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Pharmacology of the Human γ-Aminobutyric Acid$_A$ Receptor α4 Subunit Expressed in *Xenopus laevis* Oocytes

E. R. WHITTEMORE, W. YANG, J. A. DREWE, and R. M. WOODWARD

CoCensys, Inc., Irvine, California 92618

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SUMMARY

The human γ-aminobutyric acid$_A$ (GABA$_A$) receptor α4 subunit was recently cloned and characterized pharmacologically using radioligand binding techniques. These studies suggested that α4 subunits confer a novel diazepam-insensitive binding site. To further investigate the pharmacology of the α4 subunit, we expressed human α4β2γ2L subunit combinations in oocytes and compared the expression and pharmacology of these receptors with α1β2γ2L, β2γ2L, and other possible binary subunit combinations. Apparent GABA affinity was 2–3-fold higher for α4β2γ2L than for α1β2γ2L receptors. Functional modulation of receptors by benzodiazepine-site ligands and other classes of allosteric modulator were assayed over a broad concentration range (0.01–100 μM) on currents that were 10% of the maximum GABA response. Diazepam (0.01–1 μM) did not modulate GABA responses at α4β2γ2L receptors, whereas it increased α1β2γ2L responses by ~110%. Bretazenil (0.01–1 μM), a benzodiazepine partial agonist, induced higher efficacy modulation of α4β2γ2L receptors (~83%) than of α1β2γ2L (~25%). The benzodiazepine antagonist flumazenil (0.1–10 μM) unexpectedly potentiated α4β2γ2L responses up to ~41%, and the benzodiazepine partial inverse agonist Ro15-4513 (1 μM) potentiated α4β2γ2L responses by ~63%. Two other benzodiazepine-site ligands, CGS-9895 and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, had qualitatively similar effects at α1β2γ2L and α4β2γ2L. Modulators such as pentobarbital, 3α-hydroxy-5α-pregnan-20-one, mefenamic acid, and loreclezole also induced similar potentiation at both subtypes of receptor. The pharmacology conferred by the α4 subunit was similar to that conferred by the α6 subunit, to which it shows highest levels of homology, but the two subunits differ in sensitivity to the β-carboline methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate. Properties of the α4-containing receptors are consistent with diazepam-insensitive binding sites found in cerebral cortex and other forebrain structures. Characterization of these receptors should further our understanding of mechanisms underlying the behavioral effects of GABA modulators and help in the design of drugs with improved, or novel, therapeutic profiles.

Mammalian GABA$_A$ receptors are composed of structurally related polypeptide subunits assembled to form a ligand-gated chloride channel (1). Molecular cloning studies have identified 16 GABA$_A$ receptor subunits, which have been divided into five families; subunits are designated α1-6, β1-4, γ1-3, δ, and ρ1-2 (1, 2). In addition, γ subunits are found in two isoforms, designated long and short (L and S), that are generated by alternative RNA splicing (3). Native neuronal GABA$_A$ receptors are thought to be pentameric assemblies composed of three or four different types of subunit (1, 4). The subunit diversity results in a very large number of potential subunit combinations and thus in a large number of possible GABA$_A$ receptor subtypes. At present, however, the precise subunit composition and stoichiometry of the majority of native receptors remain uncertain (5). Studies on cloned GABA$_A$ receptors expressed in *Xenopus laevis* oocytes or mammalian cells have shown that different subunit combinations generate receptors that are biophysically and pharmacologically distinct (6–13). The distinctions include differences in single-channel properties and desensitization kinetics (6), differences in GABA affinity (7–9), and differences in sensitivity to allosteric modulators (10–13). Furthermore, the various GABA$_A$ receptor subunits show distinct regional and developmental distributions in the central nervous system (14), suggesting that different receptor subtypes may play different roles in brain function.

Channel gating at GABA$_A$ receptors is allosterically modulated by a wide variety of compounds, including benzodiazepines and related molecules (1, 4, 15), barbiturates (16), neuroactive steroids (17–19), and other agents (12, 20, 21). GABA$_A$ receptor modulators constitute a group of clinically significant drugs, members of which are in widespread use as anxiolytics, anticonvulsants, muscle relaxants, sedative/hypnotics, and anesthetics (22, 23). The degree to which subtype-selective modulation plays a role in side effect and therapeutic profiles of individual modulators is uncertain. To begin to address this, it is first necessary to undertake detailed pharmacological characterization of the major putative GABA$_A$

ABBREVIATIONS: GABA, γ-aminobutyric acid; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; DMSO, dimethylsulfoxide; 3α,5α-P, 3α-hydroxy-5α-pregnan-20-one; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

receptor subtypes, to identify patterns of selectivity for the modulators, and then to try to correlate this profile with behavioral and clinical studies.

Heterogeneity in $GABA_A$ receptor pharmacology was first detected in binding assays using radiolabeled benzodiazepines (10, 11, 13). Receptor subtypes were therefore classified according to differences in benzodiazepine-site pharmacology. This scheme effectively divided the receptor population into diazepam-sensitive sites (types I and II) (10, 11, 13) and diazepam-insensitive sites (24, 25). More recently, cloning and molecular pharmacological studies have pinpointed these classes to specific $GABA_A$ receptor subunits. For example, the distinction between diazepam-sensitive types I and II is dependent on the α subunit; receptors containing α1 subunits correspond to type I, whereas α2-, α3-, and α5-containing receptors correspond to type II (11, 26, 27). Diazepam-insensitive sites are generated by $GABA_A$ receptors containing α6 subunits in the cerebellum (28) and (as detailed below) α4 subunits in the forebrain (29–31).

The cDNA encoding the $GABA_A$ receptor α4 subunit was first cloned from bovine and then from rat cDNA libraries (29, 30). Characterization of receptors composed of rat α4β2γ2L subunits indicated that α4 conferred an atypical profile of sensitivity to benzodiazepines. Specifically, the α4β2γ2L receptors showed high affinity binding of the partial inverse agonist Ro15-4513, moderate affinity binding of the antagonist flumazenil, and insensitivity to the full agonists diazepam and flunitrazepam and the type I-selective compound CL218,872 (30). Cloning of the human α4 cDNA was reported only recently (31). Pharmacological characterization of human α4β2γ2L receptors using radioligand binding assays revealed a similar benzodiazepine profile to that seen in rat. In addition, these studies showed that α4β2γ2L receptors had distinctly higher affinity for some β-carbolines than did α6β2γ2L receptors, thereby distinguishing two distinct classes of diazepam-insensitive sites (31). The pharmacology of α4β2γ2L receptors resembles that of diazepam-insensitive sites found in rat forebrain (32), suggesting that this subtype of neuronal receptors is due to α4-containing subunit combinations (31).

Characterization of cloned α4-containing $GABA_A$ receptors has been accomplished mainly through radioligand binding assays (29–31). With this approach, it is difficult to predict the functional consequences of ligand binding (i.e., to determine whether the modulator is an agonist, antagonist, or inverse agonist). To address this issue, we used electrical recordings to study the pharmacology of functional human α4-containing $GABA_A$ receptors expressed in *X. laevis* oocytes. For comparison, we also assayed receptors composed of α1β2γ2L subunits, a typical diazepam-sensitive subunit combination (6, 8), and, as a control, receptors composed of the binary subunit combination β2γ2L. Portions of this work have been presented in abstract form (33).

Materials and Methods

Preparation of RNA. cDNA clones were isolated from the following sources (31). α4 was isolated from normal human cerebral cortex provided by the Alzheimer's Disease Research Center at University of Southern California. "Human β2" was engineered from rat β2. γ2L was generously provided by Dr. Peter Seeburg (University of Heidelberg). cRNA was transcribed from *Hpa*I-linearized cDNA using methylated cap analog and T7 RNA polymerase, as described in the mMessage mMachine protocol (Ambion, Austin, TX). In brief, 20 μg of the cDNA constructs encoding human α1, α4, β2, and γ2L $GABA_A$ receptor subunits were digested with *Hpa*I and treated with proteinase K. The linearized cDNAs were precipitated and resuspended in buffer (1X = 10 mM Tris, 1mM EDTA, pH 7.6). cRNA was made using T7 polymerase, 1–2 μM cDNA template, and ribonucleotide mixture (37° for 60 min). cRNA was diluted to 1 μg/μl with diethylpyrocarbonate-treated water and stored in 1–2-μl aliquots at −80°.

Expression of receptors in *Xenopus* oocytes. Surgery on *X. laevis* and dissection and defolliculation of oocytes were performed as described previously (34, 35). Oocytes were stored in Barth's medium containing 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM HEPES, pH adjusted to 7.4 with NaOH, and 0.1 mg/ml gentamycin. Immediately before injection, cRNA stocks were thawed, mixed in appropriate subunit combinations, and, where necessary, diluted 10-fold with H_2O. Individual oocytes were then microinjected with mixtures containing ~1-5 ng of cRNA encoding each $GABA_A$ receptor subunit. The ratio of α, β, and γ subunits was 1:1:1 or 5:1:1 for tertiary combinations and 1:1 or 5:1 for the binary combinations, where 1 indicates ~1 ng of cRNA/oocyte.

Electrophysiology. Membrane current responses were measured at a holding potential of −70 mV using a conventional two-electrode voltage clamp (Dagan TEV-200; Dagan, Minneapolis, MN). Recordings were made 3–14 days after cRNA injection. Individual oocytes were placed on a nylon mesh, impaled with electrodes, and superfused with Ringer's solution from a multibarreled linear array of thin-walled glass capillary tubes (o.d., 2 mm) mounted to a micromanipulator (35). The frog Ringer's solution contained 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mMHEPES, pH adjusted to 7.4 with NaOH. All drugs were dissolved in Ringer's and applied to oocytes via the linear array. GABA concentration-response data were obtained by exposing oocytes to increasing concentrations of GABA until an apparent maximal current was reached. Potency and efficacy of allosteric modulators were assayed by determining the concentration of GABA that evoked 10% of the maximum current and then using this response as a control current for measuring the effects of drugs.

