

## PROTEIN KINASE C EPSILON REGULATES GABA<sub>A</sub> RECEPTOR SENSITIVITY TO ETHANOL AND BENZODIAZEPINES THROUGH PHOSPHORYLATION OF $\gamma$ 2 SUBUNITS\*

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Running Title: PKC epsilon phosphorylation of GABA<sub>A</sub> receptors

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Ethanol enhances GABA signaling in the brain, but its actions are inconsistent at GABA<sub>A</sub> receptors, especially at low concentrations achieved during social drinking. We postulated that the epsilon isoform of protein kinase C (PKC $\epsilon$ ) regulates the ethanol sensitivity of GABA<sub>A</sub> receptors, as mice lacking PKC $\epsilon$  show an increased behavioral response to ethanol. Here we developed an ATP analog-sensitive PKC $\epsilon$  mutant to selectively inhibit the catalytic activity of PKC $\epsilon$ . We used this mutant and PKC $\epsilon$ <sup>-/-</sup> mice to determine that PKC $\epsilon$  phosphorylates  $\gamma$ 2 subunits at serine 327 and that reduced phosphorylation of this site enhances the actions of ethanol and benzodiazepines at  $\alpha$ 1 $\beta$ 2 $\gamma$ 2 receptors, which are the most abundant GABA<sub>A</sub> receptor subtype in the brain. Our findings indicate that PKC $\epsilon$  phosphorylation of  $\gamma$ 2 regulates the response of GABA<sub>A</sub> receptors to specific allosteric modulators, and, in particular, PKC $\epsilon$  inhibition renders these receptors sensitive to low intoxicating concentrations of ethanol.

Gamma-aminobutyrate type A (GABA<sub>A</sub>)<sup>2</sup> 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<sub>A</sub> receptors was first identified in synaptosomal preparations where intoxicating concentrations (10-30 mM) enhanced receptor function as measured by <sup>36</sup>Cl uptake assays (2,3). However, after thirty years of investigation, it is apparent that ethanol enhancement of synaptic GABA<sub>A</sub> receptors is variable and in some preparations cannot be detected even at anesthetic concentrations (1,4).

GABA<sub>A</sub> receptors are pentameric protein complexes of subunits from eight classes ( $\alpha$ 1-6,  $\beta$ 1-3,  $\gamma$ 1-3,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho$ 1-3) (5). Most receptors are comprised of two  $\alpha$  subunits and two  $\beta$  subunits that co-assemble with one  $\gamma$ 2 subunit, which anchors these receptors at synapses where they mediate phasic inhibition (6). A minority contain a  $\delta$  subunit instead of  $\gamma$ 2; these receptors are extrasynaptic and mediate tonic inhibition in neurons (7). Since earlier ethanol studies that focused on GABA currents carried by  $\gamma$ 2-containing receptors produced variable results, recent attention has turned to ethanol effects at receptors containing  $\delta$  subunits. Reports from

three laboratories found these receptors enhanced by low ( $\leq 30$  mM) intoxicating concentrations of ethanol (8-10). However, two recent studies were unable to demonstrate low-dose ethanol sensitivity of  $\delta$ -containing GABA<sub>A</sub> receptors (11,12), indicating that, like synaptic GABA<sub>A</sub> 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<sub>A</sub> 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 (PKC $\epsilon$ ), which show an increased behavioral response to ethanol (13). Ethanol modulation of GABA<sub>A</sub> receptors is also increased in PKC $\epsilon$ <sup>-/-</sup> mice compared with wild type mice (13,14). These results suggest that activation of PKC $\epsilon$  suppresses potentiation of GABA<sub>A</sub> receptors by ethanol.

The mechanism by which PKC $\epsilon$  regulates the ethanol sensitivity of GABA<sub>A</sub> receptors is unknown. Here, we examined this mechanism using receptors composed of  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 2$  subunits, which is the most abundant receptor subtype in the brain (15). Since there are no selective inhibitors of PKC $\epsilon$  catalytic activity available, we used a chemical-genetics approach to generate an ATP analog-sensitive mutant, as-PKC $\epsilon$ , that could be potently and selectively inhibited by a cell-permeant, small molecule inhibitor. Our findings indicate that PKC $\epsilon$  regulates the sensitivity of  $\alpha 1\beta 2\gamma 2$  receptors to ethanol and benzodiazepines through phosphorylation of serine 327 in the large intracellular loop of  $\gamma 2$ .

## EXPERIMENTAL PROCEDURES

*Animal Care and Behavior* - PKC $\epsilon$ <sup>-/-</sup> mice were generated by homologous recombination in J1 embryonic stem cells as

described (16). Male and female PKC $\epsilon$ <sup>+/-</sup> mice were maintained on inbred 129S4/SvJae and C57BL6/J backgrounds and were crossed to produce PKC $\epsilon$ <sup>+/-</sup> C57BL6/J  $\times$  129S4/SvJae F1 hybrid mice. These mice were intercrossed to generate F2 hybrid PKC $\epsilon$ <sup>+/+</sup> and PKC $\epsilon$ <sup>-/-</sup> 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  $\mu$ g protein) was incubated for 2 h at 27°C with 1  $\mu$ g of rabbit anti-GABA<sub>A</sub>- $\alpha 1$  polyclonal antibody 993-2, generated against the C-terminal decapeptide sequence common to rat, mouse, and human GABA<sub>A</sub>- $\alpha 1$  [PQLKAPTPHQ], or with purified rabbit IgG (Sigma-Aldrich, St. Louis, MO). For the reverse immunoprecipitation, lysates were incubated with 1  $\mu$ g rabbit anti-PKC $\epsilon$  (18) or purified rabbit IgG. Protein A beads (100  $\mu$ l; 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<sup>5</sup> cells on 200 mm<sup>2</sup> 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<sub>2</sub> 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<sup>6</sup> 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<sub>A</sub> receptors on the cell surface, living cultures were incubated with rabbit anti-GABA<sub>A</sub> 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'-  
GGACCGCCTCTTTTTTCGTGCGCCGAATAT  
GTAAATGGTGGAGACC-3' as the forward  
primer and

5'-  
GGTCTCCACCATTTACATATTCGGCGA  
CGAAAAGAGGCGGTCC-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 PKC $\epsilon$  in the eluate was measured in triplicate by ELISA using recombinant PKC $\epsilon$  (Invitrogen, Carlsbad, CA) as a standard, rabbit anti- PKC $\epsilon$  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-PKC $\epsilon$*  - Enzyme activity was measured by fluorescence polarization using the Protein Kinase C Assay Kit from Invitrogen. PKC $\epsilon$  (0.5 ng) or PKC $\epsilon$ -M486A (3 ng) was added to kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 0.02% NP-40, 0.03% Triton X-100, 0.1  $\mu$ g phosphatidylserine and 0.05 mM sodium vanadate) with 250 nM substrate peptide (RFARKGSLRQKNV) and 12.5 nM PMA in a final volume of 40  $\mu$ l. The mixture was incubated at 22°C for 5 min in the dark. The kinase reaction was initiated by addition of ATP (1-100  $\mu$ M) in 10  $\mu$ l and was stopped after 0-20 min by addition of 1  $\mu$ l of 0.5 mM

staurosporine (Calbiochem, San Diego, CA) in DMSO. A 50  $\mu$ l aliquot of the reaction mixture was transferred to a well of a dark-sided 96-well plate and mixed with 50  $\mu$ l 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  $P = (I_{\text{para}} - I_{\text{perp}}) / (I_{\text{para}} + I_{\text{perp}})$ , where  $I_{\text{para}}$  is the parallel emission fluorescence intensity and  $I_{\text{perp}}$  is perpendicular emission fluorescence intensity. The Michaelis-Menten constant ( $K_M$ ) and maximum velocity ( $V_{\text{max}}$ ) were calculated from the equation  $1 / V_O = K_M / V_{\text{max}}[S] + 1 / V_{\text{max}}$ , where  $V_O$  is the initial velocity of the reaction and [S] is the initial concentration of substrate. The catalytic constant ( $k_{\text{cat}}$ ) was calculated from the equation  $k_{\text{cat}} = V_{\text{max}} / [E]_O$ , where  $[E]_O$  is the concentration of enzyme added to the reaction.

