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Novel Small Molecule Inhibitors of Protein Kinase C Epsilon Reduce Ethanol Consumption in Mice

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Abstract

BACKGROUND—Despite the high cost and widespread prevalence of alcohol use disorders, treatment options are limited, underscoring the need for new, effective medications. Previous results using protein kinase C epsilon (PKCε) knockout mice, RNA interference against PKCε, and peptide inhibitors of PKCε predict that small molecule inhibitors of PKCε should reduce alcohol consumption in humans.

METHODS—We designed a new class of PKCε inhibitors based on the Rho-associated protein kinase (ROCK) inhibitor Y-27632. *In vitro* kinase and binding assays were used to identify the most potent compounds. Their effects on ethanol-stimulated synaptic transmission, ethanol, sucrose and quinine consumption, ethanol-induced loss of righting and ethanol clearance were studied in mice.

RESULTS—We identified two compounds that inhibited PKCε with $K_i < 20\text{nM}$, showed selectivity for PKCε over other kinases, crossed the blood brain barrier, achieved effective concentrations in mouse brain, prevented ethanol-stimulated GABA release in the central amygdala, and reduced ethanol consumption when administered intraperitoneally at 40mg/kg in wild type but not in *Prkce*^{-/-} mice. One compound also reduced sucrose and saccharin

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consumption, while the other was selective for ethanol. Both transiently impaired locomotion through an off-target effect that did not interfere with their ability to reduce ethanol intake. One compound prolonged recovery from ethanol-induced loss of righting but this was also due to an off-target effect since it was present in *Prkce*^{-/-} mice. Neither altered ethanol clearance.

CONCLUSIONS—These results identify lead compounds for development of PKCε inhibitors that reduce alcohol consumption.

Keywords

kinase inhibitor; alcohol; addiction; protein kinase C epsilon

INTRODUCTION

Gene targeting, RNA interference, and pharmacological studies support the conclusion that PKCε is a target for development of drugs to treat alcohol use disorder (AUD). Compared with wild type mice, *Prkce*^{-/-} mice consume 50–75% less ethanol and show markedly reduced ethanol preference (1), reduced operant ethanol self-administration (2), and increased conditioned place aversion for ethanol (3). Knockdown of PKCε in the amygdala by RNA interference (4) or administration of a peptide that inhibits PKCε translocation in the amygdala or nucleus accumbens (5) reduces ethanol consumption in mice, indicating that these two regions are important sites of PKCε action on ethanol consumption. Consistent with this conclusion, *Prkce*^{-/-} mice show markedly reduced ethanol-stimulated dopamine release in the nucleus accumbens (2) and ethanol-stimulated GABA release in the central amygdala (CeA) (6). Furthermore, *Prkce* transcripts are among the most highly expressed in the brains of inbred and selected lines of mice that drink large amounts of ethanol (7). A role for PKCε in human alcohol dependence was recently suggested by a study of lymphoblastoid cell lines, which found that *PRKCE* mRNA transcripts were increased by 1.4-fold in 21 AUD cases compared with 21 controls (8).

Recently we generated mutant mice that carry an ATP analog-specific gatekeeper mutation in the purine-binding site of PKCε (*AS*-PKCε mice) and found that administration of the selective *AS*-kinase inhibitor 1-NA-PP1 reduces their ethanol intake (9). These results predict that small-molecule inhibitors of PKCε should decrease ethanol consumption. Here we report the discovery of compounds that potently inhibit native PKCε. Two prevented ethanol-stimulated GABA release in the mouse CeA and reduced ethanol self-administration in mice. Our results identify lead small molecule PKCε inhibitors for developing a new class of therapeutics to reduce consumption of alcohol.

METHODS AND MATERIALS

Reagents

Compounds 1.0, and 1.3 were synthesized by Adesis Inc. (New Castle, DE,) or by the Center for Innovative Drug Discovery (San Antonio, TX). Compounds were dissolved at 10mg/ml in 100% DMSO for kinase assays and for the oral pharmacokinetic study, or in 10% Tween-80 (Sigma-Aldrich, St. Louis, MO) for *i.p.* administration. Y-27632 (*trans*-4-[(1*R*)-1-Aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide), DNQX (6,7-

dinitroquinoxaline-2,3-dione), AP-5 (DL-2-amino-5-phosphonovalerate), and CGP 55845A were from Tocris Bioscience (Bristol, UK). Ethanol (190 proof) was from Decon Labs (King of Prussia, PA) or Remet Alcohols Inc. (La Mirada, CA). Other chemicals were from Sigma-Aldrich (St. Louis, MO).

