Molecular Pharmacology Fast Forward. Published on July 9, 2004 as doi:10.1124/mol.104.002543

PENTOBARBITAL DIFFERENTIALLY MODULATES α1β3δ AND α1β3γ2L $GABA_A$ RECEPTOR CURRENTS

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Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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Running title: Pentobarbital modulates αβδ and αβγ $GABA_A$ receptors

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pages:	46
figures:	10
tables:	1
references:	40
words in abstract:	211
words in introduction:	440
words in discussion:	1493

abbreviations: BBS, BES buffered saline; BES, N, N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid; DMEM, Dulbecco's modified eagle medium; GABA, γ-aminobutyric acid; HEK, Human embryonic kidney; IPSCs, Inhibitory post-synaptic currents; THDOC, Tetrahydrodeoxycorticosterone

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ABSTRACT

$GABA_A$ receptors are modulated by a variety of compounds including the neurosteroids and barbiturates. Although the effects of barbiturates on αβγ isoforms, thought to dominate phasic (synaptic) GABAergic inhibition, have been extensively studied, the effects of pentobarbital on kinetic properties of αβδ $GABA_A$ receptors, thought to mediate tonic (extra or peri-synaptic) inhibition, are unknown. Utilizing ultra-fast drug delivery and single channel recording techniques, we demonstrate isoform specific pentobarbital modulation of low efficacy, minimally desensitizing α1β3δ currents and high efficacy, rapidly desensitizing α1β3γ2L currents. Specifically, with saturating concentrations of GABA, pentobarbital substantially potentiated peak α1β3δ receptor currents but failed to potentiate peak α1β3γ2L receptor currents. Also, pentobarbital had opposite effects on the desensitization of α1β3δ (increased) and α1β3γ2L (decreased) receptor currents evoked by saturating GABA. Pentobarbital increased steady-state α1β3δ receptor single channel open duration primarily by introducing a longer-duration open state, while for α1β3γ2L receptor channels, pentobarbital increased mean open duration by increasing the proportion and duration of the longest open state. The data support previous suggestions that GABA may be a partial agonist at αβδ isoforms, which may render them particularly sensitive to allosteric modulation. The remarkable increase in gating efficacy of α1β3δ receptors suggests that αβδ isoforms, and by inference tonic forms of inhibition, may be important targets for barbiturates.

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INTRODUCTION

$GABA_A$ receptors are ligand-gated chloride channels composed of five subunits with multiple subtypes, including α1-α6, β1-β4, γ1-γ3, δ, ε, π and θ (Olsen and Macdonald, 2002). The function of $GABA_A$ receptor channels is allosterically modulated by many structurally different compounds such as neurosteroids, benzodiazepines and barbiturates (Rupprecht and Holsboer, 1999; Olsen and Macdonald, 2002). The modulatory effects of these drugs are subunit selective in some cases (Pritchett et al., 1989; Thompson et al., 2002; Wallner et al., 2003). For example, neurosteroids significantly increased the maximal peak current amplitude and extent of desensitization of $GABA_A$ receptors containing a δ subunit, but these kinetic responses were not observed for receptors containing a γ2L subunit (Wohlfarth et al., 2002).

Pentobarbital is an anesthetic barbiturate and exerts its CNS effects by interacting with $GABA_A$ receptors (Olsen and Macdonald, 2002). At low concentrations pentobarbital potentiates $GABA_A$ receptor currents (Nicoll and Wojtowicz, 1980; Schulz and Macdonald, 1981) by increasing the proportion of longer single channel openings and thus increasing mean open duration. At higher concentrations, pentobarbital directly activates $GABA_A$ receptors (Nicoll and Wojtowicz, 1980; Schulz and Macdonald, 1981; Krampfl et al., 2002). At mM concentrations, pentobarbital inhibits $GABA_A$ receptor function, probably through a low affinity open channel block mechanism (Akaike et al., 1987; Rho et al., 1996; Akk and Steinbach, 2000). These properties of pentobarbital (positive allosteric modulation, direct agonism and open channel block) are similar to those reported for certain neurosteroids (Lambert et al., 1995; Rupprecht and Holsboer, 1999).

Although the effects of barbiturates on αβγ currents, thought to dominate phasic (synaptic) GABAergic inhibition, have been extensively studied, the modulatory effects of

pentobarbital on the kinetic properties of αβδ $GABA_A$ receptors are still unknown. There is increasing evidence that αβδ receptors may be selectively targeted to extra or peri-synaptic membranes, where they are thought to mediate tonic neuronal inhibition by responding to fluctuating concentrations of extracellular neurotransmitters such as GABA (Nusser et al., 1998; Bai et al., 2001; Stell et al., 2003; Wei et al., 2003). Recent studies have suggested that pentobarbital plays an important role in modulation of δ subunit-containing $GABA_A$ receptors. Chronic pentobarbital treatment evoked an alteration of $GABA_A$ receptor δ subunit mRNA in the CNS (Lin and Wang, 1996), and pentobarbital potentiated the response of δ subunit-containing $GABA_A$ receptors (Adkins et al., 2001; Brown et al., 2002). In the present study, we transfected cDNAs encoding rat α1, β3 and δ or α1, β3 and γ2L $GABA_A$ receptor subunits into human embryonic kidney (HEK 293T) cells and utilized ultra-fast drug delivery and outside-out patch single channel recording techniques to characterize the distinct effects of pentobarbital on $GABA_A$ receptors containing δ or γ2L subunits.

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MATERIALS AND METHODS

Cell culture and recombinant $GABA_A$ receptor expression

Human embryonic kidney (HEK 293T) cells (a gift from Dr. P. Connely, COR Therapeutics, San Francisco, CA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corporation, Grand Island, NY) supplemented with 10 % fetal bovine serum (Invitrogen Corporation), 100 i.u./ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). HEK cells were grown in 10-cm culture dishes (Corning Incorporated, Corning, NY) in an incubator at 37°C with 5 % CO_2 and 95 % air. The cells were passaged at 3-4 day intervals using trypsin-EDTA (Invitrogen Corporation). One day prior to transfection, the cells were seeded at a density of 400,000/dish in 60-mm culture dishes (Corning Incorporated). Cells were transfected with cDNAs encoding rat α1, β3 and δ or α1, β3 and γ2L $GABA_A$ receptor subunits using a modified calcium phosphate precipitation method (Fisher and Macdonald, 1997a). For each transfection, 2 μg of each subunit cDNA along with 2 μg of pHOOK (Invitrogen, Carlsbad, CA) were used. The cells were incubated for 4 hrs with 3 % CO_2 and shocked thereafter for 30 s with 15 % glycerol in BBS buffer (280 mM NaCl, 1.5 mM $Na_2HPO_4 \times 7H_2O$, 50 mM BES). After continuous incubation with 5 % CO_2 for 24 hrs, the cells were collected, and the transfected cells were selected using an immunomagnetic bead separation method (Greenfield et al., 1997). The selected cells were replated on 35-mm culture dishes (Corning Incorporated) for whole cell and single channel recordings 24 hr later. The percentage of cells that both bound beads and expressed $GABA_A$ receptors was 86 % (118/137).

Whole cell and single channel recordings

Whole cell macroscopic currents were obtained with the cell attached to the dish (whole cell recording) or following lifting of the cells (lifted cell recording). Single channel

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microscopic currents were recorded from excised outside-out patches. Recording electrodes were pulled on a P-87 Flaming Brown Micropipette Puller (Sutter Instrument Co., San Rafael, CA). The whole cell and lifted cell recording electrodes were pulled from the thin-wall borosilicate glass tubing (i.d. = 1.12 mm, o.d. = 1.5 mm) (World Precision Instruments Inc., Sarasota, FL) with resistances between 0.8 to 2 MΩ, and the single channel recording electrodes were pulled from the thick-wall borosilicate glass tubing (i.d. = 0.84 mm, o.d. = 1.5 mm) (World Precision Instruments Inc.) with resistances between 6 to 16 MΩ. All electrodes were fire polished on an MF-9 microforge (Narishige, Tokyo, Japan). The single channel recording electrodes were coated with polystyrene Q-dope (GC Electronics, Rockford, IL) after fire polishing to minimize the noise during recording.

Currents were recorded with either an Axopatch 1D or 200A patch clamp amplifier (Axon Instruments, Foster City, CA) and Digidata 1200 series interface (Axon Instruments). The data were stored on a PC computer hard drive for offline analysis. Series resistance was not compensated; theoretically this could lead to an underestimation of the extent of desensitization since voltage errors would be larger at the peak current than after receptor desensitization had occurred. However, we previously reported that desensitization rate and extent were not significantly affected by current size for a broad range of amplitudes, including very large current peaks (5-15 nA) that desensitized ~90 % over a 28 second application (Bianchi and Macdonald, 2002), suggesting that series resistance errors did not significantly affect our interpretations.

Solutions, drugs and drug application

All the chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). External bath solution was composed of (in mM) 142 NaCl, 1 $CaCl_2$, 6 $MgCl_2$, 8 KCl, 10 glucose and 10

HEPES that was standardized to pH 7.4 with NaOH and osmolality to 323-329 mOsm. Recording electrodes were filled with an internal solution consisting of (in mM) 153 KCl, 1 $MgCl_2$, 10 HEPES and 5 EGTA at pH 7.3 and osmolality between 301 and 309 mOsm. MgATP (2 mM) was added to the internal solution on recording days. These solutions produced an E_{Cl} near 0 mV and an E_K at –75 mV.

GABA and pentobarbital sodium salt were prepared as stock solutions and were diluted to desired concentrations with external solution on the day of the experiment. Applications of drugs were performed using an ultra-fast delivery device consisting of multi-barrel tubes (two 3-barrel square glass tubing glued together) connected to a perfusion fast-step system, a mechanical translation device (Warner Instruments Inc., Hamden, CT). Each of the 3-barrel square glasses was heated and manually pulled to the final barrel size (around 200 μm). The external bath solution and drug solutions were driven by gravity. This drug delivery device allowed rapid solution exchanges with 10-90 % rise times consistently less than 2 ms (typically .4 – 1.0 msec) determined by switches between normal and dilute external solution at an open electrode. However, solution exchanges, even when cells were lifted from the recording dish, were likely to occur with a slower rate. Applications of drugs were separated by an interval of at least 45 sec to minimize accumulation of desensitization. GABA-evoked single channel activity was recorded with and without pentobarbital application from excised outside-out patches during steady state conditions (minutes of exposure to GABA). Voltage was clamped at –75 mV during single channel recordings. All the experiments were performed at room temperature.

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Data analysis and simulations

Whole cell currents were analyzed offline using Clamp fit 8.1 (Axon Instruments) and GraphPad Prism 2.01 (GraphPad Software, San Diego, CA). Peak currents were measured directly (manually) relative to the baseline, and residual currents at "steady state" were measured from the end of 28 sec application to baseline after recovery. The extent of potentiation of GABA current by pentobarbital was measured by dividing the peak current of co-application of a given concentration of GABA and pentobarbital by the peak current evoked by GABA alone. The resulting ratio was multiplied by 100 and expressed as % of GABA control. The concentration-response data were normalized and fitted using a four-parameter logistic equation with a variable slope: $I = I_{max}/(1 + 10^{(LogEC50-Logdrug)*Hill\ slope})$. I represented the current evoked by a given concentration of GABA with or without pentobarbital co-application. I_{max} denoted the maximal GABA peak current. The extent of current desensitization was measured as a percentage of current reduction, calculated by dividing the amount of desensitized current (amplitude of peak current - amplitude of current at the end of the 4 or 28 sec GABA application) by peak current and multiplying by 100. The rate of deactivation was analyzed using standard exponential Levenberg-Marquardt methods. Deactivation currents were fitted with one or two exponential components in the form of $a_1\tau_1 + a_2\tau_2$, where a_1 and a_2 represented the relative amplitudes of the exponential components, τ_1 and τ_2 denoted the time constant. A weighted τ was calculated to compare the rates of deactivation using the formula: $a_1*\tau_1/(a_1 + a_2) + a_2*\tau_2/(a_1 + a_2)$. Single channel data were acquired at 50 μs intervals, filtered at 2 kHz and analyzed offline with Fetchan 6.0 (Axon Instruments) using 50 % threshold detection. Although most patches contained multiple channels based on overlapped openings, only single amplitude openings were included in the analysis. Kinetic analysis was performed using Interval 5 (Dr. Barry S. Pallotta,

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University of North Carolina, Chapel Hill, NC). Open duration histograms were generated and fitted by the maximum likelihood method. The number of exponential components was increased until an additional exponential component did not significantly improve the fit as determined by a log-likelihood ratio test automatically performed by the software. Events with intervals less than 1.5 times of the estimated system dead time (100 μsec) were plotted but not included in the fitting.

