0026-895X/97/010114-06$3.00/0

MOLECULAR PHARMACOLOGY, **52:**114–119 (1997).

Lactone Modulation of the γ-Aminobutyric Acid A Receptor: Evidence for a Positive Modulatory Site

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Received December 19, 1996; Accepted March 31, 1997

SUMMARY

The γ-aminobutyric acid-A ($GABA_A$) receptor complex is allosterically modulated by a variety of substances, some of clinical importance. Barbiturates and neurosteroids augment GABA-currents and also directly gate the channel. A variety of γ-butyrolactone analogues also modulate GABA-induced currents, with some potentiating and others inhibiting. Because several γ-thiobutyrolactone analogues have biphasic effects on GABA currents, experiments with wild-type and picrotoxinin-insensitive $GABA_A$ receptors were performed to analyze whether some γ-thiobutyrolactones interact with two distinguishable sites on the $GABA_A$ receptor. β-Ethyl-β-methyl-γ-thiobutyrolactone inhibited GABA-induced currents at low concentrations (0.001–1 mM), but potentiated GABA-induced currents at higher concentrations (3–10 mM) in wild-type $\alpha1\beta2\gamma2$-subunit containing ionophores. The related α-ethyl-α-methyl-γ-thiobutyrolactone potentiated submaximal GABA currents in wild-type receptors at both low and high concentrations (0.1–10 mM). Mutations in the second transmembrane domain of α1, β2, or γ2 conferred picrotoxinin-insensitivity onto $GABA_A$ receptor complexes. When these mutated α1, β2, or γ2 subunits were incorporated into the receptor complex, β-ethyl-β-methyl-γ-thiobutyrolactone potentiated GABA currents over the entire concentration range (0.1–10 mM). Neither the potentiating activity nor the EC_{50} of α-ethyl-α-methyl-γ-thiobutyrolactone changed in the mutant receptors. Further studies demonstrated that the mutations did not affect the EC_{50} of chlordiazepoxide or phenobarbital. These and our earlier results identify a modulatory site on the $GABA_A$ receptor distinct from that interacting with barbiturates, benzodiazepines, and steroids. Additionally, they show that the γ-butyrolactones probably interact at two different sites on the ionophore to produce opposite effects on GABA-mediated current.

The $GABA_A$ receptor is responsible for most fast inhibitory neurotransmission in the central nervous system. Consequently, this receptor has been targeted for the pharmacological control of anxiety, sleep, and epilepsy. Numerous natural and synthetic compounds interact with the $GABA_A$ receptor at distinct, yet incompletely defined, sites. These compounds include barbiturates, benzodiazepines, neurosteroids, and picrotoxin (1, 2).

γ-Butyrolactones and the related TBLs are a group of convulsant and anticonvulsant agents that interact with the $GABA_A$ receptor, but not at the benzodiazepine or barbiturate sites (3, 4). Behavioral studies have demonstrated the protective effects of these compounds against various seizure models (5). Displacement studies with [^{35}S]TBPS, a ligand binding at the picrotoxinin site (6), suggested an interaction between the TBLs and the picrotoxinin site on the $GABA_A$ receptor. The hypothesis that TBLs interacted with the $GABA_A$ receptor at the picrotoxinin site as either agonists or inverse agonists (i.e., blockers or potentiators of GABA-induced currents, respectively) explained most of the findings (7).

The agonist/inverse agonist concept was not novel, because benzodiazepine receptor ligands can negatively (e.g., DMCM) and positively (e.g., diazepam) modulate channel behavior (8). The benzodiazepine receptor ligands are thought to act at just one site (9, 10).

Several new observations led to a re-evaluation of the TBL agonist/inverse agonist model. Holland and colleagues (11, 12) showed biphasic effects of several TBLs on GABA currents. They found that the same compound could both block and potentiate GABA currents, albeit with different time and concentration dependencies. They also observed noncompet-

This work was supported in part by National Institutes of Health Grants NS14834, NS07027, and AA09212, the Monsanto Fund, and the Seay Fellowship.

ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid-A; TBL, γ-thiobutyrolactone; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; TBPS, *tert*-butyl-bicyclophosphorothionate; α-EMTBL, α-ethyl-α-methyl-γ-thiobutyrolactone; β-EMTBL, β-ethyl-β-methyl-γ-thiobutyrolactone; TM2, second transmembrane domain; ANOVA, analysis of variance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

itive interactions between [^{35}S]TBPS and some TBLs, suggesting two sites of interaction: a negative modulatory site (the picrotoxinin site) and a yet-to-be-defined, positive modulatory site (the lactone site). In the present studies picrotoxinin-insensitive $GABA_A$ receptor mutants were used to test this hypothesis. If the two-site model for lactone interaction with the receptor were correct, the biphasic nature of the TBLs on GABA currents should disappear, when one site was ablated.