Kinase assay

Human recombinant PKCs (Invitrogen, Carlsbad, CA,) were assayed in triplicate (see Supplemental Methods) using Lance Ultra TR-FRET technology (PerkinElmer, Waltham, MA). Compound 1.0 (200nM) was also screened against a panel of 395 non-mutant kinases by the KINOMEscan™ Profiling Service from DiscoverX (Fremont, CA).

Animals

Male C57BL/6J wild type mice, and C57BL/6J × 129S4 wild type and *Prkce*^{-/-} mice of both sexes, 2–6 months old, were housed under a 12:12 h reversed light–dark cycle (lights off 11 AM to 11 PM) with *ad libitum* access to food (Purina Mills Rodent Diet 5LL2) and water. All procedures were conducted in accordance with the University of Texas at Austin and The Scripps Research Institute (TSRI) Institutional Animal Care and Use Committee policies, and the NIH guidelines for the care and use of animals in research.

Pharmacokinetics and Plasma Protein Binding

Procedures used to determine pharmacokinetic parameters and measure protein binding for compounds 1.0 and 1.3 can be found in Supplemental Methods.

Electrophysiology

Whole-cell voltage-clamp recordings of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were performed in the medial CeA of male C57BL/6J mice (n=7; 12–14 weeks old), using DNQX (20 μM), AP-5 (30 μM), CGP 55845A (1 μM) and tetrodotoxin (0.5 μM) (6). Results were analyzed using Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) and visually confirmed. Average mIPSC characteristics were determined over a 3-min period and all drug effects were normalized to their own neuron's pre-drug baseline.

Ethanol-induced Loss of the Righting Reflex (LORR) and Ethanol Clearance

Compounds were first dissolved in 10% Tween-80. Mice received an *i.p.* injection of vehicle or inhibitor two or six hours before *i.p.* administration of ethanol. Studies using C57BL/6J mice used a dose of 3.6g/kg ethanol, and a between-subjects design. Studies with male wild type and *Prkce*^{-/-} hybrid mice used a counter-balanced, crossover, within-subjects design, in which we administered equally effective doses of ethanol (1) to wild type (3.6g/kg) and *Prkce*^{-/-} littermates (3.2g/kg). The duration of the LORR was measured as described (10). For ethanol clearance, mice were pre-treated with vehicle or inhibitor, and two hours later were administered 4g/kg ethanol. Blood samples (20 μL) were collected from the tail vein at indicated times for measurement of blood ethanol concentration (11).

Ethanol Consumption

We followed a published procedure for intermittent 24-h access, two-bottle choice drinking (12). Compounds were dissolved in 10% Tween-80 in water and administered at 10ml/kg. After 3–4 weeks, when mice were stably drinking 20% (w/v) ethanol (<10% change between consecutive drinking days), they were habituated to daily vehicle (10% Tween-80) injections given three times with one day between each injection, before they were administered compounds (0, 10, 20 and 40mg/kg, *i.p.*) using a within-subject design, two or six hours before presentation of drinking bottles. Ethanol consumption was measured during the subsequent 24h. One week later, when drinking had returned to baseline, blood ethanol concentrations (BEC) were measured in tail blood taken 4h after the beginning of a drinking session (11). Continuous access, two-bottle choice drinking was performed as described previously (1). Mice of both sexes were provided ascending concentrations of ethanol at 2, 4, and 8% (w/v) over 12 days and then 12% ethanol for the rest of the experiment. After drinking of 12% ethanol was stable, mice were administered vehicle or 40mg/kg test compound intraperitoneally in a counter-balanced, crossover design with 4d between injections. Ethanol consumption was measured for 24h after each injection.