Data analysis. GABA concentration-response data were fit to the following logistic equation (Origin, MicroCal, Northampton, MA): $I/I_{max} = 1/[\,1 + (EC_{50}/[GABA])^b\,]$, where b is the slope, EC_{50} is the concentration of GABA that produces a half-maximal response (the apparent affinity of the agonist), I is the current at a given concentration of GABA, and I_{max} is the maximum GABA response. Modulation of control (10%) GABA responses by benzodiazepines and other drugs, whether potentiation or inhibition, was measured in terms of percentage change from the control value. Numerical data given in the text are mean ± standard error, followed in parentheses by the number of separate experiments (oocytes tested) and the number of different frogs from which the oocytes were obtained (batches of oocytes injected); if the second number is not given, one frog was used.

Drugs. Diazepam was purchased from Sigma Chemical (St. Louis, MO), and Ro15-4513 was purchased from Research Biochemicals (Natick, MA). DMCM and CGS-9895 were generous gifts from Ciba-Geigy (Summit, NJ), and loreclezole was a generous gift from the Janssen Research Foundation (New Brunswick, NJ). Ro16-6028 (bretazenil) and Ro15-1788 (flumazenil) were kindly provided by Hoffman-La Roche (Nutley, NJ). The neuroactive steroid 3α-5α-P was synthesized in-house. All other chemicals were from Sigma. Benzodiazepines, steroids, and other test compounds were initially dissolved in DMSO at a concentration of 30–100 mM and then diluted to make a series of DMSO stock solutions of 0.01–100 mM. DMSO stocks were stored at 4° in the dark for 1–4 weeks without apparent changes in activity. Working solutions were made by dilution of DMSO stocks into Ringer's solution just before drug application. For

final drug concentrations of 0.01–10 μM, DMSO was 0.1% by volume. At this dilution, the vehicle had little or no effect on control GABA responses. Bretazenil, CGS-9895, DMCM, and Ro15-4513 at a final drug concentration of 100 μM required DMSO at 0.3–1% to minimize precipitation. As reported previously (36), DMSO alone (0.3% and 1%) was observed to inhibit GABA control responses as follows: at α1β2γ2L (1:1:1), 0.3% DMSO inhibited control responses by 9.0 ± 1% (three oocytes), and 1% DMSO inhibited 24 ± 2% (three oocytes). At α4β2γ2L (5:1:1), 0.3% DMSO inhibited control responses by 4.7 ± 1%, and 1% DMSO inhibited control responses by 19 ± 2% (three oocytes). At β2γ2L, 0.3% DMSO did not induce measurable inhibition, whereas 1% DMSO inhibited control responses by 14 ± 5% (four oocytes). To estimate true levels of modulation for these compounds at 100 μM, the inhibition induced by DMSO alone was factored into the value for underlying control currents.

Results

Expression of α4-Containing $GABA_A$ Receptors in *X. laevis* Oocytes

Expression of ternary subunit combinations α4β2γ2L and α1β2γ2L. Oocytes injected with the α4β2γ2L (1:1:1) combination of cRNAs showed variable levels of expression. In three batches of oocytes (each batch was taken from a different frog), maximum GABA responses ranged from 220 to 3125 nA (mean, 1560 ± 270 μM; 14 oocytes, three frogs). GABA concentration-response curves in these cells showed >3-fold variability in EC_{50} values; in two cases, the curves had distinctly low slopes (Fig. 1, *top*). EC_{50} values ranged from 23 to 74 μM (mean, 44 ± 16 μM; 14 oocytes, three frogs). Slopes ranged from 0.69 to 1.1 (mean, 0.87 ± 0.12). There was no clear correlation among EC_{50}, slope, and level of expression. The α1β2γ2L (1:1:1) combination expressed at higher levels (mean, 2450 ± 110 μM; 27 oocytes, four frogs). EC_{50} values were also variable, ranging from 29 to 255 μM (mean, 101 ± 51 μM). Slopes, however, were consistently >1.2 (mean, 1.3 ± 0.05) (Fig. 2, *bottom*). Reasons for the wide variability between batches of α1β2γ2L oocytes were not investigated.

The variable levels of expression for α4β2γ2L (1:1:1) suggested inadequate expression of α4 subunits or problems with assembly of α4-containing receptors. The low slope values measured in oocytes expressing α4β2γ2L (1:1:1) suggested two possibilities: (i) a heterogeneous population of receptors, as a result of underexpression of α4, with respect to β2 and γ2L, or (ii) inaccurate measurement of peak responses, as a result of populations of receptors with rapid desensitization kinetics.

To address these issues, we increased the ratio of α4-encoding cRNA in the injection mixture. Oocytes expressing α4β2γ2L (5:1:1) showed stronger and more consistent levels of expression than those expressing at a ratio of 1:1:1. Maximum GABA responses ranged from 1510 to 2475 nA (mean, 1780 ± 70 μM; 21 oocytes, four frogs). More importantly, the α4β2γ2L (5:1:1) combination gave GABA concentration-response curves with more-consistent GABA affinities and

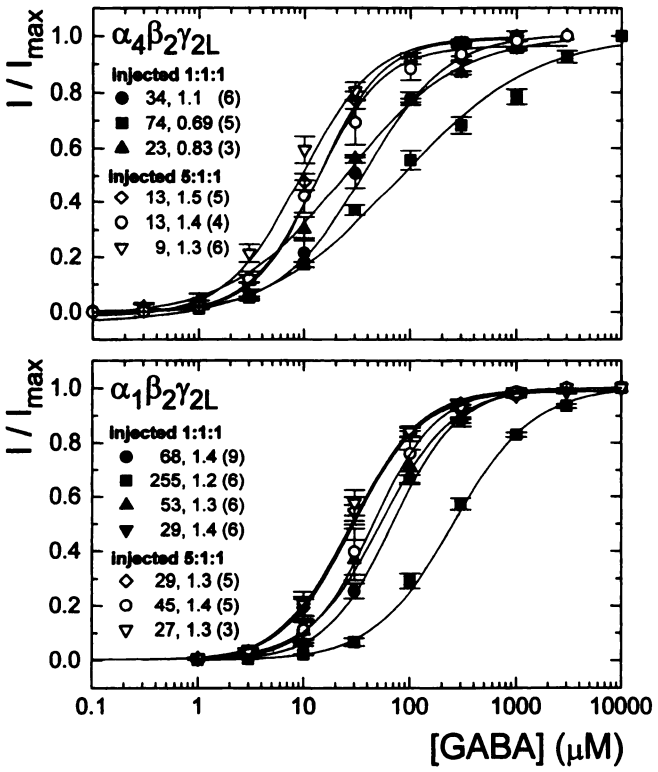

Fig. 1. Concentration-response curves for GABA in oocytes expressing α4β2γ2L (*top*) and α1β2γ2L (*bottom*) receptor combinations at the injection ratios noted. Individual curves represent best logistic fit (see Materials and Methods) to data from a batch of oocytes from a single frog. *Right of symbols*, curve parameters for individual fits [EC_{50}, slope (the number of oocytes assayed within each batch)]. Matching symbols from the *top* and *bottom* represent data obtained from oocytes taken from the same frog injected with the different subunit combinations.

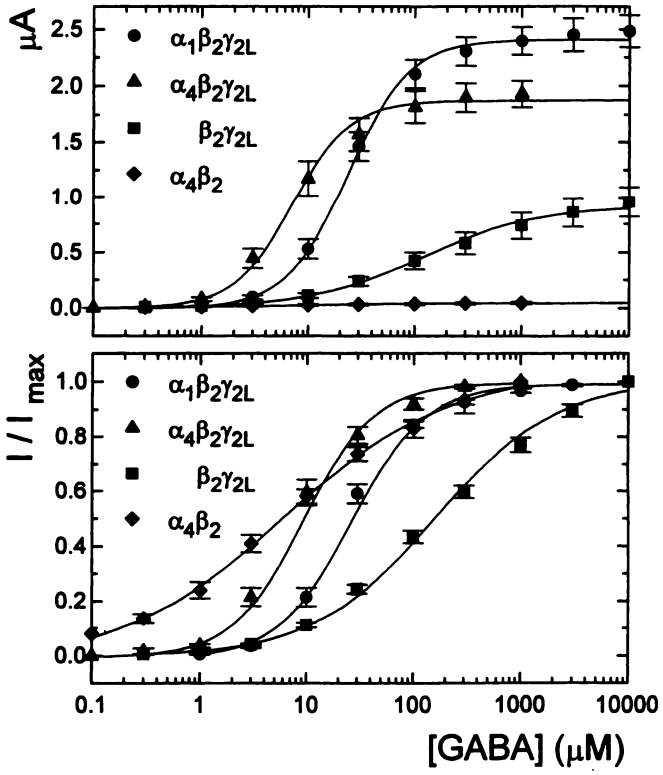

Fig. 2. Concentration-response curves for GABA from the same batch of oocytes from a single frog. *Top*, data from α1β2γ2L, α4β2γ2L, β2γ2L, and α4β2 combinations plotted as a function of total current activated by GABA. *Bottom*, same data as in *top* scaled to the maximal current for each subunit combination. As in Fig. 1, curves represent best logistic fit of data using the equation presented in Methods and Materials. Curve parameters (EC_{50} and slope) are presented in Table 1, as are the n values for each curve.

steeper slopes (Fig. 1, *top*). EC_{50} values for α4β2γ2L (5:1:1) ranged from 9 to 13 μM (mean, 11 ± 1 μM; 21 oocytes, four frogs), and slopes were 1.3–1.5 (mean, 1.4 ± 0.05 μM). Incorporation of γ-subunits into receptors expressed by the α4β2γ2L (5:1:1) combination was tested by measuring the sensitivity of maximum GABA responses to Zn^{2+}(37). Expressed in terms of a fractional response (where control = 1), the current in 100 μM $ZnCl_2$ was 0.86 ± 0.03 (10 oocytes).

