0026-895X/97/040714-11$3.00/0

MOLECULAR PHARMACOLOGY, **52:**714–724 (1997).

The Role of α1 and α6 Subtype Amino-Terminal Domains in Allosteric Regulation of γ-Aminobutyric Acid$_a$ Receptors

JANET L. FISHER, JIE ZHANG, and ROBERT L. MACDONALD

Departments of Neurology (J.L.F., J.Z., R.L.M.) and Physiology (R.L.M.), University of Michigan Medical Center, Ann Arbor, Michigan 48104-1687

Received April 15, 1997; Accepted June 19, 1997

SUMMARY

The γ-aminobutyric acid$_A$ (GABA) receptor in the mammalian central nervous system is composed of pentameric combinations of α1–6, β1–4, γ1–3, δ1, and/or ϵ1 subunit subtypes. Although each of the different subunits influences the functional properties of γ-aminobutyric acid$_A$ receptors (GABARs), the α subunit subtypes have been shown to be important for activation of the receptor by GABA and pentobarbital and the regulation of GABARs by numerous allosteric regulators, including benzodiazepines, furosemide, zinc, and lanthanum. However, with the exception of the benzodiazepines, the α subtype domain that is responsible for the action of these allosteric compounds is unknown. The α1 and α6 subtypes are among the most diverse of the α subunit family and confer a different responsiveness of GABARs to GABA and many of the allosteric modulators. These regulatory compounds act after extracellular application and therefore likely act on extracellular GABAR sites, the largest of which is the amino-terminal extracellular domain. To determine the role of this domain in the action of these allosteric regulatory agents, we constructed chimeras of the rat α1 and α6 subtypes with a splice site within the first putative transmembrane domain (TM). This separated the large extracellular amino-terminal domain from the transmembrane, intracellular, and TM2–3 and carboxyl-terminal extracellular domains of the subunit. The chimeric subtypes were expressed in L929 fibroblasts along with β3 and γ2L subtypes, and their pharmacological properties were determined with whole-cell electrophysiological recording. The α subtype amino-terminal extracellular domain was the primary determinant of GABA sensitivity and was responsible for the functional properties of activation by pentobarbital, sensitivity to diazepam, potentiation by lanthanum, and high affinity inhibition by furosemide. The remaining carboxyl-terminal domains influenced the GABA sensitivity and determined zinc sensitivity and low affinity inhibition by furosemide. Both domains were apparently required for inhibition by lanthanum.

Fast inhibitory neurotransmission in the mammalian central nervous system is mediated principally through the GABAR. The GABAR is composed of a pentameric combination of α1–6, β1–4, γ1–3, δ1, and/or ϵ1 subunit subtypes that form an intrinsic chloride ion channel. GABAR subunits are thought to have a membrane topology similar to that of nicotinic acetylcholine receptor subunits, with a large extracellular amino-terminal domain, four TMs (TM1–4), a small extracellular domain between TM2 and TM3, and a large cytoplasmic domain between TM3 and TM4 (Fig. 1A). The activity of GABARs is modulated through a large number of allosteric regulatory sites. The properties of these allosteric sites are influenced by the subunit subtype composition of the GABAR (see review in Ref. 1). The identity of the α subtype affects the receptor responsiveness to GABA and pentobarbital and to many allosteric agents, including benzodiazepines, divalent and trivalent cations, and furosemide.

The α1 and α6 subtypes are among the most divergent of the α family subtypes in their functional properties, and they share the least structural homology of the family members (59% amino acid identity) (2, 3). The differences in the amino acid sequences between α1 and α6 subtypes occur primarily in the large extracellular amino-terminal domain and the intracellular loop between TM3 and TM4, whereas the TMs and the TM2–3 extracellular domain are well conserved (83% amino acid identity). Receptors containing the α6 subtype along with β and γ2 subtypes are more sensitive to GABA and to direct activation by pentobarbital than are receptors containing the α1 subtype. Receptors containing the α1 subtype along with β and γ2 subtypes are potentiated by benzodiazepine agonists and lanthanum but are relatively insensitive to inhibition by zinc and furosemide. In contrast, receptors containing the α6 subtype along with β and γ2

This work was supported by National Institutes of Health Grant RO1-NS33300 (R.L.M.) and National Institute on Drug Abuse Grant 5T32-DA07268 (J.L.F.)

ABBREVIATIONS: GABA, γ-aminobutyric acid; GABAR, γ-aminobutyric acid receptor; TM, transmembrane domain; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

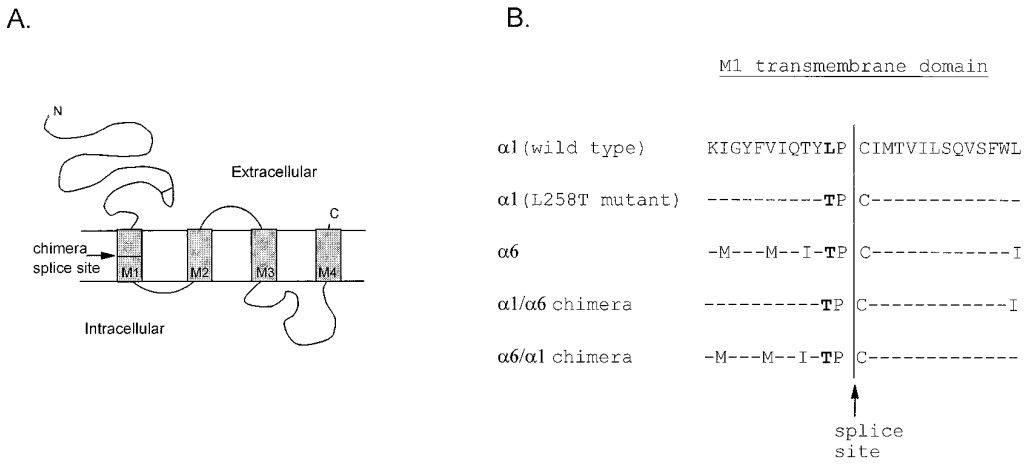

Fig. 1. Location of the chimera splice site. A, GABAR subunit. The structure of GABAR subunits consists of four TMs (TM1–4), a large amino-terminal extracellular domain, and a large intracellular domain between TM3 and TM4. *Arrow*, splice site for the chimeras was located within TM1 (not drawn to scale). B, Comparison of the amino acid sequence of TM1 of wild-type, mutant, and chimera subtypes. The full sequence of the rat α1 subtype is shown. Only differing residues and residues surrounding the splice site are shown for the other subtypes. *Dashes*, residues identical to the wild-type α1 subtype. *Bold*, leucine residue changed to a threonine residue in the mutant α1 subtype. *Solid line*, location of the splice site between the proline and cysteine residues.

subtypes are insensitive to benzodiazepines, more sensitive to inhibition by zinc, and strongly inhibited by lanthanum and furosemide (2, 4–7). The structural bases for the differences in allosteric regulation of the α1 and α6 subtypes are not known except for the benzodiazepines. Potentiation by benzodiazepine agonists requires a histidine residue in the large amino-terminal extracellular domain (H101 in rat α1); the benzodiazepine-insensitive α4 and α6 subtypes contain an arginine residue at the equivalent location (8). Other residues in this domain have also been shown to contribute to the functional properties of several benzodiazepine ligands (9). No studies on the zinc site have been reported for the GABAR, although a histidine residue located in the extracellular amino-terminal domain is required for zinc inhibition of the structurally related ρ1 subtype of GABA$_C$ receptors (10). Although structural domains important for direct activation by pentobarbital have not been localized, it has been shown that pentobarbital acts through different sites than does GABA (11). No information is available on the sites of action of lanthanum or furosemide on GABARs. All these agents act after application to the extracellular surface of the receptor and therefore likely bind to extracellular sites or the pore-forming TM. The amino-terminal domain is the largest extracellular domain, and the α1 and α6 subtypes share relatively low sequence homology in this region. Therefore, structural differences in the amino-terminal extracellular domain may be predominantly responsible for the differences in responsiveness of GABARs containing α1 and α6 subtypes to these regulatory agents.

