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Incorporation of the π Subunit into Functional γ-Aminobutyric Acid$_A$ Receptors

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ABSTRACT

mRNA encoding the recently cloned γ-aminobuytyric acid$_A$ receptor (GABAR) π subunit is expressed in the hippocampus and in several non-neuronal tissues including the uterus and ovaries. Whereas native GABARs are pentamers composed primarily of $\alpha\beta\gamma$, $\alpha\beta\delta$, or $\alpha\beta\epsilon$ subunits, it has not been demonstrated clearly that the π subunit incorporates into functional GABARs to form $\alpha\beta\pi$ receptors and, if so, with what properties. We provide electrophysiological evidence that the π subunit can coassemble with either $\alpha5\beta3$ or $\alpha5\beta3\gamma3$ subunits to produce recombinant GABARs with distinct pharmacological and biophysical properties. Compared with $\alpha5\beta3$ receptors, GABARs produced by coexpression of $\alpha5\beta3\pi$ subunits had a lower GABA EC$_{50}$ value, were enhanced to a lesser extent by loreclezole, had different IC$_{50}$ values for pregnenolone sulfate and lanthanum, and were insensitive to benzodiazepines. Incorporation of both π and $\gamma3$ subunits into an $\alpha5\beta3\gamma3\pi$ isoform was suggested by reduced enhancement by diazepam and a high zinc IC$_{50}$ value. Current-voltage relations for the $\alpha5\beta3\pi$ subunit combination outwardly rectified more than currents from $\alpha5\beta3\gamma3$ but less than $\alpha5\beta3$ combination GABARs. Single-channel $\alpha5\beta3$ GABAR currents had a main conductance state of 15.2 picoSeimens (pS). Coexpression of the π subunit with $\alpha5\beta3$ subtypes increased the conductance level to 23.8 pS, similar to the conductance level of $\alpha5\beta3\gamma3$ GABARs (26.9 pS). We conclude that the π subunit coassembles with α, β, and γ subunits to form functional $\alpha\beta\pi$ or $\alpha\beta\gamma\pi$ GABARs and, thus, could have a significant impact on the function of native GABARs expressed in the brain or non-neuronal tissue.

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate brain. Fast inhibitory postsynaptic potentials are mediated by GABA$_A$ receptors (GABARs), which contain binding sites for many modulators including benzodiazepines, barbiturates, zinc and general anesthetics including neurosteroids. GABARs have also been identified on peripheral neurons and nonneuronal cell types. The role of GABARs outside the central nervous system (CNS) is not well documented, but it has been proposed that GABARs may play a role in the motility of uterine contractions and the secretion of hormones from endocrine cells.

GABARs are composed of five subunits that together form a transmembrane chloride ion channel. Four different mammalian subunit families (α, β, γ, δ) and their subtypes ($\alpha1$–6, $\beta1$–3, $\gamma1$–3) have been studied extensively (Macdonald and Olsen, 1994). Two new subunit families, ϵ (Davies et al., 1997) and π (Hedblom and Kirkness, 1997), have recently been identified. In addition, α, β, γ, and δ subunit subtypes have been shown to be differentially expressed throughout development (Laurie et al., 1992) and in different regions of the rat brain (Wisden et al., 1992). The developmental expression of ϵ and π subunits has not been reported. Pharmacological studies of recombinant receptors have shown that individual subunits and their subtypes confer different sensitivities to such GABAR modulators as benzodiazepines (Pritchett et al., 1989; Wieland et al., 1992) and zinc (Draguhn et al., 1990). The subunit composition of the pentamer seems to be highly regulated and all of the potential subtype combinations do not assemble to create functional GABARs (Angelotti et al., 1993; Burgard et al., 1996). In addition, receptors composed of $\alpha\beta\gamma$ subunits may be further restricted by a 2:2:1 stoichiometry (Chang et al., 1996; Tretter et al., 1997).

The recently cloned π subunit is most closely related to GABAR β (37%) and δ (35%) subunits and to the GABA$_C$ receptor ρ subunit (33%) and is less similar to other GABAR or glycine receptor subunits. The π subunit was amplified from the cDNA libraries of a number of reproductive tissue (uterus, ovaries), digestive tissue (gall bladder, small intestine), and two brain regions (hippocampus and temporal cortex) with reverse transcription-polymerase chain reaction, but no transcripts were hybridized from whole brain samples with Northern analysis (Hedblom and Kirkness, 1997). In addition, the π subunit is expressed by the teratocarcinoma

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GABAR, γ-aminobutyric acid$_A$ receptors; CNS, central nervous system; alphaxalone, (3α)-hydroxy-(5α)-pregnane-11,20-dione; DMSO, dimethyl sulfoxide; PS, pregnenolone sulfate.

NT2 neuronal precursor cells (Hedblom and Kirkness, 1997) and the terminally differentiated NT2-N cells (Neelands et al., 1998). We have previously shown that the NT2 neuronal precursor cells also express high levels of mRNA for the α5 and β3 GABAR subunit subtypes and low levels of the γ3 subtype. GABAR currents in these cells are highly sensitive to inhibition by zinc, enhanced by loreclezole, and unaffected by application of the benzodiazepine diazepam (Neelands et al., 1997).

There has been some question of whether or not the π subunit coassembles with other GABAR subunits to form functional GABARs. Human embryonic kidney 293 fibroblasts transfected with the π subunit alone or in combination with either an α1 or a β1 subunit did not bind the GABAR ligands, muscimol, or *t*-butylbicyclophosphorothionate and no current was evoked by application of GABA (Hedblom and Kirkness, 1997). Muscimol and *t*-butylbicyclophosphorothionate did, however, bind to GABARs composed of the $\alpha1\beta1\pi$ combination but was indistinguishable from binding to $\alpha1\beta1$ GABARs (Hedblom and Kirkness, 1997). Transfection of cells with higher concentrations of π subunit cDNA than γ2 subtype cDNA produced GABARs with reduced binding of the benzodiazepine site ligand flumazenil, which suggests that the π subunit was interfering with the ability of the γ subunit to incorporate into functional GABARs (Hedblom and Kirkness, 1997). However, there is as yet no electrophysiological evidence that the π subunit is incorporated into functional GABARs. The aim of the present study was to determine whether coexpression of the π subunit with α and β or α, β, and γ subunits produced GABARs with properties similar to or different from those of $\alpha\beta$ or $\alpha\beta\gamma$ receptors, consistent with incorporation of the π subunit into GABARs. In addition we wanted to determine whether incorporation of the π subunit altered the pharmacological and biophysical properties of GABARs.

Materials and Methods

Transfections. Full-length cDNAs for rat GABAR α5 (obtained from A. Tobin, University of California, Los Angeles), β3 (obtained from D. Pritchett, University of Pennsylvania, Philadelphia, PA), and γ3 (obtained from P. Seeburg, Max-Planck Institute for Medical Research, Heidelberg, Germany) subtypes were subcloned into the pCMVNeo expression vector and human π (obtained from E. Kirkness, The Institute for Genomic Research, Rockville, MD) was subcloned into the pCDM8 expression vector. For selection of transfected cells, the plasmid pHook-1 (Invitrogen, San Diego, CA) containing cDNA encoding the surface antibody sFv was also transfected into the cells. L929 cells were maintained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Greenfield et al., 1997). Cells were passaged by a 5-min incubation with 0.5% trypsin/0.2% EDTA solution in PBS (10 mM Na_2HPO_4, 0.15 mM NaCl, pH 7.3).

Cells from the mouse fibroblast L929 cell line (American Type Culture Collection, Rockville, MD) were transfected with cDNAs by using a modified calcium phosphate method (Chen and Okayama, 1987; Angelotti et al., 1993). Plasmids encoding GABAR subtype cDNAs were added to the cells in 1:1 ratios of 4 μg each plus 2 to 4 μg of the plasmid encoding sFv. After a 4- to 6-h incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in buffer (50 mM *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 s. The selection procedure for sFv antibody expression was performed 20 to 28 h later as described previously (Greenfield et al., 1997). Briefly, the cells were passaged and mixed with 3 μl of magnetic beads coated with hapten (approximately 4.5×10^5 beads; Invitrogen). After 30 to 60 min of incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated with a magnetic stand. The selected cells were resuspended into Dulbecco's modified Eagle's medium, plated onto 35-mm culture dishes, and used for recording 18 to 28 h later.

Recording Solutions and Techniques. For both whole-cell and outside-out patch recording, the external solution consisted of 142 mM NaCl, 8.1 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, and osmolarity adjusted to 295 to 305 mOsM. Recording electrodes were filled with an internal solution of 153 mM KCl, 1 mM $MgCl_2$, 5 mM K-EGTA, 10 mM HEPES, and 2 mM MgATP, pH 7.4, and osmolarity adjusted to 295–305 mOsM. These solutions provided equilibrium potential for Cl^- near 0 mV. Patch pipettes for whole-cell recordings were pulled from either borosilicate glass (Fisher Scientific, Pittsburgh, PA) or Labcraft microhematocrit capillary tubes (Curtin Matheson Scientific, Inc., Houston TX) on a P-87 Flaming Brown puller (Sutter Instrument Co., San Rafael, CA) to a resistance of 8–12 MΩ. For single-channel recording, patch pipettes were pulled from thick-walled borosilicate glass with an internal filament (World Precision Instruments, Sarasota, FL), fire polished to a resistance of 5–10 MΩ, and coated with Q-dope (GC Electronics, Rockford, IL) to reduce capacitance.

Loreclezole, (3α)-hydroxy-(5α)-pregnane-11,20-dione (alphaxalone), and diazepam were first dissolved in 100% dimethyl sulfoxide (DMSO) and then added to external solution in the appropriate volume. The highest DMSO concentration applied to the cells was 0.3% (v/v) to prevent direct effects of DMSO on channel activity. All chemicals were obtained from commercial sources. Loreclezole was a gift from Janssen Laboratories (Beerse, Belgium). For whole-cell recordings, drugs were applied to cells with a modified U-tube system with a 10–90 rise time around 70 ms (Greenfield and Macdonald, 1996). Although this application rate is fast, it is too slow to capture the fastest rates of desensitization (~10 ms). For outside-out patch-clamp recordings, drugs were applied with a pressure ejection pipette. Currents were recorded with a List EPC-7 (Darmstadt, Germany) or an Axopatch 1-B (Axon Instruments, Foster City, CA) patch clamp amplifier, recorded on hard disk with the Axotape program (Axon Instruments) and stored on VHS or Beta videotape. All experiments were performed at room temperature.