Injection of α1β2γ2L at a ratio of 5:1:1 did not increase expression compared with the 1:1:1 ratio. Wide variations in GABA affinity were not detected in oocytes expressing α1β2γ2L (5:1:1); however, only three batches of oocytes were assayed. EC_{50} values for α1β2γ2L (5:1:1) ranged from 27 to 45 μM (mean, 34 ± 6 μM; 13 oocytes, three frogs), and slope values were similar to the 1:1:1 ratio, ranging from 1.3 to 1.4 (mean, 1.3 ± 0.06)(Fig. 1, *bottom*). As described previously (37), receptors expressed by α1β2γ2L were insensitive to Zn^{2+}. For example, in one batch of oocytes, the fractional maximum response in 100 μM $ZnCl_2$ was 1.01 ± 0.01 (six oocytes).

Expression of binary subunit combinations and single subunits. Injection of binary subunit combinations and single subunits served two purposes: (i) to test the expressional potential of α4 subunits in these forms, and (ii) to investigate what other subunit combinations may be present in oocytes expressing α4β2γ2L, possibly contributing to and distorting assays of allosteric modulation.

Three binary subunit combinations (α4β2, α4γ2L, and β2γ2L) and two single subunits (α4 and β2) were injected. Each subunit combination was injected on more than one occasion, and α4β2 and α4γ2L were injected at either 1:1 or 5:1 ratios. Representative data from a single batch of oocytes are given in Fig. 2 and Table 1. Expression of functional GABA-activated currents in oocytes injected with α4β2 was unexpectedly weak compared with α1β2 (data not shown), constituting only 2–11% of responses recorded for the α4β2γ2L combination (Fig. 2, *top*). Apparent GABA affinity was high for α4β2, but slope values were distinctly low (Fig. 2, *bottom*). Oocytes injected with α4γ2L failed to express receptors that could be activated by GABA. As predicted by the latter result, α4 injected alone also did not generate GABA-gated channels. The only binary subunit combination that expressed at levels approaching the ternary combinations was β2γ2L (Fig. 2, *top*). Maximum GABA responses for β2γ2L receptors ranged from 280 to 1500 nA (mean, 674 ± 67 nA; 24 oocytes, four frogs). The characteristics of GABA concentration-responses curves were inconsistent, with generally low apparent affinities and shallow slopes (Fig. 2, *bottom*). EC_{50} values for β2γ2L (1:1) ranged from 44 to 162 μM (mean, 122 ± 39 μM; 19 oocytes, three frogs), and slopes were 0.66–1.1 (mean, 0.84 ± 0.13 μM). The β2 subunit injected alone expressed very weakly, ~1% of the ternary subunit combinations, and the responses were not further characterized.

These results indicated that only β2γ2L receptors showed any real potential for generating inaccuracies in the assessment of allosteric modulation at α4β2γ2L and α1β2γ2L subunit combinations. To control for this possibility, effects of allosteric modulators on β2γ2L receptors were assayed in parallel with measurements for α4β2γ2L and α1β2γ2L.

Direct activation of α4-containing receptors by pentobarbital. In separate experiments, we found that α1β2 subunit combinations induced moderate-to-strong expression of GABA-activated currents (data not shown). Maximum GABA responses were 52–74% (six oocytes, two frogs) of responses elicited by α1β2γ2L (see also Refs. 6 and 7). As noted above, levels of expression for α4β2 combinations were therefore unexpectedly low, typically ≤10% of responses elicited by α4β2γ2L (Table 1). To test whether expression of α4-containing receptors was being underestimated due to low efficacy agonism by GABA, we assayed the effects of a high concentration of pentobarbital, applied alone or together with a saturating concentration of GABA. Sample data are given in Table 2.

In oocytes expressing α4β2γ2L combinations, 300 μM pentobarbital, applied alone, elicited currents that were ~27% of the maximum GABA response. Coapplied with 10 mM GABA, 300 μM pentobarbital caused a ~12% increase in the maximum GABA current. In contrast, for oocytes expressing α4β2 subunit combinations, 300 μM pentobarbital alone elicited currents that were ~16-fold larger than the maximum currents elicited by 10 mM GABA alone. Coapplication of pentobarbital and GABA at these concentrations generated currents that were ~25-fold larger than maximum GABA responses. The mean response activated by GABA plus pentobarbital was 1095 ± 220 nA (i.e., comparable to the current elicited by GABA in oocytes expressing α4β2γ2L) (Table 2). This type of effect was not seen in oocytes expressing α4γ2L subunit combinations or α4 alone. Pentobarbital also elicited large currents in oocytes expressing α1β2γ2L, ~73% of the maximum GABA response, but effected no increase in maximum current when coapplied with GABA. In oocytes expressing β2γ2L subunit combinations and β2 alone, pentobarbital again activated substantial currents, often showing greater efficacy than GABA; in these cases, coapplication with GABA resulted in 3–5-fold increases in current over the maximum GABA response.

TABLE 1

Expression characteristics of GABA responses in oocytes injected with various combinations of α4, α1, β2 and γ2L

Subunit combination	ratio (α:β:γ)	I_{max}	EC_{50}	Slope	*n*
		nA			
α1β2γ2L	5:1:1	2480 ± 140	27 ± 1.6	1.3 ± 0.05	4
α4β2γ2L	5:1:1	1930 ± 110	9.3 ± 0.8	1.3 ± 0.07	6
α4β2	1:1	44 ± 12	6.1 ± 1.8	0.56 ± 0.1	4
α4β2	5:1:-	212 ± 52	not tested	not tested	5
β2γ2L	-:1:1	922 ± 107	162 ± 8.3	0.76 ± 0.02	5
α4γ2L	1:-:1	no current			7
α4	5:-:-	no current			4
β2	-:1:-	23 ± 4	not tested	not tested	5

TABLE 2
Responses to pentobarbital

Subunit combination	Subunit ratio (α:β:γ)	10 mM GABA I_{max}	300 mM pentobarbital (I/I_{max})	10 mM GABA + 300 mM pentobarbital (I/I_{max})	n
		nA			
$\alpha1\beta2\gamma2L$	5:1:1	2387 ± 179	0.73 ± 0.07	1.00 ± 0.01	4
$\alpha4\beta2\gamma2L$	5:1:1	1612 ± 204	0.27 ± 0.09	1.12 ± 0.03	5
$\alpha4\beta2$	1:1:-	45 ± 11	15.6 ± 1.1	25.1 ± 3.0	4
$\beta2\gamma2L$	-:1:1	563 ± 98	2.36 ± 0.23	3.29 ± 0.37	6
$\beta2$	-:1:-	23 ± 4	0.91 ± 0.16	4.53 ± 0.83	5
$\alpha4$	1:-:-	no current	no current	no current	5
$\alpha4\gamma2L$	1:-:1	no current	no current	no current	4

Allosteric Modulation of $\alpha4\beta2\gamma2L$, $\alpha1\beta2\gamma2L$, and $\beta2\gamma2L$ Subunit Combinations

General protocol for modulation assays. The primary purpose of these experiments was to characterize the pharmacological profile of $\alpha4\beta2\gamma2L$ subunit combinations with respect to allosteric modulators. Side-by-side characterization of $\alpha1\beta2\gamma2L$ subunit combinations was mainly for comparison, and experiments on $\beta2\gamma2L$ subunit combinations were included to control for possible contamination of the $\alpha4\beta2\gamma2L$ subunit combinations with these diheteromeric receptors. Functional modulation was assessed on currents that were ~10% of the maximum GABA response. The concentration of GABA required to elicit this 10% response showed dependence on subunit combination. For $\alpha1\beta2\gamma2L$ (1:1:1), concentrations ranged from 4 to 40 μM (mean, 21 ± 2 μM; 36 oocytes, four frogs); for $\alpha4\beta2\gamma2L$ (5:1:1), the range was 1.75–10 μM (mean, 3.8 ± 0.3 μM; 35 oocytes, four frogs); and for $\beta2\gamma2L$ (1:1), the range was 4.5–11 μM (mean, 7.7 ± 0.6 μM; 23 oocytes, two frogs). Benzodiazepine-site ligands were assayed at 5 concentrations over the range of 10 nM to 100 μM. Other types of modulator were tested at a single concentration. In each case, oocytes were pretreated for 30–60 sec with the modulator before activation of receptors by coapplication of GABA (e.g., Fig. 3, *records*). All potentiation or inhibition of membrane current responses was measured in terms of percentage increase or decrease in the control 10% response. Pharmacological assays of modulators were generally made using $\alpha4\beta2\gamma2L$ at a 5:1:1 ratio of injection, whereas $\alpha1\beta2\gamma2L$ was used at either 1:1:1 or 5:1:1.

Modulation by benzodiazepine-site agonists. Three benzodiazepine-site agonists were assayed: (i) diazepam, a benzodiazepine classified as a typical full-efficacy agonist (4); (ii) bretazenil, an imidazobenzodiazepinone classified as a low-efficacy partial agonist (4, 38); and (iii) CGS-9895, a pyrazoloquinolinone also reported to be a partial agonist (39).

Diazepam, over the concentration range of 0.01–1 μM, did not induce any significant modulation of $\alpha4\beta2\gamma2L$ subunit combinations (Fig. 3). Slight potentiation was detectable with 10 μM diazepam and increased sharply to 84 ± 5% at 100 μM. In contrast, diazepam potentiated $\alpha1\beta2\gamma2L$ subunit combinations with both high and low affinity components. The high affinity potentiation had an EC_{50} value of ~50 nM and saturated at ~1 μM with an efficacy of 110 ± 16% (7). The low affinity component became evident only using 100 μM diazepam. Diazepam potentiated $\beta2\gamma2L$ subunit combinations with high potency. As seen with $\alpha1\beta2\gamma2L$, the EC_{50} value was ~50 nM and maximum efficacy was 75 ± 6%. In this case, no increase in potentiation was seen at 100 μM diazepam.

Bretazenil induced potent and surprisingly robust potentiation of $\alpha4\beta2\gamma2L$ subunit combinations (Fig. 4, *top*). At 1 μM, bretazenil caused a 83 ± 7% increase in current, which was in sharp contrast to the absence of potentiation seen with diazepam. The effects of bretazenil at $\alpha1\beta2\gamma2L$ were consistent with partial agonism. Potentiation saturated ~1 μM where the increase in current was only 25 ± 11%. EC_{50} values seemed to be ~10 nM, but they were difficult to estimate due to the low efficacy of modulation. For both ternary subunit combinations, there was an appreciable increase in potentiation, moving from 10 to 100 μM bretazenil. Interestingly, bretazenil had only inhibitory effects on $\beta2\gamma2L$ receptors, at which it behaved like a low-efficacy inverse agonist.

