ENANTIOSELECTIVITY OF α-BENZYL-α-METHYL-γ-BUTYROLACTONE - MEDIATED MODULATION OF ANTICONVULSANT ACTIVITY AND GABAA RECEPTOR FUNCTION*

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Enantioselective effects of a novel lactone

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Abbreviations:
GABA, γ-aminobutyric acid; GABA$_A$, type A GABA receptor; DMSO, dimethylsulfoxide; α-BnMeGBL, α-benzyl-α-methyl-γ-butyrolactone; EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; HEK, human embryonic kidney; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; PTZ, pentylenetetrazole; TBPS, *t*-butylbicyclophosporothionate;

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, while β-substituted lactones tend to inhibit the $GABA_A$ receptor. These compounds mediate inhibition through the picrotoxin (PTX) 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 TBPS with a higher affinity than the (-)-enantiomer (IC_{50} of 0.68 mM and 1.1 mM, respectively). Whole cell patch clamp recordings from recombinant $\alpha1\beta2\gamma2$ receptors stably expressed in HEK293 cells demonstrated that both compounds stimulated GABA-activated current. The maximal stimulation was approximately two-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 non-stereoselective 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$_A$R) 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$_A$Rs are members of the ligand-gated ion channel superfamily, which include glycine receptors, 5-HT$_3$ 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), while unsubstituted γ-butyrolactone has no significant effect on GABA current (Feigenbaum and Howard, 1996).

Following the discovery that lactones competitively inhibit binding of the picrotoxin-site ligand [^{35}S]-TBPS to the GABA$_A$R (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 or not a true lactone binding site exists on the GABA$_A$R is unknown. It is possible, for example, that the GABA current-enhancing effects of lactones are secondary to relatively non-selective alterations of the local physio-chemical environment, which could subsequently influence GABA$_A$R activity. One approach to provide evidence of a ligand binding site is demonstration of enantioselective preference. Enantiomers are non-superimposible mirror images of optically active molecules (i.e., compounds with at least one chiral center). They have identical physio-chemical properties, and thus any effects of them due to alterations of membrane lipids would be identical (Arnett et al., 1982; Guyer et al., 1983; 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$_A$Rs 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$_A$R, 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$_A$Rs.

Methods

Chemicals. α-BnMeGBL enantiomers and racemic solutions were made from a 1M DMSO stock and diluted in 5 μM GABA in normal saline. The final DMSO concentrations in the test solutions were less than 0.3% (v/v). [^{35}S]TBPS was obtained from New England Nuclear (Boston).

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$_2$Cl$_2$) was distilled over calcium hydride. Hexamethylphosphoramide (HMPA) was dried over activated 13X 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, Inc.) was utilized to monitor the progress of the reactions and to assess the purity of the products. Solvents used for extractions were dried over either Na$_2$SO$_4$ 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, Inc. 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 Perkin-Elmer 1710 FT-IR instrument as thin films on NaCl plate, and NMR spectra (^{1}H 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 in 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 diisopropylamine (LDA) [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 HMPA (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 x 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: mp: 55–56 °C (from diethyl ether–pentane at −5 °C); IR 1762 (C=O), 1604 cm^{-1} (C=C); ^{1}H NMR δ 7.33–7.18 (m, 5, PhH), 4.15–4.07 (m, 1, diastereotopic H of H-5), 3.78-3.71 (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), 2.33–2.24 (m, 1, diastereotopic H of H-4), 1.98–1.89 (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. Anal. Calcd for $C_{12}H_{14}O_2$: 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-butandiol which had $[\alpha]_D$ = (+) 3.2. Since 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 has been previously described in detail (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 µl of tissue homogenate with 50 µl [^{35}S]-TBPS (2 nM) in 1M NaBr and 50 µl Tris-citrate buffer (pH 7.5 at 0°) 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 0.9% NaCl. Radioactivity was determined using conventional liquid-scintillation counting. Non-specific binding was characterized with unlabeled 10 µM 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 [^{35}S]-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.

