

β Subunit Phosphorylation Selectively Increases Fast Desensitization and Prolongs Deactivation of $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ GABA_A Receptor Currents

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We studied the effects of phosphorylation by protein kinase A (PKA) on GABA_A receptors ($\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$) transiently expressed in HEK 293T cells. Under conditions favorable for PKA activation, currents obtained using whole-cell patch clamp of lifted cells displayed increased rate and extent of the fast phases of desensitization, decreased rate of current deactivation after GABA removal, and prolongation of brief IPSC-like currents. Mutation of serine residues ($\beta 1$ S409, $\beta 3$ S407, $\beta 3$ S408) revealed that only $\beta 1$ S409 and $\beta 3$ S408 were critical for the modulatory effect of PKA on GABA_A receptor currents. Additionally, repeated pulse inhibition was increased in receptors after mutation of the critical serine to glutamate and decreased when the serine was mutated to alanine. These data demonstrate that PKA phosphorylation modulated GABA_A receptor currents by increasing fast phases of macroscopic desensitization and suggest a role for PKA in regulating GABAergic IPSC duration.

Key words: protein kinase A; chloride ion channel; GABA_A receptor channels; rapid application; patch clamp; mutagenesis

Introduction

Both ligand- and voltage-gated ion channels have been shown to be modulated by phosphorylation (Levitan, 1999). The GABA_A receptor is a ligand-gated chloride ion channel composed of five subunit subtypes from seven families ($\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, $\delta 1$, $\epsilon 1$, $\pi 1$, $\theta 1$) (Macdonald and Olsen, 1994; Olsen and Macdonald, 2002). All β subtypes contain a conserved site in the cytoplasmic loop between M3 and M4 transmembrane domains that is a consensus phosphorylation site for multiple kinases including protein kinase A (PKA), PKC, protein kinase G, and calcium calmodulin kinase II. Moreover, purified GABA_A receptors were phosphorylated by exogenously applied PKA (Kirkness et al., 1989; Browning et al., 1990; Moss et al., 1992b). Specifically, fusion proteins containing the β subtype cytoplasmic loops were phosphorylated by PKA (Moss et al., 1992a; McDonald and Moss, 1997), whereas PKA did not phosphorylate similar $\alpha 1$ and $\gamma 2L$ subunit fusion proteins (Moss et al., 1992a). However, β subtype-containing receptors expressed in human embryonic kidney 293 (HEK293) cells were phosphorylated when $\beta 3$, but not $\beta 2$, subtype was transfected (McDonald et al., 1998). Furthermore, a kinase anchoring protein, 79/150, associated with $\beta 1$ and $\beta 3$, but not $\beta 2$, subtypes in cultured cortical neurons (Brandon et al., 2003).

Although GABA_A receptors containing β subtypes are phosphorylated by PKA, the physiological effect of the phosphorylation has been controversial. Several studies reported that PKA phosphorylation reduced GABA_A receptor current (Chen et al., 1990; Porter et al., 1990; Moss et al., 1992b; Gyenes et al., 1994), whereas others reported that PKA enhanced GABA_A receptor current (Cheun and Yeh, 1992; Angelotti et al., 1993; Feigenspan and Bormann, 1994; Cheun and Yeh, 1996; Kapur and Macdonald, 1996; Jones and Westbrook, 1997; Poisbeau et al., 1999). The different results reported in these studies are likely attributable to differences in techniques used to apply GABA, modulate phosphorylation, and control basal phosphorylation. To attempt to determine how acute PKA phosphorylation affects GABA_A receptor current, we used rapid application of GABA to transiently transfected HEK293T cells expressing rat $\alpha 1\beta 1\gamma 2L$ or $\alpha 1\beta 3\gamma 2L$ receptors. To minimize effects of cytoskeletal disruption and obtain rapid solution exchange, we used a “lifted-cell” technique; entire cells were lifted from the bottom of the dish before GABA and modulator application. We controlled phosphorylation using activators (cAMP, forskolin), phosphatase [protein phosphatase 1 (PP1)], specific inhibitory peptide (PKI), culture conditions, and β subtype mutation. We focused on the role of the serines in $\beta 1$ (S409) and $\beta 3$ (S407, S408) subtypes shown to be phosphorylated by PKA (Moss et al., 1992a,b; McDonald and Moss, 1997; McDonald et al., 1998).

Our results suggest that intracellular conditions in HEK293T cells promoted basal phosphorylation of GABA_A receptors and that S409 in $\beta 1$ and S408 in $\beta 3$ subtypes were critical residues that regulated GABA_A receptor desensitization and deactivation. Promoting PKA phosphorylation of β subtypes increased fast phases of desensitization, slowed deactivation, prolonged brief IPSP-like

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GABA_A receptor currents, and increased repeated pulse inhibition. These observations demonstrate the importance of GABA_A receptor phosphorylation by PKA in shaping GABA_A receptor currents and suggest a role for phosphorylation in regulation of GABA_Aergic IPSC duration.

Materials and Methods

Cell culture. HEK293T cells were grown in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) and 100 IU/ml each of penicillin and streptomycin (Invitrogen) at 37°C in 5% CO₂/95% air, except for serum starvation experiments in which FBS-free media were used 24 hr before recording to reduce basal levels of phosphorylation (see Results). One to 2 d before transfection, cells were passaged with 0.25% trypsin/0.2% EDTA solution and plated (200,000–400,000 cells/dish) on a 60 mm culture dish (Corning Glass Works, Corning, NY).

Transient transfection of HEK293T cells. Cells attached to the plastic surface of a 60 mm culture dish were cotransfected with pCMV α 1, pCMV β 1 or pCMV β 3, and pCMV γ 2L plasmids using a modified calcium phosphate precipitation method. pHook-1 (Invitrogen) was cotransfected with the GABA_A receptor subunits at one-half the concentration of the subunits as a marker of positively transfected cells (Greenfield et al., 1997). The plasmid constructs were added in a 1:1:1:0.5 ratio. Because it has been suggested that differing amounts of γ 2 plasmid may result in differential expression of $\alpha\beta\gamma$ and $\alpha\beta$ GABA_A receptors and altered current kinetic properties, we transfected some cells with 10 times the amount of γ 2L subunit cDNA relative to the amount of α 1 and β 3 subunit cDNA. Experiments in which GABA_A receptor α 1, β 3, and γ 2L plasmid ratios were 1:1:1:0 exhibited current kinetic properties similar to those transfected in a 1:1:1 ratio (data not shown). Furthermore, currents from cells transfected with a 1:1:1 $\alpha\beta\gamma$ ratio were insensitive to 30 μ M Zn²⁺; substantial inhibition by Zn²⁺ would be expected if the currents contained a substantial mix of $\alpha\beta\gamma$ and $\alpha\beta$ GABA_A receptors, as has been suggested (Boileau et al., 2002).

For the data presented in this study, GABA_A receptor α 1, β 3, and γ 2L plasmids and the pHook plasmid were transfected in a 1:1:1:0.5 ratio, maintaining the total amount of DNA added at 14 μ g per 60 mm culture dish. Four to 5 hr after the addition of DNA, the cells were shocked for 30 sec with 15% glycerol in 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid-buffered saline. Approximately 24 hr after transfection, cells were trypsinized and collected by centrifugation at 400 \times g (2000 rpm) for 10 min. Cells were then rotated in a 37°C incubator for 30–40 min to allow the surface-expressed pHook-1 coded antibody (sFv) to bind to magnetic hapten-coated beads. The marker-positive cells were then isolated using a magnetic stand and plated on 35 mm culture dishes. For serum-starved experiments, media in 35 mm dishes were serum free for 24–32 hr before recording. Electrophysiological analysis was performed 24–32 hr after plating on 35 mm dishes.

Mutagenesis. In some experiments, mutated β subunits were transfected in place of wild-type β subunits at the same relative concentrations. β Subunits were mutated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Serine 409 in the β 1 subunit and serines 407 and 408 in the β 3 subunit were mutated to either alanine or glutamate. β 1(S409A), β 1(S409E), β 3(S408A,S409A), β 3(S408E,S409E), β 3(S408A,S409E), and β 3(S408E,S409A) mutants were generated. These subunits have the following flanking sequence in the area of the mutation (mutated serines underlined): β 1–396, HGVPKGGRIRRRASQLKVKI 415; β 3–395, SIPHKKTHLRRRSSQLKIKI 414.

Recording solutions. Before recording, the culture dish solution was exchanged by three 2 ml washings with external recording solution containing the following (in mM): 142 NaCl, 8 KCl, 6 MgCl₂, 1 CaCl₂, 10 glucose, and 10 HEPES, pH 7.4; osmolarity, 320–325 mOsm). The intrapipette solution contained (in mM): 153 KCl, 1 MgCl₂, 5 EGTA, 10 HEPES, and 2 Mg²⁺ ATP, pH 7.3; osmolarity, 298–312 mOsm). This combination of external and intrapipette solutions produced a chloride equilibrium potential (E_{Cl}) of \sim 0 mV. Recording solutions were back-filled into pipettes for lifted cell (1–1.5 M Ω) and patch (5–10 M Ω) experiments, which were pulled from borosilicate capillary glass (World

Precision Instruments, Sarasota, FL) on a P-87 Flaming Brown or P-2000 laser puller (Sutter Instrument Co., San Rafael, CA).

Electrophysiological recording. GABA_A receptor currents were recorded using a lifted whole-cell patch-clamp technique. Currents were recorded only 42–52 hr after transfection to reduce variability observed when cells are recorded for multiple days after transfection. Small (15–20 μ m; 8–20 pF) HEK293T cells with no more than two visible processes and no processes longer than the cell diameter were selected to minimize exchange time with the lifted cell technique. We did not record from cells larger than 20 μ m, because cells with a capacitance >40 pF showed a decreased percentage contribution of the fastest phase of decay to the overall decay during macroscopic desensitization. Furthermore, we monitored the cell morphology carefully throughout the recording. If excessively rapid solution flow or bubbles caused the cell appearance to change such that the cell was pulling away from the electrode and positioned mostly behind the electrode tip, the cells were not used because of the degradation of space clamp and increase in access resistance that consistently occurs when the cell is markedly deformed in this manner. Signals were processed using a List EPC-7 amplifier (List Electronics, Darmstadt, Germany) or 200B amplifier (Axon Instruments, Union City, CA) in voltage-clamp mode. Signals were acquired simultaneously on a WR7400 chart recorder (Graphtec, Irvine, CA), on a videocassette recorder (Toshiba M-250), and on computer. Current amplitudes were measured on computer using the pClamp 8.2 software package (Axon Instruments). All cells were voltage clamped at -15 mV during recordings. With the range of current amplitudes (400–3000 pA) observed in this study, we saw no significant difference in current decay kinetics correlated with peak current size as has been reported previously (Bianchi and Macdonald, 2002). Most patch-clamp amplifiers (including the ones used in this study), although able to compensate for resting series resistance errors, are not able to dynamically compensate for series resistance errors introduced when channels open and the ratio of pipette to cell resistance increases dramatically (Axon Instruments, 1999). Furthermore, resting series resistance compensation did not affect peak current levels or I - V relationships of GABA_A receptors transfected in HEK293T cells (Bianchi and Macdonald, 2002). Finally, voltage-step protocols are sensitive to resting series resistance errors, because the voltage step occurs before the channels open; however, no voltage-step protocols were used during any of these experiments. Because the most significant series resistance error by far was the error during channel opening and not the resting error, we chose not to perform resting series resistance compensation.