*Stable transfection of HEK293 cells with as-PKC $\epsilon$*  - HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). HEK293 cells that stably express rat  $\alpha 1\beta 2\gamma 2S$  GABA<sub>A</sub> receptors (21) were provided by G. H. Dillon, (Univ. of N. Texas Health Science Center, Fort Worth, Texas). Human PKC $\epsilon$  M486A was amplified by PCR and then subcloned without the FLAG-epitope tag into pIRESpuro2 (Clontech, Palo Alto, CA). Cells were plated on poly-L-ornithine-coated 6-well plates at a density of 10<sup>5</sup> cells per well in 2 ml of growth medium (minimal essential medium with 10% fetal bovine serum, 0.2 mM L- $\alpha$ -glutamine, 200  $\mu$ g/ml G418, 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin). The cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>:95% air before transfection. Transfection complexes were prepared by mixing 2  $\mu$ g of plasmid DNA

with 6  $\mu$ l of TransIT-293 reagent (Mirus, Madison, WI) in 100  $\mu$ l 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<sub>2</sub>:95% air. Clones were selected 48 h later in 5  $\mu$ g/ml puromycin. Medium was replaced daily, and the concentration of puromycin was reduced to 3  $\mu$ g/ml after one week. Expression of PKC $\epsilon$  or PKC $\epsilon$  M486A in surviving cells was confirmed by western blot analysis.

*Generation of HEK293 cells expressing mutant GABA<sub>A</sub> receptor subunits* - Plasmids containing inserts encoding human  $\alpha$ 1 and  $\gamma$ 2L in pCDM8 were provided by Dr. P. B. Wingrove (Merck Sharp & Dohme, UK). A pCIS2 plasmid encoding rat  $\beta$ 2 was provided by Dr. N. Harrison (Cornell University, NY, NY). Phosphorylation site mutants of  $\beta$ 2 (S410A) and  $\gamma$ 2L (S327A and S343A) were generated by site directed mutagenesis 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  $\beta$ 2<sub>S410A</sub>; 5'-GTCAGCAACCGGAAACCAGCCAAGGACAAAGATAAAAAGAAGAAAACCC-3' as the forward primer and 5'-GGGTTTTTCTTCTTTTATCTTTGTCCTTGGCTGGTTCCGGTTGCTGAC-3' as the reverse primer for  $\gamma$ 2L<sub>S327A</sub>; 5'-CCTCTTCTTCGGATGTTTGCCTTCAAGGCCCTACC-3' as the forward primer and 5'-GGTAGGGGCCTTGAAGGCAAACATCCGAAGAAGAGG-3' as the reverse primer for  $\gamma$ 2L<sub>S343A</sub>. Plasmids were transiently transfected using calcium-phosphate into HEK293 cells that stably expressed PKC $\epsilon$  M486A using the TransIT-293 reagent. Cells that expressed functional GABA<sub>A</sub> receptors

were identified by measuring currents evoked by GABA and flunitrazepam using whole-cell patch clamp electrophysiology.

*Electrophysiology of transfected HEK293 cells* - GABA<sub>A</sub> receptor currents were measured in HEK293 cells stably transfected with as-PKC $\epsilon$  and native or mutant GABA<sub>A</sub> 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 M $\Omega$  when filled with pipette solution containing (in mM): 145 N-methyl-D-glucamine (NMDG)-Cl, 2 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 5 EGTA, 10 HEPES, and 2 Mg<sup>2+</sup>-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<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 10 D-glucose (pH 7.4, adjusted with NaOH).

To test the effect of inhibiting PKC $\epsilon$  on GABA<sub>A</sub> receptor function, cells were incubated with external solution containing 1  $\mu$ M 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  $\mu$ M 1Na. The response to allosteric modulators was then assessed by co-application of the modulator with an EC<sub>20</sub> 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<sub>2</sub>, 10 HEPES, 2 Mg<sup>2+</sup>-ATP, and 2 Na<sup>+</sup>-ATP (pH 7.3, adjusted with HCl; 280 mOsm). The external solution contained (in mM): 145 NaCl, 3 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 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'GTCAGCAACCGGAAGCCAGCCAAGGATAAAGACAAAAGAAGAAAACCC3' as the forward primer and 5'GGGTTTTTCTTCTTTTGTCTTTATCCTTGGCTGGCTTCCGGTTGCTGAC3' 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 0.1 μM 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<sub>2</sub>, 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-γ2S<sub>S327A</sub> loop and 10 μCi [<sup>32</sup>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 [<sup>32</sup>γ-P]ATP was determined by counting diluted aliquots of the stock [<sup>32</sup>γ-P]ATP solution to obtain a conversion value for calculating the molar ratio of <sup>32</sup>P incorporated into each fusion protein substrate.

*Detection of γ2<sub>S327</sub> 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- $\gamma$ 2-S(P)327 antibody (1:1000 dilution) generated against the phosphopeptide C<sup>317</sup>LHYFVSNRKPS(P)KDKDK<sup>332</sup> (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  $\pm$  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 PKC $\epsilon$ <sup>-/-</sup> mice* - To determine whether PKC $\epsilon$  regulates GABA<sub>A</sub> receptors that contain  $\alpha$ 1 and  $\gamma$ 2 subunits, we first examined behavioral responses in mice that lack PKC $\epsilon$ . 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<sub>A</sub> receptors that contain  $\alpha$ 1 and  $\gamma$ 2 subunits (22). We found that PKC $\epsilon$ <sup>-/-</sup> mice showed greater rotarod ataxia in response to 1 mg/kg zolpidem than wild type littermates

(Fig. 1A). 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 PKC $\epsilon$ <sup>-/-</sup> mice compared with wild type controls (Fig. 1B). These differences in behavior were not associated with altered distribution of  $\alpha$ 1 subunits in the brain (Fig. S1A), abundance of  $\alpha$ 1 or  $\gamma$ 2 subunits in frontal cortex, cerebellum, striatum, or amygdala (Fig. S1B), or altered cell surface abundance of  $\gamma$ 2 subunits (Fig. S1C) in PKC $\epsilon$ <sup>-/-</sup> mice when compared with wild type littermates. These findings suggest that the function, rather than the abundance, of zolpidem-sensitive GABA<sub>A</sub> receptors is increased in PKC $\epsilon$ <sup>-/-</sup> mice. Consistent with this conclusion, PKC $\epsilon$  and  $\alpha$ 1 subunits could be co-immunoprecipitated from frontal cortex of wild type mice (Fig. 1C and D) and were colocalized at the cell membrane in cultured hippocampal neurons from wild type animals (Fig. 1E), indicating that PKC $\epsilon$  and  $\alpha$ 1-containing GABA<sub>A</sub> receptors physically interact in a complex.

