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Enhancement of Recombinant $\alpha1\beta1\gamma2L$ γ-Aminobutyric $Acid_A$ Receptor Whole-Cell Currents by Protein Kinase C Is Mediated through Phosphorylation of Both $\beta1$ and $\gamma2L$ Subunits

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SUMMARY

The γ-aminobutyric $acid_A$ $(GABA)_A$ receptor (GABAR) $\beta1$ and $\gamma2L$ subtypes have been shown to be phosphorylated *in vitro* by protein kinase C (PKC) [*J. Biol. Chem.* **267**:14470–14476 (1992); *Neuron* **12**:1081–1095 (1994)]. To determine the physiological consequences of phosphorylation of GABAR isoforms containing the $\beta1$ and $\gamma2L$ subtypes, the specific serine residues phosphorylated by PKC ($\beta1$ S409, $\gamma2L$ S327 and S343) were changed to alanines through site-directed mutagenesis. Wild-type ($\alpha1\beta1\gamma2L$ GABARs) and three mutant GABAR isoforms [$\alpha1\beta1\gamma2L$(S327A, S343A), $\alpha1\beta1$(S409A)$\gamma2L$, and $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs) were expressed in mouse L929 fibroblasts through transient cotransfection. Recordings were obtained from each cell with the use of the whole-cell patch-clamp technique. The initial recording was made with the use of control intrapipette solution, and a second recording from the same cell was obtained with pipettes containing either constitutively active PKC [protein kinase M (PKM)] or control solution to obtain paired GABA concentration-response relationships. All GABAR isoforms studied had equivalent maximal GABA currents and similar GABA concentration-response profiles under the control condition. Intracellular PKM treatment increased the maximal current and EC_{50} value in cells expressing wild-type GABARs. However, PKM reimpalement did not significantly change these parameters in cells expressing any of the mutant GABAR isoforms, indicating that the mutation of either the $\beta1$ or $\gamma2L$ subtype alone was sufficient to prevent enhancement of GABAR current by PKM. No significant changes were obtained during control reimpalement recordings of wild-type or mutant receptors. Furthermore, PKM treatment did not alter the time constants of GABA current desensitization kinetics measured from cells expressing wild-type or mutant receptors. These data thus suggest that PKC phosphorylation of the $\beta1$ and $\gamma2L$ subtypes enhances GABAR current and that both subtypes are required for complete PKC-mediated enhancement of $\alpha1\beta1\gamma2L$ GABAR current.

The GABAR is a ligand-gated Cl^- channel that is distributed widely throughout the central nervous system (1). Different subunit families of GABAR (α, β, γ, δ, ρ) and multiple subtypes of each family ($\alpha1$–6, $\beta1$–4, $\gamma1$–4, $\rho1$–2) have been cloned (2, 3). Each subtype is thought to contain an amino-terminal extracellular domain, four membrane spanning domains, and a large and highly variable cytoplasmic domain between the third and fourth transmembrane domains, and GABARs are thought to be composed of combinations of five subunit subtypes (4). The GABAR is regulated by a number of extracellular allosteric agents, including benzodiazepines, barbiturates, steroids (4), alcohol (5), and cations (6). The GABAR also seems to be regulated by intracellular protein phosphorylation.

Protein phosphorylation is an important post-translational mechanism for regulation of voltage-gated (7) and ligand-gated (8, 9) ion channels. Many GABAR subtypes contain consensus sites for phosphorylation in the large intracellular loop between the proposed third and fourth transmembrane domains (10). Highly purified GABAR proteins, fusion proteins, or synthetic peptides composing the major intracellular domain of GABAR subunits have been shown to be phosphorylated by exogenously added PKA (11–14), PKC (11, 13, 15), CaM kinase II (15, 16), cGMP-dependent protein kinase

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ABBREVIATIONS: GABAR, γ-aminobutyric acid type A receptor; GABA, γ-aminobutyric acid; PKC, Ca^{2+}/phospholipid-dependent protein kinase; PKA, cAMP-dependent protein kinase; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; PKM, constitutively active PKC; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; I_{max}, maximal GABA response; I_{GABA}, normalized peak whole-cell current evoked at the given GABA concentration *x*; $I_{GABApeak}$, peak current amplitude evoked by 300 μM GABA; $I_{GABAend}$, remaining current at the end of 5-sec GABA application.

(16), tyrosine kinase (17), and an unknown receptor-associated kinase (18), suggesting that GABAR function may also be regulated by protein phosphorylation. *In vitro*, the large intracellular domain of the β1 subtype was phosphorylated on Ser409 by both PKA and PKC (11), and the intracellular domain of the γ2L subtype was phosphorylated on Ser327 and Ser343 by PKC (11, 19). Phosphorylation was prevented through site-directed mutagenesis of these specific serine residues to alanines (11, 20).

Although several lines of evidence point to a role of protein phosphorylation in the regulation of GABAR function, the results obtained from functional studies are conflicting. PKA has been shown to reduce GABAR function in mouse spinal cord neurons (21) and brain microsacs (22); to potentiate GABA-induced currents in cerebellar Purkinje neurons (23, 24), retinal neurons (25, 26), and transfected Cα12 cells (27); or to produce no effect in cultured spinal neurons (28). Phorbol esters that activate PKC produced no effect on $^{36}Cl^-$ uptake in cultured spinal neurons (28) but decreased $^{36}Cl^-$ uptake in mouse cerebellar membrane vesicles (29) and reduced recombinant GABAR currents expressed in *Xenopus* oocytes (20, 29–33) and in human embryonic kidney cells (20). In contrast, it has been shown that GABAR function was maintained through phosphorylation and destabilized through a Ca^{2+}-dependent dephosphorylation process (34–38). Consistent with the latter findings, we demonstrated that direct intracellular application of PKM (39) enhanced recombinant α1β1γ2L GABAR currents expressed in mouse fibroblast cells (40).

To determine whether acute enhancement of GABAR currents resulted from direct PKC phosphorylation of the β1 and/or γ2L subtypes, bovine α1 and wild-type or mutant β1 and γ2L GABAR subtypes were transiently coexpressed in mouse L929 fibroblasts, and GABAR whole-cell currents were recorded from the transfected cells. The actions of PKM on GABAR currents obtained in individual cells were determined with the use of a reimpalement protocol (40) in which sequential recordings were obtained from individual cells. The initial recording was obtained with a pipette containing a control solution, and the second recording was obtained with a control or a PKM-containing recording pipette. Thus, with this technique, each cell provided its own control. On the other hand, the maximal control current amplitudes were compared to determine whether there were functional differences among wild-type and mutant receptors. The results demonstrated that PKC phosphorylation of both β1 and γ2L subtypes was required to produce a full enhancement of GABAR current, and PKC did not alter the kinetics of GABAR desensitization. There were no significant differences in the basic functional properties among wild-type and mutant receptors.

Materials and Methods

Construction of expression vectors containing GABAR subunit cDNAs. Full-length cDNAs encoding the bovine α1, β1, and γ2L subtypes of GABARs (10, 41) were kindly provided by Dr. E. Barnard (Medical Research Council, London, UK). Expression plasmids were constructed as described previously (42). The cDNAs were released from the original plasmids with appropriate restriction enzymes (BRL, Bethesda, MD). These cDNAs contained the complete open reading frames and ~10–100 base pairs of the 5′ and 3′ untranslated sequences. Site-directed mutagenesis was performed as described by Zoller and Smith (43) using the following primers to make substitutions: Ser409 to alanine (β1 subtype), GCCGCTCAGCTCAAAGTCA; Ser343 to alanine (γ2L subtype), CCTTGAAGGCAAACATCCGAAG; and Ser327 to alanine (γ2L subtype), TTGTCTTTGTCCTTGGCTGGTTTCCGGTTGCTG. Fidelity of the final expression constructs was verified with DNA sequencing. All cDNAs were then subcloned into the *Bgl*II site of the mammalian expression vector pCMVNeo (44). A marker gene *LacZ* encoding for β-galactosidase was also subcloned into expression vector pCMVNeo to form pCMVβGal (42).

Cell line and transient transfection. A mouse fibroblast cell line L929 (American Type Culture Collection, Rockville, MD) was used in this study. Cells were grown and transfected according to a modified calcium phosphate precipitation method (45) as described previously (40). Electrophysiological recordings of cells expressing recombinant GABARs with the configurations α1β1γ2L, α1β1γ2L(S327A, S343A), α1β1(S409A)γ2L, and α1β1(S409A)γ2L(S327A, S343A) were carried out 48–72 hr after transfection.

Identification of transfected cells and whole-cell recording. To detect the expression of the marker gene (*LacZ*) product β-D-galactosidase in individual cells, fluorescein di-β-D-digalactopyranoside (Molecular Probes, Eugene, OR) staining was performed on living L929 cells immediately before electrophysiological recordings. Positively transfected cells were identified as described previously (42). The culture dish was then rinsed several times and finally bathed in 2 ml of extracellular bath solution with the following composition: 142 mM NaCl, 8 mM KCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4. The intrapipette recording solution consisted of 153 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, and 2 mM Mg^{2+}ATP, pH 7.3. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The combination of external and internal solutions resulted in an equilibrium potential of 0 mV for chloride ions and −75 mV for potassium ions. Whole-cell patch-clamp recording (46) was used to record GABAR currents from transfected L929 cells. GABAR currents were obtained with a List L/M EPC-7 amplifier (List Medical Instruments, Darmstadt, Germany) and recorded on a Sony SL-2700 VCR (modified to 0–20 kHz) via a Sony PCM-501ES digital audio processor (14-bit, 44 kHz). Responses were then low-pass filtered (3 dB, 1 kHz) with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and simultaneously recorded on a chart recorder (Gould, Cleveland, OH) and on a computer using Axotape data-acquisition software at 20 Hz (Axon Instruments, Foster city, CA). All cells were voltage-clamped at −75 mV during the recordings.

