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Spontaneous and γ-Aminobutyric Acid (GABA)-Activated $GABA_A$ Receptor Channels Formed by ϵ Subunit-Containing Isoforms

TORBEN R. NEELANDS, JANET L. FISHER,[1] MATT BIANCHI, and ROBERT L. MACDONALD

Neuroscience Program (T.R.N., M.B., R.L.M.) and Departments of Neurology (J.F., R.L.M.) and Physiology (R.L.M.), University of Michigan, Ann Arbor, Michigan

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ABSTRACT

A new γ-aminobutyric acid $(GABA)_A$ receptor (GABAR) subunit class, ϵ, has recently been cloned and shown to form functional channels when coexpressed with both α and β subunits. We report that the combination of α1β3ϵ subunit subtypes expressed in L929 cells produced functional chloride ion channels that were both spontaneously active and gated by the application of extracellular GABA. When cells were voltage-clamped at –75 mV in the whole-cell configuration, holding currents of 50 to 300 pA associated with increased noise were consistently recorded. The application of pentobarbital and loreclezole, which increase GABAR currents, increased the holding current, whereas the application of zinc and picrotoxin, which reduce GABAR currents, reduced the holding current in a concentration-dependent manner. Coexpression of α1β3γ2L, α1β3δ, α1ϵ, β3ϵ, α1β3, or ϵ subtypes did not produce holding currents that were sensitive to picrotoxin (30 μM). Cells expressing α1β3ϵ subtypes had concentration-dependent GABAR currents that were potentiated by pentobarbital, loreclezole, and lanthanum and inhibited by zinc and furosemide. Spontaneous and GABAR single-channel currents from α1β3ϵ receptors had single-channel conductances of ~24 pS. The biophysical properties and the effects of allosteric modulators were similar for spontaneous and evoked GABAR currents, suggesting that a single GABAR isoform was responsible for both currents. These data extend the pharmacological characterization of ϵ-containing GABARs and demonstrate that incorporation of the ϵ subunit permits spontaneous channel gating while preserving the structural information necessary for GABA sensitivity.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate brain, and fast inhibitory postsynaptic potentials are mediated by $GABA_A$ receptors (GABARs). GABARs belong to the superfamily of ligand-gated ion channels that includes the glycine, nicotinic cholinergic (nAChR), and 5-hydroxytryptamine receptors. Four GABAR subunit families (α1-α6, β1-β3, γ1-γ3, and δ) have been studied extensively (Macdonald and Olsen, 1994). The majority of native GABARs are thought to be heteropentamers composed of combinations of subunits from three different families (e.g., α, β, and γ) that form a chloride-selective ion channel. The potential diversity of GABAR isoforms has increased with the addition of two recently described subunits: π (Hedblom and Kirkness, 1997) and ϵ (Davies et al., 1997). The amino acid sequence of the ϵ subunit is most closely related to the γ subunits, having between 38 and 43% identical residues (Davies et al., 1997). Electrophysiological (Chang et al., 1996) and biochemical studies (Tretter et al., 1997) have suggested that the pentamer may be composed of two α subunits, two β subunits, and a single γ subunit. It is thought that the ϵ subunit, like the δ subunit (Saxena and Macdonald, 1994), is capable of replacing the γ subunit in the GABAR pentamer to form functional channels with distinct pharmacological and biophysical properties. Most GABAR isoforms require binding of GABA to initiate entry into open states. However, spontaneous channel activity has been reported in recombinant α4β1 receptors as well as β1 or β3 homomeric receptors, although these isoforms are insensitive to activation by GABA.

Naturally occurring GABAR isoforms are determined (or restricted) by the distinct regional expression of each subunit (Wisden et al., 1992). The mRNA encoding the ϵ subunit was found to be restricted to the amygdala, thalamus, and subthalamic nuclei of the human brain using Northern blot analysis (Davies et al., 1997). In contrast, in situ hybridization studies in the squirrel monkey brain localized mRNA expression in the arcuate-ventromedial area of the hypothalamus and the hilus of the hippocampus but found no detectable expression in either the amygdala or subthalamic nu-

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[1] Present address: Baylor College of Medicine, Division of Neuroscience, Houston, TX 77030.

ABBREVIATIONS: GABA, γ-aminobutyric acid; GABAR, γ-aminobutyric $acid_A$ receptor; nAChR, nicotinic acetylcholine receptor; I-V, current-voltage.

cleus (Whiting et al. 1997). The ϵ subunit gene was independently identified and mapped to a cluster of GABAR genes, including α3 and putative β4 subunit genes, on the human X chromosome (Xq28; Wilke et al., 1997). Interestingly, this location on the X chromosome is a candidate region for two neurological disorders: early-onset Parkinson's disease (Laxova et al., 1985) and X-linked mental retardation (Gedeon et al., 1991). Whether the ϵ subunit is associated with either of these disorders has yet to be determined.

Pharmacological studies of recombinant receptors have shown that individual subunits and their subtypes confer different sensitivities to GABAR modulators such as benzodiazepines (Pritchett et al., 1989) and zinc (Draguhn et al., 1990). Originally, it was reported that ϵ subunit-containing receptors were unique among GABARs in their insensitivity to the general anesthetic agents pentobarbital and propofol (Davies et al. 1997). A more recent study, however, reported that GABAR isoforms containing the ϵ subunit were directly activated by pentobarbital and that GABAR currents were enhanced by coapplication of pentobarbital (Whiting et al., 1997). Both groups showed enhancement of ϵ subunit-containing GABAR currents by the neurosteroid 5α-pregnan-3α-ol-20-one, whereas Whiting et al. (1997) also showed inhibition by zinc with a moderate affinity and rapid apparent desensitization of whole-cell currents.

The goal of this study was to characterize further the pharmacological properties and to determine the biophysical properties of GABARs containing the ϵ subunit. We found enhancement of whole-cell GABAR currents by loreclezole, lanthanum, and pentobarbital, as well as inhibition by zinc and furosemide. Whole-cell recordings consistently had large holding currents with noisy baseline values similar to that reported for spontaneously active β homopentamers (Wooltorton et al., 1997). Application of GABAR positive allosteric modulators and GABAR antagonists to the holding current produced inward and outward currents, respectively, in the absence of applied GABA. Opening frequency of single-channel currents increased during application of GABA, whereas single-channel currents with conductances of ~24 pS were recorded in the absence and presence of applied GABA. Robust effects of GABAR modulators on the holding current and the presence of single-channel openings in the absence of GABA suggested that the ϵ subunit permits spontaneous channel activity while preserving the structural information required for GABA-gated openings.

Materials and Methods

Transfection. Full-length cDNAs for rat GABAR α1, β1 (Dr. A. Tobin, University of California, Los Angeles, CA), and β3 (Dr. D. Pritchett, University of Pennsylvania) subtypes were subcloned into the pCMVNeo expression vector for transfection studies. The human ϵ cDNA was received in the pCDM8 expression vector (Dr. E. Kirkness, The Institute for Genomic Research, Rockville, MD), which was then used for transfection studies. 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 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged by a 5-min incubation with 0.5% trypsin/0.2% EDTA solution in phosphate-buffered saline (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 using a modified calcium phosphate method (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 BBS 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. Briefly, the cells were passaged and mixed with 5 μl of magnetic beads coated with hapten (~7.5×10^5 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 using 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 to 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 Co., Pittsburgh, PA) or Labcraft microhematocrit capillary tubes (Curtin Matheson Sci., Houston, TX) on a P-87 Flaming Brown puller (Sutter Instrument Co., Novato, CA) to a resistance of 8 to 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 to 10 MΩ, and coated with Q-dope (GC Electronics, Rockford, IL) to reduce capacitance.

Loreclezole and diazepam were first dissolved in 100% dimethyl sulfoxide and then added to external solution in the appropriate volume. The highest dimethyl sulfoxide concentration applied to the cells was 0.1%. All chemicals were obtained from commercial sources. Loreclezole was a gift from Janssen Laboratories (Piscataway, NJ). For whole-cell recordings, drugs were applied to cells using a modified U-tube system with a 10 to 90% rise time around 70 ms. For outside-out patch-clamp recordings, drugs were applied using 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 using the Axotape program (Axon Instruments), and stored on VHS or Beta tape. All experiments were performed at room temperature.

