

Enantioselectivity of α -Benzyl- α -methyl- γ -butyrolactone-Mediated Modulation of Anticonvulsant Activity and GABA_A Receptor Function

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Received November 14, 2003; accepted January 20, 2004

ABSTRACT

Alkyl-substituted butyrolactones have both inhibitory and stimulatory effects on GABA_A receptors. Lactones with small alkyl substitutions at the α -position positively modulate the channel, whereas β -substituted lactones tend to inhibit the GABA_A receptor. These compounds mediate inhibition through the picrotoxin site of the receptor. A distinct binding site that mediates the stimulatory actions of lactones is presumed to exist, although no definitive evidence to support this claim exists. In the present study, we used in vivo and in vitro assays to evaluate the effects of the enantiomers of a novel lactone, α -benzyl- α -methyl- γ -butyrolactone (α -BnMeGBL), on the GABA_A receptor. *R*-(-)- α -BnMeGBL was 2-fold more potent than the *S*-(+)- α -BnMeGBL in blocking pentylenetetrazol-induced seizures in

CF-1 mice. The (+)-enantiomer inhibited binding of *t*-butylbicyclophosphorothionate with a higher affinity than the (-)-enantiomer (IC₅₀ of 0.68 and 1.1 mM, respectively). Whole cell patch-clamp recordings from recombinant α 1 β 2 γ 2 receptors stably expressed in HEK293 cells demonstrated that both compounds stimulated GABA-activated current. The maximal stimulation was approximately 2-fold greater with (+)- α -BnMeGBL than that seen with (-)- α -BnMeGBL. Both enantiomers of α -BnMeGBL directly gated the GABA_A receptor at mM concentrations, in a nonstereoselective manner. Our data demonstrate the stimulatory actions of α -BnMeGBL on GABA_A receptor function display enantioselectivity and provide strong evidence for the existence of a true "lactone site" on the receptor.

The γ -aminobutyric acid_A receptor (GABA_AR) is the predominant inhibitory receptor in the nervous system (Macdonald and Olsen, 1994). The receptor is composed of protein subunits (α , β , γ , δ , ϵ , ρ , θ , and π) (Barnard et al., 1998; Bonnert et al., 1999) that are arranged in a pentameric conformation (Barnard et al., 1998). Once activated by the ligand GABA, the protein changes its conformational structure from a closed to an open state, allowing Cl⁻ ions into the cell. GABA_ARs are members of the ligand-gated ion channel superfamily, which include glycine receptors, 5-hydroxytryptamine₃ receptors, and the nicotinic acetylcholine receptors (Ortells and Lunt, 1995). Several classes of compounds bind to the receptor and influence the channel's action. These

include the benzodiazepines, steroids, barbiturates, and the convulsant picrotoxin (Hevers and Luddens, 1998).

A novel class of compounds that regulate the GABA_A receptor are the γ -butyrolactones (GBLs) and the related γ -thiobutyrolactones (TBLs). The lactones have either positive or negative modulatory activity, depending on the substitution incorporated in the lactone ring. These compounds have been substituted with alkyl groups (Mathews et al., 1996) and fluorinated groups (Canney et al., 1998). The presence of relatively small substituents at the α -position generally results in anticonvulsant activity and potentiation of GABA-gated Cl⁻ current, whereas lactones with larger substituents at this position may display convulsant activity (Canney et al., 1991; Holland et al., 1992). Alkyl substitution at the β -position results in convulsant activity and a decrease in GABA-activated current (Klunk et al., 1982b; Holland et al., 1990b; Yoon et al., 1993), whereas unsubstituted γ -butyrolactone has no significant effect on GABA current (Feigenbaum and Howard, 1996).

This work was supported by National Institutes of Health Grant ES07904 (to G.H.D.) and NS14834 (to D.F.C.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.103.063008.

ABBREVIATIONS: GABA_AR, type A GABA receptor; GBL, γ -butyrolactone; TBL, γ -thiobutyrolactone; TBPS, *t*-butylbicyclophosphorothionate; α -BnMeGBL, α -benzyl- α -methyl- γ -butyrolactone; HEK, human embryonic kidney; THF, tetrahydrofuran; IR, infrared; PTZ, pentylenetetrazole.

After the discovery that lactones competitively inhibit binding of the picrotoxin-site ligand [^{35}S]TBPS to the GABA_AR (Levine et al., 1985; Holland et al., 1990a), it was concluded that the effects of lactones are due to binding at the picrotoxin site. Convulsant (GABA current-inhibiting) or anti-convulsant (GABA current-enhancing) activity was presumed to be due the ability of lactones to act as either positive or negative modulators, respectively, of the picrotoxin site (Holland et al., 1990a). Subsequent studies led to the belief that the inhibitory effects of the lactones are in fact due to an interaction at the picrotoxin site (Yoon et al., 1993; Williams et al., 1997) but that the positive modulatory actions are due to action at a "lactone site" (Holland et al., 1995; Williams et al., 1997).