PTZ Convulsant Assay. A pentylenetetrazole (PTZ) convulsant assay was performed using female CF-1 mice (Canney et al., 1991). All procedures were

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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 non-linear curve fit procedures ($R^2 \geq 0.949$) (Sigma Plot 2000, SPSS, Chicago, IL). The ED_{50}, defined as drug concentrations affording 50% protection, were determined numerically (Calculation Center, Wolfram Research Champaign, IL). The 95% confidence intervals for ED_{50} were determined graphically by reading off the graph the minimum and maximum drug concentrations offering 50% protection 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, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1-2.5 MΩ when filled with the following pipette solution (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. Coverslips containing cultured cells were placed in a small chamber (~ 1.5 ml) on the

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stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 ml/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 MgCl$_2$, 3.0 CaCl$_2$, 20 HEPES, 10 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 was 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 EC$_{20}$ concentration (5 μM) 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 ± 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$_A$R was determined, 3 mM α-BnMeGBL was used. Concentration-response profiles for the positive modulatory actions of lactones were generated (Origin 5.0, Microcal, Inc.) using the equation I/I_{max}= [α-BnMeGBL]^n/([α-

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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 [^{35}S]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 [^{35}S]TBPS binding to GABA$_A$ receptors in isolated rat cerebral cortical membranes.   (+)-α-BnMeGBL displaced [^{35}S]TBPS with an IC$_{50}$ of 0.68 mM ± 0.01 mM. The ability of (-)-α-BnMeGBL to displace [^{35}S]TBPS was less (IC$_{50}$ = 1.1 ± 0.01 mM) that that seen with the (+) enantiomer. Compared to 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 the radioligand. α-BnMeGBL may interact with the receptor in a similar fashion 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; Klunk et al., 1982b; Canney et al., 1991). Whether or not 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 if this protection might be enantioselective. Both enantiomers of α-BnMeGBL and the racemic mixture inhibited PTZ-induced convulsions. A clear enantioselective effect was

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observed, as 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$_A$Rs, the above 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 HEK293 cells stably expressing α1β2γ2 GABA$_A$RS. (+)-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 EC$_{50}$ 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.

(-)-α-BnMeGBL also enhanced GABA-gated current in α1β2γ2 receptors (Fig. 2B). Although the apparent affinity of the negative enantiomer (EC$_{50}$ = 0.22 ± 0.02 μM) 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

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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$_A$R, 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$_A$R has not been fully explored. We thus evaluated whether enantiomers of α-BnMeGBL could directly open $\alpha1\beta2\gamma2$ GABA$_A$Rs. As shown in Figure 3, high concentrations (3 mM) of each enantiomer resulted in GABA$_A$R 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$_A$R 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$_A$R 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. While the results of Williams et al. are consistent with the suggestion that a lactone binding site is present on the GABA$_A$R, 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.

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 pentylenetetrazole-induced seizures and to enhance GABA-activated Cl$^-$ currents is comparable to that seen with other α-

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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 μM β-EMTBL were still inhibited by higher concentrations (mM) 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$_A$R 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

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support the contention that a distinct binding site exists on the GABA$_A$R that accounts for the positive modulatory actions of butyrolactones (and presumably thiobutyrolactones). The *R*-(+) enantiomer of α-BnMeGBL had a two-fold larger facilitative effect on GABA-activated Cl$^-$ currents than the *S*-(-) enantiomer. These positive modulatory effects of α-BnMeGBL at the GABA$_A$R likely underlie its anticonvulsant activity, as R-(+)-α-BnMeGBL was also two-fold more potent than *S*-(-)-αBnMeGBL 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 appears 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$_A$Rs by α-BnMeGBL. In addition to allosterically modulating GABA-activated current, we demonstrate that α-BnMeGBL can also directly activate the α1β2γ2 GABA$_A$ receptor. Several butyrolactones and thiobutyrolactones have been tested for direct receptor activating effects, at concentrations from 1-10 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 could also not fully assess if 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 μM GABA (the EC$_{20}$ concentration in α1β2γ2 receptors). Thus, compared to 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$_A$Rs by barbiturates, most noticeably between pentobarbital and isobarbital (Gonzales et al., 2001). Whether or not 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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Footnotes

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

Figure 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 α1β2γ2 receptors expressed in HEK293 cells. (+)-α-BnMeGBL caused current enhancement up to a concentration of 2 mM, then facilitated current decay. *Ab.* Mean results illustrating the effects of (+)-α-BnMeGBL. Current amplitude was maximally increased roughly 2.5 fold compared to the control. Concentration-response analysis yielded an EC_{50} 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, as current decay was enhanced at concentrations above 1 mM. *Bb.* Mean results illustrating the effects of (-)-α-BnMeGBL. n = a minimum of 4 cells tested at each concentration for both enantiomers. Calibration bars represent current amplitude in picoamperes and time in seconds.

Figure 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 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 when compared to the response to 5 μM GABA (EC$_{20}$ concentration).

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 %
(±)-α-BnMeGBL	65 (50-90)	0.92 ± 0.01	ND

[a] ED_{50} (mg/kg) against PTZ-induced seizures, 95% confidence interval in parentheses, n = 6-12 for each compound. Doses tested were 0, 25, 50, 100, 200 mg/kg for (+), 0, 25, 50, 100, 200, and 300 mg/kg for (-), and 20, 25, 40, 50, 80, 100, 200, and 250 mg/kg for (±).