Data Analysis. Whole-cell currents were analyzed off-line with the programs Axotape and Prism (Graphpad, San Diego, CA). All whole-cell current amplitudes were obtained by measuring the peak current evoked during the application of GABA or GABA plus drug. The magnitude of the enhancement or inhibition of GABAR current by a drug was measured by dividing the peak amplitude of GABAR currents elicited in the presence of a given concentration of the drug (with GABA) by the peak amplitude of control current elicited by GABA alone and multiplying the fraction by 100 to express it as percent of control. Thus, the control response was 100%. Peak GABAR currents at various drug concentrations were fitted to a sigmoidal function with a four-parameter logistic equation (sigmoidal concentration-response) with a variable slope. The equation used to fit the concentration-response relationship was:

$$I = \frac{I_{max}}{1+10^{(\text{Log EC}_{50} - \log [\text{drug}]) * \text{HillSlope}}}$$

where I is the GABAR current at a given GABA concentration, I_{max} is the maximal GABAR current and n_H is the Hill coefficient. The curve-fitting algorithm minimized the sum of the squares of the actual distance of points from the curve. Convergence was reached when two consecutive iterations changed the sum of the squares by $< 0.01\%$. Data were presented as mean $\pm$ S.E.M. All four parameters were "floating", and therefore, the maximum effect observed was not necessarily the upper limit of the fit (e.g., see Figs. 2A and 7B). The only cases in which one of the parameters was not allowed

to float were when inhibitory concentration-response curves did not plateau at the low end. In these instances, the lower limit was set at zero to prevent the fitting algorithm from extending into the undefined range below 0%. Figures include the values for the four "floating" parameters: maximal and minimal values are given on the y-axes, and the EC_{50} value and Hill coefficient are given in the insets. It is important to note that the Hill coefficient does not represent the number of molecules that bind to the receptor but is only an indication of the slope of the concentration-response relationship and is provided throughout the text only to describe the fit. For compounds that had smaller effects at higher concentrations, the fit did not include these higher points and were indicated by dashed lines in the figures. All comparisons of the effect of a compound or voltage on different subtype combinations were done with GABA concentrations that produced equivalent responses (extrapolated from Fig. 1B). Application of GABA was repeated until the peak currents had stabilized and functioned as controls for each cell. Coapplication of the same concentration of GABA with increasing concentrations of individual modulators was performed to determine the maximal effect and potency of each modulator on GABARs. All fits were made to average, normalized data with the current expressed as a percentage of the maximum current elicited by control GABA concentrations for each cell.

To quantify whole-cell current rectification, peak amplitudes of responses to GABA at equivalent effective concentrations (for example, EC_{50}) were measured at holding potentials of −75 and +75 mV. These responses exhibited no visible desensitization. An amplitude ratio (+75 mV/−75 mV) was calculated, and rectification was determined with respect to a linear ratio equal to 1 with the predicted E_{Cl} equal to 0 mV. An amplitude ratio greater than 1.0 indicated outward rectification.

Single-channel recordings were digitized with Axoscope and analyzed with pClamp6 (Axon Instruments). For analysis, the data were digitized at 20 kHz and filtered at 2 kHz.

Statistical comparisons among GABAR subunit combinations were performed with one-way ANOVAs with Newman-Keuls posthoc tests to determine which combinations differed. Post hoc tests were performed only on data sets in which the p value of the ANOVA was less than 0.05. In comparisons of individual EC/IC_{50} values, the log values of the drug concentration were used for standard parametric ANOVAs. All statistical tests were performed with the Instat program (Graphpad).

Results

GABA Sensitivity of Cells Coexpressing $\alpha5\beta3$, $\alpha5\beta3\gamma3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3\pi$ Subtype Combinations

GABA Concentration-Response Curves. To determine which combinations of $\alpha5$, $\beta3$, $\gamma3$, and π subtypes could assemble in L929 cells to form functional GABARs, we coexpressed different subtype combinations in L929 cells and obtained whole-cell recordings and GABA concentration-response relationships. In parallel transfections, no GABAR currents were recorded from cells coexpressing $\alpha5\pi\gamma3$ (n = 5), $\pi\beta3\gamma3$ (n = 5), $\alpha5\pi$ (n = 5), or $\beta3\pi$ (n = 4) subtypes or the π subunit alone (n = 4). Previous work in our laboratory has shown that L929 cells do not form functional channels when cells are cotransfected with $\alpha\gamma$ or $\beta\gamma$ subunits or transfected with an α, β, or γ subunit alone (Angelotti et al., 1993; Saxena and Macdonald, 1994; Burgard et al., 1996; Neelands et al., 1999). In subsequent experiments, concentration-dependent GABAR currents were obtained from cells cotransfected with $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ subtypes (Fig. 1A). The amplitudes of currents varied considerably among transfections and among individual cells, presumably

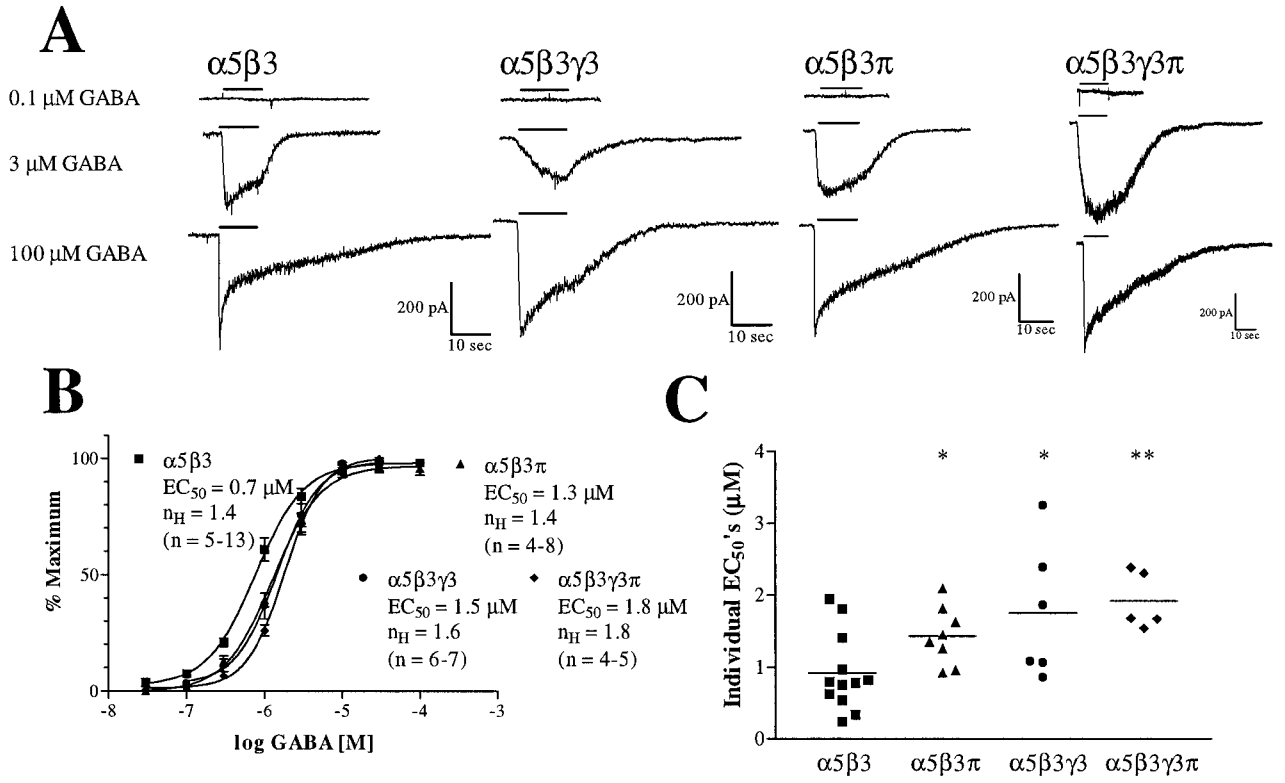

Fig. 1. Comparison of GABA concentration-response curves. A, representative current traces of 0.1, 3, and 100 μM GABA recorded from L929 cells expressing $\alpha5\beta3$, $\alpha5\beta3\gamma3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3\pi$. Horizontal bars, drug application. B, normalized averaged concentration-response curves for GABA-evoked currents from the same four subunit combinations. Data are mean ± S.E.M. C, comparison of EC_{50} values calculated from concentration-response curves of individual cells. (*$p < .05$; **$p < .01$).

because of differences in transfection efficiency or cell viability. Given this variability, there were no apparent differences in the maximum amplitudes of GABAR currents among the four subunit combinations tested ($p = .374$). $\alpha5\beta3$ currents were larger than previous reports from our laboratory on $\alpha\beta$ heterodimers. Whether this was caused by the subunit combination, the L929 cells used in this study, or some other factor was not investigated. Complete concentration-response curves were obtained for the four subunit combinations that were GABA-sensitive (Fig. 1B) to determine whether the π subunit altered GABA EC_{50} values. Peak currents were normalized to the maximum current recorded for each cell. The average maximal currents evoked by GABA were 1002 ± 450, 502 ± 177, 375 ± 103, and 274 ± 96 pA for $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$, respectively. Average amplitudes were normalized to the maximal current evoked for each cell and then plotted as a function of GABA concentration and fit with a four-parameter logistic equation (see *Materials and Methods*). Cells expressing $\alpha5\beta3$ subtypes had an EC_{50} value of 0.7 μM ($n_H = 1.4$; $n = 5$–13; Fig. 1B). GABA concentration-response curves obtained from cells expressing $\alpha5\beta3$ subtypes with either π and/or $\gamma3$ subtypes were shifted to the right (Fig. 1B). The EC_{50} values for the $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ subtype combinations were 1.3 μM (n_H, 1.4; $n = 4$–8), 1.5 μM (n_H, 1.6; $n = 6$–7), and 1.8 μM (n_H, 1.8; $n = 6$–7), respectively. GABA concentration-response curves for individual cells were also fit with logistic equations and compared across cells (Fig. 1C). The log EC_{50} values from the different subunit combinations were significantly different (one-way ANOVA, $p = .030$). Post hoc tests (see *Materials and Methods*) indicated that the $\alpha5\beta3$ subtype GABA EC_{50} values were significantly different from each of the other subtype combination GABA EC_{50} values ($p < .05$ for $\alpha5\beta3\pi$ and $\alpha5\beta3\gamma3$ and $p < .01$ for $\alpha5\beta3\gamma3\pi$ subtype combinations). There were no significant differences among the GABA EC_{50} values of $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ subtype combinations. In addition, the normalized current at 1 μM GABA was significantly larger in cells expressing the $\alpha5\beta3$ subunit combination than the $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ subtype combinations ($p < .01$, $p < .01$, $p < .001$, respectively) consistent with the shift in apparent EC_{50} value. The degree of the rightward shift in the GABA concentration-response curve (>half a log unit) for γ subunit-containing receptors was similar to that previous reported after coexpression of a γ subunit (Angelotti et al., 1993).

