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PROTEIN KINASE C EPSILON REGULATES $GABA_A$ RECEPTOR SENSITIVITY TO ETHANOL AND BENZODIAZEPINES THROUGH PHOSPHORYLATION OF γ2 SUBUNITS*

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Running Title: PKC epsilon phosphorylation of $GABA_A$ receptors

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Ethanol enhances GABA signaling in the brain, but its actions are inconsistent at $GABA_A$ receptors, especially at low concentrations achieved during social drinking. We postulated that the epsilon isoform of protein kinase C (PKCε) regulates the ethanol sensitivity of $GABA_A$ receptors, as mice lacking PKCε show an increased behavioral response to ethanol. Here we developed an ATP analog-sensitive PKCε mutant to selectively inhibit the catalytic activity of PKCε. We used this mutant and $PKC\varepsilon^{-/-}$ mice to determine that PKCε phosphorylates γ2 subunits at serine 327 and that reduced phosphorylation of this site enhances the actions of ethanol and benzodiazepines at α1β2γ2 receptors, which are the most abundant $GABA_A$ receptor subtype in the brain. Our findings indicate that PKCε phosphorylation of γ2 regulates the response of $GABA_A$ receptors to specific allosteric modulators, and, in particular, PKCε inhibition renders these receptors sensitive to low intoxicating concentrations of ethanol.

Gamma-aminobutyrate type A ($GABA_A$)[2] receptors mediate the majority of rapid inhibitory neurotransmission in the brain and are an important target for ethanol, the most widely abused drug (1). Ethanol modulation of $GABA_A$ receptors was first identified in synaptosomal preparations where intoxicating concentrations (10-30 mM) enhanced receptor function as measured by ^{36}Cl uptake assays (2,3). However, after thirty years of investigation, it is apparent that ethanol enhancement of synaptic $GABA_A$ receptors is variable and in some preparations cannot be detected even at anesthetic concentrations (1,4).

$GABA_A$ receptors are pentameric protein complexes of subunits from eight classes (α1-6, β1-3, γ1-3, δ, ε, π, θ and ρ1-3) (5). Most receptors are comprised of two α subunits and two β subunits that co-assemble with one γ2 subunit, which anchors these receptors at synapses where they mediate phasic inhibition (6). A minority contain a δ subunit instead of γ2; these receptors are extrasynaptic and mediate tonic inhibition in neurons (7). Since earlier ethanol studies that focused on GABA currents carried by γ2-containing receptors produced variable results, recent attention has turned to ethanol effects at receptors containing δ subunits. Reports from

three laboratories found these receptors enhanced by low (≤ 30 mM) intoxicating concentrations of ethanol (8-10). However, two recent studies were unable to demonstrate low-dose ethanol sensitivity of δ-containing $GABA_A$ receptors (11,12), indicating that, like synaptic $GABA_A$ receptors, ethanol modulation of extrasynaptic receptors is also variable.

The reasons for this high degree of variability are unknown. One possibility is that intracellular signaling pathways may regulate ethanol sensitivity of $GABA_A$ receptors and the activity of such pathways was not controlled for in these studies. This hypothesis is consistent with our findings in mice lacking protein kinase C epsilon (PKCε), which show an increased behavioral response to ethanol (13). Ethanol modulation of $GABA_A$ receptors is also increased in PKCε$^{-/-}$ mice compared with wild type mice (13,14). These results suggest that activation of PKCε suppresses potentiation of $GABA_A$ receptors by ethanol.

The mechanism by which PKCε regulates the ethanol sensitivity of $GABA_A$ receptors is unknown. Here, we examined this mechanism using receptors composed of α1, β2, and γ2 subunits, which is the most abundant receptor subtype in the brain (15). Since there are no selective inhibitors of PKCε catalytic activity available, we used a chemical-genetics approach to generate an ATP analog-sensitive mutant, as-PKCε, that could be potently and selectively inhibited by a cell-permeant, small molecule inhibitor. Our findings indicate that PKCε regulates the sensitivity of α1β2γ2 receptors to ethanol and benzodiazepines through phosphorylation of serine 327 in the large intracellular loop of γ2.

EXPERIMENTAL PROCEDURES

Animal Care and Behavior - PKCε$^{-/-}$ mice were generated by homologous recombination in J1 embryonic stem cells as described (16). Male and female PKCε$^{+/-}$ mice were maintained on inbred 129S4/SvJae and C57BL6/J backgrounds and were crossed to produce PKCε$^{+/-}$ C57BL6/J ×129S4/SvJae F1 hybrid mice. These mice were intercrossed to generate F2 hybrid PKCε$^{+/+}$ and PKCε$^{-/-}$ littermates for experiments. Mice were genotyped by PCR of tail biopsies (5-10 mm fragment) obtained from non-anesthetized, 10-d old pups. All animals were between 2-4 mo old at the time of experimentation. All procedures were conducted in accordance with Ernest Gallo Clinic and Research Center Institutional Animal Care and Use Committee policies. Zolpidem-induced ataxia was measured as described previously (17). The "up and down" method (17) was used to measure initial sensitivity to zolpidem with a starting dose of 0.25 mg/kg and a log dose interval of 0.0276.

Immunoprecipitation - Frontal cortex was dissected from 8-10 week-old mice and homogenized with a Teflon-glass homogenizer in ice-cold buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, Complete ™ protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN), 2 mM benzamidine chloride and 0.1 mM PMSF. The homogenate was centrifuged at 200,000 g for 30 min at 4°C. The pellet was solubilized in ice-cold TBS (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% Triton X-100 and Complete ™ protease inhibitor cocktail), and centrifuged again at 120,000 g for 15 min at 4°C. The supernatant (500 μg protein) was incubated for 2 h at 27°C with 1 μg of rabbit anti-$GABA_A$-α1 polyclonal antibody 993-2, generated against the C-terminal decapeptide sequence common to rat, mouse, and human $GABA_A$-α1 [PQLKAPTPHQ], or with purified rabbit IgG (Sigma-Aldrich, St. Louis, MO). For the reverse immunoprecipitation, lysates were incubated with 1 μg rabbit anti-PKCε (18) or purified rabbit IgG. Protein A beads (100 μl; Roche Diagnostics) were added and the suspension was incubated overnight at

4°C. Beads were washed four times with ice-cold PBS and immune complexes eluted with 100 µl of sample buffer. After being heated for 7 min at 90°C, samples were centrifuged at 6,000 g for 30 sec and proteins were separated in 4-12% polyacrylamide gels (Invitrogen, Carlsbad, CA). Proteins were analyzed using western blots with goat anti-PKCε (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution) or goat anti-α1 subunit antibody (Santa Cruz Biotechnology; 1:500 dilution) followed by peroxidase-conjugated rabbit anti-goat IgG (Roche Diagnostics, 1:1000 dilution) and enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).

Primary neuronal culture - Primary cultures of cortical and hippocampal neurons were prepared from 1 to 3 day old mice as described (19). Briefly, cells were dissociated from dissected cerebral cortex or hippocampus by treatment with papain (20 U/ml) for 45 min at 37˚C, followed by trituration through a 1 ml plastic pipette. For immunostaining, hippocampal neurons were plated at a density of 10^5 cells on 200 mm^2 chamber slides coated with poly-L-lysine in Neurobasal A media containing B27 and Glutamax (Invitrogen, Carlsbad, CA) and maintained at 37 °C in a humidified atmosphere of 6% CO_2 and 94% air. For studies of cell surface γ2 subunits, cortical neurons were plated on poly-L-lysine coated 6-well plates at 1.6 x 10^6 cells/well. For electrophysiology experiments, hippocampal neurons were plated on poly-L-ornithine coated 35-mm petri dishes. Half of the medium was changed the next day and changed every 7 d for three weeks.

Immuofluorescence staining of neurons - Mature (DIV 14-21) neurons were immunostained as described (20). To detect $GABA_A$ receptors on the cell surface, living cultures were incubated with rabbit anti-$GABA_A$ receptor α1 N-terminal peptide (1:100, Chemicon, Temecula, CA) diluted in Ringer's solution containing 0.5 µM tetrodotoxin for 90 min at 27°C. After three 10-min washes in Ringer's solution, the cells were fixed with methanol for 10 min at -20˚C. Fixed cells were washed with PBS and incubated for 90 min at 27°C with goat anti-PKCε antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS containing 10% normal donkey serum. After three washes with PBS, the cells were then incubated with donkey FITC -conjugated anti-goat and Cy3-conjugated anti-rabbit antibodies (1:200, Jackson ImmunoResearch, West Grove, PA) and cover-slipped in mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Images (1.5 µm optical sections) were acquired using a Zeiss LSM 510 META laser confocal microscope with a Plan-Apochromat 63x/NA 1.40 oil immersion objective.

Generation of as-PKCε - A pCDNA3 plasmid containing N-terminal FLAG epitope-tagged, human PKCε cDNA was provided by Dr. Alex Toker (Harvard University, Boston, MA) and used as a template to replace Met 486 with Ala by site-directed mutagenesis (QuikChange mutagenesis kit; Stratagene, La Jolla, CA) using the following primers:
5'-
GGACCGCCTCTTTTTCGTCGCCGAATAT
GTAAATGGTGGAGACC-3' as the forward primer and
5'-
GGTCTCCACCATTTACATATTCGGCGA
CGAAAAAGAGGCGGTCC-3' as the reverse primer. The PCR product was treated with Dpn I for 1 h at 37°C and then amplified in *E. coli* XL 1-Blue. The presence of the M486A mutation was confirmed by DNA sequencing.

Plasmid DNA (10 µg) was transfected into COS-7 cells using the SuperFect Transfection Reagent (Qiagen, Valencia, CA). After culture for 72 h, cells were rinsed twice with 2 ml of ice-cold PBS and incubated for 20 min at 4°C in lysis buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, and Complete

Protease Inhibitor Cocktail (Roche Diagnostics). Lysates from fifty 10-cm plates were pooled and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was incubated for 3 h at 4°C with 1 ml of anti-FLAG M2-conjugated agarose (Sigma, St Louis, MO), previously equilibrated with lysis buffer. Agarose beads were transferred to a column and washed sequentially with 15 ml of lysis buffer, 15 ml of buffer B (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and 5 ml of PKC storage buffer (0.1 mM EGTA, 20 mM HEPES, pH 7.4, 25% glycerol and 0.03% Triton X-100). To elute FLAG-tagged proteins, the beads were incubated with 1.75 ml of 0.15 mg/ml 3X FLAG peptide solution (Sigma-Aldrich, St Louis, MO) in PKC storage buffer for 30 min at 4°C and then centrifuged for 10 sec at 10,000 g. Aliquots of the supernatant were stored at -80°C. The concentration of PKCε in the eluate was measured in triplicate by ELISA using recombinant PKCε (Invitrogen, Carlsbad, CA) as a standard, rabbit anti- PKCε SN134 (18) diluted 1:1000 as the primary antibody, and horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody (1:3,500 dilution; Chemicon, Temecula, CA). Immunoreactivity was detected using 3,3′,5,5′-tetramethylbenzidine as the substrate with absorbance measured at 450 nm.