Drug application. GABA used to evoke chloride current was diluted from a frozen 10 mM stock with extracellular bath solution to the desired concentrations. To determine the GABA concentration-response relationship, a modified U-tube (multipuffer) application system was used. GABA was applied for 5–10 sec each time by stopping a suction pump via a solenoid valve driver (General Valve, Fairfield, NJ), and the application was terminated by reopening the valve. The 10–90 rise time of this system was 70 msec. During recordings, desired concentrations of GABA, ranging from 0.1–300 μM, were sequentially applied at 1-min intervals. The tip diameter of drug puffers was ~60 μm, and the tips were positioned ~50 μm from the recorded cell.

Preparation and application of PKM. PKC was isolated and purified from bovine brain (13, 47). The catalytic form of PKC, PKM, was prepared through proteolysis of PKC followed by repurification as described previously (40). PKM was stored at −70° and diluted to 40 nM with internal recording solution immediately before use. PKM was dialyzed into cells through recording pipettes after rupture of cell membranes. To allow sufficient time for PKM to enter the cell, application of GABA was delayed ~1 min after rupture of the cell membrane.

Reimpalement protocol. GABAR whole-cell current amplitudes evoked from different cells varied over a wide range. Therefore, a same-cell reimpalement recording protocol was performed to control

the variability among cells (40) and to enable paired comparisons (48). Two sequential recordings were obtained from the same cell, and GABA concentration-response relationships were obtained under control and PKM-treated condition, respectively. The initial recording was performed with a control intracellular solution-filled pipette, and increasing concentrations of GABA were applied. The recording pipette was removed gently after the initial recording. After a recovery period of ≥15 min, the reimpalement recording was carried out in the same cell with a control solution-filled or a PKM (40 nM)-containing pipette. The GABA concentration-response relationship was determined as described previously. The currents obtained from the same cell during two sequential control recordings (control/control) were then compared to determine the feasibility of reimpalement procedure, and the currents obtained from the same cell under two sequential control and PKM-treated conditions (control/PKM) were compared to determine the effects of PKM. Three to 11 transfected L929 cells expressing each of the four different compositions of GABARs were investigated according to this protocol.

Analysis and statistics. Peak whole-cell currents were normalized to the largest GABAR current response (taken as 100%) obtained during initial control recording of each cell and expressed as a percentage of the largest current. Nonlinear curve fitting (Prism, GraphPad, San Diego, CA) was used to estimate parameters by fitting to a four-parameter logistic equation: $I_{GABA} = I_{max}/[1 + (EC_{50}/x)^n]$, where I_{GABA} is the normalized peak whole-cell current evoked at the given GABA concentration x, I_{max} is the maximal GABA response, EC_{50} is the GABA concentration eliciting a half-maximal response, and n is the Hill coefficient. Fittings were made with either original data obtained in each cell or mean data pooled from all cells expressing the same GABAR compositions, whichever was appropriate. The normalized peak whole-cell current difference scores caused by PKM were obtained by subtracting data obtained under control condition from data obtained under PKM-treated condition from the same cell at the same GABA concentration. The acute desensitization rate was estimated by calculating the percentage of desensitization in control/PKM-reimpaled or control/control-reimpaled cells according to the following formula: Desensitization (%) = $\{(I_{GABApeak} - I_{GABAend})/I_{GABApeak}\} \times 100\%$, where $I_{GABApeak}$ is the peak current amplitude evoked by 300 μM GABA, and $I_{GABAend}$ is the remaining current at the end of 5-sec GABA application. On the other hand, time constants of current decay during 300 μM GABA application were obtained by fitting 9–15 cells expressing the same receptor configurations with a single exponential function. Data were averaged and presented as mean ± standard error.

The effects of PKM on I_{max}, EC_{50}, Hill coefficient, percentage of current difference, percentage of desensitization, and time constants of current decay obtained from individual cells expressing the same GABAR configuration were compared with the use of Student's paired two-tailed t tests, one-sample t tests, or unpaired t tests, depending on which was appropriate. Significance was assumed at a probability level of $p < 0.05$. The nonparametric one-way Kruskal-Wallis tests and posthoc tests corrected with a Bonferroni method ($m = 4$, where m is the number of comparisons made between groups) were used to compare the effects of PKM among wild-type and mutant GABARs; the parametric one-way analyses of variance followed by Bonferroni's multiple-comparison tests were used to compare the average maximal control current amplitudes and time constants of control current decay among receptor isoforms. The majority of the analyses were performed with Instat version 2.0 (GraphPad).

Results

PKM enhanced the maximal GABA-evoked whole-cell currents in transfected L929 cells expressing wild-type recombinant α1β1γ2L GABARs. To confirm that PKM enhanced GABAR current (40), wild-type α1β1γ2L GABARs were expressed in mouse L929 fibroblasts by transient cotransfection with α1, β1, and γ2L subtype cDNAs. For this and all subsequent experiments, cells were voltage-clamped to −75 mV in symmetrical chloride solutions at room temperature during whole-cell recordings, and a reimpalement protocol was used (see Materials and Methods for details). PKM (40 nM) was applied intracellularly via recording pipettes during the reimpalement recording in individual cells. Application of GABA (0.1–300 μM) with the multipuffer system evoked inward currents that increased in a concentration-dependent manner (Fig. 1, A and B). In the cell shown in Fig. 1A, current amplitudes recorded at low GABA concentrations during the PKM-treated reimpalement recording were comparable to the corresponding control current amplitudes. However, at GABA concentrations of >10 μM, currents

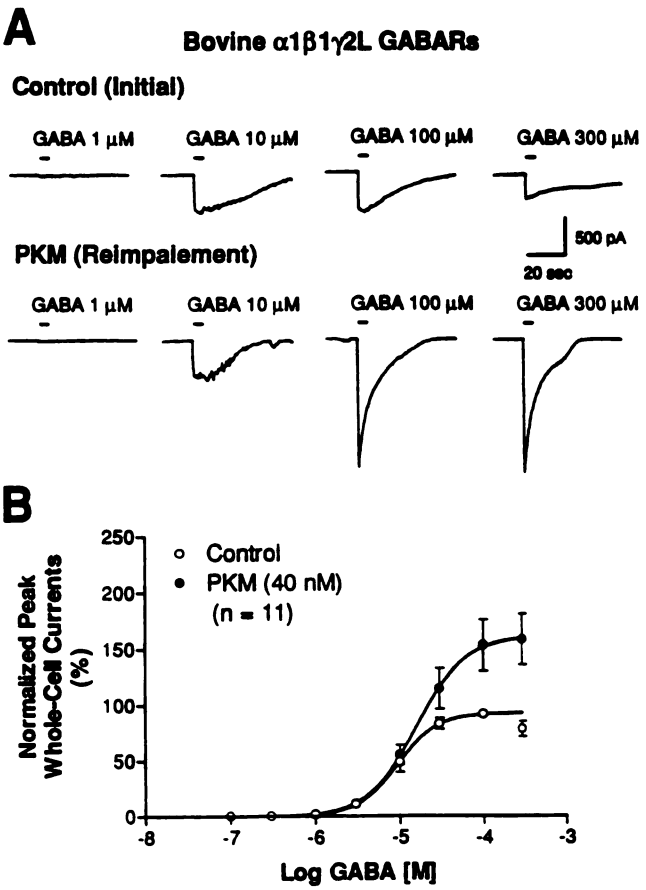

Fig. 1. Wild-type α1β1γ2L GABAR whole-cell currents were enhanced in the presence of PKM. A, GABA-evoked whole-cell current traces obtained from a transfected L929 cell expressing functional α1β1γ2L GABARs. Two sequential recordings of whole-cell GABAR currents were performed on this cell according to a reimpalement protocol. GABA (0.1–300 μM) was applied with a multipuffer drug-application system at 5-sec durations. *Top*, currents obtained during the control recording (initial). *Bottom*, currents obtained during PKM (40 nM)-treated recording (reimpalement). For this and all subsequent experiments, recordings were made 48 hr after transfection, and cells were held at −75 mV. B, Normalized concentration-response curves for GABA-evoked whole-cell currents obtained from transfected L929 cells in the absence and presence of PKM. ○, Average peak whole-cell currents obtained during the initial control recording. ●, Average currents obtained during the reimpalement recording with PKM (40 nM) (mean ± standard error, 11 cells). All currents were normalized to the largest control current in individual cells. These curves were fitted to the logistic equation with the use of nonlinear regression.

were increased in the presence of PKM (Fig. 1A). The largest whole-cell current was 540 pA at 100 μM GABA in the control condition (Fig. 1A, *top*) and 1600 pA at 300 μM GABA in the presence of PKM (Fig. 1A, *bottom*), respectively. Eleven cells expressing α1β1γ2L GABARs were investigated.

