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Dual Modulation of the γ-Aminobutyric Acid Type A Receptor/Ionophore by Alkyl-Substituted γ-Butyrolactones

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SUMMARY

Alkyl-substituted γ-butyrolactones (GBLs) and γ-thiobutyrolactones exhibit convulsant or anticonvulsant activity, depending on the alkyl substituents. α-Substituted lactones with small alkyl substituents are anticonvulsant and potentiate γ-aminobutyric acid (GABA)-mediated chloride currents, whereas β-substituted compounds are usually convulsant and block $GABA_A$ currents. We have now found that this distinction is not so clear-cut, in that some compounds can both block and augment $GABA_A$ currents, but with different time courses. For example, α,α-diisopropyl-GBL (α-DIGBL) potentiates exogenous GABA currents in cultured rat hippocampal neurons but diminishes GABA-mediated inhibitory postsynaptic currents. A more detailed analysis demonstrates a triphasic effect of α-DIGBL on GABA currents, with a rapid inhibitory phase, a slower potentiating phase, and then an "off response" when the GABA/α-DIGBL perfusion is stopped. Thus, α-DIGBL can inhibit and potentiate GABA currents with kinetically different time courses. Inhibition is more rapid, but at steady state potentiation dominates. Using a simplified model of the $GABA_A$ receptor/ionophore, we have simulated our experimental observations with α-DIGBL. Another lactone, β-ethyl-β-methyl-γ-thiobutyrolactone, also has dual actions, with inhibition predominating at low concentrations and potentiation predominating at high concentrations. We propose two distinct GBL modulatory sites on the $GABA_A$ receptor, i.e., an inhibitory "picrotoxin" site and an enhancing "lactone site." New information on the structure of the $GABA_A$ receptor/ionophore may allow the molecular dissection of these two sites.

The $GABA_A$ receptor/ionphore complex represents a family of ligand-gated ion channels that account for the majority of rapid inhibitory synaptic transmission in the mammalian brain (1). Because of its importance in regulating neuronal excitability, the $GABA_A$ receptor has been extensively characterized. In addition to a binding site for GABA, which is responsible for gating the channel and thus allowing chloride flux across the neuronal membrane, there are other binding sites that modulate the action of GABA. The receptor has regions that bind benzodiazepines, barbiturates, neuroactive steroids, and convulsant plant alkaloids such as picrotoxin. The benzodiazepine site on the $GABA_A$ receptor is capable of either enhancing or diminishing the action of GABA, depending on the nature of the ligand and the receptor subtype. Recently, alkyl-substituted GBLs and TBLs that can modulate GABA responses in opposing ways have also been described (2–4). GBLs and TBLs with ethyl and methyl alkyl substituents in the β-position diminished GABA responses and produced seizures in animals, whereas GBLs and TBLs with ethyl and methyl alkyl substituents in the α-position increased the activity of GABA and had anticonvulsant activity in a broad range of experimental seizure models in animals (5, 6). We initially thought that these compounds acted directly at the picrotoxin receptor. However, based on dissociation rates of TBPS, a ligand specific for the picrotoxin receptor site, we now have evidence that some TBLs and GBLs act at an additional, independent, site that is allosterically linked to the picrotoxin site of the $GABA_A$ receptor (7).

Although all β-, α,β-, and β,γ-alkyl-substituted GBLs and TBLs thus far studied are convulsants and inhibit GABA responses in hippocampal neurons, the α-substituted com-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GBL, γ-butyrolactone; α-DIGBL, α,α-diisopropyl-γ-butyrolactone; β-EMTBL, β-ethyl-β-methyl-γ-thiobutyrolactone; α-EIGBL, α-ethyl-α-isopropyl-γ-butyrolactone; α-EMTBL, α-ethyl-α-methyl-γ-thiobutyrolactone; TBL, γ-thiobutyrolactone; α-*t*BGBL, α-*tert*-butyl-γ-butyrolactone; IPSC, inhibitory postsynaptic current; TBPS, *t*-[^{35}S]butylbicyclophosphorothionate; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzolo[*a*,*d*]cyclohepten-5,10-amine maleate; E_{GABA}, γ-aminobutyric acid reversal potential; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; M2 region, second transmembrane region.

pounds have a more complicated structure-activity relationship. The GBLs and TBLs with small alkyl substituents are anticonvulsants, but those with larger alkyl groups, for example α-DIGBL, are convulsants (8). Because we had no information on the cellular effects of these latter compounds, we decided to investigate whether they also antagonized IPSCs and GABA-induced currents. We were surprised to discover that α-DIGBL and the related compounds α-*t*BGBL and α-EIGBL had dual effects on GABA-induced currents. They inhibited and potentiated, but with different time courses. When we reexamined the physiological effects of β-EMTBL, which has unusual concentration-dependent effects on TBPS binding (7), we found that there was a switch from block to potentiation of GABA currents at high β-EMTBL concentrations. Our results suggest that some of the GBLs interact at two distinct sites on the $GABA_A$ receptor/ionophore. This type of dual modulation has not been described for other drugs affecting ligand-gated receptors.