Current-Voltage Relationships. Current-voltage relations were generated for the four GABA-sensitive subtype combinations with EC_{35} GABA concentrations. Peak currents were measured at 25-mV increments at holding potentials ranging from −100 to +75 mV (Fig. 2A). Because of the large variation in maximal current among subunit combinations and differences in expression levels among cells, currents from individual cells were normalized to the peak current recorded at −75 mV. The average normalized current for each subtype combination was plotted as a function of the holding potential (Fig. 2B). Currents from the $\alpha5\beta3$ subtype combination were outwardly rectifying (Fig. 2A). Average peak currents evoked at positive potentials were 232 ± 14% (+50 mV) and 396 ± 33% (+75 mV) of the peak current recorded at −75 mV (Fig. 2B, inset). $\alpha5\beta3\gamma3$ (107 ± 10%, $n = 7$ at +75 mV) and $\alpha5\beta3\gamma3\pi$ ($n = 2$, data not shown) subtype currents were both linear over the membrane potentials tested (Fig. 2B). The normalized current amplitudes for these two subunit combinations superimposed. $\alpha5\beta3\pi$ currents,

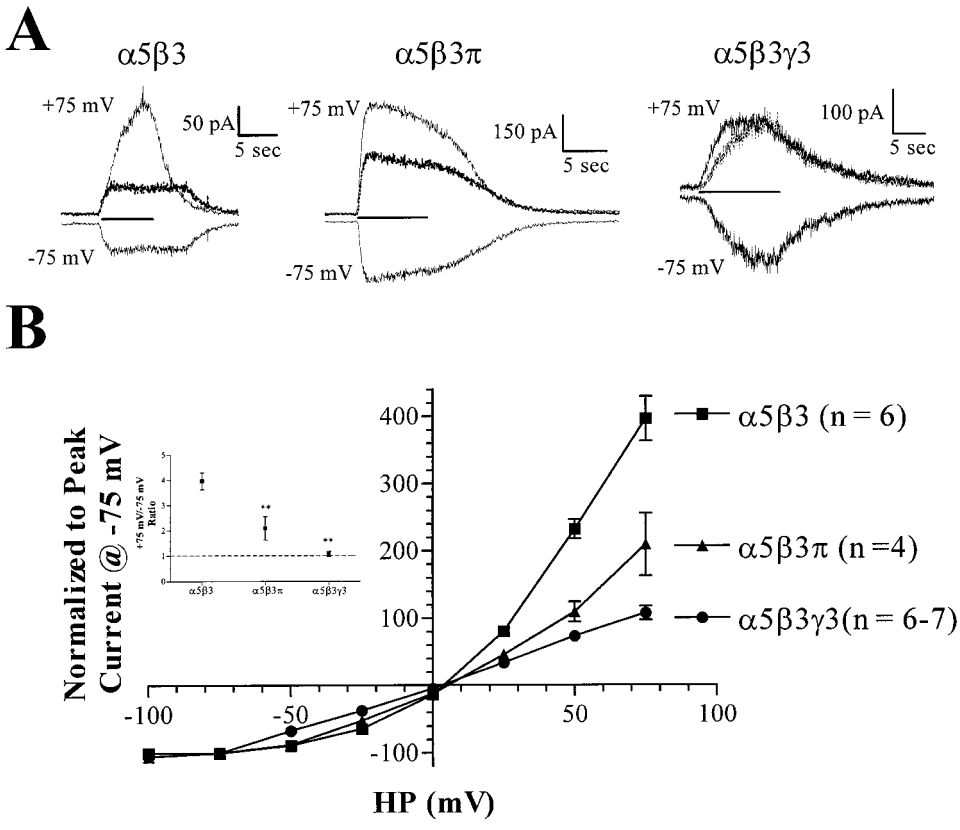

Fig. 2. Comparison of the current-voltage relations of GABA-evoked currents. A, representative current traces evoked by EC_{35} GABA concentrations at holding potentials of −75 and + 75 mV for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subunit combinations. The −75 mV current is inverted and superimposed on the + 75 mV trace. Horizontal bars, drug application. B, peak currents at each potential are normalized to the peak current at −75 mV. Average normalized currents are plotted on the *y*-axis against the membrane holding potential (HP) on the *x*-axis. Inset, amplitude ratio of peak currents evoked at +75 and −75 mV. Data are mean ± S.E.M. Pharmacology of GABAR-evoked currents from cells coexpressing $\alpha5\beta3$, $\alpha5\beta3\gamma3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3\pi$ subtypes.

however, still displayed outward rectification (209 ± 46% at +75 mV). An ANOVA performed on the degree of rectification among the $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subunit combinations was significant ($p < .001$). Post hoc tests showed the degree of rectification was significantly greater for currents from the $\alpha5\beta3$ subtype combination compared with currents from $\alpha5\beta3\pi$ or $\alpha5\beta3\gamma3$ subtype combinations ($p < .01$ at +75 mV and $p < .001$ at +50 mV for both combinations). In addition, the $\alpha5\beta3\pi$ currents had significantly greater rectification than $\alpha5\beta3\gamma3$ currents ($p < .05$).

Pharmacology of GABAR Currents from Cells Coexpressing $\alpha5\beta3$, $\alpha5\beta3\gamma3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3\pi$ Subtypes

Benzodiazepines. The effects of benzodiazepine site ligands, such as diazepam, have been shown to require a γ subunit in the GABAR to exert their effects. To determine whether a π subunit could replace a γ subunit in the formation of the benzodiazepine site we tested the ability of diazepam (1 μM) to enhance GABAR currents in cells coexpressing the π subunit with other subtypes (Fig. 3A). Control currents evoked from cells coexpressing the $\alpha5\beta3\pi$ subtypes by 1 μM GABA were not enhanced by diazepam ($n = 4$). In parallel transfections, diazepam enhanced $\alpha5\beta3\gamma3$ currents to 159 ± 13% of control ($n = 15$) but had no effect (105 ± 3%) on $\alpha5\beta3\gamma3\pi$ currents ($n = 5$; Fig. 3B, C). The differences in current enhancement by diazepam were significantly different among the subtype combinations tested ($p = .012$). The magnitude of enhancement of $\alpha5\beta3\gamma3$ currents was significantly different ($p < .05$) from that of currents from the other two subtype combinations, but there was no difference between the effect of diazepam on $\alpha5\beta3\gamma3\pi$ and $\alpha5\beta3\pi$ currents ($p > .05$).

Loreclezole. The novel anticonvulsant drug loreclezole has been shown to potentiate GABAR currents when the isoforms contained a $\beta2$ or $\beta3$ subunit subtype but not a $\beta1$ subtype (Wafford et al., 1994). Although the degree of potentiation by 1 μM loreclezole varied depending on the isoform, all currents from cells containing subunit combinations that included the $\beta3$ subtype were loreclezole-sensitive (Wingrove et al., 1994). To determine whether coexpression of the π subunit altered loreclezole sensitivity, we determined the concentration-dependence of loreclezole enhancement of currents evoked by EC_{60} GABA concentrations from the $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ subtype combinations (Fig. 4A). Coapplication of up to 10 μM loreclezole caused a concentration-dependent increase of all subtype combination currents except the $\alpha5\beta3\gamma3$ combination (Fig. 4B). The percentage of enhancement started to decrease with application of higher loreclezole concentrations, as in previous reports (Donnelly and Macdonald, 1996). Interestingly, $\alpha5\beta3\gamma3\pi$ and $\alpha5\beta3\pi$ currents were enhanced to a similar extent by loreclezole over the whole range of concentrations tested. The enhancement of loreclezole (3 μM) among subtype combinations was significantly different ($p = .221$). Enhancement of current from all three of these subunit combinations was statistically different from the loreclezole effect on the insensitive $\alpha5\beta3\gamma3$ currents at 3 μM loreclezole ($p < .05$).

To our surprise, coapplication of loreclezole produced no enhancement and only a small inhibition of $\alpha5\beta3\gamma3$ currents. We demonstrated previously that $\alpha5\beta3\gamma2L$ currents were responsive to loreclezole (Burgard et al., 1996). To confirm that the lack of a loreclezole effect was not caused by loreclezole inactivity, we repeated the experiment with a parallel transfection of $\alpha5\beta3\gamma2L$ subtypes as a positive control. During this experiment, cells expressing the $\alpha5\beta3\gamma3$ subtype combination ($n = 3$) were loreclezole-insensitive and those expressing the $\alpha5\beta3\gamma2L$ combination were enhanced in a concentration-dependent manner up to 10 μM loreclezole (390 ± 60% of control, $n = 5$, data not shown). Previous reports have shown that loreclezole enhanced currents evoked by coapplication of EC_{20} GABA concentrations from isoforms containing either an $\alpha5$ or a $\gamma3$ subunit in combination with either a $\beta2$ or $\beta3$ subunit. Consequently, it did not seem reasonable that the combination of $\alpha5$ and $\gamma3$ subtypes would eliminate loreclezole sensitivity. Therefore, in a separate experiment, GABA concentration-response curves for $\alpha5\beta3\gamma3$ currents were sequentially generated in the absence