Simulations were carried out with the Berkeley Madonna 8.0 software package (www.berkeleymadonna.com), using the 4^{th} order Runge-Kutta algorithm to solve model differential equations with a 100 μs step size. Simulated currents were imported into GraphPad Prism 2.01 (GraphPad Software) where noise was added prior to display.

Data were reported as mean ± SEM. Paired Student's *t* test was used to compare current features prior to and after pentobarbital treatment. Unpaired Student's *t* test was utilized to analyze the alterations between different treatment groups. The difference was considered to be statistically significant for $p < 0.05$.

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RESULTS

Differential sensitivity of γ2L and δ subunit-containing $GABA_A$ receptors to GABA

Whole-cell currents were recorded by rapid application of different concentrations of GABA to HEK cells transfected with cDNAs encoding rat α1, β3 and γ2L or α1, β3 and δ $GABA_A$ receptor subunits (Figure 1A). Cells were voltage clamped at –20 mV for cells transfected with α1, β3 and γ2L subunits and at –50 mV for those transfected with α1, β3 and δ subunits due to the smaller amplitude of currents recorded in these receptors (Wohlfarth et al., 2002). No voltage-dependent effects were observed between –20 mV and –50 mV in the present study (not shown) and a previous report (Wohlfarth et al., 2002). For each GABA concentration, mean peak changes in conductance (ΔG) were greater for α1β3γ2L receptors than for α1β3δ receptors (Figure 1B). Mean maximal peak ΔG for α1β3γ2L receptors (233.4 ± 41.1 nS, n = 7) was significantly higher than that for α1β3δ receptors (21.0 ± 4.0 nS, n = 6) ($p<0.001$). The mean EC_{50}s for α1β3γ2L receptors (8.5 ± 6.4 μM) and α1β3δ receptors (5.8 ± 0.7 μM) were not significantly different. The Hill slope for both α1β3γ2L and α1β3δ receptors was 1.4.

Differences in direct activation and open-channel block of γ2L or δ subunit-containing $GABA_A$ receptors by pentobarbital

As reported previously (Schulz and Macdonald, 1981; Rho et al., 1996; Thompson et al., 1996; Krampfl et al., 2002), pentobarbital directly activated $GABA_A$ receptors at high concentrations. In the present study, pentobarbital-evoked whole-cell currents were recorded from α1β3γ2L and α1β3δ receptors, followed by application of saturating GABA (0.3 mM) for each cell (Figure 2A, C). Similar to GABA, pentobarbital evoked smaller mean peak ΔG for α1β3δ receptors than for α1β3γ2L receptors (Figure 2B, D, squares). At 1000 μM pentobarbital, α1β3δ receptor mean peak ΔG (7.0 ± 2.4 nS, n = 7) was significantly smaller than α1β3γ2L

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receptor ΔG (109.4 ± 19.0 nS, n = 8) ($p<0.001$). However, at higher pentobarbital concentrations, the mean peak ΔG for α1β3γ2L receptors declined. This effect of high pentobarbital concentration was not observed for α1β3δ receptors up to 3000 μM pentobarbital (Figure 2B, D, squares). At 3000 μM pentobarbital, the mean peak ΔG was still significantly smaller for α1β3δ receptors than for α1β3γ2L receptors ($p<0.05$).

With higher pentobarbital concentrations, a "rebound" current occurred upon washout of pentobarbital (Figure 2A, C). This is consistent with rapid unbinding of pentobarbital from a low affinity open channel block site as previously suggested (Rho et al., 1996; Thompson et al., 1996; Wooltorton et al., 1997; Dalziel et al., 1999; Akk and Steinbach, 2000; Krampfl et al., 2002). For both α1β3γ2L and α1β3δ receptors, the mean peak ΔG of "rebound" current (measured from the current amplitude at the onset of the "rebound" current) increased as the pentobarbital concentrations increased from 200 μM to 3000 μM (Figure 2B, D, circles). The mean peak ΔG of "rebound" current was significantly less for α1β3δ receptors than for α1β3γ2L receptors at both 1 mM and 3 mM pentobarbital ($p<0.05$).

For α1β3γ2L receptors, maximal mean peak ΔG evoked by pentobarbital was significantly smaller than mean peak ΔG evoked by a saturating GABA concentration. Maximal mean peak ΔG evoked by pentobarbital averaged 58.5 ± 8.0 % ($p<0.01$) of GABA-evoked mean peak ΔG. For α1β3δ receptors, maximal mean peak ΔG evoked by pentobarbital was 127.1 ± 18.6 % of that evoked by a saturating GABA concentration, but this difference did not reach statistical significance. For α1β3γ2L receptors, maximal mean "rebound" ΔG evoked by pentobarbital was significantly smaller than that evoked by a saturating GABA concentration (80.2 ± 9.1 %; $p<0.05$). However, for α1β3δ receptors, maximal mean "rebound" ΔG evoked by

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pentobarbital was 1479.0 ± 590.4 % ($p<0.01$) of that evoked by a saturating GABA concentration.

For $\alpha1\beta3\gamma2L$ receptors, the pentobarbital-evoked direct current declined during the pentobarbital application (Figure 2A). This decline likely represented receptor desensitization, although a contribution from open channel block could not be ruled out. This apparent current desensitization was concentration-dependent (from 300 μM to 3000 μM pentobarbital) and was manifested differently in $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors (Figure 3A). Whereas only minimal desensitization was observed for $\alpha1\beta3\delta$ receptor currents up to 3000 μM pentobarbital (Figure 3A), more extensive desensitization was observed for $\alpha1\beta3\gamma2L$ receptors at pentobarbital concentrations greater than 300 μM (Figure 3A). It is possible, however, that a rapidly equilibrating open channel block mechanism was "masking" macroscopic manifestations of entry into desensitized states, and thus the measurements of apparent desensitization may be underestimated for both isoforms. Furthermore, the activation time with pentobarbital tended to be slower than that observed with GABA, and slow macroscopic activation would preclude observation of fast desensitization (Bianchi and Macdonald, 2002).

For $\alpha1\beta3\delta$ receptors, the deactivation rates of GABA-evoked currents were not concentration-dependent for concentrations up to 1 mM (data not shown), but significant slowing of deactivation was observed at 3000 μM pentobarbital (Figure 2C, 3B). For $\alpha1\beta3\gamma2L$ receptors, the deactivation rates of GABA-evoked current were not concentration-dependent at concentrations greater than 10 μM (data not shown). However, the deactivation rates of pentobarbital-evoked current were concentration-dependent at concentrations greater than 300 μM (Figure 2A, 3B).

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Pentobarbital enhanced currents evoked by a high concentration of GABA from δ more than γ2L subunit-containing $GABA_A$ receptors

Since low concentrations of pentobarbital (30-50 μM) caused little or no direct activation of current, they were considered modulatory concentrations. Occasionally, pentobarbital at 50 μM alone produced small direct currents for α1β3γ2L receptors, but the direct currents were minimal (<2 %) compared to maximal GABA-evoked currents. Pentobarbital concentrations in this range potentiated GABA-evoked currents for both α1β3δ and α1β3γ2L receptors. Co-application of a range of GABA concentrations with 50 μM pentobarbital slightly potentiated α1β3γ2L receptor currents (Figure 4B). The currents evoked by co-application of GABA and pentobarbital as well as GABA alone were normalized to 300 μM GABA. The normalized GABA concentration-response curve was shifted upward (Figure 4B), with a maximal extent of enhancement of 133.9 ± 19.9 % (n = 8). The mean EC_{50} for GABA + 50 μM pentobarbital (3.8 ± 1.9 μM) was not significantly different from that of GABA alone (8.5 ± 6.4 μM). Pentobarbital at 30 μM also potentiated α1β3δ receptor currents (Figure 4C). The concentration-response curve was shifted upward (Figure 4D), and the amplitude of this shift (225.9 ± 15.1 %, n = 7) was significantly greater than that observed with α1β3γ2L receptors ($p<0.001$). The mean EC_{50} for GABA + 30 μM pentobarbital (5.3 ± 1.0 μM) was not significantly different from that of GABA alone (5.8 ± 0.7 μM).

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Pentobarbital produced similar alterations in peak current, desensitization and deactivation of currents evoked by a sub-maximal concentration of GABA for δ and γ2L subunit-containing $GABA_A$ receptors

To demonstrate better the fast component of desensitization and to determine the effect of pentobarbital on $GABA_A$ receptor currents evoked by low (1 μM) GABA concentrations during co-application, cells were lifted from the recording dishes, and pentobarbital was pre-applied for 1.5 sec before a 4 sec co-application of GABA and pentobarbital. 100 μM pentobarbital (a concentration that evoked very small currents from both α1β3γ2L and α1β3δ receptors) substantially enhanced both α1β3γ2L (660.5 ± 111.9 %, n = 6) and α1β3δ (872.0 ± 105.9 %, n = 8) receptor currents, but no significant difference in enhancement was observed between these two isoforms (Figure 5A, B, C). When activated by 1 μM GABA, α1β3γ2L and α1β3δ receptor currents showed minimal apparent desensitization (<3 %; Figure 5A, B, D). Co-application of 1 μM GABA with 100 μM pentobarbital significantly increased the extent of desensitization similarly for both α1β3γ2L and α1β3δ receptors (~10 %; Figure 5A, B, D) ($p<0.01$). For these experiments, the extent of current loss during the four second applications was used to describe desensitization, rather than fitted desensitization time constants, because the rate of decay was slow relative to the application length, precluding accurate fitting of the time constants.

The currents evoked by 1 μM GABA deactivated significantly faster for α1β3δ receptors than for α1β3γ2L receptors (Figure 5A, B, E) ($p<0.001$). For α1β3δ receptors, the mean current deactivation time constants were significantly increased by pentobarbital from 86.8 ± 12.4 ms to 271.8 ± 45.9 ms ($p<0.01$). In the presence of pentobarbital, the mean current deactivation time constants were significantly increased from 321.2 ± 29.4 ms to 1257.4 ± 237.9 ms for α1β3γ2L receptors ($p<0.05$). The mean current deactivation time constants in the presence of

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pentobarbital were significantly smaller for α1β3δ receptors than for α1β3γ2L receptors ($p<0.001$) (Figure 5E).

Pentobarbital evoked a greater enhancement of peak amplitude and desensitization with a saturating concentration of GABA for δ than for γ2L subunit-containing $GABA_A$ receptors

Although pentobarbital (100 μM) modulation of currents evoked by a 4-sec application of low concentration of GABA (1 μM) was not significantly different between α1β3δ and α1β3γ2L receptors, modulation of currents evoked by a 4-sec application of a saturating concentration of GABA (1 mM) by pentobarbital (100 μM) produced a much larger effect on α1β3δ than α1β3γ2L currents (we used the same pre-application protocol as that for application of 1 μM GABA). Pentobarbital did not enhance the peak α1β3γ2L receptor current evoked by 1 mM GABA (99.4 ± 5.8 %, n = 7) (Figure 6A, C). However, pentobarbital evoked a substantial enhancement in peak α1β3δ receptor current (526.4 ± 98.3 %; Figure 6B, C). Note that in the co-application condition under which α1β3γ2L receptor GABA concentration response curves were generated, 50 μM pentobarbital potentiated currents evoked by 300 μM GABA. The basis for the difference between this enhancement compared to results with 100 μM pentobarbital pre-applied for 1.5 seconds prior to a jump into 1 mM GABA is unclear but may be related to increased direct gating of the receptors (visible during the pre-application period) that may have resulted in a small amount of "pre-desensitization", similar to what has been described for pre-incubation in low concentrations of GABA (Overstreet et al., 2000).

Currents evoked from α1β3γ2L receptors by 1 mM GABA exhibited substantial desensitization (78.5 ± 3.5 %; Figure 6A, D), consistent with previous studies on recombinant $GABA_A$ receptors containing a γ subunit (Haas and Macdonald, 1999; Bianchi and Macdonald,

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2002; Burkat et al., 2001). However, only minimal desensitization was observed for α1β3δ receptors with this concentration of GABA (14.1 ± 3.9 %; Figure 6B, D), similar to our previous reports (Haas and Macdonald, 1999). Pentobarbital differentially altered desensitization for α1β3δ and α1β3γ2L receptor currents evoked by 1 mM GABA. The mean percentage of current reduction was significantly increased to 39.1 ± 6.3 % ($p<0.01$) for α1β3δ receptors. In contrast, the mean percentage of current reduction was significantly decreased by pentobarbital by ~10 % to 69.5 ± 5.5 % for α1β3γ2L receptors ($p<0.05$) (Figure 6D).