Whether a true lactone binding site exists on the GABA_AR is unknown. It is possible, for example, that the GABA current-enhancing effects of lactones are secondary to relatively nonselective alterations of the local physiochemical environment, which could subsequently influence GABA_AR activity. One approach to provide evidence of a ligand binding site is demonstration of enantioselective preference. Enantiomers are nonsuperimposable mirror images of optically active molecules (i.e., compounds with at least one chiral center). They have identical physiochemical properties, and thus any effects of them due to alterations of membrane lipids would be identical (Guyer and Block, 1983; Arnett and Gold, 1986; Mannock et al., 2003; Westover et al., 2003). Targets that display an enantioselective preference are thus likely to have a specific binding site for the parent molecule. GABA_ARs have been shown to display enantioselective interactions with several compounds. These include the volatile anesthetics halothane (Harris et al., 1998) and isoflurane (Hall et al., 1994), barbiturates (Rho et al., 1996), and neurosteroids (Covey et al., 2001).

To more definitively evaluate the possibility that a lactone binding site exists on the GABA_AR, we have synthesized enantiomers of a novel butyrolactone, α -benzyl- α -methyl- γ -butyrolactone (α -BnMeGBL), and studied its actions using *in vitro* and *in vivo* approaches. Our results demonstrate that α -BnMeGBL enantioselectively interacts with GABA_A receptors and provide the strongest evidence to date in support of the existence of a lactone binding site on GABA_ARs.

Materials and Methods

Chemicals

α -BnMeGBL enantiomers (Fig. 1) and racemic solutions were made from a 1 M dimethyl sulfoxide stock and diluted in 5 μM GABA in normal saline. The final dimethyl sulfoxide concentrations in the

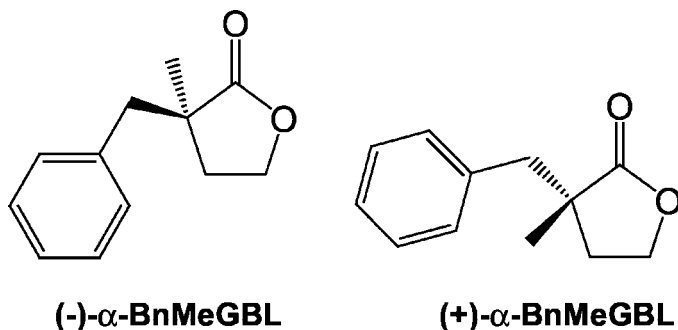


Fig. 1. Structure of α -benzyl-methyl- γ -butyrolactone enantiomers.

test solutions were less than 0.3% (v/v). [^{35}S]TBPS was obtained from PerkinElmer Life Sciences (Boston, MA).

Synthesis of Racemic α -BnMeGBL

General Methods. Unless otherwise noted, the starting materials were obtained from commercial suppliers and used without further purification. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride. Hexamethylphosphoramide was dried over activated 13 \times molecular sieves. Tetrahydrofuran (THF) was distilled over sodium-benzophenone. A Varian model 3700 or 3400 CX gas chromatograph equipped with a glass column (0.25-in. i.d., 6-ft length) packed with 15 SP2401 on 80/100 mesh Supelcoport (Supelco, Bellefonte, PA) was used to monitor the progress of the reactions and to assess the purity of the products. Solvents used for extractions were dried over either Na_2SO_4 or MgSO_4 , filtered, and removed on a rotary evaporator. Thin layer chromatography was performed on 250- μm Analtech silica gel plates containing a fluorescent indicator. Flash chromatography was carried out by using Scientific Adsorbents (Atlanta, GA) 40- μm silica gel. Bulb-to-bulb distillations were performed on an Aldrich Kugelrohr apparatus. Melting points were determined with either a Thomas-Hoover capillary or Kofler-type micro hot stage apparatus and are uncorrected. IR spectra were obtained with PerkinElmer 1710 FT-IR instrument as thin films on NaCl plate, and NMR spectra (^1H NMR and ^{13}C) were recorded in CDCl_3 solutions on a Varian Gemini-300 spectrometer. Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane as an internal standard. Elemental analyses were performed at M-H-W Laboratories (Phoenix, AZ).