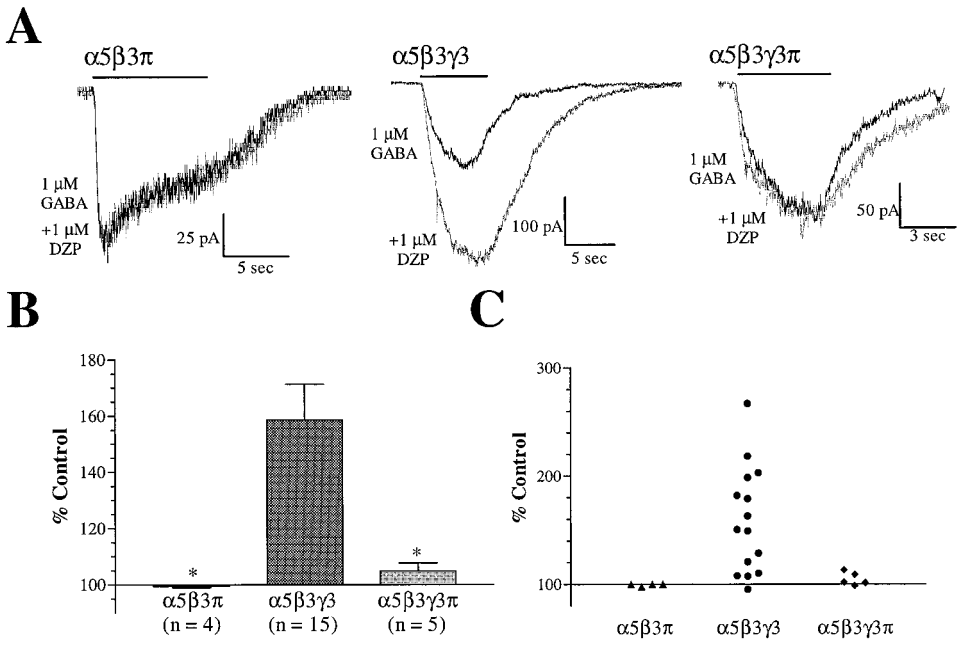

Fig. 3. Comparison of the effect of 1 μM diazepam. A, superimposed current traces of successive applications of 1 μM GABA (black trace) and 1 μM GABA plus 1 μM diazepam (gray trace) for $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$. Horizontal bars, drug application. B, average percentage of modulation of coapplication of 1 μM diazepam. Data are mean ± S.E.M. (*$p < .05$). C, effects of coapplication of 1 μM diazepam on individual cells expressing $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$.

and presence of loreclezole on individual cells. At low GABA concentrations 1 μM loreclezole produced a small but statistically insignificant increase in peak current, but at high GABA concentrations, loreclezole reduced peak currents (Fig. 5B). When concentration-response curves for loreclezole were then repeated with only 0.2 μM GABA ($\sim EC_{10}$), the ability of loreclezole to enhance the currents evoked by low GABA concentrations were more clearly demonstrated. Peak currents were enhanced by 410 ± 100% by coapplication of 10 μM loreclezole with 0.2 μM GABA (Fig. 5A). Although loreclezole (0.3–30 μM) enhanced GABA-evoked currents during the application of both drugs, the current tended to increase in amplitude immediately after the application was terminated. The degree to which this "rebound" occurred was dependent on the concentration of loreclezole (Fig. 5A, 30 μM loreclezole trace).

Barbiturates. The barbiturate pentobarbital has been shown to potentiate GABAR currents, directly activate a chloride current, and act as an open channel blocker at high concentrations (Schulz and Macdonald, 1981; Schwartz et al., 1986; Peters et al., 1988; Robertson, 1989; Thompson et al., 1996). We investigated the effect of coexpression of the π subunit with $\alpha5\beta3$ or $\alpha5\beta3\gamma3$ subtypes on the modulatory actions of pentobarbital. Coapplication of pentobarbital (0.3–100 μM) with equally effective concentrations of GABA were used to determine the EC_{50} value of pentobarbital for enhancement of $\alpha5\beta3$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\pi$ currents. Higher concentrations of pentobarbital were not used because of the direct effects of pentobarbital on GABARs (Fig. 6A). All three subtype combination currents were enhanced in a concentration-dependent manner by pentobarbital. Averaged normalized data were fit with a logistic equation with apparent EC_{50}

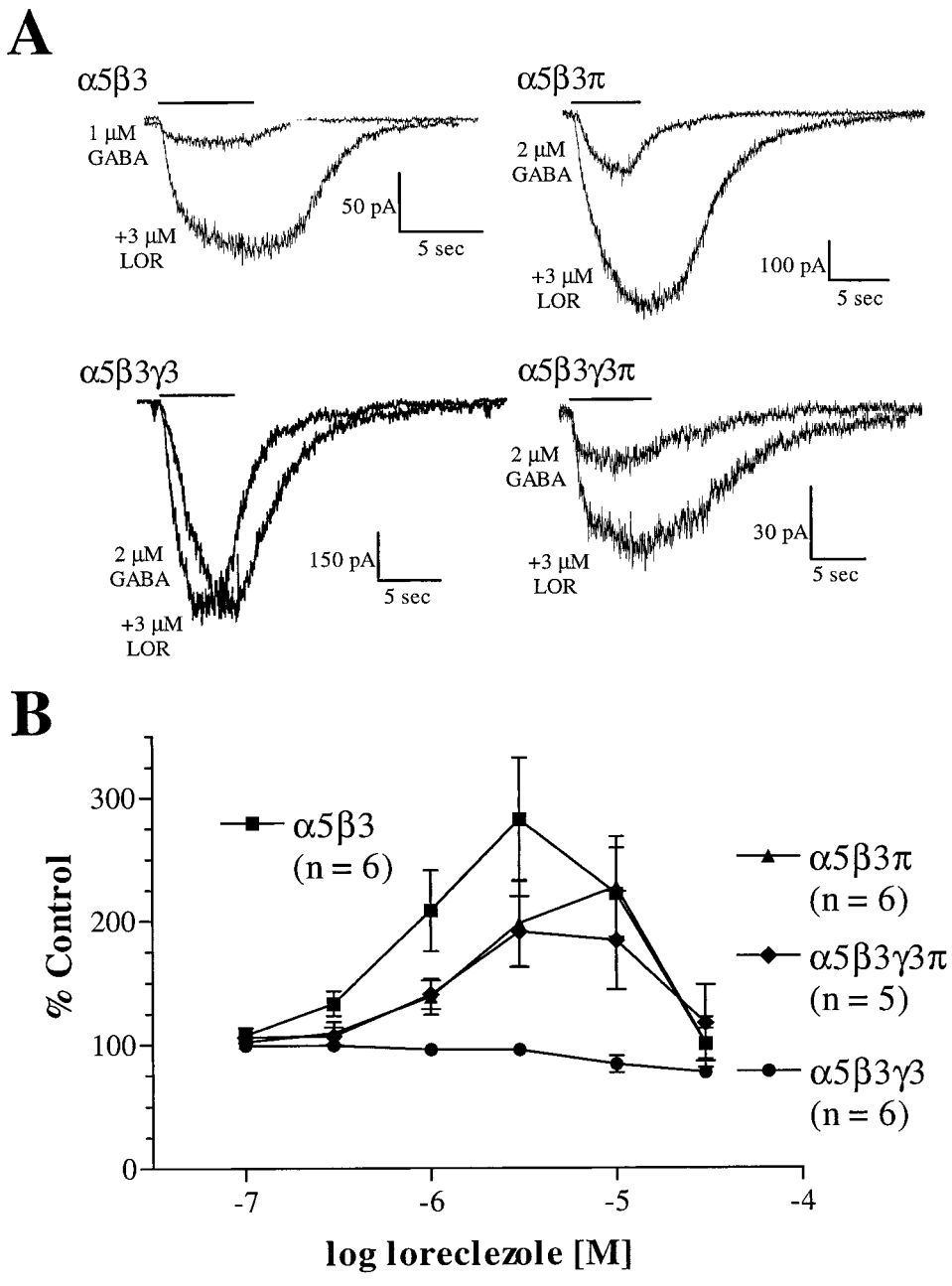

Fig. 4. Modulation of GABA-evoked currents by loreclezole. A, superimposed current traces of successive applications of EC_{60} GABA concentrations (upper trace) and coapplication of GABA plus 3 μM loreclezole (LOR; bottom trace) are shown for $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$. Horizontal bars, drug application. B, concentration-response relationship of averaged normalized data for loreclezole modulation of currents evoked by EC_{60} GABA for the same subunit combinations. Data are mean ± S.E.M.

values of 25.9 μM (n_H = 1.8, n = 3–5), 39.0 μM (n_H = 1.2, n = 4), and 59.5 μM (n_H = 1.1, n = 4) for $\alpha5\beta3$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\pi$ currents, respectively (Fig. 6B). There were no statistical differences between the three subunit combinations when individual EC_{50} values were compared ($p > .05$). The concentration range tested did not produce a plateau in the effect of pentobarbital at high concentrations. Therefore, fits were allowed to extend beyond the enhancement of 100 μM pentobarbital (see *Materials and Methods*) that resulted in maximal "effects" of 501% ($\alpha5\beta3$), 373% ($\alpha5\beta3\pi$), and 356% ($\alpha5\beta3\gamma3$). The effect of the highest concentration of pentobarbital tested (100 μM) ranged from 467 ± 130% of control for the $\alpha5\beta3$ currents to 307 ± 59% for $\alpha5\beta3\pi$ and 265 ± 61% for $\alpha5\beta3\gamma3$ currents but were not statistically different ($p > .05$; Fig. 6B).

Neurosteroids. Neurosteroids have been shown to have both positive (alphaxalone) and negative [pregnenolone sulfate (PS)] allosteric effects on recombinant GABARs. To determine whether the π subunit altered the effects of either of these compounds, concentration-response curves were generated with EC equivalent concentrations of GABA in cells expressing either the $\alpha5\beta3$, $\alpha5\beta3\pi$, or $\alpha5\beta3\gamma3$ subtype combinations.

Alphalxalone potentiated currents evoked by EC_{60} GABA concentrations for all three subtype combinations tested (Fig. 7A). Averaged normalized currents were plotted as a function of alphaxalone concentration and fit with a logistic equation with EC_{50} values of 342 μM (n_H = 1.5, n = 4), 292 μM (n_H = 2.0, n = 7) and 217 μM (n_H = 1.9, n = 8) for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ currents, respectively (Fig. 7B). The maximal effect of alphaxalone, measured at 3 μM, was significantly different among subtype combinations (p = .012). Alphaxalone enhancement of GABAR currents was greater for $\alpha5\beta3$ (327 ± 20%) and $\alpha5\beta3\pi$ (314 ± 25%) combinations than for the $\alpha5\beta3\gamma3$ combination (193 ± 27%; $p < .01$) but were not significantly different from each other ($p > .05$; Fig. 7B).