GABA and drug application system. GABA and kinase modulator application was achieved using a modified SF-77B Perfusion Fast-Step application system (Warner Instrument Corp., Hamden, CT) (Hinkle et al., 2003). The application system provided for simultaneous flow of all solutions to which the cells were exposed through three parallel glass square barrels. All step protocols began with a cell positioned in the flow of external bath solution from which the multibarreled array was repositioned such that the unmoved cell and electrode were now exposed to a drug (e.g., GABA). GABA (1 mM) was applied in all experiments, whereas forskolin (10 μ M) or dideoxyforskolin (10 μ M) were preapplied in some experiments. The drug application was initiated by an analog pulse triggered by the pClamp 8.2 software that caused the motor of the Warner Fast-Step to reposition the multibarrel array from one barrel to another (e.g., external solution to GABA). We added preapplication steps to the protocol for modulator experiments that enabled us to preapply a membrane-permeable modulator and rapidly wash in bath solution before GABA application. We modified the standard Warner Fast-Step configuration (Bianchi et al., 2002) to obtain rise times (10–90%) of 300–400 μ sec with open-tip electrodes stepped from standard bath solution to 90% bath solution. We heated the glass with a Bunsen burner to reduce the square barrel cross-sectional area as well as to reduce the septum thickness of the glass barrels. This modification allowed us to increase flow and decrease the step distance (large step distances often result in poor exchange times), both of which contributed to decreased exchange time relative to the standard Warner setup. Additionally, we improved exchange time by increasing the stability of the unit by securing all tubing to the micromanipulator and using a long post holder

(Newport, Irvine, CA) to stabilize the stepper motor unit. We repeatedly tested the open-tip electrode exchange time throughout the day to ensure that solution exchange time remained consistent for all cells from which we recorded.

Kinase activation. To assess the effect of PKA on GABA_A receptor current, PKA was activated by either intracellular cAMP (300 μ M) applied via the recording pipette or extracellular forskolin (10 μ M; 7 β -acetoxy-1 α ,6 β ,9 α -trihydroxy-8,13-epoxy-labd-14-en-11-one) applied for 5 min (Sigma, St. Louis, MO). The inactive analog of forskolin, dideoxyforskolin (10 μ M; 7 β -acetoxy-6 β -hydroxy-8,13-epoxy-labd-14-en-11-one), was applied extracellularly as a control for nonkinase-specific forskolin effects. PKA activity was blocked using intracellular application of PKI (50 μ g/ml; Sigma). β Subunit phosphorylation sites were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) to either block receptor phosphorylation entirely with alanine substitutions of the identified serines or to mimic phosphorylation with glutamate substitutions of the serines.

Analysis of rapid application currents. Lifted cell patch data were low-pass filtered at 2 kHz, digitized at 20 kHz, and analyzed with the pClamp 8.2 software. Current decay was fit for each application duration of each cell studied. The desensitization and deactivation time courses were fit to one to three exponential functions using the Levenberg–Marquardt least squares method with the form $\sum A_n e^{-t/\tau_n} + C$, where n is the number of exponential components, t is time, A is relative amplitude of a given component at time = 0, τ is the time constant for a given component, and C is a constant to account for residual (nondesensitized) current. After initial fitting to a single component, additional components were added only if they significantly improved the fit compared with the previous exponential function(s), as determined by an F test on the sum of squared residuals. Currents elicited by 400 msec GABA applications were all fitted best with two exponential functions for desensitization. Six-second GABA-induced currents were fitted best with three exponential functions in all data analyzed here. Deactivation for 6 sec, 400 msec, and 5 msec GABA applications were all fitted best with two exponential functions. For 6 sec, 400 msec, and 5 msec GABA applications, deactivation time constants were weighted $[(A_1 \tau_1)/(A_1 + A_2) * 100 + (A_2 \tau_2)/(A_1 + A_2) * 100]$, where τ_1 was the fast decay component, τ_2 was the slow decay component, and A_1 and A_2 were the corresponding relative proportion of fast and slow components at peak current (time = 0). For all three application durations, the weighted time constant was designated τ_D . Extent of desensitization was defined as (peak current – current at time of GABA offset)/(peak current). All numerical and bar graph data were expressed as mean \pm SEM. All measures of statistical significance were comparisons between wild-type control and the experimental condition being examined. In the case of forskolin and dideoxyforskolin experiments, the control was defined as the GABA-evoked current recorded before the modulator, and the experimental group was the GABA-evoked current recorded after modulator application. Statistical significance was assessed using an unpaired Student's t test: * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Macroscopic current modeling. Macroscopic currents were simulated using the Berkley Madonna 8.0.1 software package. The model used in simulations was based on a model that was developed using both single channel and rapid application data from $\alpha 1\beta 3\gamma 2L$ GABA_A receptors (Haas and Macdonald, 1999). Wild-type currents were simulated using the rate constants from the previously published model. The time constants listed here (see Fig. 9B) were corrected from the original publication that had a typographical error in which the rate constants (a1c, a2c, a3c, b1c, b2c, and b3c) for entry into distal closed states were transposed with the opening (a1o, a2o, a3o, b1o, b2o, and b3o) rates from the distal closed states. The rate constants were iteratively changed to simulate experimental data. Once a modified model was constructed to simulate experimental data, it was tested with multiple application durations and repeated pulse application to verify that the modified rate constants generated currents that were consistent with all of the acquired data.

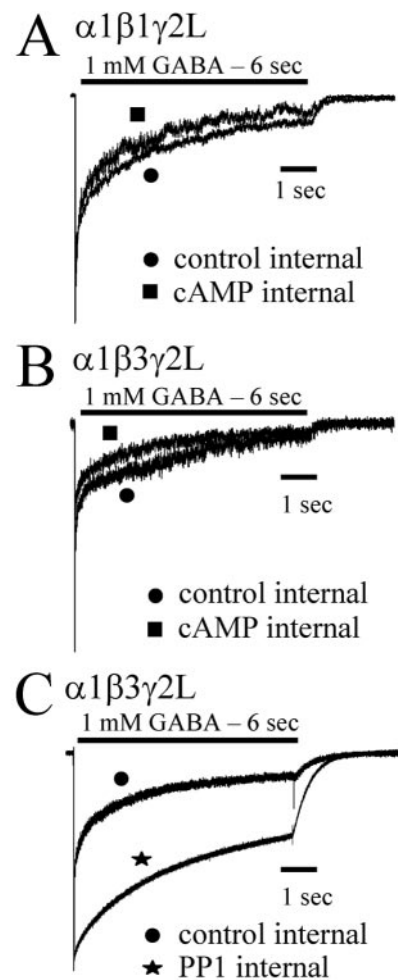


Figure 1. PKA modulation of $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ currents. GABA (1 mM) was rapidly applied for 6 sec to lifted HEK293T cells expressing $\alpha 1\beta 1\gamma 2L$ (A) or $\alpha 1\beta 3\gamma 2L$ (B, C) receptors. Data shown in all panels were normalized to peak and show GABA applications initiated 5 min after rupturing the patch and forming a whole-cell recording. Cells were then lifted from the surface of the dish and placed in the flow of bath solution from the multibarreled array of the rapid application system. Computer-initiated steps from the bath solution to the GABA solution elicited the recorded current. In separate populations of transfected cells, cAMP (300 μ M) was included in the intracellular recording pipette along with the standard intracellular solution used in controls (A, B). cAMP caused a modest increase in the rate and extent of desensitization. C, When PP1 was included in the intracellular pipette, the rate and extent of desensitization were decreased.

Results

Intracellular cAMP modestly increased desensitization of GABA_A receptor currents under standard culture conditions

When GABA (1 mM) was applied rapidly for 6 sec to cells expressing $\alpha 1\beta 1\gamma 2L$ or $\alpha 1\beta 3\gamma 2L$ GABA_A receptors, rapidly desensitizing (i.e., current decay in the persistent presence of agonist) currents were recorded (Fig. 1A, B, control internal), as described previously (Haas and Macdonald, 1999; Bianchi et al., 2001). Control $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ currents desensitized similarly with three exponential components to a “constant” current (supplemental Table A; available at www.jneurosci.org). No significant difference in any kinetic parameter was seen between $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ receptor currents (unpaired Student's t test). The addition of cAMP (300 μ M) to the intracellular recording pipette solution resulted in a modest increase in the rate and extent of desensitization for both $\beta 1$ and $\beta 3$ subunit subtype-containing receptors (Fig. 1A, B, cAMP internal). In general, all

three desensitization time constants were reduced slightly, relative amplitudes were increased, and the residual constant current was reduced by cAMP treatment. Although these data suggested that intracellular cAMP stimulated phosphorylation of an intracellular protein that increased the rate and extent of desensitization of GABA_A receptor currents, the observed effects of cAMP were quite modest and not significant (supplemental Table A). This raised the possibility that intracellular proteins, including the GABA_A receptors, might be constitutively phosphorylated in our expression system, thus occluding the effect of PKA activation.