To determine the role of the large extracellular amino-terminal domain in the functional properties of these GABAR regulatory sites, we created chimeric constructs of the rat α1 and α6 subtypes by interchanging their amino-terminal extracellular domains (Fig. 1). The chimeric subtypes contained the large extracellular domain and approximately one half of the TM1 of one of the α subtypes, with the remainder of the subunit structure from the other subtype, including TM2–4, the extracellular bridge between TM2 and TM3, and the large intracellular loop between TM3 and TM4. To generate the splice site, a restriction site for *Dra*III was created in the α1 subtype cDNA, converting a leucine residue to the threonine residue (L258T) present at this location in the α6 subtype (Fig. 1B). This mutation did not affect any of the functional properties of the α1 subtype examined in this study. Plasmids containing these constructs were transfected along with β3 and γ2L subtypes into L929 fibroblasts, and the responsiveness of the GABARs to GABA, pentobarbital, diazepam, furosemide, zinc, and lanthanum was measured with whole-cell recording. The structural differences between the α1 and α6 subtypes could cause functional differences in the response to these modulators through a large number of mechanisms, including changes in binding or transduction properties. Because we examined the functional responses to these agents, not their binding properties, we could not distinguish among these mechanisms.

Materials and Methods

Construction of mutant and chimeric α subtype cDNAs. Full-length cDNAs for the rat GABAR α1 (Dr. A. Tobin, University of California, Los Angeles) and α6 (F. Tan, University of Michigan) subtypes were subcloned into the pBluescript II KS$^{(-)}$ vector. To create a restriction site for *Dra*III in the α1 subtype, primers were created to make six nucleotide substitutions, converting the sequence TCTGCCATGCAT to CACTCCGTGCAC. This resulted in the change of a single amino acid, Leu258 to Thr258. A region between restriction sites for *Bsm*I and *Nsi*I containing the sequences to be modified was amplified using polymerase chain reaction techniques with a 5′ primer of 5′-GGATGAAAGATTAAAATTCAAAGGGACCCATGAC-3′ and a 3′ primer of 3′-GCCGATGAAACAATAAAGTTTGTATGTGAGGCACGTG-5′, which amplified the sequence from nucleotide 290 to nucleotide 783 of the open reading frame sequence of the α1 subtype. Oligonucleotide primers were synthesized at the University of Michigan DNA synthesis core facility. This region was spliced out of the wild-type sequence through digestion with *Bsm*I and *Nsi*I. The amplified fragment was digested with *Bsm*I and *Asp*HI, gel-purified, and ligated into the wild-type cDNA. Introduction of the mutation into the α1 sequence was verified with restriction mapping using *Nsi*I and *Dra*III enzymes. To form the chimeras, both the mutant α1 and wild-type α6 subtype cDNAs were digested with *Dra*III, releasing a portion of the vector and the amino-terminal region of the subtype to the restriction site within TM1. The result-

ing fragments were gel-purified, swapped, and re-ligated to the other subtype to form the chimeric sequences. The $\alpha1/\alpha6$ chimera and the $\alpha1_{(L258T)}$ subtype cDNAs were released from their vectors using *Hin*dIII and *Pst*I. The $\alpha6/\alpha1$ chimera was released using *Spe*I and *Xho*I. T4 DNA polymerase was used to fill in the ends. A *Bgl*II linker was then ligated to the ends, and the cDNAs were digested with *Bgl*II to allow ligation into the pCMVNeo vector using the *Bgl*II site. The sequence of the inserted PCR fragment was verified for all constructs with DNA sequencing.

Transfection of L929 cells. Full-length cDNAs for the rat GABAR $\alpha1$ (Dr. A. Tobin), $\beta3$ (Dr. D. Pritchett, University of Pennsylvania), and $\alpha6$ and $\gamma2L$ (F. Tan) subtypes were subcloned into the pCMVNeo expression vector (12) and transfected into the mouse fibroblast cell line L929 (American Type Culture Collection, Rockville, MD). Chimeric constructs and the $\alpha1_{(L258T)}$ mutant subtype were prepared as described above. 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, 150 mM NaCl, pH 7.3).

The cells were transfected using a modified calcium phosphate method (13, 14). Plasmids encoding GABAR subtype cDNAs were added to the cells in 1:1 ratios of 4 μg each plus 8 μg of the plasmid encoding sFv. After a 4–6-hr incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 sec. The selection procedure for sFv antibody expression was performed 20–28 hr later as described by Greenfield *et al.* (15). Briefly, the cells were passaged and mixed with 5 μl of magnetic beads coated with hapten ($\sim7.5 \times 10^5$ beads) (InVitrogen). After 30–60 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 18–28 hr later.

Electrophysiological recording solutions and techniques. For whole-cell 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 295–305 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 a chloride equilibrium potential near 0 mV. Patch pipettes were pulled from thick-walled borosilicate glass with an internal filament (World Precision Instruments, Pittsburgh, PA) on a P-87 Flaming Brown puller (Sutter Instrument Co., San Rafael, CA) and fire-polished to a resistance of 5–10 MΩ. Drugs were applied to cells using a "multipuffer" system with a 10–90% rise time of 70–150 msec (16). Currents were recorded with a List EPC-7 (List Electronics, Darmstadt, Germany) patch-clamp amplifier and stored on β videotape (Sony). All experiments were performed at room temperature.

Analysis of whole-cell currents. Whole-cell currents were analyzed off-line using the programs Axotape (Axon Instruments, Foster City CA) and Prism (GraphPAD Software, San Diego, CA). Normalized concentration-response data for the different isoforms were fit with a four-parameter logistic equation: current = maximum current/{1 + (10 (logEC_{50} − log[drug])*n)}, where n represents the Hill coefficient. All fits were made to normalized data with the current expressed as a percentage of the maximum current elicited by saturating GABA concentrations for each cell or, in the case of modulators, a percent of the response to GABA alone. Statistical tests were performed using the Instat program (GraphPAD). Comparisons of the receptor properties were performed with one-way analysis of variance and Tukey-Kramer multiple comparisons test with a statistical significance level of 0.05.

Results

Responsiveness to GABA. All α subtype constructs in combination with $\beta3$ and $\gamma2L$ subtypes produced GABA-sensitive currents in L929 fibroblasts (Fig. 2A). Cells were voltage-clamped at −50 mV, and whole-cell currents were recorded in response to varying concentrations of GABA. The amplitudes of the maximum currents evoked by GABA were similar for all GABAR isoforms, with average ± standard error values of 974.6 ± 154.7 pA ($\alpha1\beta3\gamma2L$, 13 cells), 1293.9 ± 235.9 pA ($\alpha1_{(L258T)}\beta3\gamma2L$, seven cells), 812.0 ± 179.0 pA ($\alpha6\beta3\gamma2L$, nine cells), 1123.6 ± 354.3 pA ($\alpha1/\alpha6\beta3\gamma2L$, nine cells), and 782.0 ± 226.0 pA ($\alpha6/\alpha1\beta3\gamma2L$, seven cells). The maximum currents were not significantly different among isoforms. The GABAR isoforms exhibited different GABA sensitivities (Fig. 2B). The $\alpha1\beta3\gamma2L$ isoform was ~7-fold less sensitive to GABA (average EC_{50} = 12.1 ± 2.5 μM, seven cells) than the $\alpha6\beta3\gamma2L$ isoform (average EC_{50} = 1.8 ± 0.2 μM, six cells). The $\alpha1_{(L258T)}\beta3\gamma2L$ receptor had the same sensitivity to GABA as the wild-type $\alpha1\beta3\gamma2L$ receptor, with an average GABA EC_{50} value of 12.1 ± 1.7 μM (four cells). Receptors containing the $\alpha1/\alpha6$ chimera were ~6-fold less sensitive to GABA than the $\alpha1\beta3\gamma2L$ receptor, with an average GABA EC_{50} value of 69.2 ± 6.4 μM (five cells). In contrast, receptors containing the $\alpha6/\alpha1$ chimera were ~5-fold more sensitive to GABA than the $\alpha6\beta3\gamma2L$ receptor, with an average EC_{50} value of 0.34 ± 0.055 μM (six cells). The logEC_{50} values for GABA were significantly different among all isoforms except for receptors containing the $\alpha1$(L58T) mutation or the wild-type $\alpha1$, which were not different from each other.

