Protein Kinase C Phosphorylation Regulates Membrane Insertion of GABA<sub>A</sub> Receptor Subtypes That Mediate Tonic Inhibition<sup>***</sup>

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Tonic inhibition in the brain is mediated largely by specialized populations of extrasynaptic receptors, γ-aminobutyric acid receptors (GABA<sub>A</sub>Rs). In the dentate gyrus region of the hippocampus, tonic inhibition is mediated primarily by GABA<sub>A</sub>R subtypes assembled from α4β2/3 with or without the δ subunit. Although the gating of these receptors is subject to dynamic modulation by agents such as anesthetics, barbiturates, and neurosteroids, the cellular mechanisms neurons use to regulate their accumulation on the neuronal plasma membrane remain to be determined. Using immunoprecipitation coupled with metabolic labeling, we demonstrate that the α4 subunit is phosphorylated at Ser<sup>443</sup> by protein kinase C (PKC) in expression systems and hippocampal slices. In addition, the β3 subunit is phosphorylated on serine residues 408/409 by PKC activity, whereas the δ subunit did not appear to be a PKC substrate. We further demonstrate that the PKC-dependent increase of the cell surface expression of α4 subunit-containing GABA<sub>A</sub>Rs is dependent on Ser<sup>443</sup>. Mechanistically, phosphorylation of Ser<sup>443</sup> acts to increase the stability of the α4 subunit within the endoplasmic reticulum, thereby increasing the rate of receptor insertion into the plasma membrane. Finally, we show that phosphorylation of Ser<sup>443</sup> increases the activity of α4 subunit-containing GABA<sub>A</sub>Rs by preventing current rundown. These results suggest that PKC-dependent phosphorylation of the α4 subunit plays a significant role in enhancing the cell surface stability and activity of GABA<sub>A</sub>R subtypes that mediate tonic inhibition.

γ-Aminobutyric acid type A receptors (GABA<sub>A</sub>Rs)<sup>2</sup> constitute the major inhibitory ligand-gated receptors in the adult central nervous system and are responsible for both phasic and tonic forms of inhibition (1). These receptors are pentameric, anion-selective ion channels that can be assembled from eight subunit classes: α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3(2–3). This large number of receptor subunits provides the basis for a significant degree of heterogeneity of GABA<sub>A</sub>R structure and function. However, previous studies suggest that in the brain, the majority of phasic inhibition is dependent upon a few GABA<sub>A</sub>R subunits, namely the α, β, and γ2 subunits located within synaptic sites (2, 3). In the adult brain, these receptors are specific targets for brief exposures to high concentrations of GABA, resulting in short lived, but significant, hyperpolarization. In contrast, tonic inhibition is characterized by a sustained reduction in the cell’s input resistance, effectively reducing the probability of action potential generation (1, 4). Tonic inhibition is the result of persistent activation by GABA<sub>A</sub>Rs consisting primarily of α, β, and δ subunits located within peri- or extrasynaptic sites (1). With respect to specific brain regions, extrasynaptic GABA<sub>A</sub>Rs that mediate tonic inhibition in the thalamus and dentate gyrus of the hippocampus are composed of the α4 and β2/3 subunits with or without the δ subunit (5–9). Verification of the role that the α4 subunit plays in mediating tonic inhibition comes from α4 subunit knock-out mice, which have substantially lower levels of tonic inhibition in these brain areas (10, 11).

Changes in tonic inhibition associated with GABA<sub>A</sub>Rs containing the α4 subunit have been implicated in a number of normal and pathological states in which the thalamus and the hippocampus play a role. It is apparent that tonic inhibition is essential for dynamically regulating the neuronal output, frequency of firing, and gain control of neurotransmission (12–19). In addition, extrasynaptic GABA<sub>A</sub>Rs have been further shown to be targets for a wide range of endogenous and pharmacological agents, such as neurosteroids, anesthetics, ethanol, and anticonvulsants (20–23). Finally, modifications in the efficacy of tonic inhibition arise under pathological conditions including stress, fragile X syndrome, aberrant brain activity associated with menstrual cycle, postpartum depression, schizophrenia, and temporal lobe epilepsies (20, 24–28).