*Generation of an ATP analog-sensitive mutant of PKC $\epsilon$*  - PKC $\epsilon$  can transduce signals by phosphorylation of substrates or by protein-protein interaction. The latter has been demonstrated for PKC $\epsilon$ -stimulated neurite outgrowth in human neuroblastoma cells (23). The  $\epsilon$ V1-2 peptide (EAVSLKPT), which was designed as an inhibitor of PKC $\epsilon$  translocation (24), can be used to selectively inhibit PKC $\epsilon$ . However, since  $\epsilon$ V1-2 does not bind the catalytic domain or inhibit the catalytic activity of PKC $\epsilon$ , this peptide cannot be used to distinguish between PKC $\epsilon$ -mediated effects due to protein-protein interactions or phosphorylation. Furthermore, no small molecules are known that specifically inhibit the catalytic activity of PKC $\epsilon$  over other PKC isozymes, due to the

highly conserved ATP binding sites of protein kinases. Therefore, to examine whether PKC $\epsilon$ -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 PKC $\epsilon$ , 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 PKC $\epsilon$  M486A (as-PKC $\epsilon$ ) 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-PKC $\epsilon$  ( $241.3 \pm 39.1 \mu\text{M}$ ) to be 12-fold greater than the  $K_M$  for native PKC $\epsilon$  ( $20.4 \pm 4.0 \mu\text{M}$ ). However, both enzymes had similar turnover ( $k_{cat}$ ) values ( $170,600 \pm 16,500 \text{ min}^{-1}$  for as-PKC $\epsilon$  and  $156,700 \pm 3,700 \text{ min}^{-1}$  for PKC $\epsilon$ ). The similarity in  $k_{cat}$  values indicates that as-PKC $\epsilon$  can utilize ATP as efficiently as native PKC $\epsilon$  at the high ATP concentrations (2-10 mM) found in cells.

We next examined whether commercially available kinase inhibitors inhibit as-PKC $\epsilon$ , and whether analogs of PP1 selectively inhibit as-PKC $\epsilon$ . 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-PKC $\epsilon$  compared with native PKC $\epsilon$  (Table 1). In contrast, 1-naphthyl-PP1 (1Na) and 1-naphthylmethyl-PP1 (1NM) inhibited as-PKC $\epsilon$  but not native PKC $\epsilon$ , with 1Na being 65-fold more potent than 1NM against as-PKC $\epsilon$ . 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 (PKC $\gamma$ ), novel (PKC $\delta$ ), or atypical (PKC $\iota$ ) PKC subfamilies (Table 1).

*$\gamma 2_{S327}$  is required for PKC $\epsilon$  regulation of GABA $_A$  receptors* - Next, we examined the role of subunit phosphorylation in the regulation of receptor function by PKC $\epsilon$ . Prior *in vitro* studies have suggested that there is one phosphorylation site for serine-threonine kinases on  $\beta 2$  (serine 410) and two on the long splice variant of  $\gamma 2$ ,  $\gamma 2L$  (serine 327 and 343), with only one of these (serine 327) present in the short splice variant  $\gamma 2S$  (26). To investigate whether any of these sites are required for GABA $_A$  receptor modulation by PKC $\epsilon$ , we transiently expressed  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2L$  receptor subunits carrying alanine substitutions at these residues into HEK293 cells that stably express as-PKC $\epsilon$  at a level that is 5-fold higher than endogenous PKC $\epsilon$  (Fig. 2A). In non-transfected HEK293 cells, levels of PKC $\epsilon$  are low (Fig. 2A), allowing as-PKC $\epsilon$  to dominate over endogenous PKC $\epsilon$  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. 2B and C, Table 2). This result is consistent with our prior chloride flux experiments, which showed that PKC $\epsilon$  does not affect the response to the direct GABA $_A$  receptor agonist muscimol (13). We next examined the response to a benzodiazepine since PKC $\epsilon^{-/-}$  mice showed an increased behavioral response to zolpidem (Fig. 1A and B). For receptors containing native  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2L$  subunits, flunitrazepam increased the GABA-stimulated current, which was completely blocked by 30  $\mu\text{M}$  bicuculline (Fig. 2D). Treatment with 1  $\mu\text{M}$  1Na increased the potency of flunitrazepam 4.9-fold, decreasing its  $EC_{50}$  from 98 nM to 17 nM, without affecting efficacy (Fig. 2E and F,

Table 2). Interestingly, all subunit combinations that included the  $\gamma 2L_{S327A}$  mutation showed no response to 1Na, and this mutation alone was sufficient to completely block the effect of 1Na (Fig. 2G, Table 2). In addition, without 1Na treatment, flunitrazepam potency was greater in cells expressing  $\gamma 2L_{S327A}$  when compared with cells expressing native  $\gamma 2L$  (Fig. 2H, Table 2). By contrast, receptors containing the  $\beta 2_{S410A}$  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  $\mu$ M bicuculline (Fig. 2D). These results indicate that the  $\gamma 2L_{S327A}$  mutation mimics the effect of PKC $\epsilon$  inhibition, suggesting that PKC $\epsilon$  decreases benzodiazepine potency through phosphorylation of  $\gamma 2_{S327}$ .

We next examined whether  $\gamma 2L_{S327}$  phosphorylation regulates ethanol sensitivity of GABA $_A$  receptors. In cells containing native  $\gamma 2L$  or mutant  $\gamma 2L_{S327A}$  subunits, ethanol enhanced the GABA-stimulated current, which was completely inhibited by 30  $\mu$ M bicuculline (Fig. 3A). Treatment with 1Na increased the effect of ethanol (Fig. 3A-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  $\gamma 2L_{S327A}$  (Fig. 3D). Moreover, without 1Na treatment, the response to ethanol was greater in cells expressing  $\gamma 2L_{S327A}$  when compared with cells expressing native  $\gamma 2L$  (Fig. 3E). 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  $\mu$ M bicuculline when 1 Na was present in both native receptors and receptors with  $\gamma 2L_{S327A}$  (Fig 3A). These results demonstrate that the  $\gamma 2_{S327A}$  mutation increases the response of GABA $_A$  receptors to ethanol and occludes further enhancement by a PKC $\epsilon$  inhibitor, strongly suggesting that PKC $\epsilon$  modulates the response to ethanol through phosphorylation of  $\gamma 2_{S327}$ .