To evaluate more accurately the effects of pentobarbital on the extent of desensitization, longer duration GABA applications were required for the residual currents to approach a quasi "steady state". Thus, both α1β3δ and α1β3γ2L currents were evoked by long-duration (28 sec) pulses of GABA (1 mM). The enhancement of peak currents for long-duration GABA application was within the range of that for 4-sec applications for both isoforms. However, the extent of desensitization, compared to the 4 sec applications, was increased for both isoforms. Specifically, pentobarbital significantly increased the mean residual currents from 868.0 ± 338.1 pA to 1196.0 ± 392.0 pA for α1β3γ2L receptors ($n = 5$) ($p<0.05$) as a consequence of a significant reduction in the extent of desensitization from 83.8 ± 2.9 % to 78.1 ± 2.2 % ($p<0.05$) after 28-sec GABA application (Figure 7A, B, C). For α1β3δ receptors ($n = 4$), pentobarbital induced a significant increase in apparent desensitization from 30.0 ± 9.6 % to 59.6 ± 10.6 % ($p<0.05$) after 28-sec GABA application, but the mean residual current amplitudes were significantly increased after pentobarbital treatment from 252.5 ± 60.1 pA to 512.5 ± 121.5 pA ($p<0.05$) (Figure 7D, E, F).

Although pentobarbital produced differential changes in enhancement of peak current and desensitization for α1β3δ and α1β3γ2L receptor currents evoked by a saturating concentration of

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GABA, the rates of deactivation were prolonged in the presence of pentobarbital in both of these receptors following 4-sec GABA application (Figure 6A, B). The mean current deactivation time constants following activation with 1 mM GABA alone were significantly smaller for $\alpha1\beta3\delta$ receptors than for $\alpha1\beta3\gamma2L$ receptors ($p<0.001$) (Figure 6E). Pentobarbital significantly increased the mean current deactivation time constants from 118.2 ± 32.4 ms to 471.9 ± 127.8 ms ($p<0.05$) for $\alpha1\beta3\delta$ receptors, and also significantly increased the mean current deactivation time constants from 388.8 ± 44.6 ms to 1168.9 ± 272.1 ms for $\alpha1\beta3\gamma2L$ receptors ($p<0.05$) (Figure 6E).

Pentobarbital introduced a long duration open state for δ subunit-containing $GABA_A$ receptor single channel currents

To explore possible bases for the different effects of pentobarbital in modulating macroscopic GABA-evoked currents from δ and γ2L subunit-containing $GABA_A$ receptors, single channel currents were recorded without and with pentobarbital during steady state application of GABA to outside-out membrane patches containing $\alpha1\beta3\delta$ or $\alpha1\beta3\gamma2L$ receptors. Data were analyzed from 40 to 480 seconds after the patch was excised. $\alpha1\beta3\gamma2L$ receptor single channel currents exhibited bursting openings in the presence of 1 mM GABA (Figure 8A1, A2, A3). The distribution of open states was fitted best with three exponential functions (Figure 8A4), similar to our previous reports (Fisher and Macdonald, 1997b; Haas and Macdonald, 1999). Co-application of 1 mM GABA with 100 μM pentobarbital increased the mean duration of channel openings (Figure 8B1, B2, B3), although the distribution of open states was still best described by three exponential functions (Figure 8B4). Compared with GABA alone, pentobarbital significantly increased the mean channel open duration from 1.45 ± 0.26 ms (n = 5) to 4.86 ± 1.06 ms (n = 5) for $\alpha1\beta3\gamma2L$ receptors ($p<0.05$) (Table 1). The time constant of the

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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shortest exponential function (τ_1) was not significantly altered by pentobarbital. However, pentobarbital significantly decreased the relative area of the shortest exponential function (A_1) ($p<0.05$) (Table 1). Neither the time constant (τ_2) nor the relative area (A_2) of the second exponential function was significantly altered for $\alpha1\beta3\gamma2L$ receptors (Table 1). Interestingly, the third exponential function time constant (τ_3) was significantly increased from 4.63 ± 0.81 ms to 9.37 ± 1.11 ms ($p<0.01$), and the relative area (A_3) was significantly increased from 18.2 ± 3.04 % to 41.4 ± 7.21 % ($p<0.05$) (Table 1). Pentobarbital at 100 μM alone directly activated single channel currents (Figure 8C1, C2, C3). The open duration histogram of channel activity evoked by 100 μM pentobarbital (n = 4) was similar to that evoked by 1 mM GABA (Figure 8A1, C1), except that τ_1 was significantly smaller with pentobarbital alone treatment ($p<0.05$). Compared to pentobarbital alone, the mean open duration and τ_3 were significantly increased with co-application of GABA and pentobarbital ($p<0.05$) (Table 1).

In contrast to the "high efficacy" bursting behavior of $\alpha1\beta3\gamma2L$ receptor single channel currents, $\alpha1\beta3\delta$ receptor single channel currents displayed only brief openings (Figure 9A1, A2, A3). The distribution of open states was fitted best with two exponential functions (Figure 9A4), similar to our previous reports (Fisher and Macdonald, 1997b; Haas and Macdonald, 1999). Co-application of 1 mM GABA with 100 μM pentobarbital resulted in an increased mean open duration (Figure 9B1, B2, B3). The distribution of open states in the presence of pentobarbital, however, required three exponential functions (Figure 9B4). Compared to GABA alone, co-application of GABA and pentobarbital significantly increased the mean open duration from 0.53 ± 0.04 ms (n = 6) to 1.08 ± 0.17 ms (n = 7) for $\alpha1\beta3\delta$ receptors ($p<0.05$) (Table 1). A_1 was significantly decreased by pentobarbital ($p<0.05$), although τ_1 was not significantly altered. τ_2 was significantly increased from 0.90 ± 0.10 ms to 1.27 ± 0.10 ms in the presence of

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pentobarbital ($p<0.05$). Co-application of GABA and pentobarbital introduced a third longer-duration open state for $\alpha1\beta3\delta$ receptors with a time constant of 4.03 ± 0.59 ms and relative area of 10.3 ± 2.15 % (Table 1). The single channel activity evoked by 100 μM pentobarbital alone ($n = 5$) was different from that evoked by 1 mM GABA alone because open state distributions required a third, longer-duration open state ($\tau_3 = 2.95 \pm 0.62$ ms, $A_3 = 3.50 \pm 0.57$ %) (Figure 9C1, C2, C3, C4). τ_1 was significantly smaller for pentobarbital alone treatment than for GABA alone treatment ($p<0.001$) (Table 1). Compared to pentobarbital alone, the mean open duration ($p<0.05$), τ_1 ($p<0.001$), τ_2 ($p<0.01$) and A_3 ($p<0.05$) were significantly greater with co-application of GABA and pentobarbital (Table 1).

Although it was possible that pentobarbital altered closed times or opening frequency in these experiments, we could not accurately analyze these properties because most patches in the current study exhibited overlapping openings, which indicated the presence of multiple active channels. The presence of more than one channel will result in spuriously high measures of open probability and open frequency, as well as low apparent closed duration measurements. Analysis was performed only on individual open durations; burst analysis was not performed due to the presence of multichannel patches which precluded accurate closed state analysis (required to define intraburst closed durations).

Simulation of $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptor currents

Although a detailed mechanism of action for pentobarbital could not be proposed based on the current results, we felt that simulations may shed light on two interesting features of the macroscopic data. First, pentobarbital potentiated the peak amplitude of $\alpha1\beta3\delta$ currents but did not potentiate that of $\alpha1\beta3\gamma2L$ currents evoked by a saturating GABA concentration despite the clear increase in efficacy by prolonging steady state mean open time. Although this might have

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been related to limitations of resolving the "true" peak of a rapidly activating and desensitizing current using whole cell methods, could this observation be related to underlying receptor kinetics? Second, pentobarbital exhibited opposite effects on the macroscopic desensitization of $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ currents. Although this could be related to isoform-specific pentobarbital modulation of rate constants of desensitized states, was it possible that an increase in single channel open time could partly contribute to both of these apparently opposite effects? To explore these issues, we generated simulated currents using our previously established models for $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors (Haas and Macdonald, 1999) (Figure 10A, B). For the purposes of this analysis we were limited to qualitative comparisons, particularly because the models were generated using data obtained with excised patches, a condition of substantially higher temporal resolution than the whole cell configuration (Bianchi and Macdonald, 2002).

In the first set of simulations, the currents evoked by 6 second square pulses of 1 mM agonist were modeled for the $\alpha1\beta3\gamma2L$ receptor (Figure 10A, C). Compared to the "wild-type" current (left trace), increasing the duration of O_2 by 3-fold (second trace) caused a small increase in peak current (~10 %), while the residual current increased by ~100 % (dotted lines). Combining this with a 3-fold increase in O_3 duration (third trace), no further change in peak was observed, while the residual current amplitude was increased by an additional ~100 %. Finally, β_3 and α_3 were altered to reflect the observed changes in single channel open distribution (right trace). In this case, peak amplitude changed by ~5 % while the residual current was 3-fold larger. This limited change in peak amplitude was attributed in part to rapid entry into the fast desensitized state, effectively truncating the current by preventing some channels from opening upon agonist binding. Also, the model predicts that >90 % of the peak amplitude is due to openings into the second open state (while residual current is dominated by O_3). Although we

have not evaluated this experimentally (but see Burkat et al., 2001), this kinetic arrangement predicted that increasing the duration and/or proportion of O_3 would preferentially increase residual currents. Apparent desensitization was reduced for each condition, consistent with our previous experimental and simulation results regarding increased efficacy (Bianchi and Macdonald, 2001). This general pattern of macroscopic manifestations of increasing open duration (ie, preferential increases in residual current) was observed for less complex models as well, as long as desensitized states did not proceed directly from the open state (not shown). These simulations illustrate the principle that increasing open duration can produce selective increases in residual, as opposed to peak, current amplitudes for $\alpha1\beta3\gamma2L$ receptors, in part because of the rapid entry into a fast desensitized state.

Similar simulations were also carried out with a model generated for $\alpha1\beta3\delta$ receptors (Figure 10B, D). Note that these receptors are characterized by substantially smaller open probabilities relative to $\alpha1\beta3\gamma2L$ receptors, even under conditions of saturating GABA. This was apparent in the relative increase in "noise" that was added as Gaussian noise with a constant standard deviation for both sets of simulations. Increasing the open duration via decreasing α_1 by a factor of 2 resulted in an approximate doubling of both the peak and the steady state current amplitude (Figure 10D, left trace, second trace). This can be explained by the relative contribution of O_1 to the current time course. Similar to $\alpha1\beta3\gamma2L$ receptor model, the first accessible open state (in this case O_1) contributes >90 % of the peak current amplitude. However, in contrast to the $\alpha1\beta3\gamma2L$ model, initial access to this state is not significantly compromised by rapid entry into a desensitized state (allowing peak current enhancement), and this open state continues to pass a considerable portion of the total current throughout the pulse, accounting for ~60 % of the residual amplitude (not shown). Thus, all parts of the current are

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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sensitive to changes in this open state. Consistent with this idea, when the duration of O_2 was increased by a factor of 2 (Figure 10D, third trace), the macroscopic residual current was preferentially increased, with only minor changes in peak amplitude. Interestingly, in contrast to the clear decrease in apparent desensitization observed with increased open duration for α1β3γ2L currents, apparent desensitization was slightly increased for α1β3δ currents (from ~40 % to ~50 %). This difference is attributable to isoform differences in baseline gating efficacy and desensitization, which will limit changes in peak amplitude for α1β3γ2L but not α1β3δ receptor currents. When an additional open state was included (arbitrarily made to proceed from the closed state C_4), to reflect our single channel observations, no alteration of peak current amplitudes was observed (data not shown). This is consistent with "proximal" open states (with relatively high entry rate constants) selectively contributing to the initial development of current. Again, although the exact mechanism of pentobarbital modulation remains unclear, the simulations suggested that longer duration openings could manifest differently for receptors with different baseline kinetic properties.