CGS-9895, at concentrations of ≤1 μM, induced low-level potentiation of $\alpha4\beta2\gamma2L$ combinations (Fig. 4, *bottom*). At 1 μM, the effect was 41 ± 4%. Modulation became more pronounced with 10 μM CGS-9895 and seemed to saturate ~100 μM at the point at which potentiation was 211 ± 45%. The same general pattern was observed for $\alpha1\beta2\gamma2L$ subunit combinations, except that potentiation was not detected at concentrations of <1 μM CGS-9895 and efficacy was greater at high concentrations. For the binary $\beta2\gamma2L$ combination, CGS-9895 was similarly inactive at low concentration but induced robust potentiation at 10 and 100 μM.

Modulation by benzodiazepine-site antagonists. Flumazenil (Ro15-1788), an imidazobenzodiazepinone, is generally classified as a benzodiazepine-site antagonist (4). As such, flumazenil would be expected to have no direct effects on control GABA responses but should antagonize actions of other benzodiazepine-site ligands (see discussion of blocking experiments).

Unexpectedly, flumazenil was a positive modulator of $\alpha4\beta2\gamma2L$ subunit combinations (Fig. 5). The EC_{50} value for potentiation was ~100 nM, and the effect peaked at 48 ± 3%. A decline in the level of potentiation was observed at 100 μM. For $\alpha1\beta2\gamma2L$ receptors, flumazenil evoked no significant modulatory effects at concentrations of 0.01–1 μM. At 10 μM, however, some potentiation was detected, and 100 μM flumazenil induced a 30 ± 3% increase in current. The effects of flumazenil on $\beta2\gamma2L$ receptors, if any, were weak and inconsistent.

Modulation by benzodiazepine-site inverse agonists. Two benzodiazepine-site inverse-agonists were assayed: (i) Ro15-4513, an imidazobenzodiazepinone, which is classified as a partial inverse agonist (4), and (ii) DMCM, a β-carboline that is classified as a full inverse agonist (4, 40).

Contrary to expectations, Ro15-4513 induced potentiation of $\alpha4\beta2\gamma2L$ subunit combinations (Fig. 6). The EC_{50} value for the effect was ~50 nM, and modulation seemed to saturate at

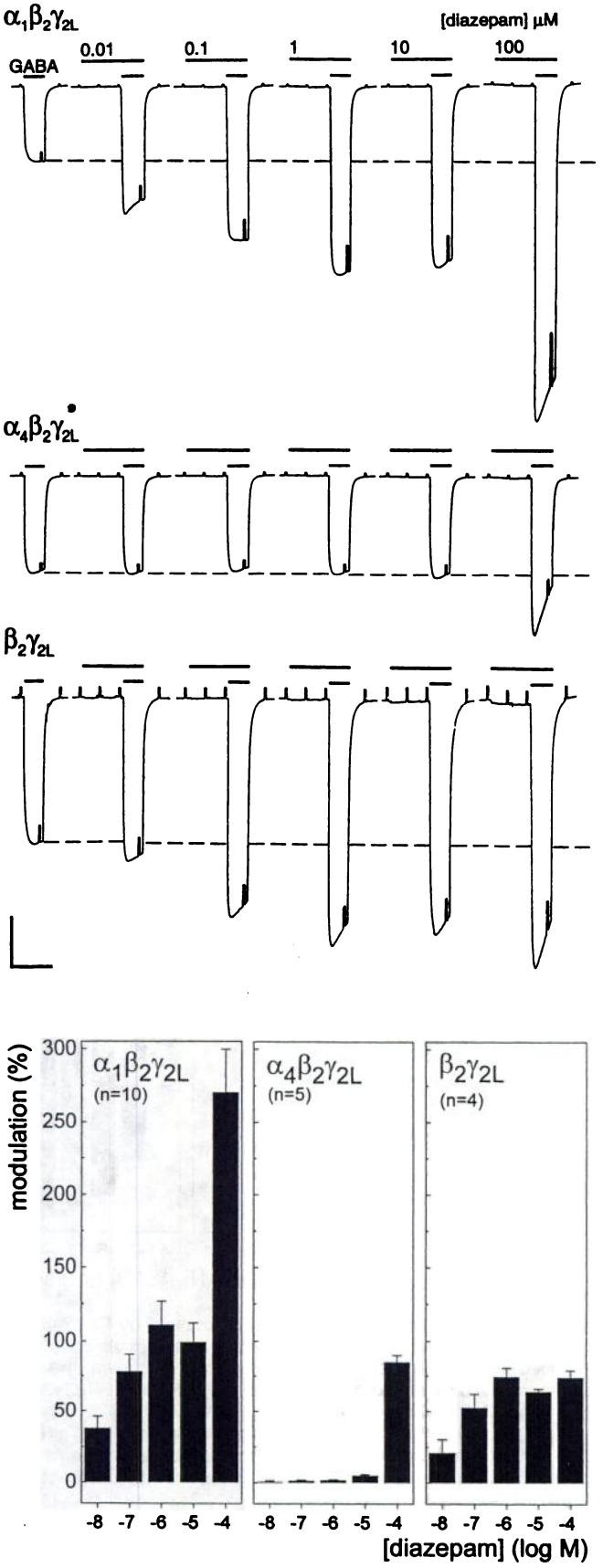

Fig. 3. *Top*, sample records of modulation evoked by diazepam on α1β2γ2L, α4β2γ2L, and β2γ2L. *Traces* at each subunit combination represent current responses evoked consecutively from a single oocyte, with gaps representing ~1-2 min. Holding potential was −70 mV and was pulsed to −60 mV (upward deflections) to time drug applications and to assess membrane conductance. *Bars*, drug applications; *downward deflection*, inward current. GABA concentrations were adjusted in each experiment to evoke control responses, which were ~10% of maximal GABA currents. In the examples shown, the GABA concentrations used were 15 μM for α1β2γ2L, 2.9 μM for α4β2γ2L, and 7.5 μM for β2γ2L. *Dashed line*, magnitude of the control response as a visual aid for modulatory effects; *vertical scale bar*, 200 nA for α1β2γ2L, 100 nA for α4β2γ2L, and 20 nA for β2γ2L; *horizontal scale bar*, 0.8 min in all cases. *Bottom*, concentration-response data from multiple experiments of the type illustrated in the top were averaged and plotted as a percentage of control (mean ± standard error). For α1β2γ2L, 10 oocytes; for α4β2γ2L, five oocytes; and for β2γ2L, four oocytes.

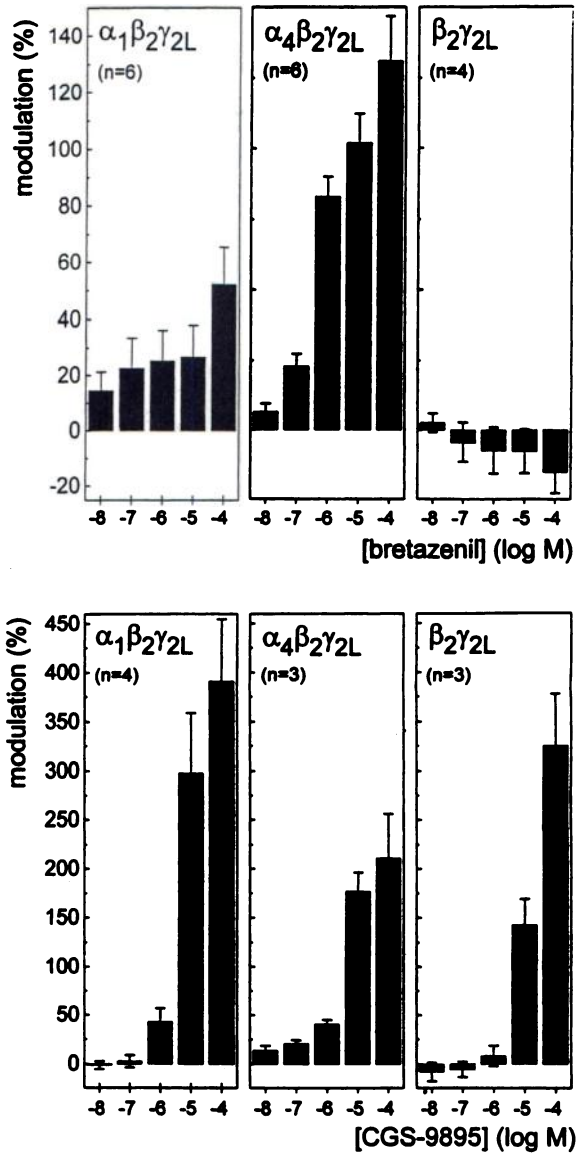

Fig. 4. Modulation of GABA control responses by (*top*) bretazenil and (*bottom*) CGS-9895. As described in the legend for Fig. 3, concentration-response data from multiple experiments of the type illustrated in Fig. 3 (*top*) were averaged and plotted as a percentage of control (mean ± standard error). For bretazenil on α1β2γ2L, six oocytes; on α4β2γ2L, six oocytes; and on β2γ2L, four oocytes. For CGS-9895 on α1β2γ2L, four oocytes; on α4β2γ2L, three oocytes; and on β2γ2L, three oocytes.