*The  $\gamma 2_{S327A}$  mutation reduces rates of inactivation and desensitization in cells treated with flunitrazepam* - By fitting the current traces activated by 100  $\mu$ M 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 ( $261 \pm 32$  vs.  $252 \pm 47$  ms), inactivation ( $292 \pm 50$  vs.  $338 \pm 50$  ms), and desensitization ( $7128 \pm 927$  vs.  $7026 \pm 1107$  ms) in receptors comprised of native  $\gamma 2L$  ( $n = 5$ ) or  $\gamma 2L_{S327A}$  ( $n = 6$ ). 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  $\gamma 2L$  or  $\gamma 2L_{S327A}$  produced currents with similar activation rates ( $776 \pm 26$ ,  $n = 8$ , vs.  $790 \pm 38$  ms,  $n = 9$ ), but  $\gamma 2L$  containing receptors had a significantly faster inactivation rate ( $117 \pm 19$ ,  $n = 8$ , vs.  $211 \pm 16$  ms,  $n = 9$ ;  $P < 0.05$  by  $t$ -test). Moreover, whereas 6 of 8  $\gamma 2L$ -transfected cells showed desensitization, only 3 of 9  $\gamma 2L_{S327A}$ -transfected cells desensitized. For those cells that showed desensitization, the desensitization rate was faster ( $P < 0.05$  by two-tailed,  $t$ -test) in  $\gamma 2L$ -transfected cells ( $1847 \pm 122$  ms) when compared with  $\gamma 2L_{S327A}$ -transfected cells ( $2892 \pm 589$  ms). For currents activated by EC $_{20}$  GABA and 100 mM ethanol, the activation rates ( $567 \pm 164$ ,  $n = 8$  vs.  $459 \pm 134$  ms,  $n = 13$ ) and deactivation rates ( $215 \pm 111$  vs.  $212 \pm 97$  ms) were similar for  $\gamma 2L$  and

$\gamma 2L_{S327A}$  containing receptors. The majority of the cells tested did not show desensitization (7 out of 8  $\gamma 2L$  cells, and 11 out of 13  $\gamma 2L_{S327A}$  cells). These findings indicate that the  $\gamma 2_{S327A}$  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.

*PKC $\epsilon$  phosphorylates  $\gamma 2_{S327}$  in vitro*  
To investigate whether PKC $\epsilon$  phosphorylates  $\gamma 2_{S327}$ , we generated two GST fusion proteins, one containing the large intracellular loop of  $\gamma 2S$ , which includes S327, and another containing the same sequence but with the S327A mutation. PKC $\epsilon$  phosphorylated the native sequence to a maximal stoichiometry of  $1.00 \pm 0.06$ , whereas the fusion protein containing the  $\gamma 2_{S327A}$  mutation was a comparatively poor substrate for PKC $\epsilon$  (Fig. 4A). 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 PKC $\epsilon$  phosphorylates  $\gamma 2_{S327}$  *in vitro*.

To examine whether PKC $\epsilon$  phosphorylates  $\gamma 2_{S327}$  in intact tissues, we used an affinity-purified antibody against a peptide derived from the large intracellular loop of  $\gamma 2$  that contains phospho- $\gamma 2_{S327}$ . This anti- $\gamma 2$ -S(P)327 antibody detected a GST- $\gamma 2$  loop fusion protein phosphorylated by PKC $\epsilon$  *in vitro*, but not the non-phosphorylated fusion protein (Fig. 4B).

*PKC $\epsilon$  regulates allosteric responses in HEK cells containing  $\gamma 2S$  and regulates  $\gamma 2S$  phosphorylation at S327* – To examine receptor phosphorylation in intact cells we transfected a HEK293 cell line (HEKGR) that stably expresses  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 2S$  subunits (21) with as-PKC $\epsilon$  and selected a subclone (HEKGR-asPKC $\epsilon$ ) that expresses as-PKC $\epsilon$  at a level 3.3-fold greater than endogenous

PKC $\epsilon$ . Since some previous studies had suggested that the long splice variant of  $\gamma 2$  ( $\gamma 2L$ ) is required to confer ethanol sensitivity to recombinant GABA $_A$  receptors (27-30), we first examined whether ethanol and PKC $\epsilon$  could regulate responses in these cells. We then used our anti- $\gamma 2$ -S(P)327 antibody to detect  $\gamma 2_{S327}$  phosphorylation in these cells before and after treatment with 1Na.

As expected, 1Na did not alter the response to GABA (Fig. 5A) in HEKGR-asPKC $\epsilon$  cells. However, 1Na increased flunitrazepam potency (Fig. 5B), decreasing the log EC $_{50}$  value from  $2.18 \pm 0.09$  nM in control cells ( $n = 10$ ) to  $1.4 \pm 0.13$  nM in 1Na-treated cells ( $n = 10$ ). Ethanol modestly enhanced GABA-stimulated currents in these cells (Fig. 5C) ( $F_{5,40} = 6.67$ ,  $P < 0.0001$  by ANOVA). The effect of ethanol was further increased by treatment with 1Na (Fig. 5C), similar to what we observed in HEK293 cells that express  $\alpha 1\beta 2\gamma 2L$  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-PKC $\epsilon$ , 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. 5D). These results indicate that receptors containing  $\gamma 2L$  or  $\gamma 2S$  subunits are both responsive to ethanol when expressed in HEK293 cells and that receptors containing either  $\gamma 2L$  or  $\gamma 2S$  show PKC $\epsilon$  regulation of sensitivity to flunitrazepam and ethanol.

Using the anti- $\gamma 2$ -S(P)327 antibody to measure  $\gamma 2_{S327}$  phosphorylation, we found that anti- $\gamma 2$ -S(P)327 immunoreactivity was decreased ( $F_{2,8} = 28.1$ ;  $P = 0.0002$ ) in HEKGR-asPKC $\epsilon$  cells after treatment with 1Na or with the PKC $\epsilon$  translocation inhibitor tat- $\epsilon V1-2$  (Fig. 5E and F). These results

demonstrate that in HEK293 cells, PKC $\epsilon$  regulates the phosphorylation of  $\gamma 2_{S327}$ .

*PKC $\epsilon$  phosphorylates  $\gamma 2_{S327}$  in vivo* - We next used the anti- $\gamma 2$ -S(P)327 antibody to investigate whether  $\gamma 2_{S327}$  phosphorylation is decreased in PKC $\epsilon^{-/-}$  mice. Anti- $\gamma 2$ -S(P)327 immunoreactivity was decreased in the hippocampus and frontal cortex of PKC $\epsilon^{-/-}$  mice compared with wild type littermates (Fig. 6A). The presence of residual immunoreactivity in PKC $\epsilon^{-/-}$  brain tissue suggested that  $\gamma 2_{S327}$  might also be phosphorylated by another kinase besides PKC $\epsilon$ . 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- $\gamma 2$ -S(P)327 immunoreactivity to levels found in PKC $\epsilon^{-/-}$  samples, whereas treatment of samples from PKC $\epsilon^{-/-}$  mice did not decrease anti- $\gamma 2$ -S(P)327 immunoreactivity further (Fig. 6B). 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 PKC $\epsilon^{-/-}$  mice is not due to  $\gamma 2$  phosphorylated at Ser327, but rather is due to cross-reactivity of the antibody with non-phosphorylated  $\gamma 2$  subunits. Consistent with this conclusion, we found that overnight incubation of the anti- $\gamma 2$ -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 PKC $\epsilon$  is the major kinase that phosphorylates  $\gamma 2_{S327}$  in vivo.

*Ethanol potentiation of GABA $_A$  currents in hippocampal neurons* - Using cultured hippocampal neurons from wild type

and PKC $\epsilon^{-/-}$  mice we examined whether absence of PKC $\epsilon$  would increase sensitivity to ethanol. In initial experiments we were unable to demonstrate any response to ethanol (Fig. 7A). However a portion of whole cell current in cultured hippocampal neurons is carried by receptors comprised of  $\alpha\beta$  subunits, which can be inhibited by 5  $\mu\text{M}$  Zn $^{2+}$  (31), and are insensitive to ethanol (28). Therefore, to eliminate currents carried by receptors containing only  $\alpha$  and  $\beta$  subunits, we measured ethanol responses in the presence of 5  $\mu\text{M}$  ZnCl $_2$ . In cells from wild type mice ( $n = 12$ ), the residual GABA current was increased  $75 \pm 11\%$  by 30 nM diazepam indicating that a substantial portion of the current was carried by receptors that contain  $\gamma 2$  subunits. In neurons from PKC $\epsilon^{-/-}$  mice ( $n = 14$ ) diazepam produced a greater effect ( $P = 0.0092$ ) enhancing the GABA-stimulated current by  $127 \pm 14\%$ . In wild type neurons, treatment with ethanol (Fig. 7B 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 PKC $\epsilon^{-/-}$  mice showed a much greater response to ethanol (Fig. 7C 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 PKC $\epsilon^{-/-}$  hippocampal neurons when compared with wild type neurons.

