Published in final edited form as:

Alcohol. 2011 September ; 45(6): 595–605. doi:10.1016/j.alcohol.2011.03.002.

Acute Ethanol Exposure Prevents PMA-mediated Augmentation of N-methyl-D-aspartate Receptor Function in Primary Cultured Cerebellar Granule Cells

Jason Reneau¹, Mary E. Reyland², and R. Lisa Popp^{1,3,4,*}

¹Department of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6592, Lubbock, TX 79430-0002

²Department of Craniofacial Biology, University of Colorado Denver, Aurora, Colorado

³South Plains Alcohol and Addiction Research Center, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6592, Lubbock, TX 79430-0002

⁴Center for Membrane Protein Research, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6592, Lubbock, TX 79430-0002

Abstract

Many intracellular proteins and signaling cascades contribute to the ethanol sensitivity of native N-methyl-D-aspartate receptors (NMDARs). One putative protein is the serine / threonine kinase, Protein kinase C (PKC). The purpose of this study was to assess if PKC modulates the ethanol sensitivity of native NMDARs expressed in primary cultured cerebellar granule cells (CGCs). With the whole-cell patch-clamp technique, we assessed if ethanol inhibition of NMDA-induced currents (I_{NMDA}) (100 μM NMDA plus 10 μM glycine) were altered in CGCs in which the novel and classical PKC isoforms were activated by phorbol-12-myristate-13-acetate (PMA). Percent inhibition by 10, 50 or 100 mM ethanol of NMDA-induced steady-state (I_{SS}) or peak current amplitudes (I_{PK}) of NMDARs expressed in CGCs in which PKC was activated by a 12.5 min, 100 nM PMA exposure at 37° C did not differ from currents obtained from receptors contained in control cells. However, PMA-mediated augmentation of I_{PK} in the absence of ethanol was abolished after brief applications of 10 or 1 mM ethanol co-applied with agonists, and this suppression of enhanced receptor function was observed for up to eight minutes post-ethanol exposure. Because we had previously shown that PMA-mediated augmentation of I_{NMDA} of NMDARs expressed in these cells is by activation of PKC α , we assessed the effect of ethanol (1, 10, 50 and 100 mM) on PKC α activity. Ethanol decreased PKC α activity by 18% for 1 mM ethanol and activity decreased with increasing ethanol concentrations with a 50% inhibition observed with 100 mM ethanol. The data suggest that ethanol disruption of PMA-mediated augmentation of I_{NMDA} may be due to a decrease in PKC α activity by ethanol. However, given the incomplete blockade of PKC α activity and the low concentration of ethanol at which this phenomenon is observed, other ethanol-sensitive signaling cascades must also be involved.

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author: Department of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6592, Lubbock, TX 79430-0002 Telephone: (806) 743-2425 x272 Fax: (806) 743-2744 lisa.popp@ttuhsc.edu .

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

N-methyl-D-aspartate receptor; Cerebellar granule cells; Protein Kinase C; Whole-cell patch-clamp; Phorbol 12-myristate 13-acetate; Ethanol

Introduction

Protein kinase C (PKC) is a family of serine-threonine, lipid-dependent kinases. Ten isoforms have been identified and are divided into three classes based on sequence homology, substrate preference and activators (Ohno and Nishizuka 2002; Newton 2001). For the classical PKC isoforms: α , β (splice variants β I and β II) (Coussens, et al., 1987) and γ activation is significantly enhanced by calcium, diacylglycerol (DAG) and phorbol esters (Newton, 2001). Activation of the novel PKC isoforms (δ , ϵ , η and θ) is also enhanced by DAG and phorbol esters but not by calcium. Activation of the atypical PKC isoforms ζ and λ is not influenced by calcium, DAG or phorbol esters. Multiple PKC isoforms are expressed throughout the central nervous system (CNS) and can modulate ligand-gated ion channel function (Swope et al., 1999). PKC modulation of the glutamatergic N-methyl-D-aspartate receptor (NMDAR) that requires the co-agonist glycine for activation has been studied extensively with neuronal NMDARs (Gerber et al., 1989; Chen and Huang, 1991; Chen and Huang, 1992; Xiong et al., 1998; Lu et al., 1999; Lan et al., 2001; Popp et al., 2008a; Lau et al., 2010) as well as with recombinant NMDARs contained in heterologous expression systems (Yamakura et al., 1993; Sigel et al., 1994; Zheng et al., 1997; Grant et al., 1998; Zheng et al., 1999; Liao et al., 2001; Lan et al., 2001).

Our laboratory has previously reported that nine (PKC α , β I, β II, δ , ϵ , θ , γ , λ and ζ) of the 10 PKC isoforms are expressed in primary cultured cerebellar granule cells (CGCs) during a time *in vitro* in which the NMDA NR2A and NR2B receptors are present (Popp et al., 1999; Popp et al., 2006; Popp et al., 2008b). We have also reported that PMA activation of PKC results in a sustained activation of agonist-induced currents acquired during whole-cell patch-clamp experiments (Popp et al., 2008a). Contrary to previous reports that PMA augmentation of NMDA-induced currents is subunit dependent when NMDARs are expressed in human embryonic kidney cells (HEK) (Grant et al., 1998; Xiong et al., 1998), native receptors containing the NR2B, NR2A or NR2ANR2B subunits expressed in our primary cultured CGCs exhibit enhanced receptor function following PMA exposure (Popp et al., 2008a). Furthermore, previous results from our laboratory are in agreement with the observations reported by Machu and co-workers (2006) in that temperature is an important variable when assessing PMA affects on ligand-gated ion channel function. Specifically, PMA-mediated alterations in NMDAR function of receptors expressed in mammalian cells (cultured CGCs), are significantly greater when PMA treatment occurs at a mammalian physiological temperature of 37° C (Popp et al., 2008a). Lastly, we have identified that augmentation of NMDAR function of receptors contained in primary cultured CGCs between 13 and 15 days *in vitro* (DIV), a time in culture in which the physiologically relevant NR2ANR2B NMDARs are present, is calcium dependent and is mediated by activation of PKC α and no other PKC isoform (Reneau et al., 2009).

In addition to PKC, NMDAR function can be attenuated or enhanced by many allosteric modulators (McBain and Mayer, 1994) including pharmacologically relevant concentrations of ethanol (Lima-Landman and Albuquerque, 1989; Hoffman et al., 1989; Lovinger et al., 1989; Simson et al., 1991). The direct effect of ethanol on NMDAR function is inhibition, but the degree of inhibition, or the ethanol sensitivity, can be modulated by intracellular proteins or signaling cascades. Furthermore, many of these intracellular signaling cascades and associated proteins have been implicated in alcohol-seeking behaviors (Newton and

Messing, 2006). While it is well established that PKC modulates NMDAR function and data from *in vivo* and *in vitro* studies with acute ethanol exposure have indicated that the classical and novel PKC isoforms may play a role in the etiology of alcohol abuse and dependence (for review see Newton and Ron, 2007), few studies have assessed the interaction of these two allosteric modulators on NMDAR function. The purpose of this study was to directly measure with the whole-cell patch-clamp technique, changes in NMDAR function mediated by activation of PKC in the presence of ethanol. We report that while PKC did not alter the direct inhibitory effect of ethanol on NMDAR function, but we did observe that ethanol prevented PKC-mediated augmentation of I_{NMDA} and loss of augmentation continued up to eight minutes post-ethanol removal.