Experimental Procedures

Materials. α-DIGBL, α-*t*BGBL, α-EIGBL, α-EMTBL, and β-EMTBL were synthesized by previously described methods and had the appropriate analytical and spectroscopic properties (Fig. 1) (8–10). cDNAs encoding $GABA_A$ receptor subunits α1 and γ2 were provided by Dr. A. Tobin (University of California, Los Angeles), and cDNA encoding the β2 subunit was provided by Dr. C. Fraser (National Institute on Alcohol Abuse and Alcoholism). MK-801 and QX-314 were gifts of Merck and Astra, respectively. All other chemicals were purchasd from Sigma, unless mentioned otherwise.

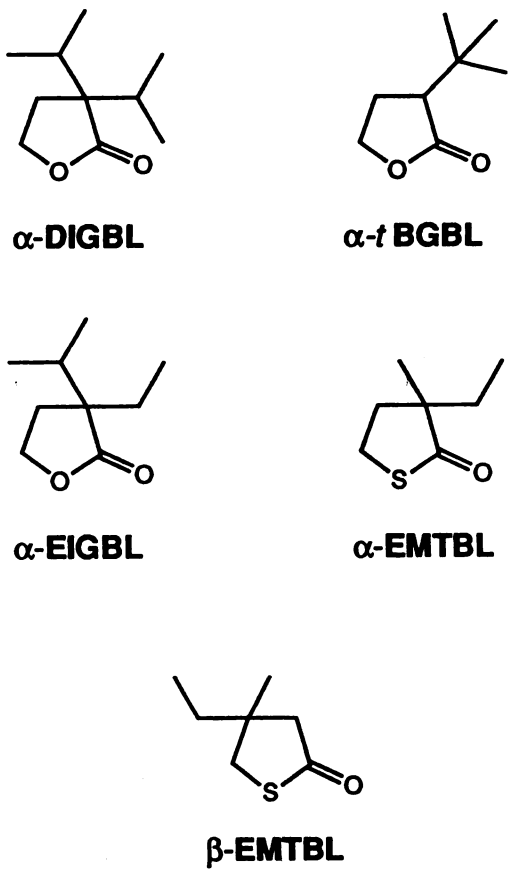

Fig. 1. Chemical structures of the five lactones used in experiments described in this paper.

Cell culture. We used two types of cultured cells for electrophysiology, namely primary cultured neurons and cell lines transfected with recombinant receptor DNA. Hippocampal neuronal cultures were prepared from neonatal rats according to previously described methods (11). Hippocampi were removed from female Sprague-Dawley rats on postnatal day 1, minced, and incubated for 20 min at 37° in 3 ml of Leibovitz's L-15 medium containing 1 mg/ml papain and 0.2 mg/ml bovine serum albumin. The hippocampi were triturated with Pasteur pipettes, in growth medium. The suspension was centrifuged through 2 ml of medium containing 10 mg/ml trypsin inhibitor and 10 mg/ml bovine serum albumin. The cells were resuspended in growth medium containing minimal essential medium (without glutamine), 10% NuSerum (Collaborative Research), 20 units/ml penicillin, and 20 μg/ml streptomycin and were plated onto a monolayer of cortical glial cells in 35-mm culture dishes (2.5×10^5 cells/dish). The culture dishes had been precoated with poly-L-lysine, followed by plating of rat cortical glial cells, which were allowed to divide for 4 days before neuron plating. HEK 293 cell lines were obtained from Washington University Tissue Culture Support Center and maintained in minimum essential medium supplemented with 5% fetal bovine serum, 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Transfection. Rat GABA receptor cDNAs were inserted into eukaryotic expression vectors containing the cytomegalovirus promoter and the antibiotic G418 resistance gene. The transfection protocol used was the calcium phosphate precipitation technique described by Chen and Okayama (12). HEK 293 cells were dispersed with trypsin and plated at a low density in 35-mm culture dishes. DNA precipitate was formed in 0.125 M $CaCl_2$ in HEPES-buffered saline at a concentration of 20 μg/ml. The pCH110 plasmid containing the *lacZ* marker gene for determination of transfection efficiency was included with the recombinant subunit DNA. The precipitate mixture was added to the medium in the culture dishes at a 1:10 ratio. Cells were incubated in the presence of the DNA precipitate for 4–8 hr, after which the cells were rinsed and the medium was replaced.