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<sup>2</sup>The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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<sup>4</sup>The abbreviations used are: GABA<sub>A</sub>R, γ-aminobutyric acid type A receptor; GABA, γ-aminobutyric acid; TM, transmembrane domain; Bgt, bungarotoxin; BBS, bungarotoxin binding site; RFP, red fluorescent protein; COS-7, CV-1 monkey cell line in origin containing SV40 genetic material; AC SF, artificial cerebral spinal fluid; NHS-SS-biotin, succinimidyl 2-(biotinamido)-ethyl-1,3′-dithiopropionate; PDBu, phorbol 12,13-dibutyrate; GFX, GF 109203X.
Little is known about the endogenous mechanism by which neurons control the functional properties of GABA$_A$R subtypes that mediate tonic inhibition. It has long been established that a direct relationship exists between the number of synaptic GABA$_A$Rs at the cell surface and the strength of inhibition at the synapse (29, 30). Therefore, modulating the insertion and removal rate of GABA$_A$Rs into or from the cell membrane has a marked affect on the amplitude of inhibitory synaptic currents (31). One way in which modulation occurs is via posttranslational modifications of the synaptic GABA$_A$R. Specifically, the phosphorylation of key residues on synaptic GABA$_A$R subunits regulates the extent to which the GABA$_A$R will interact with protein complexes responsible for endocytosis from and insertion to the cell membrane; however, the significance of these regulatory processes for subtypes that mediate tonic inhibition remains largely unknown (32, 33). Phosphorylation plays a role in regulating the functional expression of GABA$_A$Rs containing $\alpha_4$ subunits. Our results reveal that the $\alpha_4$ subunit is phosphorylated on Ser$^{443}$ within the intracellular loop between transmembrane domains 3 and 4 (TM3 and TM4) in a protein kinase C (PKC)-dependent manner. Activating PKC also resulted in higher steady state cell surface accumulation of GABA$_A$Rs containing the $\alpha_4$ subunit that was dependent on enhanced insertion into the plasma membrane when expressed in a mammalian cell line. Consistent with this, PKC-dependent phosphorylation of Ser$^{433}$ produced a robust enhancement in GABA-induced currents in this expression system. Finally, we also observed that PKC activity increased both the phosphorylation and cell surface stability of the $\alpha_4$ subunit in hippocampal slices, a phenomenon that should be correlated with an increase in tonic inhibition. Together, these experiments establish a crucial role for PKC in regulating the functional expression of GABA$_A$R subtypes that mediate tonic inhibition via direct phosphorylation of the $\alpha_4$ subunit.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Expression Constructs**—Polyclonal rabbit anti-$\alpha_4$ and anti-$\beta_3$ antibody was graciously provided to us by Dr. Verena Tretter and Dr. Werner Sieghart from Medical University Vienna. $\beta_3$ and phospho-$\beta_3$(phospho-S408A/S409A) antibodies were designed by the Moss laboratory (34). Peroxidase-conjugated IgG secondary antibody was from Jackson ImmunoResearch Laboratories. Fluorescently labeled $\alpha$-bungarotoxin ($\alpha$-Bgt) was purchased from Invitrogen. Wild-type and mutant $\alpha_4$ subunit and wild-type $\beta_3$ cDNAs were cloned into the mammalian cytomegalovirus (CMV) promoter for transgene expression. For fluorescence expression of GABA$_A$R containing subtypes that mediate tonic inhibition, it has long been established that a direct relationship exists between the number of synaptic GABA$_A$Rs at the cell surface and the strength of inhibition at the synapse (29, 30). Therefore, modulating the insertion and removal rate of GABA$_A$Rs into or from the cell membrane has a marked affect on the amplitude of inhibitory synaptic currents (31). One way in which modulation occurs is via posttranslational modifications of the synaptic GABA$_A$R. Specifically, the phosphorylation of key residues on synaptic GABA$_A$R subunits regulates the extent to which the GABA$_A$R will interact with protein complexes responsible for endocytosis from and insertion to the cell membrane; however, the significance of these regulatory processes for subtypes that mediate tonic inhibition remains largely unknown (32, 33). Phosphorylation plays a role in regulating the functional expression of GABA$_A$Rs containing $\alpha_4$ subunits. Our results reveal that the $\alpha_4$ subunit is phosphorylated on Ser$^{443}$ within the intracellular loop between transmembrane domains 3 and 4 (TM3 and TM4) in a protein kinase C (PKC)-dependent manner. Activating PKC also resulted in higher steady state cell surface accumulation of GABA$_A$Rs containing the $\alpha_4$ subunit that was dependent on enhanced insertion into the plasma membrane when expressed in a mammalian cell line. Consistent with this, PKC-dependent phosphorylation of Ser$^{433}$ produced a robust enhancement in GABA-induced currents in this expression system. Finally, we also observed that PKC activity increased both the phosphorylation and cell surface stability of the $\alpha_4$ subunit in hippocampal slices, a phenomenon that should be correlated with an increase in tonic inhibition. Together, these experiments establish a crucial role for PKC in regulating the functional expression of GABA$_A$R subtypes that mediate tonic inhibition via direct phosphorylation of the $\alpha_4$ subunit.

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**Site-directed Mutagenesis**—Mutation of the $\alpha_4$ subunit was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). The mutagenesis primers used to introduce an alanine in place of a serine at site 443 were CTTTTGGGG-GTCGGGCGGTGCTGCGGCAGATT and AAATGCGCGGCAGCAGCCGAACGGG. All mutations were verified by DNA sequencing.

**Expression of GABA$_A$R Subunit Constructs in COS-7 Cells**—$\alpha_4$ subunit cDNAs (mutant and tagged versions) and $\beta_3$ subunit cDNA (where specified) were transfected into COS-7 cells using electroporation with 2 $\mu$g of plasmid DNA per construct. COS-7 cells incubated in 60-mm dishes with 4 ml of Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Invitrogen) plus 10% fetal bovine serum (FBS) at 37 °C with a 95% O$_2$, 5% CO$_2$ gas mixture and were utilized 48 h after transfection (36).

**HEK293 Cell Culture and Transfection**—Human embryonic kidney (HEK293) cells were cultured in medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO$_2$ atmosphere. Cells were electroporated (110 V, Bio-Rad Gene Pulser Xcell) with equal ratios of cDNA encoding for GABA$_A$R receptor subunits along with GFP cDNAs (in pCDM8). Cells were used 24–72 h after transfection. Successful transfection of the cells was determined by fluorescence microscopy to identify GFP-labeled cells.

**Hippocampal Slice Preparation**—Hippocampal slices (350 $\mu$m thick) from 10–11-week-old C57BL/6 mice were prepared with a microslicer (Leica VT1000S) and pooled in ice-cold oxygenated artificial cerebral spinal fluid (ACSF). ACSF solution contents differed depending on the experiment being conducted. For $32^P$ labeling experiments (described below), ACSF contained the following: 125 mM NaCl, 4 mM KCl, 26 mM NaHCO$_3$, 1.5 mM MgSO$_4$, 1.5 mM CaCl$_2$, and 10 mM glucose, pH 7.4. For slice biotinylation experiments (described below), ACSF contained the following: 124 mM NaCl, 3 mM KCl, 25 mM NaHCO$_3$, 2 mM MgSO$_4$, 2 mM CaCl$_2$, 1.1 mM NaH$_2$PO$_4$, and 10 mM glucose, pH 7.4. The slices were transferred individually to a solution containing fresh ACSF, gassed with a mixture of 95% O$_2$, 5% CO$_2$, and equilibrated in a 30 °C water bath for 1 h. Afterward, slices were utilized for either $32^P$ labeling or slice biotinylation.