Materials and Methods

Preparation and maintenance of CGC cultures

All drugs were purchased from Sigma-Aldrich Company (St. Louis, MO) unless otherwise noted. Ethanol (190 proof) was purchased from AAper Alcohol Chemical Company (Shelbyville, KY) or Sigma-Aldrich. CGC cultures were prepared with cerebellar tissue from five to nine day-old Sprague Dawley rats (Raleigh, NC) and plated at 10^6 cells / 35 mm dish. This method has been previously published in detail (Popp et al., 1999). All procedures were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with guidelines contained in the National Institutes of Health guide for the care and use of laboratory animals, NIH Publications no. 8023, revised 1978. Cells were maintained at 37° C in a humidified atmosphere of 5% CO₂ for up to 15 DIV. Every seven DIV, existing feeding media [MEM containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM KCl (to promote CGC viability in culture; Gallo et al., 1987), penicillin/ streptomycin (100 U/100 µg/ml), and a fluorodeoxyuridine / uridine (FDU/U), 35 µm / 15 µm mixture] was supplemented with 0.5 ml of fresh feeding media. This method of nourishment minimizes excitotoxic cell death due to the introduction of fresh media and results in healthy, viable CGCs for up to five weeks in culture (Popp et al., 1999).

Drug Treatment

Experiments were conducted with CGCs between 13 and 15 DIV. Data were acquired from at least three different culture batches. PMA treatment at physiological temperatures is required when assessing PKC modulation of ligand-gated ion channels expressed in mammalian systems (Machu et al., 2006). We have previously measured changes in I_{NMDA} within the same cell pre- and post PMA treatment (12.5 min, 100 nM PMA exposure at 23° C). With this PMA exposure paradigm, small, significant increases in I_{PK} , with no significant increases in I_{SS} were observed (Popp et al., 2008a). Thus, based on the results from Machu et al., 2006, we incorporated experiments in which CGCs were treated with PMA at physiological temperatures (37° C) and observed robust and significant increases in both I_{PK} and I_{SS} (Popp et al., 2008a) and have continued to use this exposure paradigm to assess PMA-mediated alterations in I_{NMDA} (Reneau et al., 2009). PMA was diluted and applied in conditioned feeding media. CGCs were treated with 100 nM PMA and returned to the incubator (37° C) for 12.5 or 30 min prior to preparation for whole-cell patch-clamp recordings. Because this PMA exposure paradigm results in continued enhancement of I_{NMDA} during the recording period (Popp et al., 2008a), only one cell / dish was used in all PMA experiments. Sustained increases in NMDAR function are most likely due to an intercalation of PMA into the plasma membrane that is not subject to enzymatic degradation (Nishizuka, 1984) and thus PMA washout is slow and incomplete during the two-hour recording interval.

Whole-cell patch-clamp recordings

In preparation for whole-cell recordings, after drug treatment, feeding media was replaced with external recording solution at 23° C for 30 – 45 min: 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 10 mM HEPES, 10 mM D-glucose and 200 nM tetrodotoxin (Alomone Labs Ltd., Jerusalem, ISRAEL) with pH adjusted to 7.4 with NaOH and osmolality adjusted to 333-345 mOsmol with sucrose. The ionic content of the external recording solution mimics that found in normal extracellular fluid and we have found that neurons are viable for up to three hours during whole-cell recordings. The switch from a high K⁺-containing environment present in the feeding media to the lower concentration contained in the external recording solution does not result in cell death as observed by the trypan blue exclusion method. Results from these experiments indicate that less than 1 % of CGCs take up the dye even after sitting in normal external recording solution for two hours (unpublished observations). Dishes were placed on the stage of a Nikon Eclipse TE2000-U inverted microscope (NIKON Inc., Melville, NY). Detailed methods have been previously published (Popp et al., 1999; Popp et al., 2008a; Reneau et al., 2009). Briefly, all currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) at –60 mV holding potential. Cells were patched with internal patch electrode solution containing: 100 mM N-methyl-D-glucamine, 100 mM MeSO₃, 40 mM CsF, 10 mM HEPES, 1 mM MgCl₂, 3 mM Lidocaine N-ethyl bromide and 5 mM EGTA; pH adjusted to 7.4 with CsOH and osmolality adjusted to 314-317 mOsmol with sucrose.

Drug Application

All drugs were applied by gravity through microcapillary tubes (0.32 mm inner diameter) operated manually. This drug application system produces a solution exchange rate faster than the time to reach peak current amplitudes and thus can be used to measure changes in NMDA-induced peak current amplitudes (I_{PK}) (Popp and Dertien, 2008). Agonists (100 μM NMDA and 10 μM glycine) were applied alone or in combination with ethanol (concentrations noted with each experiment). All drug exposures consisted of three, five-second exposure intervals interspersed with 30 s washes with external recording solution. The number of these “drug application clusters” varied among experiments, but agonist application clusters flanked all agonist and ethanol clusters. This is a well-documented application paradigm to study the affects of ethanol on NMDAR function with the whole-cell patch-clamp technique (Lovinger et al., 1989; Popp et al., 1998; Popp et al., 1999; Popp and Lovinger, 2000; Popp et al., 2008b). All data were low pass filtered at 1 kHz with an 8-pole Bessel filter (Axopatch 200B amplifier) at a sampling rate of 10 kHz with DigiData 1200A and Clampex 9.2 (Molecular Devices). Current traces were measured with pClamp 9.2 software (Molecular Devices). Series resistance was measured throughout the entire recording period and current traces that indicated an increase of >5 % were omitted from the analysis.

PKCα activity assay

The Peptag Assay for non-radioactive Detection of Protein Kinase C (Promega, Madison, WI) was used to measure the relative kinase activity according to the manufactures' instructions. Twenty-five ngs of PKCα enzyme was either left untreated (0) or treated with 1, 10, 50 or 100 mM ethanol for the duration of the kinase assay. Enzyme aliquots were incubated with Peptag C1 peptide for 30 min at room temperature (23 - 24° C). Phosphorylated C1 peptide was resolved by SDS-PAGE and quantified by densitometry after detection by UV illumination.