Responsiveness to pentobarbital. Pentobarbital can directly activate GABARs with an efficacy dependent on the subunit subtype composition (7). The $\alpha1$-containing receptors are less responsive to pentobarbital than are $\alpha6$-containing receptors. Pentobarbital is more effective as an agonist than GABA at $\alpha6\beta3\gamma2L$ receptors, producing larger maximum currents than GABA. The structural dependence of pentobarbital activation is different from that of GABA because mutations in the β subunit that prevent activation by GABA do not affect pentobarbital activity (11). In addition, activation by pentobarbital is not prevented by bicuculline, a competitive antagonist at the GABA site, although currents are blocked by the noncompetitive antagonist picrotoxin (7). The $\alpha6\beta3\gamma2L$ isoform was highly sensitive to pentobarbital, with an EC_{50} value of 44 μM and an average maximum current evoked by 300 μM pentobarbital that is 218.5 ± 58.7% (six cells) of that evoked by 600 μM GABA (Fig. 3). In contrast, receptors containing the $\alpha1$ and $\alpha1_{(L258T)}$ subunits were nearly equally activated by 300 μM pentobarbital or 600 μM GABA, with average currents in response to 300 μM pentobarbital that were 85.2 ± 7.9% (six cells, $\alpha1\beta3\gamma2L$) and 88.2 ± 8.0% (five cells, $\alpha1_{(L258T)}\beta3\gamma2L$) of the response to GABA. EC_{50} values for pentobarbital were 101 μM ($\alpha1\beta3\gamma2L$) and 126 μM ($\alpha1_{(L258T)}\beta3\gamma2L$). The amino-terminal extracellular domain seemed to be principally responsible for the differential effect of pentobarbital. The $\alpha1/\alpha6\beta3\gamma2L$ isoform was $\alpha1$-like in its response to pentobarbital, with 300 μM pentobarbital evoking currents 95.0 ± 3.3% (five cells) of the current response to 600 μM GABA with an EC_{50} value of 131 μM. The efficacy of pentobarbital was not significantly different among the $\alpha1$-, $\alpha1_{(L258T)}$- and $\alpha1/\alpha6$-containing receptors.

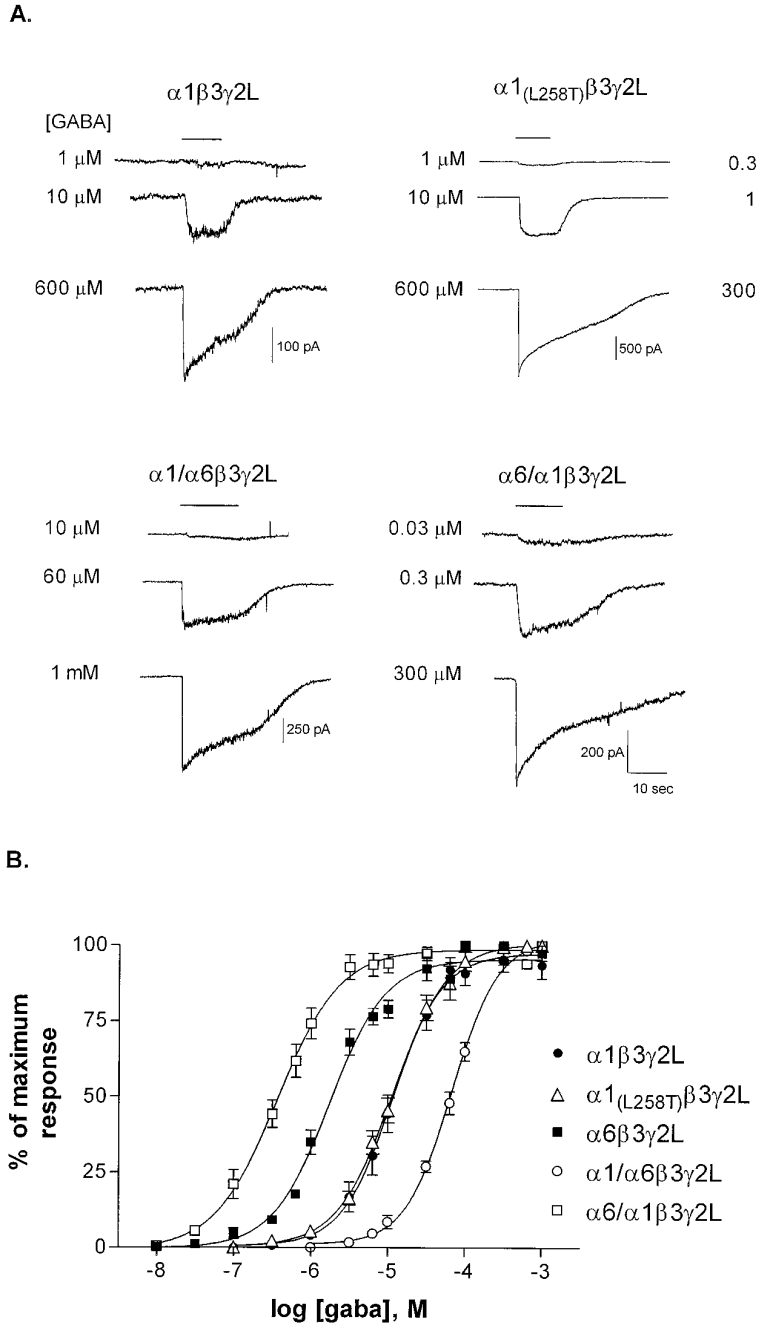

Fig. 2. Sensitivity to GABA, A, Representative whole-cell traces from transfected L929 fibroblasts. Fibroblasts were transfected with the subtypes indicated, and the peak current to varying concentrations of GABA was measured. The peak currents increased in a concentration-dependent manner for all isoforms. GABA was applied for 7–12 sec as indicated (*bar*) to cells voltage-clamped at −50 mV. The same time scale applies to all traces. B, Concentration-response relationships were constructed by normalizing the peak response to each concentration of GABA as a percentage of the maximum current response for each cell. Values are mean ± standard error. Data were fit with a four-parameter logistic equation. EC_{50} values and Hill slopes (*n*) of these fits were 0.36 μM, n = 1.1 for $\alpha6/\alpha1\beta3\gamma2L$ (six cells), 1.7 μM, n = 1.2 for $\alpha6\beta3\gamma2L$ (six cells), 11.5 μM, n = 1.3 for $\alpha1\beta3\gamma2L$ (seven cells), 11.3 μM, n = 1.3 for $\alpha1_{(L258T)}\beta3\gamma2L$ (four cells), and 67.9 μM, n = 1.4 for $\alpha1/\alpha6\beta3\gamma2L$ (five cells).

The $\alpha6/\alpha1\beta3\gamma2L$ isoform was α6-like, with 300 μM pentobarbital approximately twice as effective as 600 μM GABA (196.3 ± 15.0%, four cells) and an EC_{50} value of 35 μM. The efficacy of pentobarbital at the $\alpha6/\alpha1\beta3\gamma2L$ isoform was not significantly different from the response of the $\alpha6\beta3\gamma2L$ isoform.

Responsiveness to diazepam. A histidine residue in the amino-terminal extracellular domain of the α1 subtype (H101 in rat α1) is required for sensitivity to the benzodiazepine agonist diazepam (8). The α6 subtype contains an arginine residue in this location, which accounts for its insensitivity to benzodiazepine agonists. Consistent with these reports, the $\alpha1\beta3\gamma2L$, $\alpha1_{(L258T)}\beta3\gamma2L$, and $\alpha1/\alpha6\beta3\gamma2L$ receptor currents were all equally sensitive to potentiation by diazepam, with EC_{50} values of 38 nM ($\alpha1\beta3\gamma2L$, four cells), 38 nM ($\alpha1_{(L258T)}\beta3\gamma2L$, three cells), and 42 nM ($\alpha1/\alpha6\beta3\gamma2L$, three cells), whereas the $\alpha6\beta3\gamma2L$ (three cells) and $\alpha6/\alpha1\beta3\gamma2L$ (four cells) receptor isoform currents were insensitive to diazepam (Fig. 4). The logEC_{50} values for diazepam and the degree of current potentiation by 800 nM diazepam were not significantly different for the sensitive isoforms. The effects of 2 μM diazepam were not significantly different for the insensitive $\alpha6\beta3\gamma2L$ and $\alpha6/\alpha1\beta3\gamma2L$ isoforms.