PKM changed the concentration-response relationship of wild-type α1β1γ2L GABARs (Fig. 1B). The major effect of PKM was to increase the current amplitudes at GABA concentrations above the EC_{50} value. The I_{max}, Hill coefficient, and EC_{50} values obtained through fitting of the concentration-response curves for individual cells were averaged and were 102 ± 2%, 2.4 ± 0.3, and 13.1 ± 2.2 μM in the initial control condition and 174 ± 23%, 2.6 ± 0.8, and 21.7 ± 5.0 μM in the PKM-treated reimpalement condition, respectively (Table 1, *first row*). PKM treatment significantly increased I_{max} ($p < 0.01$) and EC_{50} ($p < 0.05$) (paired Student's *t* test; Table 1, *first row*). Significant differences between control and PKM-treated data are given in Table 1. The effect of PKM in enhancing the maximal wild-type α1β1γ2L GABAR current was consistent with our previous report (40) that PKM increased the apparent efficacy but decreased the apparent affinity of GABA for these receptors. The effect of PKM in enhancing the maximal GABAR current was thus GABA concentration dependent (Fig. 1B).

On the other hand, reimpalement recordings in the absence of PKM did not seem to produce comparable changes in GABAR current (Fig. 2, A and B). Two sequential control recordings were performed with a reimpalement protocol to determine whether the reimpalement procedure produced GABAR current enhancement independent of PKM (three cells). In the example shown (Fig. 2A), the largest whole-cell current was 1893 pA in the initial control condition (*top*) and 1065 pA in the reimpalement control condition (*bottom*). The largest GABAR whole-cell current was not increased but instead was reduced during the control reimpalement recording in this cell (Fig. 2A). The peak whole-cell currents were then normalized and fitted in individual cells. The averaged I_{max}, Hill coefficient, and EC_{50} values were 101 ± 0%, 2.4 ± 0.4, and 8.2 ± 2.7 μM in the initial control condition and 43 ± 7%, 2.1 ± 0.1, and 4.3 ± 1.1 μM in the control reimpalement condition (Fig. 2B). The I_{max} was significantly reduced in the reimpalement control condition ($p < 0.5$). Thus, the reimpalement protocol did not produce enhancement of the maximal GABAR current amplitude or EC_{50} in cells expressing wild-type receptors without the inclusion of PKM.

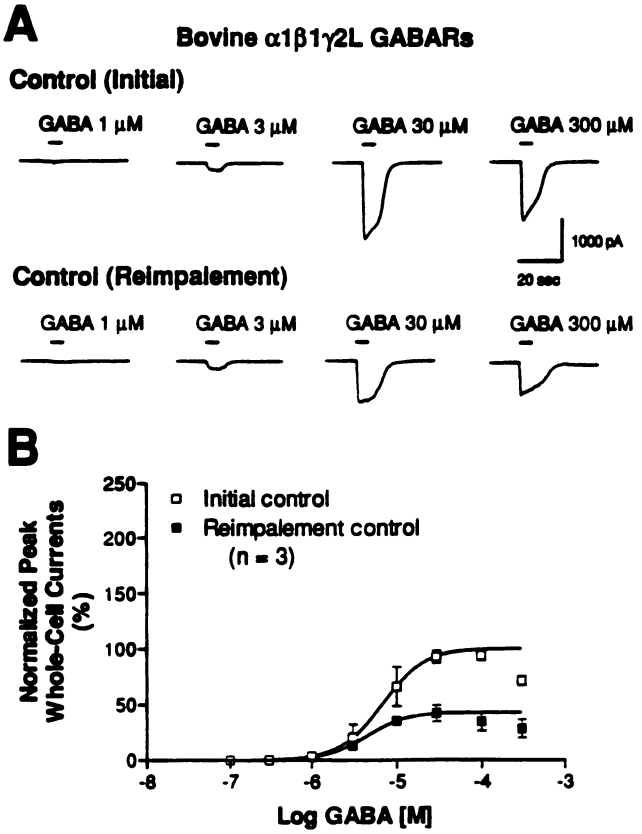

Fig. 2. Wild-type α1β1γ2L GABAR whole-cell currents were not enhanced during reimpalement recordings in the absence of PKM. A, Whole-cell current traces obtained from a transfected L929 cell expressing functional α1β1γ2L GABARs. Two sequential recordings of whole-cell GABAR currents were performed on this cell according to a reimpalement protocol. *Top*, GABAR whole-cell currents obtained during the initial control recording. *Bottom*, currents obtained during the control reimpalement recording in the absence of PKM. B, Normalized concentration-response curves for GABA-evoked whole-cell currents obtained from L929 cells expressing α1β1γ2L GABARs during the two sequential control recordings. □, Average currents obtained during the initial control recording. ■, Average currents obtained during the control reimpalement recording (means ± standard error, three cells). Reimpalement recordings in the absence of PKM did not result in increases in GABAR currents as seen in Fig. 1.

Mutation of consensus phosphorylation-site serines to alanines in both β1 and γ2L subtypes eliminated the enhancement of GABAR currents produced by PKM

TABLE 1

Effects of PKM on the normalized concentration-response relationships for GABA in transfected L929 cells expressing wild-type and mutant GABARs

All I_{max}, EC_{50}, and Hill coefficient numbers were determined by individual fits of transfected cells in each group expressing the same receptor configurations. These values were averaged and are presented as mean ± standard error. Numbers in parentheses represent the number of cells examined. PKM was applied intracellularly at a concentration of 40 nM during reimpalement recording. Parameters were compared in individual cells by paired Student's *t* tests, although the pooled, average data are shown in this table.

GABAR subunit composition	I_{max}		EC_{50}		Hill coefficient	
	Control	PKM	Control	PKM	Control	PKM
	%		μM			
α1β1γ2L (11)	102 ± 2	174 ± 23[a]	13.1 ± 2.2	21.7 ± 5.0[b]	2.4 ± 0.3	2.6 ± 0.8
α1β1 (S409A)γ2L(S327A,S343A) (7)	101 ± 0	103 ± 29	16.7 ± 5.0	15.5 ± 4.3	2.0 ± 0.2	3.0 ± 1.3
α1β1γ2L(S327A,S343A) (8)	101 ± 1	135 ± 20	20.4 ± 3.8	18.4 ± 2.4	2.3 ± 0.4	1.7 ± 0.1
α1β1(S409A)γ2L (8)	101 ± 0	118 ± 26	18.3 ± 4.7	12.8 ± 2.5	3.7 ± 0.7	2.3 ± 0.2

[a] $p < 0.01$.
[b] $p < 0.05$.

treatment in transfected L929 cells. To determine whether the enhancement of recombinant $\alpha1\beta1\gamma2L$ GABAR whole-cell current by PKM treatment was due to direct PKM phosphorylation of the GABAR, site-directed mutagenesis was performed to substitute alanine for serine residues at the three consensus phosphorylation sites in the $\beta1$ and $\gamma2L$ subtypes. Functional $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs were then expressed in L929 fibroblasts through transient cotransfection of cDNAs encoding for bovine $\alpha1$, $\beta1$(S409A), and $\gamma2L$(S327A, S343A) subtypes. Whole-cell currents from one transfected cell during the initial control recording (Fig. 3A, *top*) followed by the PKM-treated reimpalement recording (Fig. 3A, *bottom*) are shown. Application of GABA (0.1–300 μM) evoked inward currents that increased in a concentration-dependent manner (Fig. 3, A and B). In this cell, whole-cell currents recorded during the PKM-treated reimpalement recording (Fig. 3A, *bottom*) were either smaller or similar in amplitude to the corresponding control currents (Fig. 3A, *top*) for all GABA concentrations. The largest peak whole-cell current was 2500 pA in control (Fig. 3A, *top*) and 2700 pA in the presence of PKM (Fig. 3A, *bottom*). Seven cells expressing $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs were studied, and the peak whole-cell currents were normalized and fitted individually. The I_{max}, Hill coefficient, and EC_{50} values were 101 ± 0%, 2.0 ± 0.2 and 16.7 ± 5.0 μM in the control condition and 103 ± 29%, 3.0 ± 1.3, and 15.5 ± 4.3 μM in the PKM-treated reimpalement condition, respectively (Table 1, *second row*). There were no apparent differences between the GABAR currents obtained from wild-type and mutant $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs in the absence of PKM. In individual cells expressing $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs, PKM produced no effect on I_{max}, EC_{50}, or Hill coefficient values (paired Student's *t* test; Table 1, *second row*). The effect of PKM on GABAR current was eliminated through mutations made on both the $\beta1$ and $\gamma2L$ subtypes. These data indicated that the enhancement of $\alpha1\beta1\gamma2L$ GABAR current by PKM resulted from phosphorylation of one or more serines on the $\beta1$ and/or $\gamma2L$ subtypes.

