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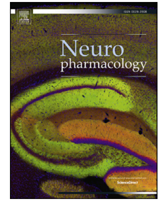

Selective chemical genetic inhibition of protein kinase C epsilon reduces ethanol consumption in mice

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ABSTRACT

Reducing expression or inhibiting translocation of protein kinase C epsilon (PKCε) prolongs ethanol intoxication and decreases ethanol consumption in mice. However, we do not know if this phenotype is due to reduced PKCε kinase activity or to impairment of kinase-independent functions. In this study, we used a chemical-genetic strategy to determine whether a potent and highly selective inhibitor of PKCε catalytic activity reduces ethanol consumption. We generated ATP analog-specific PKCε (*AS*-PKCε) knock-in mice harboring a point mutation in the ATP binding site of PKCε that renders the mutant kinase highly sensitive to inhibition by 1-tert-butyl-3-naphthalen-1-ylpyrazolo[3,4-*d*]pyrimidin-4-amine (1-NA-PP1). Systemically administered 1-NA-PP1 readily crossed the blood brain barrier and inhibited PKCε-mediated phosphorylation. 1-NA-PP1 reversibly reduced ethanol consumption by *AS*-PKCε mice but not by wild type mice lacking the *AS*-PKCε mutation. These results support the development of inhibitors of PKCε catalytic activity as a strategy to reduce ethanol consumption, and they demonstrate that the AS- PKCε mouse is a useful tool to study the role of PKCε in behavior.

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1. Introduction

Alcohol use disorder (AUD) is highly prevalent and incurs great cost to society (Rehm et al., 2009). Despite this fact, there are currently only three drugs approved in the U.S to treat AUD: Disulfuram, naltrexone, and acamprosate (Johnson, 2008). Although all are effective, disulfuram is only useful for short-term treatment of highly motivated patients in supervised settings, while naltrexone and acamprosate suffer from compliance issues and small effect sizes (Johnson, 2008). Hence, there is considerable need to develop novel therapies to combat alcoholism.

Studies from our laboratory suggest that protein kinase C epsilon (PKCε) is a target for development of drugs to reduce ethanol consumption. Previous studies found that *Prkce*$^{-/-}$ mice drink substantially less ethanol than wild type mice (Hodge et al., 1999) and show heightened aversion to ethanol (Newton and Messing, 2007), possibly because of impaired acute functional tolerance to its ataxic and hypnotic effects (Wallace et al., 2007). These behaviors do not result from developmental changes since inducible transgenic expression of PKCε in the amygdala and striatum restores normal sensitivity to intoxication and increases drinking in *Prkce*$^{-/-}$ mice to levels observed in wild type mice (Choi et al., 2002). Moreover, knockdown of PKCε in the amygdala by RNA interference (Lesscher et al., 2009) or inhibition by a peptide designed to block translocation of activated PKCε (Cozzoli et al., 2015) reduces ethanol consumption in adult wild type mice. PKCε may modulate ethanol intoxication and consumption through phosphorylation of at least 2 substrates: $GABA_A$ γ2 subunits at Ser-

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327 (Qi et al., 2007) and the N-ethylmaleimide sensitive factor at Ser-460 and Thr- 461 (Chou et al., 2010). However, all of the evidence implicating PKCε in behavioral responses to ethanol is derived entirely from genetic or shRNA-mediated reductions in PKCε expression or use of a peptide translocation inhibitor. The effect of selective, pharmacological inhibition of PKCε kinase activity with a small molecule inhibitor has not been tested. Such studies are needed to determine if PKCε is a viable drug candidate for the treatment of alcohol use disorder.

Unfortunately, there are no compounds currently available to selectively inhibit the catalytic activity of PKCε. To circumvent this problem, we have used a chemical-genetic approach to study kinase inhibition by selective, cell-permeable, small molecule inhibitors. The strategy targets the ATP-binding pocket conserved in all kinases, replacing a bulky gatekeeper residue with an alanine or glycine to generate mutant alleles that can utilize ATP analogs in addition to ATP, and that are uniquely sensitive to novel kinase inhibitors, such as analogs of PP1 (Bishop et al., 2001). We have generated such an ATP analog-sensitive PKCε (*AS*-PKCε) carrying the mutation M486A and have used it successfully to probe PKCε function in cell lines (Durgan et al., 2008; Qi et al., 2007). Here, we report the generation of an *AS*-PKCε knock-in mouse to examine the effects of PKCε on behavior. Using the *AS*-kinase inhibitor 1-Naphthyl-PP1 (1-NA-PP1) and *AS*-PKCε mice, we found that selective inhibition of *AS*-PKCε prolongs the ataxic and hypnotic effects of ethanol and reduces ethanol consumption. These results are consistent with our previous findings in *Prkce*$^{-/-}$ mice (Choi et al., 2002; Hodge et al., 1999) and validate PKCε as a candidate for drug development, while demonstrating the utility of the *AS*-PKCε mouse as a useful tool for investigating the role of PKCε in behavior.