Responsiveness to furosemide. Furosemide, a loop diuretic, is also a specific inhibitor of GABARs containing α4 or α6 subtypes (4, 17). Furosemide is much less effective at receptors containing an α1 subtype. $\alpha\beta3\gamma2L$ receptors containing wild-type α1 or the $\alpha1_{(L258T)}$ mutant showed a low sensitivity to furosemide, with average inhibition by 3 mM furosemide to 51 ± 3.6% ($\alpha1\beta3\gamma2L$, five cells), and 58 ± 4.3%

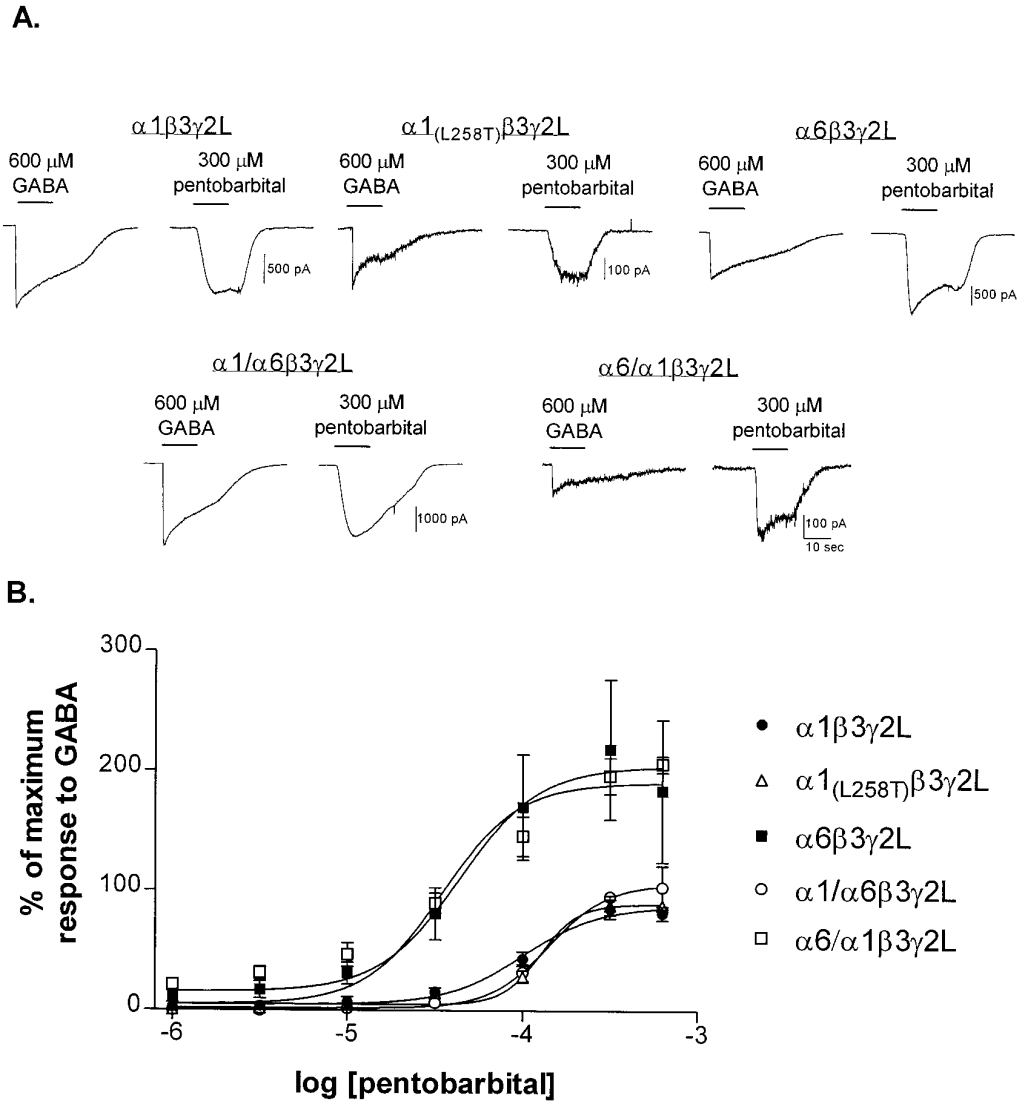

Fig. 3. Direct activation by pentobarbital. A, Representative whole-cell traces from transfected fibroblasts. The peak response to 300 μM pentobarbital alone was compared with the peak response to 600 μM GABA for fibroblasts transfected with the subtypes indicated. GABA or pentobarbital was applied for 10–15 sec as indicated (*bar*) to cells voltage-clamped at −50 mV. Traces shown are 50 sec in duration. The same time scale applies to all traces. B, Concentration-response relationships were constructed by expressing the peak current in response to pentobarbital as a percentage of the response to 600 μM GABA for each cell. *Symbols and bars*, mean ± standard error. Data were fit with a four-parameter logistic equation.

($\alpha1_{(L258T)}\beta3\gamma2L$, four cells) of the current evoked by GABA alone (Fig. 5). The degree of current inhibition by 3 mM furosemide of the mutant and wild-type α1 subtypes was not significantly different. Due to the poor solubility of furosemide in water, complete concentration-response relationships could not be determined for these isoforms. The α6β3γ2L current was highly sensitive to inhibition by furosemide, with an IC_{50} value of 27 μM and nearly complete inhibition of the current (six cells). The appearance of the currents during inhibition by furosemide also differed (Fig. 5A). For α6β3γ2L currents, higher concentrations of furosemide (≥30 μM) caused an increase in the apparent rate of desensitization, with the peak current declining rapidly. In contrast, for α1β3γ2L currents, furosemide caused a uniform decrease in the whole-cell current, with no apparent difference between peak and steady state current.

The α1/α6β3γL current had an intermediate sensitivity to inhibition by furosemide compared with the α1- and α6-subtype-containing receptors, with an IC_{50} value of 180 μM (five cells). The peak currents were less affected by furosemide than the steady state currents, causing an increase in the apparent desensitization of the current (Fig. 5). The α6/α1 chimera conferred high sensitivity to current inhibition by furosemide, with an EC_{50} value of 35 μM (five cells), similar to that for the α6β3γ2L isoform. The furosemide $logEC_{50}$ value for α1/α6β3γ2L currents was significantly different from that for α6β3γ2L and α6/α1β3γ2L currents, which were not significantly different from each other. The degree of inhibition by 3 mM furosemide of α1/α6β3γ2L current was significantly greater than that for both α1 and $\alpha1_{(L258T)}$ subtype-containing currents. Interestingly, the shape of the inhibited α6/α1β3γ2L currents was more like that of the inhibited α1β3γ2L currents, with no appearance of increased desensitization, even at concentrations of furosemide that produced nearly complete inhibition of the current (Fig. 5A). For almost all cells, high concentrations of furosemide seemed to slow the rate of return of the currents to base-line after removal of GABA.

Inhibition by zinc. The divalent cation zinc inhibits GABAR currents in a subunit subtype-dependent manner. Both αβ and αβδ currents are highly sensitive to zinc inhibition, with IC_{50} values of <5 μM (5, 18). The addition of a γ subunit decreases the sensitivity to zinc by ~10–100-fold. However, the α subtype also influences the zinc sensitivity; receptors

