Physiological Regulation of the Picrotoxin Receptor by γ -Butyrolactones and γ -Thiobutyrolactones in Cultured Hippocampal Neurons

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We examined the effects of alkyl-substituted γ -butyrolactones (GBLs), and γ -thiobutyrolactones (TBLs) on GABA currents in cultured, voltage-clamped rat hippocampal neurons. Convulsant GBLs and TBLs reversibly diminished GABA responses in a concentration-dependent manner. β -Ethyl- β -methyl GBL (β -EMGBL) completely abolished GABA responses at 3 mM (IC_{50} 390 μ M), while TBL and β -ethyl- β -methyl TBL (β -EMTBL)-induced inhibition of GABA currents was incomplete, saturating at about 50% of control at 300 μ M and 10 mM for β -EMTBL and TBL, respectively. β -EMGBL and β -EMTBL both increased the rate of decay of inhibitory post-synaptic currents (IPSCs) and β -EMGBL also decreased IPSC peak amplitude. In contrast, the anticonvulsant α -ethyl- α -methyl TBL (α -EMTBL) potentiated GABA currents at all GABA concentrations tested; maximal potentiation was 190% of control at 1 mM α -EMTBL (EC_{50} 102 μ M). Another anticonvulsant, α -ethyl- α -methyl GBL (α -EMGBL), potentiated responses to low (0.5 μ M) but not high (≥ 10 μ M) GABA. It also blocked the inhibitory effects of picrotoxin and β -EMGBL and the facilitative effect of α -EMTBL on responses to 30 μ M GABA. α -EMGBL did not interfere with other agents which augment GABA currents. Both α -EMTBL and α -EMGBL decreased the rate of IPSC decay without altering IPSC peak amplitude. None of these compounds had any direct membrane effects.

We propose that β -alkyl GBLs diminish GABA currents, and therefore, we hypothesize that these compounds are picrotoxin receptor agonists. β -Alkyl TBLs partially diminish GABA currents and may be partial agonists. α -Alkyl TBLs potentiate GABA currents and may be inverse agonists. Finally, α -EMGBL potentiates responses to low but not high GABA concentrations and may represent a mixed inverse agonist, antagonist at the picrotoxin receptor. The present results demonstrate that the picrotoxin receptor is capable of modulating GABA responses in opposing ways.

Alkyl-substituted γ -butyrolactones (GBLs) and γ -thiobutyrolactones (TBLs) have convulsant or anticonvulsant activity depending on the location of the alkyl substituents. Those GBLs

and TBLs with alkyl groups in the β -position are convulsants (Klunk et al., 1982a; Levine et al., 1986). GBLs and TBLs without β -alkyl substituents are anticonvulsant if the compounds have alkyl substituents in the α - and/or γ -position (Klunk et al., 1982b; Levine et al., 1986). A similar pattern of activity is observed with alkyl-substituted succinimides (Klunk et al., 1982c). Early structure-activity studies and computer-modeling studies of GBLs, TBLs, and succinimides suggested that these compounds may share a common mechanism of action (Klunk et al., 1982c, 1983).

The site of action of these compounds has recently been examined. Convulsant and anticonvulsant GBLs, TBLs, and succinimides all competitively displace 35 S-*t*-butylbicyclopophosphorothionate (TBPS) from the picrotoxin receptor of the GABA_A receptor complex (Weissman et al., 1984; Levine et al., 1985; Holland et al., unpublished observations). In addition, these compounds had little effect on the binding of 3 H-flunitrazepam to the benzodiazepine receptor or 3 H-muscimol to the GABA recognition site (Holland et al., unpublished observations). These data indicated that the site of action of alkyl-substituted GBLs, TBLs, and succinimides is distinct from the GABA, benzodiazepine, and barbiturate recognition sites and suggest that one of the sites of action of these compounds is the picrotoxin receptor.

Picrotoxin, a convulsant, antagonizes GABA-mediated increases in neuronal chloride conductance. The convulsant tetramethylsuccinimide (TMSM) and a convulsant GBL also had a similar effect (Barnes and Dichter, 1984; Clifford et al., 1989). In contrast, results of studies on the effects of an anticonvulsant succinimide, ethosuximide (ESM), and an anticonvulsant TBL, α -ethyl- α -methyl TBL (α -EMTBL), on GABA-mediated inhibitory processes are contradictory. Barnes and Dichter (1984) have shown that ESM antagonizes GABA responses in cultured rat cortical neurons. In contrast, Baker and his colleagues (1988) have shown that α -EMTBL potentiates GABA responses in cultured chick spinal cord neurons. The concentration of α -EMTBL used by Baker in these experiments was well below that needed to displace 35 S-*t*-butylbicyclopophosphorothionate from the picrotoxin receptor found in rat brain (Holland et al., unpublished observations). Thus, it is not clear if the observed modulation of the picrotoxin receptor by α -EMTBL in chick spinal cord neurons explains the behavioral actions of these compounds.

To determine if the opposing behavioral effects of GBLs and TBLs are mediated by opposing actions at the picrotoxin receptor, we have compared the effects of alkyl-substituted GBLs, TBLs, and succinimides on GABA currents in cultured rat hip-

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pocampal neurons using whole-cell patch voltage-clamp techniques. In addition, the effects of alkyl-substituted GBLs and TBLs on inhibitory postsynaptic currents (IPSCs) were examined. The compounds chosen for this study (Fig. 1), α -ethyl- α -methyl GBL (α -EMGBL), β -ethyl- β -methyl GBL (β -EMGBL), α -EMTBL, β -ethyl- β -methyl TBL (β -EMTBL), ESM, and TMSM, were selected because they represent the best-characterized and most potent compounds of their respective classes. Several other α -substituted GBLs and TBLs were also studied, but in less detail, for comparison to α -EMGBL and α -EMTBL.