Materials and Methods

$GABA_A$ subunits. The cDNAs encoding $GABA_A$ subunits $\alpha1$ were provided by A. Tobin (University of California, Los Angeles), $\beta2$ by P. Malherbe (Hoffman-La Roche, Switzerland), and $\gamma2_L$ by C. Fraser (National Institute on Alcohol Abuse and Alcoholism).

Mutagenesis. Mutations were created as previously described (13).

Compounds. GABA, chlordiazepoxide hydrochloride, and phenobarbital were obtained from Sigma (St. Louis, MO), and DMCM was obtained from Research Biochemicals International (Natick, MA). α-EMTBL and β-EMTBL were synthesized by previously described methods and had the appropriate analytical and spectroscopic properties (5).

cRNA preparation. The cRNA transcripts were prepared in the following manner. The 5′- and 3′-untranslated region of cDNA templates were removed to improve channel expression, and an optimal Kozak (14) sequence (CCACC) was added upstream of the initiator methionine to enhance mRNA binding to the 40 S ribosomal subunit. cDNA sequences used in the study were verified by automated nucleotide sequence analysis using the Applied Biosystems Incorporated (ABI; Foster City, CA) Prism Dye Terminator cycle sequencing system and an ABI 373A DNA sequencer. The modified cDNA templates were linearized with the appropriate restriction enzyme, and transcripts were prepared with Ambion's mMessage mMachine kit (Austin, TX). As a final check, each cRNA preparation was subjected to either polyacrylamide or agarose gel analysis to verify size and quantity.

Oocyte collection and injection. Oocyte preparation was modified from the methods of Gurley *et al.* (13). *Xenopus laevis* oocytes were collected from mature, pigmented females obtained from Xenopus One (Northland, MI). After removal, the oocytes were placed in an OR-2 solution containing 100 units of penicillin and 100 μg/ml streptomycin obtained from Sigma and 2 mg/ml collagenase I from Boehringer-Mannheim (Indianapolis, IN). The oocytes were agitated vigorously until the follicular layer was removed. Usually, the oocytes were injected the same day with a 20–60-ng mixture of α, β, and γ subunits, using the Drummond Nanojector (Broomall, PA). The oocytes were incubated for 1–3 days in L-15 medium (GIBCO, Grand Island, NY) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin sulfate (Sigma).

Electrophysiology and data analysis. The electrophysiology was carried out using Warner Instrument's 725C (Hamden, CT) two-electrode voltage clamp with a virtual ground bath clamp. Electrodes filled with 3 M KCl (resistance < 1 MΩ) were used to impale the oocytes. The oocytes were clamped at a potential of -70 mV. Peak currents were recorded, low pass filtered at 20–30 Hz by an 8-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized by a TL-1 DMA interface (Axon Instruments, Foster City, CA), and stored using Axon Instruments' AxoTape. The oocytes were perfused with a solution containing the following: 90 mM NaCl, 1 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES and 1 mM $CaCl_2$ (pH 7.3). Solutions were bath applied by a gravity-driven perfusion system until the response reached plateau or until a rapidly desensitizing response peaked, which took anywhere from 3 to 45 sec approximately. No correction in peak amplitude was made for quickly desensitizing responses that were common at high GABA concentrations, high drug concentrations, and also in mutated receptors. In the determination of the EC_{50} values of α-EMTBL, β-EMTBL, and chlordiazepoxide, the GABA EC_{10} was used as a control. Data were fit using a logistic equation (SigmaPlot; Jandel Scientific, Sausalito, CA), $y = [(a - d)/(1 + (x/EC_{50})^{nH})] + d$, where y is the response, a is the asymptotic maximum, d is the asymptotic minimum, x is the ligand concentration, and n_H is the Hill coefficient. In situations in which the 10 mM response was smaller than the 3 mM response, the 10 mM value was not used in the fitting procedure. This value was ignored, as it probably reflected some degree of both channel block and desensitization in addition to the potentiation; fitting such complex behavior is beyond the limits of the logistic equation. Even after elimination of the 10 mM response, concentration responses for α-EMTBL and β-EMTBL were fit well using the logistic equation and allowing the maximum to float.

Results

GABA sensitivity. To confirm that the double point mutations in the TM2 did not drastically alter the behavior of the channel, the GABA responses of the mutant ionophores were examined. Wild-type $\alpha1\beta2\gamma2$ $GABA_A$ receptors had an EC_{50} value of 42 μM; the GABA EC_{50} values of the channels containing mutated subunits changed by less than 5-fold (Fig. 1A). The apparent affinity of $\alpha1^M\beta2\gamma2$ and $\alpha1\beta2^M\gamma2$, but not $\alpha1\beta2\gamma2^M$, for GABA was significantly different from that of wild-type channels (ANOVA with Dunnett's test; $p < 0.05$). Although these shifts could reflect changes in GABA affinity for the receptor, they could also have resulted from altered GABA gating (15, 16).