PS inhibited currents evoked by EC_{80} GABA concentrations for all three subtype combinations tested (Fig. 8A). Averaged normalized currents were plotted as a function of PS concentration (10 nM–30 μM). Solubility of PS in DMSO prevented testing of concentrations higher than 30 μM. Logistic equations were fit to the data with IC_{50} values of 12.9 μM (n_H = −0.5, n = 2–4), 1.8 μM (n_H = −0.7, n = 3–4), and 0.7 μM (n_H = −0.6, n = 3) for $\alpha5\beta3$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\pi$ currents, respectively (Fig. 8B). Comparison of the fits for data from individual cells did not produce significant differ-

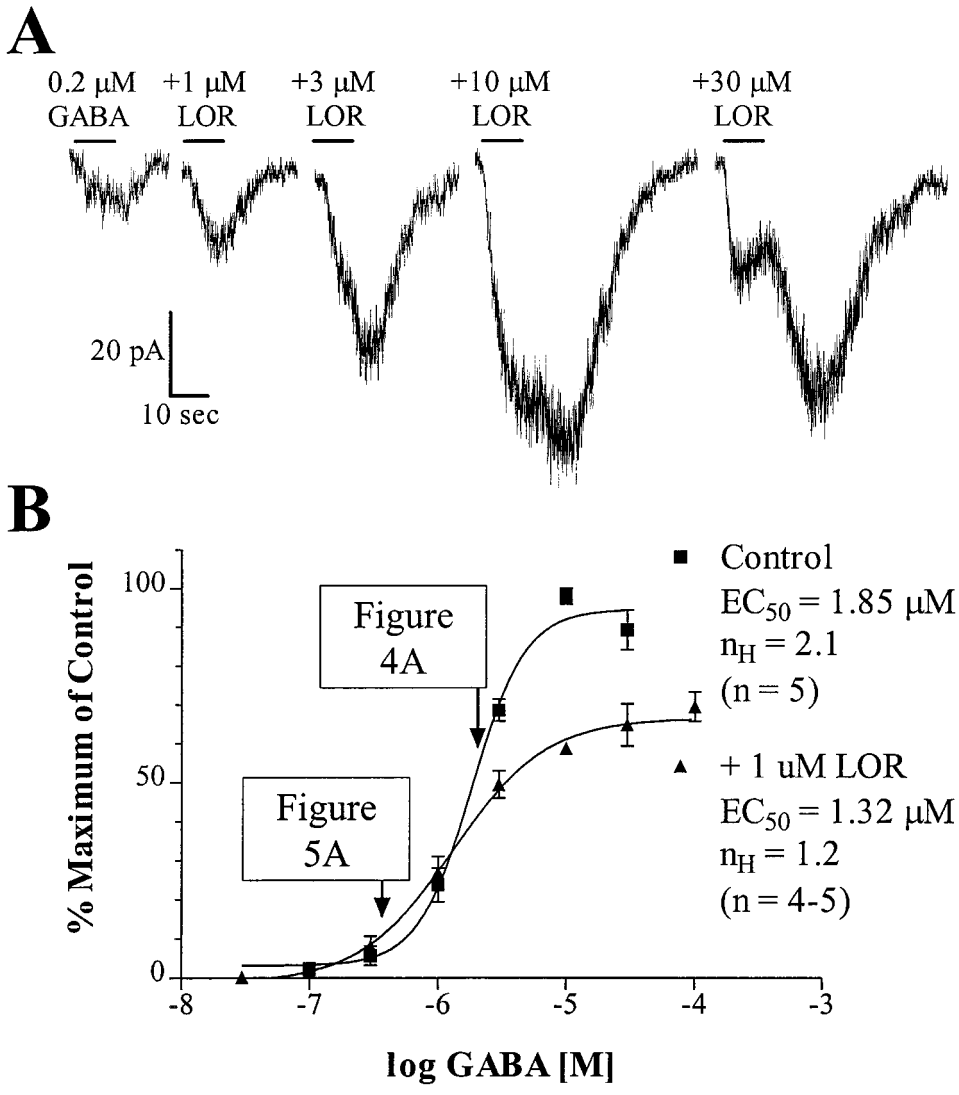

Fig. 5. Dependence on GABA concentration for the effects of loreclezole on GABA-evoked currents from the $\alpha5\beta3\gamma3$ subunit combination. A, representative current traces of the concentration dependent effects of loreclezole (1–30 μM) on currents evoked by 0.2 μM GABA from the $\alpha5\beta3\gamma3$ combination. Horizontal bars, drug application. B, average sequential concentration-response relationships for GABA alone and GABA plus 1 μM loreclezole normalized to the peak current evoked by 30 μM GABA alone for the $\alpha5\beta3\gamma3$ combination. Arrows indicate the GABA concentration used in Figs. 4A and 5A, respectively. Data are mean ± S.E.M.

ences in the apparent IC_{50} values between any pair of the subtype combinations. However, at 3 to 30 μM PS there was a difference in the percentage inhibition between the subtype combinations ($p < .05$). $\alpha5\beta3\pi$ and $\alpha5\beta3\gamma3$ currents were inhibited to a greater degree than $\alpha5\beta3$ currents ($p < .05$ and $p < 0.01$, respectively). The inhibition produced by the highest concentration of PS tested (30 μM) however, was only greater for $\alpha5\beta3\gamma3$ currents (16 ± 5% of control) compared with $\alpha5\beta3$ currents (39 ± 5% of control; $p < .01$; Fig. 8B). These differences in "maximal" effect were probably caused by the small shift in apparent IC_{50} values, rather than a change in efficacy of PS for the different subunit combinations, and were caused by our being unable to use higher PS concentrations.

Zinc. The divalent cation zinc inhibited $\alpha\beta$ and $\alpha\beta\delta$ isoform currents with low IC_{50} values (<5 μM), $\alpha\beta\epsilon$ isoforms with moderate IC_{50} values (22–42 μM), and $\alpha\beta\gamma$ isoform currents with high IC_{50} values (>100 μM; Draguhn et al., 1990; Saxena and Macdonald, 1994; Whiting et al., 1997). To determine the effect the π subunit on the sensitivity of GABAR currents to zinc, we obtained inhibition curves for zinc for the $\alpha5\beta3$, $\alpha5\beta3\gamma3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3\pi$ subtype combinations. Zinc (100

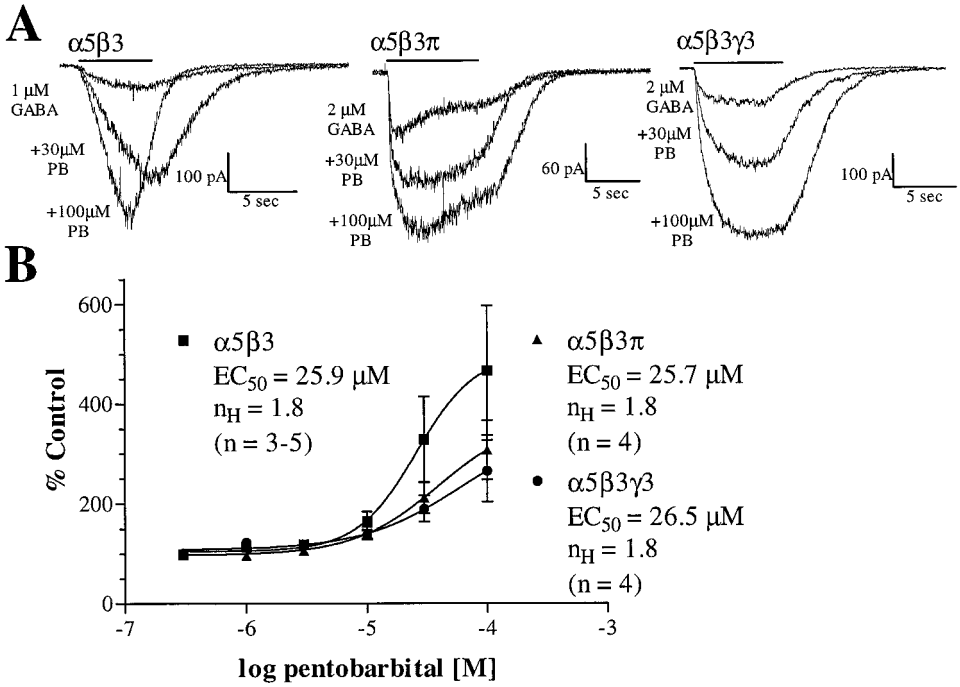

Fig. 6. Enhancement of GABA-evoked currents by pentobarbital. A, superimposed current traces of successive applications of EC_{60} concentrations of GABA (upper trace), GABA plus 30 μM pentobarbital (PB; middle trace), and GABA plus 100 μM pentobarbital (lower trace) for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subunit combinations. Horizontal bars, drug application. B, concentration-response relationship of averaged normalized data for pentobarbital enhancement of currents evoked by EC_{60} GABA for the same combinations. Data are mean ± S.E.M.