Data Analysis. Whole-cell currents were analyzed off-line using the programs Axotape and Prism (GraphPAD, San Diego, CA). Statistical tests were performed using the Instat program (GraphPAD). For initial single-population fits, the normalized concentration-response data for the different isoforms were fit with a four-parameter logistic equation: Current = maximum current/{1 + [10 (log EC_{50} − log [drug])*n]}, where n is a slope factor. 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 application of either 0.3 or 1 μM GABA (for agonists and antagonists, respectively) 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 ϵ-containing GABARs. Fits were made to normalized data with the current expressed as a percentage of the maximum current elicited for each cell.

Analysis of Single-Channel Currents. Single-channel recordings were digitized using Axoscope and analyzed using pClamp6 (Axon Instruments) and Interval5 (Dr. Barry S. Pallotta, University of North Carolina at Chapel Hill). For analysis, the data were digitized at 20 kHz and filtered at 2 kHz. Intervals were measured with

a 50% threshold detection method. Subconductance levels were occasionally observed (≤5% of openings) but were not included in the analysis. To reduce errors due to multichannel patches, recordings were included in the kinetic analysis only if overlaps of simultaneous openings occurred for less than 1% of the openings. Overlapped openings and bursts were not included in the kinetic analysis. The presence of multiple channels would decrease the apparent duration of the longer closed components but would have no effect on the open state or burst properties. Duration histograms were constructed and fit by a maximum likelihood method. The number of exponential functions required to fit the distribution was increased until additional components did not significantly improve the fit as determined by the log-likelihood ratio test. Intervals with durations less than 1.5 times the system dead-time were displayed in the histograms but were not included in the fit. For the definition of bursts, the two shortest closed components were considered as intraburst closures. A burst terminator for each patch was calculated from the closed interval distribution to equalize the proportion of misclassified events (see Fisher and Macdonald, 1997).

Results

Pharmacological Properties of the Spontaneous (Holding) Current

L929 cells were transfected with combinations of cDNAs encoding the $\alpha 1$, $\beta 1$, $\beta 3$, or ϵ subtypes and voltage-clamped in the whole-cell configuration of the patch-clamp technique. After formation of a high resistance seal and patch rupture, it was noted that cells transfected with $\alpha 1\beta x\epsilon$ subtype cDNAs required an unusually large holding current to maintain a potential of –75 mV and that the resulting baseline value was relatively unstable. The holding current reversed polarity at the chloride equilibrium potential and increased in a linear fashion as the membrane potential was made more negative (data not shown). To determine the source and specificity of the holding current, positive and negative allosteric modulators of GABARs were applied to cells transfected with $\alpha 1\beta 3\gamma$-2L, $\alpha 1\beta 3\delta$, $\alpha 1\beta 3\epsilon$, $\alpha 1\beta 3$, $\alpha 1\epsilon$, or $\beta 3\epsilon$ subtypes or the ϵ subunit alone (Fig. 1). Drugs including picrotoxin (30 μM), loreclezole (3 μM), pentobarbital (300 μM), zinc (100 μM), and GABA (30 μM) were applied for 5 to 8 s to cells held at a membrane potential of –75 mV (see *Materials and Methods*) (Fig. 1). Holding currents in cells expressing $\alpha 1\beta 3\epsilon$ subtypes (Fig. 1b), but not the other combinations (Fig. 1, c–h), were sensitive to picrotoxin, loreclezole, and zinc. This holding current was not altered by the application of glycine (data not shown). The application of pentobarbital and GABA evoked inward currents in cells expressing all of the subunit combinations except $\alpha 1\epsilon$ and ϵ alone (Fig. 1, b–h). Cells that expressed the $\beta 3\epsilon$ subtypes were slightly activated by the application of pentobarbital, but no inward current was evoked by 30 μM GABA.

L929 cells coexpressing either $\alpha 1\beta 1\epsilon$ or $\alpha 1\beta 3\epsilon$ subtypes were activated directly by pentobarbital (10–300 μM) in a concentration-dependent manner (Figs. 1b and 2A). Peak currents evoked by 300 μM barbiturate were 214.1 ± 39.8 pA for $\alpha 1\beta 3\epsilon$ and 136.6 ± 53.8 pA for $\alpha 1\beta 1\epsilon$. Averaged peak currents evoked by pentobarbital (1–300 μM) revealed similar EC_{50} values for these two isoforms. The $\alpha 1\beta 1\epsilon$ isoform had a pentobarbital EC_{50} of 211 μM (n_H = 0.9, n = 3), whereas the pentobarbital EC_{50} for the $\alpha 1\beta 3\epsilon$ isoform was 112 μM (n_H = 1.3, n = 3) (Fig. 2A).

The application of loreclezole (100 nM to 30 μM) alone to cells expressing $\alpha 1\beta 3\epsilon$ receptors evoked inward currents (Fig. 1b) in a concentration-dependent manner (Fig. 2B). Maximal currents of ~105 pA were evoked by concentrations of loreclezole greater than 3 μM (n = 6). Higher concentrations of loreclezole produced less current, likely due to open channel block (Donnelly and Macdonald, 1996). The loreclezole concentration-response curve was fit with a logistic equation with an EC_{50} of 1.0 μM and a Hill slope (n_H) of 3.2 (Fig. 2B).

Picrotoxin produced a concentration-dependent reduction of the holding current in cells transfected with $\alpha 1\beta 3\epsilon$ subtypes, with an IC_{50} of 1.8 μM (Fig. 1b). Maximal outward currents (70.7 ± 23.1 pA, n = 4) were produced by the application of 10 μM picrotoxin, which represented 80% to 90% reduction of the holding current (Fig. 3A). Bicuculline, another GABAR antagonist, also reduced the holding current but with less efficacy than picrotoxin (data not shown).

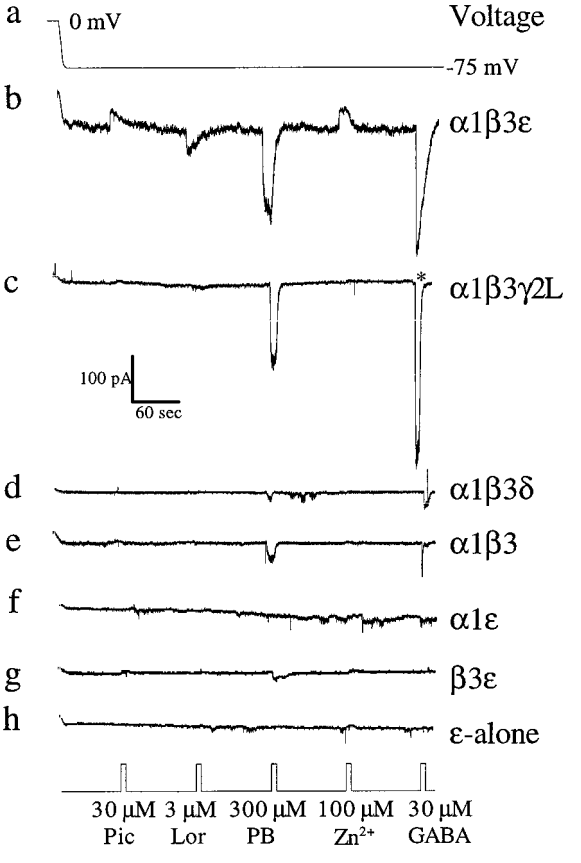

Fig. 1. Pharmacology of holding currents for various recombinant $GABA_A$ receptors. After establishing a seal and breaking into L929 cells expressing $\alpha 1\beta 3\epsilon$ channels, a large current was required to hold the membrane potential at –75 mV. To test the hypothesis that spontaneous channels were responsible for these consistently large holding currents, we examined the effects of direct drug application in the absence of GABA. a, L929 cells expressing recombinant $GABA_A$ receptors were voltage clamped to –75 mV. b–h, responses to direct applications of 30 μM picrotoxin (Pic), 3 μM loreclezole (Lor), 300 μM pentobarbital (PB), 100 μM zinc (Zn^{++}), and 30 μM GABA are shown for multiple receptor isoforms. Only $\alpha 1\beta 3\epsilon$ (b) receptors exhibited significant holding currents that were sensitive to direct application of picrotoxin, loreclezole, and zinc. Cells transfected with $\alpha 1\beta 3\epsilon$ (b), $\alpha 1\beta 3\gamma 2$L (c), $\alpha 1\beta 3\delta$ (d), or $\alpha 1\beta 3$ (e) were activated by the application of both 300 μM pentobarbital and 30 μM GABA. Cells expressing $\alpha 1\epsilon$ (f) and ϵ alone (h) were insensitive to all modulators, but the application of pentobarbital to $\beta 3\epsilon$-containing cells produced a small current (g). Bars, 6-s drug applications. *, application of 10 μM GABA for $\alpha 1\beta 3\gamma 2$L trace.