Materials and Methods

Materials. The GBLs and TBLs were synthesized according to previously described methods (Klunk et al., 1982a, b; Levine et al., 1986). Ethosuximide was provided by Parke-Davis (Morris Plains, NJ), and TMSM was obtained from ICN Pharmaceuticals (Plainville, NY). Unless noted, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Tissue culture. Hippocampal cells were cultured from 1-d-old female Sprague-Dawley rat pups by a method similar to that used by Huettnier and Baughman (1986) for cortical neurons. Hippocampi were dissected under sterile conditions, cut into pieces (<1 mm), and placed in an enzyme solution containing 1 mg/ml papain (Worthington Biochemical Corp.) and 0.2 mg/ml BSA in Leibovitz's L-15 equilibrated with 95% O_2 /5% CO_2 . After a 20 min incubation, the tissue was resuspended in approximately 2 ml of plating media (MEM with no added glutamine and 10% Nu Serum; Collaborative Research). The hippocampi were then gently triturated with a fire-polished Pasteur pipette in 2–3 ml of plating solution. The freshly dissociated cells were harvested by centrifugation ($600 \times g$ for 5 min) through 5 ml of plating media containing 10 mg/ml BSA and 10 mg/ml trypsin inhibitor. The cells (2.5×10^3) were then pipetted on to 35 mm culture dishes (Falcon) which contained 1.5 ml of plating medium.

The dishes were precoated with poly-L-lysine hydrobromide, MW $3.0\text{--}7.0 \times 10^4$, as described previously except that a solution of only 0.01% poly-L-lysine in borate buffer was used (Banker and Cowan, 1977). In some experiments hippocampal cells were plated onto dishes that had been plated with cortical glial cells (2×10^4 cells/cm 2) 4 d earlier. The glial cells were harvested according to a procedure which has been described elsewhere (Raff et al., 1979).

The cultures were maintained in a humidified incubator with 5% CO_2 at 36°C. After 3–5 d, the division of non-neuronal cells was halted by the addition of 15 μ g/ml fluorodeoxyuridine and 35 μ g/ml uridine. The plating medium was never changed and the cultures were not refed. The cells typically survived for 3–4 weeks in culture.

Electrophysiological recording. Electrophysiological experiments were carried out on the stage of an inverted microscope (Leitz) using whole-cell voltage-clamp techniques in cells that had been in culture for 10–21 d (approximately 70% of neurons examined were in cultures for 10–14 d). The growth medium was removed, and the cells were placed in an extracellular recording solution which contained 140 mM NaCl, 10 mM NaHEPES (pH 7.3), 3 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 5.5 mM glucose, and 3 μ M tetrodotoxin. Patch electrodes and drug pipettes were pulled from 1.2 mm thin-walled Omega-dot glass with a vertical pipette puller (Kopf). Electrode resistances ranged from 4–10 M Ω when filled with an intracellular recording solution that contained 130 mM K acetate, 10 mM KCl, 10 mM NaHEPES (pH 7.3), 2 mM MgATP, and 1.1 mM EGTA. When studying responses to 0.5 μ M GABA, the intracellular recording solution contained 140 mM KCl, 10 mM NaHEPES (pH 7.3), 2 mM MgATP, and 1.1 mM EGTA. The high intracellular chloride concentration amplified the otherwise negligible currents seen with such a low GABA concentration. Series resistances typically increased to 5–15 M Ω after rupturing the cell membrane, and series resistance was partially compensated accordingly.

Neurons were voltage-clamped at -60 mV using a standard patch amplifier (DAGAN) and exposed to the test compounds by a pressure application system described by Choi and Fischbach (1981). Pipettes were positioned 10–30 μ m away from the cell and drugs were applied for 100 msec by 10 psi pressure pulses every 30 sec. Drug pipettes were filled with either GABA or a mixture of GABA and drug dissolved in extracellular solution. After stable responses to GABA were obtained, GABA was applied alone 30 sec before and 30 sec after the application of the drug mixed with GABA. The data were discarded if the GABA

response after drug application was $<90\%$ of the GABA response before drug. Data from 19 of 311 cells studied were discarded on this basis. The peak current response of the drug/GABA combination was expressed as a percentage of that neuron's response to GABA alone. Thus, each cell served as its own control. Each drug concentration was tested in at least 2 different cultures. The data were digitized at 0.36 KHz and stored on disk for off-line analysis. Half-maximal effective concentrations and curve-fitting of sigmoidal dose-response curves were accomplished using probit analysis (Finney, 1971).