[b] mean IC_{50} to inhibit ^{35}[S]TBPS binding in cerebral cortex of rat; errors are SEM for duplicate experiments run in triplicate.

[c] maximal stimulation above control of GABA (5 μM) current recorded from HEK293 cells expressing rat α1β2γ2 GABA$_A$ receptors; n = 5.
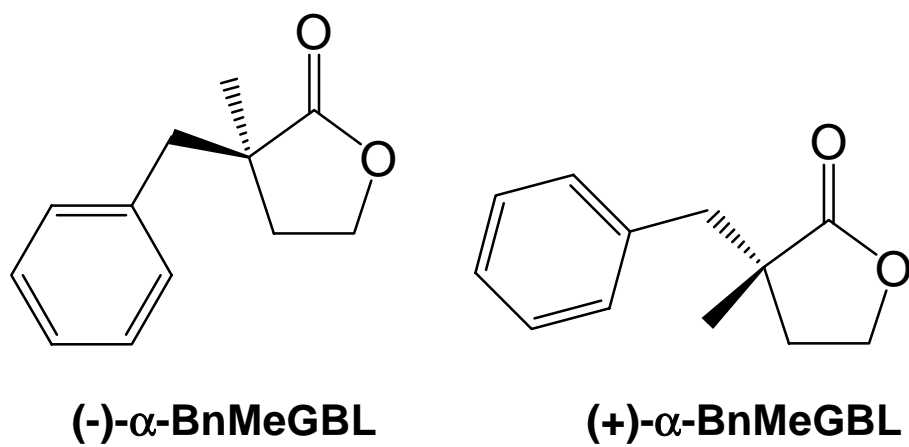
(-)-α-BnMeGBL   **(+)-α-BnMeGBL**
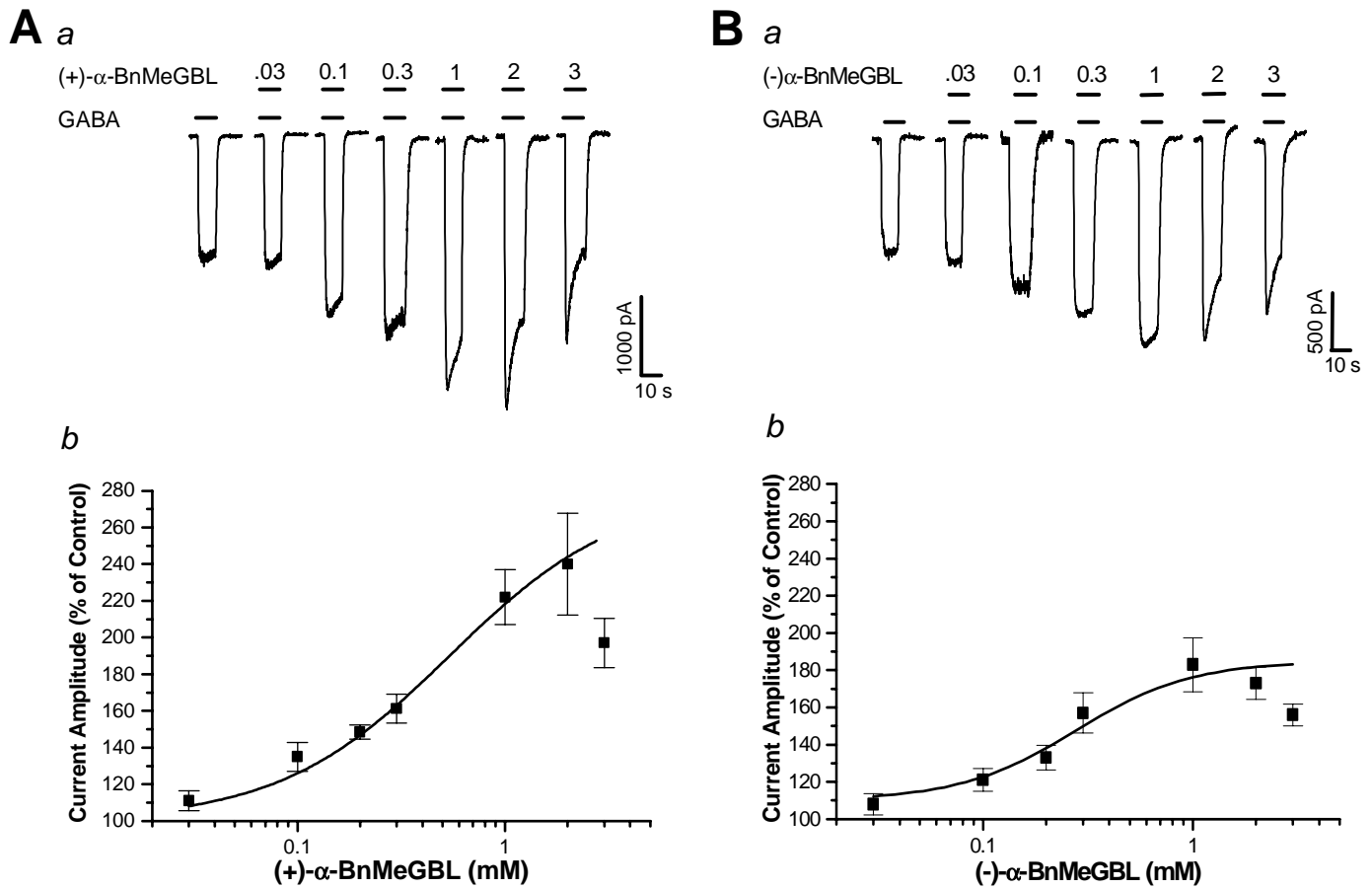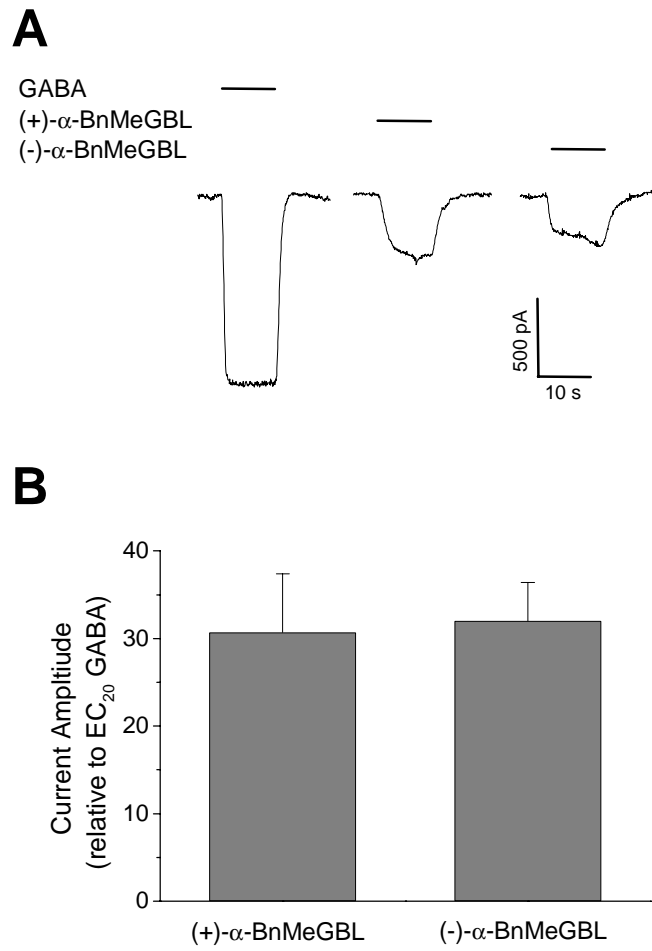
Figure 1.