Picrotoxin and picrotoxinin sensitivity of wild-type $\alpha1\beta2\gamma2$. Two point mutations in the TM2 of the $\alpha1$, $\beta2$, or $\gamma2$ GABA subunit or a single point mutation in the $\beta2$ subunit produce picrotoxin-insensitivity (13). The pharmacology of the $GABA_A$ receptor was studied using the approximate GABA EC_{10} as a control. Oocytes expressing the wild-type $GABA_A$ receptor were inhibited by both picrotoxin and picrotoxinin (the active component of picrotoxin), with IC_{50} values of 1.2 and 0.8 μM, respectively (data not shown).

Oocytes injected with $\alpha1\beta2^M\gamma2$ showed a similar insensitivity to picrotoxin and picrotoxinin (Fig. 1B) (13). The other two mutant complexes ($\alpha1^M\beta2\gamma2$ and $\alpha1\beta2\gamma2^M$) also were picrotoxinin-insensitive (Fig. 1B). Because of the small magnitude of currents observed when two or more mutated subunits were co-injected, such receptor complexes were not studied.

β-EMTBL modulation of wild-type and mutant receptors. β-EMTBL both inhibits and potentiates GABA currents in primary cultures of hippocampal neurons (12). At concentrations less than 1 mM, β-EMTBL inhibited GABA currents in wild-type receptors; at 1 mM, β-EMTBL partially relieved its own block; finally, at 3 and 10 mM β-EMTBL, the macroscopic current was potentiated relative to control (Fig. 2A). This biphasic modulation was even more clearly seen in the concentration response curve (Fig. 2C).

In contrast to its activity in wild-type receptors, β-EMTBL did not inhibit GABA currents in the picrotoxinin-insensitive channels (Fig. 2B). Below 300 μM β-EMTBL, GABA currents were no different from control or only slightly potentiated. Above this concentration range, potentiation was robust and occasionally greater than that seen in the wild-type (Fig. 2C).

α-EMTBL modulation of wild-type and mutant receptors. α-EMTBL is one of the most potent and efficacious