Pairs of neurons with monosynaptic inhibitory connections were also studied to examine the effects of various butyrolactones on GABAergic synapses. We recorded from neighboring neurons with 2 whole-cell patch electrodes which were connected to our voltage-clamp circuit and a standard bridge amplifier. Our extracellular solution lacked tetrodotoxin, and we used an intracellular solution containing 138 mM K isethionate (Kodak), 2 mM KCl, 10 mM NaHEPES (pH 7.3), 2 mM MgATP, 4 mM glucose, and 1.1 mM EGTA. Under these conditions, excitatory postsynaptic currents (EPSCs) are inward, while IPSCs are outward at a holding potential of -60 mV. Intracellular stimulation of one cell through the bridge circuit elicited an outward current in the other cell in about 50% of the successful double recordings. Pairs of cells were considered monosynaptically connected if the onset of the postsynaptic current was ≤ 5 msec after the peak of the presynaptic action potential and if all of the presynaptic action potentials resulted in postsynaptic responses. The postsynaptic current was inhibitory if stimulation of the presynaptic cell produced an outward current which was diminished by bicuculline and was insensitive to the application of CNQX, an antagonist at glutamate receptors (Honore et al., 1988; Yamada et al., 1989). Drugs were dissolved in extracellular recording solution and were applied by bath perfusion of the culture dish (volume 0.5 ml) at a rate of 1.5 ml/min for 2 min. In some experiments, 30 μ M GABA was also applied near the postsynaptic neuron from a GABA-containing pipette with 10 psi pressure pulses for 100 msec. The data were digitized at 0.5 KHz and stored on disk for off-line analysis. IPSC decay time constant, peak amplitude, and total charge transfer were determined using a commercially available analysis program (pCLAMP).

Results

Properties of neuronal responses to exogenously applied GABA

The cells used in these studies had resting membrane potentials of -57 ± 6 mV (mean $\pm$ 1 SD; $n = 292$). The typical input resistances ranged from 80 to 800 M Ω and averaged 240 ± 36 M Ω . Applications of 30 μ M GABA for 100 msec spaced 30 sec apart consistently produced currents of similar magnitude (Fig. 2). Using the K acetate/KCl intracellular solution, the measured GABA reversal potential (-53 ± 2 mV; mean $\pm$ SEM; $n = 8$; see Fig. 8B) closely matched the GABA equilibrium potential (-52 mV) calculated from the Cl $^-$ and acetate ion concentrations in the internal and external recording solutions and the relative permeabilities of these ions through the GABA-gated channel (Bormann et al., 1987). When the magnitude of the currents produced by various concentrations of GABA were normalized to the response produced by 100 μ M GABA, a sigmoidal dose-response curve was obtained. The whole-cell currents were normalized to currents produced by 100 μ M GABA because repeated brief (100 msec) applications of this concentration of GABA every 30 sec produced little GABA receptor desensitization. Currents were not normalized to higher GABA concentrations in order to minimize desensitization. The concentration of GABA that produced half-maximal response was 50 ± 5 μ M (mean $\pm$ SEM) and the responses to GABA saturated at 300 μ M (see Fig. 5A). A Hill plot of the GABA dose-response data (not shown) demonstrated cooperative activation of Cl $^-$ channels by GABA (Hill slope of 2.2 ± 0.2). Bicuculline (300 nM) had no effect on the maximal GABA response but shifted the GABA dose-response curve to the right and had no effect on the Hill coefficient (2.1 ± 0.2).

Figure 1. Comparison of the chemical structures of GBLs, TBLs, and succinimides.

Effects of alkyl-substituted GBLs, TBLs, and succinimides on responses to exogenously applied GABA

The convulsants TBL, β-EMGBL, and β-EMTBL all decreased the peak amplitude of GABA currents. In each case, this effect was immediate (<100 msec) and was completely reversible (within 5 sec). Thiobutyrolactone did not completely block GABA currents and inhibited GABA currents significantly only at concentrations of 1 mM and greater. Half-maximal inhibition was 2.5 mM, and the response saturated at 10 mM ($45 \pm 6\%$ inhibition; mean $\pm$ SEM; $n = 11$). In contrast, β-EMGBL completely blocked GABA-induced currents, with half-maximal inhibition (IC_{50}) of $388 \mu M$ (Fig. 3, A, C). Although β-EMTBL began to inhibit GABA currents at lower concentrations than β-EMGBL (10 vs $30 \mu M$), like TBL, β-EMTBL did not completely inhibit GABA responses (Fig. 3, B, C). The IC_{50} was $23 \mu M$, and the response saturated at $300 \mu M$ ($55 \pm 5\%$ inhibition; $n = 13$). α-EMTBL, an anticonvulsant, potentiated GABA currents (Fig. 4). As with the other GBLs and TBLs, this effect was immediate and was completely reversible. Saturating concentrations of α-EMTBL (1 mM) increased GABA responses to $190 \pm 17\%$ of control ($n = 11$), and the concentration of α-EMTBL that produced half-maximal potentiation was $102 \mu M$.

The effect of β-EMGBL and α-EMTBL on the GABA dose-response curve was also studied. β-EMGBL decreased the maximal response produced by GABA (Fig. 5A) without altering either the concentration of GABA that produces a half-maximal response ($51 \pm 4 \mu M$) or the nature of the cooperative interaction of GABA with its receptor (Hill coefficient, 2.1 ± 0.1). α-EMTBL

Figure 2. Successive applications of GABA for 100 msec at intervals of 30 sec produced similar currents. Three successive responses to $30 \mu M$ GABA are shown. Calibration bars: 10 pA, 400 msec.

increased the maximal response produced by saturating concentrations of GABA. Like β-EMTBL, α-EMTBL had no effect on either the GABA concentration that produced a half-maximal response ($51 \pm 4 \mu M$) or the Hill coefficient (2.0 ± 0.1). Neither the degree of inhibition of GABA responses by β-EMGBL nor the degree of potentiation produced by α-EMTBL was dependent on the GABA concentration, consistent with the lactones acting at a site distinct from the GABA receptor itself.