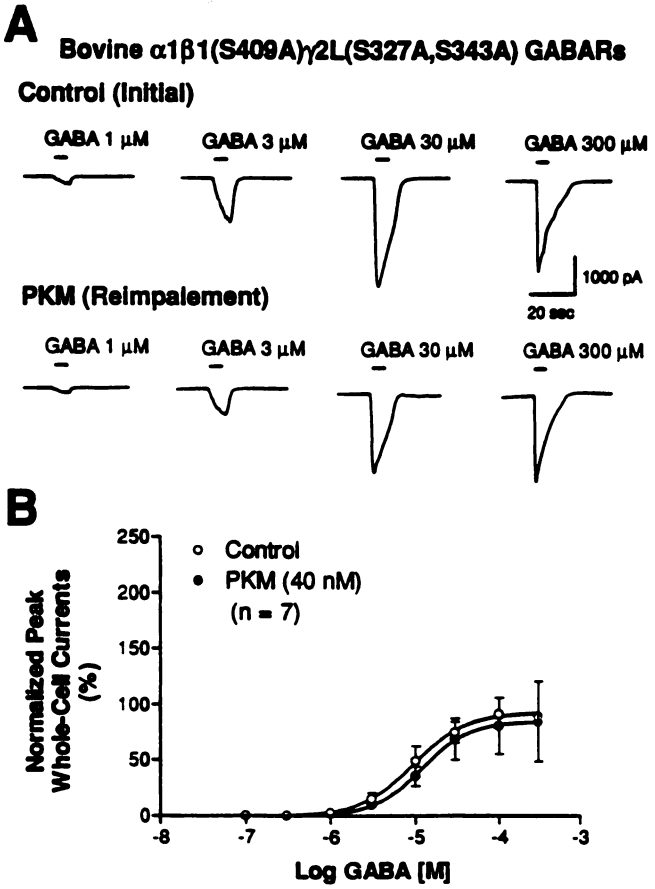

Fig. 3. Enhancement of GABAR whole-cell currents by PKM was completely prevented when all three PKC phosphorylation sites contained in the $\beta1$ and $\gamma2L$ subtypes were mutated from serine to alanine residues. A, Whole-cell current traces obtained from a transfected L929 cell expressing $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs. Two sequential recordings of whole-cell GABAR currents were performed on this cell according to a reimpalement protocol. *Top*, GABAR whole-cell currents obtained during the control recording (initial). *Bottom*, currents obtained in the presence of PKM (40 nM). B, Normalized concentration-response curves for GABA-evoked whole-cell currents obtained from L929 cells expressing $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs in the absence and presence of PKM. ○, Average currents obtained during the initial control recording. ●, Average currents obtained during the reimpalement recording with PKM (mean ± standard error, seven cells). PKM did not enhance GABAR whole-cell currents after substitution of alanines for serines at the three PKC phosphorylation sites contained in the $\beta1$ and $\gamma2L$ subtypes.

Reimpalement recordings were also performed in the absence of PKM on a separate group of four cells expressing $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs to determine whether the reimpalement procedure alone produced any effect on these mutant receptors. In the cell shown in Fig. 4A, the largest peak whole-cell current was 1839 pA in initial control condition (Fig. 4A, top) and 652 pA in the reimpalement control condition (Fig. 4A, bottom). The normalized I_{max}, Hill coefficient, and EC_{50} values were 104 ± 1%, 1.6 ± 0.2, and 20.5 ± 5.8 μM in the initial control condition and 83 ± 19%, 1.7 ± 0.2, and 14.7 ± 5.6 μM in the control reimpalement condition, respectively (Fig. 4B). No significant changes in the maximal GABAR current or EC_{50} were obtained during control reimpalement recording of mutant $\alpha1\beta1$(S409A)$\gamma2L$(S327A, S343A) GABARs.

Mutation of $\beta1$ subtype Ser409 or $\gamma2L$ subtype Ser327 and Ser343 to alanines also eliminated the enhancement of GABAR currents produced by PKM treatment in transfected L929 cells. The role of the $\gamma2L$ subtype in regulation of the GABARs by PKM phosphorylation was determined by expressing $\alpha1\beta1\gamma2L$(S327A, S343A) GABARs in L929 fibroblasts. Whole-cell currents were recorded from transfected L929 cells expressing $\alpha1\beta1\gamma2L$(S327A, S343A) GABARs. Application of GABA (0.1–300 μM) evoked inward currents that increased in a concentration-dependent manner (Fig. 5A). The currents recorded during the PKM-treated reimpalement recording were slightly increased at higher GABA concentrations compared with the corresponding control currents (Fig. 5A). However, the magnitude of the effect of PKM was smaller than that obtained in cells expressing wild-type $\alpha1\beta1\gamma2L$ GABARs (Fig. 1B). The averaged I_{max}, Hill coefficient, and EC_{50} values were 101 ± 1%, 2.3 ± 0.4, and 20.4 ± 3.8 μM in the control condition and 135 ± 20%, 1.7 ± 0.1, and 18.4 ± 2.4 μM in the PKM-treated reimpalement condition, respectively (eight cells; Table 1, *third row*). With the use of paired Student's *t* test, I_{max}, EC_{50}, and Hill coefficients were not significantly altered in these cells in the presence of PKM. These data indicated that the mutations removing the two

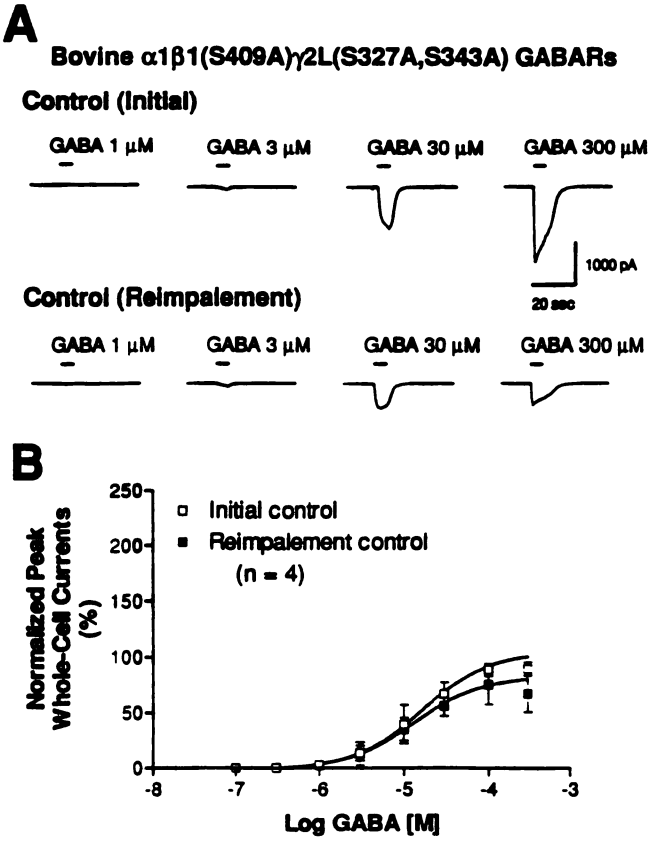

Fig. 4. Mutant $\alpha1\beta1$(S409A)$\gamma2$L(S327A, S343A) GABAR whole-cell currents were not enhanced during reimpalement recordings in the absence of PKM. A, Whole-cell current traces obtained from a transfected L929 cell expressing functional $\alpha1\beta1$(S409A) $\gamma2$L(S327A, S343A) GABARs. Two sequential recordings of whole-cell GABAR currents were performed on this cell according to a reimpalement protocol. *Top*, GABAR whole-cell currents obtained during the initial control recording. *Bottom*, currents obtained during the control reimpalement recording in the absence of PKM. B, Normalized concentration-response curves for GABA-evoked whole-cell currents obtained from cells expressing $\alpha1\beta1$(S409A)$\gamma2$L(S327A, S343A) GABARs during the two sequential control recordings. □, Average currents obtained during the initial control recording. ■, Average currents obtained during the control reimpalement recording (mean ± standard error, four cells). Reimpalement recordings of cells expressing $\alpha1\beta1$(S409A)$\gamma2$L(S327A, S343A) GABARs in the absence of PKM did not result in increases in current amplitudes or EC_{50}.