**Cell Lysis and Immunoprecipitation**—Samples collected from either COS-7 cell cultures or hippocampal slices were lysed in lysis buffer containing the following: 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na$_3$VO$_4$, 10 mM sodium pyrophosphate, 1% Triton X-100, and 0.1% SDS. In addition, the following protease inhibitors were added: 250 $\mu$g/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; 10 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, and 10 $\mu$g/ml antipain. Samples were then sonicated and spun at 16,000 $\times$ g. The supernatant was collected and then subjected to a protein assay using a standard Bradford protocol. 100–200 $\mu$g of protein were loaded per immunoprecipitation sample along with 3 $\mu$g of indicated antibody and 40 $\mu$l of protein A-Sepharose beads (1:1 slurry) (GE Healthcare). Samples were allowed to conjugate for 18–24 h at 4 °C with constant agitation. The beads were precipitated by centrifuga-
tion at 500 \times g and washed once with ice-cold Buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM NaVO₄, 10 mM sodium pyrophosphate, and 1% Triton X-100 and protease inhibitors), two times with Buffer B composed of Buffer A supplemented with 500 mM NaCl, and once again with Buffer A. After the final wash, the beads were resuspended in 25 \mu l of sample buffer and subjected to SDS-PAGE.

Whole-cell COS-7 Cell and Hippocampal Slice Metabolic \( ^{32}P \) Labeling—COS-7 cells were transfected and incubated as described above. Cells were initially incubated in 2 ml of phosphate-free DMEM (Invitrogen) for 30 min at 37 \degree C. Following this incubation, cells were labeled with 0.5 mCi/ml \( ^{32}P \)orthophosphoric acid for 4 h in phosphate-free DMEM. Hippocampal slices were prepared as described above. Slices were individually transferred to polypropylene tubes containing 2 ml of fresh ACSF; gassed with a mixture of 95% \( \text{O}_2 \), 5% \( \text{CO}_2 \), and maintained in a 30 \degree C water bath. Labeling was performed by adding 0.5 mCi/ml \( ^{32}P \)orthophosphoric acid for 1 h. For both COS-7 cells and hippocampal slices, samples were treated with drugs where indicated after the labeling period, followed by the cell lysis and immunoprecipitation procedure described above. Results were attained by SDS-PAGE followed by autoradiography.

Phosphopeptide Mapping and Phosphoamino Acid Analysis—To perform phosphopeptide mapping, gel slices from \( ^{32}P \) labeling experiments were excised from SDS-polyacrylamide gels and washed and digested with 0.1 mg/ml trypsin and subjected to two-dimensional mapping, first by electrophoresis and then by thin layer chromatography (TLC). The resulting plate was then visualized by autoradiography (37). For phosphoamino acid analysis, phosphoproteins from gel slices were hydrolyzed using 6 N HCl. The resulting phosphoamino acids, along with phosphoamino acid standards, were separated by TLC and visualized by autoradiography (37).

Metabolic \( ^{35}S \)/Methionine Labeling—Transfected COS-7 cells were incubated in methionine-free DMEM for 20 min and then pulsed with 0.5 mCi/ml \( ^{35}S \)methionine (PerkinElmer Life Sciences) for 30 min. Cells were washed and incubated in complete DMEM/F-12 with an excess amount of unlabeled methionine for the indicated time periods (chase). Cells were lysed and subjected to immunoprecipitation as described above.

COS-7 Cell and Hippocampal Slice Cell Surface Biotinylation Assay—For transfected COS-7 cells, cultures were washed once with ice-cold PBS and then incubated in 2 ml of ice-cold PBS containing 1 mg/ml NHS-SS-biotin (Pierce) for 20 min in order to label surface proteins with biotin. After labeling, the biotin was quenched by incubating cells in PBS containing 25 mM glycine and 10 mg/ml bovine serum albumin (BSA) (38, 39). Cells were then lysed in lysis buffer and sonicated. For hippocampal slice experiments, slices were incubated in ACSF described above at 30 \degree C for 1 h for recovery before experimentation. Slices were then placed on ice and incubated for 30 min with 1 mg/ml NHS-SS-biotin. Excess biotin was removed by washing slices three times in ice-cold ACSF and lysed as described above (40). For both COS-7 cells and hippocampal slices, insoluble material was removed by centrifugation. The supernatant lysates were incubated with NeutrAvidin beads (Pierce) for 18–24 h at 4 \degree C. Bound material was eluted with sample buffer and subjected to SDS-PAGE and then immunoblotted with indicated antibodies. Blots were then quantified using the CCD-based FujiFilm LAS 3000 system.

Fluorescent BBS Cell Membrane Insertion Assay—COS-7 cells were transfected with RFP- \( ^{34}S443A \) or RFP- \( ^{34}S443A \) and the \( \beta \) subunit. All surface proteins expressing the BBS were blocked with 10 \mu g/ml unlabeled \( \alpha \)-Bgt for 15 min at 18 \degree C. The cells were then washed extensively to remove unbound \( \alpha \)-Bgt. Newly inserted RFP- \( ^{34}S443A \) or RFP- \( ^{34}S443A \) was labeled with 2 \mu g/ml Alexa 647-conjugated \( \alpha \)-Bgt and fixed immediately with 4% paraformaldehyde after the indicated time points (35). Confocal images of fluorescently labeled COS-7 cells were collected using a \times 60 objective, acquired with Nikon acquisition software, and analyzed with MetaMorph.