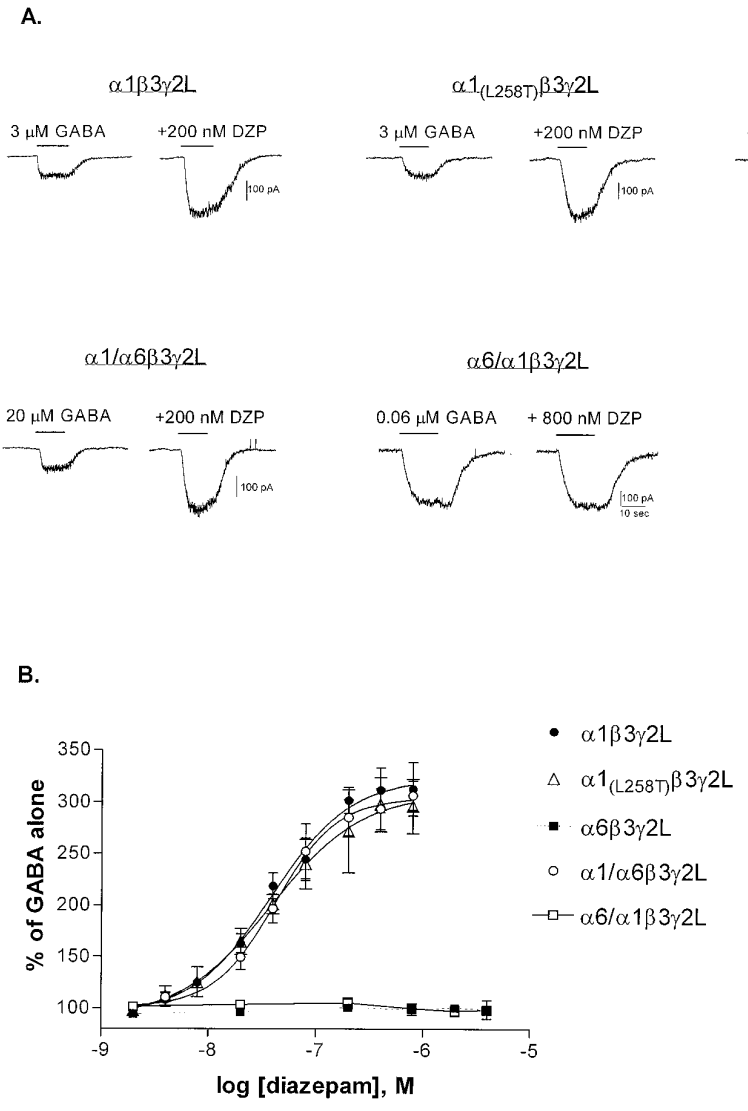

Fig. 4. Responsiveness to diazepam. A, Representative whole-cell traces from transfected L929 fibroblasts. Fibroblasts were transfected with the subtypes indicated, and the response to GABA or GABA plus diazepam was measured. The GABA concentration used was near the EC_{10} value for each isoform. GABA or GABA plus diazepam was applied for 7–12 sec as indicated (*bar*) to cells voltage-clamped at −50 mV. All traces shown are 50 sec in duration. The same time scale applies to all traces. B, Concentration-response relationships were constructed by expressing the peak current in response to diazepam as a percentage of peak current response to GABA alone for each cell. *Symbols and bars*, mean ± standard error. Data for the $\alpha1\beta3\gamma2L$, $\alpha1_{(L258T)}\beta3\gamma2L$, and $\alpha1/\alpha6\beta3\gamma2L$ isoforms were fit with a four-parameter logistic equation.

with $\alpha5$ and $\alpha6$ subtypes, even in combination with the $\gamma2L$ subtype, are more sensitive to current inhibition by zinc than are $\alpha1$ subtype-containing receptors (5, 19).

The $\alpha1\beta3\gamma2L$ and $\alpha1_{(L258T)}\beta3\gamma2L$ currents were inhibited by zinc with IC_{50} values of 111 μM (four cells) and 153 μM (five cells), respectively (Fig. 6). The $\alpha6\beta3\gamma2L$ isoform, however, was >4-fold more sensitive to inhibition by zinc, with an IC_{50} value of 27 μM (five cells). For all these isoforms, inhibition of the current was incomplete, with ~20% of the current remaining with 1 mM zinc.

The $\alpha1/\alpha6\beta3\gamma2L$ isoform was $\alpha6$ subtype-like in its sensitivity to zinc inhibition, with an IC_{50} value of 26 μM (four cells). However, the residual, unblocked current with 1 mM zinc was slightly less for the chimera, with ~13% of the current remaining (Fig. 6B). The $\alpha6/\alpha1\beta3\gamma2L$ isoform was $\alpha1$ subtype-like, with an IC_{50} value of 126 μM (five cells).

The $logEC_{50}$ values for zinc inhibition were not significantly different among the $\alpha1\beta3\gamma2L$, $\alpha1_{(L258T)}\beta3\gamma2L$, and $\alpha6/\alpha1\beta3\gamma2L$ isoforms. The $logEC_{50}$ values of the $\alpha6\beta3\gamma2L$ and $\alpha1/\alpha6\beta3\gamma2L$ isoforms were not different from one another, whereas both were significantly different from the less-sensitive isoforms. The degree of inhibition by 1 mM zinc was not significantly different among the isoforms.

Effect of lanthanum. The trivalent cation lanthanum affects GABAR currents in an α subunit subtype-dependent manner (6). $\alpha1\beta3\gamma2L$ currents are potentiated by lanthanum, whereas $\alpha6\beta3\gamma2L$ and $\alpha6\beta3\delta$ currents are inhibited. The currents from the $\alpha1\beta3\gamma2L$ and $\alpha1_{(L258T)}\beta3\gamma2L$ isoforms were potentiated by lanthanum ~2-fold, with EC_{50} values of 233 μM ($\alpha1\beta3\gamma2L$, four cells) and 263 μM ($\alpha1_{(L258T)}\beta3\gamma2L$, five cells) (Fig. 7). The $logEC_{50}$ value and degree of potentiation by lanthanum for these isoforms were not significantly different. The $\alpha6\beta3\gamma2L$ isoform was inhibited by lanthanum with an IC_{50} value of 193 μM (five cells) and a maximal inhibition to ~40% of the control current.

The $\alpha1/\alpha6\beta3\gamma2L$ currents were potentiated by lanthanum, with an EC_{50} value (190 μM, five cells) and maximum potentiation (209%) similar to those of the $\alpha1$ subtype-containing receptors. The $logEC_{50}$ value and degree of potentiation by lanthanum were not significantly different from that of the $\alpha1$ and $\alpha1_{(L258T)}$ subtype-containing receptors. The $\alpha6/\alpha1\beta3\gamma2L$ isoform showed an intermediate response to lanthanum, with very weak potentiation of the current by lanthanum (122.3 ± 8.4% of control currents by 1 mM lanthanum, four cells). The effect of 1 mM lanthanum on the $\alpha6/$