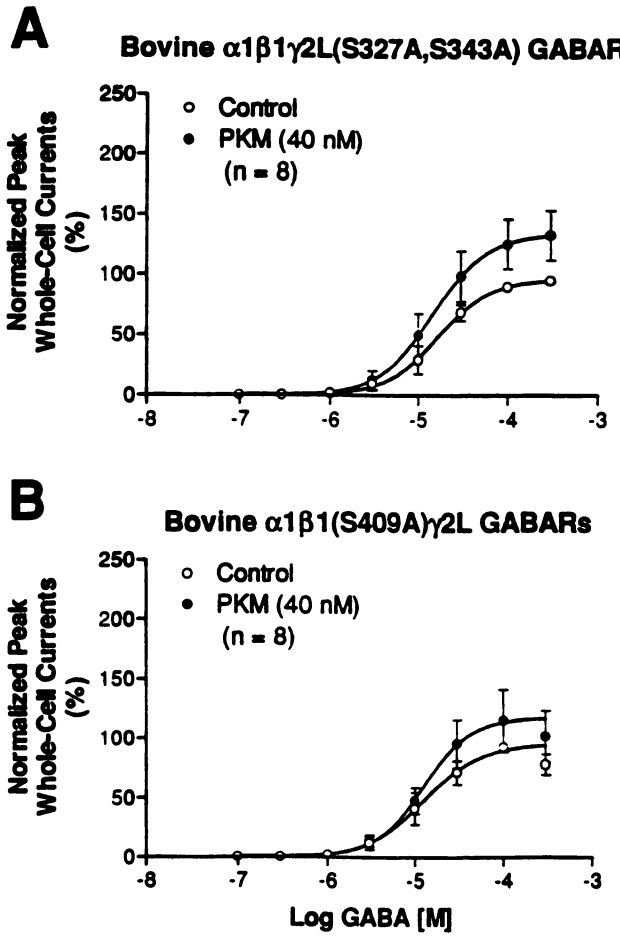

Fig. 5. Mutation of PKC phosphorylation site or sites on the $\beta1$ or $\gamma2$L subtype alone prevented GABAR current enhancement by PKM. A, Normalized concentration-response curves for GABA-evoked whole-cell currents obtained from transfected L929 cells expressing $\alpha1\beta1\gamma2$L(S327A, S343A) GABARs cells in the absence and presence of PKM (40 nM) according to a reimpalement protocol. Values are mean ± standard error of currents from individual cells (eight cells). Enhancement of GABAR whole-cell currents by PKM was blocked by mutation of the $\gamma2$L subtype phosphorylation-site serines (Ser327 and Ser343) to alanines. B, Normalized concentration-response curves for GABA-evoked whole-cell currents obtained from transfected L929 cells expressing $\alpha1\beta1$(S409A)$\gamma2$L GABARs cells in the absence and presence of PKM (eight cells). Enhancement of GABAR whole-cell currents by PKM was blocked by mutation of the $\beta1$ subtype phosphorylation-site serine (Ser409) to alanine. ○, average currents obtained during the initial control recording; ●, average currents obtained during the reimpalement recording with PKM.

potential phosphorylation sites from the $\gamma2$L subtype (Ser327 and Ser343) effectively blocked the effect of PKM even though the phosphorylation site in the $\beta1$ subtype was available (Table 1, *third row*). On the other hand, control reimpalement recording of cells that expressed $\alpha1\beta1\gamma2$L(S327A, S343A) GABARs were also performed to determine whether the intermediate but statistically insignificant increase of the maximal GABAR current would appear in the absence of PKM during reimpalement recordings (three cells). Reimpalement recordings in the absence of PKM did not significantly change the maximal GABAR whole-cell current (initial control versus reimpalement control: 101 ± 3% versus 54 ± 12%) or EC_{50} from its initial control values. The maximal GABAR whole-cell current was not increased but instead was reduced during the control reimpalement recording. These results indicated that in the absence of PKM, reimpalement recording did not produce an increase in the whole-cell current evoked from cells expressing $\alpha1\beta1\gamma2$L(S327A, S343A) GABARs.

The role of the $\beta1$ subtype in regulation of the GABARs by PKM phosphorylation was determined by expressing $\alpha1\beta1$(S409A)$\gamma2$L GABARs in L929 fibroblasts. Application of GABA (0.1–300 μM) again evoked inward currents that increased in a concentration-dependent manner (Fig. 5B). Currents recorded during the PKM-treated reimpalement recording were minimally increased compared with their corresponding controls obtained during the initial control recording (Fig. 5B). The I_{max}, Hill coefficient, and EC_{50} values were 101 ± 0%, 3.7 ± 0.7, and 18.3 ± 4.7 μM in the control condition and 118 ± 26%, 2.3 ± 0.2, and 12.8 ± 2.5 μM in the PKM-treated reimpalement condition, respectively

(eight cells; Table 1, *fourth row*). Paired Student's *t* test revealed that PKM did not significantly alter I_{max}, EC_{50}, or Hill coefficient values in these cells. These data thus indicated that the mutation removing the potential phosphorylation site (Ser409) from the β1 subtype effectively blocked the enhancement effect of PKM even though the sites in the γ2L subtype were available (Table 1, *fourth row*). Similar control reimpalement recordings as described earlier were also carried out on cells expressing these receptor isoforms to ensure that there was no enhancement produced by reimpalement procedure itself (three cells). Reimpalement recordings in the absence of PKM did not significantly alter I_{max} (initial control versus reimpalement control: 104 ± 2% versus 113 ± 18%), EC_{50}, or Hill coefficient values from its initial control values. These results indicated that in the absence of PKM, reimpalement recording was not able to cause an increase in the whole-cell current evoked from cells expressing α1β1(S409A)γ2L GABARs.

Both β1 and γ2L subtypes were required for maximal PKM-mediated enhancement of GABAR function, and the consequences of phosphorylation of the β1 and γ2L GABAR subtypes were similar. Paired comparisons between the initial control and the PKM-treated reimpalement values showed that PKM did not significantly increase the maximal GABA current or EC_{50} (Table 1 and Figs. 3 and 5) in cells expressing GABARs that contained serine-to-alanine mutations on the β1, γ2L, or both β1 and γ2L subtypes. The functional consequences of PKM phosphorylation of the β1 and γ2L subtypes were further determined by comparing the percentage changes in whole-cell currents obtained in cells expressing four configurations of α1β1γ2L GABARs. Percent changes were the difference values between normalized currents obtained during control and PKM-treated recordings at corresponding GABA concentration in the same cell (see Materials and Methods). In cells expressing wild-type α1β1γ2L GABARs, the percentage changes in whole-cell currents became statistically significant only at GABA concentrations of >30 μM but not at lower concentrations (one-sample *t* test, data not shown). The extent of the enhancement produced by PKM seemed to be GABA concentration dependent (Fig. 1, A and B). In contrast, in cells expressing α1β1(S409A)γ2L(S327A, S343A) GABARs, the percentage changes were not significant at any GABA concentration examined (one-sample *t* test, data not shown), and elimination of the PKM effect on GABAR current seemed to be complete. In cells expressing α1β1γ2L(S327A, S343A) or α1β1(S409A)γ2L GABARs, PKM produced no significant changes in their GABA concentration-response relationships (one-sample *t* test, data not shown), although there seemed to be some residual effects (Table 1 and Fig. 5). Use of a nonparametric one-way analysis (Kruskal-Wallis test) of the percentage changes in whole-cell currents followed by corrected posthoc tests revealed that percentage changes produced by PKM were indistinguishable between any two mutant receptor configurations. These results thus indicated that phosphorylation of the β1 or γ2L subtype alone did not enhance the GABAR current significantly. Full enhancement of GABAR current by PKM required phosphorylation of both β1 and γ2L subtypes, and the functional consequences of phosphorylation of the β1 and γ2L subtypes separately were indistinguishable.

The maximal currents obtained in the absence of PKM were not distinguishable among cells expressing wild-type and mutant α1β1γ2L GABARs. As shown earlier, the normalized GABA concentration-response profiles obtained from cells expressing wild-type and mutant α1β1γ2L GABARs under initial control condition were similar (Table 1). To further determine whether there were functional differences among wild-type and mutant receptor isoforms, absolute maximal control current amplitudes (in pA) measured from cells expressing these receptors were compared. Additional cells treated with or without PKM were included for this comparison. The maximal current amplitudes obtained during control recordings of individual cells were averaged and were 1285 ± 243, 1382 ± 294, 1410 ± 307, and 1565 ± 340 pA for cells expressing α1β1γ2L (17 cells), α1β1(S409A)γ2L(S327A, S343A) (17 cells), α1β1γ2L(S327A, S343A) (17 cells), and α1β1(S409A)γ2L (19 cells) GABARs, respectively. No significant differences of the control current amplitudes were found between the wild-type and any of the mutant GABARs (one-way analysis of variance followed by multiple-comparison tests).

PKM did not alter the kinetics of desensitization in cells expressing wild-type or mutant α1β1γ2L GABARs. Acute desensitization rates were determined by analyzing the percentage of current desensitization evoked by 5-sec applications of 300 μM GABA. The four groups of cells presented in Table 1 were examined. For cells expressing wild-type receptors, the percentage of desensitization of GABAR whole-cell currents was 11.6 ± 3.3% in the initial control condition and 15.6 ± 3.2% in the PKM-treated reimpalement condition, which corresponded to desensitization rates of 2.3%/sec and 3.1%/sec, respectively. No significant differences were found (paired Student's *t* test). The percentage of desensitization values were 11.6 ± 2.2% and 14.6 ± 3.3% for α1β1(S409A)γ2L(S327A, S343A) GABARs, 8.4 ± 2.3% and 15.4 ± 4.6% for α1β1γ2L(S327A, S343A) GABARs, and 12.6 ± 5.3% and 13.9 ± 3.6% for α1β1(S409A)γ2L GABARs, respectively. There were no differences among the percentage of desensitization values obtained under control and PKM-treated conditions in cells expressing any of these mutant receptor isoforms.