The simulated currents from both α1β3γ2L and α1β3δ receptor models were qualitatively in agreement with our experimental observations, regarding the differential sensitivities of peak and residual current amplitude to pentobarbital modulation. However, the quantitative differences in these amplitude measurements suggested that additional kinetic parameters, in addition to mean open duration, contributed to the alterations induced by pentobarbital. For example, the residual current amplitude was over-estimated in the case of α1β3γ2L receptor simulations (Figure 10C), while the peak current amplitude changes for α1β3δ receptors were underestimated (Figure 10D). Clearly, quantitative correlations of steady state mean open time changes evoked by pentobarbital with peak or residual macroscopic

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currents are not straightforward since occupancy of open states is only a small fraction of all bound states. This is illustrated in Figure 10E, in which a simulated $\alpha1\beta3\gamma2L$ receptor current (downward trace) is shown with the corresponding probability density functions of the two open and three desensitized states available to the fully bound receptor. For clarity, occupancy of C_3, C_4 and the intra-burst closed states is not shown, which also contributes to the overall occupancy patterns. Thus, further simulations were carried out to explore other possible mechanisms of pentobarbital modulation of macroscopic currents of $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ receptors. Because of the number of unconstrained parameters in these complex models, we did not attempt to uniquely fit all rate constants to our experimental data. Instead, focused changes were made in an attempt to generate hypotheses as to which additional parameters might be involved in pentobarbital modulation. For $\alpha1\beta3\gamma2L$ receptors, reducing the exit rate of D_i by a factor of 2 (in addition to the changes made to reflect the single channel open distribution data) reduced the residual current amplitude (by decreasing open probability) with minimal alteration of the peak amplitude (Figure 10F, left traces). Similar results were obtained with increasing entry into D_i by a factor of 1.5. For the $\alpha1\beta3\delta$ receptor model, we first altered certain parameters to render currents that more closely resembled our whole cell observations (that is, less macroscopic desensitization than observed in outside out patches) (Figure 10F; see legend for rate constant changes). From this baseline, we altered various parameters, in addition to the changes to reflect the single channel data. Specifically, increasing the opening rate β_1 by a factor of 5 (with or without corresponding 5 fold increases in β_2 and β_3) resulted in an appropriate 5-fold increase in peak current, but also a five fold increase in residual current (not shown). In order to more closely reflect our observations, we also changed desensitization by both increasing entry into and decreasing exit from the single desensitized state to obtain the current shown in Figure 10F

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(right traces). Because this entry rate was still slower than β_1, the peak amplitude was minimally affected, but the steady state occupancy of the desensitized state was increased, and thus the residual current (as well as the increase in apparent desensitization) was similar to our experimental observations. Further single channel and macroscopic experiments would be required to test the hypothesis that these kinetic parameters were altered in the presence of pentobarbital for each of these isoforms.

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DISCUSSION

Direct effects of pentobarbital on α1β3δ and α1β3γ2L GABA$_A$ receptors

Similar to many allosteric modulators, at sufficiently high concentrations pentobarbital can directly activate GABA$_A$ receptor currents. Here we report notable differences between its activity at α1β3δ and α1β3γ2L receptors. For example, like the isoform-specific GABA-evoked currents, pentobarbital evoked desensitizing currents from α1β3γ2L receptors while currents from α1β3δ receptors were essentially nondesensitizing. Although this is consistent with minimal desensitization being a property of δ subunit-containing isoforms, as we have previously suggested, we cannot rule out the possibility that desensitization could be observed upon activation by a "full" agonist. We have inferred that desensitized states are in fact accessible to αβδ isoforms because GABA-evoked currents in the presence of THDOC or pentobarbital show increased desensitization (Wohlfarth et al., 2002; this study).

Direct activation of single channel events showed isoform differences as well. Pentobarbital appeared to be a higher efficacy agonist than GABA for α1β3δ receptors, since a third open state was observed (not seen with GABA), consistent with a recent report for α4β2δ receptors (Akk et al., 2004). At the whole cell level, the amplitude of pentobarbital-evoked currents (measured at the peak of the "rebound") was significantly larger than maximal GABA-evoked currents from the same cells. These observations are consistent with the proposed idea that GABA may be a partial agonist at this isoform, which may provide the capacity for substantial modulation (relative to αβγ isoforms) by a variety of compounds (Lees and Edwards, 1998; Adkins et al., 2001; Thompson et al., 2002; Bianchi and Macdonald, 2003; Wallner et al., 2003; Akk et al., 2004).

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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Pentobarbital modulation of α1β3δ and α1β3γ2L receptor single channel behavior

Pentobarbital increased mean α1β3δ receptor open duration approximately two-fold, predominantly due to the introduction of a third, longer duration open state. This fundamental change in gating was reminiscent of that observed with neurosteroid modulation (Wohlfarth et al., 2002). Pentobarbital also increased α1β3γ2L receptor mean single channel open duration (by ~3 fold), but did so by a different mechanism than that observed for α1β3δ receptors, increasing the relative proportion and duration of the third open state. This finding is similar to reports on pentobarbital modulation of native and recombinant (α1β2γ2L) receptor currents (Macdonald et al., 1989; Steinbach and Akk, 2001), but in the spinal neuron preparations, the duration of the third open state was unaltered. Although this modulation was significant, pentobarbital did not introduce an additional open state since three open states were available to channels bound by GABA alone; pentobarbital appeared to modulate an already available open state. The differences in gating efficacy between α1β3δ and α1β3γ2L receptors in response to GABA may underlie some of the macroscopic differences in pentobarbital modulation that we observed (see below).

Pentobarbital modulation of macroscopic GABA-evoked currents from α1β3δ and α1β3γ2L receptors

Pentobarbital potentiated peak currents evoked by both low and high GABA concentration for α1β3δ receptors, but only those evoked by low GABA concentration for α1β3γ2L receptors. The selective enhancement of peak currents for low but not high GABA concentrations has been observed for many modulators, including benzodiazepines and neurosteroids. Although the precise mechanism for this difference remains unclear, we propose that it depended in part on kinetic differences in response to GABA between these isoforms. For

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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α1β3δ receptors, the peak open probability was low (Fisher and Macdonald, 1997b) and similar (within a factor of 2) throughout 28 second GABA applications (Bianchi and Macdonald, 2002; this study). In contrast, the peak open probability of the high efficacy α1β3γ2L receptors was ~10 fold higher than the steady state open probability (~90 % desensitization in 28 seconds). Simulations predicted that peak open probability (40 %) was only slightly changed with simulated increases in gating efficacy. This limited change in peak open probability was not exhibited for models of low efficacy, minimally desensitizing receptors (such as α1β3δ receptors). Pentobarbital potentiation of both α1β3γ2L and α1β3δ receptor peak currents evoked by low concentration of GABA (a condition in which both isoforms have low efficacy) is consistent with this modeling. The simulations provided a potential explanation for our observation that saturating GABA-evoked peak α1β3δ but not α1β3γ2L peak currents were potentiated by pentobarbital: low efficacy channel behavior, particularly in the context of minimal desensitization, is preferentially susceptible to positive allosteric modulation. Similar observations of selective allosteric enhancement of αβδ peak currents (relative to αβγ isoforms) have been reported for neurosteroids (Wohlfarth et al., 2002), as well as other modulators (Lees and Edwards, 1998; Thompson et al., 2002; Wallner et al., 2003).

Although pentobarbital differentially modulated the peak currents evoked by saturating GABA for α1β3δ and α1β3γ2L receptors, the residual currents for both of these isoforms were enhanced by pentobarbital. Simulations suggested that changes in opening rate and desensitization, in addition to mean open time, may also be involved in pentobarbital modulation of these isoforms. For example, pentobarbital may result in a decrease in open frequency (due to increased stability of a desensitized state) for α1β3γ2L receptors (Figure 10F). Consistent with

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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this, pentobarbital was reported to decrease single channel steady-state opening frequency in spinal neurons (Macdonald et al., 1989).

Pentobarbital modulation of desensitization and deactivation of α1β3δ and α1β3γ2L receptors

Interestingly, we found that pentobarbital differentially affected macroscopic desensitization of α1β3δ and α1β3γ2L receptor currents, resulting in increased desensitization of α1β3δ receptor currents but decreased desensitization of α1β3γ2L receptor currents. The increased mean open duration of GABA-evoked α1β3γ2L receptor single channel currents by pentobarbital might account for the apparent slowing of desensitization, as we have argued previously using a mutation that decreased apparent desensitization secondary to increased gating efficacy (Bianchi and Macdonald, 2001). However, pentobarbital might directly alter α1β3γ2L receptor desensitization. Our simulations suggested the possibility that increased gating efficacy and increased stability of the intermediate desensitized state may together account for the experimental observations. In this setting, the indirect reduction (related to efficacy) was partially balanced by an increase in microscopic desensitization, with the net result being less extensive desensitization.

In contrast, co-application of pentobarbital with a saturating GABA concentration increased desensitization of α1β3δ receptors. Similar observations of increased gating efficacy together with increased desensitization were reported for neurosteroid modulation of α1β3δ receptors (Wohlfarth et al., 2002). Although it was possible that the new longer open state observed in the presence of pentobarbital was "coupled" somehow to desensitized states, there is experimental evidence of gating and desensitization varying independently (Bianchi and Macdonald, 2001; Bianchi and Macdonald, 2003), and rapid desensitization has been reported for α1β3 and α1β3ε receptors, both of which exhibit only two open states (Fisher and

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Macdonald, 1997b; Neelands et al., 1999). As suggested by the simulations, the macroscopic manifestation of desensitization depends on the underlying gating efficacy. Interestingly, the effect is opposite for high and low efficacy receptors. When efficacy is low at baseline, increasing it may actually enhance macroscopic desensitization; when efficacy is high at baseline, further increases tend to reduce the rate and extent of desensitization. In both settings, macroscopic desensitization is changed without altering desensitized states. In addition, pentobarbital may directly alter the slow desensitized state of α1β3δ receptors since, like the α1β3γ2L receptor model, changes in the stability of the desensitized state (in combination with changes in gating efficacy) of the α1β3δ receptor model produced simulated currents that were similar to our experimental observations (Figure 10F).

We observed that the mean current deactivation time constants were prolonged for both α1β3δ and α1β3γ2L receptors, similar to reports in cultured hippocampal neurons (Rho et al., 1996). Increased desensitization is one mechanism by which deactivation may be prolonged (Jones and Westbrook, 1995; Haas and Macdonald, 1999), but this was not observed for α1β3γ2L receptor currents. We have shown that deactivation can be prolonged by increased channel open time (Bianchi and Macdonald, 2001), as well as increased agonist affinity (Haas and Macdonald, unpublished). The present data are consistent with the conclusion that prolongation of deactivation together with decreased desensitization for α1β3γ2L receptors was due in part to an increase in channel open time. In principle, it is possible that pentobarbital increased the affinity for GABA, but this (in addition to the increase in efficacy) would predict a reduction of GABA EC_{50} that was not observed here. Our previous simulations showed that, for simple models, deactivation was clearly sensitive to increased opening rate (Bianchi and

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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Macdonald, 2001), consistent with our hypothesis that opening frequency was changed for α1β3δ receptors.

Implications of pentobarbital enhancement of tonic, extra or peri-synaptic δ subunit-containing receptor currents

Barbiturates such as pentobarbital are thought to depress neuronal activity by prolongation of IPSCs (Poisbeau et al., 1997; Rovira and Ben-Ari, 1999). However, the role of CNS depressants and anesthetics on extra or peri-synaptic inhibition has remained largely unknown. The current study suggested that pentobarbital may selectively modulate δ subunit-containing $GABA_A$ receptors. Although αβγ isoforms also exist at non-synaptic locations, δ subunit-containing $GABA_A$ receptors are targeted exclusively to extra or peri-synaptic sites. Pentobarbital enhancement of tonic inhibition, and in particular that mediated by αβδ isoforms, may contribute to its anesthetic effect in the CNS.

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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ACKNOWLEDGEMENTS

We thank Luyan Song for preparing the $GABA_A$ receptor subunits cDNAs.

Molecular Pharmacology Fast Forward. Published on July 9, 2004 as DOI: 10.1124/mol.104.002543
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FOOTNOTES

This work was supported by NIH grant R01-NS33300 to Robert L. Macdonald.

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FIGURE LEGENDS

Figure 1. The differential GABA sensitivity of α1β3γ2L and α1β3δ receptors.

A, Representative whole-cell current traces evoked by different concentrations of GABA from α1β3γ2L and α1β3δ receptors are presented. B, The mean peak conductance changes (ΔG) with different GABA concentrations are plotted for α1β3γ2L and α1β3δ receptors. The squares denote the mean peak ΔG for α1β3γ2L receptors (n = 7), and the circles denote ΔG for α1β3δ receptors (n = 6). The error bars represent SEMs (the error bars are too small to be seen for α1β3δ receptors). The holding potential was –20 mV for α1β3γ2L receptors and –50 mV for α1β3δ receptors.

Figure 2. Characterization of pentobarbital direct activation and open channel block for α1β3γ2L and α1β3δ receptors.

A, Representative currents evoked from the same cell by different concentrations of pentobarbital and 0.3 mM GABA from α1β3γ2L receptors are presented. B, The mean peak conductance changes (ΔG) with different pentobarbital concentrations are plotted for direct and "rebound" currents from α1β3γ2L receptors (n = 8). C, Representative currents evoked from the same cell by different concentrations of pentobarbital and 0.3 mM GABA from α1β3δ receptors are presented. D, The mean peak ΔG for direct and "rebound" currents are plotted for α1β3δ receptors (n = 7). The solid line above each current trace denotes the duration of pentobarbital or GABA application. The squares represent the mean peak ΔG of the direct currents, and the circles represent those of the "rebound" currents. The error bars denote SEMs. The holding potential was –20 mV for α1β3γ2L receptors and –50 mV for α1β3δ receptors.

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Figure 3. Characterization of the apparent desensitization and deactivation of α1β3γ2L and α1β3δ receptors currents evoked by pentobarbital.