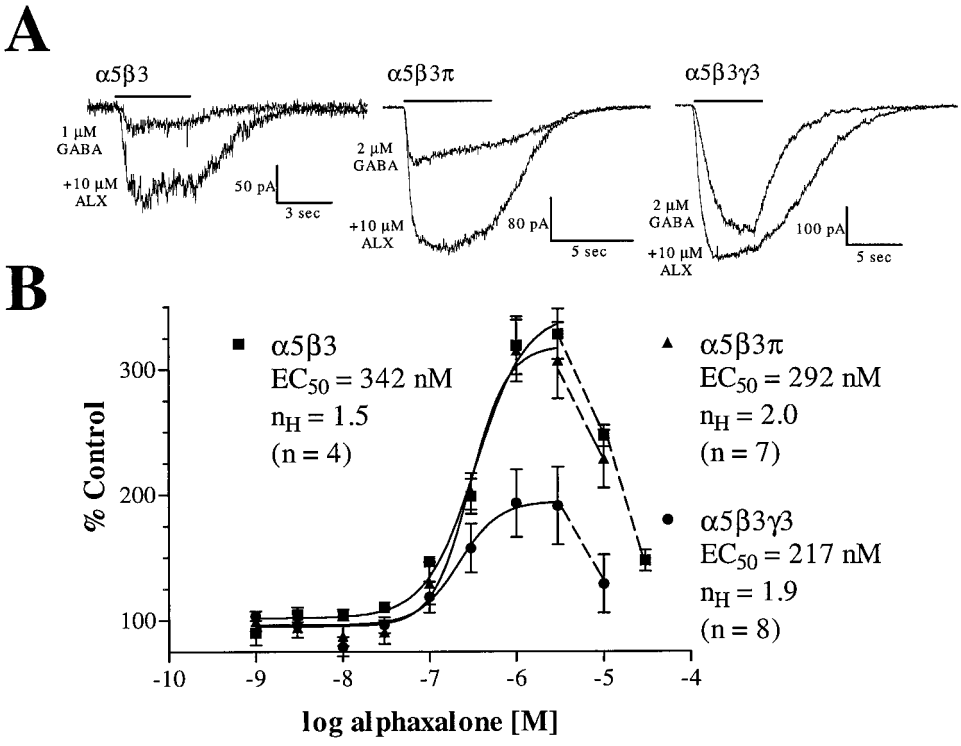

Fig. 7. Enhancement of GABA-evoked currents by alphaxalone. A, superimposed current traces of successive applications of EC_{60} concentrations of GABA (upper trace) and GABA plus 10 μM alphaxalone (ALX; lower trace) for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subunit combinations. Horizontal bars, drug application. B, concentration-response relationship of averaged normalized data for pentobarbital enhancement of currents evoked by EC_{60} GABA for the same combinations. EC_{50} value for alphaxalone enhancement (1 nM–3 μM alphaxalone) was derived independent of the apparent inhibition seen at higher concentrations (hatched line). Data are mean ± S.E.M.

nM–1 mM) inhibited currents evoked by EC_{80} GABA concentrations for all of the subtype combinations tested (Fig. 9A). Averaged normalized currents were plotted as a function of zinc concentration and fit with logistic equations with IC_{50} values of 2.1 μM ($n_H = -0.8$, $n = 6$), 2.4 μM ($n_H = -0.9$, $n = 6$), 43.3 μM ($n_H = -0.5$, $n = 4$), and 67.3 μM ($n_H = -0.6$, $n = 5$) for $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ currents, respectively (Fig. 9B). When comparing IC_{50} values obtained from individual cells, the IC_{50} values for zinc inhibition were statistically different ($p < .0001$). However, IC_{50} values for zinc inhibition of $\alpha5\beta3$ and $\alpha5\beta3\pi$ currents or $\alpha5\beta3\gamma3$ and $\alpha5\beta3\gamma3\pi$ currents were not significantly different from one another. The individual IC_{50} values for zinc inhibition of $\alpha5\beta3$ and $\alpha5\beta3\pi$ currents were statistically different from inhibition of both $\alpha5\beta3\gamma3$ and $\alpha5\beta3\gamma3\pi$ ($p < .0001$). The increase in the zinc IC_{50} for γ-containing subunit combinations was consistent with previous reports (Draguhn et al., 1990) but was of smaller magnitude, possibly because of the expression of the $\gamma3$ subtype rather than the $\gamma2$ subtype (used in most recombinant studies of GABAR pharmacology).

Lanthanum. The trivalent cation lanthanum inhibited $\alpha6$ subtype-containing receptor currents but enhanced $\alpha1$ subtype-containing receptor currents (Saxena et al., 1997), but the effects of lanthanum on $\alpha5$ subtype-containing receptor currents have not been reported. To determine the effects of π and $\alpha5$ subunits on the sensitivity of GABARs to lanthanum we obtained concentration-response curves for lanthanum for the $\alpha5\beta3$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\pi$ subtype combinations. Lanthanum inhibited currents evoked by EC_{60} GABA concentrations for all of the combinations tested (Fig. 10A). Averaged normalized currents were plotted as a function of lanthanum concentration and fit with logistic equations with IC_{50} values of 297 μM ($n_H = -1.1$, $n = 4$), 540 μM ($n_H = -1.1$, $n = 5$), and 522 μM ($n_H = -0.9$, $n = 5$) for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ currents, respectively (Fig. 10B). When comparing IC_{50} values obtained from individual cells, there was a significant difference between subunit combinations ($p = .0051$). Lanthanum inhibited $\alpha5\beta3$ currents with a significantly lower IC_{50} value than either $\alpha5\beta3\gamma3$ or $\alpha5\beta3\pi$ currents ($p < .05$, $p < .01$, respectively). In addition, the percentage inhibition produced by a high concentration of lanthanum (1 mM) was different among subunit combinations ($p = .0042$). Lanthanum (1 mM) significantly inhibited $\alpha5\beta3$ currents (82.0 ± 3.5%) more than either $\alpha5\beta3\pi$ (70.2 ± 3.2%) or $\alpha5\beta3\gamma3$ (66.9 ± 3.9%) currents ($p < .05$, $p < .01$, respectively). Similar to the inhibition by PS, this difference in "maximal" effect was most likely caused by the shift in the apparent IC_{50} value and not to a change in efficacy.

Comparison of Single-Channel GABAR Currents from Cells Expressing $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ Subtypes

Single-channel currents were recorded from outside-out patches pulled from fibroblasts transfected with $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subtype combinations to determine whether there were any differences in the conductance levels of single-channel currents when the π subunit was included in the transfection. Previously $\alpha\beta$ heterodimeric pentamers were reported to have relatively small conductance levels, ranging from 11 to 13 pS (Moss et al., 1990; Verdoorn et al. 1990; Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997) whereas heterotrimeric pentamers composed of $\alpha\beta\epsilon$, $\alpha\beta\gamma$, and $\alpha\beta\delta$ subunits had larger conductance levels ranging from 24 to 27 pS (Fisher and Macdonald, 1997; Neelands et al., 1999). $\alpha5\beta3\pi$ and $\alpha5\beta3\gamma3$ single-channel current openings were relatively long in duration and were separated into closely grouped bursts of openings in contrast to $\alpha5\beta3$ single-channel current openings which were generally brief, independent openings (Fig. 11A). Detailed kinetic analysis of the open and closed times was not performed on the limited number of openings obtained for this study. The amplitudes of single-channel openings from cells transfected with either $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subtypes were measured at holding potentials ranging from −75 to +75 mV and were fit with linear regression analysis to determine single-channel conductance. Not all holding potentials were obtained for each patch and the n values given in the figure represent the range in the number of patches recorded at each holding potential. The average conductance of channels from individ-

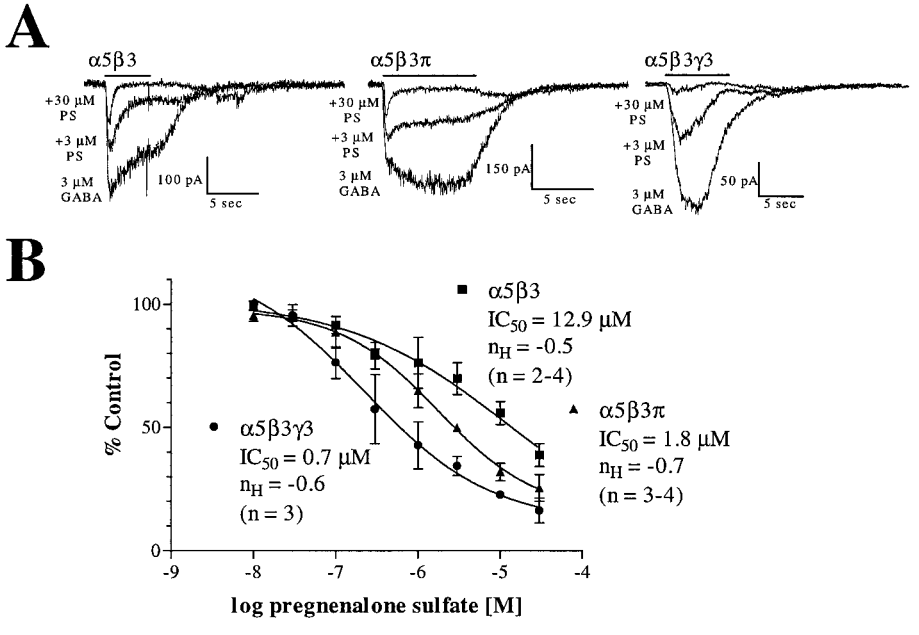

Fig. 8. Inhibition of GABA-evoked currents by PS. A, superimposed current traces of successive applications of EC_{80} concentrations of GABA (lower trace), GABA plus 3 μM PS (middle trace), and GABA plus 30 μM PS (upper trace) for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subunit combinations. Horizontal bars, drug application. B, concentration-response relationship of averaged normalized data for PS inhibition of currents evoked by EC_{80} GABA for the same combinations. Data are mean ± S.E.M.

ual patches varied depending on the subtype combination. It should be noted, however, that linear regression analysis of individual patches gave values similar to the fits of the average data (data not shown). Conductance levels of 15.2, 23.8, and 26.9 pS were calculated for channel openings from the $\alpha5\beta3$ (n = 1–3), $\alpha5\beta3\pi$ (n = 2–4), and $\alpha5\beta3\gamma3$ (n = 3) subtype combinations, respectively (Fig. 11B). Although their openings had a slightly larger conductance than previously reported heterodimers, the pattern of $\alpha5\beta3$ openings was similar to that of these studies. Further single-channel analysis should be performed to determine whether this small change in conductance was significant and represented a difference of $\alpha5\beta3$ heterodimers compared with other combinations. Because we were more interested in whether adding a π or γ subunit changed the properties of the single-channel openings, this difference was not pursued. Incorporation of either a π or $\gamma3$ subunit into the GABAR complex increased the main conductance level from $\alpha5\beta3$ alone and was in the range previously reported for heterotrimeric GABAR pentamers. $\alpha5\beta3\pi$ and $\alpha5\beta3\gamma3$ single-channel currents also displayed a bursting type behavior of openings not seen with heterodimers (Fig. 11).