α-EMGBL (10 mM) was unique because its effects were dependent upon GABA concentration. It potentiated responses to $0.5 \mu M$ GABA but did not alter currents produced by 10, 30, or $100 \mu M$ GABA (Figs. 5B, 6A). In the presence of α-EMGBL the response to $0.5 \mu M$ GABA was $202 \pm 20\%$ of the response to $0.5 \mu M$ GABA alone ($n = 12$). In contrast, the response to $10 \mu M$ GABA and 10 mM α-EMGBL was $106 \pm 6\%$ of the response to $10 \mu M$ GABA alone ($n = 6$). Similarly, current produced by $30 \mu M$ GABA and 10 mM α-EMGBL was $100 \pm 6\%$ of the response to $30 \mu M$ GABA alone ($n = 11$) and the combination

Figure 3. Inhibition of GABA responses by convulsant GBLs and TBLs. The effect of (A) $400 \mu M$ β-EMGBL and (B) $100 \mu M$ β-EMTBL on currents produced by 100 msec application of $30 \mu M$ GABA in voltage-clamped hippocampal neurons. C, Concentration-dependent inhibition of GABA currents by β-EMGBL (▲) and β-EMTBL (●). Points are mean percentage of the control response to $30 \mu M$ GABA and error bars are ± 1 SEM. A minimum of 7 cells were studied at each concentration. Calibration bars: 15 pA, 250 msec.

Figure 4. Potentiation of GABA responses by α -EMTBL. *A*, Effect of 1 mM α -EMTBL on currents produced by 100 msec application of 30 μ M GABA in voltage-clamped hippocampal neurons. *B*, Concentration-dependent potentiation of GABA currents by α -EMTBL. Points are mean percentage of the control response to 30 μ M GABA and error bars are $\pm$ 1 SEM. A minimum of 11 cells were studied at each concentration. Calibration bars: 15 pA, 250 msec.

Figure 5. Effect of α -EMTBL, α -EMGBL, and β -EMGBL on responses to various concentrations of GABA in voltage-clamped hippocampal neurons. *A*, Dose-response curves for GABA alone ($\circ$) and in the presence of 1 mM α -EMTBL ($\bullet$) or 1 mM β -EMGBL ($\blacklozenge$). Points are means $\pm$ SEM; a minimum of 4 cells were tested at each concentration. The currents produced by 1 mM GABA averaged 78 ± 42 pA (mean $\pm$ SD) in 4 cells (range, 12–109 pA). *B*, Effect of 10 mM α -EMGBL on responses to 100 msec applications of (1) 0.5 μ M GABA, (2) 10 μ M GABA. Calibration bars: (1) 3 pA, 250 msec; (2) 10 pA, 250 msec.

of 100 μ M GABA and 10 mM α -EMGBL was $99 \pm 4\%$ ($n = 7$) of the current produced by 100 μ M GABA alone. In addition, α -EMGBL antagonized the effects of other butyrolactones and picrotoxinin on GABA responses. α -EMGBL, 10 mM, completely overcame the blockade of responses to 30 μ M GABA that were produced by 400 μ M β -EMGBL (Fig. 6*B*) and 30 μ M picrotoxinin (Table 1) and decreased the potentiation of GABA responses produced by 300 μ M α -EMTBL (Fig. 6*C*). In contrast, α -EMGBL was unable to block the potentiation caused by either 500 μ M phenobarbital (Fig. 6*D*) or 25 μ M chlordiazepoxide. Two other α -substituted GBLs and 2 other α -substituted TBLs were tested for their effects on currents produced by the application of 30 μ M GABA (Table 2). All compounds were tested at 3 mM. The α -TBLs all potentiated peak GABA currents. None of the α -GBLs affected GABA responses.

Both the anticonvulsant ESM and the convulsant TMSM decreased the peak amplitude of GABA currents (Fig. 7, *A*, *B*). Ethosuximide inhibited GABA currents at concentrations exceeding 300 μ M. At 10 mM (the highest concentration tested), ESM diminished GABA currents to $64 \pm 4\%$ of control ($n = 7$). Tetramethylsuccinimide was more potent than ESM (Fig. 7*C*). It began to block GABA responses at 100 μ M and had an IC_{50} of 435 μ M. In addition, 3 mM TMSM almost completely blocked GABA currents.

In order to establish that the actions of these compounds are restricted to the GABA receptor complex, we examined their effects on other electrophysiological parameters. No currents were evoked by application of any of the succinimides, GBLs, or TBLs in the absence of GABA. The current-voltage relationship was linear between -40 and -90 mV, and this relationship was unchanged by the application of saturating concentrations of drug (4 neurons for each drug; Fig. 8*A*). Also, none of the drugs tested altered the GABA reversal potential (at least 2 cells for each drug; Fig. 8*B*). None of the drugs altered currents produced by the application of 100 μ M glutamate. When tested on current-clamped neurons in a solution that did not contain tetrodotoxin 1 mM β -EMGBL ($n = 5$), 1 mM β -EMTBL

($n = 3$), 1 mM α -EMTBL ($n = 7$), and 10 mM α -EMGBL ($n = 7$) did not change the resting membrane potential. In addition, none of these compounds altered action potential amplitude, threshold, or rise time. Thus, these compounds do not appear to have direct membrane effects. Finally, the inactive compounds succinimide (10 mM) and GBL (10 mM) did not alter GABA responses, and these compounds did not prevent the inhibitory effect of picrotoxinin.