Patch Clamp Electrophysiology—Cells were superfused, at a rate of 2 ml/min, with an extracellular solution containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 10 mM HEPES, 11 mM glucose and adjusted to pH 7.4 with NaOH. Borosilicate glass patch pipettes (resistance 2–5 megohms) were filled with an internal solution containing 140 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 1.1 mM EGTA, 10 mM HEPES, 2 mM ATP (Mg²⁺ salt), adjusted to pH 7.4 with KOH. GABA was applied once every 120 s via a fast step perfusion system (Warner Instruments, Hamden, CT). All experiments were carried out at 32–33 \degree C using a recording chamber and in-line perfusion heaters (Warner Instruments). Phorbol esters were applied to the cell either internally via the electrode solution or superfused into the recording chamber.

Data Acquisition and Analysis—For biochemical and immunofluorescent experiments, data are presented as means ± S.E. Statistical analysis was performed by using Student’s \( t \) test where a \( p \) value of <0.5 is considered significant. For electrophysiological experiments, currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 20 kHz with a Digidata 1320A data acquisition system (Molecular Devices), and analyzed using either Clampfit (pClamp, Molecular Devices) or GraphPad Prism version 4 software (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed by using one-way ANOVA with a Bonferroni post-test with statistical significance set at \( p < 0.05 \). All data are expressed as mean ± S.E.

RESULTS

Basal Phosphorylation of the GABA\( _{A} \) Receptor \( \alpha 4 \) Subunit Is Enhanced by PKC When Expressed in COS-7 Cells—Immunoprecipitation was used to examine the phosphorylation of GABA\( _{A} \) receptors in COS-7 cells transiently transfected with the GABA\( _{A} \) receptor \( \alpha 4 \) and \( \beta 3 \) subunits. Immunoprecipitation with anti-\( \alpha 4 \) from transfected COS-7 cells that had been prelabeled with \( ^{32}P \)orthophosphoric acid under basal conditions yielded a major phosphoprotein at ~64 kDa, demonstrating that the recombinant \( \alpha 4 \) subunit is basally phosphorylated (Fig. 1A). A corresponding band was not observed in

\( \textbf{PKC Activity Enhances Cell Surface Stability of GABA}_{A} \text{R Subunit } \alpha 4 \)
PKC Activity Enhances Cell Surface Stability of GABA$_4$R Subunit α4

FIGURE 1. α4 subunit phosphorylation is increased by PDBu, a specific PKC activator. A, untransfected COS-7 cells (UT) or COS-7 cells transfected with GABAA$_4$ receptor α4 and β3 subunits were labeled with 0.5 mCi/ml [32P]orthophosphoric acid and then treated with either PDBu (500 nM for 10 min) alone or following pretreatment with GFX (1 μM for 10 min), a PKC inhibitor. The α4 subunit was immunoprecipitated, subjected to SDS-PAGE, and visualized with autoradiography (top). The level of phosphorylation was normalized to the amount observed in vehicle-treated samples (bottom) (dashed line represents vehicle set at 100%, p < 0.05). B, phosphopeptide map of the α4 subunit. [32P]α4 immunopurified from transfected COS-7 cells was digested with trypsin, and the resulting phosphopeptides were blotted onto TLC plates and subjected to electrophoresis followed by ascending chromatography. The small arrow indicates the origin. C, the α4 subunit was subjected to phosphoamino acid analysis followed by autoradiography. The migration of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) standards is indicated. Error bars, S.E.

untransfected COS-7 cells. Previous studies have shown that GABA$_A$ receptor subunits are the target of PKC (41–43). To determine whether the α4 subunit is a substrate of PKC, specific kinase activators and inhibitors were utilized. Activation of PKC with 500 nM phorbol 12,13-dibutyrate (PDBu) for 10 min produced a significant increase (p < 0.05) in α4 subunit phosphorylation (223.7 ± 25.17% (n = 3)) compared with control cells treated with DMSO for 10 min (Fig. 1A). An inhibitor of PKC, GF 109203X (GFX) (10 μM for 20 min), had little effect on the basal phosphorylation of the α4 subunit (74.39 ± 15.38% of control, n = 3). However, treating transfected COS-7 cells with GFX 10 min prior to PDBu treatment prevented the increase in α4 subunit phosphorylation (81.1 ± 26.98% of control, n = 3) observed with PDBu treatment alone (Fig. 1A). Peptide mapping and phosphoamino acid analysis revealed that the PKC-dependent phosphorylation of the α4 subunit primarily occurs on serine residues (Fig. 1C) within one major phosphopeptide (Fig. 1B, circled). Together these results strongly suggest that PKC enhances basal levels of phosphorylation on the α4 subunit.