PP1 catalytic subunit decreased desensitization of GABA_A receptor currents

To explore the possibility that there was significant basal phosphorylation of proteins in HEK293T cells, we included PP1 catalytic subunit (200 U/ μ l) in the intracellular recording pipette to dephosphorylate phosphorylated serine or threonine residues. Intracellular PP1 caused a substantial decrease in the rate and extent of desensitization of $\alpha 1\beta\gamma 2L$ GABA_A receptor current (Fig. 1C). The current response to a 6 sec application of GABA in the presence of intracellular PP1 was fit to three exponential functions (supplemental Table A). Intracellular PP1 significantly increased the briefest time constant ($\tau_1 = 21.6 \pm 4.2$ msec) compared with control ($\tau_1 = 10.1 \pm 2.0$ msec). In addition, the relative contributions of each of the briefest two time constants ($A_1 = 5.3 \pm 3.7\%$; $A_2 = 6.7 \pm 1.1\%$) were decreased compared with control contributions ($A_1 = 23.5 \pm 2.9\%$; $A_2 = 19.1 \pm 3.2\%$). The extent of desensitization as indicated inversely by the constant term ($C = 45.2 \pm 4.6\%$) was significantly decreased compared with the control extent ($C = 18.2 \pm 2.6\%$). The reduction of desensitization and acceleration of deactivation by PP1 suggested that critical residues that influenced GABA_A receptor kinetic properties were constitutively phosphorylated.

GABA_A receptor currents recorded from serum-starved cells had decreased desensitization and accelerated deactivation

To explore the effects of PKA activation on GABA_A receptor currents, we expressed GABA_A receptors under conditions with reduced basal phosphorylation. *In vitro* PKA activity was reported to be downregulated in the absence of BSA (Vargas et al., 1999). PKC activity (Okuda et al., 2001) and endogenous Ca²⁺ channels (Berjukow et al., 1996) have been demonstrated to be upregulated by FBS in HEK293 cells. Furthermore, PKA activity in HEK293 cells has been indirectly shown to be downregulated by withdrawal of serum (Virdee et al., 2000). To determine whether the effects of cAMP on GABA_A receptor currents were occluded by basal phosphorylation, we incubated the cells in serum-free media for 24 hr (serum starved) to reduce basal PKA activity before recording. Because of the extensive desensitization of currents produced by 6 sec GABA applications, shorter (400 msec) GABA applications were used. Both $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ GABA_A receptor currents evoked by 400 msec GABA applications desensitized biexponentially to a constant current (supplemental Table B) (Haas and Macdonald, 1999; Bianchi et al., 2002). $\alpha 1\beta 3\gamma 2L$ GABA_A receptor currents recorded from serum-starved cells differed from those recorded from serum-fed cells (Fig. 2A). Currents from serum-starved cells had decreased the rate and extent of desensitization and accelerated deactivation (supplemental Table B). Control $\alpha 1\beta 3\gamma 2L$ currents (serum-fed conditions) desensitized with two exponential components ($\tau_1 = 10.5 \pm 0.8$ msec; $A_1 = 23.2 \pm 2.5\%$; $\tau_2 = 285 \pm 22.4$ msec; $A_2 = 29.2 \pm 1.9\%$) to a residual current ($C = 40.9 \pm 1.8\%$) (supple-

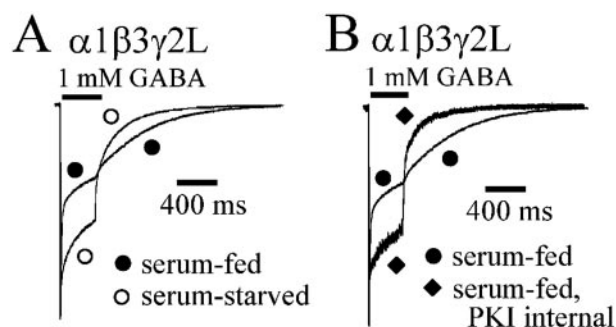


Figure 2. Modulation of $\alpha 1\beta 3\gamma 2L$ currents by culture conditions and PKI. GABA (1 mM) was applied for 400 msec to lifted HEK293T cells expressing $\alpha 1\beta 3\gamma 2L$ receptors. Serum-fed cells were cultured under standard cell conditions using DMEM with 10% FBS before recording. Serum-starved cells were cultured for 24 hr before recording in DMEM with no added FBS. In this and subsequent figures, filled symbols were used for currents from serum-fed cells, and open symbols were used for currents from serum-starved cells. Currents were normalized to peak for both panels. *A*, Rapid application currents elicited from serum-fed and serum-starved cells are overlaid for comparison. Desensitization was decreased, and deactivation was accelerated in serum-starved cells. *B*, PKI (50 μ g/ml) was included in the intracellular recording pipette of some lifted cells, which were grown under standard serum-fed culture conditions. PKI reduced desensitization and accelerated deactivation in a manner similar to serum-starved cells.

mental Table B). With serum starvation, the briefest desensitization time constant (τ_1) was increased to 17.3 ± 3.4 msec, and its relative contribution was decreased to $16.6 \pm 2.2\%$ (supplemental Table B). The slower desensitization component was unaltered, but the residual current was increased to $51.4 \pm 2.1\%$. Thus, currents recorded from serum-starved cells had slower and less extensive desensitization because of a reduction in the rate and extent of fast desensitization.

Deactivation was defined as the time course of current decay after offset of GABA application and has been shown to be substantially different for different GABA_A receptor subunit combinations (Haas and Macdonald, 1999). GABA_A receptor currents deactivate with two time constants after 400 msec applications of GABA. Deactivation of $\alpha 1\beta 3\gamma 2L$ currents was fit by two exponential functions and was reported as a weighted sum (τ_D) of the two time constants obtained for the fits. $\alpha 1\beta 3\gamma 2L$ currents deactivated more rapidly when recorded from serum-starved ($\tau_D = 117 \pm 14.6$ msec) cells than from serum-fed ($\tau_D = 205 \pm 28.2$ msec) cells.

Enhancement of desensitization and slowing of deactivation of GABA_A receptor currents from serum-fed cells was attributable to PKA phosphorylation

To determine whether the enhanced desensitization and accelerated deactivation of $\alpha 1\beta 3\gamma 2L$ currents from serum-fed cells was caused by PKA phosphorylation, PKI was included in the intracellular recording pipette (50 μ g/ml), and recordings were made from cells that were cultured normally (serum fed). When PKI was included in the recording pipette, the extent and rate of desensitization was decreased, and the rate of deactivation was increased (Fig. 2B). Desensitization was again fit with two exponential functions for cells containing PKI in the recording pipette (supplemental Table B). The changes produced by PKI were similar to those obtained with serum starvation. The briefest time constant (τ_1) was increased to 13.2 ± 2.6 msec, and its relative amplitude (A_1) was decreased to $18.2 \pm 4.8\%$, but the changes were not statistically significant (supplemental Table B). The longer time constant ($\tau_2 = 270 \pm 52.6$ msec) was not significantly

different, nor was its contribution ($A_2 = 28.7 \pm 1.5\%$) to peak amplitude. However, as with serum starvation, the current remaining at GABA offset was significantly increased with PKI ($C = 53.1 \pm 4.1\%$) compared with control ($C = 40.9 \pm 1.8\%$) (supplemental Table B). The currents recorded in the presence of PKI had an increased deactivation rate (Fig. 2*B*) similar to the effect of serum starvation (Fig. 2*A*). The mean deactivation rate (τ_D) was reduced by PKI from 205 ± 28.2 to 112 ± 11.9 msec (supplemental Table B).

These data were consistent with the premise that basal levels of PKA-mediated phosphorylation were high in HEK293T cells and that GABA_A receptors were significantly phosphorylated when grown under serum-fed conditions. The effect of PKA phosphorylation appeared to involve increased desensitization and slowed deactivation because of a selective enhancement of the fast component of desensitization.

Activation of PKA by forskolin reduced desensitization and accelerated deactivation of GABA_A receptor currents

All of the previous data were obtained from separate populations of cells. To determine directly whether GABA_A receptor currents were acutely modulated by PKA phosphorylation, we used a multibarrelled array (see Materials and Methods) to preincubate with a PKA activator (forskolin) after obtaining control currents from the same cell. We used the membrane-permeable PKA activator forskolin because it has been shown to activate PKA and increase PKA-mediated phosphorylation of GABA_A receptors in HEK293 cells (Moss et al., 1992b). Cells in which membrane-permeable activators and analogs were used were all serum starved to minimize basal phosphorylation, thus maximizing effects of the activator that would otherwise be difficult to detect under serum-fed cell culture conditions. Cells treated with forskolin were preincubated with forskolin ($10 \mu\text{M}$) for 5 min, washed in external bath solution for 1 sec, and then exposed to GABA (1 mM) for 400 msec. The wash in external solution was to eliminate any extracellular effects of forskolin on GABA_A receptor currents. To keep wash times as brief as possible, we chose to use a 1 sec wash time, because longer washes (5 and 10 sec) did not alter the postwash current (data not shown). Forskolin treatment increased the rate and extent of desensitization and slowed deactivation (Fig. 3*A*). Forskolin decreased the fast desensitization (τ_1) from 17.3 ± 3.4 to 7.1 ± 1.1 msec and increased its relative amplitude from 16.6 ± 2.2 to $39.0 \pm 5.9\%$ (supplemental Table B). The slower time constant and relative amplitude were not affected, but the residual constant component was reduced from 51.4 ± 2.1 to $30.7 \pm 7.2\%$. Forskolin also slowed deactivation (Fig. 3*A*). The weighted deactivation time constant (τ_D) was significantly increased from 117 ± 14.6 to 188 ± 8.7 msec. No significant changes in peak current were seen over the brief period of recording we examined in this study. The inactive analog of forskolin (dideoxyforskolin) did not alter GABA_A receptor current desensitization or deactivation (Fig. 3*B*).

To determine whether the forskolin effect was attributable to PKA phosphorylation, we repeated the forskolin treatment experiments but included PKI ($50 \mu\text{g/ml}$) in the intracellular recording pipette. In the presence of intracellular PKI, forskolin had no effect on current desensitization or deactivation (Fig. 3*C*). These results demonstrated that PKA modulated GABA_A receptor currents by enhancing the fast component of desensitization.