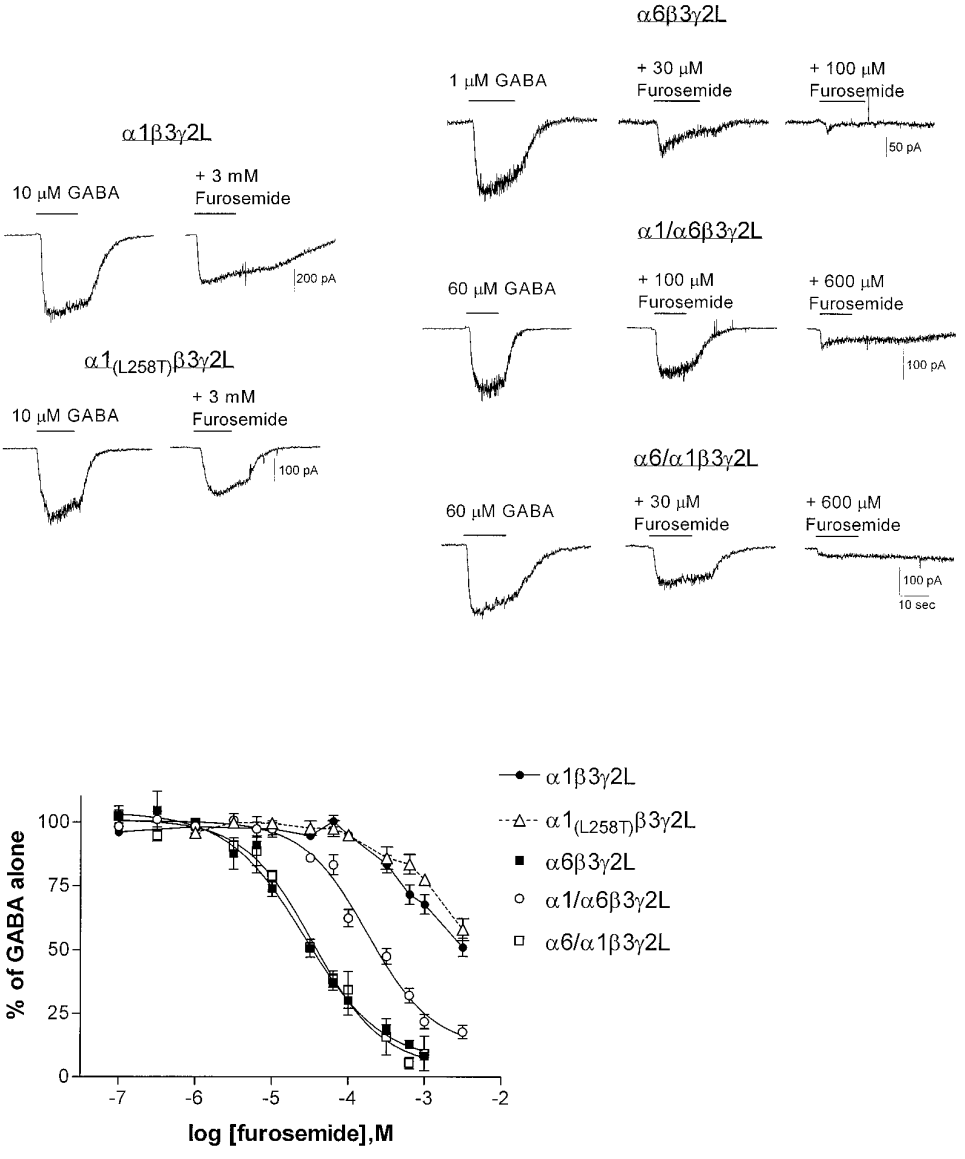

Fig. 5. Inhibition by furosemide. A, Representative whole-cell traces from transfected L929 fibroblasts. Fibroblasts were transfected with the subtypes indicated and the peak current response to GABA or GABA plus furosemide was measured. The GABA concentration used was near the EC_{50} value for each isoform. GABA or GABA plus furosemide was applied for 7–12 sec as indicated (*bar*) to cells voltage-clamped at −50 mV. All traces shown are 50 sec in duration. The same time scale applies to all traces. B, Concentration-response relationships were constructed by expressing the inhibition of the peak current by furosemide as a percentage of response to GABA alone for each cell. Symbols and bars, mean ± standard error. Data for the α6β3γ2L, α1/α6β3γ2L, and α6/α1β3γ2L isoforms were fit with a four-parameter logistic equation.

α1β3γ2L isoform was significantly different from that on all the other isoforms.

Discussion

To examine the contribution of the amino-terminal extracellular domain to the differential regulation of the α1 and α6 subtypes by several allosteric agents, we created chimeric subunits containing the entire amino-terminal extracellular domain and part of the first TM of one subtype spliced with the remaining subunit structure of the other subtype. This separated the largest extracellular portion of the subunit from the TMs. The carboxyl-terminal domains of the chimeras also included all the intracellular regions of the subtype in addition to a short extracellular sequence between TM2 and TM3. To form the chimeras, mutations were made in the α1 subtype sequence to create a restriction site; this resulted in the conversion of Leu258 to a threonine residue. This mutation had no effect on any of the pharmacological properties of the receptor examined in this study. The chimeric constructs efficiently expressed functional GABARs when cotransfected with β3 and γ2L subtypes in L929 fibroblasts; this indicates that the chimeric α subtypes assembled with β3 and γ2L subtypes to form functional GABARs. We examined the responses to GABA, pentobarbital, diazepam, furosemide, zinc, and lanthanum to determine whether the amino-terminal domain and/or the remaining carboxyl-terminal domains were involved in the actions of these agents (Table 1). Structural differences in the subtypes could cause changes in the effect or effectiveness of the allosteric modulators through many different mechanisms. The structures could form part of the binding site or the signal transduction pathways or influence either of these through remote effects on secondary, tertiary, or quaternary structures of the receptor. We can conclude from these results only whether these structural domains contribute to the differences between the properties of the α1 and α6 subtypes. It is likely that other regions of the subtypes also contribute to the binding or transduction sites but are not responsible for the functional differences among the subtypes.

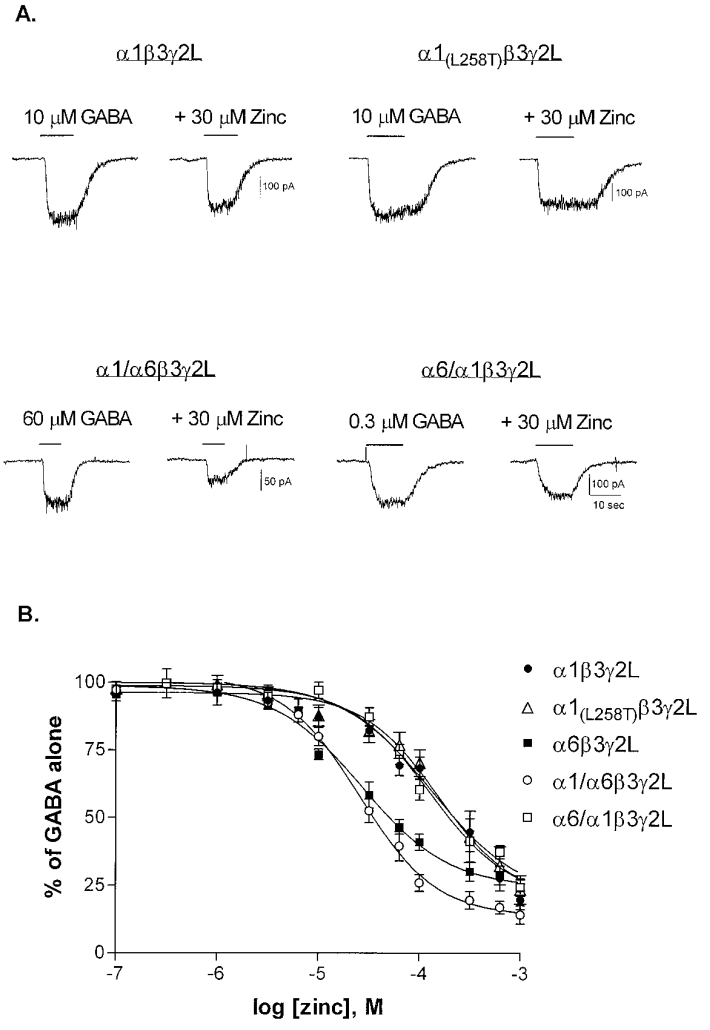

Fig. 6. Inhibition by zinc. A, Representative whole-cell traces from transfected L929 fibroblasts. Fibroblasts were transfected with the subtypes indicated and the peak current response to GABA or GABA plus zinc was measured. The GABA concentration used was near the EC_{50} value for each isoform. GABA or GABA plus zinc was applied for 7–12 sec as indicated (*bar*) to cells voltage-clamped at −50 mV. All traces shown are 40 sec in duration. The same time scale applies to all traces. B, Concentration-response relationships were constructed by expressing the inhibition of the peak current by zinc as a percentage of response to GABA alone for each cell. *Symbols and bars*, mean ± standard error. Data were fit with a four-parameter logistic equation.

GABA sites. Several amino acid residues in the large extracellular domain of many of the GABAR subunit families have been implicated in GABA binding (9, 20). Therefore, it is not surprising that the primary determinant of the GABA EC_{50} value for channel activity resided in the amino-terminal domain of the chimeric subtypes. The $\alpha1/\alpha6\beta3\gamma2L$ isoform was closer in EC_{50} value to the $\alpha1\beta3\gamma2L$ isoform than to the $\alpha6\beta3\gamma2L$ isoform, and the $\alpha6/\alpha1\beta3\gamma2L$ isoform was closer in EC_{50} value to the $\alpha6\beta3\gamma2L$ isoform than to the $\alpha1\beta3\gamma2L$ isoform. Interestingly, the EC_{50} values for the chimeras were not equal or intermediate to the wild-type subtypes. Instead, the $\alpha6/\alpha1$ chimera produced higher affinity for GABA than did the $\alpha6$ subtype, whereas the $\alpha1/\alpha6$ chimera conferred lower affinity than did the $\alpha1$ subtype. This suggests that the amino-terminal domain alone does not determine the GABA EC_{50} value. The remaining carboxyl-terminal domains of the $\alpha1$ and/or $\alpha6$ subtypes must also contribute to the ability of GABA to bind and/or activate the GABAR channel. The degree of shift in EC_{50} value was nearly identical for both chimeric constructs, although they occurred in opposite directions. Therefore, it may be that the TM2–3 or carboxyl-terminal extracellular regions of the $\alpha1$ subtype contribute to an increased sensitivity to GABA and/or that those of the $\alpha6$ subtype reduce the sensitivity to GABA.

Direct activation by pentobarbital. Pentobarbital acts as an agonist at GABARs in addition to its action as a positive allosteric modulator of GABAR activity (21, 22). Although the allosteric action is apparently independent of the subunit subtype composition of the receptor, direct activation is α subtype dependent (7, 17). At $\alpha6$-containing receptors, pentobarbital is more efficacious than GABA, producing larger maximal currents. With $\alpha1$-containing receptors, pentobarbital is as or slightly less efficacious than GABA, with a higher EC_{50} value for activation than $\alpha6$-containing receptors. Our results suggest that structural differences in the extracellular amino-terminal domain are responsible for the differences in pentobarbital activity. The $\alpha1/\alpha6$ chimera had a response similar to the $\alpha1$ subtype, whereas the $\alpha6/\alpha1$ chimera was $\alpha6$-like in both EC_{50} value and efficacy compared with GABA. It has been shown that structures that alter GABA binding do not affect activation by pentobarbital (11), but the functional properties of both of these agonists seem to be primarily determined by the same general domain, the extracellular amino terminus.