Effects of neuroactive lactones on IPSCs

Decay of the majority of IPSCs (18 of 22 postsynaptic neurons) was well fitted by a single exponential function with a time constant (τ_{IPSC}) of 34.4 ± 12.3 msec (mean $\pm$ 1 SD; range, 13.7–56.6 msec). IPSCs in the remaining 4 postsynaptic cells had a rapidly decaying component and were fit by a sum of 2 exponentials with a fast decay constant of 13.9 ± 7.0 msec and a slower time constant of 52.8 ± 13.4 msec. IPSC rise time was 3.6 ± 1.0 msec (range, 1.8–5.3 msec). There were no correlations between τ_{IPSC} , IPSC peak amplitude, and IPSC rise time ($p > 0.05$; data not shown).

Bath perfusion of 1 mM β -EMGBL decreased the peak amplitude of IPSCs and also increased the rate of decay of the inhibitory postsynaptic current (Fig. 9*A*). At a lower concentration of β -EMGBL (70 μ M), IPSC peak amplitude and τ_{IPSC} were also diminished but to a lesser extent than was seen in the presence of 1 mM β -EMGBL (Table 3). The total charge transferred (Q_{IPSC}) during IPSCs was determined by integration of the IPSCs with respect to time. The effects of β -EMGBL on the IPSC peak and τ_{IPSC} are reflected as decreases in the Q_{IPSC} . β -EMTBL, 100 μ M and 1 mM, increased the rate of decay of the IPSCs and decreased Q_{IPSC} without significantly decreasing IPSC amplitude (Fig. 9*B*). There was no qualitative or quantitative

Figure 6. Effect of α -EMGBL on responses to 30 μ M GABA alone and in combination with β -EMGBL, α -EMTBL, and phenobarbital. α -EMGBL does not directly alter GABA currents (A); however, α -EMGBL antagonizes (B) β -EMGBL-induced blockade of GABA currents and (C) α -EMTBL-induced potentiation of GABA currents but not (D) phenobarbital-induced potentiation of GABA currents. Drug concentrations: 30 μ M GABA, 10 mM α -EMGBL, 400 μ M β -EMGBL, 300 μ M α -EMTBL, and 500 μ M phenobarbital. Calibration bars: 15 pA, 250 msec.

difference between the effects of 100 μ M and 1 mM β -EMTBL on IPSCs, and as a result the data were combined. Neither β -EMGBL nor β -EMTBL affected IPSC rise time.

α -EMTBL had no effect on the rise time or peak amplitude of the IPSC in any of the 7 neuron pairs tested. However, 1 mM α -EMTBL doubled the τ_{IPSC} and increased the Q_{IPSC} to 170% of control (Table 1). The effects of 10 mM α -EMGBL were identical to those of α -EMTBL (Fig. 10A). α -EMGBL did not affect the rise time or peak amplitude of IPSCs but did increase both the τ_{IPSC} and the Q_{IPSC} (Fig. 10B). This direct effect on IPSCs contrasts with α -EMGBL's lack of an effect on responses to GABA at concentrations exceeding 10 μ M. Peak amplitude, rise time, rate of decay, and charge transfer of excitatory postsynaptic potentials were unaffected by either 1 mM α -EMTBL ($n = 4$) or 10 mM α -EMGBL ($n = 4$).

Table 1. Effect of α -EMGBL (10 mM) alone and in combination with picrotoxinin (30 μ M), β -EMGBL (400 μ M), α -EMTBL (300 μ M), phenobarbital (500 μ M), and chlordiazepoxide (25 μ M) on currents produced by the application of GABA (30 μ M) to voltage-clamped hippocampal neurons

Compound applied with GABA	% Control GABA response
α -EMGBL	101 $\pm$ 8 (8)
Picrotoxinin	44 $\pm$ 8 (8)
Picrotoxinin + α -EMGBL	102 $\pm$ 12 (8) ^a
β -EMGBL	50 $\pm$ 3 (7)
β -EMGBL + α -EMGBL	98 $\pm$ 2 (7)
α -EMTBL	176 $\pm$ 10 (12)
α -EMTBL + α -EMGBL	114 $\pm$ 7 (12) ^a
Phenobarbital	153 $\pm$ 8 (8)
Phenobarbital + α -EMGBL	156 $\pm$ 12 (8)
Chlordiazepoxide	168 $\pm$ 15 (7)
Chlordiazepoxide + α -EMGBL	163 $\pm$ 4 (7)

Means $\pm$ SEM; the number of cells tested is given in parentheses.

^a $p < 0.01$ compared with responses without α -EMGBL (Student's t test).

Discussion

The picrotoxin receptor is thought to be the site of action of several convulsant compounds including picrotoxin and related plant alkaloids, pentyleneetetrazol, and cage convulsants (Ticku, 1986). Recently, Clifford and his colleagues (1989) have shown that the convulsant β -isopropyl GBL inhibits GABA currents in a manner similar to picrotoxinin. In addition, binding studies have shown that alkyl-substituted GBLs and TBLs compete with TBPS, a ligand specific for the picrotoxin receptor (Levine et al., 1985; Holland et al., unpublished observations). Here we have demonstrated that β -EMGBL, TBL, and β -EMTBL, like picrotoxinin and β -isopropyl GBL, diminished GABA responses. At inhibitory synapses, β -EMGBL decreased both IPSC peak amplitude and duration, while β -EMTBL diminished τ_{IPSC} more significantly than it affected IPSC peak amplitude.