To determine the effects of PKM on the kinetics of GABA current desensitization, time constants of current decay during applications of 300 μM GABA were determined. Due to the limitation of the drug-application system, we were able to detect only the slower phase or phases of desensitization. For wild-type and mutant receptors, the current decays were best fitted with a single exponential function. The time constants obtained under the initial control and PKM-treated reimpalement conditions were 8.5 ± 2.3 sec (11 cells) and 8.2 ± 2.8 sec (11 cells) for cells expressing wild-type α1β1γ2L GABARs, 7.9 ± 1.9 sec (12 cells) and 9.2 ± 1.9 sec (nine cells) for α1β1(S409A)γ2L(S327A, S343A) receptors, 6.5 ± 0.9 sec (13 cells) and 7.9 ± 2.1 sec (10 cells) for α1β1γ2L(S327A, S343A) receptors, and 8.8 ± 1.8 sec (15 cells) and 6.9 ± 1.3 sec (13 cells) for α1β1(S409A)γ2L receptors, respectively (Table 2). There were no significant differences in the time constants of current decay under the control condition among wild-type and mutant receptors (one-way analysis of variance followed by multiple-comparison tests). With the use of unpaired Student's *t* tests, PKM produced no significant effects on the current decay time constants in any receptor isoform exam-

TABLE 2

Analysis of the decay kinetics of GABA-evoked currents expressed in transfected L929 cells

Decay time constants (sec) were determined by single exponential fitting after multipuffer application of 300 μM GABA at 5–10-sec durations to cells recorded with a control intracellular solution-filled (initial control) or a PKM (40 nM)-contained (PKM reimpalement) pipette. Time constants are presented as mean ± standard error. Numbers in parentheses represent the number of cells examined. All values obtained from individual cells were compared by unpaired Student's *t* tests.

GABAR subunit composition	Control (*n*)	PKM (*n*)
α1β1γ2L	8.6 ± 2.3 (11)	8.2 ± 2.8 (11)
α1β1(S409A)γ2L(S327A,S343A)	7.9 ± 1.9 (12)	9.2 ± 1.9 (9)
α1β1γ2L(S327A,S343A)	6.5 ± 0.9 (13)	7.9 ± 2.1 (10)
α1β1(S409A)γ2L	8.8 ± 1.8 (15)	6.9 ± 1.3 (13)

ined. These results indicated that PKM did not alter kinetics of GABAR desensitization under these experimental conditions.

Discussion

Phosphorylation by PKC of both β1 and γ2L subtypes of recombinant α1β1γ2L GABARs was required for full enhancement of GABAR current. GABAR subunits have been demonstrated to be substrates for a number of protein kinases, including PKC (9, 12, 13, 15, 16). Although other potential phosphorylation sites for PKC are present in the GABAR subunit families, the major substrates for PKC are the β and γ2L subtypes (9). Ser409 in the β1 subtype and Ser327 and Ser343 in the γ2L subtype have been identified as high affinity substrates for PKC *in vitro* (11). However, phosphorylation of a fusion protein *in vitro* does not ensure that the protein can be phosphorylated *in vivo*. If phosphorylation does occur, it still does not prove that phosphorylation is produced only by a specific protein kinase (49). As reported previously, intracellular PKM treatment increased GABAR currents in transfected L929 cells expressing wild-type α1β1γ2L GABARs (40). The apparent efficacy of GABA was increased, but the apparent affinity was decreased by PKM. Furthermore, the effect of PKM on GABAR currents was GABA concentration dependent. The ineffectiveness of PKM at low GABA concentration may simply reflect a mixed action of PKM to decrease affinity and to increase efficacy of GABA for the GABAR. Enhancement of α1β1γ2L GABAR currents produced by PKM could have resulted from direct phosphorylation of the GABAR at predicted serine residue or residues or at serine/threonine residues not predicted from the primary amino acid sequence or resulted indirectly from phosphorylation of some cellular protein or proteins that regulate GABAR function. Site-directed mutagenesis was thus performed to substitute alanine for specific serine residues contained in the consensus PKC phosphorylation sites on bovine β1 and γ2L subtypes to determine the functional consequences of phosphorylation of these two subtypes. We have demonstrated that in fibroblasts expressing α1β1(S409A) γ2L(S327A, S343A) GABARs, enhancement of GABAR currents by PKM is eliminated. In fibroblasts expressing GABARs containing only one of the mutant subtypes [i.e., α1β1(S409A)γ2L GABARs or α1β1γ2L(S327A, S343A) GABARs], PKM did not significantly increase the maximal GABAR currents. Removal of the PKC phosphorylation site or sites contained in either the β1 or γ2L subtype was sufficient to block PKM enhancement of the maximal GABAR whole-cell currents. If the increase of GABAR whole-cell currents was due to phosphorylation of some regulatory protein or proteins associated with the GABARs, which in turn modified GABAR function, the mutations in β1 and/or γ2L subtypes should not have affected the current enhancement produced by PKM. The results thus suggested that phosphorylation of both β1 and γ2L subtypes was directly involved in the enhancement of GABAR current by PKC and that phosphorylation of both subtypes was required to produce full enhancement of GABAR whole-cell currents.

Another line of evidence to support this finding arose from the reimpalement studies performed in the absence of PKM and from the analysis of average control current amplitudes among different receptor isoforms. Because no enhancement of GABAR whole-cell current was obtained during the control reimpalement recordings from cells expressing each of the four GABAR isoforms, the reimpalement procedure itself was reliable, and any increase observed between the initial and reimpalement recordings should be due to the given treatment, such as the 40 nM PKM applied in the control/PKM reimpalement experiments. In other words, the enhancement of I_{max} and EC_{50} of cells expressing wild-type GABARs in the presence of PKM was a result of the specific actions of PKM. In contrast, the insignificant increase of I_{max} in mutant receptors suggested an insensitivity or only partial sensitivity of these mutant receptors to PKM. The reduced sensitivity to PKM was produced by mutation or mutations made on specific serine or serines through site-directed mutagenesis without altering their basic GABA concentration-response profiles.

PKC phosphorylation had no significant effect on the GABAR desensitization. It has been reported that PKA phosphorylation reduced the fast component of desensitization of recombinant α1β1 GABARs without changing the rate of desensitization (14). Meanwhile, the desensitization kinetics of α1β1γ2L GABARs were only slightly affected (14). In a study in which phorbol esters were used to activate PKC phosphorylation, no changes were identified in the desensitization kinetics of recombinant α1β1γ2L GABARs (20). By analyzing the percentage of desensitization and current decay time constants, we have demonstrated that under the current experimental conditions, GABAR desensitization was not altered by PKM in cells expressing wild-type or mutant GABARs. Our data thus suggest that the major effect of PKC phosphorylation on α1β1γ2L GABARs expressed in transfected L929 cells was to increase their maximal currents without causing detectable changes in receptor desensitization. Nevertheless, it remains possible that PKC phosphorylation affects other desensitization states of GABARs that can be resolved only through the use of a very rapid application system. The enhancement of the maximal whole-cell currents and reduction in affinity for GABA by PKM in cells expressing wild-type GABARs imply a role of PKC phosphorylation in modulating GABAR channel gating (open/closure) mechanism or, alternatively, in reversing some nonconducting desensitization states. To differentiate these possibilities requires further study.

Functional significance of PKC phosphorylation of the GABAR: comparison with previous studies. Most ligand-gated ion channels have been shown to contain consensus phosphorylation sites for protein kinases and to be regulated by protein phosphorylation. The functional conse-

quences of GABAR phosphorylation include regulation of receptor desensitization rate, subunit assembly, receptor aggregation at the synapse, and open-channel probability (see Ref. 10 for review). Several laboratories have reported that active phorbol esters inhibited recombinant GABAR currents (20, 29–33). In contrast, phorbol esters produced no effect on GABAR function in cultured neurons (28, 50, 51). Run down is a progressive decline of currents during repeated ligand application, and run down of GABAR current is associated with decreases in both the maximal GABAR current and the GABAR EC_{50}. Gyenes *et al.* (35) demonstrated that in cultured chick spinal cord neurons, inclusion of magnesium adenosine-5′-*O*-(3-thio)triphosphate in the intracellular solution prevented the decline in the maximal GABA current, but the GABA EC_{50} still decreased, suggesting that protein phosphorylation maintained GABAR function and that phosphorylation may affect the potency and efficacy of GABA. In our previous study, we demonstrated that PKM enhanced recombinant GABAR currents in transfected L929 cells and that the effects of PKM included an increase in the apparent efficacy and a decrease in the apparent potency of GABA (40). Recently, coexpression of tyrosine kinases with GABAR subunits has been shown to enhance recombinant GABAR whole-cell currents (17), suggesting that protein phosphorylation by kinases other than PKC can also increase GABAR function. Furthermore, in the current study, we demonstrated the direct involvement of specific serine residues in the GABAR β1 and γ2L subtypes in this PKC-mediated GABAR regulation. Our data suggest that although phosphorylation of either β1 and γ2L subtypes may increase GABAR current, phosphorylation of both β1 and γ2L subtypes is required for complete PKC-mediated up-regulation of α1β1γ2L GABAR currents expressed in transfected L929 cells.