*Ethanol-induced phosphorylation of  $\gamma 2_{S327}$  in vivo requires PKC $\epsilon$*  - 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 PKC $\epsilon^{-/-}$  mice (17). Since increased

phosphorylation at  $\gamma 2_{S327}$  is associated with diminished receptor sensitivity to ethanol, we investigated whether *in vivo* exposure to ethanol increases phosphorylation of  $\gamma 2_{S327}$ . 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,  $\gamma 2S(P)327$  immunoreactivity was increased in the cerebellum of wild type ( $n = 6$ ) relative to  $PKC\epsilon^{-/-}$  mice ( $n = 6$ ). In wild type mice immunoreactivity was increased after injection of 4g/kg ethanol (Fig. 8). In contrast, ethanol did not alter  $\gamma 2S(P)327$  immunoreactivity in cerebellar tissue from  $PKC\epsilon^{-/-}$  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  $PKC\epsilon$  and selectively inhibit the catalytic activity of an individual PKC. Using this mutant we identified a specific mechanism by which  $PKC\epsilon$  regulates the function of  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors. We demonstrated that  $PKC\epsilon$  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  $\gamma 2$  subunits, but not phosphorylation at other predicted PKC phosphorylation sites on  $\beta 2$  or  $\gamma 2$  subunits, is required for  $PKC\epsilon$  modulation of GABA<sub>A</sub> receptor function. Finally, we demonstrated that  $PKC\epsilon$  directly

phosphorylates  $\gamma 2_{S327}$  *in vitro*,  $\gamma 2_{S327}$  phosphorylation is reduced in mice lacking  $PKC\epsilon$ , and exposure to ethanol *in vivo* increases  $\gamma 2_{S327}$  phosphorylation in the cerebellum of wild type mice but not  $PKC\epsilon^{-/-}$  mice. These findings establish that  $PKC\epsilon$  phosphorylation of S327 in  $\gamma 2$  subunits regulates the response of  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors to specific allosteric modulators. Moreover, our results show that concentrations of ethanol achieved during social drinking can increase the function of  $\alpha 1\beta 2\gamma 2$  receptors by 40-60% when  $PKC\epsilon$  phosphorylation of  $\gamma 2_{S327}$  is prevented.

Our results clearly identify a role for  $\gamma 2_{S327}$  phosphorylation in the regulation of GABA<sub>A</sub> receptor function. Prior studies investigated whether  $\gamma 2_{S327}$  phosphorylation modulates direct responses to GABA and showed minimal or negative results. In receptors containing  $\alpha 1, \beta 1,$  and  $\gamma 2$  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  $\beta 1_{S409}, \gamma 2_{L_{S327}},$  and  $\gamma 2_{S_{343}}$  (33). Phosphorylation of the  $\beta 1$  subunit was responsible for this effect as a single  $\beta 1_{S409A}$  mutation was sufficient to eliminate phorbol ester-induced down-regulation. Another study using  $\alpha 1\beta 2\gamma 2S$  receptors expressed in *Xenopus* oocytes found a very short-lived and modest effect of  $\gamma 2_{S_{S327A}}$  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  $\gamma 2_{S_{S327A}}$ , though this 10% difference disappeared after 10 min (34). Our studies with HEK293 cells expressing  $\gamma 2_{S_{S327A}}$  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,  $\gamma 2_{S327}$  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  $\gamma 2$  subunits,  $\gamma 2L$ , in conferring PKC-mediated sensitivity to low doses (< 50 mM) of ethanol (27-30). However, subsequent studies failed to observe a requirement for  $\gamma 2L$  in receptor responses to ethanol (35-37) and mice expressing  $\gamma 2S$  but not  $\gamma 2L$  showed normal behavioral responses to ethanol (38). We found that receptors containing the long or short splice variant of  $\gamma 2$  respond similarly to ethanol and that inhibition of PKC $\epsilon$  enhances the effect of ethanol in both receptor types. These findings add to the body of evidence indicating that splice variation in  $\gamma 2$  or phosphorylation at S343 in  $\gamma 2L$  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  $\mu$ M), contributes to the tonic current, and is likely comprised of  $\alpha\beta$  receptors (39). In stably transfected L(tk-) mouse fibroblasts, ethanol enhances GABA-stimulated chloride uptake through  $\alpha 1\beta 1\gamma 2$  receptors but not  $\alpha 1\beta 1$  receptors, suggesting a requirement for  $\gamma 2$  subunits for response to ethanol (28). Therefore, we used 5  $\mu$ M Zn $^{2+}$  to inhibit such ethanol-insensitive currents, since in HEK293 cells expressing recombinant GABA $_A$  receptors, currents carried by  $\alpha 1\beta 3$  receptors are inhibited by more than 90% by 5  $\mu$ M Zn $^{2+}$ , whereas  $\alpha 1\beta 3\gamma 2$  receptors are inhibited by less than 10% (31). Our finding that 5  $\mu$ M 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 PKC $\epsilon$  inhibition alone was sufficient to increase allosteric sensitivity indicates that there is an active endogenous phosphatase associated with  $\gamma 2$  subunits. One candidate is calcineurin (protein phosphatase 2B) which can be co-immunoprecipitated with  $\gamma 2$  subunits and which dephosphorylates  $\gamma 2_{S327}$  in mouse hippocampus (40). Therefore, PKC $\epsilon$  and calcineurin may act in concert to reciprocally regulate GABA $_A$  receptor phosphorylation and function in the brain.

Genetic variation in the  $\gamma 2$  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  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 2$  and  $\gamma 2$  subunits (41). DBA/2J mice show particularly severe alcohol withdrawal seizures and possess a unique  $\gamma 2$  allele resulting in an alanine to threonine substitution at residue 11 in the mature  $\gamma 2$  protein. Moreover,  $\gamma 2^{+/-}$  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  $\gamma 2$  is associated with susceptibility to ethanol-induced ataxia (42). It is therefore interesting that compared with wild type mice, PKC $\epsilon^{-/-}$  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 PKC $\epsilon$  modulates the intensity and duration of alcohol intoxication and the severity of alcohol withdrawal through phosphorylation of GABA $_A$  receptors at  $\gamma 2_{S327}$

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## FOOTNOTES

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<sup>1</sup>These authors contributed equally to this work.