Statistical Analysis

All data values are expressed as mean $\pm$ s.e.m. All statistical analyses were done within each component of NMDA-induced currents: NMDA-induced peak current amplitudes (I_{pk}) and NMDA-induced steady-state current amplitudes (I_{ss}) and steady-state to peak current ratios (I_{ss} / I_{pk}). Data are expressed as percent inhibition of control or as normalized to cell capacitance (pA / pF). Single or two-way analysis of variance (ANOVA) was used to identify significant main effects. The Multiple t-Test or the Bonferroni post hoc tests were used to determine individual group differences when F values resulted in $P \leq 0.05$ for one- and two-ANOVA, respectively. Differences in PKC α activity due to ethanol were analyzed by Student's paired t-test, each concentration of ethanol compared to control values.

Results

PMA exposure results in sustained, significant increases in I_{pk}

We previously reported that a 30 min, 100 nM PMA exposure at 37° C resulted in significant increases in both I_{pk} and I_{ss} of NMDARs expressed in primary cultured CGCs 13 - 15 DIV (Popp et al., 2008a). We also reported that similar results could be observed with briefer (12.5 min) PMA exposures, and that increases in I_{pk} could be observed for up to 20 min with repeated agonist exposure clusters during whole-cell recordings (Reneau et al., 2009). In this current study, we corroborate our previous findings in that 100 nM PMA treatment at 37° C resulted in sustained augmentation of I_{pk} (Figure 1). A 30 min, 100 nM PMA exposure at 37° C resulted in significant increases of approximately 22 % in I_{pk} due to repeated agonist exposure clusters applied every two minutes ($F_{(3, 28)} = 3.8$, $P \leq 0.05$) and increases were observed for up to 12 min during whole-cell recordings. In addition, similar to our previous findings with repeated agonist exposures every two minutes, while I_{ss} did not significantly increase over the time of the recordings ($F_{(3, 28)} = 0.19$, $P \geq 0.05$) moderate increases in I_{ss} prevented changes in receptor desensitization as indicated by steady-state to peak current ratios (I_{pk} / I_{ss}). Steady state to peak current ratios were 0.47 ± 0.03 at the start of the experiment, 0.44 ± 0.04 at 4 min, 0.4 ± 0.03 at 8 min and 0.4 ± 0.04 at the end of the experiment; ($F_{(3, 28)} = 1.1$, $P \geq 0.05$). In addition to confirming that this PMA exposure paradigm *always* results in sustained increases in I_{pk} , data from these experiments served as positive controls to verify positive PMA effects for experiments presented in Figures 2 and 3. Representative current traces and summarized data are shown in Figure 1A and B, respectively.

Activation of PKC by PMA does not modify ethanol inhibition of I_{NMDA}

Historically ethanol inhibition of NMDA-induced currents (I_{NMDA}) was reported on the peak component (I_{pk}) of macroscopic currents that is more representative of receptor gating (Lovinger et al., 1989; Lovinger, 1995; Peoples et al., 1997). Although single channel experiments supported that ethanol was an allosteric modulator that altered receptor gating, results from these experiments did not exclude the possibility that ethanol altered the rate of fast desensitization (Wright et al., 1991). We were one of the first laboratories to report that factors which confer the ethanol sensitivity of native NMDA receptors can differentially affect the two components of macroscopic currents: I_{pk} and I_{ss} , (stable inward current that no longer changes and reflects desensitization of individual channels) (Popp et al., 1999; Popp and Dertien, 2008). Therefore, when studying factors that modulate the ethanol sensitivity of NMDA-induced currents, we always measure both components. We compared the ethanol inhibition of I_{NMDA} from receptors in CGCs in which PKC had been activated by a 12.5 min, 100 nM PMA treatment at 37° C with data acquired from CGCs that received no PMA treatment. Results from two-way ANOVA indicated that the ethanol inhibition of NMDARs contained in CGCs treated with PMA did not differ from receptors contained in control CGCs (cells that received no PMA) for I_{pk} ($F_{(treatment; 1, 48)} = 0.4$, $P \geq 0.05$) or for

I_{SS} ($F_{(\text{treatment}; 1, 38)} = 0.71$, $P \geq 0.05$). Summarized data are shown in Figure 2. Increasing concentrations of ethanol significantly increased inhibition of I_{PK} ($F_{(\text{ethanol}; 2, 48)} = 79.2$, $P \leq 0.001$) as well as I_{SS} ($F_{(\text{ethanol}; 2, 48)} = 13.0$, $P \leq 0.001$) and this was not affected by PMA treatment: $F_{(\text{interaction}; 2, 48)} = 0.009$, $P \geq 0.05$ and $F_{(\text{interaction}; 2, 48)} = 0.73$, $P \geq 0.05$ for I_{PK} and I_{SS} , respectively.

Comparison of currents (I_{PK} and I_{SS}) normalized to capacitance within the ethanol concentration also indicated no significant differences attributed to PKC activation by PMA (data not shown). However, given that this PMA exposure paradigm always resulted in a *robust and continuous* augmentation of I_{NMDA} currents (Popp et al., 2008a), specifically the peak component of NMDA-induced macroscopic currents (I_{PK}) (Reneau et al., 2009), we had predicted that potentiation of I_{PK} by PMA would result in larger currents in the absence of ethanol throughout the remainder of the whole-cell patch-clamp experiments. Results from a one-way ANOVA of I_{PK} after ethanol exposure indicated differently: $F_{(3, 38)} = 0.45$; $P \geq 0.05$ for I_{PK} . Agonist-induced currents were identical pre- and post-ethanol application: I_{PK} was 55.4 ± 2.14 pA / pF, $n = 15$ for agonist application clusters at the start of the experiment; 58.3 ± 1.6 pA / pF, $n = 15$; and 56.9 ± 2.7 pA / pF, $n = 8$ for agonists clusters flanked by ethanol and agonist application clusters and 55.5 ± 4.0 pA / pF, $n = 4$ at the termination of the experiments.

Acute, repeated ethanol exposure blocks PMA-mediated potentiation of I_{PK}

Because the order of ethanol concentration was varied among cells in the previous experiments, it was impossible to determine which ethanol concentration was responsible for negating the PMA-mediated potentiation of I_{PK} once ethanol was removed. Therefore, we repeated these experiments with the lowest concentration of ethanol (10 mM). Results from one-way ANOVA comparing normalized currents indicated a significant difference among peak current amplitudes ($F_{(4, 55)} = 6.4$; $P \leq 0.001$) that was attributed to ethanol in that normalized I_{PK} were significantly smaller than I_{PK} in the absence of ethanol. However, this brief 10 mM ethanol exposure completely blocked PMA augmentation of I_{PK} as indicated by an insignificant difference among agonist-induced currents in the absence of ethanol ($P \geq 0.05$). Normalized I_{SS} while inhibited by 10 mM EtOH were not significantly different from I_{SS} in the absence of ethanol ($F_{(4, 55)} = 2.4$; $P \geq 0.05$) and currents generated by agonists did not differ from one another. Despite the lack of significant inhibition by 10 mM ethanol on normalized I_{SS} , the 14 % inhibition is similar to data previously published from our laboratory (Popp et al., 2008b). Representative current traces are shown in Figure 3A and summary graphs in Figure 3B.