Zinc also reduced the holding current (Fig. 1b). As increasing concentrations of zinc were applied to the cell, the total holding current diminished in amplitude ($n = 3$). To control for zinc-induced current rundown, we measured the ability of each concentration of zinc to inhibit the holding current recorded just before its application. The averaged percent block calculated by this method had an IC_{50} of 22.3 μM with an n_H of −0.9 ($n = 3$) (Fig. 3B).

It should be noted that the benzodiazepine site agonist, diazepam (1 μM), did not enhance GABAR current or alter the holding current (data not shown). This was consistent with reports that benzodiazepine sensitivity required inclusion of a γ subunit in association with an α1, α2, α3, or α5 subtype and a β subunit.

Characterization of GABAR Currents in ϵ-Containing GABARs

Inward currents evoked by GABA (10 nM to 100 μM) in cells expressing $\alpha1\beta3\epsilon$ receptors increased in a concentration-dependent manner, with faster apparent activation rates and greater apparent desensitization with higher GABA concentrations (Fig. 4A). Currents were normalized to the maximal current for each cell, averaged, and fit with a logistic equation with an EC_{50} = 0.8 μM and n_H = 0.9 (n = 9) (Fig. 4B). The half-maximal response to GABA was lower, and the n_H was reduced compared with cells transfected with $\alpha1\beta3$ subtypes as previously reported by our laboratory.

The current-voltage (I-V) relation for $\alpha1\beta3\epsilon$ receptors was obtained by repeated applications of 1 μM GABA at holding potentials ranging from −100 to +75 mV in 25-mV increments ($n = 4$) (Fig. 5A). The I-V relation was linear at negative holding potentials, whereas potentials above +25 mV revealed inward rectification for all cells tested (Fig. 5B). To verify that current rundown was not responsible for this apparent rectification, GABA was reapplied at –75 mV following each I-V protocol. Rundown was not detected in any cells during this protocol, suggesting this rectification reflected an intrinsic property of the channel.

Pharmacological Properties of ϵ-Containing GABARs

Barbiturate Sensitivity of ϵ Subunit-Containing GABARs. The effect of the barbiturate pentobarbital on ϵ subunit-containing GABARs has been controversial. Pentobarbital had no effect on GABAR currents when $\alpha1\beta3\epsilon$ receptors were expressed in human embryonic kidney 293 cells (Davies

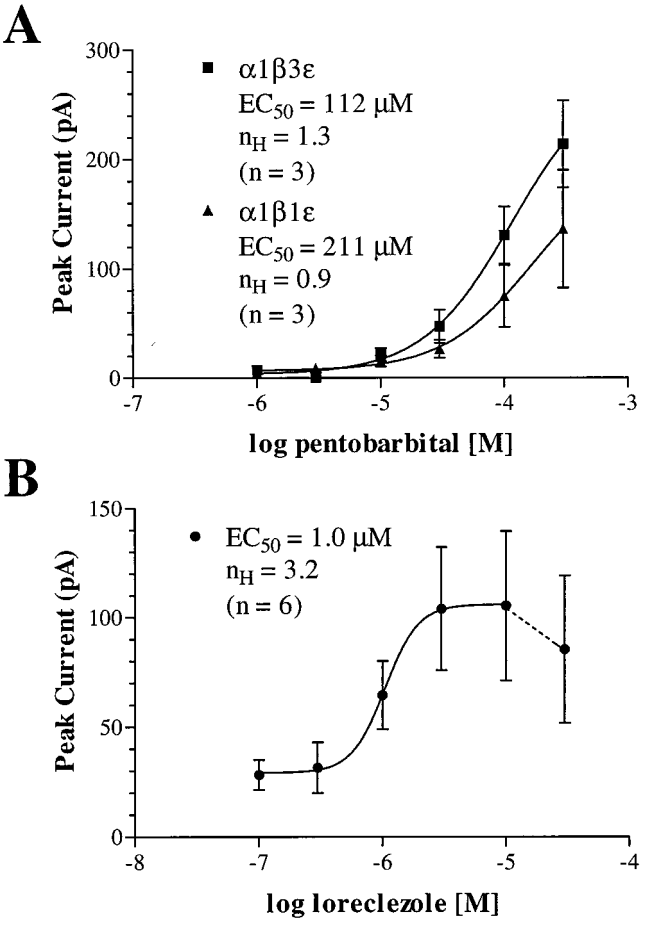

Fig. 2. Direct activation of GABA receptors by pentobarbital and loreclezole. A, concentration-response relationships for direct activation of GABA receptors by pentobarbital. ■, Responses of $\alpha1\beta3\epsilon$ receptors (n = 3). ▲, $\alpha1\beta1\epsilon$ responses (n = 3). B, concentration-response relationship of loreclezole on the holding current (n = 6). Data are mean ± S.E.M. The EC_{50} values and n_H were derived from fitting a four-parameter logistic equation but did not include the highest loreclezole concentration (30 μM; hatched line).

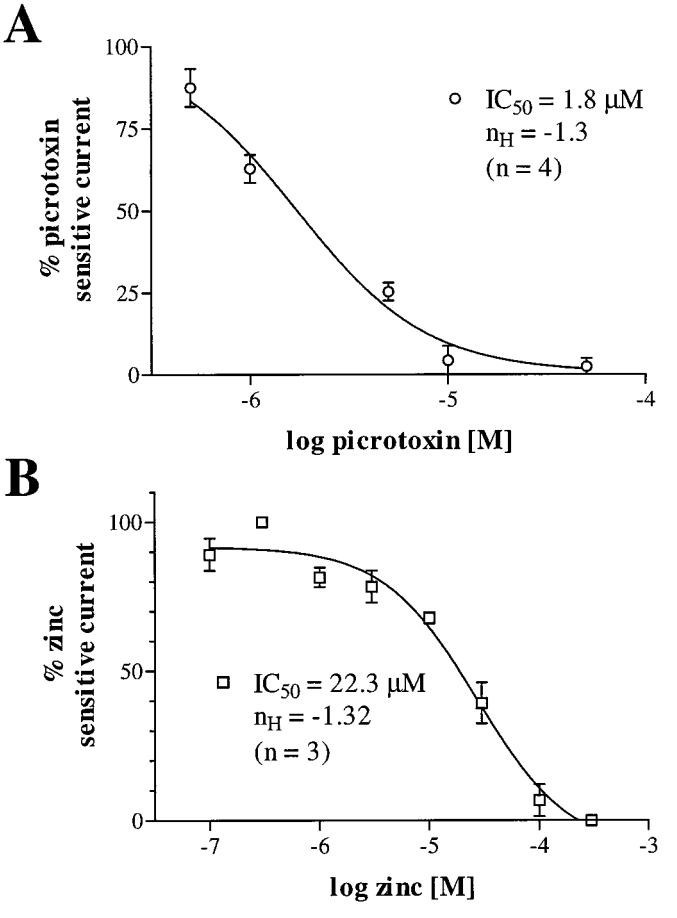

Fig. 3. Inhibition of $\alpha1\beta3\epsilon$ holding currents by picrotoxin and zinc. A, concentration-response relationship for picrotoxin inhibition of the holding current (n = 4), normalized in each cell to the maximum amplitude block of holding current and plotted as the percent picrotoxin-sensitive current remaining after each application. B, concentration-response relationship of zinc on the holding current (n = 3), normalized to the maximum amplitude zinc block of holding current in each cell and plotted as percent of zinc-sensitive current remaining after zinc application. Data are mean ± S.E.M.