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Figure 2.

Figure 3.

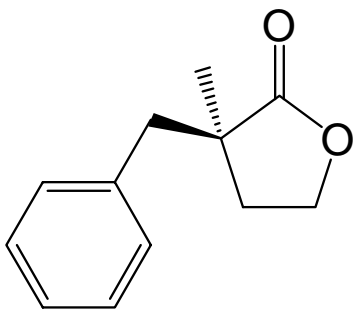**Table 1.** Effects of α-BENZYL-α-METHYL-γ-BUTYROLACTONE on seizure activity, ^{35}[S]TBPS displacement and GABA-gated Cl^- current.

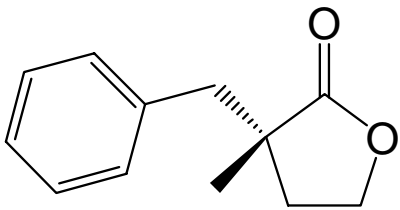| Compound	Anticonvulsant potency [a]	^{35}[S]TBPS displacement[b]	GABA current potentiation[c]
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[c] maximal stimulation above control of GABA (5 μM) current recorded from HEK293 cells expressing rat α1β2γ2 $GABA_A$ receptors; n = 5.

(-)-α-BnMeGBL　　　　**(+)-α-BnMeGBL**

A

GABA
(+)-α-BnMeGBL
(-)-α-BnMeGBL

500 pA

10 s

B