Characterization of as-PKCε - Enzyme activity was measured by fluorescence polarization using the Protein Kinase C Assay Kit from Invitrogen. PKCε (0.5 ng) or PKCε-M486A (3 ng) was added to kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.02% NP-40, 0.03% Triton X-100, 0.1 µg phosphatidylserine and 0.05 mM sodium vanadate) with 250 nM substrate peptide (RFARKGSLRQKNV) and 12.5 nM PMA in a final volume of 40 µl. The mixture was incubated at 22°C for 5 min in the dark. The kinase reaction was initiated by addition of ATP (1-100 µM) in 10 µl and was stopped after 0-20 min by addition of 1 µl of 0.5 mM staurosporine (Calbiochem, San Diego, CA) in DMSO. A 50 µl aliquot of the reaction mixture was transferred to a well of a dark-sided 96-well plate and mixed with 50 µl of a solution containing 2X fluorescein-labeled phosphopeptide and 2X anti-phosphoserine antibody, according to the manufacturer's instructions. After incubation for 30 min at 22°C in the dark, plane-polarized fluorescence was measured using an Analyst HT (Molecular Devices, Sunnyvale, CA) with excitation at 485 nm and emission at 530 nm. Polarization (P) was calculated by the equation $P = (I_{para} - I_{perp}) / (I_{para} + I_{perp})$, where I_{para} is the parallel emission fluorescence intensity and I_{perp} is perpendicular emission fluorescence intensity. The Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) were calculated from the equation $1 / V_O = K_M/V_{max}[S] + 1 / V_{max}$, where V_O is the initial velocity of the reaction and [S] is the initial concentration of substrate. The catalytic constant (k_{cat}) was calculated from the equation $k_{cat} = V_{max} / [E]_O$, where $[E]_O$ is the concentration of enzyme added to the reaction.

Stable transfection of HEK293 cells with as-PKCε – HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). HEK293 cells that stably express rat α1β2γ2S $GABA_A$ receptors (21) were provided by G. H. Dillon, (Univ. of N. Texas Health Science Center, Fort Worth, Texas). Human PKCε M486A was amplified by PCR and then subcloned without the FLAG-epitope tag into pIRESpuro2 (Clonetech, Palo Alto, CA). Cells were plated on poly-L-ornithine-coated 6-well plates at a density of 10^5 cells per well in 2 ml of growth medium (minimal essential medium with 10% fetal bovine serum, 0.2 mM L-α-glutamine, 200 µg/ml G418, 100 µg/ml streptomycin and 100 U/ml penicillin). The cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2:95% air before transfection. Transfection complexes were prepared by mixing 2 µg of plasmid DNA

with 6 µl of TransIT-293 reagent (Mirus, Madison, WI) in 100 µl of serum-free medium. Complexes were added drop-wise to each well and the cells were incubated for 24 h at 37°C in 5% CO_2:95% air. Clones were selected 48 h later in 5 µg/ml puromycin. Medium was replaced daily, and the concentration of puromycin was reduced to 3 µg/ml after one week. Expression of PKCε or PKCε M486A in surviving cells was confirmed by western blot analysis.

Generation of HEK293 cells expressing mutant $GABA_A$ receptor subunits - Plasmids containing inserts encoding human α1 and γ2L in pCDM8 were provided by Dr. P. B. Wingrove (Merck Sharp & Dohme, UK). A pCIS2 plasmid encoding rat β2 was provided by Dr. N. Harrison (Cornell University, NY, NY). Phosphorylation site mutants of β2 (S410A) and γ2L (S327A and S343A) were generated by site directed mutagensis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: 5'-CGCCTGAGGAGACGTGCCGCCCAACTGAAAATCACC-3' as the forward primer and 5'-GGTGATTTTCAGTTGGGCGGCACGTCTCCTCAGGCG-3' as the reverse primer for $\beta2_{S410A}$; 5'-GTCAGCAACCGGAAACCAGCCAAGGACAAAGATAAAAAGAAGAAAAACCC-3' as the forward primer and 5'-GGGTTTTTCTTCTTTTTATCTTTGTCCTTGGCTGGTTTCCGGTTGCTGAC-3' as the reverse primer for $\gamma2L_{S327A}$; 5'-CCTCTTCTTCGGATGTTTGCCTTCAAGGCCCCTACC-3' as the forward primer and 5'-GGTAGGGGCCTTGAAGGCAAACATCCGAAGAAGAGG-3' as the reverse primer for $\gamma2L_{S343A}$. Plasmids were transiently transfected using calcium- phosphate into HEK293 cells that stably expressed PKCε M486A using the TransIT-293 reagent. Cells that expressed functional $GABA_A$ receptors were identified by measuring currents evoked by GABA and flunitrazepam using whole-cell patch clamp electrophysiology.

Electrophysiology of transfected HEK293 cells - $GABA_A$ receptor currents were measured in HEK293 cells stably transfected with as-PKCε and native or mutant $GABA_A$ receptors using a conventional whole-cell patch clamp technique. The recording electrodes were pulled from borosilicate glass capillary tubing (World Precision Instruments, Sarasota, FL) using a micropipette puller (P-97, Sutter Instruments, Novato, CA), to achieve a resistance of 5-8 MΩ when filled with pipette solution containing (in mM): 145 N-methyl-D-glucamine (NMDG)-Cl, 2 $MgCl_2$, 0.1 $CaCl_2$, 5 EGTA, 10 HEPES, and 2 Mg^{2+}-ATP (pH 7.3, adjusted with HCl). The external solution used to perfuse the cells continuously during the experiment contained (in mM): 145 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 D-glucose (pH 7.4, adjusted with NaOH).

To test the effect of inhibiting PKCε on $GABA_A$ receptor function, cells were incubated with external solution containing 1 µM 1Na or vehicle (0.1% DMSO) for 30 min prior to stimulation with GABA. Cells were then voltage-clamped at -75 mV and the GABA dose-response relationship was determined in the presence or absence of 1 µM 1Na. The response to allosteric modulators was then assessed by co-application of the modulator with an EC_{20} concentration of GABA. Flunitrazepam was applied 5 sec prior to co-application with GABA and ethanol was co-applied with GABA by local perfusion using a Perfusion Fast-Step SF-77B system (Warner Instruments, Inc., Hamden, CT) driven by pClamp 9 software (Axon Instruments, Union City, CA). Unless otherwise stated, there was a 1-min washout period between each drug application. Whole-cell currents were recorded using an Axopatch 200B patch amplifier (Axon CNS-Molecular Devices,

Union City, CA), filtered at 2 kHz, and digitized at 5 kHz with a Digidata 1322A interface and pClamp 9 software (Axon CNS). The serial resistance was monitored continuously during each experiment, and data from cells that showed a >30% change in resistance were discarded. All recordings were obtained at 27°C.

Electrophysiology of primary hippocampal neurons – Cultures of hippocampal neurons were prepared as described above and used at 3-10 DIV. The pipette solution contained (in mM): 145 N-methyl-D-glucamine (NMDG)-Cl, 2 $MgCl_2$, 10 HEPES, 2 Mg^{2+}-ATP, and 2 Na^{+}-ATP (pH 7.3, adjusted with HCl; 280 mOsm). The external solution contained (in mM): 145 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 D-glucose (pH 7.4, adjusted with NaOH). 1 µM TTX and 10 µM DNQX were freshly added to the external solution each day. All recordings were made at 32°C.

Phosphorylation of GST-γ2 intracellular loop fusion proteins - A plasmid (provided by R. Olsen, UCLA) containing the cDNA sequence encoding the mouse γ2S large intracellular loop (amino acids 318-404) in pGEX-2T (GE Healthcare, Piscataway, NJ) was used to generate an alanine mutation at S327 with a QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) and the primers:

5'GTCAGCAACCGGAAGCCAGCCAAGG ATAAAGACAAAAAGAAGAAAAACCC3' as the forward primer and 5'GGGTTTTTCTTCTTTTTGTCTTTATCC TTGGCTGGCTTCCGGTTGCTGAC3' as the reverse primer. BamHI and EcoRI fragments encoding the native and S327A mutant loops were subcloned into pGEX-6P-2 (GE Healthcare).

Fusion proteins were produced in *E. coli* BL21(DE3)pLysS cells (Invitrogen, Calsbad, CA) and purified by affinity chromatography using Glutathione Sepharose 4B (GE Healthcare). Phosphorylation was performed using 0.1µM human recombinant PKCε (Invitrogen) in 10 µl kinase buffer containing 20 mM HEPES, pH 7.4, 0.1 mM EGTA, 0.03% TritonX-100, 10 mM $MgCl_2$, 0.48 µg/µl L-α-phosphatidylserine (Avanti Polar Lipids, Alabaster, AL), 1 µM PMA and 0.5 mM ATP. PKCε was preincubated with kinase buffer for 3h at 27°C to stimulate autophosphorylation and thereby maximize the activity of PKCε. Then 1.25 pmole purified GST-γ2S loop or GST-$\gamma2S_{S327A}$ loop and 10 µCi [^{32}γ-P]ATP were added to the reaction mixture, which was incubated at 37°C for the indicated times. At each time point, 10 µl of reaction mixture were removed and mixed with 2.5 µl 5X SDS sample buffer to stop the reaction. Proteins were separated by SDS-PAGE on 4-12% gradient gels (Invitrogen) and the amount of radioactivity incorporated was quantified by phosphorimaging (Typhoon 9410; GE Healthcare). Specific radioactivity per mole of [γ^{32}P]ATP was determined by counting diluted aliquots of the stock [γ^{32}P]ATP solution to obtain a conversion value for calculating the molar ratio of ^{32}P incorporated into each fusion protein substrate.

Detection of $\gamma2_{S327}$ phosphorylation by western analysis - GST-γ2S loop proteins were phosphorylated *in vitro* in the presence of non-radioactive ATP. Samples of frontal cortex and hippocampus were homogenized in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, and Complete ™ protease inhibitor cocktail (Roche Diagnostics). Samples were adjusted to a concentration of 2 mg/ml and some were incubated with 8,000 U of lambda protein phosphatase (Upstate Biotechnology, Lake Placid, NY) at 30°C for 30 min. Control samples not treated with phosphatase were incubated with Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich).

Brain lysates, cell lysates, or GST-γ2S loop proteins were subjected to SDS-PAGE

using 4-12% gradient gels (Invitrogen). The proteins were detected by western blot analysis using affinity-purified, polyclonal rabbit anti-γ2-S(P)327 antibody (1:1000 dilution) generated against the phosphopeptide C^{317}LHYFVSNRKPS(P)KDKDK^{332} (PhosphoSolutions, Aurora, CO), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution; Chemicon, Temecula, CA) for 1 h at 27°C. Immunoreactivity was detected by enhanced chemiluminescence and quantified by scanning densitometry.