Sucrose, Saccharin, and Quinine Consumption

Drug and ethanol naive mice were singly housed and provided one bottle containing 4% (w/v) sucrose and the other tap water for five consecutive days. The bottles were weighed daily and positions alternated to account for side preferences. Over the next 6d, mice were acclimated to three *i.p.* injections of vehicle (10% Tween-80). Two days later, mice received vehicle or 40mg/kg of compound 1.0 *i.p.* using a counter-balanced, crossover design with 2d between injections. Three days later, mice were administered vehicle or 40mg/kg of compound 1.3 using the same counter-balanced, crossover protocol. Two days later, mice were presented with 0.015mM quinine or tap water, and the concentration of quinine was increased daily to 0.03 and then 0.05mM. After three more days of access to 0.05mM quinine, mice were administered vehicle or compounds using a counter-balanced, crossover protocol. A second cohort of mice was provided 0.03% (w/v) saccharin or water by the same protocol used for sucrose consumption. After three days of acclimation to *i.p.* injections of vehicle, these mice were administered vehicle or compounds (40mg/kg) using the same counter-balanced, crossover design.

Statistical Analyses

Data were expressed as mean $\pm$ SEM values and analyzed using Prism 7.0 (GraphPad Software, La Jolla, CA). Kinase assays were analysed by nonlinear regression to determine IC_{50} values for each inhibitor. Michaelis-Menten constants (K_m) for novel PKCs were calculated using non-linear curve fitting with equation $V_0 = V_{max}[S]/(K_m + [S])$, where V_0 is initial velocity, V_{max} is maximum initial velocity, and $[S]$ is ATP concentration. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (13) using the following ATP K_m values: $1.67 \pm 1.25 \mu M$ (PKC ϵ), $3.11 \pm 1.14 \mu M$ (PKC δ), and $7.08 \pm 2.19 \mu M$ (PKC θ). Additional results were first tested for normality using a D'Agostino-Pearson omnibus normality test and if normally distributed were analyzed by two-tailed *t*-test or

ANOVA with a *post-hoc* Dunnett's or Sidak's multiple comparisons test. Data that were not normally distributed were analyzed by a Wilcoxon matched-pairs signed rank test.

RESULTS

Design of Novel PKC ϵ Inhibitors

The widely used ROCK inhibitor Y-27632 inhibits PKC ϵ 10- to 50-fold less potently than ROCK1 (14). Compound 397 (described in patent WO 2007/006546) was developed from Y-27632 as a PKC inhibitor (Figure 1A). Molecular modeling guided structural modifications around these two lead compounds and led us to design the novel hybrid analogs 1.0 and 1.3, which possessed very favorable physical chemical properties for CNS drugs (Figure 1A) (15). *In vitro* PKC ϵ kinase assays revealed the following rank order of inhibitory potency: 397 (2nM) > 1.0 (19nM) $\approx$ 1.3 (18nM) > Y-27632 (141nM).

Because individual PKC isozymes participate in different biochemical pathways (16), we screened our compounds for PKC isozyme specificity to determine if they were selective for PKC ϵ . Unlike compound 397, compound 1.0 showed little activity against conventional PKC γ at 10 μ M, while compound 1.3 showed little activity against PKC γ and atypical PKC ζ at that high concentration (Table 1). All three compounds inhibited the highly-related novel PKCs, PKC δ and PKC θ , but compounds 1.0 and 1.3 showed some selectivity within the novel PKC subfamily being 14- to 20-fold less potent in inhibiting PKC δ or PKC θ than PKC ϵ (Figure 1B). Using a test concentration of 200nM, we had compound 1.0 screened against a panel of 395 non-mutant kinases in a binding competition assay (17). Compound 1.0 inhibited PKC ϵ and only ten other kinases to <35% of control: CDK7, CLK1, CLK2, CLK4, PKN1, PKN2, PRKCH, PRKCQ, ROCK1, and ROCK2. We also had compounds 1.0 and 1.3 screened by the NIMH Psychoactive Drug Screening Program (<http://pdspdb.unc.edu/pdspWeb/>) at 10 μ M for inhibition of radioligand binding to several G-protein coupled receptors (5-HT 1A, 1B, 1D, 1E, 2A, 2B, 2C, 3, 6, and 7; alpha adrenergic 1A, 1B, 1D, 2A, 2B, 2C; beta adrenergic 1 and 3; dopamine 1–5; delta, mu and kappa opioid, histaminergic 1–4, muscarinic 1–4; and sigma 1 and 2) and monoamine transporters. Compound 1.0 inhibited ligand binding at five targets with the following K_i values: 5-HT1A (2.09 μ M) and 5-HT7 (1.18 μ M) receptors; and dopamine (2.07 μ M), norepinephrine (693nM) and serotonin (36nM) transporters. Compound 1.3 was more selective, inhibiting binding only at dopamine (1.71 μ M) and norepinephrine (2.64 μ M) transporters.