Ser$^{443}$ Is a Major Site for PKC-dependent Phosphorylation of the α4 Subunit When Expressed in COS-7 Cells—To further analyze α4 subunit phosphorylation, site-directed mutagenesis was utilized to convert candidate serine residues within the α4 subunit intracellular domain to alanines. Based on the consensus PKC motif of (R/K)X$_1$–$_4$(S/T)X$_5$–$_7$(R/K) (44–45), a mutant version of the α4 subunit was produced in which Ser$^{443}$ was changed to an alanine (α4S$_{443}$A subunit) (Fig. 2A). COS-7 cells transfected with wild-type α4 or α4S$_{443}$A and β3 subunits were subjected to [32P]orthophosphoric acid labeling and treated with either DMSO or PDBu. PDBu significantly enhanced (p < 0.05) levels of phosphorylation of wild-type α4 336 ± 50.03% of control (Fig. 2B). In contrast to wild type, PDBu did not significantly enhance the phosphorylation of the α4S$_{443}$A subunit (Fig. 2B). These results strongly suggest
PKC Activity Enhances Cell Surface Stability of GABA<sub>4</sub>R Subunit α4

PKC-dependent phosphorylation on Ser<sup>443</sup> regulates the cell surface expression of the α4 subunit in COS-7 cells. A, COS-7 cells transfected with GABA<sub>4</sub>R receptor α4 and β3 subunits were treated with either PDBu (500 nm for 10 min) alone or following pretreatment with GFX (1 μM for 10 min) and then labeled with NHS-SS-biotin. Detergent-soluble extracts were then purified on NeutrAvidin. The purified cell surface (Surface) and total (Total) fractions were also blotted with actin to ensure the integrity of the cell surface assay. The amount of α4 subunit on the cell surface was then measured for each condition and normalized to the amount observed in vehicle-treated samples (lower panel) (dashed line represents vehicle set at 100%; p < 0.05). B, COS-7 cells co-transfected with either wild-type (α4-WT) or S443A mutant (α4-S443A) GABA<sub>4</sub>R receptor α4 and β3 subunits were treated with either vehicle or PDBu (500 nm for 10 min) and then subjected to biotinylation. Histograms show the proportion of cell surface α4 protein expressed as a percentage of vehicle-treated controls (dashed line represents vehicle set at 100%; p < 0.05). Error bars, S.E.

Insignificant amounts of actin protein were pulled down in our biotinylated samples, ensuring that only cell surface proteins were being collected (Fig. 3A). In contrast to these results, PDBu treatment did not significantly increase (p < 0.05) the cell surface expression level of α4<sup>S443A</sup> (Fig. 3B). Together, these biochemical experiments indicate that the PKC-dependent phosphorylation of the α4 subunit on Ser<sup>443</sup> increases receptor cell surface expression.

**Analyzing the Phosphorylation of the β3 and δ Subunits**—In the brain, the α4 subunit assembles with the β2/3 subunit with or without the δ subunit (5–9, 48). Thus, we examined if these subunits are also subject to PKC-dependent phosphorylation. To do so, the α4, β3, and δ subunits were co-expressed in COS-7 cells and then labeled with [32P]orthophosphoric acid. After treatment with 500 nm PDBu for 10 min, the δ subunit was isolated via immunoprecipitation after denaturing lysis. Under these conditions, minimal levels of phosphorylation of the δ subunit were seen under basal conditions or after the activation of PKC (Fig. 4B). However, robust immunoprecipitation of the δ subunit was seen as measured via immunoblotting (Fig. 4B).

Previous studies have revealed that the β3 subunit is predominantly phosphorylated on serines 408/409 (Ser<sup>408/409</sup>) in neurons upon activation of PKC (34, 49). To examine if these residues are phosphorylated in COS-7 cells, lysates were immunoblotted with a phosphospecific antibody against these residues, phosphoserines 408/409 (phospho-Ser<sup>408/409</sup>). PDBu treatment produced a robust enhancement of Ser<sup>408/409</sup> phosphorylation as measured via immunoblotting with Ser<sup>(P)408/409</sup> antibody (Fig. 4A). Thus, this experiment suggests that the δ subunit is not a PKC substrate at least when expressed in COS-7 cells and suggests that the principle sites for PKC phosphorylation within GABA<sub>4</sub>R subtypes that mediate tonic inhibition are Ser<sup>443</sup> in the α4 subunit and Ser<sup>408/409</sup> in β3.
PKC Activity Enhances Cell Surface Stability of GABA$_{\alpha}$R Subunit $\alpha$4

FIGURE 4. Analyzing PKC phosphorylation of GABA$_{\alpha}$R subunits that mediate tonic inhibition. A, COS-7 cells expressing the $\alpha$4 and $\beta$3 subunits were treated with 500 nM PDBu for 10 min and then immunoblotted with phospho-S408A/S409A (pS408/9) or $\beta$3 antibodies, and the ratio of pS408/9/$\beta$3 immunoreactivity was determined and normalized to control (dashed line represents vehicle set at 100%; p < 0.05). B, COS-7 cells expressing $\alpha$4, $\beta$3, and $\delta$ subunits were labeled with 0.5 mCi/ml $[^{32}$P]orthophosphoric acid and treated with 500 nM PDBu for 10 min. The $\delta$ subunit was isolated by denaturing immunoprecipitation followed by SDS-PAGE (IP/$[^{32}$P]). Parallel cultures were immunoprecipitated and immunoblotted with $\delta$ antibodies (IP/ WB $\delta$).

Mutation of Ser$^{443}$ Increases the Rate of Insertion of the $\alpha$4 Subunit on the Cell Membrane—To further evaluate the mechanism underlying PKC-dependent modulation of the $\alpha$4 subunit cell surface stability, we determined what effect mutating the PKC site on the $\alpha$4 subunit has on the level of insertion of $\alpha$4 subunit-containing receptors. To do so, we utilized a Bgt binding assay that has previously been used to measure the rates of insertion of various receptor types (35, 50). To analyze the insertion of the $\alpha$4 subunit, we engineered the $\alpha$4 subunit with the BBS peptide, WRYYESSLEPYPD. The BBS is derived from the $\delta$ subunit of the muscle nicotinic receptor and has been established to bind Bgt with an affinity of $\sim$3 nM (51, 52). The BBS together with a red fluorescent protein reporter were added to the N-terminal region of both the $\alpha$4 and $\alpha$4$^{S443A}$ subunit.