Statistical Analysis - Data shown are mean ± SEM values. Data were first tested for normality using the Kolmogorov-Smirnov test. Since all data were normally distributed, mean values were compared by either two-tailed, unpaired t-tests or ANOVA with indicated post-hoc tests. Differences between pairs of means were considered significant where $P < 0.05$. Except where indicated, dose-response relationships were analyzed by non-linear regression analysis using Prism 4 (GraphPad Software, Inc., San Diego, CA). Potentiation of the GABA response by allosteric modulators was calculated as the ratio of the current elicited by modulator plus GABA divided by the current elicited by GABA alone.

RESULTS

Increased response to zolpidem in PKCε$^{-/-}$ mice - To determine whether PKCε regulates $GABA_A$ receptors that contain α1 and γ2 subunits, we first examined behavioral responses in mice that lack PKCε. Since behavioral responses to benzodiazepines are increased in these mice (13), we tested their response to zolpidem, an imidazopyridine that selectively acts at the benzodiazepine site on $GABA_A$ receptors that contain α1 and γ2 subunits (22). We found that PKCε$^{-/-}$ mice showed greater rotarod ataxia in response to 1 mg/kg zolpidem than wild type littermates (Fig. 1*A*). Two-way repeated measures ANOVA showed main effects of genotype ($F_{1,48} = 5.66$, $P = 0.044$) and time ($F_{6,48} = 11.19$, $P < 0.001$) with an interaction between these factors ($F_{6,48} = 2,87$, $P = 0.018$). The threshold dose of zolpidem for producing ataxia was also lower in PKCε$^{-/-}$ mice compared with wild type controls (Fig. 1*B*). These differences in behavior were not associated with altered distribution of α1 subunits in the brain (Fig. S1*A*), abundance of α1 or γ2 subunits in frontal cortex, cerebellum, striatum, or amygdala (Fig. S1*B*), or altered cell surface abundance of γ2 subunits (Fig. S1*C*) in PKCε$^{-/-}$ mice when compared with wild type littermates. These findings suggest that the function, rather than the abundance, of zolpidem-sensitive $GABA_A$ receptors is increased in PKCε$^{-/-}$ mice. Consistent with this conclusion, PKCε and α1 subunits could be co-immunoprecipitated from frontal cortex of wild type mice (Fig. 1*C* and *D*) and were colocalized at the cell membrane in cultured hippocampal neurons from wild type animals (Fig. 1*E*), indicating that PKCε and α1-containing $GABA_A$ receptors physically interact in a complex.

Generation of an ATP analog-sensitive mutant of PKCε - PKCε can transduce signals by phosphorylation of substrates or by protein-protein interaction. The latter has been demonstrated for PKCε-stimulated neurite outgrowth in human neuroblastoma cells (23). The εV1-2 peptide (EAVSLKPT), which was designed as an inhibitor of PKCε translocation (24), can be used to selectively inhibit PKCε. However, since εV1-2 does not bind the catalytic domain or inhibit the catalytic activity of PKCε, this peptide cannot be used to distinguish between PKCε-mediated effects due to protein-protein interactions or phosphorylation. Furthermore, no small molecules are known that specifically inhibit the catalytic activity of PKCε over other PKC isozymes, due to the

highly conserved ATP binding sites of protein kinases. Therefore, to examine whether PKCε-mediated phosphorylation is required for modulation of $GABA_A$ receptors, we used a chemical-genetics approach to develop a selective small molecule inhibitor. We generated a single amino acid substitution at methionine 486 in the ATP binding pocket of PKCε, which we predicted would permit binding of ATP analogs with bulky side groups attached to the purine base. Such a strategy has been used to successfully generate ATP analog-sensitive kinase mutants, which can also be selectively inhibited by analogs of the kinase inhibitor 4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) at concentrations that do not affect native kinases (25).

We generated human PKCε M486A (as-PKCε) containing a FLAG-epitope tag at the amino terminus using site-directed mutagenesis. Kinetic analysis using different concentrations of ATP revealed the K_M for as-PKCε (241.3 ± 39.1 μM) to be 12-fold greater than the K_M for native PKCε (20.4 ± 4.0 μM). However, both enzymes had similar turnover (k_{cat}) values (170,600 ± 16,500 min^{-1} for as-PKCε and 156,700 ± 3,700 min^{-1} for PKCε). The similarity in k_{cat} values indicates that as-PKCε can utilize ATP as efficiently as native PKCε at the high ATP concentrations (2-10 mM) found in cells.

We next examined whether commercially available kinase inhibitors inhibit as-PKCε, and whether analogs of PP1 selectively inhibit as-PKCε. The IC_{50} for the non-specific kinase inhibitor staurosporine was 14-fold higher and for the general PKC inhibitor bisindolylmaleimide-I (Bis) was 2-fold higher for as-PKCε compared with native PKCε (Table 1). In contrast, 1-naphthyl-PP1 (1Na) and 1-naphthylmethyl-PP1 (1NM) inhibited as-PKCε but not native PKCε, with 1Na being 65-fold more potent than 1NM against as-PKCε. 1Na did not inhibit the activity of a commercially available mixture of native PKC isozymes. In addition, 1Na did not inhibit representative members of the conventional (PKCγ), novel (PKCδ), or atypical (PKCι) PKC subfamilies (Table 1).

$\gamma2_{S327}$ is required for PKCε regulation of $GABA_A$ receptors - Next, we examined the role of subunit phosphorylation in the regulation of receptor function by PKCε. Prior *in vitro* studies have suggested that there is one phosphorylation site for serine-threonine kinases on β2 (serine 410) and two on the long splice variant of γ2, γ2L (serine 327 and 343), with only one of these (serine 327) present in the short splice variant γ2S (26). To investigate whether any of these sites are required for $GABA_A$ receptor modulation by PKCε, we transiently expressed α1, β2 and γ2L receptor subunits carrying alanine substitutions at these residues into HEK293 cells that stably express as-PKCε at a level that is 5-fold higher than endogenous PKCε (Fig. 2*A*). In non-transfected HEK293 cells, levels of PKCε are low (Fig. 2*A*), allowing as-PKCε to dominate over endogenous PKCε in these stably transfected cells.

As expected, 1Na did not alter the response to GABA alone in cells expressing native receptors or any of the mutant receptor combinations (Fig. 2*B* and *C*, Table 2). This result is consistent with our prior chloride flux experiments, which showed that PKCε does not affect the response to the direct $GABA_A$ receptor agonist muscimol (13). We next examined the response to a benzodiazepine since $PKC\varepsilon^{-/-}$ mice showed an increased behavioral response to zolpidem (Fig. 1*A* and *B*). For receptors containing native α1, β2 and γ2L subunits, flunitrazepam increased the GABA-stimulated current, which was completely blocked by 30 μM bicuculline (Fig. 2*D*). Treatment with 1 μM 1Na increased the potency of flunitrazepam 4.9-fold, decreasing its EC_{50} from 98 nM to 17 nM, without affecting efficacy (Fig. 2*E* and *F*,

Table 2). Interestingly, all subunit combinations that included the $\gamma2L_{S327A}$ mutation showed no response to 1Na, and this mutation alone was sufficient to completely block the effect of 1Na (Fig. 2*G*, Table 2). In addition, without 1Na treatment, flunitrazepam potency was greater in cells expressing $\gamma2L_{S327A}$ when compared with cells expressing native γ2L (Fig. 2*H*, Table 2). By contrast, receptors containing the $\beta2_{S410A}$ mutant responded to flunitrazepam and 1Na in a manner indistinguishable from native receptors (Table 2). Like currents in cells expressing native receptors, currents elicited with mutant receptors or in the presence of 1Na were completely blocked by 30 μM bicucculine (Fig. 2*D*). These results indicate that the $\gamma2L_{S327A}$ mutation mimics the effect of PKCε inhibition, suggesting that PKCε decreases benzodiazepine potency through phosphorylation of $\gamma2_{S327}$.

We next examined whether $\gamma2L_{S327}$ phosphorylation regulates ethanol sensitivity of $GABA_A$ receptors. In cells containing native γ2L or mutant $\gamma2L_{S327A}$ subunits, ethanol enhanced the GABA-stimulated current, which was completely inhibited by 30 μM bicuculline (Fig. 3*A*). Treatment with 1Na increased the effect of ethanol (Fig. 3*A*-C) in cells expressing native receptors. Two-way, repeated measures ANOVA showed a main effect of ethanol concentration ($F_{4,48} = 38.87$, $P < 0.001$) and 1Na treatment ($F_{1,48} = 20.26$, $P < 0.001$) with a significant interaction between these factors ($F_{4,48} = 4.59$, $P < 0.003$). By contrast, 1Na did not increase the response to ethanol in cells containing $\gamma2L_{S327A}$ (Fig. 3*D*). Moreover, without 1Na treatment, the response to ethanol was greater in cells expressing $\gamma2L_{S327A}$ when compared with cells expressing native γ2L (Fig. 3*E*). Two-way repeated measures ANOVA showed main effects of ethanol concentration ($F_{4,60} = 35.98$, $P < 0.001$) and subunit type ($F_{1,60} = 8.16$, $P < 0.012$) with a significant interaction between these factors ($F_{4,60} = 4.54$, $P < 0.003$). Ethanol-enhanced GABA-stimulated currents were completely blocked by 30 μM bicuculline when 1 Na was present in both native receptors and receptors with $\gamma2L_{S327A}$ (Fig 3*A*). These results demonstrate that the $\gamma2_{S327A}$ mutation increases the response of $GABA_A$ receptors to ethanol and occludes further enhancement by a PKCε inhibitor, strongly suggesting that PKCε modulates the response to ethanol through phosphorylation of $\gamma2_{S327}$.

The $\gamma2_{S327A}$ mutation reduces rates of inactivation and desensitization in cells treated with flunitrazepam - By fitting the current traces activated by 100 μM GABA to a one-component exponential function and comparing the time constants, we found that currents activated by GABA alone showed similar rates of activation (261 ± 32 vs. 252 ± 47 ms), inactivation (292 ± 50 vs. 338 ± 50 ms), and desensitization (7128 ± 927 vs. 7026 ± 1107 ms) in receptors comprised of native γ2L ($n = 5$) or $\gamma2L_{S327A}$ ($n = 6$). In contrast, we found differences in channel kinetics when currents were stimulated by GABA plus flunitrazepam. For currents activated by EC_{20} GABA and 300 nM flunitrazepam, receptors containing γ2L or $\gamma2L_{S327A}$ produced currents with similar activation rates (776 ± 26, $n = 8$, vs. 790 ± 38 ms, $n = 9$), but γ2L containing receptors had a significantly faster inactivation rate (117 ± 19, $n = 8$, vs. 211 ± 16 ms, $n = 9$; $P < 0.05$ by *t*-test). Moreover, whereas 6 of 8 γ2L-transfected cells showed desensitization, only 3 of 9 $\gamma2L_{S327A}$-transfected cells desensitized. For those cells that showed desensitization, the desensitization rate was faster ($P < 0.05$ by two-tailed, *t*-test) in γ2L-tranfected cells (1847 ± 122 ms) when compared with $\gamma2L_{S327A}$-transfected cells (2892 ± 589 ms). For currents activated by EC_{20} GABA and 100 mM ethanol, the activation rates (567 ± 164, n = 8 vs. 459 ± 134 ms, n = 13) and deactivation rates (215 ± 111 vs. 212 ± 97 ms) were similar for γ2L and

$\gamma2L_{S327A}$ containing receptors. The majority of the cells tested did not show desensitization (7 out of 8 γ2L cells, and 11 out of 13 $\gamma2L_{S327A}$ cells). These findings indicate that the $\gamma2_{S237A}$ mutation does not alter channel kinetics in response to GABA alone or with ethanol, but instead selectively reduces inactivation and desensitization rates when benzodiazepines are present.