A, The mean amounts of desensitization evoked by pentobarbital for α1β3γ2L and α1β3δ receptors were determined. The % of current reductions (desensitization) was increased in a concentration-dependent manner from 300 μM to 3000 μM pentobarbital for α1β3γ2L receptors. For the α1β3δ receptors, only minimal desensitization (2-3 % current reduction) was observed at 1000 μM and 3000 μM pentobarbital. B, The mean rates of deactivation evoked by pentobarbital for α1β3γ2L and α1β3δ receptors were determined. The rate of deactivation was increased in a concentration dependent manner from 300 μM to 3000 μM pentobarbital for α1β3γ2L receptors. For α1β3δ receptors, the mean weighted deactivation time constant was significantly greater at 3000 μM than at 1000 μM pentobarbital. The blank bars represent the mean apparent desensitization or deactivation of α1β3γ2L receptors (n = 8), and the filled bars represent those of α1β3δ receptors (n = 7). Absence of bars indicates that the measurements could not be made due to either small amplitude currents or lack of measurable desensitization. The error bars denote the SEMs.

** Significantly different from 1000 μM pentobarbital at $p<0.01$, # $p<0.05$, +++ $p<0.001$

*** Significantly different from 300 μM pentobarbital at $p<0.001$

Figure 4. Pentobarbital at modulatory concentrations produced greater enhancement of α1β3δ receptor than α1β3γ2L receptor currents.

A, Representative current traces evoked by 300 μM GABA and co-application of 300 μM GABA and 50 μM pentobarbital from α1β3γ2L receptors are presented. B, The concentration-

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response curves for GABA alone (n = 7) and co-application of GABA with 50 μM pentobarbital (n = 8) are plotted for α1β3γ2L receptors. C, Representative current traces evoked by 300 μM GABA and co-application of 300 μM GABA and 30 μM pentobarbital from α1β3δ receptors are presented. D, The concentration-response curves for GABA alone (n = 6) and co-application of GABA with 30 μM pentobarbital (n = 7) are plotted for α1β3δ receptors. The solid line above each representative current trace denotes the duration of drug application. The squares represent the mean normalized response of co-application of GABA and pentobarbital, and the circles represent that of GABA alone. The error bars denote the SEMs (the error bars in D are too small to be seen).

Figure 5. Co-application (4 sec) of 1 μM GABA with 100 μM pentobarbital produced similar alterations in enhancement, desensitization and deactivation for α1β3γ2L and α1β3δ receptors.

A, Representative current traces evoked by 1 μM GABA and co-application of 1 μM GABA with 100 μM pentobarbital from α1β3γ2L receptors are presented. The GABA control trace (gray trace) was normalized to the pentobarbital enhanced trace to show the changes in desensitization and deactivation evoked by co-application of GABA and pentobarbital. B, Representative current traces evoked by 1 μM GABA and co-application of 1 μM GABA with 100 μM pentobarbital from α1β3δ receptors are presented. C, Pentobarbital potentiation of GABA currents are plotted for both α1β3γ2L (n = 6) and α1β3δ receptors (n = 8). D, Mean apparent desensitization evoked by 1 μM GABA and co-application of 1 μM GABA with 100 μM pentobarbital is plotted. E, Mean rate of current deactivation evoked by 1 μM GABA and co-application of 1 μM GABA with 100 μM pentobarbital is plotted. The solid line above each

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current trace denotes the duration of GABA application, and the dashed line denotes that of pentobarbital application. The blank bars represent the mean desensitization or deactivation of GABA alone treatment, and the filled bars represent that of co-application of GABA and pentobarbital. The error bars denote the SEMs.

* Significantly different from corresponding GABA control at $p<0.05$, ** $p<0.01$

Significantly different from GABA control in δ isoform at $p<0.001$

+ Significantly different from co-application of GABA and pentobarbital in δ isoform at $p<0.001$

Figure 6. Co-application (4 sec) of 1 mM GABA with 100 μM pentobarbital produced differential alterations in enhancement and desensitization for α1β3γ2L and α1β3δ receptors.

A, Representative current traces evoked by 1 mM GABA and co-application of 1 mM GABA with 100 μM pentobarbital from α1β3γ2L receptors. The GABA control trace (gray trace) was normalized to the trace evoked by co-application of GABA and pentobarbital to show the changes in desensitization and deactivation. B, Representative current traces evoked by 1 mM GABA and co-application of 1 mM GABA with 100 μM pentobarbital from α1β3δ receptors. C, Pentobarbital differentially affected the mean GABA peak currents for α1β3γ2L (n = 7) and α1β3δ (n = 8) receptors. The dashed line indicates 100 %. D, Comparison of the mean apparent desensitization evoked by 1 mM GABA and co-application of 1 mM GABA with 100 μM pentobarbital. E, Comparison of the mean rate of current deactivation evoked by 1 mM GABA and co-application of 1 mM GABA with 100 μM pentobarbital. The solid line above each representative current trace denotes the duration of GABA application, and the dashed line denotes that of pentobarbital application. The blank bars represent the mean apparent

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desensitization or deactivation of GABA alone treatment. The filled bars represent that of co-application of GABA and pentobarbital. The error bars denote the SEMs.

* Significantly different from corresponding GABA control at $p<0.05$

** Significantly different from γ2L isoform or GABA control at $p<0.01$

Significantly different from GABA control in δ isoform at $p<0.001$

+ Significantly different from co-application of GABA and pentobarbital in δ isoform at $p<0.05$, ++ $p<0.01$

Figure 7. Pentobarbital significantly enhanced the residual currents for both α1β3γ2L and α1β3δ receptors after long-duration (28 sec) application of GABA

A, Representative current traces evoked by 1 mM GABA (28 sec) and co-application of 1 mM GABA with 100 μM pentobarbital from α1β3γ2L receptors. B, Pentobarbital significantly enhanced the mean residual currents at "steady state" for α1β3γ2L receptors (n = 5). C, Pentobarbital significantly decreased the apparent desensitization for α1β3γ2L receptors (n = 5). D, Representative current traces evoked by 1 mM GABA (28 sec) and co-application of 1 mM GABA with 100 μM pentobarbital from α1β3δ receptors. E, Pentobarbital significantly enhanced the mean residual currents at "steady state" for α1β3δ receptors (n = 4). F, Pentobarbital significantly increased the apparent desensitization for α1β3δ receptors (n = 4). The solid line above each representative current trace denotes the duration of GABA application, and the dashed line denotes that of pentobarbital application. The gray dashed line indicates the level of residual current for GABA controls. The error bars denote the SEMs.

* Significantly different from GABA control at $p<0.05$

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Figure 8. Single channel $\alpha1\beta3\gamma2L$ receptor currents evoked by 1 mM GABA, co-application of 1 mM GABA and 100 μM pentobarbital or 100 μM pentobarbital alone

The portion of each single channel current trace (A1, B1, C1) below the hatched bar was expanded and shown in A2, B2 and C2, and a portion of each trace (A2, B2, C2) was shown in A3, B3 and C3. Compared to the single channel current evoked by 1 mM GABA or 100 μM pentobarbital, the open duration of single current was prolonged with co-application of GABA and pentobarbital. The distributions of open states for each treatment are plotted in A4, B4 and C4. The open events for A4, B4 and C4 were 7351, 3976 and 4229, respectively. Each histogram contained the data from a single patch. Note that the time constants and relative areas of the third open states were increased by co-application of GABA and pentobarbital (the x-axis values for B4 are different than those of A4 and C4).

Figure 9. Single channel $\alpha1\beta3\delta$ receptor currents evoked by 1 mM GABA, co-application of 1 mM GABA and 100 μM pentobarbital or 100 μM pentobarbital alone

The portion of each single current trace (A1, B1, C1) below the hatched bar was expanded proportionally and shown in A2, B2 and C2, and a portion of each trace (A2, B2, C2) was shown in A3, B3 and C3. Compared to the single channel current evoked by 1 mM GABA or 100 μM pentobarbital, the open duration of single current was prolonged with co-application of GABA and pentobarbital. The distributions of open states for each treatment are plotted in A4, B4 and C4. The open events for A4, B4 and C4 were 4890, 3767 and 4257, respectively. Each histogram contained the data from a single patch. Note that co-application of GABA and pentobarbital introduced an additional open state, which was not seen with GABA alone. Three open states were observed with pentobarbital treatment alone.

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Figure 10. Simulations of α1β3δ and α1β3γ2L receptor currents

A, Kinetic model of α1β3γ2L receptor behavior (from Haas and Macdonald, 1999). Rate constants (in units of s^{-1}, except k_{on} in $s*M^{-1}$) were: k_{on} = 7.0e+6; k_{off} = 170; C_{34} = 710; C_{43} = 58; β_1 = 50; β_2 = 1800; β_3 = 76; α_1 = 3100; α_2 = 280; α_3 = 150; D_f = 960; R_f = 22; D_i = 8; R_i = 0.81; D_s = 0.75; R_s = 0.49; for clarity, intra-burst closed states proceeding from each open state were omitted from the figure (see Haas and Macdonald, 1999). B, Kinetic model of α1β3δ receptor current response to 1 mM GABA. Rate constants (in units of s^{-1}, except k_{on} in $s*M^{-1}$) were: k_{on} = 6.5e+6; k_{off} = 18; C_{34} = 12; C_{43} = 11; β_1 = 80; β_2 = 9; α_1 = 2400; α_2 = 600; D_s = 0.35; R_s = 0.35; for clarity, intra-burst closed states proceeding from each open state were omitted from the figure (see Haas and Macdonald, 1999). C, Simulations of α1β3γ2L receptor current response to 1 mM GABA under control condition (left trace), as well as decreasing α_2 by 3 (second trace), decreasing both α_2 and α_3 by a factor of 3 (third trace), and decreasing α_3 by a factor of 2 and increasing β_3 by a factor of 2 to reflect the observed changes in O_3 time constant and relative proportion (right trace). For each trace, the current was generated using a 6 second square pulse of 1 mM GABA. Gaussian "noise" was added to each current with a standard deviation of 0.5 (on a scale of 0-100 open probability percentage). Dotted lines show the level of the peak and residual current for the control (left) trace for comparison with other conditions. D, Simulations of α1β3δ receptors under control conditions (left trace), with α_1 decreased by a factor of 2 (second trace), with α_2 decreased by a factor of 2 (third trace), or both (right trace). For each trace, the current was generated using a 6 second square pulse of 1 mM GABA. Gaussian "noise" was added to the currents with a standard deviation of 0.5 (on a scale of 0-100 open probability percentage). The apparent increase in noise reflects the decreased open probability of

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this model relative to the α1β3γ2L model. The horizontal time scale is the same as that in panel C. E, Simulation showing the occupancy of various states of the α1β3γ2L kinetic model during a simulated 6 sec application of 1 mM GABA. The total open probability (ie, the sum of states O_1, O_2 and O_3) were shown as downward going and represented the shape of the predicted current. Overlaid upward going lines were the probability density functions of the three desensitized states, O_2 and O_3 (O_1 and other closed states were not shown). F, Simulations of α1β3γ2L (left) and α1β3δ receptors (right) generated to reflect observed macroscopic currents in the presence of pentobarbital. For the α1β3γ2L model, the following rate constants were changed: β_2 = 1450; β_3 = 150; α_2 = 200; α_3 = 75; R_i = 0.4. The resulting trace was overlaid, and shifted to the right for comparison (but was not normalized). For the α1β3δ model, the "control" rate constants were altered in the following way to more closely represent our whole cell observations: C_{43} = 22; β_2 = 18. The following additional changes were made to reflect the presence of pentobarbital: β_1 = 400; β_2 = 90; β_3 = 10; α_3 = 250; α_2 = 450; D_s = 0.17; R_s = 0.07. No intraburst closed states were added to state O_3. * Indicates simulations that reflect the presence of pentobarbital.