Racemic α -BnMeGBL was prepared by benzylation of α -methyl- γ -butyrolactone. A solution of α -methyl- γ -butyrolactone (10.0 g, 100 mmol) in THF (15 ml) was added slowly (ca. 15 min) to a solution of lithium diisopropylamide [prepared from diisopropylamine (21.0 ml, 150 mmol) and *n*-butyllithium in hexanes (2.5 M, 57.2 ml, 143 mmol)] in THF (175 ml) at 0°C in a N_2 atmosphere, and the mixture was stirred for 30 min. The temperature was then decreased to -78°C, and a solution of benzyl bromide (26.9 g, 157 mmol) in THF (10 ml) and hexamethylphosphoramide (15 ml, 86.6 mmol) was added over a period of 10 min. After 2 h at -78°C, the reaction mixture was allowed to warm to room temperature (ca. 3 h), and stirring was continued overnight (ca. 11 h). The reaction was quenched by addition of HCl (3 N, 200 ml) at 0°C. The layers were separated, the aqueous phase was further extracted with diethyl ether (3 \times 75 ml), and the combined organic extract was washed with 100-ml portions of water, saturated NaHCO_3 , water, and brine. Removal of the solvent gave 27.7 g of pale yellow-colored viscous residue, which upon flash chromatography over silica gel (EtOAc/hexanes, 1:9) followed by bulb-to-bulb distillation [pot temperature 125–130°C (0.4 mm Hg)], afforded the previously described (Jakovac and Jones, 1979) α -BnMeGBL (17.6 g, 92%) as a colorless solid: melting point 55–56°C (from diethyl ether-pentane at -5°C); IR 1762 ($\text{C}=\text{O}$), 1604 cm^{-1} ($\text{C}=\text{C}$); ^1H NMR δ 7.33 to 7.18 (m, 5, PhH), 4.15 to 4.07 (m, 1, diastereotopic H of H-5), 3.78 to 3.71 (m, 1, diastereotopic H of H-5), 3.04 (d, 1, J = 13.4 Hz, diastereotopic H of CH_2Ph), 2.74 (d, 1, J = 13.4 Hz, diastereotopic H of CH_2Ph), 2.33 to 2.24 (m, 1, diastereotopic H of H-4), 1.98 to 1.89 (m, 1, diastereotopic H of H-4), 1.30 (s, 3, CH_3); ^{13}C NMR δ 181.71, 136.67, 129.93, 128.39, 126.94, 64.98, 43.84, 43.37, 33.27, 23.60. Analysis Calculated for $\text{C}_{12}\text{H}_{14}\text{O}_2$: C, 75.76; H, 7.42. Found: C, 75.61; H, 7.50.

Separation of Racemic α -BnMeGBL into (+)- and (-)-Enantiomers. The racemic α -BnMeGBL was reacted with *S*(-)- α -methylbenzylamine using an established literature procedure to obtain a 65% yield of a mixture of the diastereomeric ring opened γ -hydroxyamides (Bigg and Lesimple, 1992). The diastereomers were separated by column chromatography using hexanes/ethyl acetate (1:4) as solvents. Each pure diastereomer was obtained as white crystals. Each pure diastereomer was then hydrolyzed with 1 N H_2SO_4 in refluxing ethylene glycol to give the γ -hydroxyacids, which lactonized *in situ* to give pure (+)- and (-)- α -BnMeGBL in 96% yields. The

(+)- α -BnMeGBL had $[\alpha]_D = (+) 60$ and the (-)- α -BnMeGBL had $[\alpha]_D = (-) 59$.

Assignment of R and S Absolute Configurations to the (+)- and (-) Enantiomers. The S absolute configuration for the chiral center in the (+) enantiomer was established by the following reaction sequences. The (+)-BnMeGBL was reduced with diisobutylaluminum hydride to give 2-benzyl-2-methyl-1,4-butanediol, which had $[\alpha]_D = (+) 4.1$. An authentic sample of (S)-*n*-butyl 2-benzyl-2-methyl-3-benzoylpropionate was then prepared according to a literature procedure (Meyers et al., 1990). The benzoyl group of this ketoester was then oxidized with sodium perborate in refluxing trifluoroacetic acid to a phenyl ester group, yielding (S)-2-benzyl-2-methylsuccinic acid phenyl ester, *n*-butyl ester. Reduction of this diester with lithium aluminum hydride gave (S)-2-benzyl-2-methyl-1,4-butanediol, which had $[\alpha]_D = (+) 3.2$. Because the same diol when obtained from the (+)- α -BnMeGBL also had a positive optical rotation, (+)- α -BnMeGBL is established as (S)-(+)- α -BnMeGBL.

Cloned GABA_A Receptors. Human embryonic kidney cells stably transfected with rat $\alpha 1\beta 2\gamma 2$ GABA_A receptors were studied in this investigation. Cells expressing these receptors were generously supplied by Pharmacia (Kalamazoo, MI). Preparation and culture of these cells have been described in detail previously (Hamilton et al., 1993).