PKCε phosphorylates $\gamma2_{S327}$ in vitro- To investigate whether PKCε phosphorylates $\gamma2_{S327}$, we generated two GST fusion proteins, one containing the large intracellular loop of γ2S, which includes S327, and another containing the same sequence but with the S327A mutation. PKCε phosphorylated the native sequence to a maximal stoichiometry of 1.00 ± 0.06, whereas the fusion protein containing the $\gamma2_{S327A}$ mutation was a comparatively poor substrate for PKCε (Fig. 4*A*). Two-factor ANOVA showed main effects of substrate ($F_{1,30}$ = 119.5, $P < 0.0001$) and time ($F_{4,30}$ = 60.55, $P < 0.0001$) with an interaction between these factors ($F_{4,30}$ = 13.03, $P < 0.0001$). These findings indicate that PKCε phosphorylates $\gamma2_{S327}$ *in vitro*.

To examine whether PKCε phosphorylates $\gamma2_{S327}$ in intact tissues, we used an affinity-purified antibody against a peptide derived from the large intracellular loop of γ2 that contains phospho-$\gamma2_{S327}$. This anti-γ2-S(P)327 antibody detected a GST-γ2 loop fusion protein phosphorylated by PKCε *in vitro*, but not the non-phosphorylated fusion protein (Fig. 4*B*).

PKCε regulates allosteric responses in HEK cells containing γ2S and regulates γ2S phosphorylation at S327 – To examine receptor phosphorylation in intact cells we transfected a HEK293 cell line (HEKGR) that stably expresses α1, β2, and γ2S subunits (21) with as-PKCε and selected a subclone (HEKGR-asPKCε) that expresses as-PKCε at a level 3.3-fold greater than endogenous PKCε. Since some previous studies had suggested that the long splice variant of γ2 (γ2L) is required to confer ethanol sensitivity to recombinant $GABA_A$ receptors (27-30), we first examined whether ethanol and PKCε could regulate responses in these cells. We then used our anti anti-γ2-S(P)327 antibody to detect $\gamma2_{S327}$ phosphorylation in these cells before and after treatment with 1Na.

As expected, 1Na did not alter the response to GABA (Fig. 5*A*) in HEKGR-asPKCε cells. However, 1Na increased flunitrazepam potency (Fig. 5*B*), decreasing the log EC_{50} value from 2.18 ± 0.09 nM in control cells (n = 10) to 1.4 ± 0.13 nM in 1Na-treated cells (n = 10). Ethanol modestly enhanced GABA-stimulated currents in these cells (Fig. 5*C*) ($F_{5,40}$ = 6.67, $P < 0.0001$ by ANOVA). The effect of ethanol was further increased by treatment with 1Na (Fig. 5*C*), similar to what we observed in HEK293 cells that express α1β2γ2L receptors (Fig. 3). Two-way repeated measures ANOVA showed main effects of 1Na treatment ($F_{1,64}$ = 26.08, P = 0.0001) and ethanol concentration ($F_{4,64}$ = 40.48, $P < 0.0001$), with an interaction between these factors ($F_{4,64}$ = 4.85, P = 0.0018). The effect of 1Na required the presence of as-PKCε, since in the parent HEKGR cell line, 1Na had no effect on potentiation of $GABA_A$ current by ethanol or by the benzodiazepine diazepam (Fig. 5*D*). These results indicate that receptors containing γ2L or γ2S subunits are both responsive to ethanol when expressed in HEK293 cells and that receptors containing either γ2L or γ2S show PKCε regulation of sensitivity to flunitrazepam and ethanol.

Using the anti-γ2-S(P)327 antibody to measure $\gamma2_{S327}$ phosphorylation, we found that anti-γ2-S(P)327 immunoreactivity was decreased ($F_{2,8}$ = 28.1; P = 0.0002) in HEKGR-asPKCε cells after treatment with 1Na or with the PKCε translocation inhibitor tat-εV1-2 (Fig. 5*E* and *F*). These results

demonstrate that in HEK293 cells, PKCε regulates the phosphorylation of $\gamma2_{S327}$.

PKCε phosphorylates $\gamma2_{S327}$ in vivo - We next used the anti-γ2-S(P)327 antibody to investigate whether $\gamma2_{S327}$ phosphorylation is decreased in PKCε$^{-/-}$ mice. Anti-γ2-S(P)327 immunoreactivity was decreased in the hippocampus and frontal cortex of PKCε$^{-/-}$ mice compared with wild type littermates (Fig. 6*A*). The presence of residual immunoreactivity in PKCε$^{-/-}$ brain tissue suggested that $\gamma2_{S327}$ might also be phosphorylated by another kinase besides PKCε. To investigate this possibility, we treated samples from frontal cortex with lambda protein phosphatase for 30 min before performing western analysis. Phosphatase treatment of wild type samples decreased anti-γ2-S(P)327 immunoreactivity to levels found in PKCε$^{-/-}$ samples, whereas treatment of samples from PKCε$^{-/-}$ mice did not decrease anti-γ2-S(P)327 immunoreactivity further (Fig. 6*B*). Analysis of these data by two-factor ANOVA showed main effects of genotype ($F_{1,18}$ = 13.20, P = 0.0019) and treatment ($F_{1,18}$ = 11.40, P = 0.0034) with an interaction between genotype and treatment ($F_{1,18}$ = 5.51, P = 0.030). These findings suggest that the residual immunoreactivity present in samples from PKCε$^{-/-}$ mice is not due to γ2 phosphorylated at Ser327, but rather is due to cross-reactivity of the antibody with non-phosphorylated γ2 subunits. Consistent with this conclusion, we found that overnight incubation of the anti-γ2-S(P)327 antibody with a dephosphorylated version of the immunizing peptide prevented its ability to detect a protein band at 48 kDa (data not shown). Taken together, these results indicate that PKCε is the major kinase that phosphorylates $\gamma2_{S327}$ *in vivo*.

Ethanol potentiation of $GABA_A$ currents in hippocampal neurons – Using cultured hippocampal neurons from wild type and PKCε$^{-/-}$ mice we examined whether absence of PKCε would increase sensitivity to ethanol. In initial experiments we were unable to demonstrate any response to ethanol (Fig. 7*A*). However a portion of whole cell current in cultured hippocampal neurons is carried by receptors comprised of αβ subunits, which can be inhibited by 5 μM Zn^{2+} (31). and are insensitive to ethanol (28). Therefore, to eliminate currents carried by receptors containing only α and β subunits, we measured ethanol responses in the presence of 5 μM $ZnCl_2$. In cells from wild type mice (n = 12), the residual GABA current was increased 75 ± 11 % by 30 nM diazepam indicating that a substantial portion of the current was carried by receptors that contain γ2 subunits. In neurons from PKCε $^{-/-}$ mice (n = 14) diazepam produced a greater effect (P = 0.0092) enhancing the GABA-stimulated current by 127 ± 14%. In wild type neurons, treatment with ethanol (Fig. 7*B* and *D*) produced a small but significant increase in the Zn^{2+}-resistant current ($F_{5,30}$ = 6.94, P < 0.0001 by repeated measures ANOVA). In contrast, neurons from PKCε$^{-/-}$ mice showed a much greater response to ethanol (Fig. 7*C* and *D*). Analysis of these data by two-way repeated measures ANOVA showed main effects of genotype ($F_{1,53}$ = 6.87, P = 0.02) and treatment ($F_{4,53}$) = 12.01, P < 0.001) with an interaction between these factors ($F_{4,53}$ = 2.72, P = 0.039). These findings indicate that Zn^{2+}-resistant GABA currents are enhanced to a greater extent by diazepam and ethanol in PKCε$^{-/-}$ hippocampal neurons when compared with wild type neurons.

Ethanol-induced phosphorylation of γ2S327 in vivo requires PKCε - In mice, exposure to 4.0 g/kg ethanol *in vivo* produces $GABA_A$ receptor resistance to ethanol when measured 1h later in cerebellar microsacs (3,17). This acute tolerance of $GABA_A$ receptors to ethanol occurs in wild type mice but not in PKCε$^{-/-}$ mice (17). Since increased

phosphorylation at $\gamma2_{S327}$ is associated with diminished receptor sensitivity to ethanol, we investigated whether *in vivo* exposure to ethanol increases phosphorylation of $\gamma2_{S327}$. Mice were injected with saline or with 4 g/kg ethanol *i.p.* and then the cerebellum was removed and analyzed by western blot analysis 1h later. After saline injection, γ2S(P)327 immunoreactivity was increased in the cerebellum of wild type (n = 6) relative to PKCε$^{-/-}$ mice (n = 6). In wild type mice immunoreactivity was increased after injection of 4g/kg ethanol (Fig. 8). In contrast, ethanol did not alter γ2S(P)327 immunoreactivity in cerebellar tissue from PKCε$^{-/-}$ mice. Analysis of these data by two factor ANOVA showed main effects of genotype ($F_{1,20}$ = 47.64, P < 0.0001) and treatment ($F_{1,20}$ = 6.46, P 0.0194) with a significant interaction between these factors ($F_{1,20}$ = 4.93, P = 0.0381).

DISCUSSION

It has been difficult to identify PKC isozyme-specific targets because the catalytic domains of PKC isozymes are very similar and there are no isozyme-selective inhibitors of catalytic activity available. Here we used a chemical-genetics approach to generate an ATP analog-sensitive mutant of PKCε and selectively inhibit the catalytic activity of an individual PKC. Using this mutant we identified a specific mechanism by which PKCε regulates the function of α1β2γ2 $GABA_A$ receptors. We demonstrated that PKCε regulates the allosteric effect of benzodiazepines and ethanol at these receptors through phosphorylation rather than by a protein-protein interaction. Importantly, we found that phosphorylation at S327 on γ2 subunits, but not phosphorylation at other predicted PKC phosphorylation sites on β2 or γ2 subunits, is required for PKCε modulation of $GABA_A$ receptor function. Finally, we demonstrated that PKCε directly phosphorylates $\gamma2_{S327}$ *in vitro*, $\gamma2_{S327}$ phosphorylation is reduced in mice lacking PKCε, and exposure to ethanol *in vivo* increases $\gamma2_{S327}$ phosphorylation in the cerebellum of wild type mice but not PKCε$^{-/-}$ mice. These findings establish that PKCε phosphorylation of S327 in γ2 subunits regulates the response of α1β2γ2 $GABA_A$ receptors to specific allosteric modulators. Moreover, our results show that concentrations of ethanol achieved during social drinking can increase the function of α1β2γ2 receptors by 40-60% when PKCε phosphorylation of $\gamma2_{S327}$ is prevented.