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Table 1. Pentobarbital (100 μM) modulated the open states of $GABA_A$ receptors

	α1β3γ2L			α1β3δ		
	GABA (1 mM)	+PB (+100 μM)	PB (100 μM)	GABA (1 mM)	+PB (+100 μM)	PB (100 μM)
N	5	5	4	6	7	5
Mean	1.45±0.26	4.86±1.06[a,c]	1.41±0.19	0.53±0.04	1.08±0.17[a,c]	0.48±0.07
τ_1 (ms)	0.31±0.02	0.32±0.07	0.24±0.01[f]	0.33±0.02	0.35±0.03[e]	0.18±0.02[g]
A_1 (%)	49.2±3.33	33.2±5.34[a]	46.0±6.39	65.0±2.87	54.4±2.81[a]	61.1±3.26
τ_2 (ms)	1.55±0.39	2.28±0.58	1.11±0.13	0.90±0.10	1.27±0.10[a,d]	0.75±0.10
A_2 (%)	32.7±3.01	25.4±3.33	32.9±2.92	35.1±2.88	35.3±1.97	35.4±2.87
τ_3 (ms)	4.63±0.81	9.37±1.11[b,c]	4.66±0.67		4.03±0.59	2.95±0.62
A_3 (%)	18.2±3.04	41.4±7.21[a]	21.2±4.33		10.3±2.15[c]	3.50±0.57

For α1β3γ2L receptors the average number of openings were 3758 (GABA), 3899 (GABA + pentobarbital) and 3645 (pentobarbital), and for α1β3δ receptors 5309 (GABA), 4320 (GABA+ pentobarbital) and 3499 (pentobarbital).

a significantly different from corresponding GABA control at $p<0.05$, *b* $p<0.01$;

c significantly different from corresponding PB at $p<0.05$, *d* $p<0.01$, *e* $p<0.001$;

f significanlty different from corresponding GABA at $p<0.05$, *g* $p<0.001$

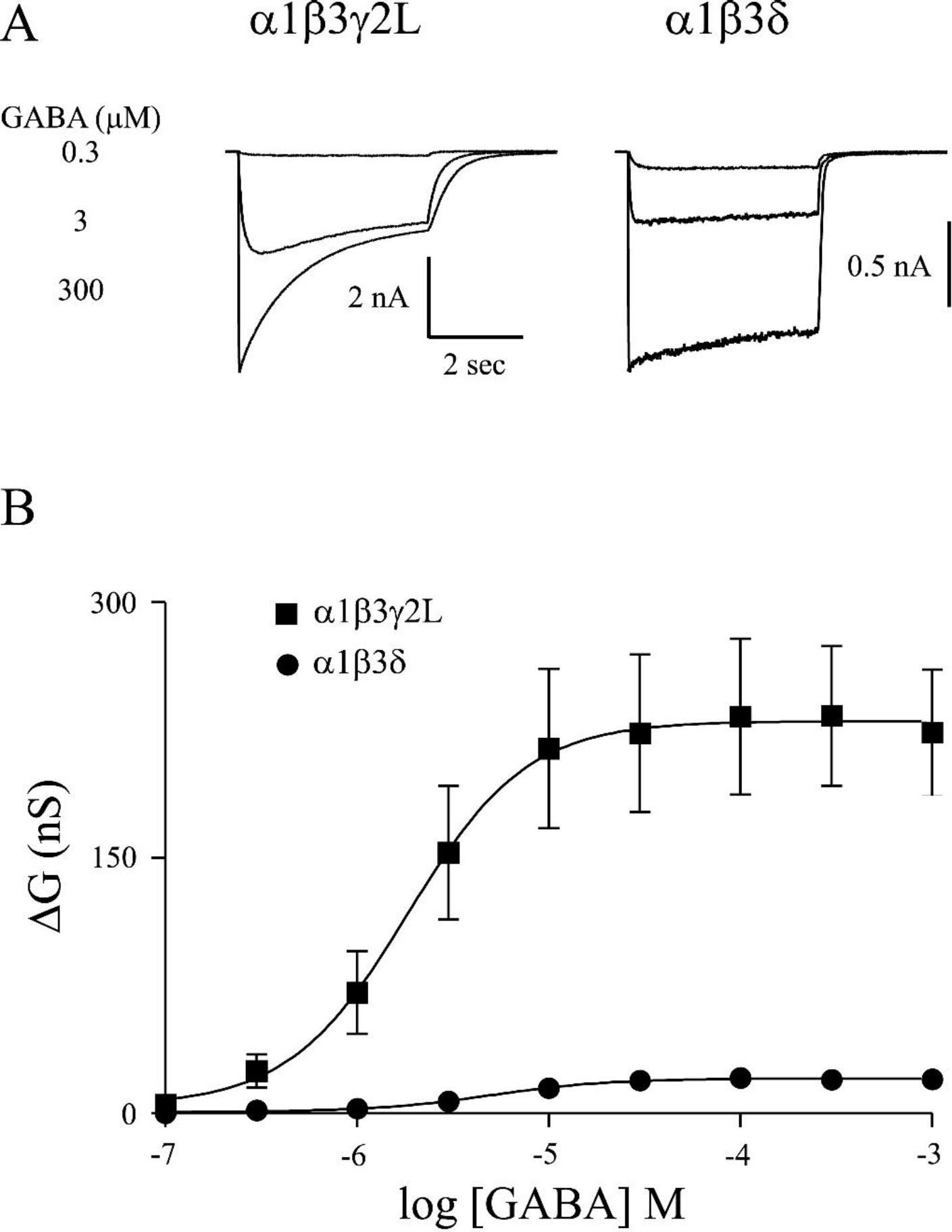

A
α1β3γ2L
α1β3δ
GABA (μM)
0.3
3
300
2 nA
2 sec
0.5 nA
B
300
150
0
ΔG (nS)
α1β3γ2L
α1β3δ
-7
-6
-5
-4
-3
log [GABA] M

Fig. 1

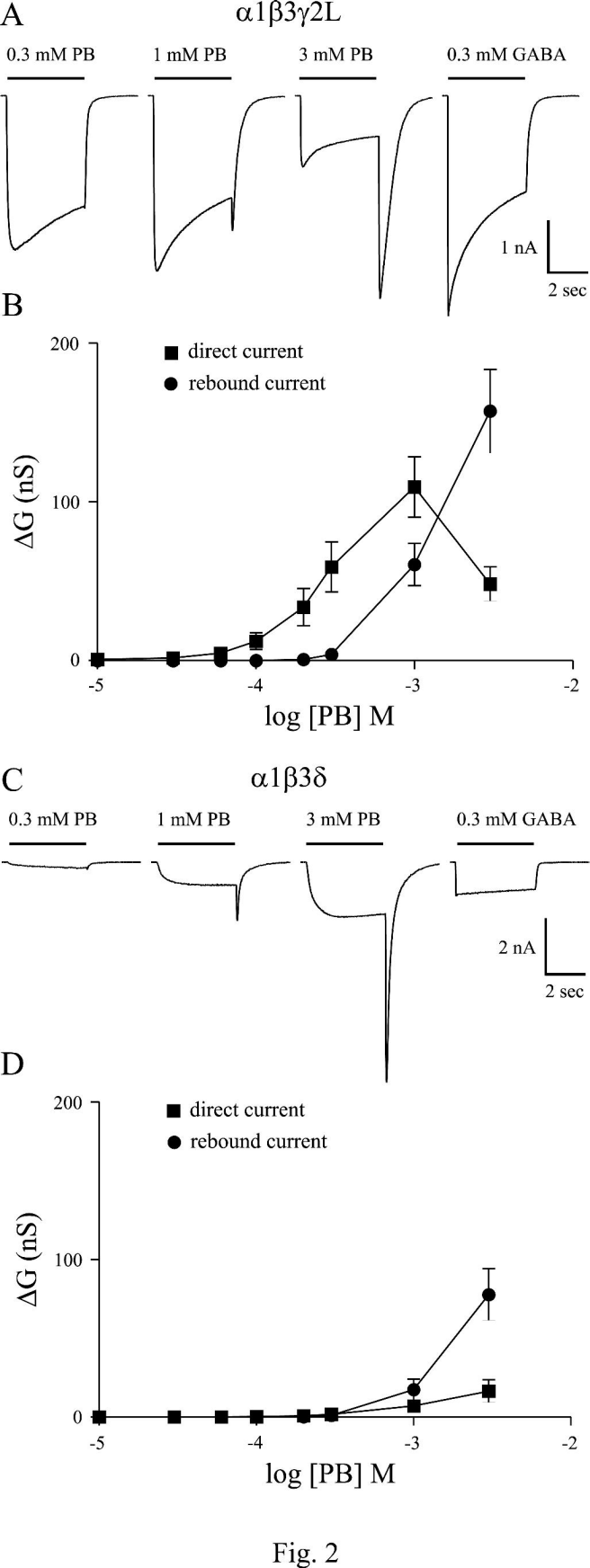

A
α1β3γ2L
0.3 mM PB
1 mM PB
3 mM PB
0.3 mM GABA
1 nA
2 sec
B
200
100
0
ΔG (nS)
direct current
rebound current
-5
-4
-3
-2
log [PB] M
C
α1β3δ
0.3 mM PB
1 mM PB
3 mM PB
0.3 mM GABA
2 nA
2 sec
D
200
100
0
ΔG (nS)
direct current
rebound current
-5
-4
-3
-2
log [PB] M

Fig. 2

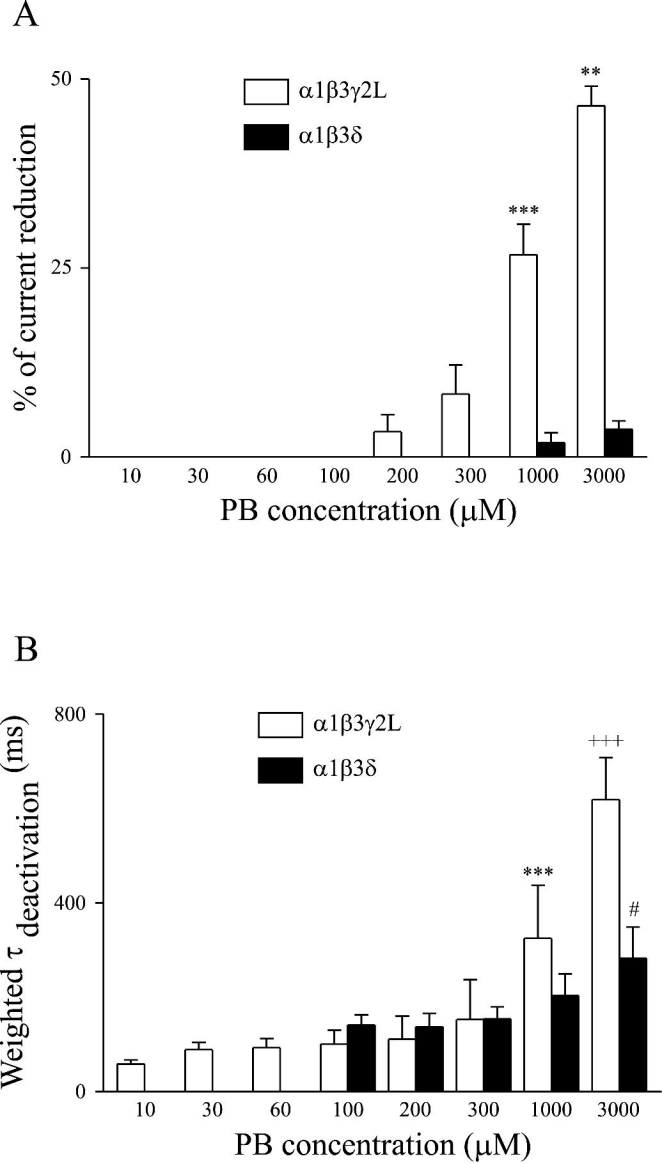

A
50
25
0
% of current reduction
α1β3γ2L
α1β3δ
**
***
10
30
60
100
200
300
1000
3000
PB concentration (μM)
B
800
400
0
Weighted τ deactivation (ms)
α1β3γ2L
α1β3δ
+++
***
#
10
30
60
100
200
300
1000
3000
PB concentration (μM)