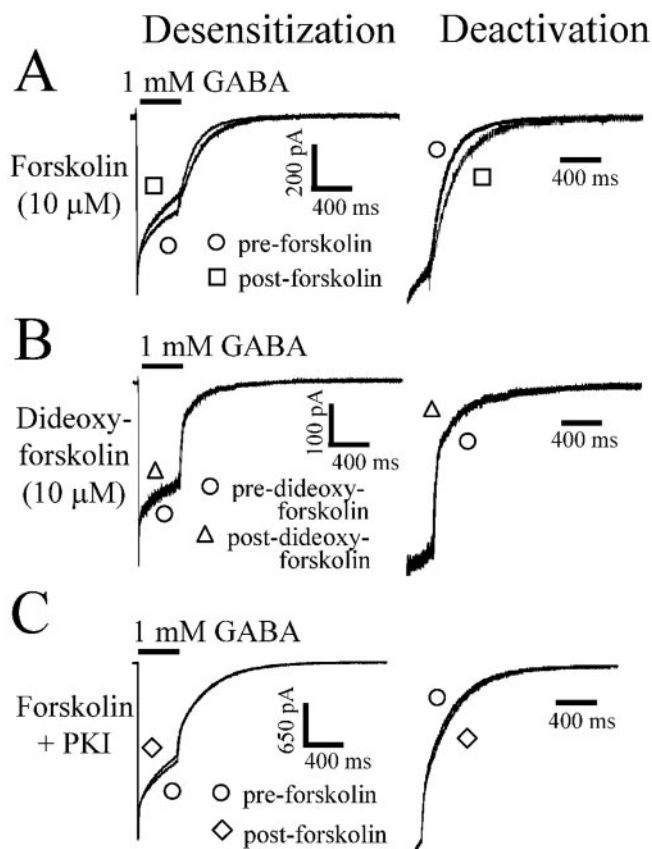


Figure 3. Modulation of $\alpha 1\beta 3\gamma 2L$ currents by forskolin. GABA was applied to lifted HEK293T cells expressing $\alpha 1\beta 3\gamma 2L$ GABA receptors. *A*, GABA (1 mM) was applied for 400 msec for all traces. After formation of a gigaohm seal, each cell was lifted from the bottom of the dish, and GABA was applied as an initial control (pre-forskolin). After the control GABA application, the lifted cell was incubated in forskolin ($10 \mu\text{M}$) for 5 min and was then washed for 1 sec in external bath solution before another 400 msec GABA application (post-forskolin). The extent of current desensitization was greater after incubation in forskolin (left). The right panel shows the same deactivation shown on the left, except the amount of current at the time of GABA offset is normalized to aid comparison. Deactivation was accelerated after forskolin application. *B*, No change in desensitization or deactivation was seen after dideoxyforskolin application. *C*, When the forskolin paradigm in *A* was repeated with PKI ($50 \mu\text{g/ml}$) in the intracellular recording pipette, no changes in desensitization or deactivation were observed.

Modification of desensitization and deactivation of $\alpha 1\beta 1\gamma 2L$ currents by PKA was caused by phosphorylation of GABA_A receptor $\beta 1$ subunit serine 409

Although the previous results demonstrated that PKA modulated GABA_A receptor currents, they could not distinguish between PKA phosphorylation of a receptor-coupled protein and direct receptor phosphorylation. To determine whether the effect of PKA was because of GABA_A receptor phosphorylation, the $\beta 1$ subtype serine 409 phosphorylation site was mutated to an alanine (S409A) to prevent phosphorylation of that serine by PKA. GABA (1 mM) was applied for both 400 msec and 6 sec durations. The briefer 400 msec applications allowed better resolution of deactivation and fast components of desensitization (Fig. 4*A*), whereas the longer 6 sec applications allowed resolution of the third phase of desensitization (Fig. 4*B*). All currents recorded from receptors containing mutated subunits were performed using normal (serum fed) culture conditions. Qualitatively, $\alpha 1\beta 1\gamma 2L$ receptors containing the mutated $\beta 1$ subtype [$\beta 1(S409A)$ receptors] had decreased the extent of desensitization and accelerated deactivation, similar to $\alpha 1\beta 1\gamma 2L$ currents recorded from serum-starved cells (Fig. 4*A, B*).

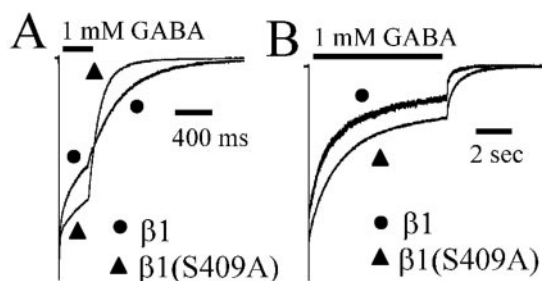


Figure 4. Mutation of the $\beta 1$ subtype serine 409 to alanine decreased desensitization and accelerated deactivation. Wild-type $\alpha 1\beta 1\gamma 2L$ receptor currents were compared with $\alpha 1\beta 1(S409A)\gamma 2L$ receptor currents. *A*, Currents evoked by a 400 msec application of GABA (1 mM) to $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 1(S409A)\gamma 2L$ receptors were normalized to peak current. $\alpha 1\beta 1(S409A)\gamma 2L$ receptor currents had decreased desensitization and accelerated deactivation compared with currents from $\alpha 1\beta 1\gamma 2L$ receptors. *B*, Currents evoked by a 6 sec application of GABA (1 mM) to $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 1(S409A)\gamma 2L$ receptors were normalized to peak current. $\alpha 1\beta 1(S409A)\gamma 2L$ receptor currents had decreased desensitization and accelerated deactivation compared with currents from $\alpha 1\beta 1\gamma 2L$ receptors. Desensitization of $\alpha 1\beta 1(S409A)\gamma 2L$ receptor currents was greater than desensitization observed with $\alpha 1\beta 1\gamma 2L$ receptor currents evoked by 400 msec and 6 sec GABA application. Deactivation after time of GABA offset was accelerated with $\alpha 1\beta 1(S409A)\gamma 2L$ receptor currents compared with $\alpha 1\beta 1\gamma 2L$ receptor currents for evoked by 400 msec and 6 sec GABA application.

Desensitization of $\alpha 1\beta 1\gamma 2L$ receptor currents elicited with 400 msec applications of GABA were fit to two exponential functions and a constant. Desensitization of $\beta 1(S409A)$ receptor currents elicited with 400 msec applications of GABA were also fit to two exponential functions and a constant (supplemental Table B). When compared with wild-type current desensitization, the briefest $\beta 1(S409A)$ receptor desensitization time constant ($\tau_1 = 12.6 \pm 2.0$ msec) was longer than wild type ($\tau_1 = 8.7 \pm 0.9$ msec) (supplemental Table B). The $\beta 1(S409A)$ receptor slower time constant (τ_2) was not significantly different than wild type. Furthermore, the relative contribution of both exponential components ($A1 = 5.7 \pm 1.1\%$; $A2 = 18.2 \pm 4.8\%$) of desensitization were reduced for the $\beta 1(S409A)$ receptor compared with wild-type receptors ($A1 = 21.8 \pm 2.3\%$; $A2 = 31.4 \pm 3.5\%$), whereas the contribution of the constant term was increased in $\beta 1(S409A)$ receptors ($C = 74.4 \pm 5.2\%$) versus wild type ($C = 46.8 \pm 4.9\%$). The deactivation time courses of 400 msec currents were fit to two exponential functions and converted to a weighted time constant (τ_D). The $\beta 1(S409A)$ receptor deactivation ($\tau_D = 90.9 \pm 13.3$ msec) was faster than that of wild-type currents ($\tau_D = 158 \pm 14.3$ msec).

Similar results were obtained with 6 sec GABA applications (Fig. 4*B*). Desensitization of $\beta 1$ receptor currents elicited with 6 sec GABA applications were fit to three exponential functions and a constant (supplemental Table A). Deactivation for this and all 6 sec applications were fit with two exponential functions and reported as weighted deactivation (τ_D). When compared with $\alpha 1\beta 1\gamma 2L$ receptor currents, the briefest $\beta 1(S409A)$ receptor time constant ($\tau_1 = 25.6 \pm 7.3$ msec) was significantly longer than wild type ($\tau_1 = 10.3 \pm 2.5$ msec) (supplemental Table A). Furthermore, the relative contribution ($A1 = 9.6 \pm 1.5\%$) of the briefest time constant (τ_1) to desensitization was reduced for the $\beta 1(S409A)$ receptor compared with wild type ($A1 = 22.8 \pm 4.3\%$), whereas the contribution of the constant term ($C = 34.9 \pm 5.5\%$) was increased in $\beta 1(S409A)$ receptor compared with wild type ($C = 19.3 \pm 5.3\%$). The intermediate (τ_2) and longest (τ_3) time constants and relative amplitudes ($A2$ and $A3$) were not changed by the $\beta 1(S409A)$ mutation. Mutant receptor current deactivation was faster ($\tau_D = 119 \pm 9.9$ msec) than wild-

type current deactivation ($\tau_D = 219 \pm 57.6$ msec). These data suggested that PKA phosphorylation affected GABA_A receptor current kinetic properties by direct receptor phosphorylation.

Modification of desensitization and deactivation of $\alpha 1\beta 3\gamma 2L$ currents by PKA was attributable to phosphorylation of $\beta 3$ subunit serine 408 but not serine 407

In contrast to the $\beta 1$ subunit, $\beta 3$ subunits contain two adjacent amino acids (S407 and S408) that are phosphorylated by PKA (McDonald et al., 1998). Because both amino acids are phosphorylated by PKA, we mutated both amino acids to either alanine ($\beta 3AA$), to block receptor phosphorylation, or glutamate ($\beta 3EE$), to mimic a tonically phosphorylated state. $\beta 3AA$ receptor currents evoked by 400 msec GABA applications desensitized biexponentially and had a larger brief time constant and faster deactivation ($\tau_1 = 18.3 \pm 3.6$ msec; $\tau_D = 145 \pm 14.3$ msec) than wild-type receptor currents ($\tau_1 = 10.5 \pm 0.8$ msec; $\tau_D = 205 \pm 28.2$ msec) (Fig. 5; supplemental Table B). Additionally, the $\beta 3AA$ receptor fast component contributed less to the desensitization ($A1 = 12.9 \pm 2.6\%$) and had a greater proportion of residual current ($C = 53.6 \pm 4.6\%$) than wild-type receptor currents ($A1 = 23.2 \pm 2.5\%$; $C = 40.9 \pm 1.8\%$). Neither the longer time constant (τ_2) nor its contribution ($A2$) to peak current was altered in $\beta 3AA$ receptors (supplemental Table B). No significant differences were found between $\beta 3EE$ and wild-type receptors for any parameter for currents evoked by 400 msec GABA applications (supplemental Table B).