Our results clearly identify a role for $\gamma2_{S327}$ phosphorylation in the regulation of $GABA_A$ receptor function. Prior studies investigated whether $\gamma2_{S327}$ phosphorylation modulates direct responses to GABA and showed minimal or negative results. In receptors containing α1, β1, and γ2 subunits heterologously expressed in HEK293 cells or in *Xenopus* oocytes, treatment for 10-60 min with phorbol esters, which activate several PKC isozymes and other proteins (32), reduced receptor activation by GABA, and this effect was absent in receptors with alanine mutations at $\beta1_{S409}$, $\gamma2L_{S327}$, and $\gamma2_{S343}$ (33). Phosphorylation of the β1 subunit was responsible for this effect as a single $\beta1_{S409A}$ mutation was sufficient to eliminate phorbol ester-induced down-regulation. Another study using α1β2γ2S receptors expressed in *Xenopus* oocytes found a very short-lived and modest effect of $\gamma2S_{S327A}$ expression; after 1.5 min of phorbol ester treatment the current amplitude decreased by 29% in native receptors but only by 19% in receptors carrying $\gamma2S_{S327A}$, though this 10% difference disappeared after 10 min (34). Our studies with HEK293 cells expressing $\gamma2S_{S327A}$ indicate no effect of this mutation on current stimulated for up to 10 min by GABA alone (data not shown). Therefore, in contrast to its clear regulation of allosteric responses shown

in our present study, $\gamma2_{S327}$ phosphorylation does not appear to play a role in regulating the direct response to GABA.

Previous expression studies using *Xenopus* oocytes or mouse L(tk-) cells suggested a requirement for the long splice variant of γ2 subunits, γ2L, in conferring PKC-mediated sensitivity to low doses (< 50 mM) of ethanol (27-30). However, subsequent studies failed to observe a requirement for γ2L in receptor responses to ethanol (35-37) and mice expressing γ2S but not γ2L showed normal behavioral responses to ethanol (38). We found that receptors containing the long or short splice variant of γ2 respond similarly to ethanol and that inhibition of PKCε enhances the effect of ethanol in both receptor types. These findings add to the body of evidence indicating that splice variation in γ2 or phosphorylation at S343 in γ2L does not modulate $GABA_A$ receptor responses to ethanol.

In primary hippocampal neurons, we found that it was not possible to demonstrate ethanol enhancement of whole cell GABA currents. However, in cultured rat hippocampal neurons there is a benzodiazepine-insensitive tonic current that exhibits high sensitivity to Zn^{2+} (IC_{50} ~ 1.89 μM), contributes to the tonic current, and is likely comprised of αβ receptors (39). In stably transfected L(tk-) mouse fibroblasts, ethanol enhances GABA-stimulated chloride uptake through α1β1γ2 receptors but not α1β1 receptors, suggesting a requirement for γ2 subunits for response to ethanol (28). Therefore, we used 5 μM Zn^{2+} to inhibit such ethanol-insensitive currents, since in HEK293 cells expressing recombinant $GABA_A$ receptors, currents carried by α1β3 receptors are inhibited by more than 90% by 5 μM Zn^{2+}, whereas α1β3γ2 receptors are inhibited by less than 10% (31). Our finding that 5 μM Zn^{2+} unmasked a response to ethanol indicates the presence of an ethanol-insensitive current that contributes significantly to GABA responses in these cells and underscores the difficulty in using whole cell patch clamp recording to detect ethanol sensitivity in primary neurons that express both ethanol-sensitive and ethanol-insensitive receptors.

Our finding that PKCε inhibition alone was sufficient to increase allosteric sensitivity indicates that there is an active endogenous phosphatase associated with γ2 subunits. One candidate is calcineurin (protein phosphatase 2B) which can be co-immunoprecipitated with γ2 subunits and which dephosphorylates $\gamma2_{S327}$ in mouse hippocampus (40). Therefore, PKCε and calciuneurin may act in concert to reciprocally regulate $GABA_A$ receptor phosphorylation and function in the brain.

Genetic variation in the γ2 subunit plays an important role in behavioral responses to ethanol. Quantitative trait locus analysis has revealed two loci associated with severity of acute alcohol withdrawal in mice within a 3-cM interval on mouse chromosome 11 that includes the $GABA_A$ receptor gene cluster encoding α1, α6, β2 and γ2 subunits (41). DBA/2J mice show particularly severe alcohol withdrawal seizures and posses a unique γ2 allele resulting in an alanine to threonine substitution at residue 11 in the mature γ2 protein. Moreover, $\gamma2^{+/-}$ mutant mice show more severe alcohol withdrawal seizures than wild type littermates (41). In addition to alterations in alcohol withdrawal seizure severity, allelic variation in γ2 is associated with susceptibility to ethanol-induced ataxia (42). It is therefore interesting that compared with wild type mice, $PKC\varepsilon^{-/-}$ mice show less severe alcohol withdrawal seizures (43) and less acute tolerance to alcohol-induced ataxia (17). Together with our present results, these findings suggest that PKCε modulates the intensity and duration of alcohol intoxication and the severity of alcohol withdrawal through phosphorylation of $GABA_A$ receptors at $\gamma2_{S327}$

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FOOTNOTES

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[2]The abbreviations used are: GABA; gamma-aminobutyrate; PKC, protein kinase C; PP1, 4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; 1Na, 1-naphthyl-PP1; 1NM, 1-naphthylmethyl-PP1.

FIGURE LEGENDS

Fig. 1. PKCε$^{-/-}$ mice show increased sensitivity to zolpidem, and PKCε interacts with α1 subunits of $GABA_A$ receptors. (*A*) Compared with wild type mice (+/+, *n* = 5), PKCε$^{-/-}$ mice (-/-, *n* = 5) showed a shorter latency to fall from a constant velocity rotarod 15, 30 and 45 min after injection of 1 mg/kg zolpidem (*$P < 0.05$ compared with wild type mice at the same time by *post-hoc* Newman Keuls test). (*B*) The zolpidem ED_{50} for inducing rotarod ataxia was decreased in PKCε$^{-/-}$ mice (-/-) compared with wild type (+/+) littermates. Data are mean values ± 95% confidence intervals from 6 mice per genotype. (*C*) α1 subunit immunoreactivity was detected by western analysis of whole brain lysates from PKCε$^{+/+}$ mice immunoprecipitated with anti-PKCε antibody. No α1 subunit immunoreactivity was detected in immunopreciptates from PKCε$^{-/-}$ brain lysates or when rabbit IgG was used instead of anti-PKCε antibody. (*D*) PKCε immunoreactivity was detected by western analysis of whole brain lysates from PKCε$^{+/+}$ mice immunopreciptated with anti-α1 antibody. No PKCε immunoreactivity was seen when lysates were immunoprecipitated with rabbit IgG. (*E*) PKCε (left, green) and α1 subunits (middle, red) colocalize (right, merge) at the cell surface of cultured hippocampal neurons from wild type mice. Boxes refer to enlarged regions shown in the bottom panels. Scale bar = 20 μm.

Fig. 2. 1Na increases flunitrazepam potency in HEK293 cells expressing as-PKCε and native α1β2γ2L $GABA_A$ receptors, but not in cells expressing receptors containing $\gamma 2L_{S327A}$. (*A*) Expression of PKCε-like immunoreactivity in vector-transfected (V) and as-PKCε-transfected HEK293 cells. (*B*) Current tracings from a representative experiment showing similar responses to GABA in control and 1Na-treated cells transiently transfected with native α1β2γ2L subunits. (*C*) Combined results from 5 cells for each condition showing that the response to GABA was not altered by 1Na in these cells. (*D*) Current tracings showing the response to 3 μM GABA and increasing concentrations of flunitrazepam in cells expressing native or mutant receptors. 1Na (1 μM) increased the response to flunitrazepam and this effect was inhibited by bicuculline (BMI) and was completely reversed after a final 1-min washout period. (*E*) A representative experiment showing that 1 Na shifts the dose-response curve for flunitrazepam enhancement of GABA currents to the left in cells expressing native receptors. (*F*) Combined results showing that 1Na increases the potency of flunitrazepam (*n* = 9) when compared with cells treated with control vehicle (*n* = 8). (*G*) 1Na did not affect the flunitrazepam response in receptors that contained the

$\gamma 2L_{S327A}$ subunit (n = 8 for each condition). (*H*) Comparison of flunitrazepam dose-response curves in native γ2L- and $\gamma 2L_{S327A}$-containing receptors in the absence of 1Na revealed that flunitrazepam potency was greater in receptors containing $\gamma 2L_{S327A}$ subunits (n = 8 for each condition).

Fig. 3. 1Na increases the effect of ethanol in native receptors, but not in receptors containing $\gamma 2L_{S327A}$. (*A*) Representative current tracings in cells treated with 3 μM GABA and increasing concentrations of ethanol, showing that 1 Na increased current amplitude (middle tracing) when compared with control cells (top tracing) and this effect was blocked by bicuculline (BMI) and reversed by a final 2-min washout period. (*B*) Representative tracings showing that ethanol potentiation of GABA elicited current in α1β2γ2L receptors was increased by 1Na. (*C*) Combined results from 1Na treated (n = 6) and vehicle-treated cells (n = 8) showing that 1Na increases the ethanol response by shifting the ethanol dose-response curve upwards. (*D*) 1Na did not alter ethanol sensitivity in receptors containing $\gamma 2L_{S327A}$ (n = 9 for control and n = 8 for 1Na-treated cells). (*E*) In the absence of 1Na, potentiation by ethanol was greater in $\alpha 1\beta 2\gamma 2L_{S327A}$ receptors (n = 8) compared with native receptors (n = 9). * $P < 0.05$ compared with control cells (*C*) or native receptors (*E*) by *post-hoc* Newman Keuls tests.

Fig. 4. PKCε phosphorylates $\gamma 2_{S327}$ *in vitro* (*A*) GST fusion proteins, containing the large intracellular loop of γ2S with or without the $\gamma 2_{S327A}$ mutation, were subjected to phosphorylation for up to 4h using recombinant human PKCε. PKCε preferentially phosphorylated the non-mutant GST-γ2S loop. Shown are an autoradiogram (top panel) and silver-stained gel (bottom panel) from a representative experiment. GST alone was not a substrate for PKCε (not shown). The graph shows results from 4 experiments. $^{*}P < 0.05$ by Bonferroni posttests. (*B*) An affinity-purified rabbit antibody (anti-γ2-S(P)327) detected PKCε-phosphorylated (P), but not non-phosphorylated (N) GST-γ2S loop fusion protein. The antibody was not immunoreactive against PKCε-phosphorylated GST-$\gamma 2S_{S327A}$ loop fusion protein.