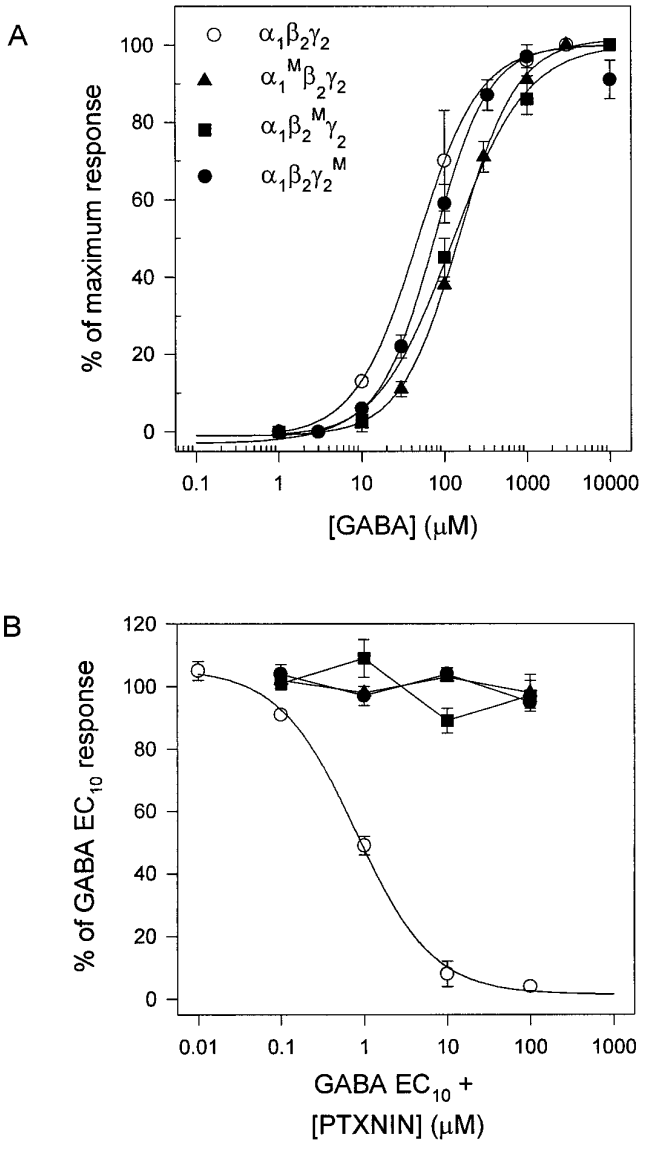

Fig. 1. A, GABA concentration-response curves in $\alpha1\beta2\gamma2$, $\alpha1^{M}\beta2\gamma2$, $\alpha1\beta2^{M}\gamma2$, and $\alpha1\beta2\gamma2^{M}$-subunit containing ionophores. The EC_{50} values and Hill coefficients were, respectively, 40 μM and 1.6 ($n = 9$); 172 μM and 1.3 ($n = 5$); 114 μM and 1.0 ($n = 3$); 78 μM and 1.4 ($n = 3$). The $\alpha1^{M}\beta2\gamma2$ and $\alpha1\beta2^{M}\gamma2$ EC_{50} values were significantly different ($p < 0.05$) from $\alpha1\beta2\gamma2$ by a one-way ANOVA with Dunnett's test; the EC_{50} for $\alpha1\beta2\gamma2^{M}$ was not significantly different ($p > 0.05$). The GABA EC_{10} concentrations for each receptor combination are: 10 μM ($\alpha1\beta2\gamma2$), 27 μM ($\alpha1^{M}\beta2\gamma2$), 18 μM ($\alpha1\beta2^{M}\gamma2$), and 16 μM ($\alpha1\beta2\gamma2^{M}$). B, Picrotoxinin (*PTXNIN*) concentration-response in wild-type $\alpha1\beta2\gamma2$ (○) and mutant $\alpha1^{M}\beta2\gamma2$ (▲), $\alpha1\beta2^{M}\gamma2$ (■), and $\alpha1\beta2\gamma2^{M}$ (●).

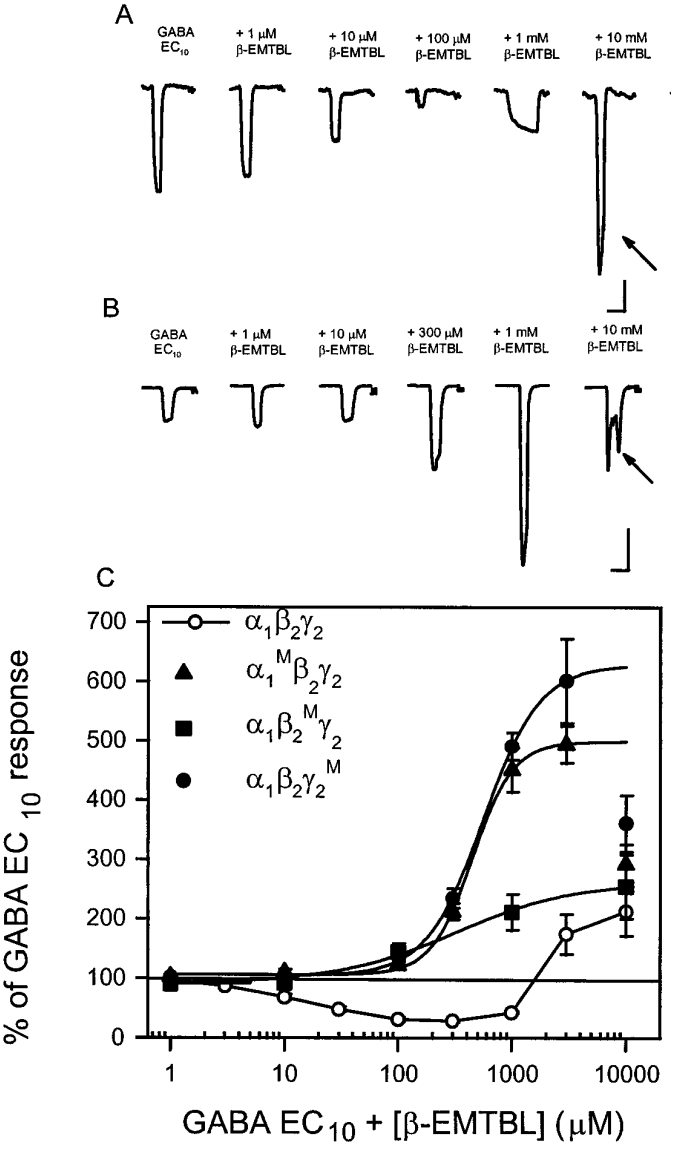

Fig. 2. β-EMTBL concentration response in wild-type and picrotoxinin-insensitive $\alpha1\beta2\gamma2$-subunit containing ionophores. *Arrows,* small off-currents seen at 10 mM drug. A, GABA currents modulated by β-EMTBL in $\alpha1\beta2\gamma2$, demonstrating its ability to inhibit and potentiate a GABA-gated current. Calibration bars: 25 nA, 1 min. B, GABA currents modulated by β-EMTBL in $\alpha1^{M}\beta2\gamma2$, demonstrating its ability to only potentiate a GABA-gated current. Calibration bars: 100 nA, 1 min. C, The β-EMTBL concentration-response in wild-type and $\alpha1^{M}\beta2\gamma2$, $\alpha1\beta2^{M}\gamma2$, and $\alpha1\beta2\gamma2^{M}$. Note that the biphasic modulation is lost in the picrotoxinin-insensitive ionophores.