et al., 1997), whereas $\alpha1\beta1\epsilon$ receptors expressed in *Xenopus laevis* oocytes were potentiated by pentobarbital (Whiting et al., 1997). Effects of pentobarbital on GABAR currents have not been shown to depend on either β subunit subtype or expression system, so the basis for the different results was unclear. In our study, pentobarbital enhanced both $\alpha1\beta1\epsilon$ and $\alpha1\beta3\epsilon$ currents (Fig. 6, A and B) by 150.5 ± 23.8% (n = 4) and 83.7 ± 25.1%, respectively, with EC_{50} values of ~40 μM (n = 6) (Fig. 6C). A normalized, averaged concentration-response curve for each isoform was fit with a logistic equation with an EC_{50} of 40 μM and an n_H between 1.8 and 1.9 (Fig. 6C).

Loreclezole Sensitivity of ϵ Subunit-Containing GABARs. Loreclezole enhancement of GABAR currents depends on the presence of $\beta2$ or $\beta3$ subtypes (Wingrove et al., 1994). Loreclezole (30 nM to 30 μM) enhanced $\alpha1\beta3\epsilon$ currents evoked by 0.3 μM GABA in a concentration-dependent manner, with a maximal enhancement of 55.6 ± 18.6% at 3 μM (n = 8) (Fig. 7A). With higher loreclezole concentrations, an apparent inhibition of currents to below control levels was observed. The range over which enhancement occurred was fit with a logistic equation with EC_{50} = 0.9 μM and n_H = 8.8. This inhibition was more dramatic than that seen with $\alpha\beta\gamma$ receptor currents, in which loreclezole produced only slight inhibition at 30 μM after maximal enhancement at 10 μM (Donnelly and Macdonald, 1996). Inclusion of the ϵ subunit in the GABAR appeared to increase the ability of high concentrations of loreclezole to inhibit GABAR currents.

Lanthanum Sensitivity of ϵ Subunit-Containing GABARs. The trivalent cation lanthanum has been shown to have subunit-specific effects on recombinant GABARs. GABAR currents from receptors containing the $\alpha6$ subtype were inhibited by lanthanum, whereas $\alpha1$ subtype-containing receptor currents were enhanced (Saxena et al., 1997). Lanthanum (1 μM to 3 mM) produced a concentration-dependent increase in $\alpha1\beta3\epsilon$ currents evoked by 0.3 μM GABA. Significant enhancement of the GABAR current was first observed at 100 μM lanthanum and increased through 3 mM lanthanum (148.1 ± 5.9%) (n = 5) (Fig. 7B). Normalized data were fit with a logistic equation (see *Materials and Methods*) with an EC_{50} value of 500 μM and an n_H of 0.9 (Fig. 7B).

Zinc Sensitivity of ϵ Subunit-Containing GABARs. The inhibitory effects of the divalent cation zinc have been well characterized for recombinant and native GABARs (Draguhn et al., 1990; Kapur and Macdonald, 1996). Control $\alpha1\beta3\epsilon$ currents evoked by 1 μM GABA were inhibited in a concentration-dependent manner by zinc. Zinc maximally inhibited 96 ± 2.6% (n = 7) of GABAR currents, with an IC_{50} of 32.5 μM and an n_H of –1.0 (Fig. 8A), similar to that reported by Whiting et al. (41.9 μM).

Furosemide Sensitivity of ϵ Subunit-Containing GABARs. The anthranilic acid derivative furosemide inhibited recombinant GABAR currents with IC_{50} values in the micromolar range only when an $\alpha4$ or $\alpha6$ subtype was expressed

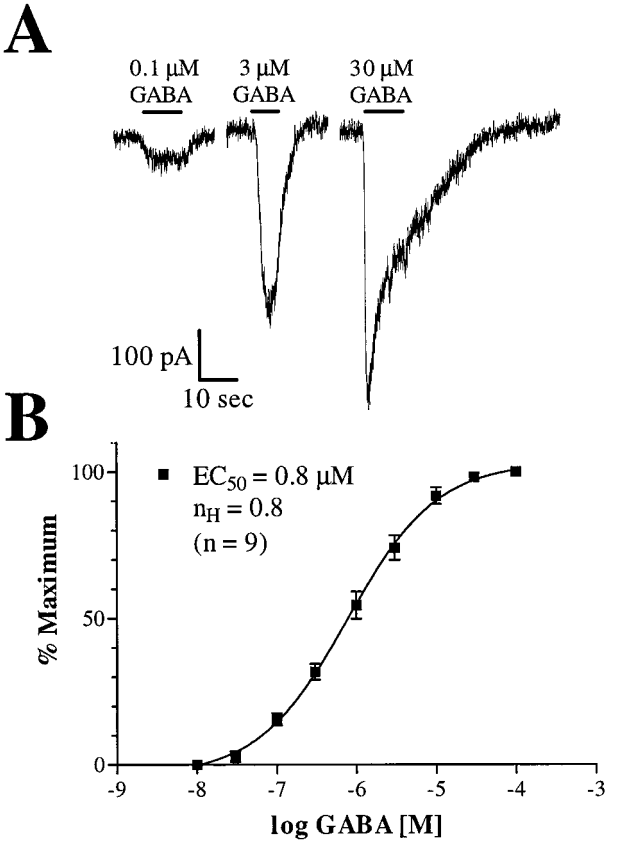

Fig. 4. GABA concentration-response profile for $\alpha1\beta3\epsilon$ receptors. A, representative current traces from application of 0.1, 3, and 30 μM GABA to a single cell. Horizontal bar, drug application. B, normalized concentration-response curve for GABA-evoked currents (n = 9). Data are mean ± S.E.M.

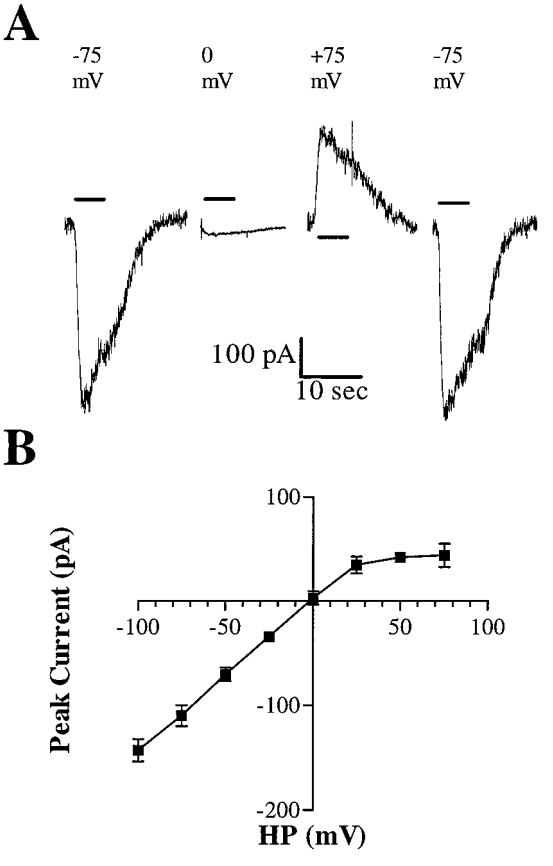

Fig. 5. Voltage-related properties of GABA-evoked $\alpha1\beta3\epsilon$ currents. A, representative current traces evoked by 1 μM GABA in a single cell clamped at –75, 0, and +75 mV. Subsequent reapplication of 1 μM GABA at –75 mV demonstrates the absence of current rundown. Horizontal bars, drug application. B, peak currents evoked by 1 μM GABA are plotted versus membrane holding potential (n = 4). Data are mean ± S.E.M.