Fig. 5. PKCε regulates allosteric sensitivity to flunitrazepam and ethanol, and phosphorylates $\gamma 2_{S327}$ in HEK293 cells that stably express $\alpha 1\beta 2\gamma 2S$ $GABA_A$ receptors and as-PKCε. (*A*) GABA elicited similar currents in control (n = 10) and 1Na-treated cells (n = 8). (*B*) 1Na produced a leftward shift in the dose-response curve for flunitrazepam assayed in the presence of an EC_{20} concentration of GABA (P = 0.005 by non-linear regression analysis). (*C*) Ethanol increased GABA-elicited currents in cells incubated without 1Na ((n = 10; $^{*}P < 0.05$ compared with GABA alone by Dunnett's test). Treatment with 1 μM 1Na (n = 10) increased ethanol potentiation of GABA-stimulated current when compared with cells treated without 1Na (n = 10). $^{\dagger}P < 0.05$ compared with control by Newman Keuls test. (*D*) 1Na had no effect on potentiation of EC_{20} GABA responses by 30 nM diazepam or 30 mM ethanol in cells that had not been transfected with as-PKCε. (*E-F*) γ2-S(P)327 immunoreactivity was reduced after treatment for 1h with 1 μM 1Na or 500 nM tat-εV1-2 peptide. A representative experiment is shown in (*E*). Results from 4 experiments are shown in (*F*). *$P < 0.05$ compared with control by Tukey test.

Fig. 6. PKCε phosphorylates $\gamma 2_{S327}$. *in vivo* (*A*) Anti-γ2-S(P)327 antibody detected an immunoreactive band at 46 kDa in frontal cortex (Ctx; n=9 for both genotypes) and hippocampus (Hip; n=3 for both genotypes). Immunoreactivity was reduced in tissue from $PKC\varepsilon^{-/-}$ mice (-/-)

compared with wild type (+/+) littermates. *$P < 0.05$ by two-tailed *t*-tests. (*B*) Lambda protein phosphatase (λPtase) decreased anti-γ2-S(P)327 immunoreactivity in cortical samples from wild type but not from PKCε$^{-/-}$ mice. Shown is a representative western blot demonstrating a γ2-S(P)327 immunoreactive band at 46 kDa. Without phosphatase treatment, immunoreactivity was greater in samples from wild type mice (+/+; *n*=10) compared with samples from PKCε$^{-/-}$ mice (-/-; *n*=9). Following treatment with lambda phosphatase, immunoreractivity in wild type mice (*n*=4) was decreased and was similar to immunoreactivity in PKCε$^{-/-}$ ($n = 3$) samples. *$P < 0.05$ compared with phosphatase-treated wild type (+/+) samples; †$P < 0.05$ compared with wild type control samples but $P > 0.05$ compared with phosphatase-treated PKCε$^{-/-}$ (-/-) samples by Tukey test.

Fig. 7. PKCε regulates ethanol responses in cultured hippocampal neurons. (*A*) Current trace from wild type hippocampal neurons showing no response to ethanol at concentrations up to 100 mM. (*B*) Addition of 5 μM Zn^{2+} to block αβ receptors that are not ethanol sensitive allowed detection of a small ethanol response. (*C*) Greater enhancement of GABA currents by ethanol was seen in neurons cultured from PKCε$^{-/-}$ mice assayed in 5 μM Zn^{2+}. (*D*) Combined results from studies using 5 μM Zn^{2+} showing a greater effect of ethanol in neurons from wild type mice ($n = 8$) when compared with neurons from PKCε$^{-/-}$ mice ($n = 9$). *$P < 0.05$ compared with wild type cells treated with GABA alone by Dunnett's test. †$P < 0.05$ compared with wild type cells treated with the same concentration of ethanol by Tukey test.

Fig. 8. Intraperitoneal injection of ethanol *in vivo* increases γ2-S(P)327 immunoreactivity in wild type but not in PKCε$^{-/-}$ cerebellum. (*A*) Representative western blots and (*B*) combined results showing increased γ2-S(P)327 immunoreactivity in wild type compared with PKCε$^{-/-}$ cerebellum after saline injection. Immunoreactivity increased after ethanol injection in wild type but not in PKCε$^{-/-}$ cerebellum ($n = 8$ for each condition). *$P < 0.05$ when compared with saline-treated, PKCε$^{-/-}$ samples and †$P < 0.05$ compared with saline-treated, wild type samples by Bonferroni tests.

Table 1. Selectivity of kinase inhibitors

Enzyme	Bis	Staurosporine	1Na
PKCε	6±1	1±1	$>10^5$
as-PKCε	12±1	21±3	21±1
PKCδ	32±3	41±1	$>10^5$
PKCγ	30±2	18±1	$>10^5$
PKCι	3,463±317	364±29	$>10^5$

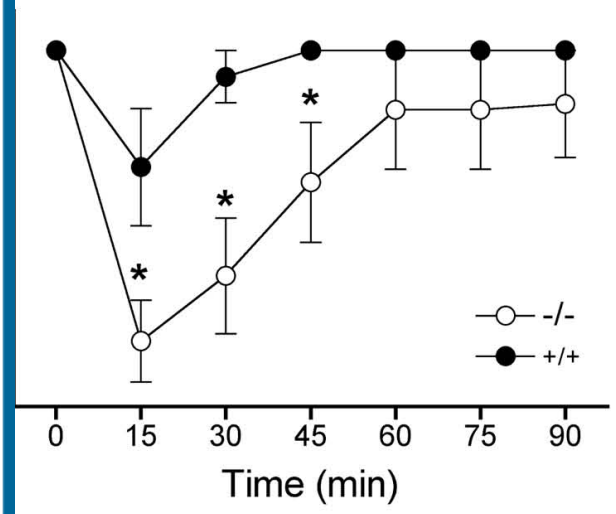
Kinase activity was measured by fluorescence polarization using 0.012-2x10^5 nM inhibitor, 2.5 μM ATP, and incubation for 90 min at 27°C. Data are from 3 triplicate experiments and IC_{50} values (nM) were calculated by nonlinear regression analysis.

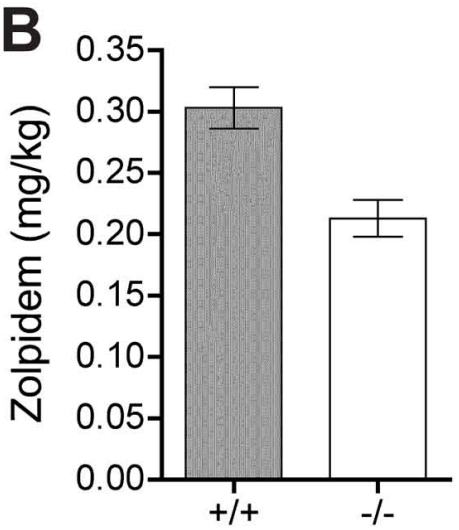
Table 2. Effect of as-PKCε inhibition on GABA and flunitrazepam potency at mutant $GABA_A$ receptors

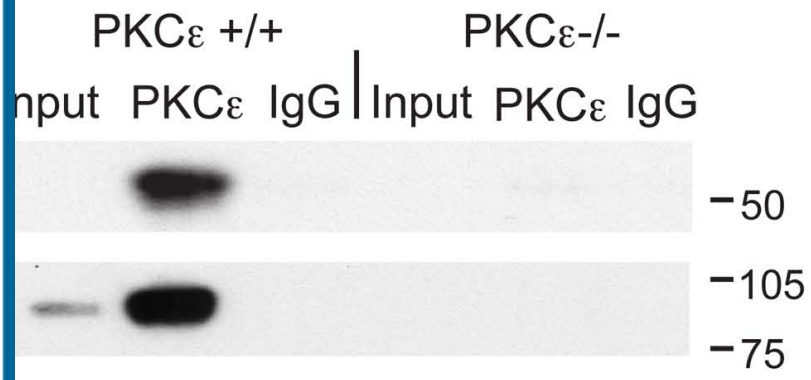
	GABA				Flunitrazepam			
	Control		1Na		Control		1Na	
Subunit Composition	log EC_{50}	n	log EC_{50}	n	log EC_{50}	n	log EC_{50}	n
α1β2γ2L	1.50±0.02	5	1.50±0.01	5	1.99±0.11	8	1.22±0.09*	9
$\alpha1\beta2_{S410A}\gamma2L_{S327A,S343A}$	1.43±0.04	5	1.36±0.07	4	1.59±0.06	11	1.51±0.07	12
$\alpha1\beta2\gamma2L_{S327A,S343A}$	1.56±0.02	9	1.58±0.03	7	1.46±0.08	7	1.57±0.10	5
$\alpha1\beta2\gamma2L_{S327A}$	1.52±0.06	6	1.59±0.02	4	1.46±0.09	8	1.50±0.11	8
$\alpha1\beta2_{S410A}\gamma2L$	1.58±0.09	3	1.63±0.11	4	1.80±0.08	8	1.05±0.11*	8

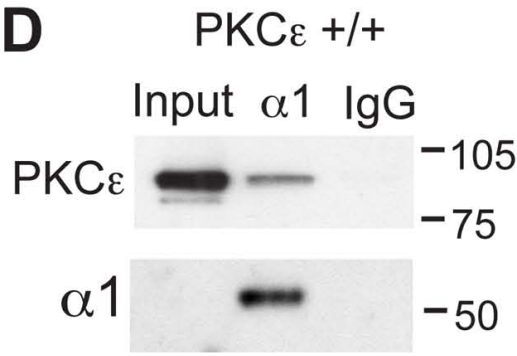
GABA currents were recorded from HEK293 cells stably transfected with as-PKCε and transiently transfected with indicated receptor subunits. The logEC_{50} values were calculated for GABA (μM) and flunitrazepam (nM) by non-linear regression analysis. *P<0.005 compared with vehicle-treated control cells.