There are several interpretations of the discrepancies in the literature. First, in all functional studies of GABAR phosphorylation involving PKC except our previous study (40) and the current study, phorbol esters or diacylglycerol analogues instead of PKM (a constitutively active form of PKC) were used to stimulate PKC phosphorylation. Some membrane-permeant second messenger modulators have phosphorylation-independent effects (52), and chronic phorbol ester treatment has been shown to attenuate cellular PKC levels (47, 53, 54). Second, the effects of PKC observed in native neuron preparations could differ from those obtained with the use of heterologous expression systems because the GABARs isoforms may be different. In recombinant receptor experiments, the possible subunit composition of GABARs is under experimental control, whereas the distribution and structure of native GABARs are variable throughout the central nervous system (55). Third, differences in cell types used (kidney cell, fibroblast or *Xenopus* oocyte) might affect the results due to their endogenous protein kinase levels, basal kinase activities, other differences in post-translational modifications, or, alternatively, differences in cytoskeleton interaction with membrane-bound proteins (56). Fourth, the experimental time courses for PKC phosphorylation differed among studies. For example, Krishek *et al.* (20) examined the slow inhibitory effect of phorbol esters on GABA concentration-response relationships starting 30–90 min after drug application. In our previous study (40) and the current study, however, the acute effects of PKC phosphorylation on GABAR currents were determined at 2–15 min after PKM exposure. Finally, in our study, a reimpalement protocol was used to determine the effect of PKM phosphorylation in the same cell. In contrast, in most of previous reports, the effects of PKC activation were interpreted based on population data, with control and treatment data obtained from different groups of cells. The paired experimental design controls for factors that cause variability between cells. In turn, paired statistical tests allow one to distinguish between-cell variability from within-cell variability. For studies involving observations with high variability among subjects (e.g., whole-cell current measurement), this approach ensures more reliable results. Nevertheless, this difference in experimental design might explain the failure of other studies to demonstrate significant enhancement of GABAR current by PKC treatment but cannot account for the studies that show a significant reduction in GABAR current.

Significance of differential phosphorylation of GABAR subunits. Either the total number or the specific location of amino acid residues of the PKC phosphorylation sites in GABAR subunits could affect the extent of enhancement of GABAR current produced through PKC phosphorylation. Krishek *et al.* (20) concluded that PKC phosphorylation of the β1 and γ2L subtypes differentially inhibited GABAR function and that phosphorylation of Ser343 in the γ2L subtype produced a greater reduction in GABAR current than did phosphorylation of Ser327 in γ2L or Ser409 in β1. In the current study, even though PKM seemed to produce small and insignificant increases in the whole-cell current evoked from GABARs containing mutation or mutations in the β1 or γ2L subtype alone, [i.e., α1β1(S409A)γ2L and α1β1γ2L(S327A, S343A) GABARs], the increases were not significant. It is possible that this represents a type II error (failure to demonstrate a significant difference) due to an insufficient number of cells, although the small enhancements and the high variability of current magnitudes would make the demonstration of significant differences difficult. Furthermore, although PKM produced an apparently greater enhancement of GABAR current after the γ2L mutations than after the β1 mutations, we were unable to distinguish statistically the functional consequences of PKC phosphorylation of the β1 and γ2L subtypes. Thus, although there was a suggestion that phosphorylation of the β1 subunit was more efficient in enhancing GABAR current than was phosphorylation of the γ2L subunit, our data did not support the view that the phosphorylation sites were functionally nonequivalent (20). Instead, our data suggest that for PKC enhancement of GABAR current, phosphorylation of a certain number of sites rather than phosphorylation of a specific site may be more important and that the phosphorylation sites may have relatively equivalent impacts on GABAR current. To produce the full enhancement of maximal GABAR current by PKM treatment, phosphorylation of at least three serines was necessary.

In contrast, differential regulation of the GABAR may exist among protein kinases on the basis that kinases have distinct, although sometimes overlapping, substrate specificity (49). Different kinases may also have different efficacy for phosphorylation. Activation of PKC decreases but activation of PKA increases glycine-evoked currents in *Xenopus* oocytes injected with brain mRNA (57). Because the structure and distribution of GABARs in the brain are heterogeneous (55),

regulation produced by phosphorylation may depend on the presence of available phosphorylation sites on the specific receptor isoforms and the relative abundance or activity of different protein kinases. The same GABAR isoforms could be subject to differential phosphorylation by different kinases, or alternatively, differential phosphorylation could occur on different GABAR subunits with similar protein kinases. For example, the β1 subtype (Ser409) can be phosphorylated by a number of protein kinases, including PKA, PKC, cGMP-dependent protein kinase, and CaM kinase II (11, 14, 16, 20), but CaM kinase II also phosphorylates Ser384 in the β1 subtype (16). The γ2 subunit has a long (γ2L) and a short (γ2S) form; the γ2L form has an eight-amino acid insert in the major cytoplasmic domain (19). CaM kinase II can phosphorylate Ser343, Ser348, and Thr350 in γ2L and Ser348 and Thr350 in γ2S, whereas PKC phosphorylates Ser327 and Ser343 in γ2L and Ser327 in γ2S (16). Tyrosine kinase also phosphorylates γ2L at tyrosine residues 365 and 367 (17). Differential regulation of GABAR function through sequential or simultaneous activation of multiple protein kinases may occur in the central nervous system under physiological conditions, allowing fine control of GABAR function and neuronal excitability.

Activation of GABAR phosphorylation via physiological pathways. cAMP-dependent protein phosphorylation has been shown to induce long-lasting potentiation of GABA-mediated whole-cell currents and miniature inhibitory synaptic currents (24), supporting the view that protein phosphorylation of amino acid receptors in the central nervous system may underlie some form of synaptic plasticity (58). Extracellular signals released from presynaptic neurons, including neurotransmitters, neuropeptides, or extracellular matrix proteins, may regulate phosphorylation of ligand-gated ion channels (59). Phosphorylation of the GABAR may be initiated by neuropeptides or neurotransmitters that are coreleased with GABA via activation of specific second messenger-mediated protein kinase cascades. Vasoactive intestinal polypeptide has been demonstrated to potentiate GABAR currents via a PKA-mediated signal transduction pathway in freshly dissociated ganglion cells of rat retina (25). Furthermore, Feigenspan and Bormann (26) identified a convergent pathway in retinal amacrine cells in which the neuromodulators dopamine, histamine, adenosine, and vasoactive intestinal polypeptide stimulate PKA phosphorylation to facilitate GABA-mediated inhibition. Somatostatin is colocalized with GABA in some neurons in the CA1 area of the hippocampus (60) and has been shown to modify GABAergic response (60). Also, it has been shown that glutamate induces a long-lasting depression of GABA-activated whole-cell currents that could be blocked by PKC inhibitors (i.e., sphingosine, staurosporine, and specific inhibitory peptide) in hippocampal CA1 pyramidal neurons (61). The interaction between postsynaptic neurotransmitter receptors via protein phosphorylation may play an important role in both the short and long term modulation of synaptic efficacy.

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References

1. Olsen, R. W., and J. C. Venter. *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties.* Alan R. Liss, New York (1986).
2. Burt, D. R., and G. L. Kamatchi. $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–2923 (1991).
3. Harvey, R. J., H. C. Kim, and M. G. Darlison. Molecular cloning reveals the existence of a fourth gamma subunit in the vertebrate brain $GABA_A$ receptor. *FEBS Lett.* **331**:211–216 (1993).
4. Macdonald, R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17**:569–602 (1994).
5. Deitrich, R. A., T. V. Dunwiddie, R. A. Harris, and V. G. Erwin. Mechanism of action of ethanol: initial central nervous system actions. *Pharmacol. Rev.* **41**:491–537 (1989).
6. Celentano, J. J., M. Gyenes, T. T. Gibbs, and D. H. Farb. Negative modulation of the γ-aminobutyric acid responses by extracellular zinc. *Mol. Pharmacol.* **40**:766–773 (1991).
7. Dascal, N., T. P. Snutch, H. Lubbert, N. Davidson, and H. A. Lester. Expression and modulation of voltage-gated calcium channels after RNA injection in *Xenopus* oocytes. *Science (Washington D. C.)* **321**:1147–1150 (1986)
8. Nestler, E. J., and P. Greengard. *Protein Phosphorylation in the Nervous System.* John Wiley and Sons, New York (1984).
9. Swope, S. L., S. J. Moss, C. D. Blackstone, and R. L. Huganir. Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. *FASEB J.* **6**:2514–2523 (1992).
10. Schofield, P. R., M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriguez, L. M. Rhee, J. Ramachandran, V. Reale, T. Glencorse, P. H. Seeburg, and E. A. Barnard. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature (Lond.)* **328**:221–227 (1987).
11. Moss, S. J., C. A. Doherty, and R. L. Huganir. Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the β1, γ2S, and γ2L subunits of the γ-aminobutyric acid type A receptor. *J. Biol. Chem.* **267**:14470–14476 (1992).
12. Kirkness, E. F., C. F. Bovenkerk, T. Ueda, and A. J. Turner. Phosphorylation of γ aminobutyric (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. *Biochem. J.* **259**:613–616 (1989).
13. Browning, M. D., M. Bureau, E. M. Dudek, and R. W. Olsen. Protein kinase C and cAMP-dependent protein kinase phosphorylate the β subunit of the purified γ-aminobutyric acid A receptor. *Proc. Natl. Acad. Sci. USA* **87**:1315–1318 (1990).
14. Moss, S. J., T. G. Smart, C. D. Blackstone, and R. L. Huganir. Functional modulation of $GABA_A$ receptors by cAMP-dependent protein phosphorylation. *Science (Washington D. C.)* **257**:661–665 (1992).
15. Machu, T. K., J. A. Firestone, and M. D. Browning. Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C phosphorylate a synthetic peptide corresponding to a sequence that is specific for the γ2L subunit of the $GABA_A$ receptor. *J. Neurochem.* **61**:375–377 (1993).
16. McDonald, B. J., and S. J. Moss. Differential phosphorylation of intracellular domains of γ-aminobutyric acid type A receptor subunits by Ca^{2+}/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase. *J. Biol. Chem.* **269**:18111–18117 (1994).
17. Moss, S. J., G. H. Gorrie, A. Amato, and T. G. Smart. Modulation of $GABA_A$ receptors by tyrosine phosphorylation. *Nature* **(Lond.)377**:344–348 (1995).
18. Sweetnam, P. M., J. Lloyd, R. Gallombardo, R. T. Madison, D. W. Gallager, J. F. Tallman, and E. J. Nestler. Phosphorylation of the $GABA_A$/benzodiazepine receptor α subunit by a receptor-associated protein kinase. *J. Neurochem.* **51**:1274–1284 (1988).
19. Whiting, P., R. M. McKernan, and L. L. Iversen. Another mechanism for creating diversity in γ-aminobutyric type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. USA* **87**:9966–9970 (1990).
20. Krishek, B. J., X. Xie, C. Blackstone, R. L. Huganir, S. J. Moss, and T. G. Smart. Regulation of $GABA_A$ receptor function by protein kinase C phosphorylation. *Neuron* **12**:1081–1095 (1994).
21. Porter. N. M., R. E. Twyman, M. D. Uhler, and R. L. Macdonald. Cyclic AMP-dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons. *Neuron* **5**:789–796 (1990).
22. Leidenheimer, N. J., T. K. Machu, S. Endo, R. W. Olsen, R. A. Harris, and M. D. Browning. Cyclic AMP-dependent protein kinase decreases γ-aminobutyric $acid_A$ receptor-mediated $^{36}Cl^-$ uptake by brain microsacs. *J. Neurochem.* **57**:722–725 (1991).
23. Sessler, F. M., R. D. Mouradian, J.-T. Cheng, H. H. Yeh, W. Liu, and B. D. Waterhouse. Noradrenergic potentiation of cerebellar Purkinje cell re-