anticonvulsant TBL analogues. It does not produce biphasic modulation like β-EMTBL, suggesting it does not interact strongly with the picrotoxinin receptor. With oocytes expressing wild-type $\alpha1\beta2\gamma2$ $GABA_A$ receptors, α-EMTBL potentiated submaximal GABA currents. At 10 mM, α-EMTBL exhibited less potentiation than at 3 mM and also exhibited off-currents (Fig. 3A).

α-EMTBL's activity was unchanged in the mutated receptor complexes (Fig. 3B). Potentiation was observed over the entire concentration range, even though the 10 mM response once again fell off. Quantitatively, the EC_{50} values were relatively unaffected: α-EMTBL's EC_{50} in the picrotoxinin-insensitive channels differed by less than 20% from wild-type. Yet, the efficacy for one of the complexes ($\alpha1^{M}\beta2\gamma2$) was 50% greater than wild-type (Fig. 3C).

Chlordiazepoxide and phenobarbital modulation. Double point mutations in the TM2 sufficed to eliminate picrotoxinin modulation of the channel; moreover, GABA concentration responses from two types of picrotoxinin-insensitive channels were significantly different from wild-type channels. To address whether these mutations affected more than picrotoxinin and β-EMTBL modulation, we examined three other allosteric modulators of the channel: a benzodiazepine agonist, benzodiazepine inverse agonist, and a barbiturate. Each compounds' activity was qualitatively unaf-

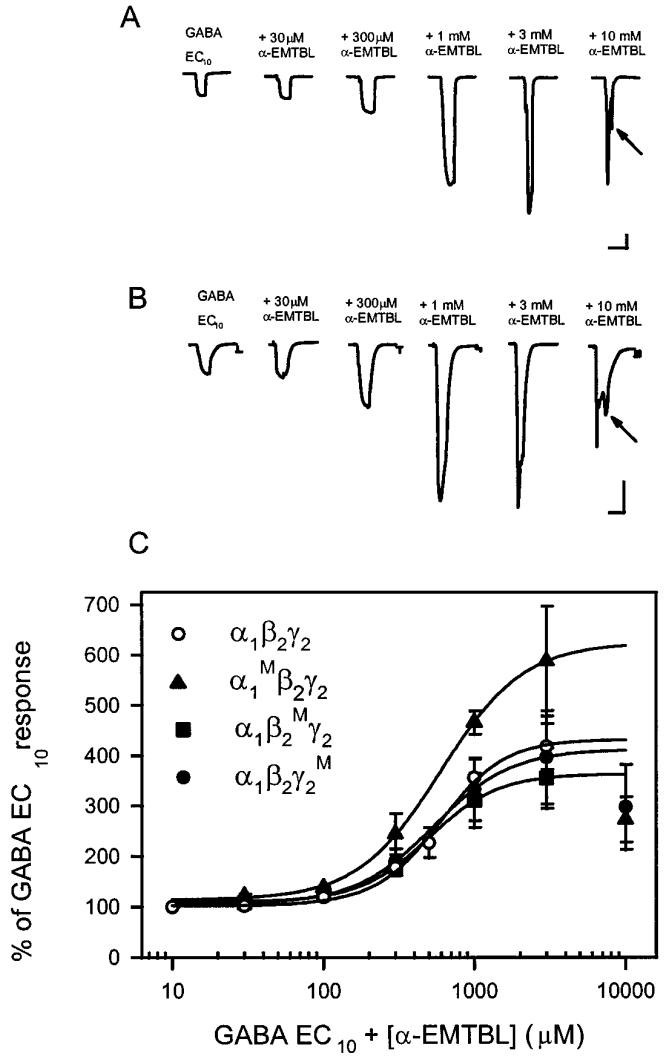

Fig. 3. α-EMTBL concentration response in wild-type and picrotoxinin-insensitive α1β2γ2-subunit containing ionophores. *Arrows,* off-currents seen at 10 mM drug. A, GABA currents modulated by α-EMTBL in α1β2γ2, demonstrating its ability to potentiate a GABA-gated current. *Calibration bars*, 100 nA, 1 min. B. GABA currents modulated by α-EMTBL in $\alpha1^M\beta2\gamma2$, demonstrating its ability to only potentiate a GABA-gated current. *Calibration bars*, 100 nA, 1 min. C, The α-EMTBL concentration response in α1β2γ2, $\alpha1^M\beta2\gamma2$, $\alpha1\beta2^M\gamma2$, and $\alpha1\beta2\gamma2^M$. The EC_{50} values and Hill coefficients are: 597 μM and 2.0, 600 μM and 1.6, 501 μM and 1.9, and 537 μM and 1.7, respectively. The differences in EC_{50} values of the mutants were not significantly different from wild-type values, as assessed by a one-way ANOVA ($p = 0.20$).