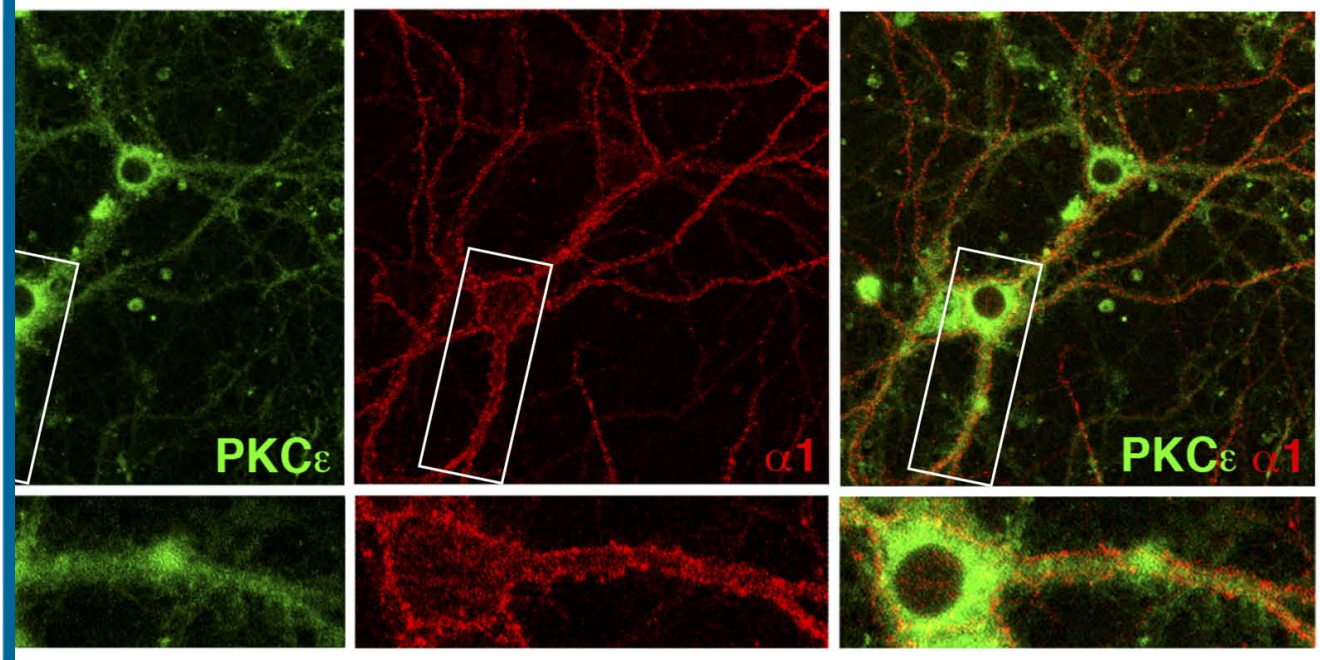
Figure 1, Qi et al.

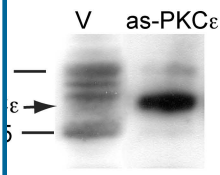


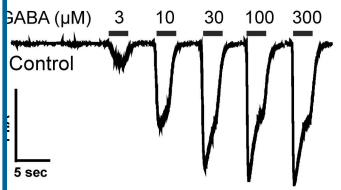


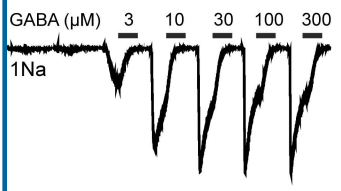







V
as-PKCε
ε


GABA (μM) 3 10 30 100 300
Control
5 sec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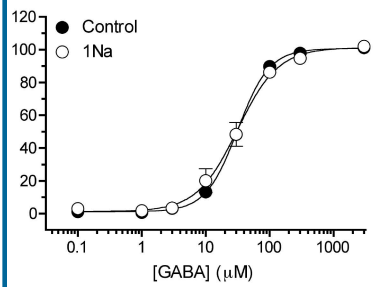
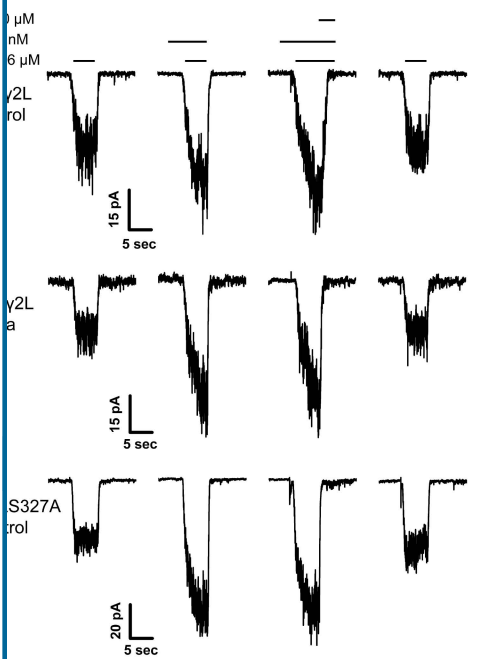
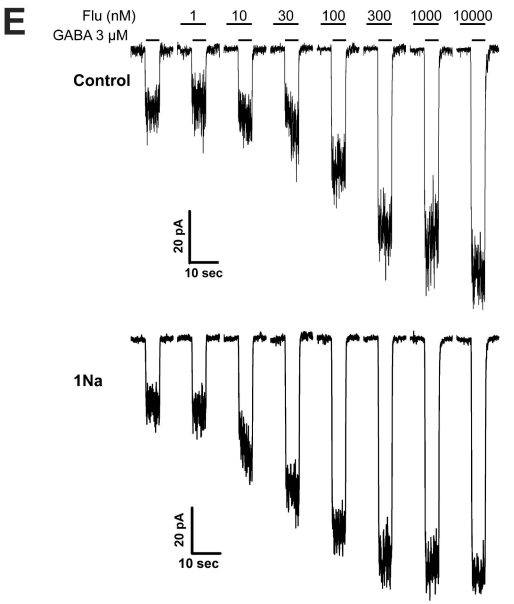
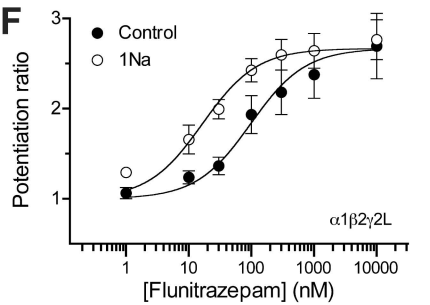
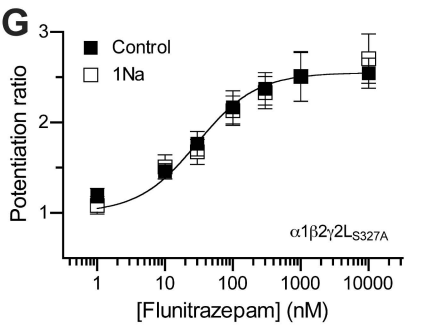
1Na


Control
1Na
120
100
80
60
40
20
0
0.1
1
10
100
1000
[GABA] (μM)


μM
nM
6 μM
γ2L
rol
15 pA
5 sec
γ2L
a
15 pA
5 sec
S327A
rol
20 pA
5 sec


E
Flu (nM) 1 10 30 100 300 1000 10000
GABA 3 μM
Control
20 pA
10 sec
1Na
20 pA
10 sec


F
Control
1Na
Potentiation ratio
α1β2γ2L
[Flunitrazepam] (nM)


G
Control
1Na
Potentiation ratio
α1β2γ2L$_{S327A}$
[Flunitrazepam] (nM)

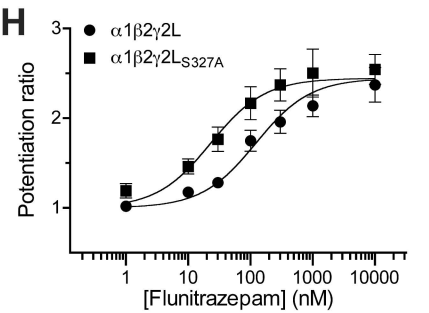

H
α1β2γ2L
α1β2γ2L$_{S327A}$
Potentiation ratio
[Flunitrazepam] (nM)

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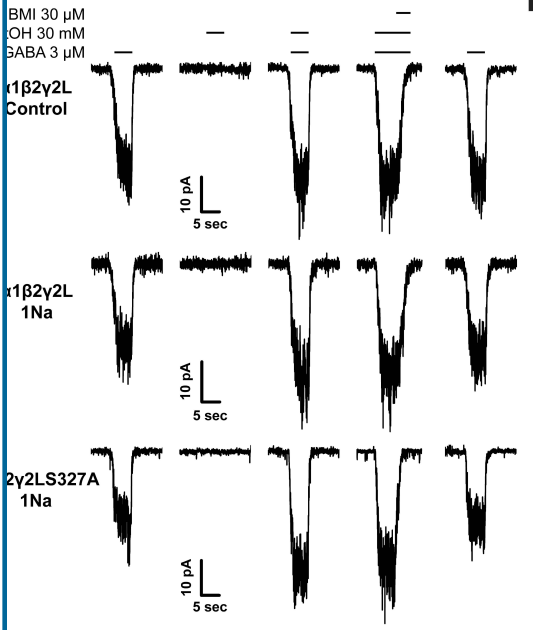
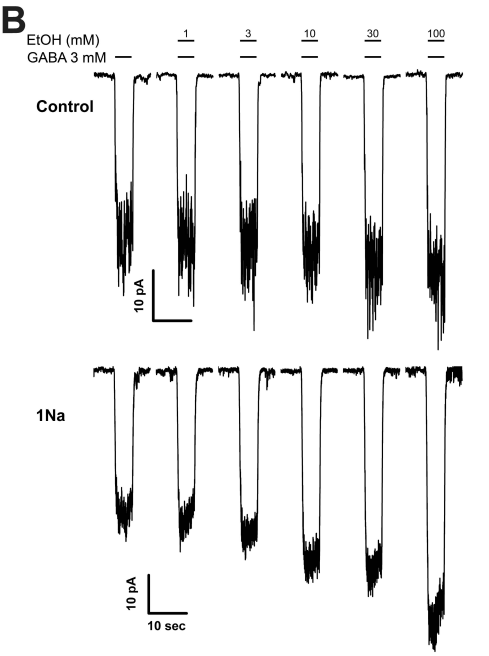
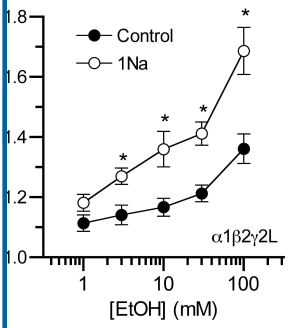
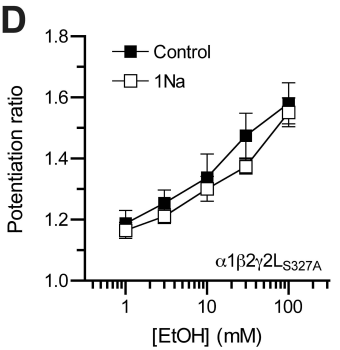
Figure 3, Qi et al.









