileptogenesis (Gonzalez and Brooks-Kayal, 2011).

Although our present results demonstrate modulation of GABA_A receptor $\alpha 1$ subunit abundance by PKA in vitro, our previous results suggest the same processes occur in vivo (Kumar et al., 2012). Recently, we found that GABA_A $\alpha 1$ levels in rat cerebral cortex were increased following an acute ethanol injection, concurrent with an increase in PKA, whereas PKC was unchanged. Further, behavioral studies have well established that PKA is a key mediator of ethanol sensitivity. Infusion of Sp-cAMP into the lateral ventricles prior to systemic ethanol administration increased the duration of loss of righting reflex (Kumar et al., 2012). Conversely, inhibition of PKA prior to ethanol administration decreased loss of righting reflex duration (Lai et al., 2007) and withdrawal-induced anxiety (Pandey et al., 2003). Studies utilizing mutant mouse lines with reduced PKA activity mimic

results found with pharmacological inhibition of PKA (Thiele et al., 2000; Naassila et al., 2002; Kim et al., 2011). The precise role of PKA in mediating these behaviors is complex, however, as some studies have found increased ethanol sensitivity in animals with diminished PKA activity (Wand et al., 2001; Yang et al., 2003; Maas et al., 2005). Although the precise role remains equivocal, collectively these studies clearly establish the importance of PKA activation in mediating the behavioral effects of ethanol, potentially through GABA_A receptor regulation. The present results underscore the clinical relevance of understanding the role of PKA in modulating the effects of ethanol for the development of drugs for the treatment of alcoholism.

The relatively rapid time course over which changes in zolpidem potentiation were observed suggests a posttranslational mechanism of GABA_A receptor regulation (Brandon et al., 2002). Previous studies in cultured cerebral cortical neurons have found changes in GABA_A receptor activity via phosphorylation and dephosphorylation to be due entirely to changes in channel gating and conductance rather than channel number (Brandon et al., 2000). The fact that the functional changes were observed prior to alterations in surface receptor expression suggest that multiple mechanisms of GABA regulation may be involved, including both direct effects on channel conductance as well as receptor trafficking. Further studies investigating single-channel conductance states and utilizing phosphorylation state-dependent antibodies will help to elucidate these mechanisms. Additionally, the observation that whole-cell GABA-evoked current amplitude was unchanged would suggest bidirectional regulation of GABA receptor subtypes by PKA, as well as ethanol. Thus, whereas PKA activation leads to increased surface expression of GABA_A $\alpha 1$ receptors, there is likely a concurrent decrease in other subtypes. Previous studies have observed bidirectional regulation of synaptic GABA_A $\alpha 1$ - and $\alpha 4$ -containing receptors by PKC γ (Kumar et al., 2010; Werner et al., 2011), and studies are currently under way to investigate bidirectional regulation of GABA_A $\alpha 4$ receptors by PKA.

Overall, the present study elucidates a novel role for PKA in the subcellular pathways mediating the effects of ethanol. Although PKC and PKA play important roles in altered GABA_A receptor trafficking following ethanol exposure, it is possible that other kinases or phosphatases are also active. In particular, ethanol-induced adaptations in other brain regions may be governed by different subcellular mechanisms, which could, in part, account for some of the brain region-specific effects of ethanol. This area requires further

study to fully elucidate the physiology of ethanol intoxication and withdrawal. Nonetheless, the current results suggest that PKA activation may delay some ethanol-induced adaptations in GABAergic signaling. Additionally, PKA may provide a promising target for the development of drugs aimed at restoring normal GABA_A receptor functioning for the treatment of multiple pathologies involving similar GABA_A receptor adaptations, including alcohol dependence, epilepsies, and benzodiazepine abuse.

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Authorship Contributions

Participated in research design: Carlson, Kumar, Morrow.

Conducted experiments: Carlson, Kumar, Comerford.

Performed data analysis: Carlson, Kumar, Comerford.

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

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