<sup>2</sup>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.** PKC $\epsilon^{-/-}$  mice show increased sensitivity to zolpidem, and PKC $\epsilon$  interacts with  $\alpha 1$  subunits of GABA<sub>A</sub> receptors. (A) Compared with wild type mice (+/+,  $n = 5$ ), PKC $\epsilon^{-/-}$  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<sub>50</sub> for inducing rotarod ataxia was decreased in PKC $\epsilon^{-/-}$  mice (-/-) compared with wild type (+/+) littermates. Data are mean values  $\pm$  95% confidence intervals from 6 mice per genotype. (C)  $\alpha 1$  subunit immunoreactivity was detected by western analysis of whole brain lysates from PKC $\epsilon^{+/+}$  mice immunoprecipitated with anti-PKC $\epsilon$  antibody. No  $\alpha 1$  subunit immunoreactivity was detected in immunoprecipitates from PKC $\epsilon^{-/-}$  brain lysates or when rabbit IgG was used instead of anti-PKC $\epsilon$  antibody. (D) PKC $\epsilon$  immunoreactivity was detected by western analysis of whole brain lysates from PKC $\epsilon^{+/+}$  mice immunoprecipitated with anti- $\alpha 1$  antibody. No PKC $\epsilon$  immunoreactivity was seen when lysates were immunoprecipitated with rabbit IgG. (E) PKC $\epsilon$  (left, green) and  $\alpha 1$  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  $\mu$ m.

**Fig. 2.** 1Na increases flunitrazepam potency in HEK293 cells expressing as-PKC $\epsilon$  and native  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptors, but not in cells expressing receptors containing  $\gamma 2L_{S327A}$ . (A) Expression of PKC $\epsilon$ -like immunoreactivity in vector-transfected (V) and as-PKC $\epsilon$ -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  $\alpha 1\beta 2\gamma 2L$  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  $\mu$ M GABA and increasing concentrations of flunitrazepam in cells expressing native or mutant receptors. 1Na (1  $\mu$ M) 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  $\gamma 2L$ - 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  $\mu$ M 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  $\alpha 1\beta 2\gamma 2L$  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.** PKC $\epsilon$  phosphorylates  $\gamma 2S_{327}$  *in vitro* (A) GST fusion proteins, containing the large intracellular loop of  $\gamma 2S$  with or without the  $\gamma 2S_{327A}$  mutation, were subjected to phosphorylation for up to 4h using recombinant human PKC $\epsilon$ . PKC $\epsilon$  preferentially phosphorylated the non-mutant GST- $\gamma 2S$  loop. Shown are an autoradiogram (top panel) and silver-stained gel (bottom panel) from a representative experiment. GST alone was not a substrate for PKC $\epsilon$  (not shown). The graph shows results from 4 experiments. \*  $P < 0.05$  by Bonferroni posttests. (B) An affinity-purified rabbit antibody (anti- $\gamma 2$ -S(P)327) detected PKC $\epsilon$ -phosphorylated (P), but not non-phosphorylated (N) GST- $\gamma 2S$  loop fusion protein. The antibody was not immunoreactive against PKC $\epsilon$ -phosphorylated GST- $\gamma 2S_{327A}$  loop fusion protein.

**Fig. 5.** PKC $\epsilon$  regulates allosteric sensitivity to flunitrazepam and ethanol, and phosphorylates  $\gamma 2S_{327}$  in HEK293 cells that stably express  $\alpha 1\beta 2\gamma 2S$  GABA $_A$  receptors and as-PKC $\epsilon$ . (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  $\mu$ M 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-PKC $\epsilon$ . (E-F)  $\gamma 2$ -S(P)327 immunoreactivity was reduced after treatment for 1h with 1  $\mu$ M 1Na or 500 nM tat- $\epsilon$ V1-2 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.** PKC $\epsilon$  phosphorylates  $\gamma 2S_{327}$  *in vivo* (A) Anti- $\gamma 2$ -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 $\epsilon^{-/-}$  mice (-/-)

compared with wild type (+/+) littermates.  $*P < 0.05$  by two-tailed *t*-tests. (B) Lambda protein phosphatase ( $\lambda$ Ptase) decreased anti- $\gamma 2$ -S(P)327 immunoreactivity in cortical samples from wild type but not from PKC $\epsilon^{-/-}$  mice. Shown is a representative western blot demonstrating a  $\gamma 2$ -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 PKC $\epsilon^{-/-}$  mice (-/-;  $n=9$ ). Following treatment with lambda phosphatase, immunoreactivity in wild type mice ( $n=4$ ) was decreased and was similar to immunoreactivity in PKC $\epsilon^{-/-}$  ( $n=3$ ) samples.  $*P < 0.05$  compared with phosphatase-treated wild type (+/+) samples;  $^{\dagger}P < 0.05$  compared with wild type control samples but  $P > 0.05$  compared with phosphatase-treated PKC $\epsilon^{-/-}$  (-/-) samples by Tukey test.

**Fig. 7.** PKC $\epsilon$  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  $\mu$ M Zn $^{2+}$  to block  $\alpha\beta$  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 PKC $\epsilon^{-/-}$  mice assayed in 5  $\mu$ M Zn $^{2+}$ . (D) Combined results from studies using 5  $\mu$ M Zn $^{2+}$  showing a greater effect of ethanol in neurons from wild type mice ( $n=8$ ) when compared with neurons from PKC $\epsilon^{-/-}$  mice ( $n=9$ ).  $*P < 0.05$  compared with wild type cells treated with GABA alone by Dunnett's test.  $^{\dagger}P < 0.05$  compared with wild type cells treated with the same concentration of ethanol by Tukey test.

**Fig. 8.** Intraperitoneal injection of ethanol *in vivo* increases  $\gamma 2$ -S(P)327 immunoreactivity in wild type but not in PKC $\epsilon^{-/-}$  cerebellum. (A) Representative western blots and (B) combined results showing increased  $\gamma 2$ -S(P)327 immunoreactivity in wild type compared with PKC $\epsilon^{-/-}$  cerebellum after saline injection. Immunoreactivity increased after ethanol injection in wild type but not in PKC $\epsilon^{-/-}$  cerebellum ( $n=8$  for each condition).  $*P < 0.05$  when compared with saline-treated, PKC $\epsilon^{-/-}$  samples and  $^{\dagger}P < 0.05$  compared with saline-treated, wild type samples by Bonferroni tests.

**Table 1. Selectivity of kinase inhibitors**

Enzyme	Bis	Staurosporine	1Na
PKC $\epsilon$	6 $\pm$ 1	1 $\pm$ 1	>10 <sup>5</sup>
as-PKC $\epsilon$	12 $\pm$ 1	21 $\pm$ 3	21 $\pm$ 1
PKC $\delta$	32 $\pm$ 3	41 $\pm$ 1	>10 <sup>5</sup>
PKC $\gamma$	30 $\pm$ 2	18 $\pm$ 1	>10 <sup>5</sup>
PKC $\iota$	3,463 $\pm$ 317	364 $\pm$ 29	>10 <sup>5</sup>

Kinase activity was measured by fluorescence polarization using 0.012-2x10<sup>5</sup> nM inhibitor, 2.5  $\mu$ M ATP, and incubation for 90 min at 27°C. Data are from 3 triplicate experiments and IC<sub>50</sub> values (nM) were calculated by nonlinear regression analysis.