There is an important difference in the effects of the convulsant GBLs and the effects of TBL and β -EMTBL. The β -substituted GBLs (β -EMGBL and β -isopropyl GBL) fully block GABA responses, while TBL and β -EMTBL only partially inhibit GABA currents. This suggests that β -EMTBL may elicit a less pronounced conformational change in the structure of the GABA receptor complex than does picrotoxin or β -EMGBL.

Table 2. Comparison of the effects of various α -substituted GBLs and TBLs on currents produced by 30 μ M GABA

Compound	% Control GABA current
α -EMGBL	100 $\pm$ 7 (8)
α - <i>t</i> -Butyl GBL	106 $\pm$ 11 (8)
α -Isopropyl- α -methyl GBL	95 $\pm$ 7 (9)
α -EMTBL	190 $\pm$ 17 (11) ^a
α -Ethyl TBL	181 $\pm$ 23 (9) ^a
α -Isopropyl- α -methyl TBL	174 $\pm$ 18 (9) ^a

Means $\pm$ SEM; the number of cells tested is given in parentheses. All drugs were tested at a concentration of 3 mM.

^a $p < 0.01$ as compared with responses to 30 μ M GABA alone; Student's t test.

Figure 7. Inhibition of GABA responses by alkyl-substituted succinimides. The effect of (A) 10 mM ESM and (B) 300 μM TMSM on currents produced by the application of 30 μM GABA in voltage-clamped hippocampal neurons. C, Concentration-dependent inhibition of GABA currents by ESM ($\blacktriangle$) and TMSM ($\bullet$). Points are mean percentage of the control response to 30 μM GABA and error bars are ± 1 SEM. At least 5 cells were studied at each concentration. Calibration bars: 15 pA, 250 msec.

Thus, β -EMTBL appears to have only partial efficacy at the picrotoxin receptor site. A similar observation has been made for certain ligands acting at the benzodiazepine receptor, which also appear to only partially modulate GABA responses (Bracstrup and Nielsen, 1986). The differential action of β -EMGBL and β -EMTBL on inhibitory synapses may be a reflection of the partial efficacy of β -EMTBL when compared with β -EMGBL. Although β -EMTBL is only partially effective, it is 6-fold more potent as a convulsant compared with β -EMGBL. The relative convulsant potencies of β -EMGBL and β -EMTBL suggest that only slight antagonism of GABA-mediated inhibitory processes is required to produce convulsions.

Figure 8. Alkyl-substituted TBLs have no effect when applied without GABA and do not alter the GABA reversal potential. A, Typical whole-cell current-voltage relationship alone ($\circ$) and in the presence of 1 mM α -EMTBL ($\square$) or 1 mM β -EMTBL ($\triangle$). The resting potential of this cell was -60 mV. B, Typical determination of the GABA reversal potential using 30 μM GABA alone ($\bullet$) and in the presence of 1 mM α -EMTBL ($\blacksquare$) or 1 mM β -EMTBL ($\blacktriangle$).

The relative potencies of the convulsants, β -EMGBL, TBL, and β -EMTBL in the ^{35}S -TBPS binding assays (Levine et al., 1985; Holland et al., unpublished observations) correlate with relative potencies as inhibitors of GABA currents. However, higher concentrations of these convulsants were required to inhibit GABA currents than to inhibit ^{35}S -TBPS binding to rat brain membrane preparations. In contrast, the anticonvulsant α -EMTBL was slightly more effective in potentiating GABA currents than in displacing TBPS from the picrotoxin receptor.

Table 3. Effects of β -EMGBL, β -EMTBL, α -EMTBL, and α -EMGBL on the rise time, peak amplitude, decay time constant (τ_{IPSC}), and charge transfer (Q_{IPSC}) of inhibitory postsynaptic potentials

Compound ^a	% Control			
	Rise time	Peak	τ_{IPSC} ^b	Q_{IPSC}
β -EMGBL (70 μM)	105 $\pm$ 9	86 $\pm$ 2 ^d	76 $\pm$ 5 ^d	69 $\pm$ 5 ^d
β -EMGBL (1 mM)	88 $\pm$ 5	43 $\pm$ 2 ^e	22 $\pm$ 2 ^e	13 $\pm$ 2 ^e
β -EMTBL (100 μM , 1 mM) ^c	92 $\pm$ 8	86 $\pm$ 8	36 $\pm$ 8 ^d	36 $\pm$ 5 ^e
α -EMTBL (1 mM)	105 $\pm$ 8	101 $\pm$ 4	192 $\pm$ 19 ^d	174 $\pm$ 11 ^e
α -EMGBL (10 mM)	118 $\pm$ 12	97 $\pm$ 3	159 $\pm$ 11 ^d	170 $\pm$ 17 ^d

Means $\pm$ SEM.

^a Number of inhibitory pairs studied was 5 for 70 μM and 1 mM β -EMGBL, 5 for β -EMTBL, and 8 for α -EMTBL and α -EMGBL.

^b Only IPSCs that decayed with a single exponential were used; 70 μM and 1 mM β -EMGBL, $n = 3$; β -EMTBL, $n = 5$; α -EMTBL and α -EMGBL, $n = 6$.

^c 100 μM ($n = 3$) and 1 mM ($n = 2$) have the same qualitative and quantitative effects on IPSCs and the data were combined.

^d $p < 0.01$ vs control (Student's t test).