As in previous experiments, three exponential functions were used to fit the desensitization in response to a 6 sec GABA application for $\alpha 1\beta 3\gamma 2L$ wild-type receptor currents, $\beta 3AA$ receptor currents, and $\beta 3EE$ receptor currents (supplemental Table A). $\beta 3AA$ receptor currents had a larger brief desensitization time constant ($\tau_1 = 34.4 \pm 2.5$ msec) and decreased contribution of that exponential to the overall desensitization ($A1 = 11.5 \pm 1.7\%$) compared with wild-type receptors ($\tau_1 = 10.1 \pm 2.0$ msec; $A1 = 23.5 \pm 2.9\%$). There was an increase in the relative amplitude of current at GABA offset for a 6 sec application to $\beta 3AA$ receptors ($C = 31.7 \pm 2.7\%$) compared with wild-type receptors ($C = 18.2 \pm 2.6\%$). The deactivation of $\beta 3AA$ receptors ($\tau_D = 162 \pm 12.4$ msec) was accelerated compared with wild type ($\tau_D = 244 \pm 43.7$ msec). Finally, the intermediate (τ_2) and slowest time constant (τ_3) of the $\beta 3AA$ receptor were not significantly different than wild type, nor were the percentage contributions of these time constants ($A2$ and $A3$) significantly different (supplemental Table A). No significant kinetic differences between wild-type and $\beta 3EE$ receptor currents were found for 6 sec application durations.

Although both $\beta 3$ phosphorylation sites are likely phosphorylated in HEK293 cells under our standard culture conditions (McDonald et al., 1998), we sought to determine whether S407, S408, or both were critical for PKA-induced changes in the current time course. To this end, we generated two $\beta 3$ mutations [(S407A,S408E) and (S407E,S408A)] in which one phosphorylated serine was mutated to alanine and the other to glutamate to mimic phosphorylation at one site and to block it at the other site. These experiments used mutant or wild-type $\beta 3$ subunits cotransfected with $\alpha 1$ and $\gamma 2L$ subunits. With 400 msec GABA applications, $\alpha 1\beta 3(S407E, S408A)\gamma 2L$ ($\beta 3EA$ receptor) receptor current time courses were similar to those of $\beta 3AA$ receptor current time courses, having an increased brief desensitization time constant ($\tau_1 = 26.7 \pm 2.4$ msec), a decreased contribution of the fast desensitization component to peak amplitude ($A1 = 16.0 \pm 2.9\%$), an increased residual ($C = 56.9 \pm 2.2\%$) contribution,

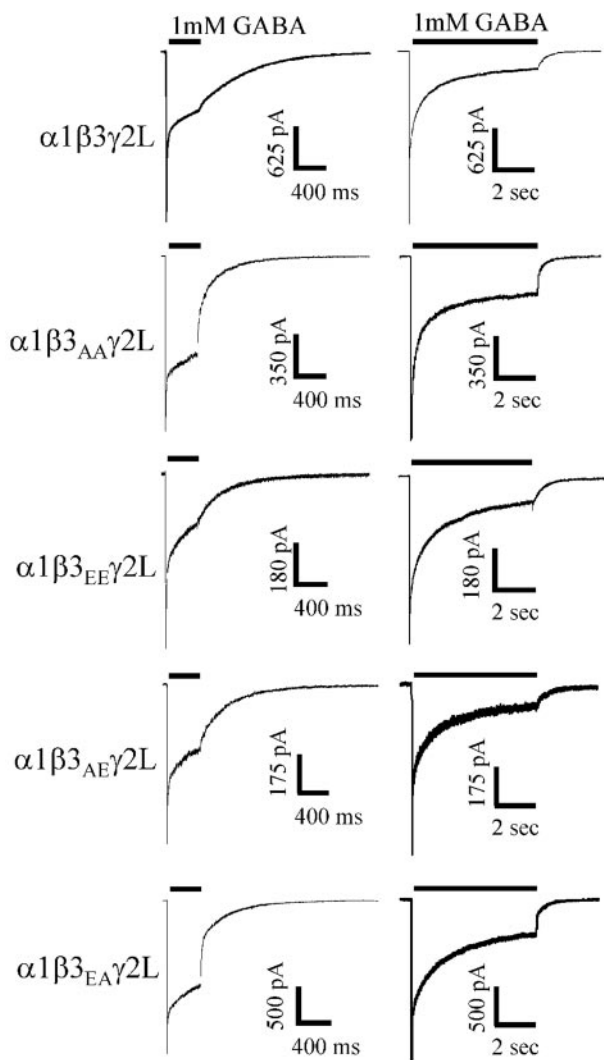


Figure 5. Mutation of $\beta 3$ (S407A,S408A) decreased desensitization and accelerated deactivation, whereas $\beta 3$ (S407E, S408E)-containing receptors did not differ from wild type. Wild-type $\alpha 1\beta 3\gamma 2L$ receptors were compared with $\alpha 1\beta 3$ (S407A,S408A) $\gamma 2L$ ($\beta 3AA$ receptors), $\alpha 1\beta 3$ (S407E,S408A) $\gamma 2L$ ($\beta 3EA$), $\alpha 1\beta 3$ (S407A,S408E) $\gamma 2L$ ($\beta 3AE$), and $\alpha 1\beta 3$ (S407E,S408E) $\gamma 2L$ ($\beta 3EE$ receptors) mutant receptors. Currents were normalized to peak. Initially, we compared $\beta 3AA$ receptors and $\beta 3EE$ receptors with wild type. A 400 msec GABA (1 mM) application to $\beta 3AA$ and $\beta 3EA$ receptors resulted in a decreased desensitization and accelerated deactivation compared with wild-type, $\beta 3EE$, and $\beta 3AE$ GABA receptors. A 6 sec GABA (1 mM) application to $\beta 3AA$ and $\beta 3EA$ receptors resulted in an decreased desensitization and accelerated deactivation compared with wild-type $\beta 3EE$, and $\beta 3AE$ GABA receptors.

and an accelerated deactivation ($\tau_D = 119 \pm 10.3$ msec) (Fig. 5; supplemental Table B) compared with wild type (above). The $\beta 3EA$ receptor long time constant (τ_2) and relative contribution (A2) of that time constant to peak were not significantly different than wild type (supplemental Table B).

Although qualitatively similar to $\alpha 1\beta 3\gamma 2L$ receptor currents (Fig. 5), $\beta 3AE$ receptor currents were quantitatively different with 400 msec GABA applications. The $\beta 3EA$ receptor brief time constant was decreased ($\tau_1 = 6.8 \pm 1.9$ msec), and the slower time constant contributed less to the current ($A_2 = 16.9 \pm 5.4\%$) than in wild-type receptor currents (Fig. 5). However, we were unable to resolve these or any other significant differences between $\beta 3AE$ receptors and wild-type receptors with 6 sec applications (Fig. 5; supplemental Table A). Six-second GABA applications to $\beta 3EA$ receptors resulted in differences similar to those

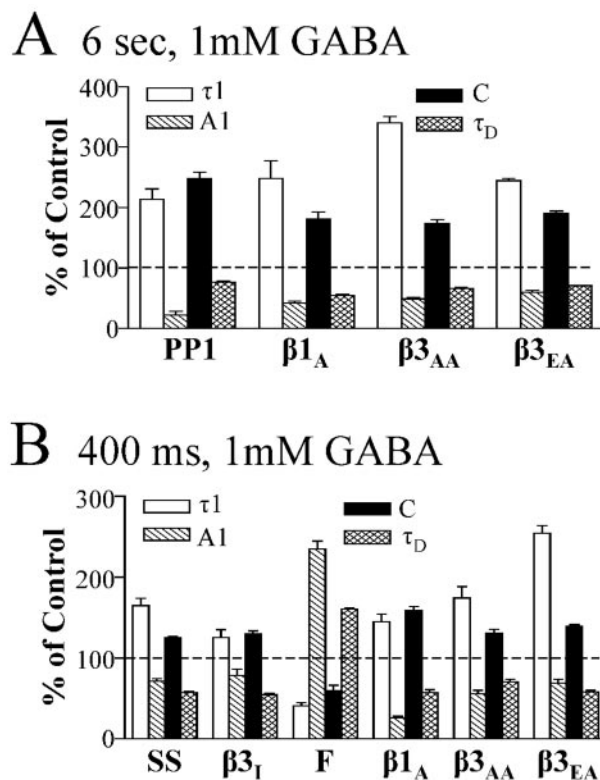


Figure 6. Altering the β phosphorylation site primarily altered τ_1 , A1, C, and τ_D . GABA (1 mM) was applied to lifted cells for 6 sec (A) or 400 msec (B). Selected kinetic parameters (τ_1 , A1, C, and τ_D) were expressed as a mean percentage of the value of the appropriate β subunit control. Control was defined as 100% and is indicated by a dotted line. In the case of forskolin, the percentage was calculated using the mean of the paired controls recorded before forskolin application. Only groups with significant differences from control were displayed. Groups transfected with $\alpha 1\beta 3\gamma 2L$ GABA_A receptors include PP1, serum starved (SS), PKI treated ($\beta 3_I$), forskolin treated (F), $\beta 3$ (S407A, S408A), and $\beta 3$ (S407E, S408A). One group transfected with $\alpha 1\beta 1\gamma 2L$ GABA_A receptors is the $\beta 1$ (S409A) mutant ($\beta 1_A$). All data displayed were significantly different from control, except for the A1 parameter for PP1 (A) and τ_1 and A1 for $\beta 3_I$ (supplemental Tables A and B).

seen for $\beta 3AA$ receptors. The briefest $\beta 3EA$ receptor time constant was longer ($\tau_1 = 24.7 \pm 0.8$ msec) and contributed less to the peak amplitude of the current ($A_1 = 14.1 \pm 1.6\%$) than for wild-type currents (Fig. 5; supplemental Table A). Additionally, the $\beta 3EA$ receptor residual current ($C = 34.7 \pm 1.5\%$) was larger than wild-type current ($C = 18.2 \pm 2.6\%$), and deactivation was faster ($\tau_D = 173 \pm 3.4$ msec) than wild-type deactivation ($\tau_D = 244 \pm 43.7$ msec) (Fig. 5). Finally, neither $\beta 3EA$ receptor intermediate (τ_2) and long (τ_3) time constants nor the relative contribution of these time constants (A2 and A3) to the peak current were significantly different than wild-type receptors.