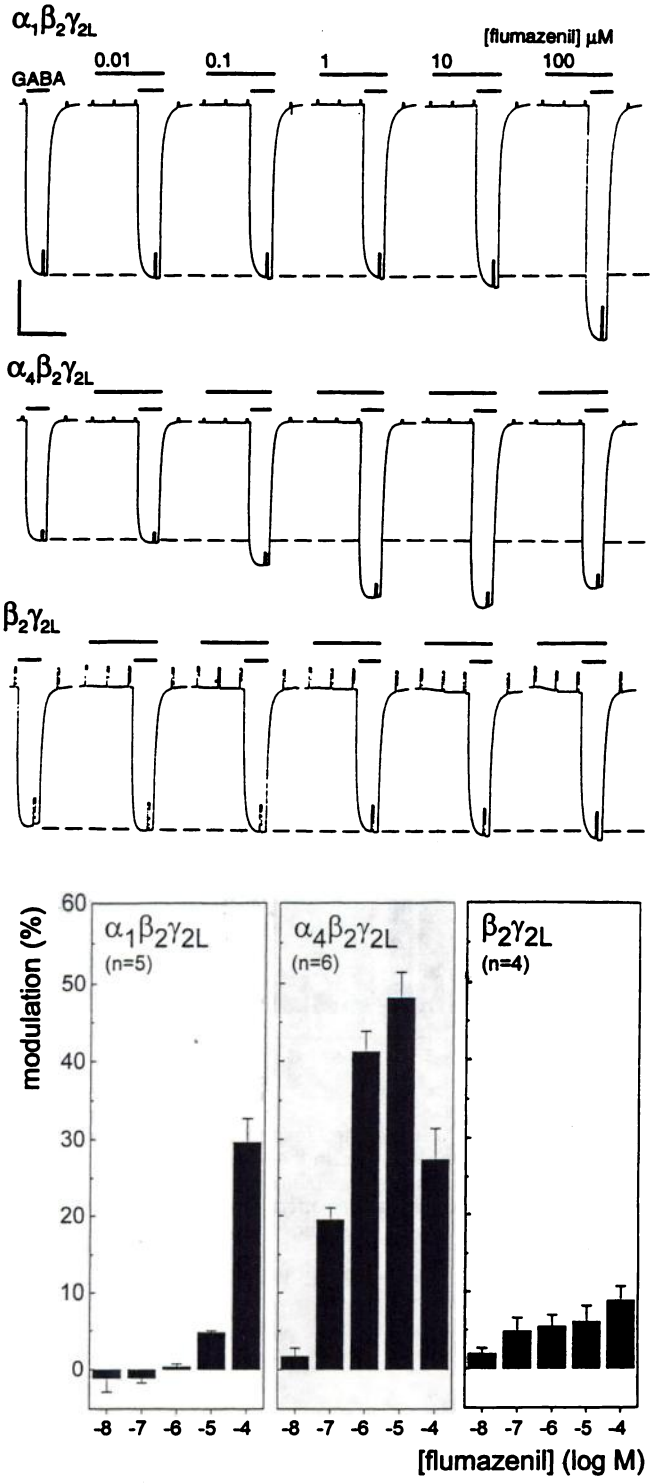

Fig. 5. *Top*, sample records of modulation evoked by flumazenil on α1β2γ2L, α4β2γ2L, and β2γ2L. The methods are similar to those described in the legend to Fig. 3. In the examples shown, the GABA concentrations used were 18 μM for α1β2γ2L, 2.2 μM for α4β2γ2L, and 7.5 μM for β2γ2L. *Vertical scale bar*, 100 nA for α1β2γ2L and α4β2γ2L, and 50 nA for β2γ2L; *horizontal scale bar*, 0.8 min in all cases. *Bottom*, concentration-response data from multiple experiments of the type illustrated in the *top* were averaged and plotted as a percentage of control (mean ± standard error). For α1β2γ2L, five oocytes; for α4β2γ2L, six oocytes; and for β2γ2L, four oocytes.

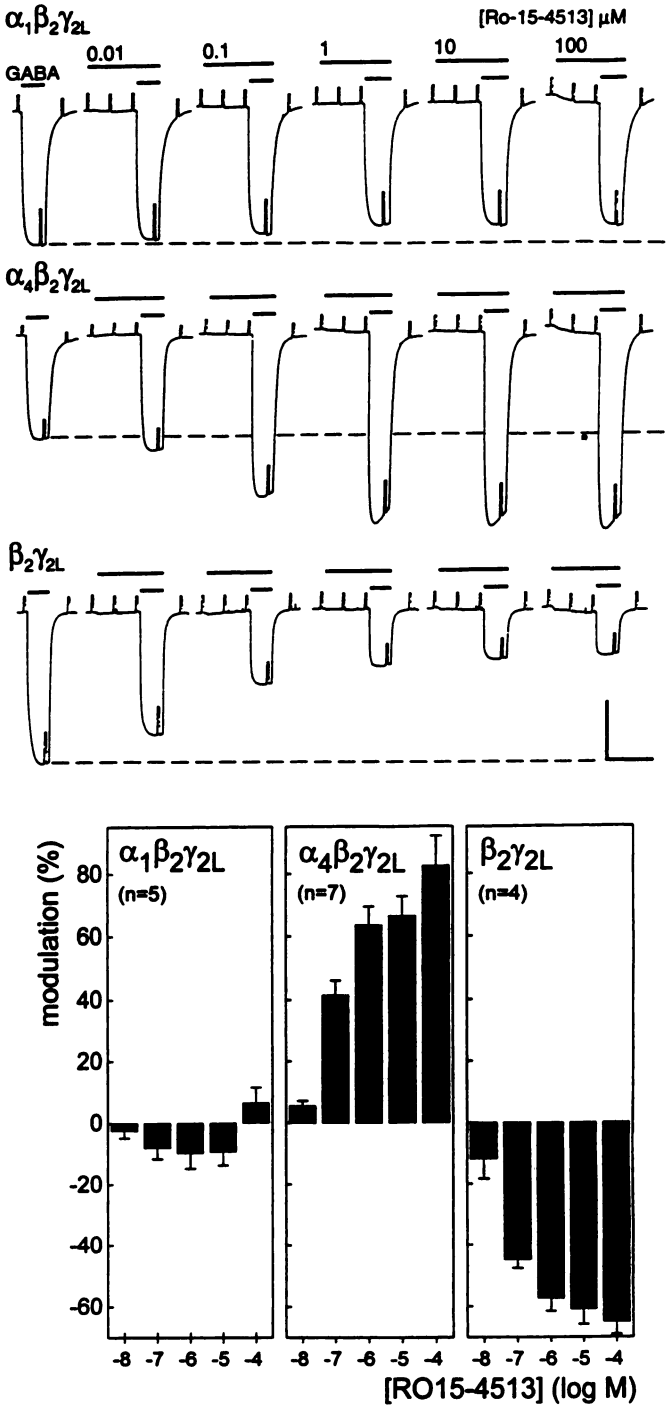

Fig. 6. *Top*, sample records of modulation evoked by Ro15-4513 at α1β2γ2L, α4β2γ2L, and β2γ2L. The methods are very similar to those described in the legend to Fig. 3. In the examples shown, the GABA concentrations used were 5.5 μM for α1β2γ2L, 3.5 μM for α4β2γ2L, and 5.5 μM for β2γ2L. *Vertical scale bar*, 100 nA for α1β2γ2L, 50 nA for α4β2γ2L, and 20 nA for β2γ2L; *horizontal scale bar*, 0.8 min in all cases. *Bottom*, concentration-response data from multiple experiments of the type illustrated in the *top* were averaged and plotted as a percentage of control (mean ± standard error). For α1β2γ2L, six oocytes; for α4β2γ2L, seven oocytes; and for β2γ2L, four oocytes.

1–10 μM. At 1 μM, Ro15-4513-induced potentiation was 63 ± 6%. Raising concentrations to 100 μM caused only a modest increase in current. As expected, 0.01–10 μM Ro15-4513 induced low-efficacy inhibition of α1β2γ2L receptors, which is

consistent with partial inverse agonism. In some cases, inhibition inverted into slight potentiation at 100 μM, but this was not consistent (e.g., Fig. 6, *records*). Interestingly, Ro15-4513 induced robust inhibition of β2γ2L receptors. The EC_{50} value for this effect was ~50 nM, and the maximum level of inhibition was 65 ± 4%.

In contrast to Ro15-4513, low concentrations of DMCM induced inhibition of α4β2γ2L receptors (Fig. 7). Peak effect was seen at 100 nM, where the reduction in current was 26 ± 2%. At concentrations of >1 μM, however, there was a sharp inversion to net potentiating effects (7, 40). Potentiation induced by 10 and 100 μM DMCM was 58 ± 12% and 125 ± 13%, respectively. The EC_{50} value for potentiation seemed to be >10 μM. The same pattern of modulation was evident for α1β2γ2L and β2γ2L receptors, but maximum levels of inhibition were slightly higher: 44 ± 5% for α1β2γ2L and 55 ± 9% for β2γ2L.

Sensitivity of modulation to blockade by flumazenil. Previous electrophysiological studies indicated that DMCM interacts at distinct high and low affinity sites on cloned α1β2γ2L receptors expressed in HEK 293 cells (41). Thus, DMCM evokes inhibition at a high affinity site and potentiation at a low affinity site; the high affinity inverse agonist effect of DMCM is blocked by flumazenil, whereas the lower affinity potentiation is insensitive (41). Biphasic modulatory effects were also observed in the current study. In addition to the inhibition/potentiation observed with DMCM (Fig. 7), a number of benzodiazepine-site ligands showed potentiation with clear biphasic character, also suggesting distinct high and low affinity effects (Figs. 3 and 4). To begin to investigate this possibility, we performed a limited series of experiments addressing four questions: Were the HEK 293 cell experiments with DMCM described by Im *et al.* (41) reproducible in oocytes? Were the low affinity components of diazepam modulation mediated by flumazenil-insensitive sites? Was the potentiation induced by CGS 9895 inhibited by flumazenil? Was potentiation of α4β2γ2L receptors by flumazenil and Ro15-4513 additive?

As outlined above, in control experiments DMCM induced a biphasic modulation of α1β2γ2L, with inverse agonism observed at ≤1 μM and potentiation observed at ≥10 μM (Fig. 7). Inverse agonism induced by 1 μM DMCM was reduced by 1 μM flumazenil (not shown) and reversed to potentiation by 10 μM flumazenil (Fig. 8, *top*). In contrast, potentiation induced by 100 μM DMCM was unaffected by 10 and 100 μM flumazenil. These experiments confirmed the results described previously in HEK 293 cells (41), supporting the idea of high and low affinity sites for DMCM and further suggesting that flumazenil is not an effective antagonist at the low affinity site.