fected by the double point mutations in the TM2. Chlordiazepoxide's EC_{50} was not changed by more than 4-fold by the mutations (Table 1); only the EC_{50} for $\alpha1^M\beta2\gamma2$ was statistically different from the wild-type EC_{50}. The efficacy, however, was quite variable, with the α1 mutant giving the most robust response (Table 2).

DMCM negatively modulates the GABA receptor through the benzodiazepine site, which is demonstrated by flumazenil antagonism (17). In the γ2 mutant DMCM's efficacy was unchanged, confirming that the point mutations abolished only picrotoxinin's negative modulation (Table 2).

Phenobarbital could not be modeled using the logistic equation, because its concentration response reflects not only potentiation of GABA-gated currents, but also direct gating

TABLE 1

Determination of EC_{50} values of chlordiazepoxide and phenobarbital in wild-type and picrotoxinin-insensitive mutants

Ionophore composition	EC_{50}	
	Chlordiazepoxide	Phenobarbital
	μM	
α1β2γ2 (wt)	3.1	ND[a]
$\alpha1^M\beta2\gamma2$	11.4[b]	ND
$\alpha1\beta2^M\gamma2$	1.0	ND
$\alpha1\beta2\gamma2^M$	1.2	ND

[a] ND, not determined.
[b] Different ($p < 0.05$) from wild-type mutants by ANOVA followed by Dunnett's test.

by phenobarbital. Hence, only its efficacy is reported, but there were no significant differences (one-way ANOVA: $p = 0.42$) in phenobarbital efficacy by mutations in the TM2 (Table 2).

Flumazenil inhibition. Last, flumazenil was used to test whether the lactone interacted with the ionophore at the benzodiazepine receptor. Flumazenil was unable to block α-EMTBL potentiation in a wild-type α1β2γ2-containing ionophore, providing additional evidence that the lactone site is distinct from the benzodiazepine site (Fig. 4).

Discussion

The results in this study alter our early view that lactones modulate $GABA_A$ currents through action at a single site on $GABA_A$ channels, the picrotoxinin receptor. Because picrotoxinin contains a γ-butyrolactone ring as part of its structure, it seemed reasonable that the neuroactive γ-butyrolactone analogues could interact at the $GABA_A$ receptor through the picrotoxinin binding site (18, 19). Supporting evidence for this hypothesis came from [^{35}S]TBPS displacement studies that revealed a high affinity, competitive interaction between β-substituted TBLs and the picrotoxinin receptor. Moreover, the anticonvulsant α-substituted TBLs also displayed an ability to displace TBPS, albeit with less potency (20). Furthermore, modeling of picrotoxinin and different lactones demonstrated potential regions for interaction with the channel interior (19). Therefore, the agonist/inverse agonist model of lactone activity seemed firmly grounded both experimentally and theoretically.

Recent findings prompted us to consider the hypothesis that lactones may interact at two sites (11, 12). To demonstrate clearly that some TBLs interact with two sites, one of the sites had to be functionally, even if not physically, absent. With the picrotoxinin-like effect absent, β-EMTBL only potentiated GABA currents. The affinity and potentiation activity of α-EMTBL was unchanged, suggesting that the α-substituted lactones have, at best, weak interactions with the picrotoxinin site.

Two issues were considered in interpreting the data. Inasmuch as the mutations were introduced into the TM2, a region of the $GABA_A$ subunits critical for proper channel function, it is possible that these mutations could have conformational consequences that led to the loss of the biphasic activity of β-EMTBL. Although the observed picrotoxinin insensitivity could have resulted from disruption of the picrotoxinin binding site, it could also be explained by restricted access to a binding site, or altered channel conformation after

TABLE 2
Efficacy as percentage of GABA EC_{10} response

Ionophore composition	Chlordiazepoxide[a]	DMCM[a,b]	Phenobarbital[a,c]
		%	
$\alpha1\beta2\gamma2$	397 ± 23	69 ± 5	1129 ± 87
$\alpha1^{M}\beta2\gamma2$	594 ± 16	ND[d]	823 ± 113[c]
$\alpha1\beta2^{M}\gamma2$	196 ± 9	ND	935 ± 78[c]
$\alpha1\beta2\gamma2^{M}$	282 ± 10	60 ± 4	1113 ± 236[c]

[a] Mean ± SEM (n = 3).
[b] The mutant channel efficacy was not significantly different from wild-type channel as determined by a *t* test ($p = 0.23$).
[c] The mutant channels' efficacies were not significantly different from wild-type channels as determined by one-way ANOVA ($p = 0.42$).
[d] ND, not determined.