(Wafford et al., 1996). However, in this study, furosemide potently inhibited $\alpha1\beta3\epsilon$ GABAR currents evoked by 1 μM GABA with an apparent IC_{50} of 167 μM ($n_H = -0.7$, $n = 5$) (Fig. 8B). Maximal inhibition of control currents was 83.9 ± 4.4% at 3 mM furosemide, which was the solubility limit of furosemide in 0.1% dimethylsulfoxide. The affinity and efficacy of furosemide block for this isoform were much greater than those reported for $\alpha1\beta x\gamma2L$ receptors but was similar to the 162 μM IC_{50} reported for the $\alpha4\beta3\gamma2L$ isoform (Wafford et al., 1996).

Single-Channel Properties of ϵ-Containing GABARs

To examine the single-channel properties of these receptors, 1 μM GABA was applied to outside-out patches pulled from transfected fibroblasts. $\alpha1\beta3\epsilon$ single-channel openings were relatively long in duration and were separated into closely grouped bursts of openings (Fig. 9A, top trace). Openings occurred primarily to a single conductance level (average amplitude = 1.6 pA at –70 mV). The amplitudes of single-channel openings were measured at holding potentials ranging from −80 to +80 mV and were fit with linear regression analysis to determine single-channel conductance (Fig. 9B, ●). From fits of individual patches, the average conductance (±S.E.M.) of $\alpha1\beta3\epsilon$ channel openings was 23.7 ± 0.13 pS ($n = 4$). This was similar to the main conductance level reported for both $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ channels (27 pS) (Fisher and Macdonald, 1997) and was larger than the main conductance level reported for $\alpha1\beta1$, $\alpha1\beta2$, or $\alpha1\beta3$ channels (11–13 pS) (Verdoorn et al., 1990; Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997). The average reversal potential for the individual patches was near 0 mV (2.9 ± 0.8 mV), as predicted for a chloride ion-selective channel.

To confirm that single-channel openings of the ϵ-containing GABARs were responsible for the spontaneous current, outside-out patches were pulled from fibroblasts transfected with $\alpha1\beta3\epsilon$ subunits. Brief, infrequent single-channel openings were recorded in the absence of applied GABA (Fig. 9A, bottom trace). The amplitudes of single-channel openings were measured at holding potentials ranging from −80 to +80 mV and were fit with linear regression analysis to determine single-channel conductance (Fig. 9B, ○). From fits of individual patches, the average conductance of $\alpha1\beta3\epsilon$ channel openings was 22.2 pS ($n = 2$). The average reversal potential for the two patches was near 0 mV (2.6 ± 2.3 mV) as predicted.

The kinetic properties of GABAR single-channel openings and closings were examined by constructing open and closed duration histograms from data obtained during long (5–10 min) applications of 1 μM GABA. Openings occurred with low frequency with an average percent open time of 1.04 ± 0.33% ($n = 4$). Open interval histograms were fitted best with the sum of two exponential functions with nearly equal relative proportions (Fig. 10A). The time constants and relative areas

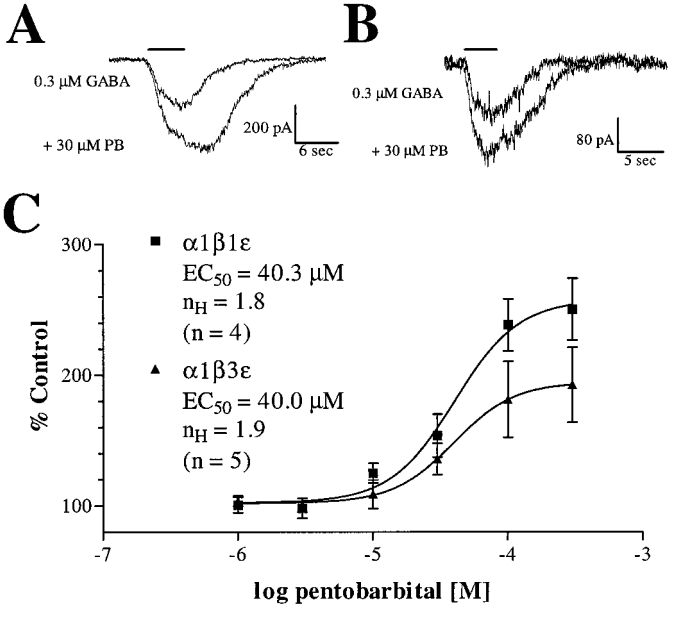

Fig. 6. Enhancement of GABA-evoked currents by pentobarbital. A and B, superimposed current traces of successive application of 0.3 μM GABA (upper trace in A and B) and 0.3 μM GABA plus 30 μM pentobarbital (lower trace in A and B) for $\alpha1\beta1\epsilon$ receptors (A) and $\alpha1\beta3\epsilon$ receptors (B). Horizontal bars, drug application. C, concentration-response relationship for pentobarbital enhancement of currents evoked by 0.3 μM GABA. ■, $\alpha1\beta1\epsilon$ currents ($n = 5$). ▲, $\alpha1\beta3\epsilon$ currents ($n = 4$). Data are mean ± S.E.M.

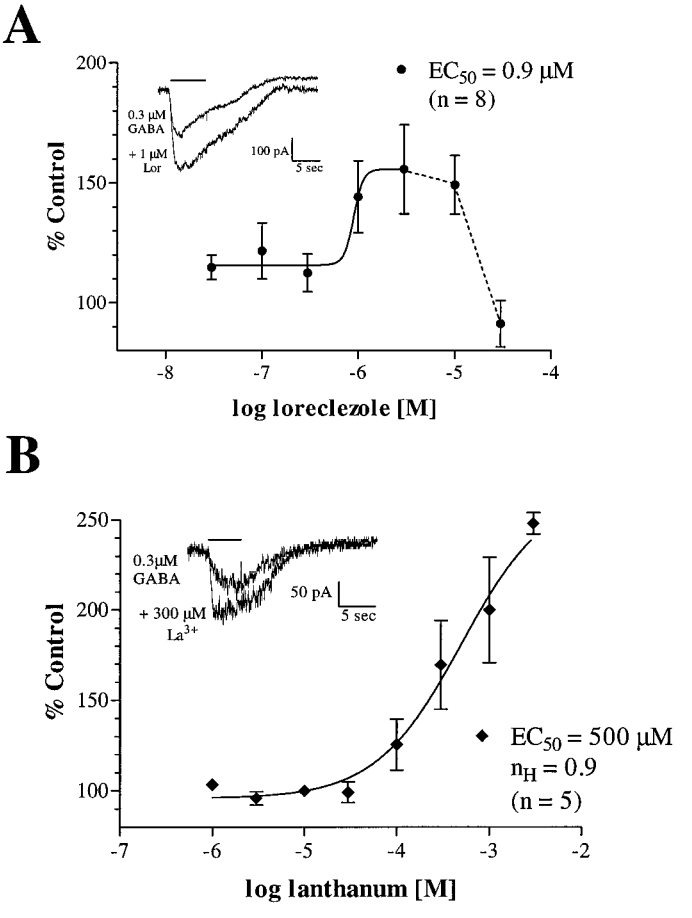

Fig. 7. Modulation of GABA-evoked $\alpha1\beta3\epsilon$ currents by loreclezole and lanthanum. A, concentration-response relationship for loreclezole modulation of currents evoked by 0.3 μM GABA ($n = 8$). EC_{50} value for loreclezole enhancement (0.03–10 μM loreclezole) was derived independent of the apparent inhibition seen at higher concentrations (hatched line). Inset, superimposed current traces of successive application of 0.3 μM GABA current (upper trace) and 0.3 μM GABA plus 1 μM loreclezole (lower trace) are shown. Horizontal bar, drug application. B, concentration-response relationship for lanthanum enhancement of 0.3 μM GABA-evoked currents ($n = 5$). Inset, superimposed current traces of successive application of 0.3 μM GABA (upper trace) and 0.3 μM GABA plus 300 μM lanthanum (lower trace). Horizontal bar, drug application. Data are mean ± S.E.M.