Diazepam site. Our results are consistent with the finding that H101 (in the rat $\alpha1$ subtype), which is located in the amino-terminal extracellular domain, is required for diazepam sensitivity (8). The $\alpha1/\alpha6$ chimera conferred complete sensitivity to diazepam with an EC_{50} value and maximum potentiation indistinguishable from those of the wild-type $\alpha1$ subtype. The $\alpha6/\alpha1$ chimera was completely insensitive to diazepam. This suggests that the amino-terminal extracellu-

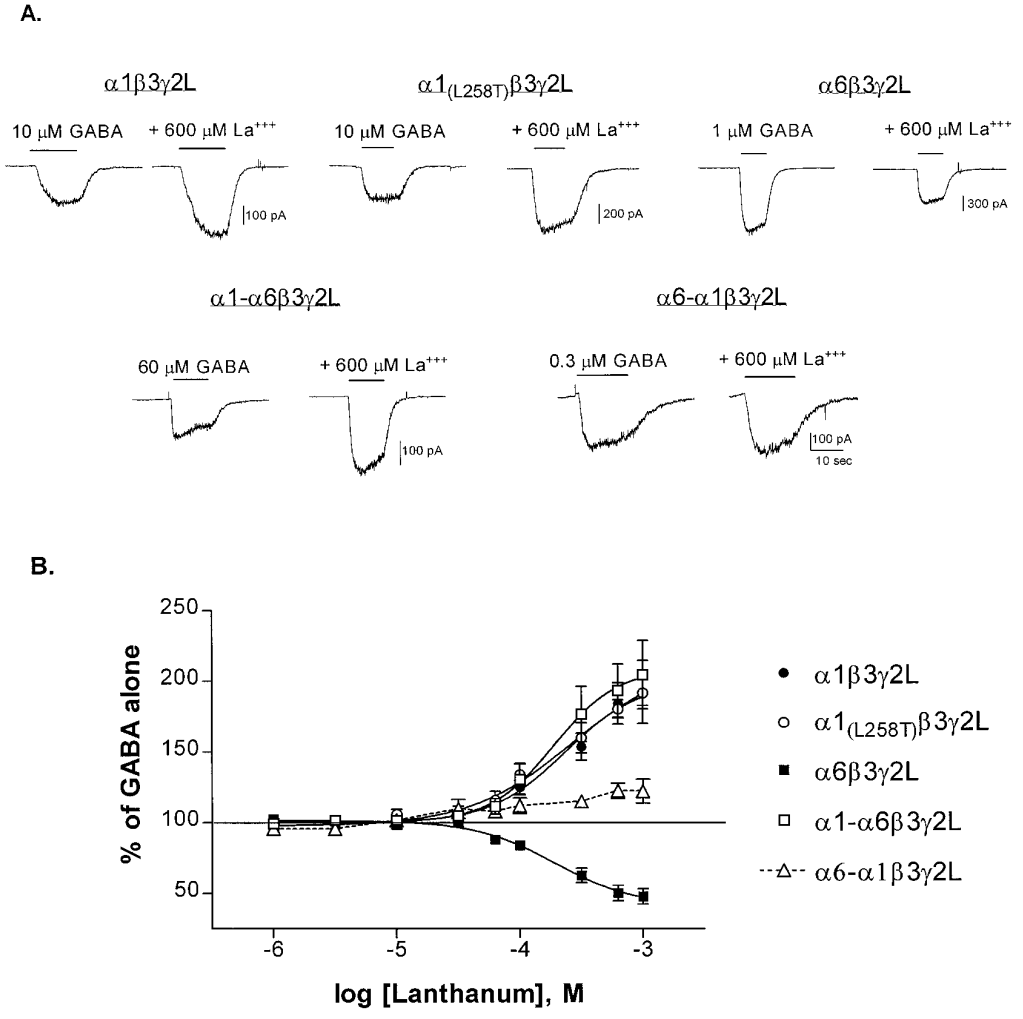

Fig. 7. Effect of lanthanum. A, Representative whole-cell traces from transfected L929 fibroblasts. Fibroblasts were transfected with the subtypes indicated, and the peak current response to GABA or GABA plus lanthanum was measured. The GABA concentration used was near the EC_{50} value for each isoform. GABA or GABA plus lanthanum was applied for 7–12 sec as indicated (*bar*) to cells voltage-clamped at −50 mV. All traces shown are 60 sec in duration. The same time scale applies to all traces. B, Concentration-response relationships were constructed by expressing the peak current in response to GABA plus lanthanum as a percentage of response to GABA alone for each cell. *Symbols and bars*, mean ± standard error. Data for all isoforms except the α6/α1β3γ2L isoform were fit with a four-parameter logistic equation.

TABLE 1
Summary of pharmacological properties

	α1	α1/α6	α6	α6/α1
GABA (EC_{50})	12 μM	68 μM	1.7 μM	0.4 μM
Pentobarbital (EC_{50} for direct activation)	101 μM	131 μM	44 μM	35 μM
Diazepam (EC_{50})	38 nM	42 nM	Insensitive	Insensitive
Furosemide (IC_{50})	≥3 mM	180 μM	27 μM	35 μM
Zinc (IC_{50})	111 μM	26 μM	27 μM	126 μM
Lanthanum (EC_{50} or IC_{50})	233 μM (100% enhancement)	190 μM (100% enhancement)	193 μM (60% inhibition)	Slight enhancement (22% by 1 mM)

lar domain is principally responsible for the contribution of the α1 subtype to the functional domains for diazepam and that the remaining carboxyl-terminal domains do not contribute to the difference in diazepam sensitivity between the α1 and α6 subtypes.

Furosemide sites. Furosemide inhibits α6 subtype-containing receptors with high affinity, whereas α1 subtype-containing receptors are almost 100-fold less sensitive. A high affinity site seemed to require the amino-terminal extracellular domain of the α6 subtype because the α6/α1 chimera conferred the same sensitivity to furosemide as the wild-type α6 subtype. However, receptors containing the α1/α6 chimera were also somewhat sensitive to furosemide, with an intermediate IC_{50} value. This suggests that a second, lower affinity inhibitory site for furosemide was associated with the carboxyl-terminal domains of the α6 subtype. The appearance of the inhibited currents was also different for the two chimeras. The high affinity inhibition seen with the α6/α1 chimera was uniform, with equal block of early and late currents. The lower affinity inhibition of the α1/α6 chimera increased through the time of drug application, causing an increase in the degree of apparent desensitization. This is a common characteristic of open-channel blockers, which would be consistent with the involvement of the TMs in the formation of this inhibitory site. Although receptors containing the α6 subtype also showed this enhanced apparent desensitization, it appeared at lower concentrations of furosemide than required for receptors containing the α1/α6 chimera. This could result from a positive interaction between the two proposed furosemide sites, resulting in an

increased sensitivity at the blocking site when the high affinity site is bound. Interestingly, the α4 subtype, which shares many functional characteristics with the α6 subtype, is less sensitive to furosemide than the α6 subtype, with an IC_{50} value of 162 μM when coexpressed with β3 and γ2S subtypes in *Xenopus laevis* oocytes (17). This is similar to results seen with the α1/α6 chimera (IC_{50} = 180 μM) and suggests that the α4 subtype may contain the lower affinity site for furosemide but not the higher affinity site associated with the extracellular amino-terminal domain.

Zinc site. The α6 subtype confers a greater sensitivity to zinc inhibition than the α1 subtype. This characteristic was associated with the carboxyl-terminal portion of the chimeras. Receptors containing the α1/α6 chimera had α6 subtype-like affinity for zinc, whereas receptors containing the α6/α1 chimera had α1 subtype-like affinity. This is in contrast to the findings with the ρ1 subtype of the $GABA_C$ receptor in which a histidine in the amino-terminal extracellular domain was responsible for zinc sensitivity (10). The inhibition of GABAR currents by zinc is noncompetitive and voltage independent. The primary effect of zinc on single-channel kinetics is to reduce the frequency of channel opening rather than to reduce channel open time or conductance (23, 24). This is consistent with zinc acting through an extracellular allosteric regulatory site rather than as a channel blocker. It has been suggested that the TM2–3 extracellular domain may be important in the regulation of zinc binding by the γ subunit (24). The α6 subtype contains a histidine residue (H292) in this region, whereas the α1 subtype contains an asparagine residue (N301) in the equivalent location. Because histidine residues frequently contribute to zinc binding sites, this residue may play an important role in the zinc sensitivity of receptors containing the α6 subtype.