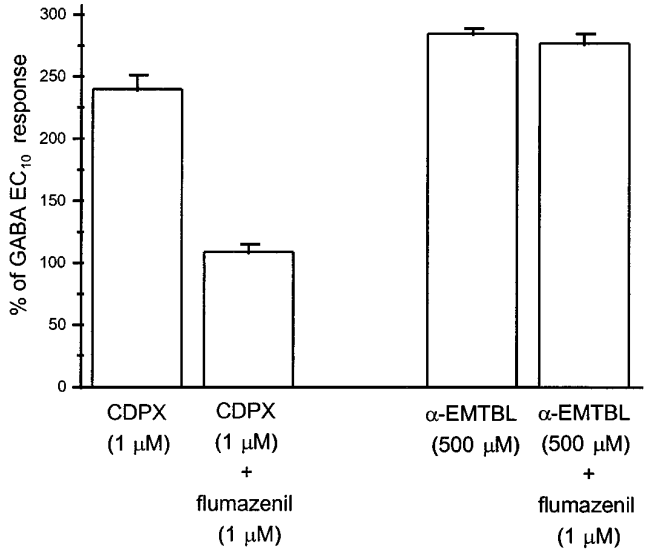

Fig. 4. Comparison of the ability of 1 μM flumazenil to inhibit the approximate EC_{50} of both chlordiazepoxide and α-EMTBL. Flumazenil did inhibit chlordiazepoxide (*CDPX*) (two-sided *t* test: $p < 0.0001$), but not α-TBL (two-sided *t* test: $p = 0.2$).

picrotoxinin binding. If conformational changes were disturbed by the mutations, then the loss of β-EMTBL's biphasic activity would be difficult to ascribe to two binding sites. If this were the case, the activity of other modulators might be expected to change as well. Given that the mutants' responses to GABA, α-EMTBL, chlordiazepoxide, DMCM, and phenobarbital are qualitatively similar to responses in the wild-type channel, these picrotoxinin-insensitive channels do not appear to alter the conformational equilibria. Therefore, these mutations appear to target specifically the picrotoxinin site and do not alter macroscopic responses to other modulators of $GABA_A$ currents.

A second concern in interpreting the data was the decrease in potentiation seen at 10 mM lactone (Figs. 2B and 3, A and B). The fall-off in potentiation can be attributed to desensitization or to block at a second site at high drug concentrations (12). The contribution of desensitization can be inferred from similar fall-offs in current seen with high concentration GABA responses (10 mM), chlordiazepoxide (100 μM; data not shown), and phenobarbital (3 mM; data not shown). The other consideration, a second block site, is reflected by the off-currents observed with the washout of high lactone concentrations (*arrows* in Figs. 2, A and B, and 3, A and B). Similar block has also been seen with washout of 3 mM phenobarbital, so this is not necessarily unique to the lactones.

We believe that our data can be most parsimoniously explained by a lactone site that is distinct from the barbiturate, benzodiazepine, and steroid sites (4, 7, 21–23). Our next goal is to localize the lactone site and determine the physical explanation for its influence on $GABA_A$ channel conductance. We believe that this site represents a logical target for drugs that will be effective therapy for epilepsy, spasticity, and sleep disorders.