**Table 2. Effect of as-PKC $\epsilon$  inhibition on GABA and flunitrazepam potency at mutant GABA<sub>A</sub> receptors**

Subunit Composition	GABA				Flunitrazepam			
	Control		1Na		Control		1Na	
	log EC <sub>50</sub>	n	log EC <sub>50</sub>	n	log EC <sub>50</sub>	n	log EC <sub>50</sub>	n
$\alpha 1\beta 2\gamma 2L$	1.50±0.02	5	1.50±0.01	5	1.99±0.11	8	1.22±0.09*	9
$\alpha 1\beta 2_{S410A}\gamma 2L_{S327A,S343A}$	1.43±0.04	5	1.36±0.07	4	1.59±0.06	11	1.51±0.07	12
$\alpha 1\beta 2\gamma 2L_{S327A,S343A}$	1.56±0.02	9	1.58±0.03	7	1.46±0.08	7	1.57±0.10	5
$\alpha 1\beta 2\gamma 2L_{S327A}$	1.52±0.06	6	1.59±0.02	4	1.46±0.09	8	1.50±0.11	8
$\alpha 1\beta 2_{S410A}\gamma 2L$	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-PKC $\epsilon$  and transiently transfected with indicated receptor subunits. The logEC<sub>50</sub> values were calculated for GABA ( $\mu$ M) and flunitrazepam (nM) by non-linear regression analysis. \* $P$ <0.005 compared with vehicle-treated control cells.

Figure 1, Qi et al.

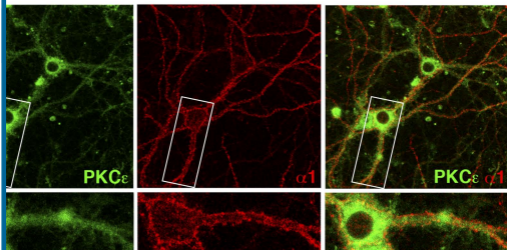
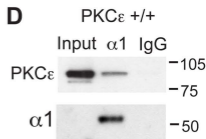
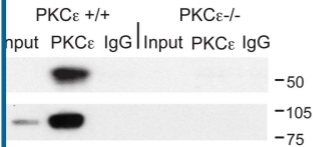
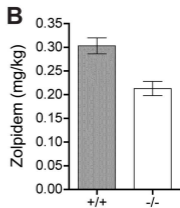
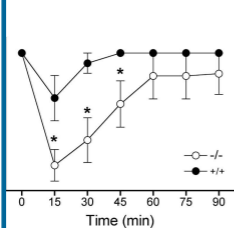


Figure 2, Qi et al.

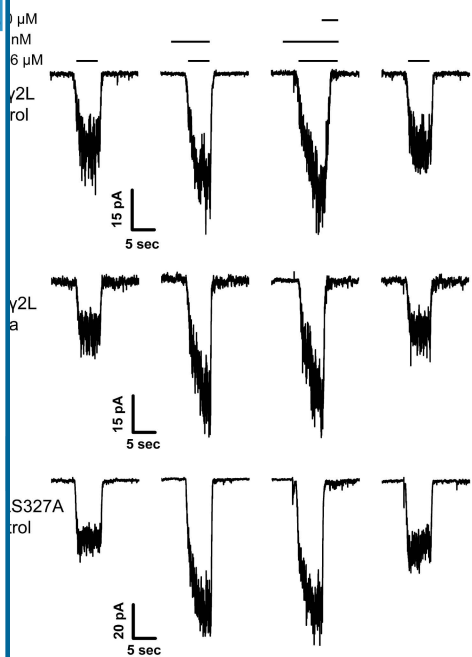
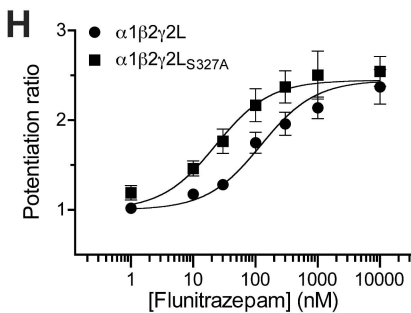
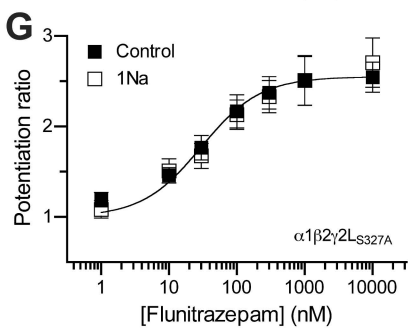
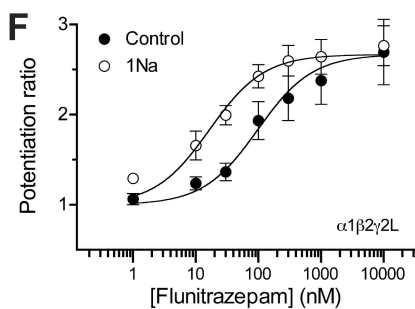
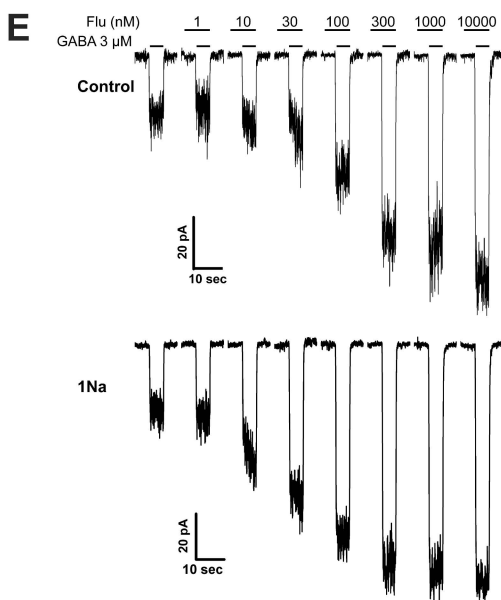
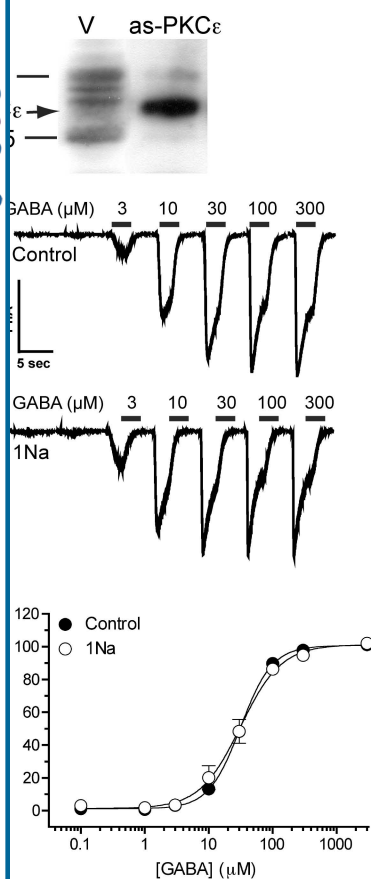


Figure 3, Qi et al.