Lanthanum sites. Lanthanum affects the activity of α1 and α6 subtype-containing receptors in opposite directions, enhancing the activity of the α1β3γ2L isoform while inhibiting the activity of the α6β3γ2L isoform. The positive modulatory site seemed to be associated with the amino-terminal extracellular domain of the α1 subtype, with the α1/α6 chimera conferring the same sensitivity and degree of enhancement as the α1 subtype. However, the α6/α1β3γ2L isoform showed an intermediate sensitivity, with only a slight enhancement by lanthanum. Neither receptor containing a chimera exhibited the inhibition seen with receptors containing the α6 subtype. The simplest explanation for this result is that structural domains from both regions of the α6 subtype are required for the formation of the inhibitory site.

Structure-function relationships of GABAR subunits. The structure of GABARs is complex, with five different subunits contributing to the functional properties of the receptor. We examined the structural domains responsible for contribution of the α1 and α6 subtypes to several allosteric regulatory sites of the GABAR. Whether the same or homologous structures form these sites for the other α subtypes is not known. In addition, the α subunit is clearly not solely responsible for the functional properties of these sites. The identity of the β subtype also influences the response of the receptor to GABA, pentobarbital, and furosemide (4, 7, 19, 25), and the γ subunit contributes to the functional properties of the GABA, diazepam, zinc, and lanthanum sites (5, 6, 18, 26, 27). For the benzodiazepine site, a threonine residue (T142 in human γ2) in the amino-terminal extracellular region of the γ2 subtype has been found to be important for sensitivity (28). This is in the same general domain as the histidine residue that confers benzodiazepine sensitivity in the α1 subtype. The regions of the β, γ, and δ subunits that contribute to the functional properties of the other allosteric modulators are not necessarily structurally homologous to those for the α subunit. To completely understand the structural properties of the regulatory sites of the GABAR, the contributions that the other subunits make to these sites must also be examined.

References

1. Sieghart, W. Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmacol. Rev.* **47:**181–234 (1995).
2. Lüddens, H., D. B. Pritchett, C. Köhler, I. Killisch, K. Keinänen, H. Monyer, R. Sprengel, and P. H. Seeburg. Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature (Lond.)* **346:**648–651 (1990).
3. Tyndale, R. F., R. W. Olsen, and A. J. Tobin. $GABA_A$ receptors, in *Ligand- and Voltage-Gated Ion Channels* (R. A. North, ed.). CRC Press, Boca Raton, 265–290 (1995).
4. Korpi, E. R., T. Kuner, P. H. Seeburg, and H. Lüddens. Selective antagonist for the cerebellar granule cell-specific γ-aminobutyric acid type A receptor. *Mol. Pharmacol.* **47:**283–289 (1997).
5. Saxena, N. C., and R. L. Macdonald. Properties of putative cerebellar γ-aminobutyric $acid_A$ receptor isoforms. *Mol. Pharmacol.* **49:**567–579 (1996).
6. Saxena, N. C., T. R. Neelands, and R. L. Macdonald. Contrasting actions of lanthanum on different recombinant γ-aminobutyric acid receptor isoforms expressed in L929 fibroblasts. *Mol. Pharmacol.* **51:**328–335 (1997).
7. Thompson, S. A., P. J. Whiting, and K. A. Wafford. Barbiturate interactions at the human $GABA_A$ receptor: dependence on receptor subunit combination. *Br. J. Pharmacol.* **117:**521–527 (1996).
8. Wieland, H. A., H. Lüddens, and P. H. Seeburg. A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.* **267:**1426–1429 (1992).
9. Im, W. B., J. F. Pregenzer, J. A. Binder, G. L. Alberts, and H. K. Im. Alterations of the benzodiazepine site of rat $\alpha6\beta2\gamma2$-$GABA_A$ receptor by replacement of several divergent amino-terminal regions with the α1 counterparts. *Br. J. Pharmacol.* **120:**559–564 (1997).
10. Wang, T. L., A. Hackam, W. B. Guggino, and G. R. Cutting. A single histidine residue is essential for zinc inhibition of GABA ρ1 receptors. *J. Neurosci.* **15:**7684–7691 (1995).
11. Amin, J., and D. S. Weiss. $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature (Lond.)* **366:**565–569 (1993).
12. Huggenvik, J. I., M. W. Collard, R. E. Stofko, A. F. Seasholtz, and M. D. Uhler. Regulation of the human enkephalin promoter by two isoforms of the catalytic subunit of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase. *Mol. Endocrinol.* **5:**921–930 (1991).
13. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:**2745–2752 (1987).
14. Angelotti, T. P., M. D. Uhler, and R. L. Macdonald. Assembly of $GABA_A$ receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J. Neurosci.* **13:**1418–1428 (1993).
15. Greenfield, L. J., Jr., F. Sun, T. R. Neelands, E. C. Burgard, J. L. Donnelly, and R. L. Macdonald. Expression of functional $GABA_A$ receptors in transfected L929 cells isolated by immunomagnetic bead separation. *Neuropharmacology* **36:**63–73 (1997).
16. Greenfield, L. J., Jr., and R. L. Macdonald. Whole cell and single channel $\alpha1\beta1\gamma2s$ $GABA_A$ receptor currents elicited by a 'multipuffer' drug application device. *Pflueg. Arch. Eur. J. Physiol.* **432:**1080–1090 (1996).
17. Wafford, K. A., S. A. Thompson, D. Thomas, J. Sikela, A. S. Wilcox, and P. J. Whiting. Functional characterization of human γ-aminobutyric $acid_A$ receptors containing the α4 subunit. *Mol. Pharmacol.* **50:**670–678 (1996).
18. Draguhn, A., T. A. Verdoorn, M. Ewert, P. H. Seeburg, and B. Sakmann. Functional and molecular distinction between recombinant rat $GABA_A$ receptor subtypes by Zn^{2+}. *Neuron* **5:**781–788 (1990).
19. Burgard, E. C., E. I. Tietz, T. R. Neelands, and R. L. Macdonald. Properties of recombinant γ-aminobutyric $acid_A$ receptor isoforms containing the α5 subunit subtype. *Mol. Pharmacol.* **50:**119–127 (1996).
20. Smith, G. B., and R. W. Olsen. Functional domains of $GABA_A$ receptors. *Trends Pharmacol. Sci.* **16:**162–168 (1995).
21. Macdonald, R. L., and J. L. Barker. Different action of anticonvulsant and anesthetic barbiturates revealed by use of cultured mammalian neurons. *Science (Washington D. C.)* **200:**775–777 (1978).
22. Nicoll, R. A., and J. M. Wojtowicz. The effects of pentobarbital and related compounds on frog motoneurons. *Brain Res.* **191:**225–237 (1980)
23. Legendre, P., and G. L. Westbrook. Noncompetitive inhibition of γ-aminobutyric acid channels by Zinc. *Mol. Pharmacol.* **39:**267–274 (1990).

24. Smart, T. G. A novel modulatory binding site for zinc on the $GABA_A$ receptor complex in cultured rat neurones. *J. Physiol. (Lond.)* **447:**587–625 (1992).
25. Hadingham, K. L., P. B. Wingrove, K. A. Wafford, C. Bain, J. A. Kemp, K. J. Palmer, A. W. Wilson, A. S. Wilcox, J. M. Sikela, C. I. Ragan, and P. J. Whiting. Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **44:**1211–1218 (1993).
26. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor subunit for benzodiazapine pharmacology. *Nature (Lond.)* **338:**582–585 (1989).
27. Im, M. S., B. J. Hamilton, D. B. Carter, and W. B. Im. Selective potentiation of GABA-mediated Cl^- current by lanthanum ion in subtypes of cloned $GABA_A$ receptors. *Neurosci. Lett.* **144:**165–168 (1992).
28. Mihic, S. J., P. J. Whiting, R. L. Klein, K. A. Wafford, and R. A. Harris. A single amino acid of the human γ-aminobutyric acid type A receptor γ_2 subunit determines benzodiazepine efficacy. *J. Biol. Chem.* **269:**32768–32773 (1994).

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