Fig. 3

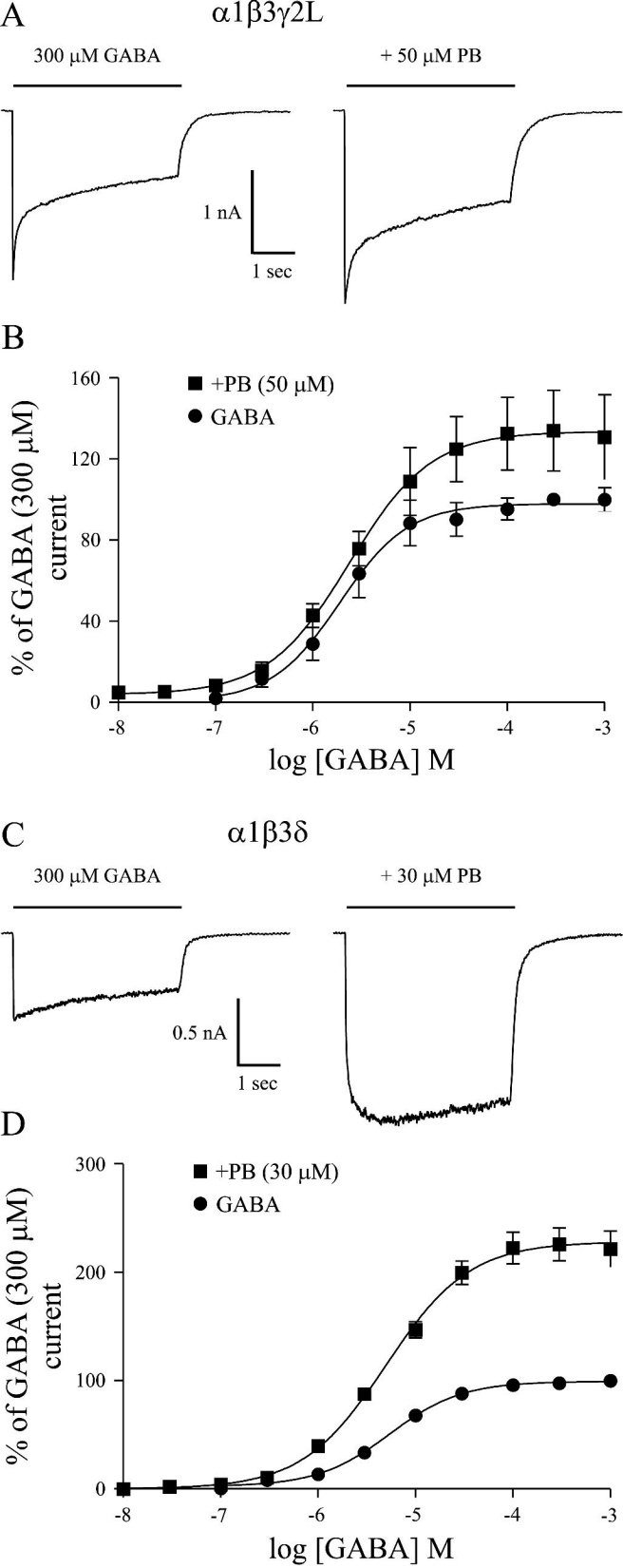

A
α1β3γ2L
300 μM GABA
+ 50 μM PB
1 nA
1 sec
B
+PB (50 μM)
GABA
% of GABA (300 μM) current
log [GABA] M
C
α1β3δ
300 μM GABA
+ 30 μM PB
0.5 nA
1 sec
D
+PB (30 μM)
GABA
% of GABA (300 μM) current
log [GABA] M

Fig. 4

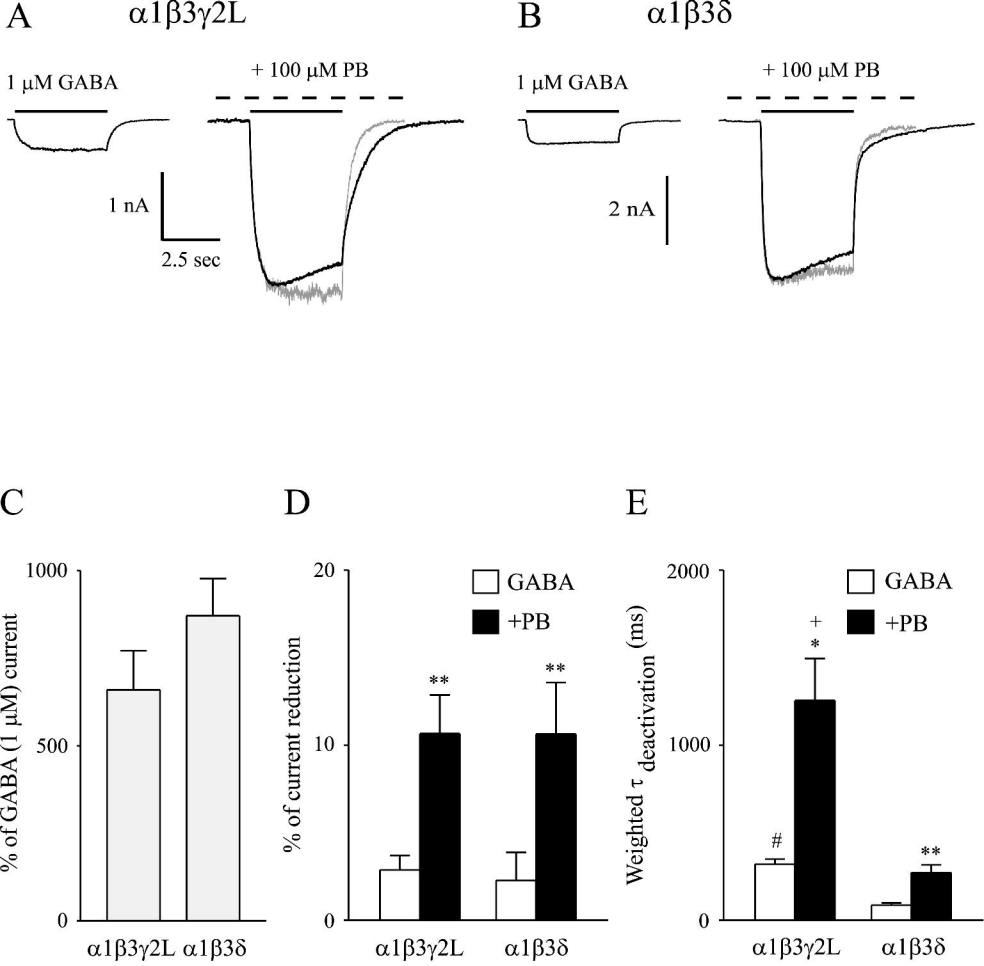
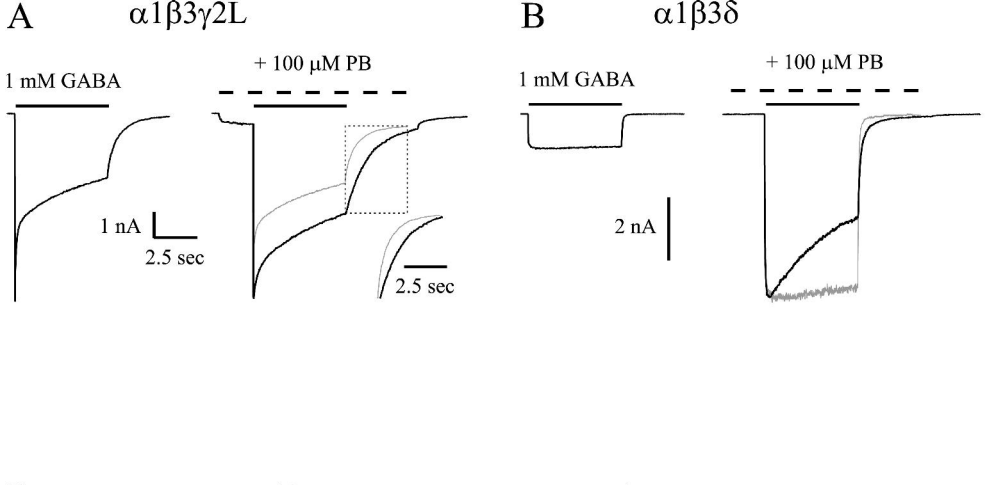

A
α1β3γ2L
1 μM GABA
+ 100 μM PB
1 nA
2.5 sec
B
α1β3δ
1 μM GABA
+ 100 μM PB
2 nA
C
% of GABA (1 μM) current
1000
500
0
α1β3γ2L
α1β3δ
D
% of current reduction
20
10
0
GABA
+PB
**
**
α1β3γ2L
α1β3δ
E
Weighted τ deactivation (ms)
2000
1000
0
GABA
+PB
+
*
#
**
α1β3γ2L
α1β3δ

Fig. 5


A
α1β3γ2L
1 mM GABA
+ 100 μM PB
1 nA
2.5 sec
2.5 sec
B
α1β3δ
1 mM GABA
+ 100 μM PB
2 nA

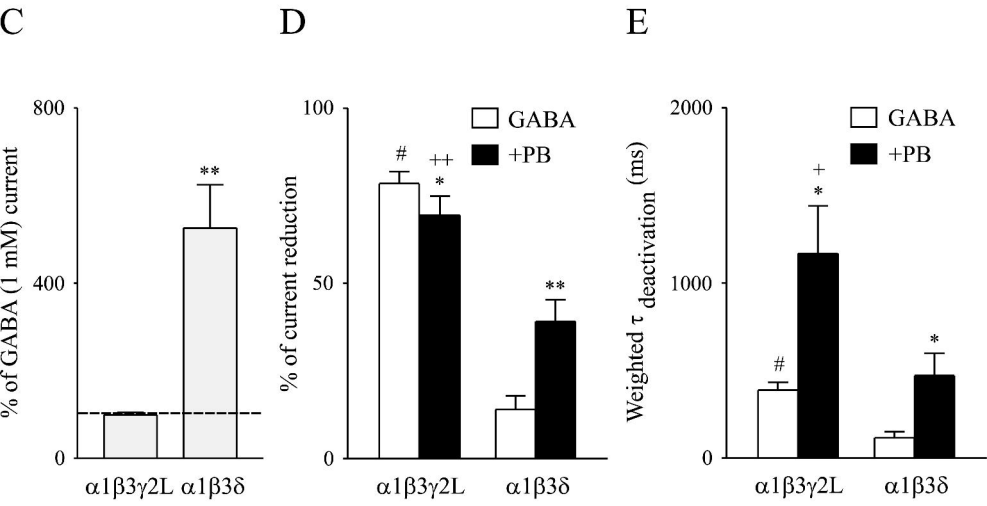

C
800
400
0
% of GABA (1 mM) current
**
α1β3γ2L
α1β3δ
D
100
50
0
% of current reduction
GABA
+PB
#
++
*
**
α1β3γ2L
α1β3δ
E
2000
1000
0
Weighted τ deactivation (ms)
GABA
+PB
#
+
*
*
α1β3γ2L
α1β3δ

Fig. 6

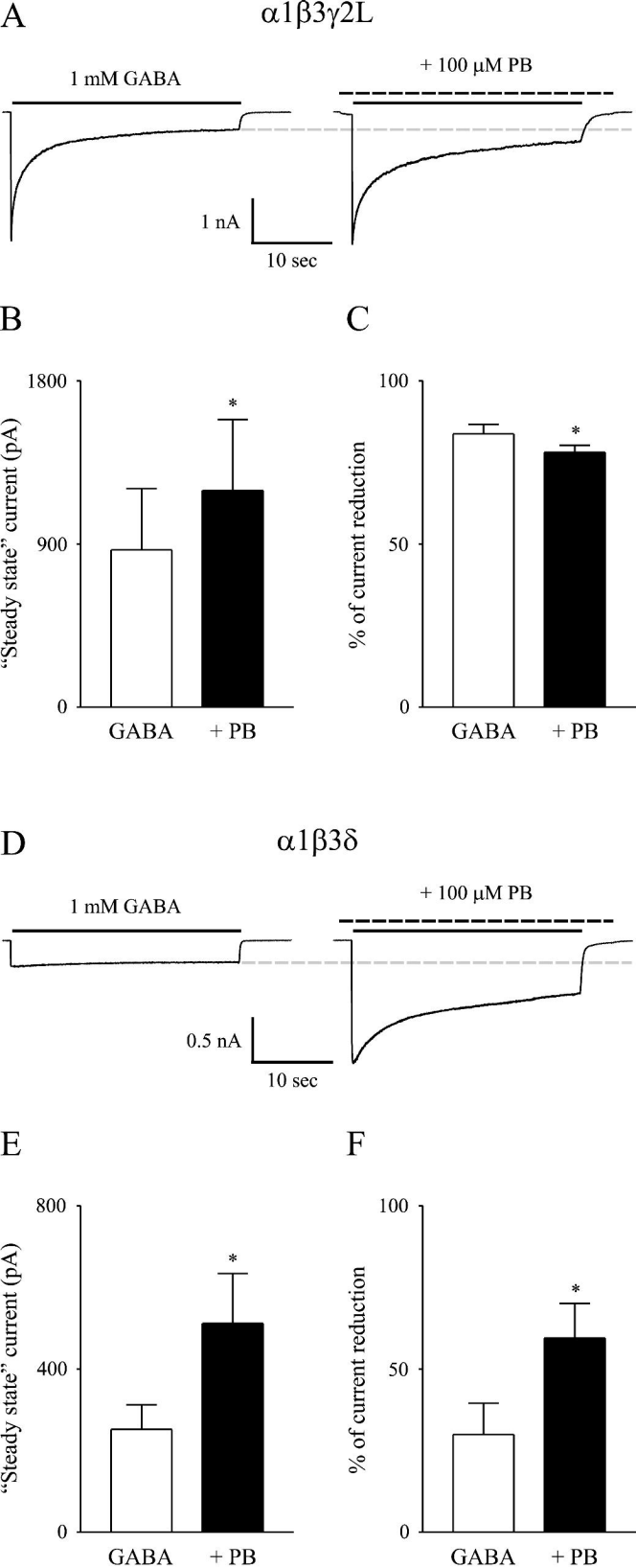

A
α1β3γ2L
1 mM GABA
+ 100 μM PB
1 nA
10 sec
B
C
1800
900
0
"Steady state" current (pA)
*
GABA
+ PB
100
50
0
% of current reduction
*
GABA
+ PB
D
α1β3δ
1 mM GABA
+ 100 μM PB
0.5 nA
10 sec
E
F
800
400
0
"Steady state" current (pA)
*
GABA
+ PB
100
50
0
% of current reduction
*
GABA
+ PB