Some small molecule kinase inhibitors act non-selectively by forming aggregates that sequester enzymes from substrates, resulting in apparent inhibition (18). This mechanism contrasts with that of selective inhibitors, such as Y-27632 (19), which compete with substrates for kinase binding. Since our compounds are derived from Y-27632, we predicted they should compete with ATP for binding to PKC ϵ . Therefore, we assayed PKC ϵ activity in the presence of increasing ATP concentrations (Figure 1C). The IC₅₀ for compound 1.0 was 61.7nM (57.8 – 65.9nM 95% C.I.) with 2.5 μ M ATP and increased 15-fold to 940nM (775 – 1135nM, 95% C.I.) with 50 μ M ATP. This rightward shift in potency indicates that compound 1.0 inhibits PKC ϵ by competing with ATP.

Pharmacokinetics and Protein Binding

Compounds were administered intraperitoneally (20mg/kg for compound 1.0 and 40mg/kg for compound 1.3) or by oral gavage (100mg/kg for both compounds), and plasma and brain samples were collected at various times for up to 24 hours (Figure 2 and Table 2). Both compounds entered the brain. Two hours after intraperitoneal injection (Figure 2A and B) of compound 1.0, the brain/plasma ratio was 0.56 ± 0.12 , which was ~4 fold greater ($P=0.022$; $t=3.648$; $n=3$ for each compound) than the ratio for compound 1.3 (0.13 ± 0.03), even though compound 1.3 was given at a higher dose. By contrast, two hours following oral gavage of 100mg/kg, brain concentrations (Figure 2C and D) and brain/plasma ratios (0.17 ± 0.04 for compound 1.0 and 0.10 ± 0.03 for compound 1.3) were similar. When incubated at 5 μ M with plasma for six hours, compound 1.0 was 85.9% and compound 1.3 was 95% protein bound. The binding data suggest that free, unbound plasma drug levels of compound 1.0 and 1.3 two hours post *i.p.* administration were 313nM and 278nM, respectively, providing a brain/free plasma ratio of 3.8 for compound 1.0 and 2.3 for compound 1.3. These results indicate that these compounds enter the brain extremely well, are more slowly eliminated from brain than plasma, and provide free plasma drug levels and brain levels sufficient for PKC ϵ occupancy and *in vivo* efficacy despite high plasma protein binding.

PKC ϵ Inhibitors Reduce Basal and Ethanol-stimulated GABA Release in the CeA

Ethanol application enhances GABA release in the medial CeA, and this response does not occur in *Prkce*^{-/-} mice (6). To examine whether compounds 1.0 and 1.3 also prevent ethanol enhancement of GABA release, we first assessed the effects of compound 1.0 and 1.3 on CeA GABA mIPSCs (baseline properties: frequency= 0.82 ± 0.22 Hz, amplitude= 44.2 ± 2.0 pA, rise time= 2.57 ± 0.10 ms and decay time= 6.26 ± 0.41 ms). Both compounds (2 μ M, 15 min) rapidly and persistently decreased mIPSC frequencies [1.0: $t(8)=3.89$, $P=0.0046$ and 1.3: $t(9)=3.98$, $P=0.0032$, one-sample *t*-test] (Figure 3A and C), without altering their amplitudes or kinetics (Figure 3A and B). These results indicate that PKC inhibition decreases vesicular GABA release at CeA synapses, without affecting local GABA_A receptor function (20). To ensure that there were no delayed effects of either compound on mIPSC frequencies, we tested each compound (2 μ M) for 24 min in a second set of cells and found no difference after 15 min versus 24 min of drug exposure [1.0: $t(4)=0.304$ and 1.3: $t(6)=1.437$, two-tailed paired *t*-test].