For diazepam at α1β2γ2L receptors, the high affinity potentiation induced at 1 μM was inhibited 97% by equimolar flumazenil, whereas potentiation induced by 100 μM diazepam was only partially blocked (Fig. 8, *top*). This partial inhibition was probably due, at least in part, to blockade of the underlying high affinity component. The low affinity potentiation induced by diazepam (100 μM) on α4β2γ2L was only weakly blocked by 1–100 μM flumazenil (Fig. 8, *bottom*). Modulation of both types of receptor by 10 μM CGS 9895 was only partially inhibited by 10–100 μM flumazenil. Finally, 1 μM flumazenil had essentially no effect on the level of potentiation induced by 1 μM Ro15-4513 on α4β2γ2L receptors (Fig. 8, *bottom*).

Modulation by other classes of drugs. Four additional compounds were tested for modulation of α4β2γ2L, α1β2γ2L, and β2γ2L subunit combinations: pentobarbital, a sedative barbiturate (7); 3α,5α-P, a neuroactive steroid (33); loreclezole, an anticonvulsant triazole (12, 23, 42–44); and mefenamic acid, a nonsteroidal anti-inflammatory drug (20). These modulators were assayed at a single concentration in experiments designed to test whether α4β2γ2L receptors were sensitive to modulation and whether there were large differences in sensitivity among the three subunit combinations. GABA control responses at α4β2γ2L receptors were potentiated by all four drugs (Fig. 9). For pentobarbital (10 μM) and 3α,5α-P (0.03 μM), potentiation was slightly more pronounced for α4β2γ2L compared with α1β2γ2L and β2γ2L, whereas for mefenamic acid (3 μM) and loreclezole (1 μM), the relative sensitivities were reversed. At the concentrations tested, both pentobarbital and mefenamic acid elicited appreciable membrane current responses when applied alone (not shown).

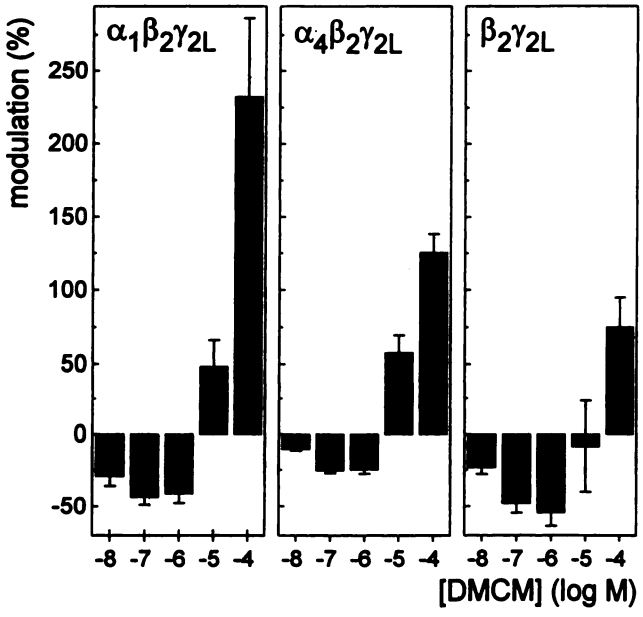

Fig. 7. Modulation of GABA control responses by DMCM. Concentration-response data from multiple experiments of the type illustrated in Fig. 3 (*top*) were averaged and plotted as a percentage of control (mean ± standard error). At α1β2γ2L, six oocytes; at α4β2γ2L, eight oocytes; and at β2γ2L, four oocytes.

Discussion

Functional pharmacology at α4-containing receptors. GABA receptors containing α4 subunits are thought to be a physiologically relevant diazepam-insensitive subtype in mammalian forebrain (14, 29–31, 45). Our results are functional confirmation for binding data suggesting that α4-containing receptors exhibit a unique and novel benzodiazepine-site pharmacology that resembles, but is distinct from, that of receptors containing the structurally related α6 subunit.

Expression of α4-containing receptors in oocytes. Expressional potency and the properties of GABA concentration-response curves for α4-containing receptors show dependence on the ratio of α4-encoding cRNA coinjected with cRNA

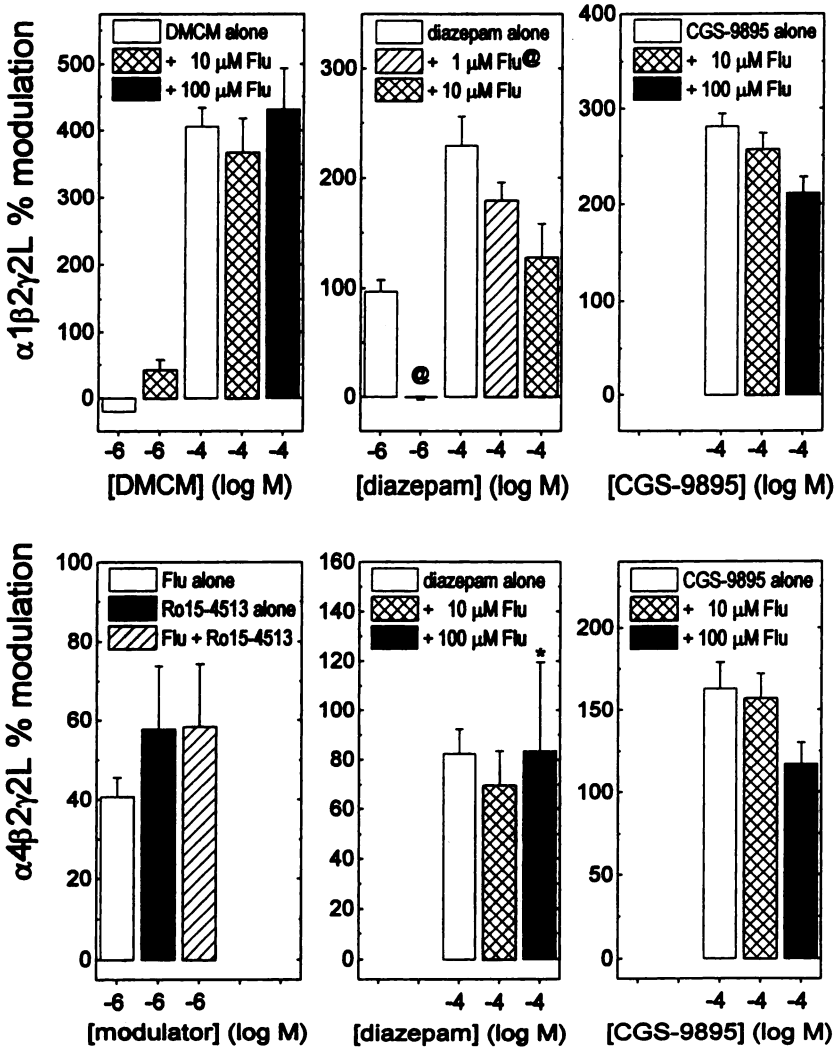

Fig. 8. Sensitivity of modulation to blockade by flumazenil at $\alpha1\beta2\gamma2L$ (*top*) and $\alpha4\beta2\gamma2L$ (*bottom*). As above, GABA responses that were ~10% of maximal GABA currents were used as a basal response. Modulators were then coapplied with GABA either alone (control) or coapplied with flumazenil at the concentrations shown. The general method of drug application is as outlined in legend to Fig. 3 (*top*). As in the preceding figures, the data are plotted as a percentage of the GABA basal response (mean ± standard error from three to five experiments using different oocytes, except in the case noted by *, which represents only two experiments).

encoding $\beta2$ and $\gamma2L$. Our experiments suggest that the $\alpha4$-encoding cRNA expresses at lower levels than cRNAs encoding $\alpha1$, $\beta2$, and $\gamma2L$ or that $\alpha4$ subunits do not assemble efficiently into the ternary complex. Pentobarbital induces direct currents from $\alpha4\beta2\gamma2L$ that are ~25% of maximal GABA current. This pattern is also observed for ternary receptors containing $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ subunits (46). In contrast, pentobarbital induces direct currents from $\alpha6\beta2\gamma2S$ that are significantly larger that the maximal currents elicited by GABA (46). Thus, with respect to direct modulation by pentobarbital, $\alpha4\beta2\gamma2L$ receptors are distinct from $\alpha6\beta2\gamma2S$ receptors.

The binary combination $\alpha4\beta2$ exhibits very low expression of GABA current and very low slopes in our experiments, as reported previously for $\alpha4\beta1$ (29). Pentobarbital induces much more substantial direct currents from $\alpha4\beta2$, suggesting either that GABA is a low-efficacy agonist or that desensitization kinetics of $\alpha4\beta2$ receptors are more rapid than our slow drug application can resolve. Because $\alpha4$ and $\beta2$ subunits colocalize in regions of the thalamus that exhibit very low levels of $\gamma2$ (14), it may be worthwhile to characterize $\alpha4\beta2$ receptors in more detail, using excised patches and fast drug application techniques.

Modulation of $\alpha4\beta2\gamma2L$ receptors by benzodiazepine-site ligands. Interaction of benzodiazepine-site ligands with receptors containing the $\alpha4$ subunit has been investigated in some detail using radioligand binding assays (29–31) but only superficially by electrophysiological techniques (29). Binding studies show that $\alpha4\beta2\gamma2$ receptors do not have high affinity sites for diazepam (30, 31) but do exhibit high affinity for the partial or inverse agonists bretazenil, CGS-9895, Ro15-4513, and DMCM and moderate affinity for the benzodiazepine-site antagonist flumazenil (31). This profile is similar to that observed for $\alpha6$-containing receptors, with subtle differences in affinity for some ligands (28, 31, 32).