Radioligand Binding. Competitive radioligand binding studies were conducted as described previously (Levine et al., 1985). Briefly, Sprague-Dawley male rats were dissected and their cerebral hemispheres were isolated. Membrane fractions were made and stored at -70° until time of experiment. At the time of experiment, fractions were resuspended with a Polytron and protein concentration was determined. The assay consisted of 100 μ l of tissue homogenate with 50 μ l [³⁵S]TBPS (2 nM) in 1 M NaBr and 50 μ l of Tris-citrate buffer (pH 7.5 at 0°C) containing either no additive or varying concentrations of either enantiomer of α -BnMeGBL. The samples were incubated at 25°C for 90 min., followed by dilution with 3 ml 0.9% NaCl. Samples were filtered through Whatman GF/B microfiber filter discs with slight vacuum applied and washed twice with 3 ml of 0.9% NaCl. Radioactivity was determined using conventional liquid scintillation counting. Nonspecific binding was characterized with unlabeled 10 μ M TBPS and was subtracted to compute specific binding. α -BnMeGBL concentration-response data were fitted to the equation $I/I_{\max} = [\alpha\text{-BnMeGBL}]^n / ([\alpha\text{-BnMeGBL}]^n + IC_{50}^n)$, where *I* is the degree of inhibition of [³⁵S]TBPS binding at a given concentration of α -BnMeGBL, *I*_{max} is maximal inhibition observed with α -BnMeGBL, *IC*₅₀ is the half-maximal effective concentration of α -BnMeGBL, and *n* is the Hill coefficient.

Pentylenetetrazole (PTZ) Convulsant Assay. A PTZ convulsant assay was performed using female CF-1 mice (Canney et al., 1991). All procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Seizures were induced with PTZ (85 mg/kg) dissolved in 0.9% NaCl. (+)- α -BnMeGBL, (-)- α -BnMeGBL or a racemic mixture was dissolved in varying concentrations of 30% polyethylene glycol, and a volume of 0.01 ml/g body weight was administered via intraperitoneal injection 30 min before the PTZ injection. Mice were monitored for seizure activity for up to 30 min after PTZ administration. Protection from PTZ-induced seizures was defined as an absence of convulsions with application of test compound. Ability of either enantiomer of α -BnMeGBL to block PTZ-induced seizures was recorded at the observed concentration. The experimental data pooled from two independent experiments for each tested drug were described as dose-response curves together with 95% confidence intervals by fitting them into Hill equation using nonlinear curve fit procedures ($R^2 \geq 0.949$) (SigmaPlot 2000; SPSS Science, Chicago, IL). The ED₅₀ values, defined as drug concentrations affording 50% protection, were determined numerically (Calculation Center, Wolfram Research, Champaign, IL). The 95% confidence intervals for ED₅₀ values were determined graphically by reading off the graph the minimum and maximum drug concentrations offering 50% pro-

tection with 95% confidence (dose-response curve confidence intervals at 50% response).

Electrophysiology. The whole-cell patch-clamp technique was used to record GABA-induced Cl⁻ currents. Patch pipettes of borosilicate glass (1B150F; WPI Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument Co., Novato, CA) to a tip resistance of 1 to 2.5 M Ω when filled with the following pipette solution: 140 mM CsCl, 10 mM EGTA, 10 mM HEPES, and 4 mM Mg-ATP; pH 7.2. Coverslips containing cultured cells were placed in a small chamber (~1.5 ml) on the stage of an inverted light microscope (IMT-2; Olympus, Tokyo, Japan) and superfused continuously (5–8 ml/min) with the following external solution containing 125 mM NaCl, 5.5 mM KCl, 0.8 mM MgCl₂, 3.0 mM CaCl₂, 20 mM HEPES, and 10 mM D-glucose, pH 7.3. GABA-induced Cl⁻ currents were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. GABA currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0; Axon Instruments) for subsequent analysis. Initial patch characteristics were used as reference throughout the experiment. If a change in access resistance was observed during the recording period, the patch was aborted and the data were not included in the analysis. All recordings were collected at room temperature with cells voltage-clamped at -60 mV.

Experimental protocol. Modulatory effects of α -BnMeGBL enantiomers were assessed using an EC₂₀ concentration (5 μ M) of GABA. GABA with or without α -BnMeGBL was prepared in the extracellular solution and applied (10 s) to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. Control responses were established by observing two consecutive GABA-induced currents that varied in amplitude by no more than $\pm 10\%$. After establishing the control, effects of lactones at varying concentrations were determined. Recovery from the lactones was often fully obtained, thus a complete concentration-response profile could generally be obtained from a single cell. In experiments where the ability of α -BnMeGBL enantiomers to directly activate the GABA_AR was determined, 3 mM α -BnMeGBL was used. Concentration-response profiles for the positive modulatory actions of lactones were generated (Origin 5.0; OriginLab Corp., Northampton, MA) using the equation $I/I_{\max} = [\alpha\text{-BnMeGBL}]^n / ([\alpha\text{-BnMeGBL}]^n + EC_{50}^n)$, where *I* is normalized current amplitude at a given concentration of α -BnMeGBL, *I*_{max} is the maximum GABA current induced by α -BnMeGBL, *EC*₅₀ is the half-maximal effective concentration of α -BnMeGBL, and *n* is the Hill coefficient.