We next investigated whether these compounds prevent the ability of ethanol to enhance CeA GABA release. As previously reported (6), application of a maximally effective concentration of ethanol (44mM EtOH; 9 min) significantly increased mIPSC frequency [$t(8)=4.28$, $P=0.0027$, one-sample *t*-test], without altering other mIPSC properties (Figure 3D). Notably, both compounds (2 μ M; 15 min) blocked ethanol enhancement of mIPSC frequency [$F(2,25)=15.93$, $P<0.0001$] (Figure 3D). Therefore, PKC ϵ activity mediates the acute effects of ethanol on CeA GABA release.

PKC ϵ Inhibitors Reduce Ethanol Consumption and Preference

To determine whether compounds 1.0 and 1.3 reduce ethanol intake, we first used an intermittent access ethanol drinking procedure (12) in which C57BL/6J mice have 24-hour access to 20% (w/v) ethanol three days per week for several weeks and which leads to high

levels of ethanol consumption. After 4 weeks, mice achieved a stable level of ethanol consumption that reached 16.97 ± 1.11 g/kg/day, and during the first 4 hours, they consumed 5.4 ± 0.2 g/kg to achieve a mean BEC of 104 ± 10 mg/dL (Supplemental Figure S1). We administered vehicle, or 10, 20, or 40 mg/kg of test compound two hours before an ethanol drinking session using a counterbalanced design. Compound 1.0 (Figure 4A and B) reduced ethanol consumption [$F(3,33)=8.87$, $P=0.0024$] and preference [$F(3,33)=7.36$, $P=0.0064$]. Compound 1.3 (Figure 4C and D) also reduced consumption [$F(3,30)=22.65$, $P<0.0001$] and preference [$F(3,30)=10.13$, $P=0.0006$].

Since ethanol tastes both bitter and sweet and there is an association between sweet taste and ethanol consumption in several inbred rodent strains (21), we examined whether compounds 1.0 and 1.3 alter consumption of solutions containing quinine or sucrose (Supplemental Figure S2). Neither compound altered quinine consumption. Compound 1.0 also did not alter sucrose consumption, but compound 1.3 reduced it. To investigate whether this effect of compound 1.3 occurred with a sweet substance that has no caloric value, we also measured saccharin consumption and found that compound 1.3 reduced it as well. Thus, while compound 1.0 selectively reduced ethanol intake, compound 1.3 reduced consumption of ethanol and sweet substances.

To determine whether these compounds reduce drinking by inhibiting PKC ϵ , we examined ethanol consumption in 2 cohorts of C57BL/6J $\times$ 129S4 *Prkce*^{-/-} mice and wild type littermates of both sexes. For this experiment, we used a two-bottle choice continuous access procedure to replicate our prior finding with these mice (1). *Prkce*^{-/-} mice drank less ethanol than wild type mice (Figure 4E), and compound 1.0 (40 mg/kg, *i.p.*) reduced ethanol consumption in wild type but not in *Prkce*^{-/-} mice [$F_{\text{genotype} \times \text{treatment}}(1,25)=16.87$, $P=0.0004$]. Compound 1.0 (40 mg/kg, *i.p.*) also reduced ethanol preference (Figure 4F) in wild type but not in *Prkce*^{-/-} mice [$F_{\text{genotype} \times \text{treatment}}(1,25)=5.52$, $P=0.027$].

PKC ϵ Inhibitors Transiently Decrease Locomotor Activity in Mice

We noticed that some mice moved less in their cages shortly after compound administration, but moved readily when touched. To quantify a potential sedative effect of these compounds, we pretreated C57BL/6J mice with vehicle, compound 1.0, or compound 1.3 before placing them in a locomotor activity chamber. Male C57BL/6J mice showed reduced activity when administered compound 1.0 [$F_{\text{Treatment} \times \text{Time}}(1,24)=4.40$, $P=0.0467$] or compound 1.3 [$F_{\text{Treatment} \times \text{Time}}(1,26)=6.84$, $P=0.0146$] 2h before, but not 6h before locomotor testing (Supplemental Figure S3A and B). This transient suppression of activity appeared to be due to an off-target effect since we detected it in both wild type and *Prkce*^{-/-} littermates 2h after administering compound 1.0 [$F_{\text{Treatment}}(1,19)=13.97$, $P=0.0014$; $F_{\text{Genotype}}(1,19)=0.212$, $P=0.6503$; $F_{\text{Treatment} \times \text{Genotype}}(1,19)=0.001$, $P=0.9725$] (Supplemental Figure S3C). To test whether decreased activity impacted our results for ethanol consumption, we repeated this experiment but administered compounds 6h before testing, when locomotor activity would have recovered. We found that 40mg/kg compound 1.0 and 1.3 still reduced ethanol consumption and preference (Supplemental Figure S3D and E) in C57BL/6J mice when administered 6h before the test.