To summarize, $\beta 3$ mutant receptors in which S408 was mutated ($\beta 3AA$ and $\beta 3EA$ receptors) had current time courses that were similar to those of the serum-starved, PKI-treated, and $\beta 1$ (S409A) receptors, and to each other. The key macroscopic kinetic parameters that changed significantly for these groups were τ_1 (increased), A1 (decreased), C (increased), and τ_D (decreased) (Fig. 6A, B). $\beta 3EE$ currents were not statistically different from wild-type currents in any measured kinetic parameter. $\beta 3AE$ currents were very similar to wild-type currents, but with 400 msec GABA application, the briefest time constant (τ_1) was smaller (i.e., the opposite direction of the shift in $\beta 3$ S408 alanine mutants) and the relative contribution (A2) of the slow time constant (τ_2) was decreased.

Mutation of $\beta 3$ subunit serine 408 altered brief “IPSC-like” GABA_A receptor currents

Because brief applications of GABA mimic the kinetic behavior of IPSCs (Jones and Westbrook, 1995), we compared brief $\beta 3$ AA receptor currents to $\alpha 1\beta 3\gamma 2L$ receptor and $\beta 3EE$ receptor currents to explore how unphosphorylatable GABA_A receptors might differ from GABA_A receptors mimicking full phosphorylation in response to very brief (5 msec) GABA (1 mM) applications (Fig. 7*A, B*). The time courses of deactivation after 5 msec GABA applications were fit with two exponential functions. Deactivation after a 5 msec application of GABA (1 mM) was accelerated in $\beta 3AA$ receptors compared with wild type, whereas $\beta 3EE$ receptors had slowed deactivation similar to wild-type receptors (supplemental Table C). Deactivation was also accelerated in $\beta 3AA$ for both 400 msec and 6 sec applications (Fig. 7*C*). The overall weighted time constant (τ_D) for 5 msec applications was significantly different for $\beta 3AA$ ($\tau_D = 46 \pm 3.3$ msec) and $\beta 3EA$ ($\tau_D = 49 \pm 3.1$ msec) receptors compared with control receptors ($\tau_D = 66$ msec). When individual time constants and relative contributions were analyzed, only the longer time constant (τ_2) was significantly different for any group (supplemental Table C). $\beta 3AA$ ($\tau_2 = 132 \pm 11$ msec) and $\beta 3EA$ ($\tau_2 = 135 \pm 8.0$ msec) time constant were decreased compared with wild type ($\tau_2 = 184 \pm 13$ msec). Although the weighted deactivation for $\beta 3EE$ ($\tau_D = 95 \pm 17$ msec) and $\beta 3AE$ ($\tau_D = 81 \pm 8.1$ msec) receptor deactivation was slower than deactivation of wild-type receptors, neither the weighted deactivation nor individual time constants and relative contributions reached statistical significance. Based on these observations, one would expect IPSCs evoked from GABA_A receptors containing phosphorylated $\beta 3$ subunits to be prolonged.

Mutation of $\beta 3$ subunit serine 408 modulated repeated pulse inhibition of brief IPSC-like GABA_A receptor currents

Repeated pulse protocols have been used to examine changes in microscopic desensitization in GABA_A receptors (Jones and Westbrook, 1995; Bianchi and Macdonald, 2002). We performed repeated pulse experiments during which GABA (1 mM) was applied for 5 msec twice at varying interapplication intervals. The peak in response to the second GABA application was normalized (peak second current/peak initial current \times 100) and displayed as a percentage of the peak of that was generated by the first GABA application. Peak currents in response to the second application of GABA for $\beta 3AA$ ($66.9 \pm 3.7\%$) and $\beta 3EA$ ($69.3 \pm 3.5\%$) receptors at 100 msec interapplication intervals were larger than wild-type currents ($46.2 \pm 6.0\%$) (Fig. 8*B, C*). The second pulse with $\beta 3EE$ ($47.6 \pm 0.5\%$) or $\beta 3AE$ ($44.0 \pm 2.0\%$) receptors were not significantly different than wild type at any interpulse interval (Fig. 8*A, C*). These results suggest that phosphorylated receptors spend more time in desensitized states than dephosphorylated receptors.

Simulated macroscopic currents closely approximated experimental data when entry into desensitized states was decreased

We used 6 sec, 400 msec, and 5 msec agonist application durations in simulating the time course of the GABA_A currents using a model based on both single channel and macropatch rapid application data (Fig. 9*A, B*) (Haas and Macdonald, 1999). Macroscopic rapid application currents were simulated using the Berkley Madonna 8.0.1 software package. Because much of the data shown here were from experiments using lifted cells rather than macropatches, the simulated data are qualitatively the same,

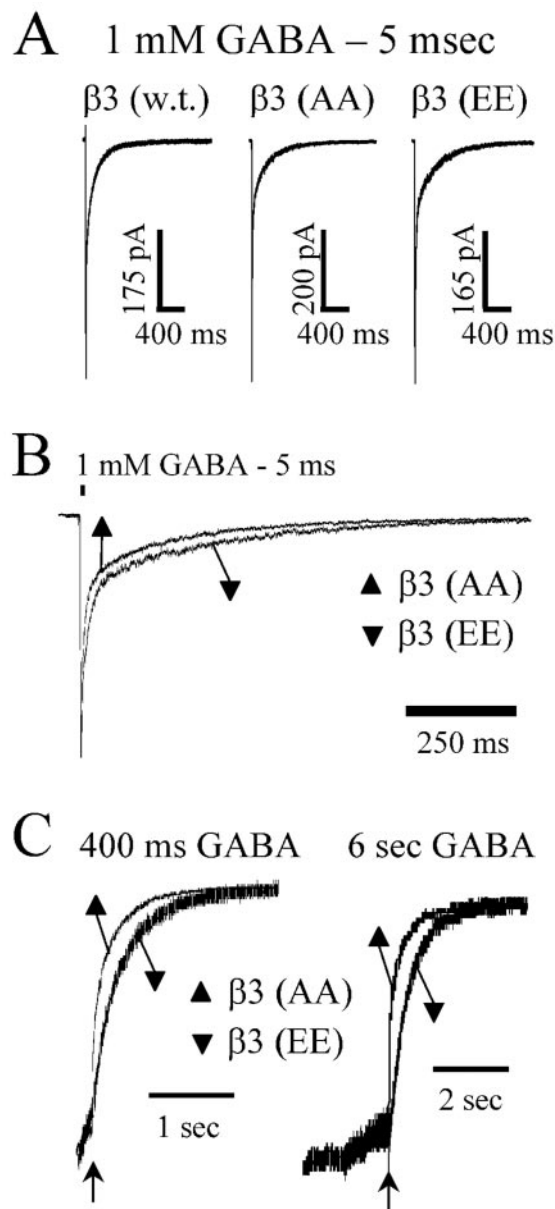


Figure 7. Brief (5 msec) GABA application response durations were modulated by mutation of $\beta 3$ subtype serine 407 and 408 to alanine. GABA (1 mM) was applied to excised macropatches for 5 msec. *A*, Representative data traces are displayed for $\alpha 1\beta 3\gamma 2L$, $\alpha 1\beta 3(S407A, S408A)\gamma 2L$ ($\beta 3AA$), and $\alpha 1\beta 3(S407A, S408A)\gamma 2L$ ($\beta 3EE$) receptors. *B*, Data from *A* are normalized to peak and overlaid for comparison showing faster deactivation for $\beta 3AA$ receptors and slower deactivation for $\beta 3EE$ receptors compared with wild-type receptors. Deactivation was accelerated in $\beta 3AA$ receptor currents compared with $\beta 3EE$ and wild-type receptor currents. *C*, Representative data showing current deactivation after 400 msec and 6 sec GABA application. For purposes of comparison, the level of current at offset of GABA (arrow) is normalized, and most current in the presence of GABA has been excluded from the display. Deactivation was accelerated in $\beta 3AA$ receptor currents compared with $\beta 3EE$ receptor currents.

yet quantitatively different in some respects. When recording from lifted cells, the fast phase of desensitization is somewhat underrepresented in lifted-cell currents compared with excised macropatch currents, because of slower rise times (Bianchi and Macdonald, 2002). Consequently, when comparing lifted-cell data to simulated data, we fitted the simulated current by filtering the peak of the simulated data at 250 Hz to approximate the amount of effective peak filtering introduced by the lifted cell

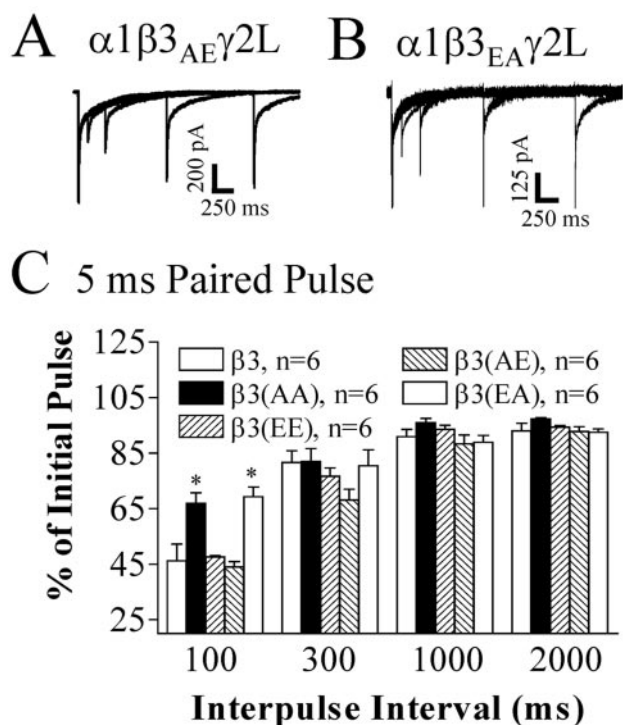


Figure 8. Brief (5 msec) GABA repeated pulse inhibition was decreased by of $\beta 3$ subtype serine 408 to alanine. GABA (1 mM) was applied for 5 msec twice in succession to excised macropatches at varying interpulse intervals (100 msec, 300 msec, 1 sec, 2 sec). Shown are representative data traces for $\alpha 1\beta 3(S407A,S408E)\gamma 2L$ ($\beta 3AE$) receptors (A) and $\alpha 1\beta 3(S407E,S408A)\gamma 2L$ ($\beta 3EA$) receptors (B). Each panel of representative data are four overlaid raw data traces aligned such that the initial currents were superimposed and each subsequent GABA current represented the second pulse of repeated pulse protocols. Each second pulse represented a separate trial on the same macropatch. C, Repeated pulse inhibition was measured for $\alpha 1\beta 3\gamma 2L$ ($\beta 3$), $\alpha 1\beta 3(S407A,S408A)\gamma 2L$ ($\beta 3AA$), $\alpha 1\beta 3(S407E,S408E)\gamma 2L$ ($\beta 3EE$), $\alpha 1\beta 3(S407A,S408E)\gamma 2L$ ($\beta 3AE$), and $\alpha 1\beta 3(S407E,S408A)\gamma 2L$ ($\beta 3EA$) receptors and was displayed as the percentage of second pulse peak to initial pulse peak ($\text{amplitude}_{\text{second current}}/\text{amplitude}_{\text{initial current}} \times 100$). Repeated pulse inhibition was decreased for $\beta 3AA$ (66.9%) and $\beta 3EA$ (69.3%) receptors at a 100 msec interpulse interval compared with wild-type (46.2%), $\beta 3EE$ (47.6%), and $\beta 3AE$ (44.0%) receptors.