^e $p < 0.001$ vs control (Student's t test).

Figure 9. Effects of β -EMGBL and β -EMTBL on inhibitory postsynaptic currents. In each part, the *top trace* is the IPSC elicited by stimulating a nearby neuron; *bottom trace* is the presynaptic action potential. *A*, An IPSC before, during, and after bath perfusion with 1 mM β -EMGBL. The *thin line* is the β -EMGBL trace multiplied by a factor of 2.25. A comparison of this line with the control traces shows β -EMGBL shortens IPSC duration. *B*, An IPSC before, during, and after bath perfusion with 1 mM β -EMTBL.

These differences may be a reflection of the different methods of tissue preparation (intact cells in the electrophysiological experiments versus membrane preparation in ligand binding experiments), drug application (localized application to cell versus exposure of the entire membrane preparation), the presence of GABA in the electrophysiological experiments but not in the TBPS binding experiments, and different ionic compositions of the solutions used in the 2 experimental paradigms (Tris-citrate buffer with 250 mM NaBr was used in the ligand binding protocol).

β -EMGBL-induced antagonism of GABA response is non-competitive. High concentrations of GABA could not overcome blockade by β -EMGBL. Also the degree of inhibition of GABA responses by this compound was independent of GABA concentration, and β -EMGBL did not alter the half-maximal GABA

concentration. These characteristics are all indicative of non-competitive inhibition (Segel, 1975). Akaike and his colleagues (1985) have reported that picrotoxin has an identical effect in bullfrog dorsal root ganglion cells. In contrast, both β -isopropyl GBL and TBPS have been reported to act as mixed-type inhibitors of GABA responses (Van Renterghem et al., 1987; Clifford et al., 1989). These studies used chick GABA receptors which also quantitatively differ from hippocampal neurons in their responses to alkyl-substituted GBLs and TBLs (Clifford et al., 1989). Classically, mixed-type inhibition results when the inhibitor-receptor complex has a different affinity for the ligand than does the uninhibited receptor (Segel, 1975). ^3H -muscimol binding studies with rat brain membranes have shown that β -EMGBL does not affect the affinity of the GABA_A-recognition site (Holland et al., unpublished observations). This observation

Figure 10. α -EMTBL and α -EMGBL diminish the rate of decay of inhibitory postsynaptic currents. In each part, the *top trace* is the IPSC elicited by stimulating a nearby neuron; *bottom trace* is the presynaptic action potential. *A*, An IPSC before, during, and after bath perfusion with 1 mM α -EMTBL. *B*, An IPSC before, during, and after bath perfusion with 10 mM α -EMGBL.

is in agreement with the physiological data obtained in this study and supports the hypothesis that β -EMGBL, like picrotoxin, is a noncompetitive inhibitor of GABA responses in rat hippocampal neurons.

In voltage-clamped hippocampal neurons α -EMTBL, an anticonvulsant, potentiated GABA currents. This potentiation was not caused by a direct GABA mimetic action because α -EMTBL did not alter membrane conductances in the absence of GABA. These observations qualitatively agree with those seen by Baker et al. (1988) in chick spinal cord neurons. In addition, we show that α -EMTBL decreases the rate of decay of IPSCs. Thus, this agent is capable of modulating neuronal inhibition under physiological conditions, and our results are consistent with radioligand binding and behavioral experiments that indicate the binding of α -EMTBL with the picrotoxin receptor is a low-affinity interaction (Klunk et al., 1982b; Weissman et al., 1984; Levine et al., 1985, 1986; Holland et al., unpublished observations). This interaction is distinct from that of picrotoxinin. Instead of decreasing GABA-mediated inhibition like picrotoxinin, α -EMTBL acts in an opposite fashion by potentiating GABA responses. All of the other α -alkyl-substituted TBL derivatives examined in this report potentiated GABA currents as well.

The effects of α -EMTBL on the GABA dose-response curve are opposite to those of β -EMGBL. α -EMTBL increased the maximal response to GABA but did not change the concentration of GABA that produced a half-saturating response. The action of α -EMTBL on the GABA dose-response curve is different from that of benzodiazepine receptor agonists, which produce a parallel leftward shift of the GABA dose-response curve but do not increase responses to saturating concentrations of GABA (Choi et al., 1981; Farb et al., 1984; Chan and Farb, 1985). Instead, the actions of α -EMTBL more closely resemble those of barbiturates (Twyman et al., 1988). However, radioligand binding experiments and electrophysiological experiments with α -EMGBL suggest that α -EMTBL and barbiturates act at different sites on the GABA receptor (see below; Holland et al., unpublished observations).

The effects of α -EMGBL were initially very confusing. This compound did not alter currents produced by 10–100 μ M GABA but did prevent picrotoxinin and other butyrolactones from modulating GABA responses. This suggested that α -EMGBL was devoid of activity alone but eliminated the ability of other compounds to act at the picrotoxin site. However, it was difficult to reconcile this hypothesis with the decrease in the rate of decay of evoked IPSCs produced by α -EMGBL. Recent information about the kinetics of the GABA channel provides a plausible explanation for these apparently contradictory results (Mathers and Wang, 1988; MacDonald et al., 1989; Weiss and Magleby, 1989). The main conductance state of the GABA-gated channel probably has 2 or more open states; an early, short open state produced when one GABA molecule binds to the receptor/channel complex and later, longer open states produced by 2 molecules binding to the receptor. Rapid application or low GABA concentrations favor the short open state, while prolonged drug application or high GABA concentrations favor the later, double-liganded state of the GABA receptor/channel complex. We think that the opening or closing rate constants are selectively affected by α -EMGBL. This explains why IPSCs (which occur on a relatively rapid time scale) are affected by α -EMGBL, while exogenous applications of GABA between 10–100 μ M (which occur on a slower time scale than IPSCs) are unaffected by α -EMGBL. This hypothesis is also consistent with

the observation that α -EMGBL augments currents produced by extremely low GABA (0.5 μ M).