PKC ϵ Inhibitors Prolong Ethanol-Induced LORR in Wild Type and in *Prkce*^{-/-} Mice

Since the duration of the ethanol-induced LORR is longer in *Prkce*^{-/-} mice than in wild type littermates (1), we tested whether PKC ϵ inhibitors prolong the duration of the ethanol-induced LORR (Figure 5A and B). We administered equally effective doses of ethanol (1) to wild type (3.6mg/kg) and *Prkce*^{-/-} littermates (3.2mg/kg) and unexpectedly found that compound 1.0 (40mg/kg) prolonged the LORR in both genotypes when administered 2h [$F_{\text{Treatment}}$ (1,18)=23.48, $P=0.0001$; F_{Genotype} (1,18)=0.266, $P=0.6123$; $F_{\text{Treatment} \times \text{Genotype}}$ (1,18)=0.734, $P=0.4023$], but not 6h [$F_{\text{Treatment}}$ (1,18)=2.43, $P=0.1365$; F_{Genotype} (1,18)=1.085, $P=0.3115$; $F_{\text{Treatment} \times \text{Genotype}}$ (1,18)=0.0748, $P=0.7876$], before ethanol. These results indicate that, similar to its effects on locomotion in an open field, the ability of compound 1.0 to prolong the duration of the LORR is transient and due to an off-target effect.

PKC ϵ Inhibitors Do Not Alter Ethanol Clearance

To determine whether any of the effects of these PKC ϵ inhibitors could be explained by altered ethanol clearance, we measured blood ethanol concentrations following intraperitoneal administration of 4.0 g/kg ethanol. Neither compound 1.0 [$F_{\text{treatment}}$ (1, 26)=0.58, $n.s$] nor compound 1.3 [$F_{\text{treatment}}$ (1, 28) = 0.61, $n.s$] altered ethanol clearance (Figure 5C) when administered at 40mg/kg, 2h prior to ethanol.

DISCUSSION

Here we describe novel small molecule inhibitors of PKC ϵ that reduce ethanol consumption in mice. These compounds competitively inhibit PKC ϵ with respect to ATP with K_i values below 20nM. They possess favorable CNS drug-like, physical chemical properties based on ranges correlated with successful, clinically active CNS drugs by CNS multi-parameter optimization analysis (15). When screened against a panel of 395 non-mutant kinases and a series of G protein-coupled receptors and monoamine transporters, concentrations of compound 1.0 200nM interacted with only novel PKCs, ROCK 1 and 2, six other kinases, and the serotonin transporter. Both compounds were brain penetrant, orally bioavailable, and had a long CNS half-life. As predicted for a PKC ϵ inhibitor (6), both compounds reduced ethanol-stimulated GABA release in CeA slices. Importantly, both compounds reduced ethanol consumption and preference in mice. Neither drug altered the rate of ethanol clearance, suggesting that they do not reduce ethanol consumption by interfering with ethanol metabolism.

Compound 1.0 inhibited ethanol but not sucrose consumption indicating that its effects were selective for ethanol. In contrast, compound 1.3 reduced consumption of ethanol, sucrose and saccharin. This effect is like what has been observed for naltrexone, which reduces sucrose consumption in several strains of rats and mice (22, 23) and reduces preference for sucrose solutions in humans (24). However, it is worth noting that compound 1.3 did not reduce preference for sucrose or saccharin, suggesting that it does not have a general effect on hedonic reward, but rather it instead reduces the perception of sweetness.