Results

Influence of α -BnMeGBL Enantiomers on [³⁵S]TBPS Binding. The lactone class of compounds has been shown previously to bind at or near the site of action of convulsants, particularly TBPS and picrotoxin (Canney et al., 1991). The binding affinity for this class of compounds to the convulsants site ranges from low micromolar to millimolar dissociation constants (Canney et al., 1991; Holland et al., 1993). Thus, we investigated the ability of α -BnMeGBL isomers to inhibit [³⁵S]TBPS binding to GABA_A receptors in isolated rat cerebral cortical membranes. (+)- α -BnMeGBL displaced [³⁵S]TBPS with an *IC*₅₀ of 0.68 ± 0.01 mM. The ability of (-)- α -BnMeGBL to displace [³⁵S]TBPS was less (*IC*₅₀ = 1.1 ± 0.01 mM) that that seen with the (+)-enantiomer. Compared with α -IMGBL, the (+)-isomer of α -BnMeGBL displayed higher affinity (Holland et al., 1993). Holland et al. (1993) demonstrated that alkyl-substituted γ -butyrolactones do not compete for the TBPS site. These lactones act at a distinct, allosteric site that increases the dissociation rate of

TABLE 1

Effects of α -benzyl- α -methyl- γ -butyrolactone on seizure activity, ^{35}S]TBPS displacement, and GABA-gated Cl^- current

Compound	Anticonvulsant Potency ^a	^{35}S]TBPS Displacement ^b	GABA Current Potentiation ^c
(+)- α -BnMeGBL	65 (53–84)	0.68 ± 0.01	149 ± 32
(-)- α -BnMeGBL	158 (97–250)	1.1 ± 0.01	80 ± 8.7
(\pm)- α -BnMeGBL	65 (50–90)	0.92 ± 0.01	ND

ND, not determined.

^a ED₅₀ (milligrams per kilogram) against PTZ-induced seizures, 95% confidence interval in parentheses, $n = 6$ to 12 for each compound. Doses tested were 0, 25, 50, 100, and 200 mg/kg for (+)-BnMeGBL; 0, 25, 50, 100, 200, and 300 mg/kg for (-)-BnMeGBL; and 20, 25, 40, 50, 80, 100, 200, and 250 mg/kg for (\pm)-BnMeGBL.^b Mean IC₅₀ to inhibit ^{35}S]TBPS binding in cerebral cortex of rat; errors are S.E.M. for duplicate experiments run in triplicate.^c Maximal stimulation above control of GABA (5 μM) current recorded from HEK293 cells expressing rat $\alpha 1\beta 2\gamma 2$ GABA_A receptors; $n = 5$.

the radioligand. α -BnMeGBL may interact with the receptor in a similar manner to inhibit TBPS binding.

Anticonvulsant Activity of α -BnMeGBL Enantiomers. γ -Butyrolactones have been characterized as convulsant or anticonvulsant, depending in large part on the location of the substituent on the lactone ring (Klunk et al., 1982a,b; Canney et al., 1991). Whether an enantioselective effect of the anticonvulsant actions of γ -butyrolactones exists has never been studied. We thus tested the hypothesis that α -BnMeGBL would protect against PTZ-induced convulsions, and whether this protection might be enantioselective. Both enantiomers of α -BnMeGBL and the racemic mixture inhibited PTZ-induced convulsions. A clear enantioselective effect was observed, because the potency of the (+)- α -BnMeGBL was more than 2-fold greater than that observed with (-)- α -BnMeGBL (Table 1).

Effects of α -BnMeGBL Enantiomers on GABA-Activated Cl^- Current. If the anticonvulsant effects of α -BnMeGBL are due to their interaction with GABA_ARs, the above-mentioned results suggest α -BnMeGBL should also modulate GABA-gated currents in an enantioselective manner. We thus evaluated the actions of (+)- α -BnMeGBL and (-)- α -BnMeGBL on GABA-activated currents recorded from human embryonic kidney 293 cells stably expressing $\alpha 1\beta 2\gamma 2$ GABA_ARs. (+)- α -BnMeGBL facilitated GABA current in a concentration-dependent manner (Fig. 2A). Maximal enhancement of GABA current was roughly 2.5-fold of the control and occurred at 2 mM (+)- α -BnMeGBL. The EC₅₀ value and Hill coefficient were estimated to be 0.5 ± 0.08 mM and 1.3 ± 0.25 , respectively. At the highest concentrations (at and above 1 mM), enhancement of current decay became apparent.