2. Materials and methods

2.1. Generation of AS-PKCε mice

Knock-in mice were generated by Caliper Discovery Alliances and Services (Hanover, MD). The Ensembl database was used to identify the BAC clone RP23-75J18 containing the genomic sequence of mouse chromosome 17 from nt # 86451480 to 86613962. This sequence includes the exon encoding *Prkce* M486. 5′ arm (~1.9 kb) and 3′ homology arms (~6.0 kb) were generated by PCR and cloned into the targeting vector pLoxNwCD, which contains a floxed neo expression cassette for positive selection and a DTA expression cassette for negative selection. The M486A mutation was introduced into the 5′ arm by site-directed mutagenesis. The final vector was confirmed by restriction digestion and end sequencing analysis, and then linearized and electroporated into C57BL/6 ES cells. Approximately 192 ES clones that survived selection were screened using a 5′ external probe and 4 clones were expanded. Southern analysis of the ES cell DNA using 5′ external, 3′ external and neo cassette probes identified three correctly aligned clones with a single neo insertion. Two were transfected with Cre recombinase and one was confirmed to be neo deleted by PCR. Presence of the mutation in that clone was confirmed by PCR and sequencing. These ES cells were injected into tyrosinase deficient blastocysts and transplanted into pseudo-pregnant mice. Germ line transmission of the mutation from chimeras was confirmed by PCR using the following primers; CAGCACGGAGTGATCTACAGGTATTCTC (forward primer) and CGGACACAAACAGCAGGTCAAATCT (reverse primer). Heterozygous mutant progeny were then intercrossed to generate homozygous *AS*-PKCε mice, which were subsequently intercrossed and maintained as an inbred line on a C57BL/6NTac background. Mice used for experiments were housed under a reverse light dark cycle (lights off at 10 a.m.; lights on at 10 p.m.). Only male mice were tested so that we could compare results with prior studies that used male *Prkce*$^{-/-}$ mice (Choi et al., 2002; Hodge et al., 1999). All procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 2011) and were approved by the Institutional Animal Care and Use Committees of the Ernest Gallo Clinic and Research Center and the University of Texas at Austin. All efforts were made to minimize animal suffering and to reduce the number of animals used in experiments.

2.2. Administration of 1-NA-PP1

1-NA-PP1 was obtained from Dr. Kevin Shokat (UCSF) or from Tocris Biosciences (Bristol, UK). For ethanol, saccharin, and quinine consumption studies, we dissolved 1-NA-PP1 in 100% DMSO at 20 or 30 mg/ml and then diluted it 20-fold in de-ionized water containing 10% Tween-80 with sonication. For studies using oral administration, we prepared 1-NA-PP1 as a 100 mM stock solution in 100% DMSO by gentle heating and sonication. This stock was diluted to 500 μM in water containing 1% cremophor-RH40 (Sigma-Aldrich, St. Louis, MO) and 2 g/L sucralose (Sigma-Aldrich) to increase palatability. Control animals received an equivalent amount of DMSO vehicle in cremophor-sucralose-water. 1-NA-PP1 food pellets (1 g/kg) were obtained from Research Diets (New Brunswick, NJ). Control food pellets contained an equivalent amount of vehicle (DMSO). To determine the effects of 1-NA-PP1 on protein phosphorylation, we dissolved 1-NA-PP1 in vehicle containing 5% DMSO and 20% Cremophor EL (Sigma-Aldrich).

2.3. Western blot analysis

Animals were sacrificed with CO_2 asphyxiation or cervical dislocation and the amygdala and striatum were rapidly dissected on ice. Brain regions were homogenized with a glass Dounce homogenizer using 20 strokes in 0.1–0.5 ml of ice-cold extraction buffer (25 mM HEPES–pH7.8, 300 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 0.1 mM DTT) containing Phosphatase Inhibitor 1 and 3 (Sigma-Aldrich) and Protease inhibitor Complete ™ (Roche Diagnostics USA, Indianapolis, IN.). Lysates were clarified by centrifugation at 10,621 × g for 15 min at 4 °C and then resolved by SDS-PAGE using 4–12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes which were blocked with Tris buffered saline (TBS: 50 mM Tris, pH 7.6, 150 mM NaCl) containing 0.1%Tween-20 (TBS-T) and 5% BSA. The blots were incubated with anti-phospho-$GABA_A$ γ2-S(P)327 antibody (Qi et al., 2007) at 1:1000 dilution in 5% BSA overnight at 4 °C. Blots were washed 3 times in TBS-T, 8 min per wash, incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA; 1:1000 in 5% nonfat dry milk) for 1 h at room temperature, washed again, and visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher, Waltham, MA). Immunoreactive bands were quantified by densitometric scanning using Image J (Schneider et al., 2012). Blots were stripped and re-probed with total $GABA_A$ γ2 antibody (Alamone labs, Jerusalem, Israel; 1:1000 dilution) or GAPDH (Cell Signaling, Danvers, MA; 1:10,000 dilution).

2.4. Behavioral screen

We examined mice for morphological abnormalities, startle response, righting reflex, and body weight (Crawley, 2008). Strength was measured using the hanging wire test. Motor learning and coordination were assessed as in prior work (Lee et al., 2013) using a rotarod treadmill (AccuRotor Rota-Rod; Omnitech Electronics, Columbus, OH) that accelerated from 0 to 40 rpm in 5 min. Locomotor activity was recorded as the distance traveled in an open

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