Three limitations to this study deserve mention. Although both compounds reduced ethanol consumption in a dose-dependent fashion, the effect was statistically significant by *post-hoc* analysis only at the highest dose (40mg/kg). The relatively low potency of these compounds may be due to their high degree of protein binding, which could limit their access to brain PKC ϵ . Also, we have not yet assayed these compounds for effects on ethanol reinforcement or ethanol-induced place conditioning. Although our studies with *Prkce*^{-/-} mice predict that these compounds will reduce ethanol reward and enhance ethanol aversion (3), this hypothesis remains to be tested. Finally, we have not yet determined whether tolerance develops to repeated doses of these compounds.

Both compounds prolonged the duration of the ethanol-induced LORR and reduced locomotor activity, when administered 2h but not when administered 6h prior to testing, in both wild type and *Prkce*^{-/-} mice, indicating an off-target effect. This off-target effect is likely due to an action outside of the central nervous system, since it had resolved 6h after drug administration, when plasma levels were falling, yet brain levels remained stable and the compound still suppressed ethanol consumption. Current efforts are directed at developing novel analogs of these lead compounds that lack such an off-target effect and have improved specificity and potency against PKC ϵ , while maintaining favorable CNS drug-like properties.

Ethanol evokes presynaptic release of GABA in the central amygdala through a PKC ϵ -dependent mechanism (6) that may contribute to ethanol's reinforcing effects (25). Consistent with this concept, we previously found that knockdown of PKC ϵ in the amygdala through RNA interference reduced drinking in mice (4). Here we found that compound 1.0 or 1.3 alone reduced mIPSC frequency in the medial CeA. This finding stands in contrast to our previous results, in which basal mIPSC frequency was increased in *Prkce*^{-/-} mice relative to wild type littermate controls (6). We had previously interpreted this finding as suggesting that PKC ϵ normally has a dual function, acting to limit baseline GABA release while facilitating ethanol-induced GABA release. In light of our present findings, the elevation in baseline GABA release that we previously observed in *Prkce*^{-/-} mice may reflect a compensatory response to lifelong absence of PKC ϵ in knockout animals. Critically, our present data reveal that PKC ϵ promotes GABA release in the CeA and that acute inhibition of PKC ϵ blocks ethanol-induced facilitation of GABA release in that brain region.

There are some concerns about potential side effects of PKC ϵ inhibitors. We had found that the hypnotic effect of ethanol is prolonged in *Prkce*^{-/-} mice (1). Such an effect could complicate clinical use of PKC ϵ inhibitors in treating AUD. However, as noted above, we found that the ability of our compounds to prolong the ethanol-induced LORR was due to an off-target effect since it was evident in *Prkce*^{-/-} mice. This finding leads us to conclude that the enhanced response of *Prkce*^{-/-} mice to high doses of ethanol may involve compensatory changes in other proteins due to lifelong absence of PKC ϵ in knockout mice. It will be important in future studies to confirm that more selective PKC ϵ inhibitors do not enhance the sedative-hypnotic effects of ethanol.

There are additional concerns worthy of mention. Several investigators have identified a role for PKC ϵ in ischemic preconditioning (26–28) raising the possibility that PKC ϵ inhibitors could impair that process. Some studies with one line of *Prkce*^{-/-} mice have reported delayed wound healing (29) and impaired macrophage activation (30) in these animals, although we strongly believe that these two phenotypes need further confirmation using complementary approaches and other animal models before concluding that PKC ϵ plays an important role in these biological processes.

In conclusion, we have developed brain penetrant, small molecule inhibitors of PKC ϵ that are relatively selective and reduce ethanol consumption in mice. Ongoing work is directed towards improving the drug-like properties of these compounds to reduce off target effects, achieve greater bioavailability and potency, and investigate their effect on drug reward and aversion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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R.O.M. and M.A.P. are inventors on Patent Nos. US 8,785,648 B1 and 9,376,423 entitled *PKC-Epsilon Inhibitors*.

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Figure 1.

Development of novel PKC ϵ inhibitors. **(A)** Structures of Y-27632 and compounds 397, 1.0 and 1.3. **(B)** Dose-response curves for inhibition of novel PKC isozymes. K_i values for compound 1.0 were 19nM (PKC ϵ), 391nM (PKC δ), and 277nM (PKC θ). K_i values for compound 1.3 were 18nM (PKC ϵ), 290nM (PKC δ), and 270nM (PKC θ). **(C)** Dose-response curves for inhibition of PKC ϵ by compound 1.0 showing a rightward shift at higher [ATP] ($n=3$ for each condition in B and C).