technique without additional external filtering. The time courses (or shapes) of the simulated currents (Fig. 9C–E, w.t.) were quantitatively similar (Table 1; supplemental Table C) to those of wild-type currents (Figs. 4–6; supplemental Tables A–C) recorded in this study. Because conditions favorable to phosphorylation resulted in current time courses that were similar to control time courses, the time course of currents simulated with the Haas and Macdonald (1999) time constants also closely approximated $\beta 3$ EE and $\beta 3$ AE receptor currents (Fig. 9C–E; Table 1; supplemental Table C).

Because the significant differences we observed experimentally were between currents recorded under conditions favorable for or simulating phosphorylation (i.e., wild-type currents recorded from serum-fed cells, $\beta 3AE$ currents, or $\beta 3EE$ currents) and currents recorded under conditions unfavorable to receptor phosphorylation [i.e., wild-type currents recorded from serum-starved cells, $\beta 1(S409A)$, $\beta 3AA$, and $\beta 3EA$ currents], we altered rate constants in the Haas and Macdonald (1999) model, which were presumably based on phosphorylated receptor currents, to generate simulated currents that had time courses similar to those observed experimentally for low phosphorylation conditions (Figs. 2A, 5). Increasing the opening rates ($\beta 1$, $\beta 2$, $\beta 3$) (Fig.

9B) either individually or all together to all open states (O1, O2, and O3) (Fig. 9A) decreased the magnitude of macroscopic desensitization but did not accelerate deactivation for simulated currents having 6 sec, 400 msec, or 5 msec agonist application durations (data not shown). Similarly, decreasing the closing rates ($\alpha 1$, $\alpha 2$, $\alpha 3$) (Fig. 9B) also decreased macroscopic desensitization without accelerating deactivation (data not shown). Increasing the GABA unbinding rate (k_{off}) (Fig. 9B) by 5000% ($k_{\text{off}} = 8500$) or decreasing desensitization rates (d_f , d_i) (Fig. 9B) by 50% ($d_f = 48$ and $d_i = 4$) (Figs. 9C, D) both decreased macroscopic desensitization and accelerated deactivation for 6 sec, 400 msec and 5 msec applications (Table 1). It was necessary to increase both d_f and d_i to simulate “dephosphorylated” currents, because decreasing d_f alone produced simulated currents without an increased residual at time of agonist offset (column C in tables) and decreasing d_i alone did not alter deactivation. Decreasing d_s (entry rate into the longest-lasting desensitization state, Ds) to 0 or increasing d_s 3 orders of magnitude either alone or in addition to decreases in d_f and d_i did not significantly affect simulated brief (5 msec) or intermediate (400 msec) current traces. Currents simulated by increasing k_{off} or decreasing d_f and d_i were qualitatively similar to those obtained experimentally under dephosphorylating conditions. The change in macroscopic desensitization was very similar for both sets of simulated currents (Table 1; supplemental Table C); however, the weighted deactivation time constant τ_D with increased k_{off} was reduced 70–98% relative to the τ_D from the unaltered model, whereas the decrease in d_i and d_f only resulted in a 22–26% reduction in the τ_D .

Because increasing GABA unbinding and decreasing entry into fast and intermediate microscopic desensitized states both qualitatively reproduced the alterations in current time course recorded under conditions favoring dephosphorylation (i.e., macroscopically, both reduced desensitization and accelerated deactivation), we attempted to determine whether one or both of these changes were sufficient to explain the effect of dephosphorylation. One approach was to compare the effects of changing unbinding rate and decreasing entry into brief and intermediate microscopic desensitization states on repeated pulse inhibition. One would expect both changes to decrease repeated pulse inhibition; however, one would not necessarily predict that the amount of loss of repeated pulse inhibition would be the same in both cases. We simulated currents obtained with repeated pulse experiments (100 msec interpulse interval) and found that the increase in unbinding rate ($k_{\text{off}} = 8500$) that was necessary to simulate changes in macroscopic desensitization and deactivation produced by 6 sec and 400 msec GABA application durations experimentally resulted in virtually no repeated pulse inhibition (repeated pulse = 97% of control) (Fig. 9E). When the desensitization rate constants were sufficiently decreased to simulate changes in macroscopic desensitization and deactivation produced by 6 sec and 400 msec GABA applications, the amount of repeated pulse inhibition (repeated pulse = 77% of control) (Fig. 9E) was similar to that obtained experimentally (repeated pulse = 69.3% of control) (Fig. 8C) (i.e., repeated pulse inhibition was decreased, but not eliminated). Entry into slow desensitized states has been shown to decrease peak current response with multiple repetitive stimulation (Bianchi and Macdonald, 2002). However, increasing the rate of entry (d_s) into the slowest desensitization state (D_s) produced <1% change in peak current, a single repeated pulse with an interpulse interval of 100 msec. Furthermore, simulated 5 msec currents obtained (Fig. 9F; supplemental Table C) using an appropriately decreased unbinding rate constant had a macroscopic deactivation rate that was faster

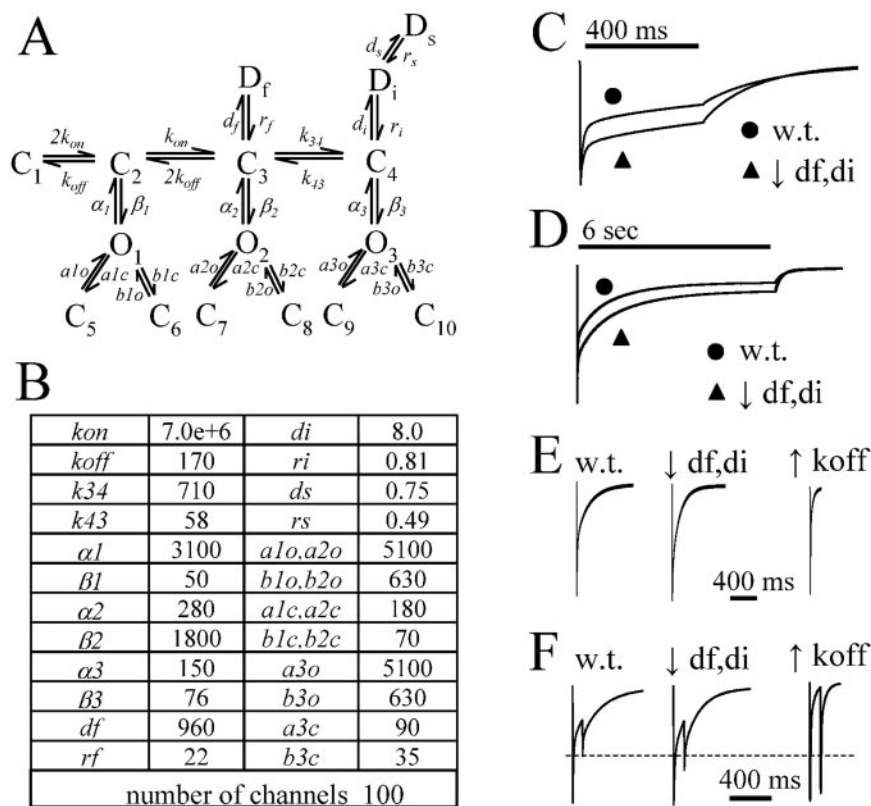


Figure 9. Simulated currents approximated experimentally observed changes in current time course when rate of entry into desensitization was increased. *A*, Wild-type current time courses were simulated using the Haas and Macdonald (1999) model. *B*, The time constants listed here were corrected from the original publication that had a typographical error in which the rate constants (*a1c*, *a2c*, *a3c*, *b1c*, *b2c*, and *b3c*) for entry into distal closed states were transposed with the opening (*a1o*, *a2o*, *a3o*, *b1o*, *b2o*, and *b3o*) rates from the distal closed states. Units for all rate constants were (s^{-1}), except for *k_{off}* ($M^{-1}s^{-1}$). Currents with decreased rate and extent of rapid macroscopic desensitization and accelerated macroscopic deactivation observed under conditions unfavorable to receptor phosphorylation were simulated by increasing both fast (*d_f*) and intermediate (*d_i*) entry rates into microscopic desensitized states in by 50% or by increasing the unbinding rate (*k_{off}*) by 5000%. No significant change was seen when the entry rate (*d_i*) into the slowest microscopic desensitization state (*D_s*) was increased or decreased. (*C*, *D*) Decreasing *d_f* and *d_i* resulted in a simulated current time course qualitatively identical to experimental data for 6 sec, 400 msec, and 5 msec application of agonist, as did increasing *k_{off}* (data not shown). *E*, Changing both *k_{off}* and *d_f* and *d_i* resulted in simulated data with faster deactivation with 5 msec agonist applications. However, the reduction seen with increased *k_{off}* was much greater than observed experimentally, whereas decreasing *d_f* and *d_i* by 50% approximated the prolongation in brief IPSC-like current well. *F*, When *d_f* and *d_i* were decreased 50%, the degree of repeated pulse inhibition was similar to that observed under dephosphorylating experimental conditions. A dashed line shows the peak current level of the second simulated agonist pulse using the unaltered model (w.t.) to aid comparison with the other simulations. Increasing *k_{off}* resulted in simulated currents with a nearly complete loss of repeated pulse depression that was not observed experimentally. w.t., Wild type.

than the more modest macroscopic acceleration of desensitization observed experimentally (Fig. 7*A*, *B*; supplemental Table C). When entry into microscopic desensitized states was decreased, the time course of simulated 5 msec currents closely approximated the experimental 5 msec currents obtained under “dephosphorylated” conditions. Our simulations suggested that the effect of dephosphorylation could be explained by decreased entry into fast and intermediate microscopic desensitization states and the effect of phosphorylation by increased entry into fast and intermediate microscopic desensitized states. Although an effect on unbinding was not required to explain our data, we cannot completely rule out a minor effect on unbinding in addition to the altered desensitization. It should be noted that despite construction of an alternative model, Jones and Westbrook (1995) also came to the conclusion that increased repeated pulse inhibition was indicative of increased occupancy of desensitized states. When taken together, these simulations support the conclusion

that PKA-mediated phosphorylation alters macroscopic currents by increasing entry into fast and intermediate desensitized states.