sponses to GABA: evidence for mediation through the β-adrenoceptor-coupled cyclic AMP system. *Brain Res.* **499**:27–38 (1989).
24. Kano, M., and A. Konnerth. Potentiation of GABA-mediated currents by cAMP-dependent protein kinase. *Neuroreport* **3**:563–566 (1992).
25. Veruki, M. L., and H. H. Yeh. Vasoactive intestinal polypeptide modulates $GABA_A$ receptor function through activation of cyclic AMP. *Visual Neurosci.* **11**:899–908 (1994).
26. Feigenspan, A., and J. Bormann. Facilitation of GABAergic signaling in the retina by receptors stimulating adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **91**:10893–10897 (1994).
27. Angelotti, T. P., M. D. Uhler, and R. L. Macdonald. Enhancement of recombinant γ-aminobutyric acid type A receptor currents by chronic activation of cAMP-dependent protein kinase. *Mol. Pharmacol.* **44**:1202–1210 (1993).
28. Ticku, M. K., and A. K. Mehta. γ-Aminobutyric $acid_A$ receptor desensitization in mice spinal cord cultured neurons: lack of involvement of protein kinases A and C. *Mol. Pharmacol.* **38**:719–724 (1990).
29. Leidenheimer, N. J., S. J. McQuilkin, L. D. Hahner, P. Whiting, and R. A. Harris. Activation of protein kinase C selectively inhibits the γ-aminobutyric $acid_A$ receptor: role of desensitization. *Mol. Pharmacol.* **41**:1116–1123 (1992).
30. Sigel, E., and R. Baur. Activation of protein kinase C differentially modulates neuronal Na^+, Ca^{2+}, and γ-aminobutyrate type A channels. *Proc. Natl. Acad. Sci. USA* **85**:6192–6196 (1988).
31. Moran, O., and N. Dascal N. Protein kinase C modulates neurotransmitter responses in *Xenopus* oocytes injected with rat brain RNA. *Mol. Brain Res.* **5**:193–202 (1989).
32. Sigel, E., R. Baur, and P. Malherbe. Activation of protein kinase C results in down-modulation of different recombinant $GABA_A$-channels. *FEBS Lett.* **291**:150–152 (1991).
33. Kellenberger, S., P. Malherbe, and E. Sigel. Function of the α1β2γ2S γ-aminobutyric acid type A receptor is modulated by protein kinase C via multiple phosphorylation sites. *J. Biol. Chem.* **267**:25660–25663 (1992).
34. Gyenes, M., M. Farrant, and D. H. Farb. "Run-down" of γ-aminobutyric $acid_A$ receptor function during whole-cell recording: a possible role for phosphorylation. *Mol. Pharmacol.* **34**:719–723 (1988).
35. Gyenes, M., Q. Wang, T. L. Gibbs, and D. H. Farb. Phosphorylation factors control neurotransmitter and neuromodulator actions at the γ-aminobutyric acid type A receptor. *Mol. Pharmacol.* **46**:542–549 (1994).
36. Stelzer, A., A. R. Kay, and R. K. S. Wong. $GABA_A$-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science (Washington D. C.)* **241**:339–341 (1988).
37. Chen, Q. X., A. Stelzer, A. R. Kay, and R. K. S. Wong. $GABA_A$ receptor function is regulated by phosphorylation in acutely dissociated guinea-pig hippocampal neurons. *J. Physiol.* **420**:207–221 (1990).
38. Chen, Q. X., and R. K. S. Wong,. Suppression of $GABA_A$ receptor responses by NMDA application in hippocampal neurones acutely isolated from the adult guinea-pig. *J. Physiol.* **482**:353–362 (1995).
39. Huang, K. P., and F. L. Huang. Conversion of protein kinase C from a Ca^{2+}-dependent to an independent form of phorbol ester-binding protein by digestion with trypsin. *Biochem. Biophys. Res. Commun.* **139**:320–326 (1986).
40. Lin, Y. F., M. D. Browning, E. M. Dudek, and R. L. Macdonald. Protein kinase C enhances recombinant α1β1γ2L $GABA_A$ receptor whole-cell currents expressed in L929 fibroblasts. *Neuron* **13**:1421–1431 (1994).
41. Shingai, R., M. L. Sutherland, and E. A. Barnard. Effects of subunit types of cloned $GABA_A$ receptor on the response to a neurosteroid. *Eur. J. Pharmacol.* **206**:77–80 (1991).
42. Angelotti, T. P., M. D. Uhler, and R. L. Macdonald. Assembly of $GABA_A$ receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J. Neurosci.* **13**:1418–1428 (1993).
43. Zoller, M. J., and M. Smith. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods Enzymol.* **154**:329–351 (1987).
44. Huggenvik, J. I., M. W. Collard, R. E. Stofko, A. F. Seasholtz, and M. D. Uhler. Regulation of the human enkephalin promoter by two isoforms of the catalytic subunit of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase. *Mol. Endocrinol.* **5**:921–930 (1991).
45. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
46. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
47. Woodgett, J. R., and T. Hunter. Isolation and characterization of two distinct forms of protein kinase C. *J. Biol. Chem.* **262**:4836–4843 (1987).
48. Bolton, S. *Pharmaceutical Statistics: Practical and Clinical Applications.* Marcel Dekker, New York (1990).
49. Kennelly, P. J., and E. G. Krebs. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**:15555–15558 (1991).
50. Malenka, R. C., G. S. Ayoub, and R. A. Nicoll. Phorbol esters enhance transmitter release in rat hippocampal slices. *Brain Res.* **403**:198–203 (1987).
51. Harrison, N. L., and N. A. Lambert. The effects of protein kinase activators on $GABA_A$ receptor function in cultured rat hippocampal neurones. *J. Physiol.* **412**:17P (1989).
52. Leidenheimer, N. J., M. D. Browning, and R. A. Harris. $GABA_A$ receptor Phosphorylation: multiple sites, actions and artifacts. *Trends Pharmacol. Sci.* **12**:84–87 (1991).
53. Rodriguez-Pena, A., and E. Rozengurt. Disappearance of Ca^{2+}-sensitive, phospholipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.* **120**:1053–1059 (1984).
54. Wolf, M., N. Sahyoun, H. LeVine III, and P. Cuatrecasas. Protein kinase C: rapid enzyme purification and substrate-dependence of the diacylglycerol effect. *Biochem. Biophys. Res. Commun.* **122**:1268–1275 (1984).
55. Vicini, S. Pharmacologic significance of the structural heterogeneity of the $GABA_A$ receptor-chloride ion channel complex. *Neuropsychopharmacol.* **4**:9–15 (1991).
56. Sachs, F. Mechanical transduction by membrane ion channels: a mini review. *Mol. Cell. Biochem.* **104**:57–60 (1991).
57. Vaello, M. L., A. Ruiz-Gomez, J. Lerma, and F. Mayor Jr. Modulation of inhibitory glycine receptor by phosphorylation by protein kinase C and cAMP-dependent protein kinase. *J. Biol. Chem.* **269**:2002–2008 (1994)
58. Raymond, L. A., C. D. Blackstone, and R. L. Huganir. Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. *Trends Neurosci.* **16**:147–153 (1993).
59. Miles, K., P. Greengard, and R. L. Huganir. Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes. *Neuron* **2**:1517–1524 (1989).
60. Xie, Z., and B. R. Sastry. Actions of somatostatin on GABA-ergic synaptic transmission in the CA1 area of the hippocampus. *Brain Res.* **591**:239–247 (1992).
61. Ragozzino, D., and F. Eusebi. Inhibition of GABA and glycine responses by glutamate in rat hippocampal neurons. *Brain Res.* **628**:115–120 (1993).

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