Figure 2. Pharmacokinetics of PKCε inhibitors. (**A** and **B**) Compounds 1.0 (20mg/kg) and 1.3 (40mg/kg) were administered *i.p.*, and plasma and brain concentrations were measured over a 24-hour period ($n = 3$ for each time point). (**C** and **D**) Compounds 1.0 (100mg/kg) and 1.3 (100mg/kg) were administered by oral gavage, and plasma and brain concentrations were measured over a 12-hour period ($n = 3-12$ for each time point).

Figure 3.

PKC ϵ inhibitors reduce basal and ethanol-stimulated GABA release. Representative mIPSC traces (**A**) and histograms (**B**) indicate that compounds 1.0 and 1.3 (2 μ M for 15 min) each decreased mIPSC frequencies, but had no effect on amplitudes or kinetics (9–10 cells from 5 mice). ** $P < 0.01$, one-sample t-test. (**C**) Time course of the decrease in mIPSC frequencies produced by both compounds. (**D**) Pretreatment with compound 1.0 or 1.3 (2 μ M for 15 min) blocked the ethanol (EtOH) enhancement of mIPSC frequency compared with EtOH alone (9–10 cells from 5–8 mice). ** $P < 0.01$, one-sample t-test; ### $P < 0.001$, Sidak's multiple comparisons test.

Figure 4.

PKCe inhibitors reduce ethanol consumption in mice. Compounds 1.0 (A and B, $n=12$) and 1.3 (C and D, $n=11$) reduced intermittent access 2 bottle choice ethanol consumption (A and C) and preference (B and D). ** $P<0.01$, *** $P<0.001$ compared with control, Dunnett's test. Compound 1.0 also reduced continuous access 2-bottle choice ethanol consumption (E) and preference (F) in wild type ($n=17$), but not in *Prkce*^{-/-} mice ($n=10$ per group).

**** $P<0.0001$, ** $P=0.0046$, Sidak's multiple comparisons test.

Figure 5.

Ethanol intoxication and clearance. (A) Compound 1.0 prolonged the duration of the ethanol-induced LORR in wild type ($n=12$) and *Prkce*^{-/-} ($n=10$) mice when administered 2h before injection of ethanol. * $P=0.011$, ** $P=0.0034$, Sidak's multiple comparisons test. (B) Compound 1.0 did not alter the duration of the ethanol-induced LORR when administered 6h before ethanol in either genotype ($n=11$ wild type and 9 *Prkce*^{-/-} mice). (C) Compounds 1.0 and 1.3 (40mg/kg) did not alter clearance of 4g/kg ethanol ($n=8$ per group).

Table 1Inhibition of PKC activity (%) by 10 μ M of each compound ($n=3$)

Compound	Conventional	Novel		Atypical
	PKC γ	PKC δ	PKC θ	PKC ζ
Y-27632	6.8 $\pm$ 0.3	82.5 $\pm$ 1.4	82.7 $\pm$ 0.3	7.4 $\pm$ 2.7
397	80.5 $\pm$ 3.4	101.3 $\pm$ 2.4	100.8 $\pm$ 0.7	12.8 $\pm$ 2.7
1.0	16.7 $\pm$ 3.8	96.3 $\pm$ 0.7	96.9 $\pm$ 1.1	55.9 $\pm$ 3.8
1.3	17.0 $\pm$ 1.3	96.4 $\pm$ 1.0	94.4 $\pm$ 1.0	6.3 $\pm$ 2.1

Table 2

Pharmacokinetics of compounds 1.0 and 1.3.

Compound and route	Plasma		Brain	
	t _{1/2} (h)	AUC(h-μM)	t _{1/2} (h)	AUC(h-μM)
1.0 i.p.	1.90	17.45	15.11	17.31
1.3 i.p.	1.37	48.53	49.21	11.38
1.0 p.o.	ND	8.01	ND	2.03
1.3 p.o.	1.4	10.79	ND	2.16

AUC, area under the curve

t_{1/2}, half-life

ND, could not be determined