References

1. Sieghart, W. $GABA_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug-binding sites. *Trends Pharmacol. Sci.* **13:**446–450 (1992).
2. Smith, G. B., and C. E. Olsen. Functional domains of $GABA_A$ receptors. *Trends Pharmacol. Sci.* **16:**162–168 (1995).
3. Klunk, W. E., A. McKeon, D. F. Covey, and J. A. Ferrendelli. α-Substituted γ-butyrolactones: new class of anticonvulsant drugs. *Science (Washington D. C.)* **217:**1040–1042 (1982).
4. Mathews, G. C., A. M. Bolos-Sy, D. F. Covey, S. M. Rothman, and J. A. Ferrendelli. Physiological comparison of α-ethyl-α-methyl-γ-thiobutyrolactone with benzodiazepine and barbiturate modulators of $GABA_A$ receptors. *Neuropharmacology* **35:**123–136 (1996).
5. Canney, D. J., K. D. Holland, J. A. Levine, A. C. McKeon, J. A. Ferrendelli, and D. F. Covey. Synthesis and structure-activity studies of alkyl-substituted γ-butyrolactones and γ-thiobutyrolactones for the picrotoxin receptor. *J. Med. Chem.* **34:**1460–1467 (1991).
6. Squires, R. F., J. E. Casida, M. Richardson, and E. Saederup. [^{35}S]*t*-Butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to gamma-aminobutyric acid-A and ion recognition sites. *Mol. Pharmacol.* **23:**326–336 (1983).
7. Holland, K. D., J. A. Ferrendelli, D. F. Covey, and S. M. Rothman. Physiological regulation of the picrotoxin receptor by γ-butyrolactones and γ-thiobutyrolactones in cultured hippocampal neurons. *J. Neurosci.* **10:** 1719–1727 (1990).
8. Chan, C. Y., and D. H. Farb. Modulation of neurotransmitter action: control of the γ-aminobutyric acid response through the benzodiazepine receptor. *J. Neurosci.* **5:**2365–2373 (1985).
9. Braestrup, C., R. Schmiechen, G. Neef, M. Nielsen, and E. N. Petersen. Interaction of convulsive ligands with benzodiazepine receptors. *Science (Washington D. C.)* **216:**1241–1243 (1982).
10. Hunkeler, W., H. Moehler, L. Pieri, P. Polc, E. P. Bonetti, R. Cumin, R. Schaffner, and W. Haefely. Selective antagonists of benzodiazepines. *Nature (Lond.)* **290:**514–516 (1981).
11. Holland, K. D., M. G. Bouley, D. F. Covey, and J. A. Ferrendelli. Alkyl-substituted γ-butyrolactones act at a distinct site allosterically linked to the TBPS/picrotoxinin site on the $GABA_A$ receptor complex. *Brain Res.* **615:**170–174 (1993).
12. Holland, K. D., G. C. Mathews, A. M. Bolos-Sy, J. B. Tucker, P. A. Reddy, D. F. Covey, J. A. Ferrendelli, and S. M. Rothman. Dual modulation of the γ-aminobutyric acid type A receptor/ionophore by alkyl-substituted γ-butyrolactones. *Mol. Pharmacol.* **47:**1217–1223 (1995).
13. Gurley, D. A., J. Amin, P. C. Ross, D. S. Weiss, and G. White. Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. *Recept. Channels* **3:**13–20 (1995).
14. Kozak, M. An analysis of 5′ non-coding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15:**8125–8148 (1987).
15. 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).
16. Colquhoun, D., and M. Farrant. The binding issue. *Nature (Lond.)* **366:** 510–511 (1993).
17. Puia, G., M. R. Santi, S. Vicini, D. B. Pritchett, P. H. Seeburg, and E. Costa. Differences in the negative allosteric modulation of γ-aminobutyric acid receptors elicited by 4′-chlorodiazepam and by a β-carboline-3-carboxylate ester: a study with natural and reconstituted receptors. *Proc. Natl. Acad. Sci. USA* **86:**7275–7279 (1989).
18. Klunk, W. E., D. F. Covey, and J. A. Ferrendelli. Comparison of epileptogenic properties of unsubstituted and β-alkyl-substituted γ-butyrolactones. *Mol. Pharmacol.* **22:**431–437 (1982).
19. Klunk, W. E., B. L. Kalman, J. A. Ferrendelli, and D. F. Covey. Computer-assisted modeling of the picrotoxinin and γ-butyrolactone receptor site. *Mol. Pharmacol.* **23:**511–518 (1983).
20. Holland, K. D., A. C. McKeon, D. F. Covey, and J. A. Ferrendelli. Binding interactions of convulsant and anticonvulsant γ-butyrolactones and γ-thiobutyrolactones with the picrotoxin receptor. *J. Pharmacol. Exp. Ther.* **254:**578–583 (1990).
21. Mathews, G. C., A. M. Bolos-Sy, K. D. Holland, K. E. Isenberg, D. F. Covey, J. A. Ferrendelli, and S. M. Rothman. Developmental alteration in $GABA_A$

receptor structure and physiological properties in cultured cerebellar granule neurons. *Neuron* **13:**149–158 (1994).
22. Baker, K., J. Yang, D. F. Covey, D. B. Clifford, and C. F. Zorumski. α-Substituted thiobutyrolactones potentiate GABA currents in voltage-clamped chick spinal cord neurons. *Neurosci. Lett.* **87:**133–138 (1988).
23. Rodgers-Neame, N. T., D. F. Covey, Y. Hu, K. E. Isenberg, and C. F. Zorumski. Effects of a benz[*e*]indene on γ-aminobutyric acid-gated chloride currents in cultured postnatal rat hippocampal neurons. *Mol. Pharmacol.* **42:**952–957 (1992).

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