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Acute ethanol exposure prevents PMA-mediated augmentation of *N*-methyl-D-aspartate receptor function in primary cultured cerebellar granule cells

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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 current amplitudes (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 coapplied with agonists, and this suppression of enhanced receptor function was observed for up to 8 min 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 PKCα, we assessed the effect of ethanol (1, 10, 50, and 100 mM) on PKCα activity. Ethanol decreased PKCα 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 PKCα activity by ethanol. However, given the incomplete blockade of PKCα activity and the low concentration of ethanol at which this phenomenon is observed, other ethanol-sensitive signaling cascades must also be involved.

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 (Newton, 2001; Ohno and Nishizuka, 2002). For the classical PKC isoforms: α, β (splice variants bI and bII) (Coussens et al., 1987) and g 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 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 (Chen and Huang, 1991, 1992; Gerber et al., 1989; Lan et al., 2001; Lau et al., 2010; Lu et al., 1999; Popp et al., 2008a; Xiong et al., 1998) and with recombinant NMDARs contained in heterologous expression systems (Grant et al., 1998; Lan et al, 2001; Liao et al., 2001; Sigel et al., 1994; Yamakura et al, 1993; Zheng et al., 1997, 1999).

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Our laboratory has previously reported that nine (PKC α, βI, βII, 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, 2006, 2008b). We have also reported that phorbol-12-myristate-13-acetate (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 (I_{NMDA}) is subunit dependent when NMDARs are expressed in human embryonic kidney cells (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 et al. (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 PKCα 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 (Hoffman et al., 1989; Lima-Landman and Albuquerque, 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). Although 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, we did observe that ethanol prevented PKC-mediated augmentation of I_{NMDA} and loss of augmentation continued up to 8 min 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 5- to 9-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% CO_2 for up to 15 DIV. Every seven DIV, existing feeding media (MEM containing 5% fetal bovine serum, 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, 35 μm/15 μm mixture) was supplemented with 0.5 mL of fresh feeding media. This method of nourishment minimizes excitotoxic cell death because of 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 because of an intercalation of PMA into the plasma membrane that is not subject