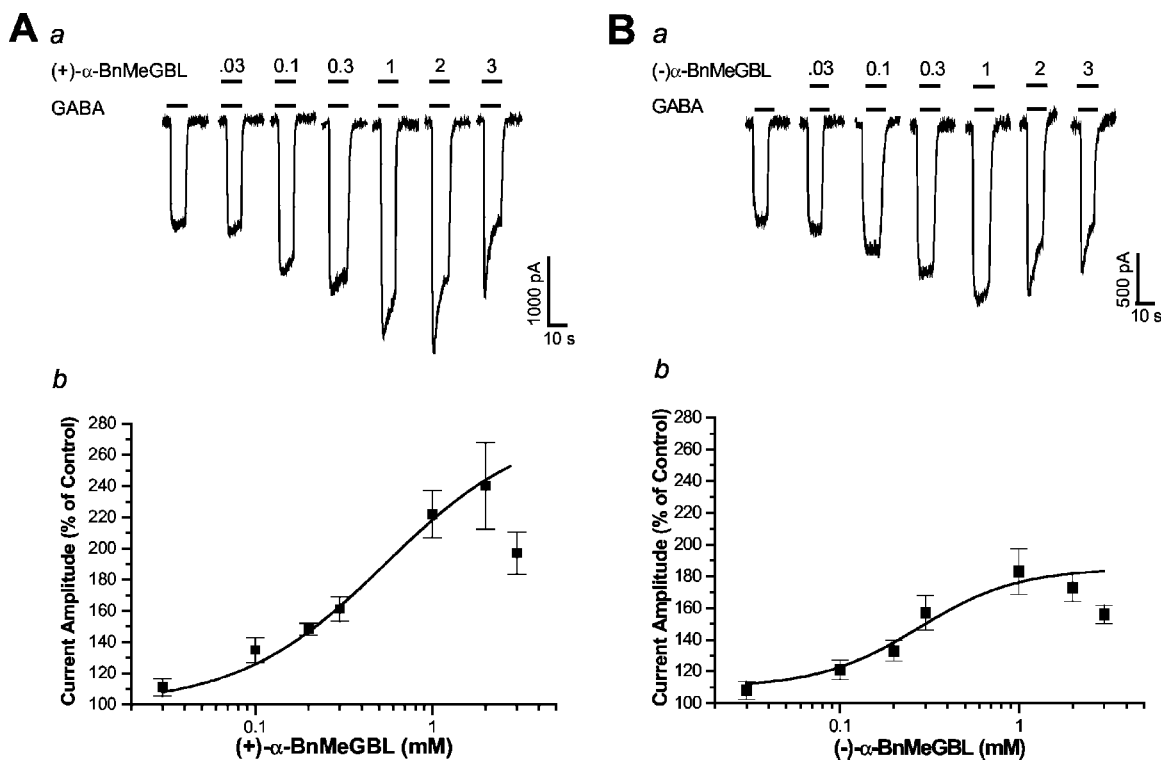


Fig. 2. Enantioselective modulation of GABA-activated Cl^- current by α -BnMeGBL. Aa, typical response of GABA-activated currents to increasing concentrations of (+)- α -BnMeGBL. Currents were recorded from rat $\alpha 1\beta 2\gamma 2$ receptors expressed in HEK293 cells. (+)- α -BnMeGBL caused current enhancement up to a concentration of 2 mM and then facilitated current decay. Ab, mean results illustrating the effects of (+)- α -BnMeGBL. Current amplitude was maximally increased roughly 2.5-fold compared with the control. Concentration-response analysis yielded an EC₅₀ value of 0.5 ± 0.08 mM and a Hill coefficient of 1.3 ± 0.25 . The data point recorded at the highest concentration of (+)- α -BnMeGBL was not included in the curve fitting analysis due to the onset of current inhibition. Ba, typical response of GABA-activated currents to increasing concentrations of (-)- α -BnMeGBL. Whereas (-)- α -BnMeGBL also facilitated GABA-gated current, the magnitude of enhancement was approximately 50% of that observed with the (+) enantiomer. (-)- α -BnMeGBL also displayed a biphasic effect, because current decay was enhanced at concentrations above 1 mM. Bb, mean results illustrating the effects of (-)- α -BnMeGBL. $n =$ a minimum of four cells tested at each concentration for both enantiomers. Scale bars represent current amplitude in picoamperes and time in seconds.

(-)- α -BnMeGBL also enhanced GABA-gated current in $\alpha 1\beta 2\gamma 2$ receptors (Fig. 2B). Although the apparent affinity of the negative enantiomer ($EC_{50} = 0.22 \pm 0.02 \mu\text{M}$) was higher than that of (+)- α -BnMeGBL, it had roughly 50% of the efficacy observed with the (+)-enantiomer. As seen with the (+)-enantiomer, high concentrations of (-)- α -BnMeGBL elicited enhancement of current decay (Fig. 2B, traces at 2 and 3 mM). It should be noted that the presence of both inhibitory and direct activating effects of lactones at high concentrations (below) make accurate determination of EC_{50} and Hill coefficient values difficult. Nevertheless, the estimated values do provide some useful comparative value. The enantioselectivity seen with both anticonvulsant actions and GABA current-enhancing actions strongly supports the existence of a lactone binding site on the GABA_A receptor. Table 1 summarizes the data for binding, convulsant, and patch-clamp experiments.