Before conducting our insertion assay, we verified that the BBS-tagged versions of our $\alpha$4 constructs were capable of forming a functional GABA$_{\alpha}$R. To do so, we expressed RFP-BBS$^{\alpha}$4 and non-tagged $\alpha$4 separately with the $\beta$3 subunit in HEK293 cells and measured the current responses to 1 mM and 1 mM GABA. Utilizing this method, we demonstrated that the RFP-BBS$^{\alpha}$4$\beta$3 GABA$_{\alpha}$R subtype forms a functional receptor and has GABA-mediated currents similar to those of the $\alpha$4$\beta$3 GABA$_{\alpha}$R subtype (Fig. 5).

To measure the role that Ser$^{443}$ plays in regulating the cell surface accumulation of $\alpha$4, RFP-BBS$^{\alpha}$4 and RFP-BBS$^{\alpha}$4$^{S443A}$ cDNAs were separately transfected into COS-7 cells along with the GABA$_{\alpha}$ $\beta$3 subunit and subjected to the BBS insertion assay. To perform this assay, we first masked the surface RFP-BBS$^{\alpha}$4 and RFP-BBS$^{\alpha}$4$^{S443A}$ with unlabeled Bgt by incubating the transfected COS-7 cells with native Bgt at 18 °C for 15 min. Under these conditions, the unlabeled Bgt completely blocked the existing cell surface population of RFP-BBS$^{\alpha}$4 and RFP-BBS$^{\alpha}$4$^{S443A}$ subunits. Next, the cells were incubated at 37 °C with Alexa 647-Bgt in order to fluorescently label newly inserted RFP-BBS$^{\alpha}$4 and RFP-BBS$^{\alpha}$4$^{S443A}$. Visually, it is apparent that there is a higher amount of RFP-BBS$^{\alpha}$4$^{S443A}$ inserted after 10 min compared with RFP-BBS$^{\alpha}$4 (Fig. 6A). To quantify these results, we calculated the ratio of the level of Alexa 647-Bgt staining to the level of RFP fluorescence. This ratio was significantly higher ($p < 0.05$) in COS-7 cells transfected with RFP-BBS$^{\alpha}$4$^{S443A}$ than in cells transfected with RFP-BBS$^{\alpha}$4 after 10 min of labeling (1.35 ± 0.33 versus 0.78 ± 0.16, n = 3; Fig. 6B). We also tested the rate of endocytosis of the RFP-BBS$^{\alpha}$4 and RFP-BBS$^{\alpha}$4$^{S443A}$ in a similar BBS assay and found no significant difference between the wild-type and mutant $\alpha$4 subunit (data not shown). Together, these results strongly suggest that phosphorylation of Ser$^{443}$, the major site of PKC-dependent phosphorylation within the $\alpha$4 subunit, regulates the rate of insertion of the $\alpha$4 subunit in COS-7 cells.

Mutation of Ser$^{443}$ Increases the Protein Stability of the $\alpha$4 Subunit—To determine the role phosphorylation plays in the production and stability of the $\alpha$4 subunit, COS-7 cells transfected with $\alpha$4 or $\alpha$4$^{S443A}$ alone were subjected to a $[^{35}$S]methionine pulse-chase assay. Transfected COS-7 cells were labeled with 100 $\mu$Ci/ml $[^{35}$S]methionine for 30 min and chased for 0 and 4 h with excess cold methionine. Cell lysates were then prepared and subjected to immunoprecipitation with anti-$\alpha$4 and resolved on SDS–polyacrylamide gels and quantified on a Bio-Rad isotope imager (Fig. 7). Data at 4 h are presented as a percentage of $[^{35}$S]methionine-labeled protein existing at time 0. $\alpha$4 subunit protein does not reach the cell surface unless a $\beta$3 subunit is also present (supplemental Fig. 1); therefore, under these conditions, we are measuring the stability of protein that is retained in the endoplasmic reticulum. Using this technique, we determined that 47.40% of newly synthesized $\alpha$4 subunit protein remained after 4 h (Fig. 7). Interestingly, this reduction was markedly less robust for the $\alpha$4$^{S443A}$ subunit protein, reducing to 80.20% ± 7.00% of newly synthesized protein after 4 h (Fig. 7). Therefore, the $\alpha$4$^{S443A}$ subunit is more stable than the wild-type $\alpha$4 subunit, suggesting that PKC phosphorylation of the Ser$^{443}$ site regulates the $\alpha$4 subunit protein half-life.

Protein Kinase C Activation Reverses Current Run-down—The functional effects of protein kinase C activation were de-
The α4S443A subunit mutation prevents run-down—GABA-mediated currents in cells expressing α4S443Aβ3 resulted in functional channels that had GABA-evoked EC50 values of 1.7 ± 0.7 and 2.6 ± 0.9 μM (n = 3–10), with both Hill coefficients of 0.8 ± 0.2 (data not shown). To examine the run-down of GABA-mediated currents, 1 mM GABA was applied to the cells once every 2 min. In the absence of PKC activation, the GABA-mediated current amplitude decreased over time but appeared to plateau after 16 min of recording. After 20 min of recording, the GABA-mediated current was 37 ± 12% (n = 5) of the initial response. Inclusion of 100 nM PDBu in the recording pipette solution prevented the GABA-mediated current amplitude run-down. At 20 min after the start of the experiment, the GABA-mediated current amplitude was 97 ± 9% (n = 3) of the initial GABA-mediated response. Similarly, external application of 100 nM PDBu also reverses GABA-mediated current amplitude run-down with current amplitude being 107 ± 12% (n = 4) compared with that at the start of the experiment (Fig. 8).