(±S.E.M.) averaged 0.388 ± 0.057 ms (57.4 ± 5.9%) and 2.24 ± 0.08 ms (42.6 ± 5.9%) with an average mean open time of 1.18 ± 0.08 ms (n = 4 patches) (Table 1). Closed duration histograms were fitted best with the sum of five exponential functions (Fig. 10B). The average durations of the longer closed components were relatively variable, probably due to differences in the number of channels in the patches. The short-duration closed times, however, represented intraburst channel closings and therefore were not affected by multiple-channel patches. The averages for time constants (and relative areas) of the components for the four patches were 0.128 ± 0.004 ms (0.538 ± 0.021), 1.123 ± 0.07 ms (0.194 ± 0.024), 12.63 ± 1.55 ms (0.113 ± 0.005), 138.7 ± 41.3 ms (0.083 ± 0.007), and 1596.3 ± 481.6 ms (0.072 ± 0.014) (Fig. 10B; Table 1). The average mean shut time was 133 ± 55 ms. The relatively high proportion of the shortest closed components was consistent with the bursting behavior of the channels, whereas the long duration of the longer components was consistent with entry into desensitized states.

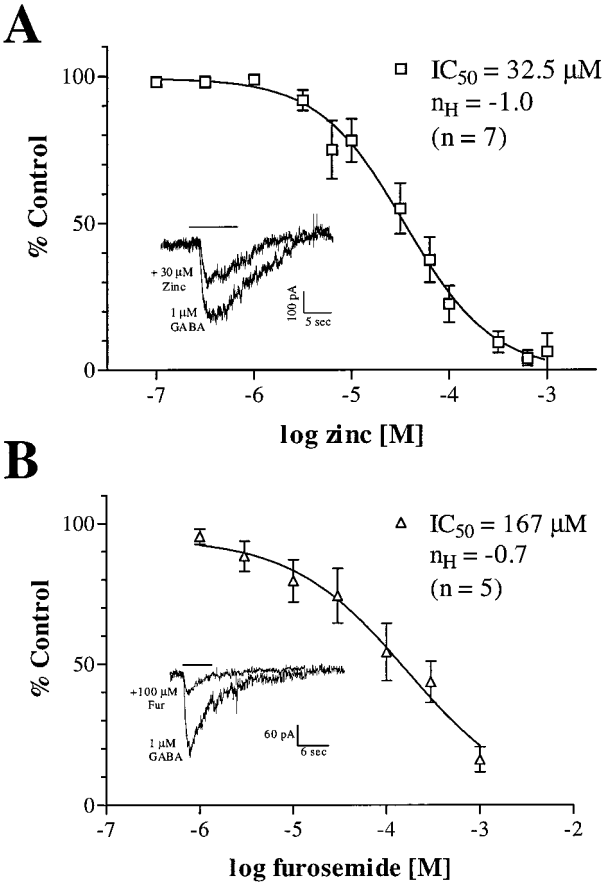

Fig. 8. Inhibition of GABA-evoked $\alpha1\beta3\epsilon$ currents by zinc and furosemide. A, concentration-response relationship for zinc inhibition of currents evoked by 1 μM GABA (n = 7). Inset, superimposed current traces of successive application of 1 μM GABA (lower trace) and 1 μM GABA plus 30 μM zinc (upper trace). Horizontal bar, drug application. B, concentration-response relationship for furosemide inhibition of currents evoked by 1 μM GABA (n = 5). Inset, superimposed current traces of successive application of 1 μM GABA (lower trace) and 1 μM GABA plus 100 μM furosemide (upper trace). Horizontal bar, drug application. Data are mean ± S.E.M.

Discussion

Incorporation of the ϵ Subunit Produced a Spontaneously Active GABAR Channel. L929 cells expressing the $\alpha1\beta3\epsilon$ GABAR isoform consistently produced a large holding current when voltage clamped at –75 mV that was sensitive to both positive and negative allosteric modulators of GABARs but was not affected by the application of glycine. Other subunit combinations, including $\alpha1\beta3\gamma2L$, $\alpha1\beta3\delta$, $\alpha1\beta3$, $\alpha1\epsilon$, and $\beta3\epsilon$, and ϵ alone failed to produce significant holding currents that were sensitive to enhancement by loreclezole or block by picrotoxin or zinc. Specifically, the negative results obtained with transfections of $\alpha1\epsilon$ or $\beta3\epsilon$ subtypes or the ϵ subunit alone strongly suggested that channels potentially composed of only one or two of the three transfected subunits were not contributing to the observed holding current.

To date, the only wild-type GABAR isoforms reported to result in spontaneously active currents are $\beta1$ (Sigel et al., 1989; Krishek et al., 1996), $\beta3$ (Wooltorton et al., 1997), and

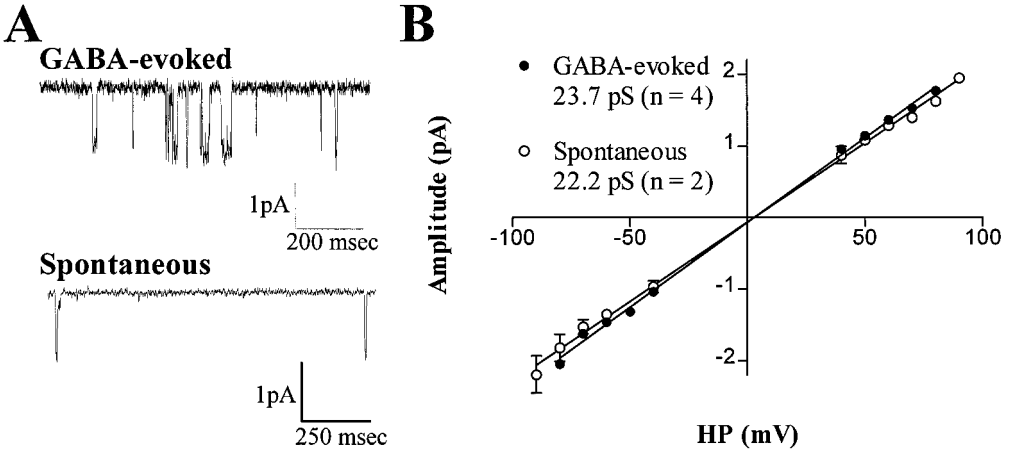

Fig. 9. Single-channel GABA-evoked $\alpha1\beta3\epsilon$ currents A, representative single-channel current traces evoked by 1 μM GABA applied to an outside-out patch (upper trace) or spontaneous openings recorded in the absence of applied GABA (lower trace). B, single-channel current-voltage relationships are shown for GABA-evoked openings (●, n = 4) or spontaneous openings (○, n = 2). Data are mean ± S.E.M.

$\alpha 4\beta 1$ GABAR isoforms (Khrestchatisky et al., 1989). The spontaneous currents from GABARs composed only of β subunits were potentiated by pentobarbital and inhibited by picrotoxin and zinc. Although the apparent affinity of picrotoxin for the $\alpha 1\beta 3\epsilon$ isoform holding current was similar to that observed with the β subunit homomers, pentobarbital and zinc exhibited much lower affinities for the holding currents in our study. The holding current was also partially reduced by the competitive GABA antagonist bicuculline, which has recently been shown to act as an allosteric inhibitor at the GABA binding site (Ueno et al., 1997). In addition, the spontaneous murine β homomeric channels, unlike the $\alpha 1\beta 3\epsilon$ receptors, were not responsive to GABA. Pentobarbital, but not GABA, activated an inward current from cells transfected with $\beta 3\epsilon$ subunits, although we did not determine whether this current was carried by a $\beta 3$ homomer that shares these properties or from a novel $\beta 3\epsilon$ channel. In any case, the pharmacology of this current differed substantially from, and thus was likely not contributing to, the holding current observed with $\alpha 1\beta 3\epsilon$ GABARs. Combined, the pharmacological data provide strong evidence that the holding current was carried by $\alpha 1\beta 3\epsilon$ channels. The $\alpha 1\beta 3\epsilon$ isoform reported here is the first example of a GABAR composed of three different subunits that exhibits both spontaneous and GABA-activated currents.