Frequency of agonist application does not affect PMA-mediated potentiation of I_{PK}

With the ethanol application paradigm used throughout this study, NMDARs are pulsed with a five second agonist or agonist plus ethanol application every 30 s for a maximum of 12 to 13 min (total recording time for all three ethanol concentrations used in experiments for which data are shown in Figure 2.). We had previously reported that frequency of NMDA agonist application could prevent significant increases in I_{SS} by PMA (Popp et al., 2008a; Reneau et al., 2009). Therefore, to verify that the loss in PMA-mediated augmentation of I_{NMDA} observed in the above experiments was not due to frequent agonist exposure, we conducted experiments in PMA-treated CGCs with this more frequent agonist application paradigm. Unlike our previous studies (Reneau et al., 2009 and data presented in Fig. 1) in which NMDA clusters were applied every two minutes, we now assessed changes in I_{NMDA} generated by agonist application clusters every 30 s in PMA-treated CGCs. In the absence of ethanol, treatment with 100 nM PMA for 12.5 min at 37° C resulted in significant increases in I_{PK} amplitudes ($F_{(2, 27)} = 12.0$; $P \leq 0.001$). Post-hoc analysis indicated that peak current amplitudes at the start of the recording 58.7 ± 1.4 pA / pF increased to 65.1 ± 1.7 at the four

minute time point ($t = 2.6$, $P \leq 0.05$) and continued to increase to 70.5 ± 2.1 pA / pF; ($t = 4.8$, $P \leq 0.001$) until the end of the experiment (eight minutes). With this agonist exposure paradigm, PMA did not increase I_{SS} amplitudes in that I_{SS} was relatively unchanged throughout the recording interval ($F_{(2, 27)} = 0.003$; $P \geq 0.05$). But, as previously noted (Popp et al., 2008a; Reneau et al., 2009), the desensitized state of the receptor did not change as indicated by no significant changes ($F_{(2, 27)} = 2.8$; $P \geq 0.05$) in the I_{SS} / I_{PK} over the eight minutes of recording: 0.39 ± 0.2 , 0.35 ± 0.2 and 0.32 ± 0.2 , from start to finish, respectively. Representative current traces are shown in Figure 4A and summary graphs are shown in Figure 4B. Data from these experiments were collected with data reported in Figures 5 and 6 and served as control experiments to verify positive PMA effects.

Brief, low ethanol exposure prevents PMA augmentation of I_{PK} currents for up to eight minutes post-ethanol exposure

Results from the previous experiment confirmed that loss of PMA-mediated enhancement of I_{PK} amplitudes when ethanol was washed away, was not caused by frequency of agonist application but must be due to ethanol. We then determined if one ethanol application cluster (three, five-second ethanol and agonist applications interspersed with a 30 s wash with normal external recording solution) could attenuate PMA augmentation of I_{NMDA} in the absence of ethanol. Very brief, 10 mM ethanol exposures blocked PMA-mediated potentiation of I_{PK} for up to eight min post ethanol exposure, $F_{(3,32)} = 2.3$; $P \geq 0.05$ as indicated by similar I_{PK} amplitudes in the absence of ethanol: normalized I_{PK} was 56.3 ± 2.3 pA / pF at the start of the experiment, 57.8 ± 3.2 pA / pF at the four minute time point and 57.1 ± 3.3 pA / pF at the end of the experiment. I_{PK} was significantly decreased (49.0 ± 2.4 pA / pF) by 10 mM ethanol when compared to the mean I_{PK} of pre- and post-ethanol administrations ($t = 2.1$; $P \leq 0.05$). Normalized I_{SS} amplitudes did not differ from one another in the absence or presence of 10 mM ethanol, $F_{(3,32)} = 0.9$; $P \geq 0.05$, and there was no change in I_{SS} / I_{PK} ($F_{(3,32)} = 1.2$; $P \geq 0.05$). Percent inhibition of I_{SS} by 10 mM ethanol was 17.6 %. Representative current traces are shown in Figure 5A and summarized data in 5B.

We then wanted to assess if ethanol could negate PMA-mediated augmentation of I_{PK} with a concentration of ethanol that had no affect on NMDAR function. Results from one-way ANOVA comparing normalized currents acquired in the presence and absence of 1 mM ethanol did not differ from one another, thus indicating that this low concentration of ethanol had no direct effect on receptor function ($F_{(3, 24)} = 1.0$; $P \geq 0.05$ for I_{PK} ; $F_{(3, 24)} = 0.5$; $P \geq 0.05$ for I_{SS} and $F_{(3, 24)} = 0.7$; $P \geq 0.05$ for I_{SS} / I_{PK}) and, that 1 mM ethanol blocked PMA-induced potentiation of I_{PK} amplitudes. Representative current traces are shown in Figure 6A and summarized data in Figure 6B. However, assessing alterations in NMDAR function at the level of the individual cell yielded different results. We assessed the effect of ethanol on I_{NMDA} amplitudes of receptors in PMA-treated CGCs pre- and post-ethanol exposure for each individual cell. Since PMA augmentation is more robust for I_{PK} we assessed only this component of the macroscopic current. ***Currents were considered significantly potentiated by PMA if I_{PK} was $> 10\%$ above the initial baseline values at the 8 min time point to. The reduction of PMA potentiation was more consistent at 10 than at 1 mM ethanol. After three, five-second exposures to 1 mM ethanol, 43% of the neurons still exhibited potentiation of I_{PK} by PMA, while only 22.2% showed detectable potentiation after exposure to the 10 mM concentration. Potentiation was comparable in both groups: 17.6 ± 5.4 % and 15.2 ± 2.2 % for 10 and 1 mM ethanol, respectively.

Ethanol decreases PKC α activity in a dose-dependent manner in vitro

We have previously reported (Reneau et al., 2009) that PMA-mediated augmentation does not occur if both intracellular and extracellular calcium are removed. Thus suggesting that a

classical PKC isoform(s) was mediating this effect. Furthermore, through a series of experiments with PKC-isoform-specific antibodies (Popp et al., 2006) introduced into the cell, that resulted in no PMA-mediated potentiation, as well as experiments in which a regain of function was achieved (augmentation of I_{NMDA} lost in PKC-depleted CGCs) by introducing activated classical PKC isoforms into the cell, we confirmed that PMA-mediated augmentation of I_{NMDA} was mediated by PKC α and no other isoform. Ethanol attenuation of PMA-mediated augmentation of NMDAR function is most likely due to multiple mechanisms (see discussion). But based on our results, we hypothesized that one of these mechanisms might be by attenuating PKC α activation. We therefore, conducted activity assays on PKC α with increasing concentrations of ethanol. To replicate conditions similar to our whole-cell patch-clamp experiments, PKC α kinase activity assays were conducted at room temperature (23 – 24° C) in the presence and absence of ethanol. PKC α activity was significantly decreased ($16.3 \pm 6.3 \%$) by 1 mM ethanol and attenuation of activity increased with increasing concentrations of ethanol. Summarized data are shown in Fig. 7.