Discussion

It has become clear that phosphorylation has multiple, controversial physiological effects on GABA_A receptor function (Brandon et al., 2002). Exogenously applied cAMP decreased GABA_A receptor current over time in HEK293 cells transiently transfected with rat $\alpha 1$, $\beta 1$, and $\gamma 2L$ subunits (Moss et al., 1992*b*). In contrast, larger $\alpha 1\beta 1\gamma 2S$ GABA_A receptor peak currents were observed in L929 mouse fibroblast cell lines constitutively expressing high amounts of catalytic subunit of PKA ($C\alpha 12$ cells) compared with cell lines expressing intermediate (L929 cells) or low (RAB 10 cells) amounts of active PKA (Angelotti et al., 1993). In cultured hippocampal neurons, IPSC decay was shortened, and macroscopic desensitization of GABA-evoked currents was increased in the rate and extent with calcineurin inhibition and no exogenous kinase (Jones and Westbrook, 1997). However, Jones and Westbrook (1997) did not see prolonged deactivation with phosphorylation, as was observed here. CA1 hippocampal neurons were reported to be tonically phosphorylated by PKA, which caused a decrease in miniature IPSC (mIPSC) amplitude and an increase in the proportion of mIPSCs with a double exponential decay (Poisbeau et al., 1999).

Many studies have investigated changes in GABA_A receptor function associated with phosphorylation. However, the results obtained were very dependent on the hypotheses being tested and the methodology used to test them. For example, many studies used peak current as a functional assay of GABA_A receptor function (Kano and Konnerth, 1992; Moss et al., 1992*b*; Angelotti et al., 1993; Robello et al., 1993; Cheun and Yeh, 1996; McDonald et al., 1998). However, measurements of peak current alone do not permit an accurate assessment of changes in desensitization and deactivation, which may have a significant effect on inhibition. Furthermore, true peak currents were often missed by the slow (>50 msec 10–90% rise time) application systems that were available. Additionally, membrane-permeable PKA activators were frequently coapplied with GABA, which did not allow investigators to distinguish between direct effects of the activator on GABA_A receptor currents and effects attributable to PKA activation (Schwartz et al., 1991; Cheun and Yeh, 1992; Robello et al., 1993). Some investigators have reported changes that occur slowly (increasing effects over 10–30 min) (McDonald et al., 1998), whereas we have observed the full effect of PKA-induced change in desensitization in as little as 3 min using intracellular cAMP and extracellular forskolin. It is likely that PKA phosphorylation modu-

Table 1. Desensitization and deactivation time constants for simulated currents (400 msec)

Simulation group	τ_1	A1	τ_2	A2	τ_3	A3	C	τ_D
6 sec agonist								
Unaltered model	9.3	24	353	21	1240	38	16	269
Decreased d_i and d_r	15.1	16	395	18	1378	43	23	198
Increased k_{off}	15.4	23	361	20	1276	39	17	79
400 msec agonist								
Unaltered model	9.5	28	333	33			37	236
Decreased d_i and d_r	13	16	382	36			47	184
Increased k_{off}	13	22	341	37			40	34

All data were simulated using the Haas and Macdonald model (1999). Although effects on τ_1 and A1 were similar with both model perturbations (decreased d_i and d_r , increased k_{off}), only the change in d_i and d_r resulted in a similar change in C and τ_D as was seen experimentally with serum starvation, PKI, and mutations [$\beta 1$ (S409A), $\beta 3$ (S408A), and $\beta 3$ (E408A)].

lates GABA_A receptor currents somewhat differently when activated acutely and chronically. Because the modulation of GABA_A receptor currents is more complicated than a simple increase or decrease in peak, we have examined GABA_A receptor currents more rigorously by analyzing transient kinetic properties observed with rapid application of GABA to lifted cells. Because receptor subtypes other than the ones we transfected were not present in our preparation, changes in current shape and time course were attributable to functional changes in the receptor not altered trafficking of different subunit combinations.

Phosphorylation of $\beta 1$ and $\beta 3$ GABA_A receptor subunits increased desensitization and decreased deactivation

Rapid GABA application to lifted cells revealed an increase in the rate and extent of desensitization and slowed deactivation under serum-fed culture conditions or with β subunit serine to glutamate mutations that mimic receptor phosphorylation. When cells were serum starved or transfected with β subunit serine to alanine mutations of the critical serine [$\beta 1$ (S409), $\beta 3$ (S408)] that mimic receptor dephosphorylation, both the rate and extent of desensitization were decreased, and the rate of deactivation was increased. Mutation of $\beta 3$ (S407) had no effect on desensitization or deactivation, supporting the conclusion that the phosphorylation state of S408 in $\beta 3$ is sufficient to modulate of kinetics by β subunit phosphorylation. McDonald et al. (1998) have reported that the site we have identified in $\beta 3$ as crucial for affecting receptor kinetics causes a decreased peak current over time. Although their application system was inadequate to rigorously assess kinetic changes, their $\beta 3$ mutant sample records show changes in desensitization similar to ours, even under control conditions. Whereas increased desensitization is consistent with a previous study (Jones and Westbrook, 1997) of hippocampal neurons and endogenous kinases, we observed prolonged deactivation and Jones and Westbrook did not. This may be because of different receptor subunit combinations present in these neurons or direct receptor effects of calcineurin inhibitors, which could not be assessed with the drug application systems available at that time.

Our results are consistent with findings that constitutive levels of kinase activity are present in both cultured neurons (Brandon et al., 2000) and HEK293 cells (Moss et al., 1992b; McDonald et al., 1998), which result in basal phosphorylation of GABA_A receptor β subunits. Although the previous studies in HEK293 cells only showed moderate levels of receptor phosphorylation under basal conditions, our results suggest that those levels are sufficient for near maximal PKA modulation of GABA_A receptor kinetic properties. However, because the phosphorylation site we have identified is also phosphorylated by PKC and we observed a smaller change in τ_1 with PKI experiments than with mutant experiments, we cannot rule out the possibility that part of the

effect of serum starvation was attributable to a reduction of other constitutive kinase activities. Another explanation of the PKI data are that the amount of PKI and its incubation time were not sufficient to fully block basal PKA activity, but we cannot conclude that based on our data. If another kinase was responsible for the basal level of GABA_A receptor phosphorylation, it likely was not PKC, because the intracellular recording solution contained a calcium-chelating agent (EGTA). Furthermore, these data underscore the importance of cell culture conditions and intracellular environment in determining receptor function for any experimental paradigm. Because other investigators have found changes in peak GABA_A receptor currents over the course of 30 min when adding PKA activators to an already moderately phosphorylated background, it is possible that very high levels of PKA could affect receptor trafficking and play a more important role in longer-term modification of receptor and synapse function, whereas more moderate levels are responsible for more rapid modification of GABA_A receptor kinetic properties, which could exert a faster and finer control over receptor function.

Phosphorylation of $\beta 3$ subunit serine 408 prolonged brief IPSC-like currents

Mutation of the $\beta 3$ subtype serine 408 to glutamate to simulate phosphorylation conferred a deactivation rate similar to that of wild-type receptors. These findings are contrary to a report that promotion of phosphorylation by inhibition of the phosphatase calcineurin (protein phosphatase 2B) shortened IPSCs and GABA-evoked currents in excised patches from hippocampal neurons (Jones and Westbrook, 1997). Because the GABA_A receptor subunit composition was not known for the neuronal cells and calcineurin may serve to dephosphorylate residues other than the specific ones we mutated, the effects observed with calcineurin were likely a result of phosphorylation of other residues on the GABA_A receptor, direct inhibitor effects, differing GABA_A receptor subunit composition, or phosphorylation of different intracellular proteins that interact with GABA_A receptors. It is more difficult to compare directly the kinetic properties reported by Poisbeau et al. (1999) to our results, because they recorded from neurons at physiological temperature compared with our recombinant experiments with slower kinetics at room temperature. Nevertheless, our findings are consistent with their finding of an increase in the proportion of CA1 mIPSCs with a double exponential decay. Because we have found a prolongation of the slower deactivation time constant in recombinant cells, it is possible that the increase in double exponentially decaying mIPSCs was the result of an increased ability to resolve the two time constants as the second time constant was prolonged. The fact that with biexponential decays one time constant was shorter and the other longer than the single exponential decay time constant allowed for the possibility that the single exponentially decaying

time constant was actually two time constants that were too close in value to be resolved at physiological temperature, as opposed to our experiments at room temperature. In our system, we likely always see both time constants of decay because room temperature recordings slow the kinetics of deactivation sufficiently to permit consistent resolution of both time constants.

PKA-induced alteration in macroscopic kinetics was attributable to increased microscopic desensitization

Data presented here indicate that PKA phosphorylation of GABA_A receptors causes an increase in fast macroscopic desensitization and a slowing of macroscopic deactivation. This effect would prolong IPSPs, as we are reporting (Jones and Westbrook, 1995; Haas and Macdonald, 1999). Based on longer GABA application experiments (400 msec and 6 sec) as well as brief (5 msec) and repeated pulse experiments, we propose that PKA causes more rapid entry into the fast and intermediate microscopic desensitized states compared with unphosphorylated receptors. We have identified S409 in β 1 and S408 in β 3 as critical phosphorylated residues, which mediate the effect on GABA_A receptor function. These findings demonstrate the importance of GABA_A receptor phosphorylation to its functional characteristics and may have implications for GABA_A receptors function when kinase activity is altered in disease states (Tehrani and Barnes, 1995; Sik et al., 2000; Yechikhov et al., 2001).

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