Spontaneously active channels have been reported for native GABARs (Mathers, 1985) and nAChRs (Jackson et al., 1990; Franco-Obergon and Lansman, 1995), but the presence of low concentrations of endogenous agonist could not be ruled out. Single-channel recordings from neurons of mice carrying at least one copy of the gene for dystrophia muscularis (*dy*) exhibited increased spontaneous openings relative to wild-type mice (Franco-Obergon and Lansman, 1995). The subunit composition of these receptors was unknown; however, the biophysical properties of the spontaneous openings were similar to the embryonic form of nAChR. Whether the ϵ subunit-containing isoforms represent an embryonic GABAR cannot be addressed at this time because no information on the developmental expression of ϵ has been reported.

The functional consequences of a spontaneously active GABAR channel would depend on developmental stage, the regional distribution of the channel, the neuronal circuitry where the channel is expressed, and the local chloride ion gradient across the membrane. The resting membrane potential of most neurons is primarily determined by a potassium conductance. The addition of a significant resting chloride conductance, in the form of a spontaneous GABAR, offers a mechanism to adjust the baseline excitability of neurons. Tonic activation of GABARs on certain neurons, such as inhibitory interneurons, may actually result in a net increase in output from a system, and tonic activation of GABARs early in development may also be excitatory because GABA-

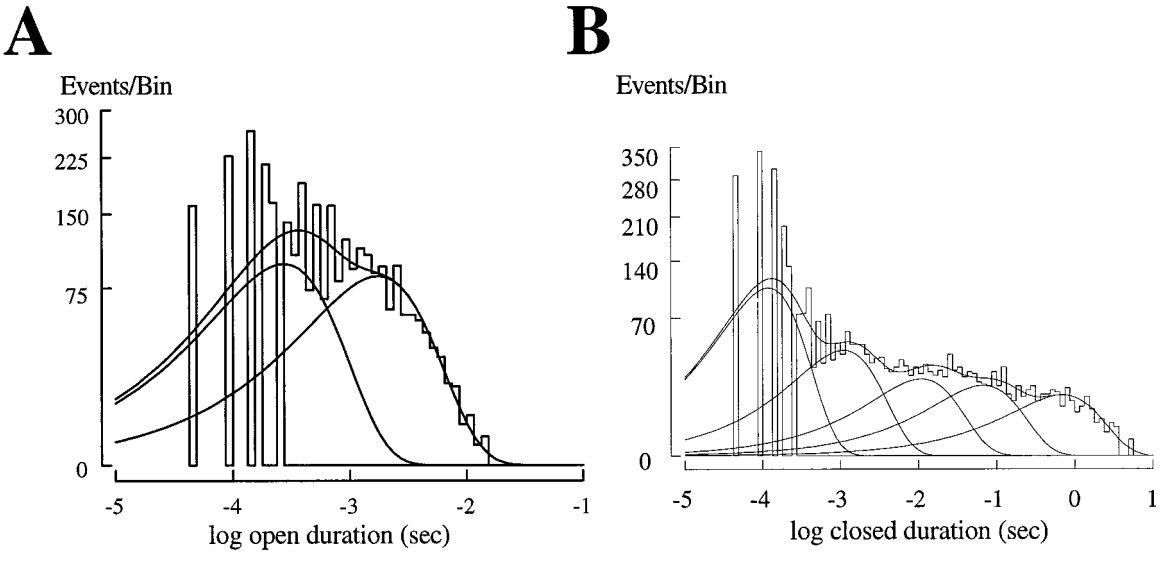

Fig. 10. Open- and closed-duration histograms of single-channel $\alpha 1\beta 3\epsilon$ currents evoked by 1 μM GABA. A, open-time histograms were best fit by two exponential distributions with means (±S.E.M.) and relative areas (±S.E.M.) of 0.388 ± 0.057 ms (57.4 ± 5.9%) and 2.24 ± 0.08 ms (42.6 ± 5.9%). B, closed times were best fit with five exponential distributions, with means and relative areas of 0.128 ± 0.004 ms (53.8 ± 2.1%), 1.123 ± 0.07 ms (19.4 ± 2.4%), 12.63 ± 1.55 ms (11.3 ± 0.5%), 138.7 ± 41.3 ms (8.3 ± 0.78%), and 1596.3 ± 481.6 ms (7.2 ± 1.47%).

TABLE 1

Summary of single-channel properties of recombinant $GABA_A$ receptor isoforms

Major kinetic properties of single-channel openings were represented as the mean ± S.E.M. for the number of patches indicated. The burst properties were combined from the data obtained from all analyzed patches.

	$\alpha 1\beta 3^a$	$\alpha 1\beta 3\epsilon^b$	$\alpha 1\beta 3\gamma 2L^a$	$\alpha 1\beta 3\delta^a$
No. of patches	6	4	8	7
NP_0(*100)	0.28 ± 0.07	1.04 ± 0.33	3.74 ± 0.81	0.69 ± 0.27
Mean open time (ms)	0.46 ± 0.04	1.18 ± 0.08	2.10 ± 0.28	0.43 ± 0.05
Mean shut time (ms)	230 ± 42	132 ± 55	70.0 ± 17.4	151.2 ± 52.9
Mean burst duration (ms)	2.48 ± 0.51	3.72 ± 0.39	7.32 ± 1.11	1.15 ± 0.26
Mean openings/burst	2.70 ± 0.27	2.94 ± 0.13	3.36 ± 0.23	1.55 ± 0.06

[a] From Fisher and Macdonald (1997).
[b] Present study.

activated currents are often depolarizing early in development (Ben-Ari et al., 1997).

The structural basis for the spontaneous activity of ϵ-containing GABARs was unclear. A number of studies have reported that mutations to either the M2 or M3 transmembrane domains of ligand-gated heteromeric ion channels result in spontaneously active currents. For example, a tonically active glycine receptor was generated by mutation of the α1 subunit (A288W) (Mihic et al., 1997). This alanine is conserved in the homologous position of GABAR ϵ subunits. Interestingly, wild-type *rho* GABAR are not spontaneously active even though there is a tryptophan in the corresponding position. Point mutations of the rat *rho*-1 subunit T314A, L317A (Pan et al., 1997), or mutation of the analogous human residue L301 (Chang and Weiss, 1998) have also been reported to produce receptors with spontaneous channel activity. However, these mutant isoforms were unique in that they were inhibited by GABA. These sites are conserved in the ϵ subunit, and therefore, it is unlikely that any of these sites are solely responsible for the spontaneous activity of ϵ-containing GABARs.

Effects of Pentobarbital on ϵ-Containing GABARs. Pentobarbital and other general anesthetic agents have been shown to have three actions on GABARs: 1) GABAR currents were potentiated by coapplication of pentobarbital (Schulz and Macdonald, 1981); 2) pentobarbital directly activated GABARs (Schulz and Macdonald, 1981); and 3) application of high concentrations of pentobarbital alone or in combination with GABA resulted in an open-channel block of the ion pore (Schwartz et al., 1986).

The potency of pentobarbital potentiation of GABAR currents was not dependent on the α or β subtype when coexpressed with the γ2S subunit (Thompson et al., 1996). The extent of potentiation, however, was significantly greater for α6 subtype-containing isoforms (536%) compared with other α subtype-containing isoforms (240–400%) (Thompson et al., 1996). In our study, pentobarbital potentiated GABAR currents with EC_{50} values similar to those for αxβxγ2S isoforms (α1β1ϵ = 40.3 μM; α1β3ϵ = 40.0 μM), and 100 μM pentobarbital produced a maximal enhancement of ~200% for both isoforms. Whiting et al. (1997) reported a 200 to 300% potentiation of GABAR currents for the α1β1ϵ isoform expressed in *X. laevis* oocytes. In contrast, Davies et al. (1997) reported that pentobarbital did not enhance GABAR current. The cDNA used by Whiting et al. (1997) was subcloned into a different vector and had one amino acid difference in position 102 (serine for alanine), but the expression vector and sequence in our experiments were identical to those used by Davies et al. (1997). The reason for this discrepancy remains unclear but could depend on factors related to the expression system, such as differences in receptor stoichiometry or post-translational modifications of the protein.