Discussion

The initial purpose of this study was to more clearly define the role that PKC plays in the ethanol sensitivity of native NMDARs expressed in primary cultured CGCs with the whole-cell patch-clamp method. We assessed the ethanol inhibition of agonist-induced currents of receptors in CGCs in which all classical and novel PKC isoforms had been activated by PMA. The inhibitory effects of acute ethanol exposure on I_{PK} and I_{SS} were unchanged by a 12.5 min, 100 nM PMA treatment at 37° C. These data suggest that PKC α , βI , βII , δ , ϵ , γ and θ , (novel and classical isoforms contained in this DIV of CGCs; Popp et al., 2006) do not modulate ethanol sensitivity of NMDARs expressed in these cells. Few studies have assessed the interaction of PKC and acute ethanol exposure on native NMDAR function. The PKC inhibitor Calphostin C mitigated ethanol-mediated decreases in intracellular calcium in primary cultured CGCs (Snell et al., 1994). Thus suggesting that the inhibitory effects on NMDAR function are in part, mediated by activation of PKC. However, this was not observed in primary cultured cortical neurons (Hoffman et al., 1994). Thus, suggesting that PKC modulation by ethanol is brain region specific. While different brain regions cannot explain the discrepant results between our studies and those of Snell and co-workers (1994), other factors including differences in culture methodology, the age of the cultures at the time the studies were conducted and method used to measure receptor function could individually or collectively explain the observed differences. Other plausible explanations include that the atypical isoforms PKC ζ and PKC λ (the rat orthologue of PKC I) may modulate ethanol inhibition of NMDARs or it might be possible that ethanol modulates multiple PKC isoforms in a manner that leads to opposite effects (augmentation and attenuation) of NMDAR function and thus resulting in a negative effect.

Similar to our previous findings (Popp et al., 2008a; Reneau et al., 2009) we observed a robust and sustained enhancement of I_{PK} of NMDARs contained in primary cultured CGCs that were treated with 100 nM PMA at physiological temperature. Also, similar to our previous observations, while PMA augmentation of I_{SS} was not significant, I_{SS} increased sufficiently so that $I_{\text{SS}} / I_{\text{PK}}$ ratios did not change. The lack of change in receptor desensitization was observed despite the increase in frequency of agonist exposure. A most intriguing finding of this study was the observation that a very brief (cumulative 15 s) and a very low (1 mM) ethanol exposure blocked PMA-mediated augmentation of I_{PK} for up to 8 min post-ethanol exposure. A similar phenomenon has been observed during slice electrophysiological experiments assessing ethanol alterations in NMDA-mediated synaptic transmission and long-term potentiation (LTP) in that the amount of time to reach pre-ethanol baseline levels after removal of ethanol can be hours (Izumi et al., 2005) or 10 - 20

min (Ariwodola et al., 2003; Roberto et al., 2004; Weitlauf et al., 2004). In one of these studies, the incomplete recovery of NMDA-mediated currents from ethanol was only observed in the whole-cell recordings and this phenomenon was attributed to intracellular dialysis via the patch electrode (Weitlauf et al., 2004). However, other possible explanations are the disruption of protein-protein interactions that occur in the whole-cell configuration that could disrupt the intracellular second messenger signaling cascades relevant to ethanol and NMDA transmission and or LTP (Chandler, 2003).

We have previously shown that PMA-mediated augmentation of I_{PK} of receptors contained in 13 – 15 DIV CGCs is by activation of PKC α and no other isoform. (Reneau et al., 2009). Therefore, our data suggest that one possible mechanism for the observed ethanol attenuation of PMA-enhanced NMDAR function might be by a decrease in PKC α activity by ethanol. In support of this hypothesis are results from *in vitro* studies conducted in lipid vesicles with a lipid composition similar to that found in synaptic vesicles and with DAG as activators. Results indicated that small chain alcohols, such as ethanol, inhibited PKC α activity in a dose-dependent manner and decreased PKC α membrane association (Slater et al., 1997). Because these kinase activity assays were conducted at elevated temperatures and with concentration of ethanol greater than 1 mM, we repeated these experiments with conditions similar to those used during whole-cell recordings. We conducted activity assays with PKC α in the presence and absence of ethanol with concentrations that resulted in the loss of PMA augmentation of I_{NMDA} at room temperature (23 – 24° C). In support of our hypothesis, we observed that 1 mM ethanol decreased PKC α activity by approximately 16 %. Furthermore, activity decreased with an increase in ethanol concentration. Yet, at the highest concentration of ethanol tested, a concentration approaching the upper limit of pharmacological relevance (100 mM), PKC α activity was not completely blocked. An inhibition of approximately 41 % was observed. Interestingly, at the level of the individual cell, not every cell contained NMDARs in which ethanol attenuated PMA-mediated augmentation of I_{PK} . Furthermore, a greater percentage of PMA-treated cells contained receptors in which I_{PK} continued to increase after 1 mM ethanol exposure compared to CGCs that were treated with 10 mM ethanol. Also the percent increase observed in these cells was similar to that observed with the frequent application protocol (17.1% and data shown in Figure 4). This suggests that other mechanisms, in addition to attenuation of PKC α activity by ethanol, are responsible for our observations.

There are many different ethanol-sensitive, intracellular signaling cascades that could converge in a manner that enhances inhibition of NMDAR function and yet individually, the effect is minimal or much smaller. Many intracellular proteins and signaling cascades have been identified as ethanol-sensitive targets (Newton and Messing, 2006) and most likely vary among brain regions and cell type. Data strongly support two primary mechanisms by which PKC activation augments NMDAR function and both of these mechanisms include ethanol-sensitive targets. One proposed mechanism by which PKC augments NMDAR function is by direct phosphorylation of NR1, NR2A and NR2B NMDAR subunits (Leonard and Hell, 1997) and specifically phosphorylation of S896 on the NR1 subunit (Tingley et al., 1997). Phosphorylation of S896 results in enhanced trafficking of NMDARs to the plasma membrane in hippocampal neurons (Scott et al., 2001). Additionally, in primary cultured hippocampal neurons, PMA-mediated increased insertion of NMDARs at the cell surface is mediated through a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent exocytosis (Lan et al., 2001). More recently, data indicate that the SNARE protein synaptosomal-associated protein with a molecular weight of 25 kDa (SNAP-25) is phosphorylated by PKC and phosphorylated SNAP-25 plays a critical role in the insertion of NMDARs into the plasma membrane and the resultant potentiation of I_{NMDA} at hippocampal CA3 synapses (Lau et al., 2010). Thus, ethanol attenuation of PKC α activity would result not only in a decrease in NMDAR subunit phosphorylation but also in

a decrease in SNAP-25 phosphorylation, both of which would result in a loss of NMDAR recruitment to the plasma membrane.