Our experiments confirm these data functionally and demonstrate unexpected modulatory effects for certain benzodiazepine-site ligands on $\alpha4\beta2\gamma2L$ receptors. As expected, diazepam (≤1 μM) does not evoke potentiation of $\alpha4\beta2\gamma2L$. However, bretazenil (≤1 μM) evokes robust potentiation at $\alpha4\beta2\gamma2L$. Similarly, flumazenil evokes moderate potentiation of $\alpha4\beta2\gamma2L$, as recently reported for $\alpha6\beta2\gamma2L$ (47). Strong potentiation of $\alpha4\beta2\gamma2L$ receptors by bretazenil could contribute to reported sedative effects of bretazenil in humans (48), and potentiation of $\alpha4$- and $\alpha6$-containing receptors by flumazenil may explain certain behavioral effects of this drug in animal studies. Although the pyrazoloquinoline CGS-9895 exhibits high affinity binding at $\alpha4\beta2\gamma2L$ (31), it evoked only low efficacy potentiation at concentrations of ≤1

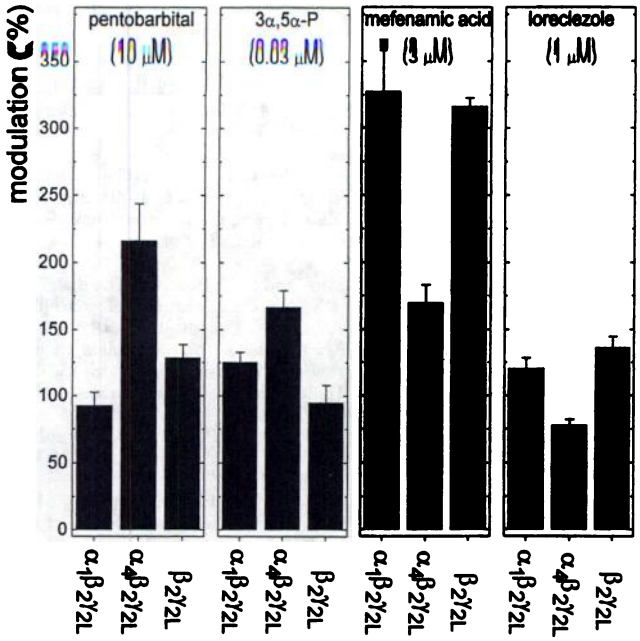

Fig. 9. Modulation of GABA control responses by pentobarbital, 3α,5α-P, mefenamic acid, and loreclezole at α1β2γ2L, α4β2γ2L, and β2γ2L. Experiments were performed as described in the legend to Fig. 3, except that only a single concentration of each modulator was assayed at each subunit combination. Data from four oocytes for each data point were averaged and plotted as a percentage of control (mean ± standard error).

μM in our oocyte assays. Thus, the functional significance of the reported high affinity binding is unclear.

The inverse agonists DMCM and Ro15-4513 exhibit distinctly different effects with respect to functional modulation of α4β2γ2L receptors. As previously reported for α1β2γ2 (7, 40), DMCM evokes biphasic inhibition/potentiation of α4β2γ2L, whereas it has only potentiating effects on α6β2γ2L (40, 44). In contrast, Ro15-4513 evokes only potentiation at both α4β2γ2L and α6β2γ2S (46). Thus, although α4 and α6 are closely related structurally and both are diazepam insensitive, the current experiments confirm that benzodiazepine sites on α4 and α6 can be distinguished pharmacologically.

Modulation by other drugs. Our experiments demonstrate that the GABA$_A$ modulators pentobarbital, 3α,5α-P, mefenamic acid, and loreclezole do not show pronounced differences in modulation of α4β2γ2L and α1β2γ2L receptors. These data suggest that the sites at which these other modulators act are not dependent on the type of α subunit in the receptor or are conserved between the α1 and α4 subunits. The former point is reinforced by the observation that all four compounds induce strong modulation of β2γ2L receptors.

Potentiation of α4β2γ2L receptors by imidazobenzodiazepines. The three benzodiazepine-site ligands that evoke potentiation of α4β2γ2L, bretazenil, flumazenil, and Ro15-4513, are all imidazobenzodiazepines (Fig. 10). This is consistent with binding studies that demonstrate that these compounds have high affinity for diazepam-insensitive sites (30, 31, 49). We suspect that other 6-oxoimidazobenzodiazepines (e.g., Ro15-3505 and Ro19-4603) will also be positive modulators of α4- and α6-containing receptors (47). Binding studies further suggest that 6-phenyl-substituted imidazobenzodiazepines (e.g., triazolam and midazolam) have low affinity for diazepam-insensitive sites (49) and therefore should not potentiate α4β2γ2L. To test this hypothesis, we assayed midazolam (0.01–1 μM) for modulation of α4β2γ2L and, as predicted, found no potentiation.[1] Together with previous studies (47, 49), our results indicate that imidazobenzodiazepines lacking a 6-phenyl substitution represent a good structural lead for the design of potent GABA$_A$ receptor modulators that show selectivity for diazepam-insensitive subtypes.

High and low affinity modulation by benzodiazepine-site ligands. The current experiments demonstrate distinct high- and low-potency modulatory effects of benzodiazepine-site ligands on both α1β2γ2L and α4β2γ2L receptors. This is exemplified by two component modulation evoked by diazepam and bretazenil and most clearly by the biphasic inhibitory/potentiating actions of DMCM (41). Pharmacological separation of these effects is convincingly dem-

[1] E. R. Whittemore, W. Yang, J. A. Drewe, and R. M. Woodward, unpublished observations.

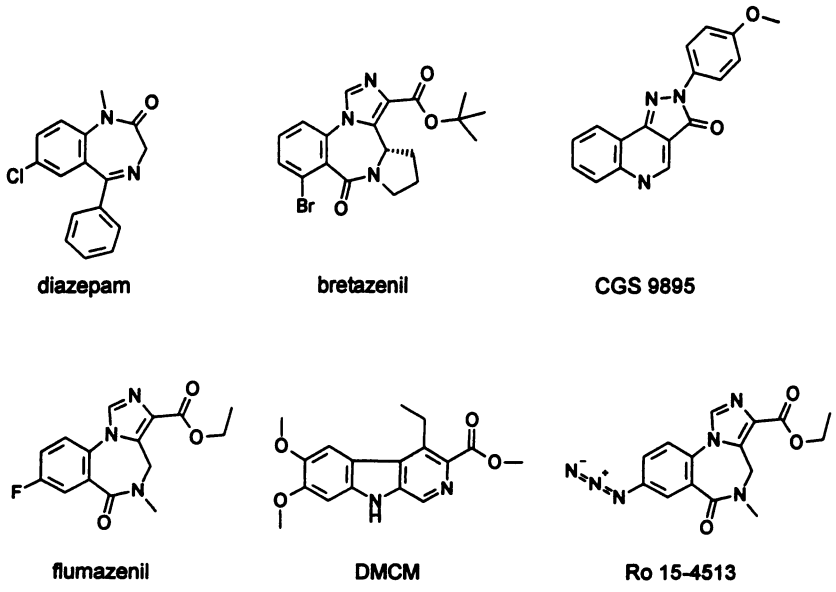

Fig. 10. Structures of selected molecules assayed for modulation of GABA$_A$ receptors in oocytes.

onstrated for $\alpha1\beta2\gamma2L$, for which flumazenil does not have agonist activity. Attempts to separate these two effects pharmacologically for $\alpha4\beta2\gamma2L$ are confounded by the direct potentiating activity of flumazenil for $\alpha4\beta2\gamma2L$.

Im *et al.* (41) proposed that the low affinity agonist site for DMCM is distinct from modulatory sites for barbiturates and neuroactive steroids. Furthermore, Stevenson *et al.* (44) presented evidence that the low affinity site for β-carbolines like DMCM is the same site at which loreclezole evokes modulation, or at least that signal transduction for the two effects is mechanistically related, both being dependent on an amino acid residue (Asn289) within the β2 subunit. These authors further predict that other benzodiazepine-site ligands may also have actions at the loreclezole site (44).

The simplest explanation for our results is that the high affinity site present on $\alpha4\beta2\gamma2L$ receptors is a modified form of the classic diazepam-binding site. This site no longer binds diazepam and other 1,4-benzodiazepines (31) but remains accessible to select imidazobenzodiazepines and to β-carbolines such as DMCM (Fig. 10). The $\alpha4\beta2\gamma2L$ receptors also have a distinct low affinity modulatory site. Diazepam and DMCM are positive modulators at this site, and low potency effects of CGS-9895 may also be mediated via this mechanism. We did not test the β-subunit dependence of these effects to investigate whether they share the same profile as loreclezole (44). Nevertheless, $\alpha4\beta2\gamma2L$ receptors are robustly potentiated by loreclezole, leaving open the possibility that low-potency modulation of $\alpha4\beta2\gamma2L$ receptors by diazepam and DMCM is mediated by the loreclezole-binding site.

Conclusions. $GABA_A$ receptors containing α4 subunits are thought to represent a major diazepam-insensitive subtype in mammalian forebrain (31). Our data show that the human $\alpha4\beta2\gamma2L$ receptor exhibits a distinct and novel pharmacological profile to benzodiazepine-site ligands. The unique pharmacology of α4-containing receptors may lead to a more precise understanding of the mechanisms underlying behavioral effects of $GABA_A$ modulators and to the design of drugs with novel or improved therapeutic profiles.