Direct application of moderate to high concentrations of pentobarbital have been shown to evoke chloride currents independent of subunit composition, although pentobarbital potency and efficacy were greater for α6 subtype-containing isoforms (Thompson et al., 1996). EC_{50} values for direct activation by pentobarbital ranged from around 50 μM (α6βxγ2s) to 540 μM (α1β1γ2S), whereas efficacies ranged from 33 to 160% of the maximal GABA current (Thompson et al., 1996). Cells coexpressing either α1β1ϵ or α1β3ϵ subunits were directly activated by pentobarbital (10–300 μM). The pentobarbital EC_{50} values for the isoforms were 211 and 112 μM, respectively, and were 4 to 5 times higher than those for the potentiation of GABA-evoked currents by pentobarbital, similar to previous reports for other GABAR isoforms (Thompson et al., 1996). In addition, very high concentrations of pentobarbital (1 mM) produced less direct activation and enhancement (data not shown), presumably due to open-channel block. Interestingly, Davies et al. found that 1 mM pentobarbital directly activated receptors containing the ϵ subunit but failed to enhance GABAR currents. It has been

TABLE 2
Summary of pharmacological properties of recombinant $GABA_A$ receptor isoforms
Values represent IC_{50} or EC_{50} calculations for each compound, as appropriate. Where these values were dependent on subunit subtype, a range is reported.

Agonist/Antagonist	αβ	αβϵ (evoked)	αβϵ (spontaneous)	αβγ	αβδ
GABA	1–16 μM[i,c]	0.8[n]–11 μM[h,i]	N.A.	6–30 μM[c,k]	0.2–8 μM[b,c]
Diazepam	No effect	No effect	No effect	10–100 nM[l]	No effect
+ Pentobarbital	26 μM[m]	40 μM[n]	N.A.	20–35 μM[j]	S
Pentobarbital (alone)		N.A.	112 μM[n]	50–540 μM[j]	S
Loreclezole	~1 μM[m]	~1 μM[n]	1.0 μM[n]	1 μM[d,e]	N.P.
Lanthanum (α1)	N.P.	(+) 500 μM[n]	N.P.	(+) 207 μM[a]	N.P.
Lanthanum (α6)	N.P.	N.P.	N.P.	(−) 85 μM[a]	(−) 21.8 μM[a]
Zinc	<5 μM[f,h]	32–42 μM[h]	22 μM[n]	>100 μM[f,h]	1–5 μM[b]
Picrotoxin	N.P.	N.P.	1.8 μM[n]	N.P.	N.P.
Furosemide (α1)	N.P.	167 μM[n]	N.P.	>5 mM[g]	N.P.
Furosemide (α4)	N.P.	N.P.	N.P.	162 μM[g]	N.P.
Furosemide (α6)	N.P.	N.P.	N.P.	6 μM[g]	N.P.

S, sensitivity of receptor subtype to drug application; N.P., experiments were not performed; N.A., experimental conditions were not applicable to this study. Regarding the lanthanum experiments, (+) and (−) referred to lanthanum enhancement or inhibition of GABAR currents, respectively.
[a] Saxena et al. (1997).
[b] Saxena et al. (1994).
[c] Fisher et al. (1997).
[d] Donnelly et al. (1996).
[e] Wingrove et al. (1994).
[f] Draguhn et al. (1990).
[g] Wafford et al. (1996).
[h] Whiting et al. (1997).
[i] Davies et al. (1997).
[j] Thompson et al. (1996).
[k] Burgard et al. (1996).
[l] Pritchett et al. (1989).
[m] Neelands and Macdonald, unpublished data.
[n] This study.

postulated that different sites exist in GABARs for activation, potentiation, and inhibition by pentobarbital (Sanna et al., 1995), and thus it was possible that the site of pentobarbital enhancement was altered in receptors studied by Davies et al., ourselves, and Whiting et al.

Additional Pharmacological Properties of GABARs Containing the ϵ Subunit. Functional $\alpha\beta\epsilon$ GABAR isoforms had pharmacological properties that were different from those of $\alpha\beta$, $\alpha\beta\gamma$, and $\alpha\beta\delta$ GABARs (Table 2). Notably, benzodiazepine insensitivity corroborates the proposed requirement of a γ subunit to form a benzodiazepine site. Zinc inhibited both the holding and GABA-evoked $\alpha1\beta3\epsilon$ currents with similar IC_{50} values, suggesting that a single GABAR isoform was responsible for both currents. Furosemide affinity, which depended on the α subtype in previous experiments, was dramatically altered. Further experiments are required to determine whether the ϵ subunit increased the affinity of furosemide for all α subunits or changed the rank order of potency across different α-containing isoforms. Lanthanum potentiated $\alpha1\beta3\epsilon$ currents with a lower affinity than it potentiated $\alpha1\beta3\gamma2L$ currents. Finally, the ϵ subunit conferred a greater sensitivity to the inhibitory effects of high concentrations of loreclezole. These data highlight the notion that the context of intersubunit interactions can significantly affect "subunit-specific" properties.

Biophysical Properties of GABARs Containing the ϵ Subunit. Spontaneous and evoked single-channel currents recorded from cells expressing the $\alpha1\beta3\epsilon$ isoform were not significantly different from each other but had properties that distinguished them from other isoforms. The single-channel conductance of $\alpha1\beta3\epsilon$ channels was similar to conductances of isoforms containing the $\gamma2L$ and δ subunits (Angelotti et al., 1993; Fisher et al., 1997) but were larger than $\alpha\beta$ single-channel conductances (Verdoorn et al., 1990; Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997). Unlike $\alpha\beta\gamma$ isoforms that had three open states and five closed states (Macdonald et al., 1989), the $\alpha1\beta3\epsilon$ isoform had only two open states. The second open state was similar to the O2 state recorded from native neurons in that it showed long bursts of multiple openings (Twyman et al., 1990) but was longer and more frequent than those seen with $\alpha1\beta3$ or $\alpha1\beta3\delta$ isoforms (Fisher and Macdonald, 1997). Interestingly, three open states, as seen in spinal cord, were observed in recombinant channels only when a γ subunit was present, raising the possibility that the O3 open state may be a γ subunit-dependent property.

The I-V relation of single-channel $\alpha1\beta3\epsilon$ currents was linear in contrast to the inward rectification of $\alpha1\beta3\epsilon$ whole-cell currents. Weiss et al. (1988) also reported that native single channels from cultured chick neurons did not rectify but had an increase in opening frequency at positive holding potentials. In-depth kinetic analysis was not performed at different holding potentials for the $\alpha1\beta3\epsilon$ isoform, but a reduction in opening frequency at positive potentials may underlie the rectification of whole-cell currents. Outward rectification of the whole-cell currents evoked from $\alpha\beta$ heterodimers (Draguhn et al., 1990; Davies et al., 1997) was abolished by inclusion of either a γ or δ subunit. Davies et al. (1997) reported that expression of the $\alpha2\beta1\epsilon$ isoform in human embryonic kidney 293 cells also resulted in a linear I-V relation. The inward rectification we report for the $\alpha1\beta3\epsilon$ isoform was unique among recombinant GABARs.

Summary. In this study, we established the existence of robust spontaneous channel activity and extend the pharmacological characterization of GABARs containing the ϵ subunit. This is the first example of a heterotrimeric GABAR that exhibits both spontaneous and GABA-gated channel openings. It is apparent that the ϵ subunit can influence the contributions of other subunits to GABAR pharmacology, yet the specific mechanisms remain obscure. Structure-function studies might provide insight into the relationship between ligand binding and gating and its partial uncoupling in this isoform. Although spontaneous chloride conductances present an attractive potential mechanism to modulate the basal excitability of neurons, further studies in vivo are necessary to establish the role of the ϵ subunit in native systems.

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Send reprint requests to: Robert L. Macdonald, M.D., Ph.D., Neuroscience Laboratory Building, 1103 East Huron St., Ann Arbor, MI 48104-1687. E-mail: rlmacd@umich.edu