Alternatively, ethanol inhibition of PMA-mediated augmentation of NMDAR function may be downstream of PKC activation. It is well documented that activation of Src tyrosine kinase results in augmentation of NMDAR function (for review see Lee, 2006). Further evidence suggests that tyrosine kinase augmentation of NMDARs is mediated by PKC activation (Lu et al., 1999; Grosshans et al., 2002). Activation of PKC results in the phosphorylation and activation of cell adhesion kinase β (CAK β) followed by CAK β phosphorylation and activation of Src tyrosine kinase. Activated Src then phosphorylates tyrosine residues on the NMDAR resulting in augmentation of receptor function. Ethanol could inhibit any of the kinases involved in this cascade, or ethanol could promote tyrosine dephosphorylation through enhancement of phosphatases activity. Thus, a potential ethanol sensitive target responsible for the lack of PMA-mediated augmentation of I_{NMDA} could be tyrosine phosphatases. This premise is supported by the observation that acute ethanol exposure can result in dephosphorylation of amino acids mediated by Src tyrosine kinase (Ferrari-Kile and Leslie, 2005). In addition, ethanol decreases in NMDA-mediated neurotransmission may be via tyrosine phosphatases since ethanol sensitivity of NMDARs expressed in hippocampal CA1 pyramidal neurons is decreased when tyrosine phosphatases are inhibited (Alvestad et al., 2003). Both of these mechanisms involve multiple protein-protein interactions and include complicated intracellular signaling cascades and thus, can easily account for the prolonged ethanol affect as well as amplification of downstream second messengers. Ethanol modulation of PKC activity is brain region specific (for review see Newton and Ron, 2007). Therefore, while our results suggest that ethanol blocking of PMA-mediated augmentation of I_{NMDA} is in part due to a decrease in PKC α activity, this may not be true for other brain regions. However, the important finding of the current experiments is that a very brief, low ethanol exposure prevents augmentation of NMDAR function due to PKC activation long after ethanol is removed.

Acknowledgments

This work was supported by grants from the National Institute on Alcohol Abuse and Alcoholism (NIAAA): RO1AA13436 and the TTUHSC Seed Grant Program (R. L. P.) and the National Institute of Dental and Craniofacial Research (NIDCR): R01DE015648 (M.E.R.). We would like to thank Dr. Michael Blanton for helpful scientific discussions and editorial advice and Angela Ohm for conducting the kinase activity assays.

References

- Alvestad RM, Grosshans DR, Coultrap SJ, Nakazawa T, Yamamoto T, Browning MD. Tyrosine dephosphorylation and ethanol inhibition of N-methyl-D-aspartate receptor function. *J. Biol. Chem.* 2003; 278:11020–11025. [PubMed: 12536146]
- Ariwodola OJ, Crowder TL, Grant KA, Daunais JB, Friedman DP, Weiner JL. Ethanol modulation of excitatory and inhibitory synaptic transmission in rat monkey dentate granule neurons. *Alcohol Clin. Exp. Res.* 2004; 27:1632–1639. [PubMed: 14574234]
- Chandler LJ. Ethanol and brain plasticity: receptors and molecular networks of the postsynaptic density as targets of ethanol. *Pharmacol. Ther.* 2003; 99:311–326. [PubMed: 12951163]
- Chen L, Huang LM. Sustained potentiation of NMDAR-mediated glutamate responses through activation of protein kinase C by a μ opioid. *Neuron.* 1991; 7:319–326. [PubMed: 1678615]
- Chen L, Huang L-YM. Protein kinase C reduces Mg^{2+} block of NMDA-receptor channels as a mechanism of modulation. *Nature.* 1992; 356:521–523. [PubMed: 1373227]
- Coussens L, Rhee L, Parker PJ, Ullrich A. Alternative splicing increases the diversity of the human protein kinase C family. *DNA.* 1987; 5:389–394. [PubMed: 3677994]

- Ferrari-Kile K, Randall PK, Leslie SW. Acute ethanol affects phosphorylation state of the NMDAR complex: implication of tyrosine phosphatases and protein kinase A. *Mol. Brain Res.* 2003; 115:78–86. [PubMed: 12824058]
- Gallo V, Kingsbury A, Balázs R, Jørgensen OS. The role in depolarization in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* 1987; 7:2203–2213. [PubMed: 2886565]
- Gerber G, Kangra I, Ryu PD, Larew JSA, Randic M. Multiple effects of phorbol esters in the rat spinal dorsal horn. *J. Neurosci.* 1989; 9:3606–3617. [PubMed: 2571684]
- Grant ER, Bacskaí BJ, Aneqawa NJ, Pleasure DE, Lynch DR. Opposing contributions of NR1 and NR2 to protein kinase C modulation of NMDARs. *J. Neurochem.* 1998; 71:1471–1481. [PubMed: 9751180]
- Grosshans DR, Clayton DA, Coultrap SJ, Browning MD. LTP leads to rapid surface expression of NMDA but not AMPA receptors in adult rat CA1. *Nature Neurosci.* 2002; 5:27–33. [PubMed: 11740502]
- Hoffman PL, Rabe CS, Moses F, Tabakoff B. *N*-methyl-d-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J. Neurochem.* 1989; 52:1937–1940. [PubMed: 2542453]
- Izumi Y, Nagashima K, Murayama K, Zorumski CF. Acute effects of ethanol on hippocampal long-term potentiation and long-term depression are mediated by different mechanisms. *Neuroscience.* 2005; 136:509–517.
- Lan J-Y, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennet MVL, Zukin SR. Protein kinase C modulates NMDAR trafficking and gating. *Nature.* 2001; 4:382–390.
- Lau CG, Takayasu Y, Rodenas-Ruano A, Paternain AV, Lerma J, Bennett MVL, Zukin RS. SNAP-25 is a target of protein kinase C phosphorylation critical to NMDA receptor trafficking. *J. Neurosci.* 2010; 30:242–254. [PubMed: 20053906]
- Lee H-K. Synaptic plasticity and phosphorylation. *Pharmacol. Ther.* 2006; 112:810–832. [PubMed: 16904750]
- Leonard AS, Hell JW. Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate *N*-methyl-d-aspartate receptors at different sites. *J. Biol. Chem.* 1997; 272:12107–12115. [PubMed: 9115280]
- Liao G-Y, Wagner DA, Hsu MH, Leonard JP. Evidence for direct protein kinase-C mediated modulation of *N*-methyl-d-aspartate receptor. *Current. Mol. Pharmacol.* 2001; 59:960–964. [PubMed: 11306676]
- Lima-Landman MT, Albuquerque EX. Ethanol potentiates and blocks NMDA-activated single-channel currents in rat hippocampal pyramidal cells. *FEBS Lett.* 1989; 247:61–67. [PubMed: 2468533]
- Lovinger DM, White G, Weight FF. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science.* 1989; 243:1721–1724. [PubMed: 2467382]
- Lovinger DM. Developmental decrease in ethanol inhibition of *N*-methyl-d-aspartate receptors in rat neocortical neurons: Relation to the actions of ifenprodil. *J. Neurosci.* 1995; 15:274:164–172.
- Lu W-Y, Xiong Z-G, Lei S, Orser BA, Dudek E, Browning MD, MacDonald JF. G-protein-coupled receptors act via protein kinase C and Src to regulate NMDARs. *Nat. Neurosci.* 1999; 2:331–338. [PubMed: 10204539]
- Machu TK, Dillon GH, Huang R, Lovinger DM, Leidenheimer NJ. Temperature: An important experimental variable in studying PKC modulation of ligand-gated ion channels. *Brain Res.* 2006; 1086:1–8. [PubMed: 16626662]
- McBain CJ, Mayer ML. *N*-methyl-d-aspartic acid receptor structure and function. *Physiol. Rev.* 1994; 74:723–760. [PubMed: 8036251]
- Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev.* 2001; 101:2353–2364. [PubMed: 11749377]
- Newton PM, Messing RO. Intracellular signaling pathways that regulate behavioral responses to ethanol. *Pharmacol. Ther.* 2006; 109:227–237. [PubMed: 16102840]
- Newton PM, Ron D. Protein kinase C and alcohol addiction. *Pharmacol. Res.* 2007; 55:570–577. [PubMed: 17566760]

- Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumor production. *Nature*. 1984; 308:693–697. [PubMed: 6232463]
- Ohno S, Nishizuka Y. Protein kinase C isotypes and their specific functions: prologue. *J. Biochem*. 2002; 132:509–511. [PubMed: 12359062]
- Peoples RW, White G, Lovinger DM, Weight FF. Ethanol inhibition of N-methyl-d-aspartate-activated current in mouse hippocampal neurons: whole-cell patch-clamp analysis. *Brit. J. Pharmacol*. 1997; 122:1035–1042. [PubMed: 9401766]
- Popp RL, Lickteig R, Browning MD, Lovinger DM. Ethanol sensitivity of N-methyl-d-aspartate receptors in cultured striatal neurons. *Neuropharmacol*. 1998; 37:45–56.
- Popp RL, Lickteig R, Lovinger DM. Factors that enhance ethanol inhibition of N-methyl-d-aspartate receptors in cerebellar granule cells. *J. Pharmacol. Exp. Therapeutics*. 1999; 289:1564–1574.
- Popp RL, Lovinger DM. Interaction of acamprosate with EtOH and spermine on NMDARs expressed in primary cultured neurons. *Euro. J. Pharmacol*. 2000; 394:221–231.
- Popp RL, Velasquez O, Bland J, Hughes P. Characterization of protein kinase C isoforms in primary cultured cerebellar granule cells. *Brain Res*. 2006; 1083:70–84. [PubMed: 16546140]
- Popp RL, Dertien JS. Actin depolymerization contributes to ethanol inhibition of NMDARs in primary cultured cerebellar granule cells. *Alcohol*. 2008; 42:525–539. [PubMed: 18789629]
- Popp RL, Reneau JC, Brotherton BJ. Activation of Protein Kinase C enhances NMDA-induced currents in primary cultured cerebellar granule cells: Effect of temperature and NMDA NR2 subunit composition. *Euro. J. Pharmacol*. 2008a; 599:1–10.
- Popp RL, Reneau JC, Dertien JS. Cerebellar granule cells cultured from adolescent rats express functional N-methyl-d-aspartate receptors: an *in vitro* model for studying the developing cerebellum. *J. Neurochem*. 2008b; 106:900–911. [PubMed: 18466339]
- Reneau JC, Kindy C, Phillips J, Reyland ME, Popp RL. Phorbol 12-myristate 13-acetate potentiation of N-methyl-d-aspartate-induced currents in primary cultured cerebellar granule cells is mediated by Protein Kinase C α . *J. Pharmacol. Exp. Ther*. 2009; 330:641–649. [PubMed: 19429793]
- Roberto M, Scheitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR. Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an *in vitro* and *in vivo* analysis. *J. Neurosci*. 2004; 24:1594–1603. [PubMed: 14973247]
- Scott DB, Blanpied TA, Swanson GT, Zhang C, Ehlers MD. An NMDAR ER retention signal regulated by phosphorylation and alternative splicing. *J. Neurosci*. 2001; 21:3063–3072. [PubMed: 11312291]
- Sigel E, Baur R, Malherbe P. Protein kinase C transiently activates heteromeric N-methyl-d-aspartate receptor channels independent of the phosphorylatable C-terminal splice domain and of consensus phosphorylation sites. *J. Biol. Chem*. 1994; 269:8204–8208. [PubMed: 7510681]
- Simson PE, Criswell HE, Johnson KB, Hicks RE, Breese GE. Ethanol inhibits NMDA-evoked electrophysiological activity *in vivo*. *J. Pharmacol. and Exp. Therapeutics*. 1991; 257:225–231.
- Slater SJ, Kelly MB, Larkin JD, Ho C, Mazurek A, Taddo FJ, Yeager MD, Stubbs CD. Interaction of alcohols and anesthetics with protein kinase C α . *J. Biol. Chem*. 1997; 272:6167–6173. [PubMed: 9045629]
- Snell LD, Tabakoff B, Hoffman PL. Involvement of protein kinase C in ethanol-induced inhibition of NMDAR function in cerebellar granule cells. *Alcohol Clin. Exp. Res*. 1994; 18:81–85. [PubMed: 8198231]
- Swope SL, Moss SJ, Raymond LA, Haganir RL. Regulation of ligand-gated ion channels by protein phosphorylation. *Advances Sec. Messenger Phosphoprotein Res*. 1999; 33:49–78.
- Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, Haganir RL. Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-d-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J. Biol. Chem*. 1997; 272:5157–5166. [PubMed: 9030583]
- Weitlauf C, Egli RE, Grueter BA, Winder DG. High-frequency stimulation induces ethanol-sensitive long-term potentiation at glutamatergic synapses in the dorsolateral bed nucleus of the stria terminalis. *J. Neurosci*. 2004; 24:5741–5747. [PubMed: 15215296]