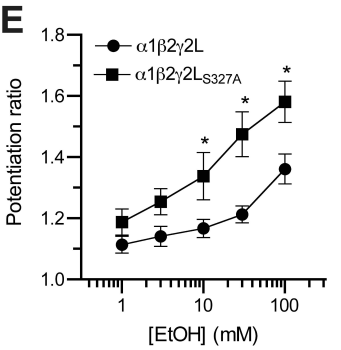
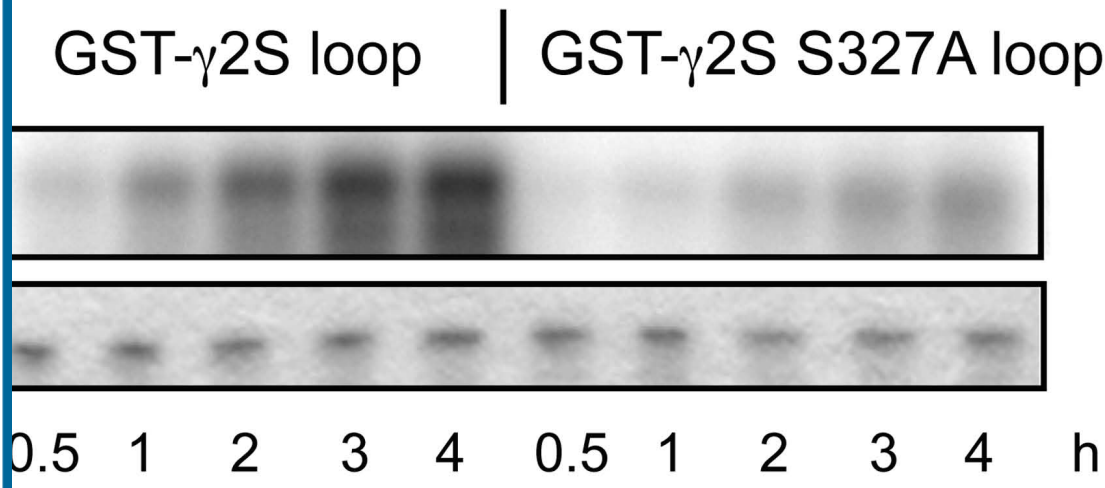
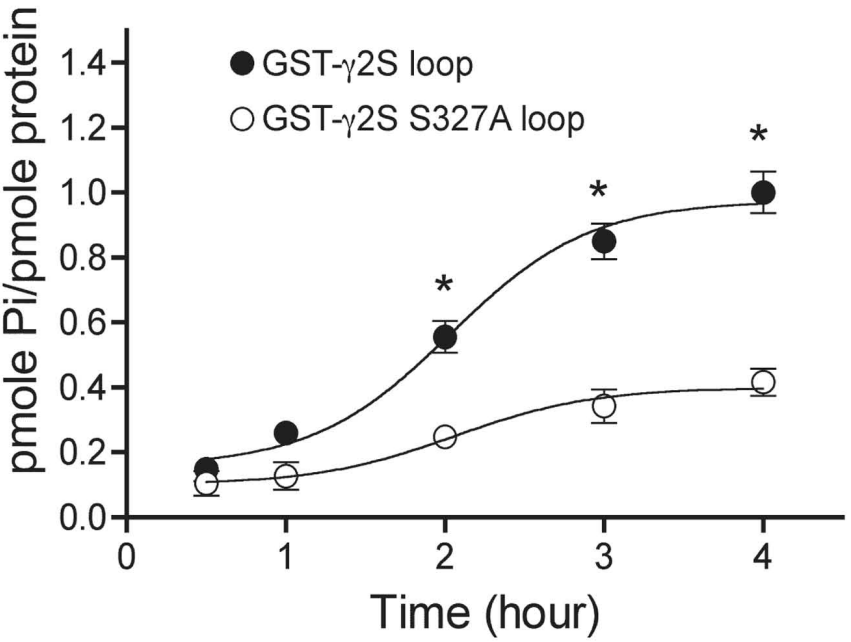


Figure 4, Qi et al.





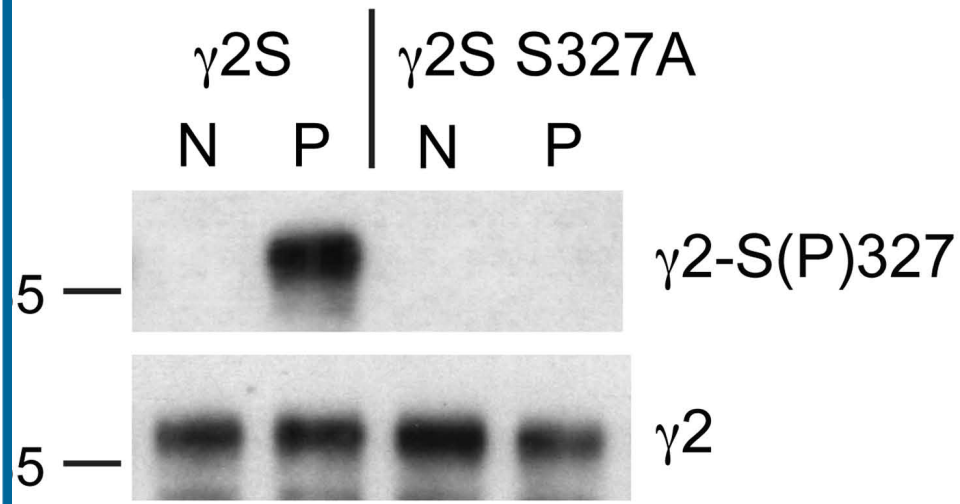


Figure 5, Qi et al.

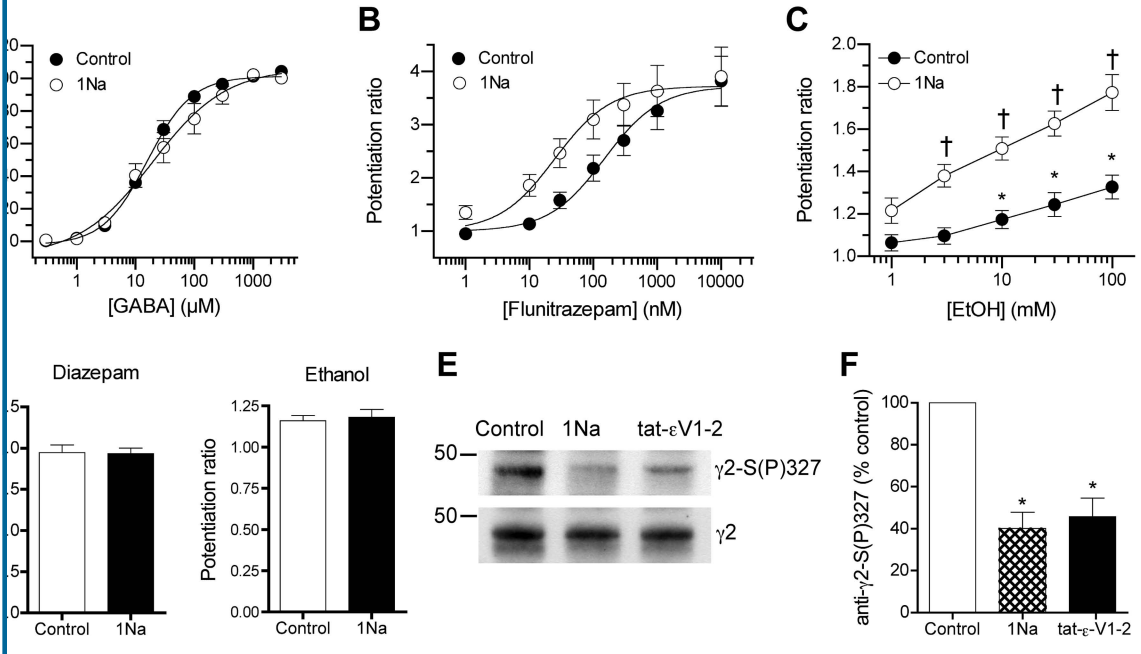
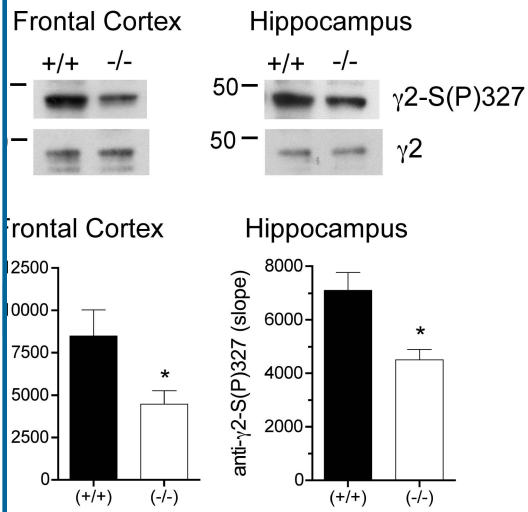


Figure 6, Qi et al.



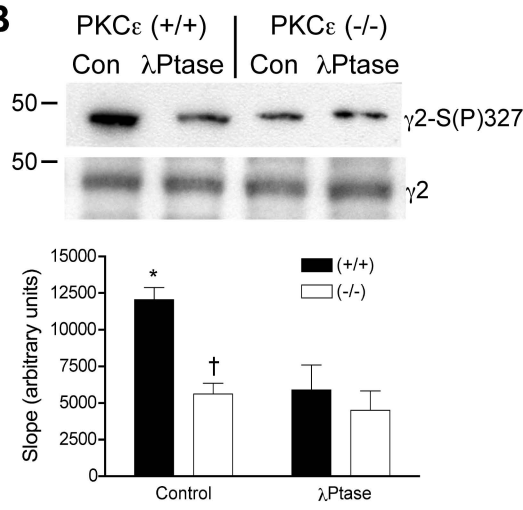
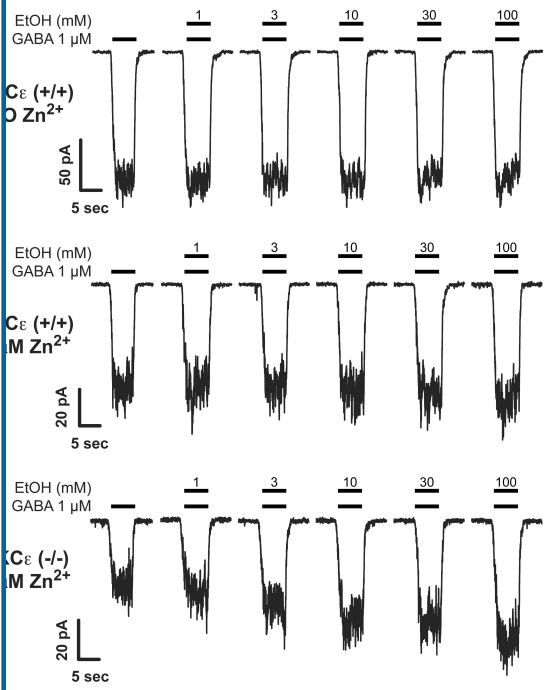


Figure 7, Qi et al.



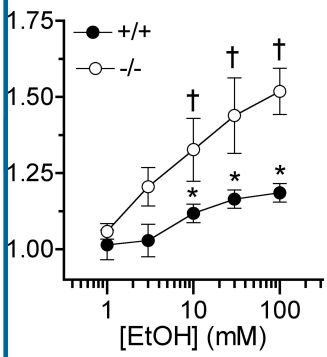


Figure 8, Qi et al.