References

1. MacDonald, R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17**:569–602 (1994).
2. Olsen, R. W., and A. J. Tobin. Molecular biology of $GABA_A$ receptors. *FASEB J.* **4**:1469–1480 (1990).
3. Gutiérrez, A., Z. U. Khan, and A. L. De Blas. Immunocytochemical localization of γ_2 short and γ_2 long subunits of the $GABA_A$ receptor in the rat brain. *J. Neurosci.* **14**:7168–7179 (1994).
4. Haefely, W. E. Allosteric modulation of the $GABA_A$ receptor channel: a mechanism for interaction with a multitude of central nervous system functions, in *The Challenge of Neuropharmacology* (H. Möhler and M. Da Prada, eds.). Roche, Basel, 15–39 (1994).
5. McKernan, R. M., K. Quick, R. Prince, P. A. Cox, N. P. Gillard, C. I. Ragan, and P. Whiting. $GABA_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties. *Neuron* **7**:667–676 (1991).
6. Verdoorn, T. A., A. Draguhn, S. Ymer, P. H. Seeburg, and B. Sakmann. Functional properties of recombinant rat $GABA_A$ receptors depend on subunit composition. *Neuron* **4**:919–928 (1990).
7. Sigel, E., R. Baur, G. Trube, H. Möhler, and P. Malherbe. The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron* **5**:703–711 (1990).
8. Ebert, B., K. A. Wafford, P. J. Whiting, P. Krogsgaard-Larsen, and J. A. Kemp. Molecular pharmacology of γ-aminobutyric acid $type_A$ receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor subunit combinations. *Mol. Pharmacol.* **46**:957–963 (1994).
9. Ducic, I., H. J. Caruncho, W. J. Zhu, S. Vicini, and E. Costa. γ-Aminobutyric acid gating of Cl^- channels in recombinant $GABA_A$ receptors. *J. Pharmacol. Exp. Ther.* **272**:438–445 (1994).
10. Pritchett, D. B., and P. H. Seeburg. γ-Aminobutyric acid A receptor α5-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.* **54**:1802–1804 (1990).
11. Lüddens, H., and W. Wisden. Function and pharmacology of multiple $GABA_A$ receptor subunits. *Trends Pharmacol. Sci.* **12**:49–51 (1991).
12. Wafford, K. A., C. J. Bain, K. Quirk, R. M. McKernan, P. B. Wingrove, P. J. Whiting, and J. A. Kemp. A novel allosteric modulatory site on the $GABA_A$ receptor β subunit. *Neuron* **12**:775–782 (1994).
13. 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).
14. Wisden, W., D. J. Laurie, H. Monyer, and P. H. Seeburg. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **12**:1040–1062 (1992).
15. Sieghart, W. $GABA_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug binding sites. *Trends Pharmacol. Sci.* **13**:446–450 (1992).
16. Smith, M. C., and B. J. Riskin. The clinical use of barbiturates in neurological disorders. *Drugs* **42**:365–378 (1991).
17. Puia, G., M. Santi, S. Vicini, D. B. Pritchett, R. H. Purdy, S. M. Paul, P. H. Seeburg, and E. Costa. Neurosteroids act on recombinant human $GABA_A$ receptors. *Neuron* **4**:759–765 (1990).
18. Hawkinson, J. E., C. L. Kimbrough, D. Belelli, J. J. Lambert, R. H. Purdy, and N. C. Lan. Correlation of neuroactive steroid modulation of [^{35}S]*t*-butylbicyclophosphorothionate and [^{3}H]flunitrazepam binding and γ-aminobutyric $acid_A$ receptor function. *Mol. Pharmacol.* **46**:977–985 (1994).
19. Gee, K. W., L. D. McCauley, and N. C. Lan. A putative receptor for neurosteroids on the $GABA_A$ receptor complex: the pharmacological properties and therapeutic potential of epalons. *Crit. Rev. Neurobiol.* **9**:207–227 (1995).
20. Woodward, R. M., L. Polenzani, and R. Miledi. Effects of fenamates and other nonsteroidal anti-inflammatory drugs on rat brain $GABA_A$ receptors expressed in *Xenopus* oocytes. *J. Pharmacol. Exp. Ther.* **268**:806–817 (1994).
21. Harris, B. D., G. Wong, E. J. Moody, and P. Skolnick. Different subunit requirements for volatile and nonvolatile anesthetics at γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **47**:363–367 (1995).
22. Sivilotti, L., and A. Nistri. GABA receptor mechanisms in the central nervous system. *Prog. Neurobiol.* **36**:35–92 (1991).
23. Sieghart, W. Structure and pharmacology of *gamma*-aminobutyric $acid_A$ receptor subtypes. *Pharmacol. Rev.* **47**:181–234 (1995).
24. Malminiemi, O., and E. R. Korpi. Diazepam-insensitive [^{3}H]Ro15–4513 binding in intact cultured cerebellar granule cells. *Eur. J. Pharmacol.* **169**:53–60 (1989).
25. Turner, D. M., D. W. Sapp, and R. W. Olsen. The benzodiazepine/alcohol antagonist Ro 15–4513: binding to a $GABA_A$ receptor subtype that is insensitive to diazepam. *J. Pharmacol. Exp. Ther.* **257**:1236–1242 (1995).
26. Pritchett, D. B., H. Lüddens, and P. H. Seeburg. Type I, and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
27. Pritchett, D. B., and P. H. Seeburg. γ-Aminobutyric acid A receptor α5-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.* **54**:1802–1804 (1990).
28. Lüddens, H., D. B. Pritchett, M. Köhler, I. Killisch, K. Keinänen, H. Monyer, R. Sprengel, and P. H. Seeburg. Cerebellar $GABA_A$ receptor selective for a behavioral alcohol antagonist. *Nature (Lond.)* **346**:648–651 (1990).
29. Ymer, S., A. Draguhn, M. Köhler, P. R. Schofield, and P. H. Seeburg. Sequence and expression of a novel $GABA_A$ receptor α subunit. *FEBS Lett.* **258**:119–122 (1989).
30. Wisden, W., A. Herb, H. Wieland, K. Keinänen, H. Lüddens, and P. H. Seeburg. Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α4 subunit. *FEBS Lett.* **289**:227–230 (1991).
31. Yang, W., J. A. Drewe, and N. C. Lan. Cloning and characterization of the human $GABA_A$ receptor α4 subunit: identification of a unique diazepam-insensitive binding site. *Eur. J. Pharmacol.* **291**:319–325 (1995).
32. Ito, Y., E. Abiko, K. Mitaani, and H. Fukuda. Characterization of diazepam-insensitive [^{3}H]Ro15–4513 binding in rodent brain and cultured cerebellar neuronal cells. *Neurochem. Res.* **19**:289–295 (1994).
33. Whittemore, E. R., W. Yang, J. A. Drewe, and R. M. Woodward. Functional and pharmacological characterization of the human $GABA_A$ receptor α4 subunit expressed in *Xenopus* oocytes. *Soc. Neurosci. Abstr.* **21**:850 (1995).
34. Woodward, R. M., L. Polenzani, and R. Miledi. Effects of steroids on γ-aminobutyric acid receptors expressed in *Xenopus* oocytes by $poly(A)^+$ RNA from mammalian brain and retina. *Mol. Pharmacol.* **41**:89–103 (1992).
35. Hawkinson, J. A., J. A. Drewe, C. L. Kimbrough, J. S. Chen, D. J. Hogenkamp, N. C. Lan, K. W. Gee, K. Z. Shen, E. R. Whittemore, and R. M. Woodward. 3α-Hydroxy-3β-trifluoromethyl-5α-pregnan-20-one (Co 2–1970): a partial agonist at the neuroactive steroid site of the γ-aminobutyric $acid_A$ receptor. *Mol. Pharmacol.* **49**:897–906 (1996)
36. Nakahiro, M., O. Arakawa, T. Narahashi, S. Ukai, Y. Kato, K. Nishinuma, and T. Nishimura. Dimethyl sulfoxide (DMSO) blocks GABA-induced current in rat dorsal root ganglion neurons. *Neurosci. Lett.* **138**:5–8 (1992).
37. Draguhn, A., T. A. Verdorn, 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).
38. Haefely, W., M. Fackham, P. Schoch, J. R. Martin, E. P. Bonetti, J.-L. Moreau, F. Jenck, and J. G. Richards. Partial agonists of benzodiazepine receptors for the treatment of epilepsy, sleep, and anxiety disorders, in *GABAergic Synaptic Transmission* (G. Biggio, A. Concas, and E. Costa, eds.). Raven Press, New York, 379–394 (1992).
39. Bennett, D. A. Pharmacology of the pyrazolo-type compounds: agonist, antagonist and inverse agonist actions. *Physiol. Behav.* **41**:241–245 (1987).
40. Capogna, M., N. Berretta, F. Berton, R. Bianchi, M. Brunelli, and W. Francesconi. The β-carboline derivative DMCM decreases γ-aminobutyric acid responses and Ca^{2+}-mediated K^+-conductance in rat neocortical neurons *in vitro*. *Neuropharmacology* **33**:875–883 (1994).
41. Im, H. K., W. B. Im, D. B. Carter, and D. D. McKinley. Interaction of β-carboline inverse agonists for the benzodiazepine site with another site on $GABA_A$ receptors. *Br. J. Pharmacol.* **114**:1040–1044 (1995).
42. Zhang, C. L., and U. Heinemann. Effects of the triazole derivative loreclezole (R72063) on stimulus induced ionic and field potential responses and on different patterns of epileptiform activity induced by low magnesium in rat entorhinal cortex-hippocampal slices. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346**:581–587 (1992).
43. Wingrove, P. B., K. A. Wafford, C. Bain, and P. J. Whiting. The modulatory action of loreclezole at the *gamma*-aminobutyric acid type A receptor is determined by a single amino acid in the β_2 and β_3 subunit. *Proc. Natl. Acad. Sci. USA* **91**:4569–4573 (1994).
44. Stevenson, A., P. B. Wingrove, P. J. Whiting, and K. A. Wafford. β-Carboline γ-aminobutyric $acid_A$ receptor inverse agonists modulate γ-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. *Mol. Pharmacol.* **48**:965–969 (1995).
45. Kern, W., and W. Sieghart. Polyclonal antibodies directed against an epitope specific for the α4-subunit of $GABA_A$ receptors identify a 67-kDa protein in rat brain membranes. *J. Neurochem.* **62**:764–769 (1994).
46. 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).
47. Hadingham, K. L., E. M. Garrett, K. A. Wafford, C. Bain, R. P. Heavens, J. S. Sirinathsinghji, and P. J. Whiting. Cloning of cDNAs encoding the human γ-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6-containing receptors. *Mol. Pharmacol.* **49**:253–259 (1996).
48. Busto, U., H. L. Kaplan, L. Zawertailo, and E. M. Sellers. Pharmacologic effects and abuse liability of bretazenil, diazepam, and alprazolam in humans. *Clin. Pharmacol. Ther.* **55**:451–463 (1994).
49. Wong, G., K. F. Koehler, P. Skolnick, Z. Q. Gu, D. Ananthan, P. Schonholzer, W. Hunkeler, W. Zhang, and J. M. Cook. Synthetic and computer-assisted analysis of the structural requirements for selective, high-affinity ligand binding to diazepam-insensitive benzodiazepine receptors. *J. Med. Chem.* **36**:1820–1830 (1993). [

Send reprint requests to: Dr. Edward R. Whittemore, CoCensys, Inc., 1003 Health Sciences Rd. W., Irvine, CA 92715.