α -BnMeGBL Directly Activates the GABA_A Receptor.

Barbiturates and other anesthetics, in addition to allosterically modulating the GABA_AR, can also directly activate the channel (Rho et al., 1996; Krasowski et al., 1998). The ability of γ -butyrolactone compounds to directly activate the GABA_AR has not been fully explored. We thus evaluated whether enantiomers of α -BnMeGBL could directly open $\alpha 1\beta 2\gamma 2$ GABA_ARs. As shown in Fig. 3, high concentrations (3 mM) of each enantiomer resulted in GABA_AR activation. The direct activating effect was relatively modest, i.e., about 30% of the amplitude of the response seen with application of 5 μM GABA (GABA EC_{20}). Due to solubility considerations, we did not evaluate direct activating effects of α -BnMeGBL at higher concentrations. At a concentration of 3 mM, however, where a clear enantioselective effect was seen for modulatory effects of GABA_AR activity, no enantioselective effect was observed for direct activating effects of α -BnMeGBL.

Discussion

Despite extensive study, definitive evidence for the existence of a lactone binding site on the GABA_AR has proven to be elusive. Whereas early studies led to the conclusion that both positive modulatory (GABA current-enhancing) and negative modulatory (GABA current-inhibiting) actions of the lactone compounds were due to an interaction at the picrotoxin site of the receptor (Holland et al., 1991), subsequent work led to the suggestion that positive modulatory actions of lactones resulted from modulation of a novel site (Yoon et al., 1993; Holland et al., 1995). The fact that both GABA current stimulatory and inhibitory effects of lactones are not due to differential modulation of a single site is best supported by the results of Williams et al. (1997). They showed that the ability of convulsant lactones to inhibit GABA-activated current was abolished by a mutation known to also abolish sensitivity to picrotoxin. However, the GABA current-enhancing effects of lactones could not be abolished by this mutation. Although the results of Williams et al. (1997) are consistent with the suggestion that a lactone binding site is present on the GABA_AR, alternative explanations are not precluded. Thus, the conclusion that a true lactone site exists on the receptor requires additional verification. Results of the present studies provide additional evidence in support of this conclusion.

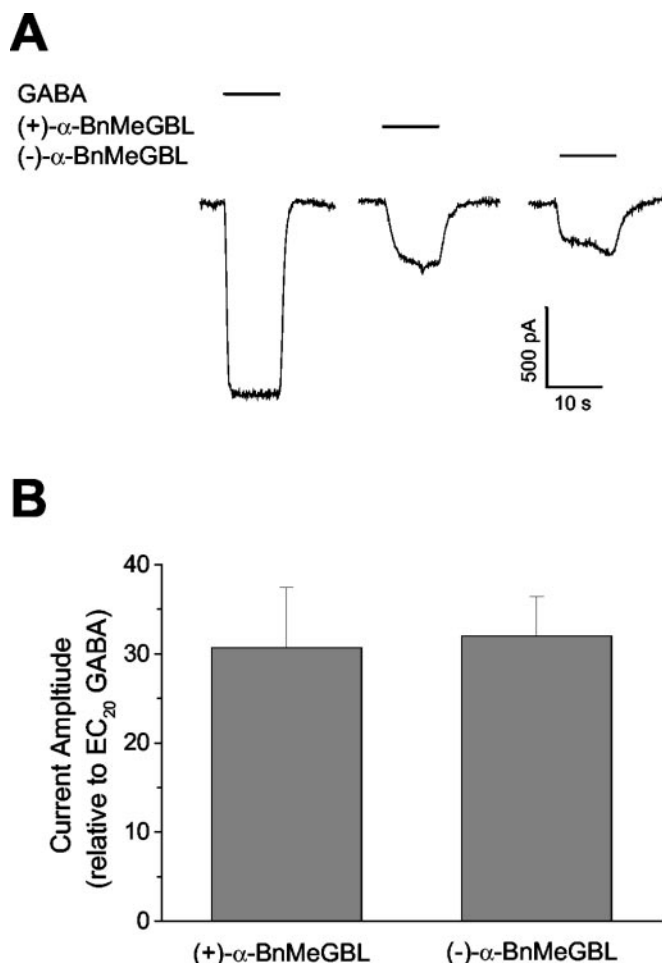


Fig. 3. Direct activation of GABA_A receptors by α -BnMeGBL. A, initial trace is response to application of 5 μM GABA. Subsequent traces demonstrate the response to each enantiomer when applied in the absence of GABA. Application of either enantiomer of α -BnMeGBL (3 mM) resulted in opening of GABA_A receptors. B, mean results from above-mentioned experiments ($n = 3$ cells for each enantiomer). The direct channel activating effects were the same for both enantiomers, and the magnitude of resulting currents was modest compared with the response to 5 μM GABA (EC_{20} concentration).