When the PKC-inactive phorbol ester, 4-α-phorbol 12,13-didecanoate (100 nM), was included in the intracellular solution of the recording pipette, the GABA-mediated current amplitude run-down was no different from control. Cells rarely remained healthy past 16 min of recording. After 16 min of recording, the GABA-evoked currents were 17 ± 6% (n = 3) of the first current in the presence of 100 nM 4-α-phorbol 12,13-didecanoate compared with 33 ± 10% (n = 7) in control (Fig. 9). At this time point, minimal run-down was observed in cells internally perfused with 100 nM PDBu (98 ± 9% of control, n = 3).

The α4S443A Subunit Mutation Prevents Run-down—GABA-mediated currents in cells expressing α4S443Aβ3 remained healthy after 20 min of recording. Inclusion of 100 nM PDBu in the recording pipette solution prevented the GABA-mediated current amplitude run-down. At 20 min after the start of the experiment, the GABA-mediated current amplitude was 97 ± 9% (n = 3) of the initial GABA-mediated response. Similarly, external application of 100 nM PDBu also reverses GABA-mediated current amplitude run-down with current amplitude being 107 ± 12% (n = 4) compared with that at the start of the experiment (Fig. 8).

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FIGURE 7. S443A point mutation reduces turnover of the α4 subunit in transfected COS-7 cells. Untransfected COS-7 cells (UT) or COS-7 transfected with either wild-type (α4-WT) or S443A mutant (α4-S443A) GABA_4 receptor subunits subjected to a pulse-chase with [35S]methionine. Cells were lysed and immunoprecipitated with anti-α4 subunit antibody and then subjected to SDS-PAGE. Bands were then analyzed by autoradiography (top). Turnover levels are presented as a percentage of levels at time 0 (bottom) (p < 0.05). Error bars, S.E.

FIGURE 8. Run-down of GABA_4 receptor α4/β3-mediated responses are prevented with protein kinase C activation. A, 1 μM GABA-activated currents recorded at 0, 10, and 20 min after the start of the experiment (defined as t = 0 min and 100%), recorded 3–5 min after achieving the whole-cell configuration. Whole-cell currents were recorded from HEK293 cells expressing α4 and β3 subunits in the absence (control, upper currents) and presence (+[PDBu]; lower currents) of 100 nM internal PDBu. Holding potential was −60 mV at 32 °C. B, time dependence relationship for 1 μM GABA-activated currents recorded from α4β3 receptors without (open squares) or with (solid squares) 100 nM PDBu internally perfused or with 100 nM PDBu externally perfused (solid diamonds). All data points are mean ± S.E. (error bars).

FIGURE 9. Inclusion of the inactive phorbol ester, 4α-Phorbol 12,13-didecanoate, does not prevent run-down of GABA_4 receptor α4/β3-mediated responses. A, overlaid GABA-evoked currents from HEK293 cells expressing α4β3 receptors recorded at t = 0 (gray) and t = 16 (black) min after the start of the experiment. Significant run-down of current amplitude at t = 16 compared with t = 0 is observed in control and in the presence of 4α-phorbol 12,13-didecanoate (4α-phorbol). In comparison, the current at t = 16 min in the presence of internal 100 nM PDBu was not different from that at t = 0 min. B, bar graph of the relative current at t = 16 min compared with current at t = 0 min for cells in control conditions (n = 8), perfused internally with 100 nM PDBu (n = 3) or 100 nM 4α-phorbol (n = 3). Values are mean ± S.E. (error bars).

Receptors did not show the typical time-dependent run-down phenomena. Unlike that observed with wild-type α4-containing receptors in control conditions, after 20 min of recording, the GABA-mediated current from α4S443A containing receptors was 84 ± 13% (n = 4) of the initial GABA-mediated response. In the presence of internal 100 nM PDBu, GABA-mediated current was 98 ± 9% (n = 4) of the initial response after 20 min of recording (Fig. 10).

PKC Activity Modulates the Phosphorylation and Cell Surface Stability of Endogenous α4 Subunit in Hippocampal Slices—To determine the relevance of our recombinant studies, we examined the phosphorylation of the endogenous α4 subunit and its effects on cell surface stability of this protein in hippocampal slices. We began by examining the PKC-dependent phosphorylation of the α4 subunit. Hippocampal slices were cut from brains dissected from 10–11-week-old C57BL/6 mice. Slices were labeled with [32P]orthophosphoric acid for 4 h in ACSF continuously being bubbled with a 95% O_2, 5% CO_2 gas mixture. Toward the end of the 4 h, control slices were treated with DMSO for 10 min, whereas PKC slices were treated with 500 nM PDBu for 10 min. Afterward, slices were lysed and subjected to immunoprecipitation with anti-α4. Under control conditions, a very faint band at ~64 kDa was detected, representing the basal level of α4 subunit phosphorylation (Fig. 11A). Treatment of slices with PDBu significantly increased (p < 0.05) α4 subunit phosphorylation to 2016 ± 1260% of control (Fig. 11A).