- Wright JM, Peoples RW, Weight FF. Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons. *Brain Res.* 1996; 738:249–256. [PubMed: 8955520]
- Xiong Z-G, Raouf R, Lu W-Y, Wang L-Y, Orser BA, Dudek EM, Browning MD, Macdonald JF. Regulation of N-Methyl-d-Aspartate receptor function by constitutively active protein kinase C. *Mol. Pharmacol.* 1998; 54:1055–1063. [PubMed: 9855634]
- Yamakura T, Mori H, Shimoji K, Mishina M. Phosphorylation of the carboxyl-terminal domain of the $\zeta 1$ subunit is not responsible for potentiation by TPA of the NMDAR channel. *Biochem. Biophys. Res. Commun.* 1993; 196:1537–1544. [PubMed: 7504480]
- Zheng X, Zhang L, Wang AP, Bennet MVL, Zukin RS. Ca^{2+} Influx amplifies protein kinase C potentiation of recombinant NMDARs. *J. Neurosci.* 1997; 17:8676–8686. [PubMed: 9348336]
- Zheng X, Zhang L, Wang AP, Bennet MVL, Zukin RS. Protein kinase C potentiation of N-methyl-d-aspartate receptor activity is not mediated by phosphorylation of N-methyl-d-aspartate receptor subunits. *PNAS.* 1999; 96:15262–15267. [PubMed: 10611373]

Fig. 1.

Sustained PMA potentiation of I_{PK} amplitudes. (a) Representative current traces from a 15 DIV CGC that was treated with 100 nM PMA for 30 min at 37° C. Data was collected, one cell / day, as this was used to verify positive PMA affects on agonist-induced currents (NMDA) during whole-cell recordings conducted in experiments in which data is represented in Figures 2 and 3. NMDA-induced peak current amplitudes (I_{PK}) increased over the time of recording from 48.9 ± 2.9 pA / pF at the start of recording (0 min) with significant increases observed after 8 min ($t = 2.2$, $df = 28$, $P \leq 0.05$) and 12 min of recording ($t = 2.8$, $df = 28$, $P \leq 0.01$). NMDA-induced steady-state current amplitudes (I_{SS}) did not significantly increase over time. (b) Summarized data consists of currents

normalized to cell capacitance (mean $\pm$ s.e.m.) for I_{p_k} and I_{SS} ; $P^* \leq 0.05$ and $P^{**} \leq 0.01$ compared to the zero time point (0 min); $n = 9$ cells.

Fig. 2.

Activation of PKC by PMA treatment does not alter ethanol inhibition of I_{NMDA} of NMDARs expressed in CGCs. The percent ethanol inhibition of NMDA-induced peak currents (I_{pk}) and NMDA-induced steady state currents (I_{ss}) were assessed in CGCs that had received a 12.5 min, 100 nM PMA at 37° C. Ethanol sensitivity of I_{NMDA} did not differ between control CGCs or CGCs in which PKC was activated by PMA for the three ethanol concentrations tested: 10, 50 and 100 mM. Summary graphs depict mean $\pm$ s.e.m and $n = 9$ in all groups.

Fig. 3. Repeated 10 mM ethanol exposure blocks PMA-augmentation of I_{P_k} . (a) Representative NMDA and glycine (NMDA)-induced current traces in the presence and absence of 10 mM ethanol taken from a 13 DIV CGC that received a 100 nM PMA treatment for 12.5 min at 37° C. Each trace is one of three traces generated during a cluster of agonist applications (NMDA #1, 2 and 3) interspersed with agonist co-applied with 10 mM ethanol (EtOH #1 and 2). (b) Summary graph of currents normalized to cell capacitance (pA / pF) for NMDA-induced peak current amplitudes (I_{P_k}) or NMDA-induced steady-state current amplitudes (I_{SS}); $n = 12$ cells. Ethanol significantly decreased I_{P_k} ($t = 2.6$; $P \leq 0.01$ for EtOH #1) and ($t = 2.7$; $P \leq 0.01$ for EtOH #2) compared to control currents from NMDA #1; ** $P \leq 0.01$. I_{SS}

did not significantly differ from control currents in the presence or absence of ethanol.
Graphed data are mean $\pm$ s.e.m

Figure 4.

PMA potentiation of I_{PK} amplitudes is observed when NMDAR agonists are applied every thirty seconds. (a) Representative current traces obtained from a 13 DIV CGC treated with 100 nM PMA for 12.5 min at 37° C. A representative NMDA (100 μ M NMDA and 10 μ M glycine)-induced trace is shown at the start of the experiment (1+ min) and then at the 4 and 8 min time points during whole-cell recordings. I_{NMDA} were collected every 30 s for 8 – 10 min. (b) Summarized data for I_{PK} and I_{SS} are shown as mean $\pm$ s.e.m.; $P^* \leq 0.05$ and $P^{***} \leq 0.001$, $n = 10$ cells.

Fig. 5.

PMA-mediated potentiation of I_{pk} amplitudes is blocked by a brief 10 mM exposure. (a) Representative current traces acquired from a 14 DIV PMA-treated CGC indicating that the amplitude of NMDA (100 μ M NMDA and 10 μ M glycine)-induced currents in the absence of ethanol [Pre E, Post E (4 min) and Post E (8 min)] are not statistically different after brief (three, five-second) co-applications of agonists and 10 mM ethanol. Thus, indicating that PMA-mediated potentiation of I_{pk} is negated by ethanol exposure. (b) Summarized data (mean $\pm$ s.e.m) for normalized NMDA-induced peak current amplitudes (I_{pk}) and NMDA-induced steady-state current amplitudes (I_{ss}) acquired from 9 CGCs. Inhibition of I_{pk} by 10 mM EtOH was significant, * $P \leq 0.05$.

Fig. 6.

PMA-mediated potentiation of I_{pk} amplitudes is blocked by a brief 1 mM exposure. Presentation of data is similar to that presented in Fig. 6. (a) Representative current traces acquired from a 13 DIV PMA-treated CGC indicating that 1 mM ethanol blocks PMA-mediated potentiation of I_{pk} after ethanol is removed. (b) Summarized data (mean $\pm$ s.e.m) for normalized NMDA-induced peak current amplitudes (I_{pk}) and NMDA-induced steady-state current amplitudes (I_{ss}) acquired from 7 CGCs. Note, this concentration of ethanol did not inhibit I_{pk} or I_{ss} .

Fig. 7.

Ethanol dose-dependently decreases PKC α activity. Kinase activity for PKC α enzyme was assessed in the absence (0) or presence of 1, 10, 50 or 100 mM ethanol at (23-24C). The graph represents the average of the normalized values of 8 replicates from 4 separate experiments. Error bars are $\pm$ s.e.m. Significant differences for each ethanol concentration compared to the 0 condition are indicated: * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$.