BMI 30  $\mu$ M  
OH 30 mM  
GABA 3  $\mu$ M

$\alpha$ 1 $\beta$ 2 $\gamma$ 2L  
Control

$\alpha$ 1 $\beta$ 2 $\gamma$ 2L  
1Na

$\alpha$ 2 $\gamma$ 2LS327A  
1Na

10 pA  
5 sec

10 pA  
5 sec

10 pA  
5 sec

**B**

EtOH (mM) 1 3 10 30 100  
GABA 3 mM

Control

1Na

10 pA

10 pA  
10 sec

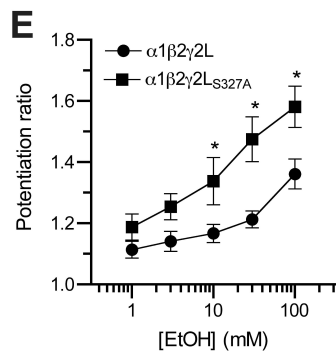
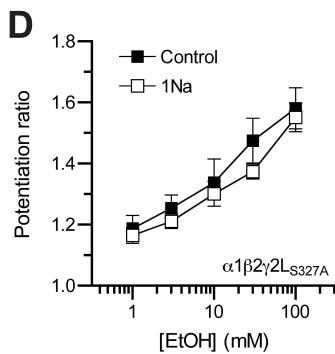
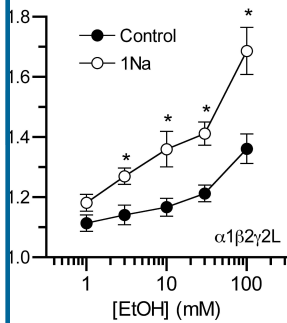


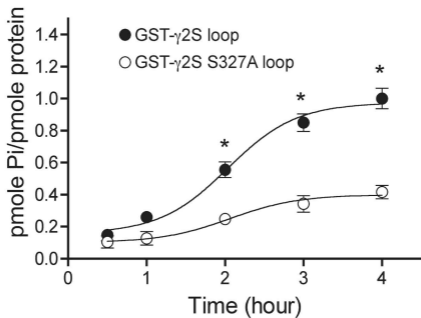
Figure 4, Qi et al.

GST- $\gamma$ 2S loop

GST- $\gamma$ 2S S327A loop



0.5 1 2 3 4 0.5 1 2 3 4 h



$\gamma$ 2S

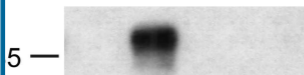
$\gamma$ 2S S327A

N

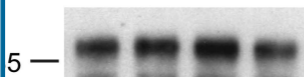
P

N

P



$\gamma$ 2-S(P)327



$\gamma$ 2

Figure 5, Qi et al.

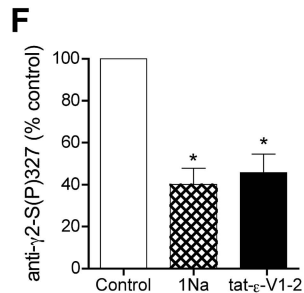
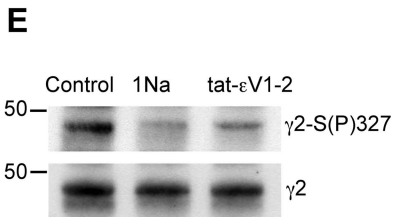
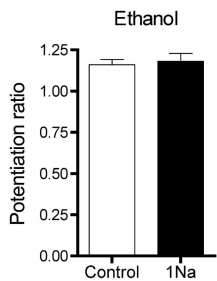
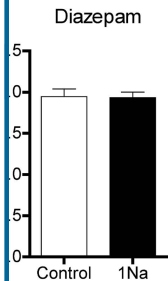
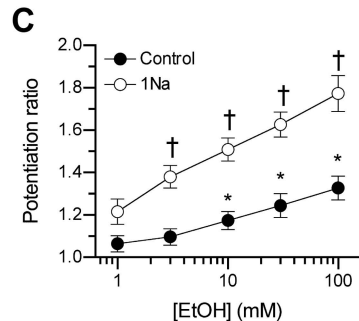
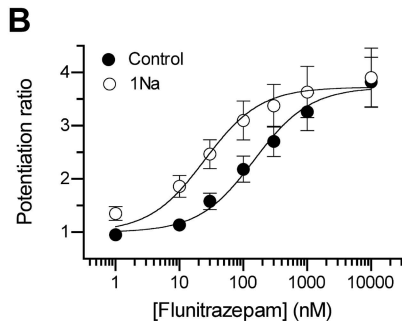
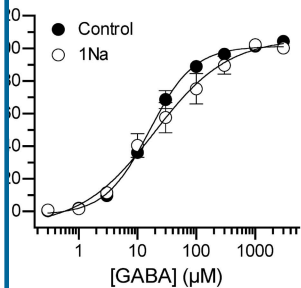


Figure 6, Qi et al.

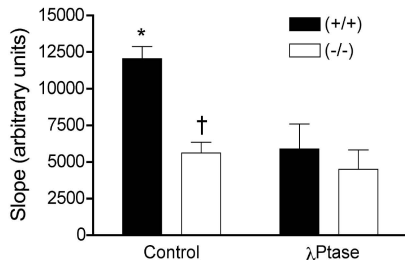
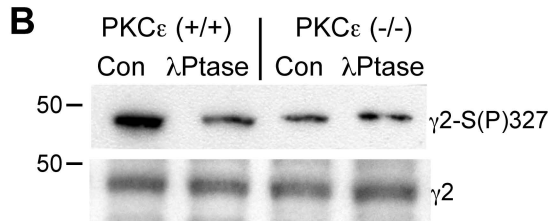
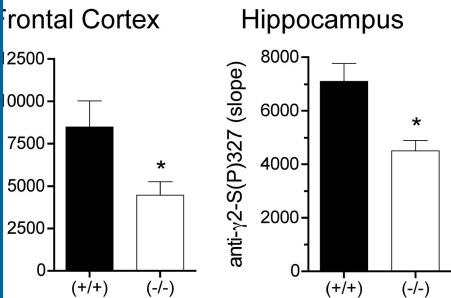
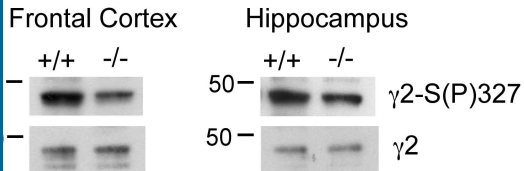


Figure 7, Qi et al.

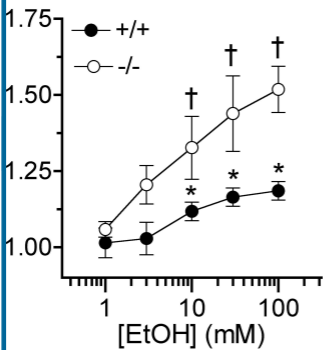
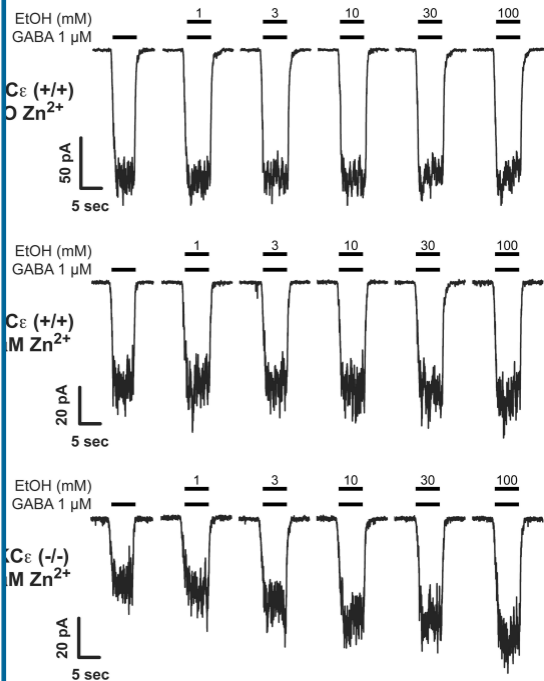


Figure 8, Qi et al.