We further evaluated phosphorylation of the α4 subunit in hippocampal slices by performing phosphoamino acid analysis. We determined that PKC activation with PDBu resulted in the phosphorylation of the α4 subunit principally on serine...
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Fig. 10. Run-down is prevented with the inclusion of the \( \alpha4^{443A} \) mutation. A, whole-cell currents recorded from HEK293 cells expressing \( \alpha4^{443A}/\beta3 \) receptors. 3–5 min after achieving the whole cell configuration (defined as \( t = 0 \) and 100%), mediated by 1 \( \mu \)M GABA were recorded at 0, 10, and 20 min after the start of the experiment and recorded. Current was recorded in the absence (control, upper currents) and presence of 100 \( \mu \)M PDBu (lower currents). B, time dependence relationship for 1 \( \mu \)M GABA-activated currents recorded from \( \alpha4/\beta3 \) receptors without (open squares) or with (solid squares) 100 \( \mu \)M PDBu internally perfused. All data points are mean \( \pm \) S.E. (error bars).

Fig. 11. PKC increases the level of phosphorylation and cell surface expression of the \( \alpha4 \) subunit in hippocampal slices. A, hippocampal slices from 10–11-week-old C57BL/6 male mice were labeled with \( ^{32}P \)orthophosphoric acid and treated with either vehicle or PDBu (500 \( \mu \)M for 10 min). Detergent-soluble extracts were immunoprecipitated with either rabbit IgG or anti-\( \alpha4 \), resolved by SDS-PAGE, and then visualized by phosphorimaging (top). Histograms are presented as \( ^{32}P \) incorporation expressed as a percentage of vehicle-treated control (bottom) (dashed line, \( p < 0.05 \)). B, the immunoprecipitated \( \alpha4 \) subunit from \( ^{32}P \)orthophosphoric acid-treated hippocampal slices was subjected to phosphoamino acid analysis followed by autoradiography. The migration of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) standards is indicated. C, hippocampal slices from 10–11-week-old C57BL/6 male mice treated with either vehicle or PDBu (500 \( \mu \)M for 10 min) were labeled with NHS-S-S-biotin and detergent-soluble extracts were purified on NeutrAvidin. Cell surface (Surface) and 10% of total fractions (Total) were analyzed by immunoblotting with anti-\( \alpha4 \) (top). Histograms show the proportion of cell surface \( \alpha4 \) protein expressed as a percentage of vehicle-treated controls (bottom) (dashed line, vehicle set at 100%; \( p < 0.05 \)). Error bars, S.E.
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from adult male mice. We also analyzed the phosphorylation of the \(\delta\) and \(\beta_3\) subunits in our study. Consistent with studies on GABA\textsubscript{A}R subtypes that mediate phasic inhibition, Ser\textsuperscript{408/409} in the \(\beta_3\) subunit were phosphorylated by PKC activity when expressed with \(\alpha_4\). However, at least in COS-7 cells, only low levels of \(\delta\) subunit phosphorylation were seen. Thus, these results suggest that the primary PKC substrates with GABA\textsubscript{A}R subtypes that mediate tonic inhibition are Ser\textsuperscript{443} in the \(\alpha_4\) subunit and Ser\textsuperscript{408/409} in \(\beta_3\).

To begin ascertaining the functional consequences of phosphorylation on tonic inhibition, we looked at the effect PKC activation had on the cell surface stability of the \(\alpha_4\) subunit. The activation of PKC leads to a dramatic increase in the amount of \(\alpha_4\) subunit protein at the cell surface in both transfected COS-7 cells and hippocampal slices, as measured by biotinylation. The Ser\textsuperscript{443} phosphorylation site plays a crucial role in this enhancement because mutation of this residue did not result in elevated levels of \(\alpha_4\) subunit protein at the cell surface of COS-7 cells. At this point, it was clear that Ser\textsuperscript{443} is essential in mediating the effects of PKC activity on \(\alpha_4\) subunit cell surface accumulation. To begin to address the underlying mechanism, we measured the rate of insertion of the \(\alpha_4\) subunit into the cell membrane using a BBS fluorescent insertion assay. Here we discovered that over a 10-min period, more mutant \(\alpha_4\) was being inserted into the COS-7 cell membrane than wild type \(\alpha_4\) subunit. This increased rate of insertion was also paralleled with an increase in stability of newly translated \(\alpha_4\) subunit compared with wild type \(\alpha_4\) when expressed alone in COS-7 cells. Given that the \(\alpha_4\) subunit is retained within the endoplasmic reticulum in homomeric expression, this result suggests that phosphorylation of Ser\textsuperscript{443} acts to regulate the stability of the \(\alpha_4\) subunit in this intracellular compartment, which would be predicted to increase receptor assembly, leading to increased insertion into the plasma membrane.

To investigate this possibility, we measured the amount of protein degradation using an \(^{35}\text{S}\)methionine pulse-chase assay and found that the \(\alpha_4\) subunit Ser\textsuperscript{443} mutant was more stable in the endoplasmic reticulum over a 4-h period. Taking these results together, we see a situation in which the mutant version of the \(\alpha_4\) subunit is not only degraded less but is inserted faster into the cell membrane. At first glance, this seems at odds with our results showing that Ser\textsuperscript{443} is a critical residue for the PKC-dependent phosphorylation of the \(\alpha_4\) subunit that leads to higher levels of this protein on the cell surface. How is it then that ablating this phosphorylation site prevents \(\alpha_4\) from being phosphorylated but still causes the mutant protein to be inserted at a faster rate? One answer to this question is that mutation of Ser\textsuperscript{443} to an alanine results in phosphorylation mimic of the \(\alpha_4\) subunit. That is to say that masking the hydroxyl group that is normally found in a serine residue by removing it, which is what we do when we replace this residue with an alanine, is tantamount to masking it by covalently attaching a phosphate group. Both situations may lead to similar protein conformational changes that result in the \(\alpha_4\) subunit being more stable and therefore being inserted at a faster rate. We can further draw this conclusion from our electrophysiological studies, which suggest a similar occur-

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