We believe that α -EMGBL is an antagonist at the picrotoxin receptor but that its antagonist effect is restricted to the longer open state(s) of the GABA-gated ionophore. This property is seen with GABA concentrations ≥ 10 μ M and long (≥ 100 msec) application times. Preliminary results suggest that another compound, α -isopropyl- α -methyl GBL, appears to be a complete picrotoxin receptor antagonist. This agent fails to prolong the duration of IPSCs and does not augment responses to 0.5 μ M GABA but does block effects of other drugs which act at the picrotoxin receptor (Holland et al., unpublished observations).

α -EMGBL is able to block the effects of picrotoxinin, α -EMTBL, and β -EMGBL but not phenobarbital or chlordiazepoxide (a benzodiazepine receptor agonist) on GABA responses in voltage-clamped hippocampal neurons. Neither phenobarbital nor chlordiazepoxide were used at saturating concentrations; when higher concentrations of either compound were tested, larger potentiations of GABA currents were observed. Thus, the inability of α -EMGBL to overcome the effects of these agents cannot be attributed to saturation of a common receptor by the active agent. Therefore, α -EMGBL selectively blocks the effects of ligands thought to act at the picrotoxin receptor (picrotoxinin, β -EMGBL, and α -EMTBL) but has no effect on agents that act at other modulatory sites on the GABA receptor. These data indicate that α -EMGBL, α -EMTBL, β -EMGBL, and picrotoxin share a common site of action which is distinct from the barbiturate and benzodiazepine binding sites.

Our data demonstrate that the picrotoxin receptor can modulate the GABA-gated chloride channel in opposing ways. Picrotoxinin and β -substituted GBLs decrease the effect of GABA. Alpha-substituted TBLs act at this receptor to enhance the action of GABA. α -EMGBL has no direct effect on GABA responses but blocks the actions of agents which act at the picrotoxin receptor. A similar type of modulation has previously been described for ligands that act at the benzodiazepine receptor (Braestrup and Nielsen, 1986). At this receptor, benzodiazepine agonists enhance the effects of GABA, while benzodiazepine receptor-inverse agonists decrease GABA responses. This type of modulation, where opposing effects can be mediated by different ligands acting at the same receptor, was thought to be unique to the benzodiazepine receptor. However, here we report that the other major modulatory site on the GABA-gated Cl^- channel, the picrotoxin receptor, can also affect the gating of the channel in opposing ways.

Like Barnes and Dichter (1984), we also conclude that the convulsant effects of TMSM may involve a blockade of GABAergic neurotransmission, but the anticonvulsant effect of ESM is unrelated to its action at the picrotoxin receptor. The behavioral effects of ESM and TMSM differ substantially. Tetramethylsuccinimide is a potent convulsant, while ESM is an anticonvulsant which is used clinically for the treatment of absence seizures. However, although TMSM is about 10-fold more potent than ESM, both TMSM and ESM bind to the picrotoxin receptor and decrease GABA-mediated neuronal inhibition. High concentrations of ESM partially inhibit the binding of ^3H -muscimol to the GABA recognition site and ^3H -flunitrazepam to the benzodiazepine receptor (Holland et al., unpublished observations). This has also been described with the convulsants picrotoxinin, β -EMGBL, β -EMTBL, and TMSM but not with the anticonvulsants α -EMGBL and α -EMTBL. The concentrations of ESM required to block GABA currents and to displace

³⁵S-TBPS from the picrotoxin receptor are 5- to 10-fold higher than those that are needed to produce anticonvulsant effects (Chang et al., 1972). Recently, Coulter and his colleagues (1989) have shown that therapeutic concentrations of ESM but not TMSM inhibit low-threshold calcium currents in thalamic neurons. They hypothesize that this effect is responsible for the anticonvulsant properties of ESM.

In summary, our data show that β -substituted GBLs block GABA currents and therefore we hypothesize that these compounds may act as picrotoxin receptor agonists. The convulsant TBLs only partially diminish GABA currents and may be partial agonists. Alpha-alkyl-substituted TBLs have the opposite effect and therefore may be picrotoxin receptor inverse agonists. α -EMGBL enhances responses to a low GABA concentration but does not directly affect currents produced by higher concentrations of GABA. However, at high GABA concentrations, α -EMGBL prevents the effects of α -EMTBL, β -EMGBL, and picrotoxin. Thus, α -EMGBL may represent a mixed inverse agonist/antagonist at the picrotoxin receptor. These results demonstrate that alkyl-substituted GBLs and TBLs represent a class of chemical compounds that have multiple and different effects on GABA-mediated chloride conductances and neuronal activity. Moreover, our results are the first demonstration that the picrotoxin receptor is capable of modulating GABA responses in opposing ways. It has previously been reported that ligands acting at the benzodiazepine receptor can also have opposite effects on GABA responses. Thus, both modulatory sites of the GABA-gated ionophore are capable of regulating GABA responses in complex ways.

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