Discussion

Coexpression of the π Subunit-Altered GABAR Properties. By using electrophysiological techniques, we demonstrated that coexpression of the π subunit with $\alpha5$, $\beta3$, or $\gamma3$ subtypes alone did not result in expression of functional GABARs. However, coexpression of the π subunit with $\alpha5\beta3$ or $\alpha5\beta3\gamma3$ subtypes resulted in GABARs with pharmacological and biophysical properties that were different from those of $\alpha5\beta3$ and $\alpha5\beta3\gamma3$ GABARs. Expression of the $\alpha5\beta3\pi$ subunit combination in L929 cells resulted in GABAR currents that had a higher GABA EC_{50} value (Table 1), less outward rectification, and larger single-channel conductance than $\alpha5\beta3$ receptors. $\alpha5\beta3\pi$ channels tended to open into bursts of longer duration than single, brief openings typically recorded from patches containing $\alpha5\beta3$ subunit channels. In addition, $\alpha5\beta3\pi$ currents were inhibited by lanthanum with a higher IC_{50} value and were inhibited by PS with a lower IC_{50} value than $\alpha5\beta3$ currents (Table 1). In contrast, there were no significant differences in the effects of zinc, loreclezole, pentobarbital, diazepam, or alphaxalone on $\alpha5\beta3$ and $\alpha5\beta3\pi$ GABAR currents (Table 1). No multiple component inhibition curves were obtained for $\alpha5\beta3\pi$ GABAR currents, and single-channel recordings did not reveal low $\alpha5\beta3$ conductance levels, which suggests that it was unlikely that a significant proportion of $\alpha5\beta3$ receptor channels were also expressed with $\alpha5\beta3\pi$ receptor channels.

When the π subunit was coexpressed with $\alpha5\beta3\gamma3$ subtypes, the resulting GABAR currents had a linear current-voltage relation (which was qualitatively similar to the

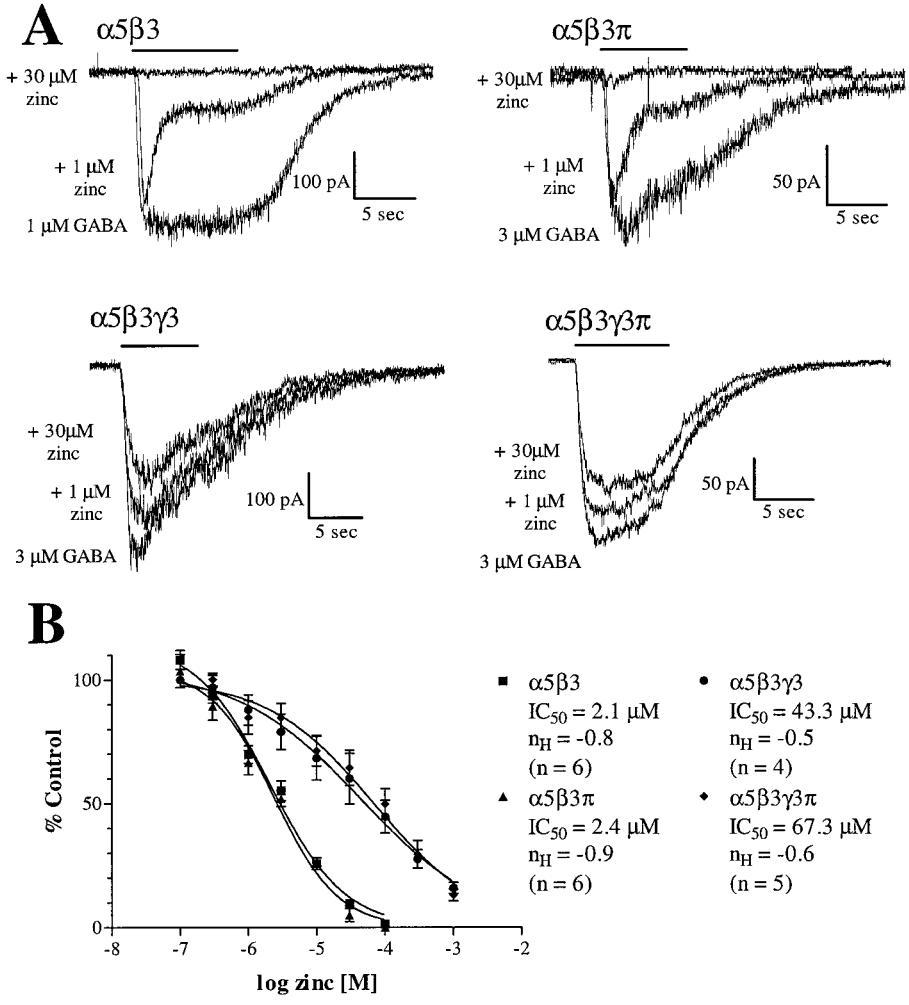

Fig. 9. Inhibition of GABA-evoked currents by zinc. A, superimposed current traces of successive applications of EC_{80} concentrations of GABA (lower trace), GABA plus 1 μM zinc (middle trace), and GABA plus 30 μM zinc (upper trace) for $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ subunit combinations. Horizontal bars, drug application. B, concentration-response relationship of averaged normalized data for zinc inhibition of currents evoked by EC_{80} GABA for the same combinations. Data are mean ± S.E.M.

$\alpha5\beta3\gamma3$ subunit combination), were less sensitive to enhancement by diazepam and were more sensitive to potentiation by loreclezole then when $\alpha5\beta3\gamma3$ subtypes were coexpressed. The virtual absence of diazepam sensitivity of $\alpha5\beta3\gamma3\pi$ receptors strongly suggested that currents recorded after cotransfection of $\alpha5$, $\beta3$, $\gamma3$, and π subunits were $\alpha5\beta3\gamma3\pi$ receptor currents with little if any $\alpha5\beta3\gamma3$ receptor currents. These data suggest that the π subunit can be incorporated into GABARs to form $\alpha\beta\pi$ and $\alpha\beta\gamma\pi$ isoforms.

Coexpression of the π subunit with $\alpha5\beta3\gamma3$ subtypes resulted in relatively diazepam-insensitive receptors, despite the presence of a γ subunit, which implies that the π subunit replaced the γ subunit but was incapable of forming a benzodiazepine binding site or that it was incorporated into the receptor with α, β, and γ subunits but disrupted the formation of the site by the α and γ subunits. This is consistent with the disruption of flumazenil binding to GABARs coexpressing γ and π subunits (Hedblom and Kirkness, 1997). The π subunit did not appear to alter the sensitivity of zinc inhibition of GABAR currents (currents from $\alpha\beta$ and $\alpha\beta\pi$ combinations were highly zinc-sensitive and currents from $\alpha\beta\gamma$ and $\alpha\beta\gamma\pi$ combinations were relatively zinc-insensitive). Taken together, the zinc and diazepam results indicate that the π subunit was most likely incorporated into the receptor to form an $\alpha5\beta3\gamma3\pi$ receptor. Interestingly, the δ GABAR subunit seemed to follow similar rules for assembly (Saxena and Macdonald, 1996). Only $\alpha\beta\delta$ or $\alpha\beta\delta\gamma$ GABARs were expressed after different subunit combinations were coexpressed. It remains to be determined, however, if receptors that combine four different subunits (including π and δ subunits) are actually expressed in the CNS. Thus these results suggest that the π subunit can coassemble with $\alpha\beta$ or $\alpha\beta\gamma$ GABAR subunits to form GABAR isoforms that would result GABA-ergic inhibitory postsynaptic potentials in the brain with different properties.

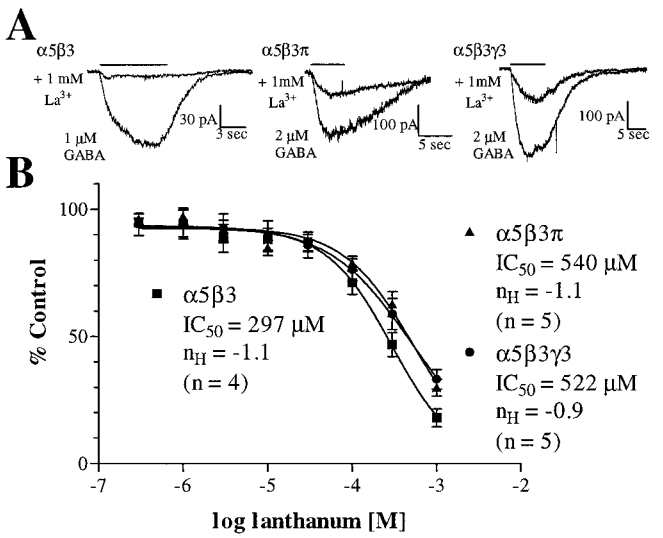

Fig. 10. Inhibition of GABA-evoked currents by lanthanum. A, superimposed current traces of successive applications of EC_{80} concentrations of GABA (lower trace), GABA plus 1 mM lanthanum (La^{3+}; upper trace) for $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ subunit combinations. Horizontal bars, drug application. B, concentration-response relationship of averaged normalized data for lanthanum inhibition of currents evoked by EC_{80} GABA for the same combinations. Data are mean ± S.E.M.

Preferred Assembly of $\alpha5\beta3$ Isoforms in NT2 Neuronal Precursor Cells. The results of this study imply that the π subunit can coassemble with other GABAR subunits to form functional recombinant GABARs. However, as noted above, it is not known if this coassembly actually occurs in neurons. Interestingly, NT2 neuronal precursor cells, a cell line that can be differentiated into a neuronal cell type by retinoic acid treatment, have been shown to express mRNA encoding the π subunit (Hedblom and Kirkness, 1997) along with $\alpha5$, $\beta3$, and $\gamma3$ GABAR subtype mRNAs (Neelands et al., 1997). Thus, comparisons of the pharmacological and biophysical properties of NT2 neuronal precursor cell and recombinant GABARs were made to determine whether functional evidence of incorporation of the π subunit into a GABAR could be demonstrated in a neuronal precursor cell type (Neelands et al., 1997). The pharmacological and biophysical properties of NT2 neuronal precursor cell GABAR currents, however, were most similar to those of recombinant $\alpha5\beta3$ GABARs, suggesting that although π subunit mRNA was expressed by NT2 neuronal precursor cells, the π subunit protein was not incorporated into functional GABARs in these cells. Thus expression of the π subunit in a population of native neurons has not been clearly demonstrated and whether the π subunit is incorporated into native GABARs remains unclear.