Effects of α -BnMeGBL Are Similar to Other α -Substituted Lactones. The actions of the novel butyrolactone α -BnMeGBL were evaluated using both in vivo (anticonvulsant) and in vitro (whole-cell patch-clamp recording, radioligand binding) assays. Its ability to protect against pentyletetrozole-induced seizures and to enhance GABA-activated Cl^- currents is comparable with that seen with other α -substituted lactones (Canney et al., 1991). α -BnMeGBL also inhibited binding of the picrotoxin site ligand [^{35}S]TBPS. Like with other α -substituted lactones, displacement of [^{35}S]TBPS requires much higher concentrations than necessary with β -substituted lactones (Canney et al., 1991).

At higher concentrations of α -BnMeGBL (above 1 mM), inhibition of GABA-activated current became apparent. This effect, which has also been observed for other α -substituted lactones (Williams et al., 1997), can be explained by several possibilities. Our radioligand binding studies demonstrate that α -BnMeGBL inhibits [^{35}S]TBPS with IC_{50} values near 1 mM. Thus, an obvious possibility is that the inhibition could reflect effects due to binding at the convulsant site predominating over effects due to binding at the positive modulatory

lactone site. Alternatively, as noted by Williams et al. (1997), the decrease in GABA current induced by α -BnMeGBL may be due to its facilitation of entry into the desensitized state. Finally, the existence of another low affinity inhibitory site for lactones cannot be ruled out. Williams et al. (1997) found that GABA_A receptors expressing a mutation that rendered them insensitive to the inhibitory effects of μ M β -EMTBL were still inhibited by higher concentrations (millimolar) of β -EMTBL.

Enantioselectivity of α -BnMeGBL. Although in the present studies we have characterized a novel anticonvulsant lactone, the major goal of this research was to evaluate whether enantiomers of this lactone had differential anticonvulsant and GABA_AR-modulating properties. Because enantioselective preference is typically considered to be evidence of a specific ligand-protein interaction (Bahr and Parsons, 1986; Bertucci et al., 1997), assessment of this trait is a useful approach for evaluating whether a binding site for a particular molecule exists on a particular receptor. Our data support the contention that a distinct binding site exists on the GABA_AR that accounts for the positive modulatory actions of butyrolactones (and presumably thiobutyrolactones). The *R*-(-) enantiomer of α -BnMeGBL had a 2-fold larger facilitative effect on GABA-activated Cl⁻ currents than the *S*-(+)-enantiomer. These positive modulatory effects of α -BnMeGBL at the GABA_AR likely underlie its anticonvulsant activity, because *R*-(-)- α -BnMeGBL was also 2-fold more potent than *S*-(+)- α -BnMeGBL in protecting animals from pentylenetetrazole-induced seizures. The fact that the degree of enantioselectivity was the same in both the anticonvulsant and patch-clamp assays confirms the significance of the stereoselective effect. Although the overall magnitude of enantioselective preference seems relatively modest, this degree of enantioselectivity has been reported for other molecules and does result in distinct functional effects (Hall et al., 1994; Cordato et al., 1999; Covey et al., 2000).

Direct Activation of GABA_ARs by α -BnMeGBL. In addition to allosterically modulating GABA-activated current, we demonstrate that α -BnMeGBL can also directly activate the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. Several butyrolactones and thiobutyrolactones have been tested for direct receptor activating effects, at concentrations from 1 to 10 mM, and they have not shown this characteristic (Holland et al., 1990a; Mathews et al., 1996). Because of solubility issues, we did not determine the maximal efficacy for the direct gating effects of α -BnMeGBL and thus also could not fully assess whether enantioselectivity for this effect existed. At 3 mM, the (+) and (-) enantiomers of α -BnMeGBL activated a current less than one-third the amplitude of current generated in response to 5 μ M GABA (the EC₂₀ concentration in $\alpha 1\beta 2\gamma 2$ receptors). Thus, compared with other ligands that directly activate the receptor, the efficacy of α -BnMeGBL for direct channel activation may be modest (Rho et al., 1996; Gonzales et al., 2001). Considering that other lactones previously reported not to directly activate the receptor had smaller alkyl substituents than those present in α -BnMeGBL, the presence of a bulky side chain may be crucial in altering the GABA_A receptor structure in the closed state. Size and orientation of side chain alkyl substitutions influence efficacy of direct activation of GABA_ARs by barbiturates, most noticeably between pentobarbital and isobarbital (Gonzales et al., 2001). Whether the direct activation effects

of barbiturates and α -BnMeGBL occurs through a similar domain of the receptor remains to be determined.

The results of the present study provide further evidence that positive modulatory actions of butyrolactones and thiobutyrolactones are due to interaction with a distinct protein binding site. Although we presume the binding site is located on the GABA_A receptor itself, the possibility that lactones may be binding to an associated protein that regulates GABA_A receptor function cannot be dismissed. Distinguishing between these two possibilities will require additional studies.

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