Fig. 7

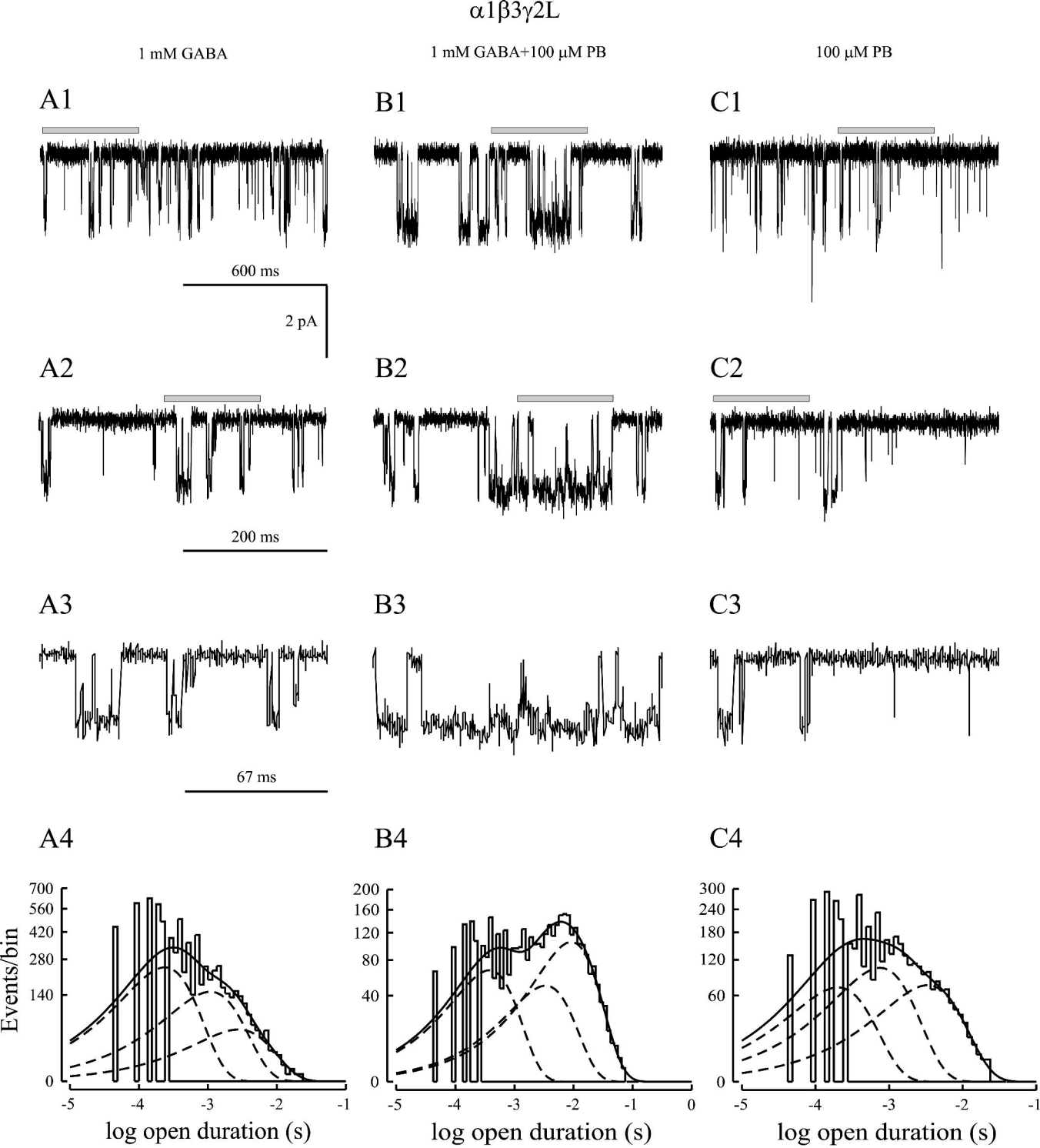

α1β3γ2L
1 mM GABA
1 mM GABA+100 μM PB
100 μM PB
A1
B1
C1
600 ms
2 pA
A2
B2
C2
200 ms
A3
B3
C3
67 ms
A4
B4
C4
Events/bin
log open duration (s)
log open duration (s)
log open duration (s)

Fig. 8

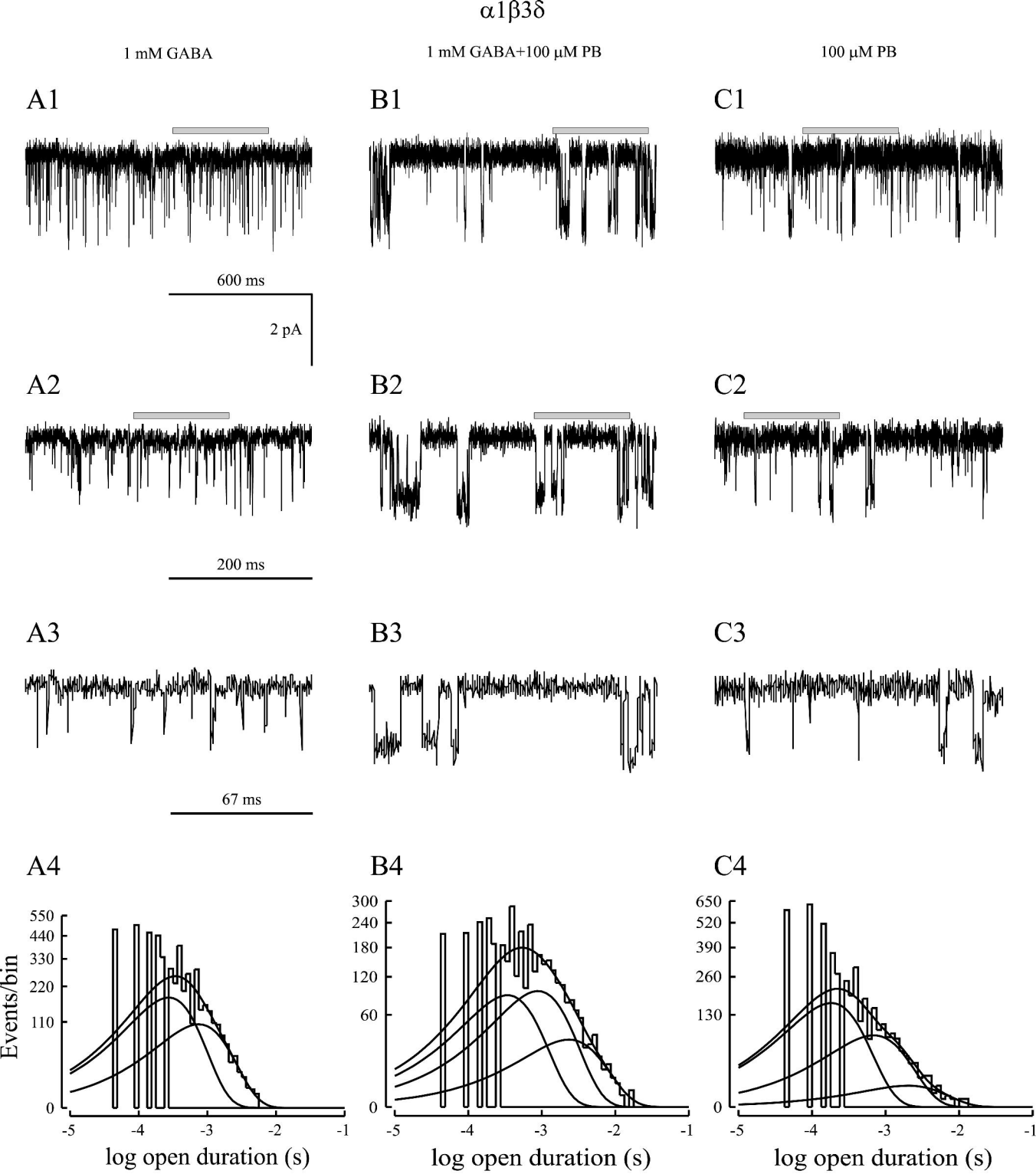

α1β3δ
1 mM GABA
1 mM GABA+100 μM PB
100 μM PB
A1
B1
C1
600 ms
2 pA
A2
B2
C2
200 ms
A3
B3
C3
67 ms
A4
B4
C4
Events/bin
550
440
330
220
110
0
300
240
180
120
60
650
520
390
260
130
-5
-4
-3
-2
-1
log open duration (s)

Fig. 9

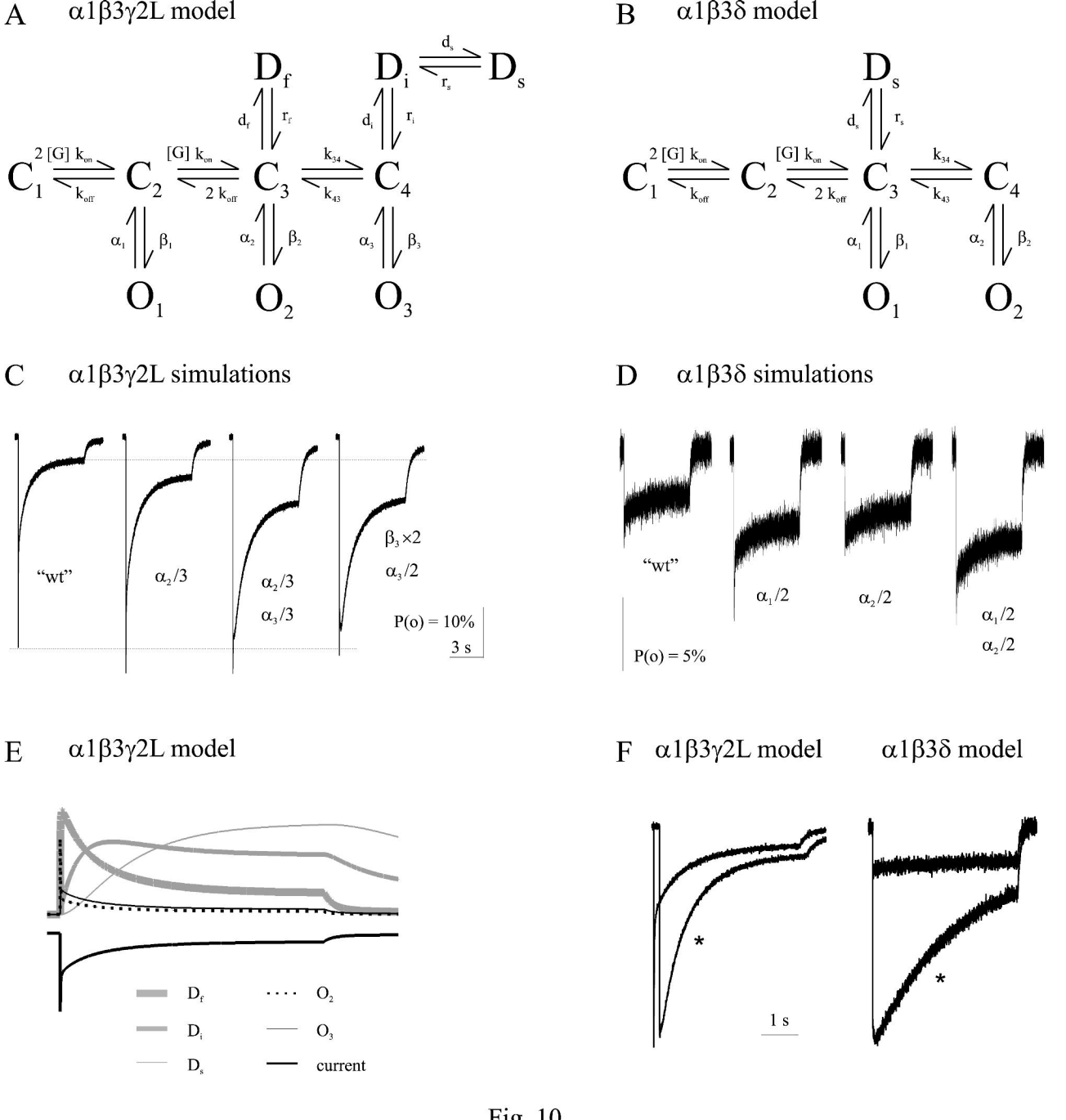

A
α1β3γ2L model
B
α1β3δ model
C
α1β3γ2L simulations
"wt"
α2/3
α2/3
α3/3
β3×2
α3/2
P(o) = 10%
3 s
D
α1β3δ simulations
"wt"
α1/2
α2/2
α1/2
α2/2
P(o) = 5%
E
α1β3γ2L model
Df
Di
Ds
O2
O3
current
F
α1β3γ2L model
α1β3δ model
1 s

Fig. 10