Effects of Loreclezole on $\alpha5$ and π Subtype-Containing GABARs. Loreclezole enhanced $\alpha5\beta3\gamma3\pi$ and $\alpha5\beta3\pi$ subunit combination currents with similar EC_{50} values. In contrast, $\alpha5\beta3\gamma3$ subunit combination currents were not enhanced by loreclezole but were inhibited by higher loreclezole concentrations. Interestingly, the $\alpha5\beta3\gamma3\pi$ combination was enhanced

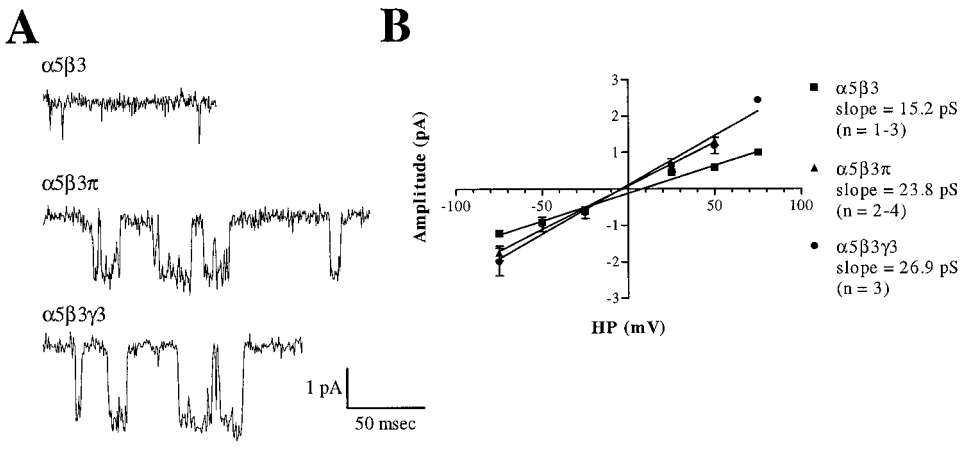

Fig. 11. Single-channel currents recorded from $\alpha5\beta3$, $\alpha5\beta3\pi$, and $\alpha5\beta3\gamma3$ isoforms. A, representative outside-out single-channel openings recorded from fibroblast cells transfected with either $\alpha5\beta3$, $\alpha5\beta3\pi$, or $\alpha5\beta3\gamma3$. B, current-voltage relationships are shown for each combination. Data are mean ± S.E.M.

less by 10 μM loreclezole than the $\alpha5\beta3\pi$ combination. This could have been caused by expression of two distinct populations of GABAR isoforms. However, the degree of potentiation of the current was not attenuated at lower concentrations, which would have been expected if a proportion of the channels were insensitive. The $\beta2$ and $\beta3$ subunit subtypes have been shown to have a single amino acid necessary for loreclezole enhancement (Wingrove et al., 1994), and no GABAR isoform containing either of these subtypes has been reported to be loreclezole-insensitive (Wafford et al., 1994). At lower GABA concentrations, however, $\alpha5\beta3\gamma3$ currents were enhanced. It is possible that the $\alpha5\beta3\gamma3$ isoform has a higher affinity for loreclezole at the inhibitory site than other isoforms. Therefore, at high GABA concentrations, loreclezole potentiation of $\alpha5\beta3\gamma3$ GABAR currents was masked by inhibition of the current by loreclezole. Regardless, the coexpression of the π subunit with $\alpha5\beta3\gamma3$ changed the concentration-dependent effects of loreclezole. These changes in loreclezole sensitivity provide further evidence for assembly of a recombinant $\alpha5\beta3\gamma3\pi$ isoform.

Effects of Lanthanum on $\alpha5$ and π Subtype-Containing GABARs. Lanthanum has been shown to potentiate currents from GABARs containing the $\alpha1$ subtype and to inhibit currents from GABARs containing the $\alpha6$ subtype (Fisher et al., 1997; Saxena et al., 1997). The effect of lanthanum on GABAR isoforms containing the other α subunits has not been reported. In this study, we showed that GABARs containing an $\alpha5$ subtype were inhibited by lanthanum but with a much higher IC_{50} value than for inhibition of GABARs containing an $\alpha6$ subtype (Table 2). The coexpression of π or $\gamma3$ subtype with $\alpha5$ and $\beta3$ subtypes slightly increased the IC_{50} value for lanthanum inhibition compared with the IC_{50} value for lanthanum inhibition of $\alpha5\beta3$ currents. Although the enhancing effect of lanthanum has been shown to be restricted to the amino-terminal extracellular domain (Fisher et al., 1997), the exact site or sites of action of lanthanum have not been determined. It is possible that the inhibitory and enhancing effects of lanthanum act at completely different sites on the GABAR complex. Different α subunits may have only one site or may have both sites but with a different rank order of potency for lanthanum. Determining the

TABLE 1
Pharmacological properties of $\alpha5\beta3$, $\alpha5\beta3\pi$, $\alpha5\beta3\gamma3$, and $\alpha5\beta3\gamma3\pi$ GABARs

Agonist/Antagonist	$\alpha5\beta3$	$\alpha5\beta3\pi$	$\alpha5\beta3\gamma3$	$\alpha5\beta3\gamma3\pi$
GABA				
EC_{50}, μM	0.7	1.3*	1.5*	1.8*
Diazepam				
% effect of 1 μM		None*	+59 ± 13%$^+$	+5 ± 3*
Loreclezole[a]				
EC_{50}, μM	~1	1		~1
% effect of 10 μM	+122 ± 37	+127 ± 41	None$^+$	+83 ± 40
Pentobarbital				
EC_{50}, μM	26	39	59	
% effect of 100 μM	+367	+207 ± 59	+165 ± 61	
Alphaxalone				
EC_{50}, nM	342	292	217	
% effect of 3 μM	+227 ± 20	+214 ± 25	+93 ± 27**	
Pregnenolone				
IC_{50}, μM	13	1.8	0.7	
% effect of 30 μM	−61 ± 5	−75 ± 6*	−84 ± 5**	
Zinc				
IC_{50}, μM	2	2^{+++}	43**	67**
% maximal effect	−99 ± 1	−99 ± 3	−86 ± 2	−87 ± 2
Lanthanum				
IC_{50}, μM	297	522	540*	
% effect of 1 mM	−82 ± 4	−70 ± 3**	−67 ± 4**	

[a] At EC_{60} GABA concentration.
* Level of significance between $\alpha5\beta3$ isoform and the other isoforms as follows: * $p < .05$; ** $p < .01$ (see *Materials and Methods* for statistical test used). Statistically significant differences between $\alpha5\beta3\gamma3\pi$ and $\alpha5\beta3\pi$ or $\alpha5\beta3\gamma3$ isoforms were denoted by plus signs as follows: $^+p < .05$; $^{+++}p < .001$. No entry indicates that no data or fit was acquired for the subunit combination for a given compound.

TABLE 2
Pharmacological properties of $\alpha\beta$, $\alpha\beta\gamma$, $\alpha\beta\delta$, and $\alpha\beta\pi$ GABARs

Agonist/Antagonist	$\alpha\beta$	$\alpha\beta\gamma$	$\alpha\beta\delta$	$\alpha\beta\pi$
GABA (μM)	1–16[a]	6–30[b,c]	0.2–8[d,e]	>1*
Diazepam (nM)	No effect[f]	10–100[f,g]	No effect[e]	No effect*
Pentobarbital (μM)	26*	20–35[h]	+[e]	26*
Loreclezole (μM)	~1*	1[c,i]		~1*
Lanthanum ($\alpha1$) (μM)		(+) 207[j]		
Lanthanum ($\alpha5$) (μM)	(−) 297*	(−) 522*		(−) 540*
Lanthanum ($\alpha6$) (μM)		(−) 85[j]	(−) 21.8[e]	
Alphaxalone (nM)	340*	220*		290*
Pregnenolone (μM)	13*	0.7*		2*
Zinc (μM)	<5[k,l]	>100[k,l]	1–5[e]	2*

Apparent EC/IC_{50} values for different compounds ability to affect recombinant GABARs. + indicates pentobarbital was able to enhance GABA-evoke currents but a full concentration response curve was not reported. No entry indicates data that has not been reported.
[a] Davies et al., 1997; [b] Burgard et al., 1996; [c] Donnelly and Macdonald, 1996; [d] Fisher and Macdonald, 1997; [e] Saxena and Macdonald, 1994; [f] Pritchett et al., 1989; [g] Wieland et al., 1992; [h] Thompson et al., 1996; [i] Wafford et al., 1994; [j] Saxena et al., 1997; [k] Draguhen et al., 1990; [l] Whiting et al., 1997.
* Present study.

effects of lanthanum on the other α subunits in combination with studies of chimeric or mutant receptors may give insights into the mechanism(s) of action of lanthanum.

Potential Biological Roles for the π Subunit. We have demonstrated that coexpression of the π subunit with $\alpha\beta$ or $\alpha\beta\gamma$ subunits in a non-neuronal expression system produced GABAR isoforms with different properties consistent with assembly of $\alpha\beta\pi$ or $\alpha\beta\gamma\pi$ receptors (Table 1). In addition, the pharmacological properties of $\alpha\beta\pi$ receptors were different from those of $\alpha\beta\gamma$, $\alpha\beta\delta$, and $\alpha\beta\epsilon$ receptors, which suggests that incorporation of the π subunit into GABARs could alter regulation of native GABAR currents by GABAR modulators (Table 2). The other heterotrimeric pentameric receptors confer specific pharmacological properties to recombinant GABARs [such as benzodiazepine sensitivity ($\alpha\beta\gamma$)], eliminate the sensitivity to neurosteroids ($\alpha\beta\delta$), or produce spontaneously active channels that are sensitive to allosteric modulation ($\alpha\beta\epsilon$; Pritchett et al., 1989; Zhu et al., 1996; Neelands et al., 1999). Currents from π subunit-containing GABARs, which have a high sensitivity to inhibition by zinc, insensitivity to diazepam, and differential modulation by neurosteroids, might play a critical role in neuronal development or regulation of neuronal excitability. It remains to be determined, however, if the π subunit is incorporated into native GABARs. The GABAR subunits expressed along with the π subunit in NT2 neuronal precursor cells are predominately expressed in the brains of perinatal rats and not well expressed in the adult rat brain (Laurie et al., 1992). It is possible that the π subunit is incorporated primarily into "immature" GABAR isoforms, similar to the γ subunit of the nicotinic acetylcholine receptor (Witzemann et al., 1990). On the other hand, the expression of π subunit mRNA is highest in non-neuronal tissue (Hedblom and Kirkness, 1997), which indicates that it may be more critical in the function of peripheral GABARs than central GABARs. Future work on the π subunit will be required to determine its role in GABA-mediated inhibition in the brain.

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