Electrophysiology. Electrophysiological experiments were carried out at 22° on the stage of an inverted microscope (Nikon), using whole-cell patch-clamp techniques (13). We recorded from hippocampal neurons that had been in culture for 10–21 days or HEK 293 cells that had been transfected 24–72 hr previously. The growth medium was removed and the cells were placed in an extracellular recording solution that contained 140 mM NaCl, 10 mM Na-HEPES, pH 7.3, 3 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 5.5 mM glucose, and 3 μM tetrodotoxin. Electrodes (4–10-MΩ resistance) were filled with an intracellular recording solution that contained 130 mM CsCl, 10 mM tetraethylammonium chloride, 10 mM Na-HEPES, pH 7.3, 2 mM MgATP, 1.1 mM EGTA, and 5.5 mM glucose. This solution also contained 2 mM QX-314 to block $GABA_B$ receptor responses that might complicate data interpretation (14). Cells were voltage clamped at −30 mV, using a standard commercial amplifier that allowed series resistance compensation and capacitative transient cancellation (Dagan 8900). GABA and test compounds, dissolved in extracellular solution, were applied at a rate of 30 μl/sec from a linear array of six flow tubes (340-μm diameter) positioned 50 μm from the cell. Flow was gravity driven and gated by a solenoid valve under the control of the data acquisition system. As soon as the drug application was terminated, the culture dish was perfused with extracellular solution, at 30 μl/sec, from a 340-μm-diameter glass tube positioned within 100 μm of the cell body, to prevent accumulation of drug in the extracellular solution. The effects of drugs on GABA-mediated currents were determined by measuring the control GABA current, followed by the current with GABA and drug and then the control GABA current again. Only data in which control current (±10%) was recovered were used. The control current amplitude was taken as the mean of the pre- and post-drug responses. The data were digitized and stored on disk for off-line analysis using a commercially available program (pClamp; Axon Instruments). Concentration-response curves were fit, using a commercially avail-

able program (SigmaPlot; Jandel Scientific), to the logistic function $y = MX^n/(X^n + K^n)$, where M is the maximal response, K is the EC_{50}, and n is equivalent to the Hill coefficient. For recording from HEK 293 cells, only single cells were used; the data were excluded if responses were recorded from several electrically coupled cells.

Pairs of neurons with monosynaptic connections were studied by recording from neighboring neurons with two whole-cell patch electrodes. We recorded current through a voltage-clamp circuit and stimulated cells intracellularly through a bridge amplifier. For these experiments, we omitted tetrodotoxin from the extracellular bath and added 20 μM MK-801, which reduced polysynaptic activity. The intracellular solution contained 138 mM potassium isethionate (Kodak), 2 mM KCl, 10 mM Na-HEPES, pH 7.3, 2 mM MgATP, 1.1 mM EGTA, and 4 mM glucose. With these solutions, excitatory postsynaptic connections were inward and IPSCs were outward at a holding potential of −60 mV. Pairs of cells were considered monosynaptic if the onset of the current was ≤5 msec after the peak of the presynaptic action potential and if all of the presynaptic action potentials resulted in postsynaptic responses. α-DIGBL was dissolved in extracellular solution and applied for 5–10 sec by local perfusion, at a rate of 2 ml/min, from a large-bore glass tube positioned 100 μm from the cell. After α-DIGBL application, the culture dish was perfused for 30 sec with extracellular solution at 2 ml/min, to prevent accumulation of drug. Control IPSCs were measured before and after the IPSC recorded in the presence of α-DIGBL, and the data were excluded if the second control current was not within 10% of the initial response. The data were digitized and stored on disk for analysis. IPSC decay time constants, peak amplitude, and total charge transfer were determined using the computer program pClamp.

Current simulations. We used a computer simulation program (TUTSIM; Tutsim Products) to model the interaction of α-DIGBL with the $GABA_A$ receptor/ionophore.

Results

As expected for a convulsant, α-DIGBL diminished the IPSC peak amplitude and charge transfer (Fig. 2). α-DIGBL at 10 mM completely obliterated IPSCs ($n = 2$), whereas 1 mM α-DIGBL decreased the peak amplitude of IPSCs to 51 ± 4% of control and the charge transfer to 54 ± 4% of control ($n = 7$). Interestingly, α-DIGBL prolonged the duration of IPSCs to 120 ± 8% of control ($n = 7$; $p < 0.05$). We doubt that this is a presynaptic effect of α-DIGBL, because the drug had no effect on the peak amplitude or decay of excitatory postsynaptic currents.

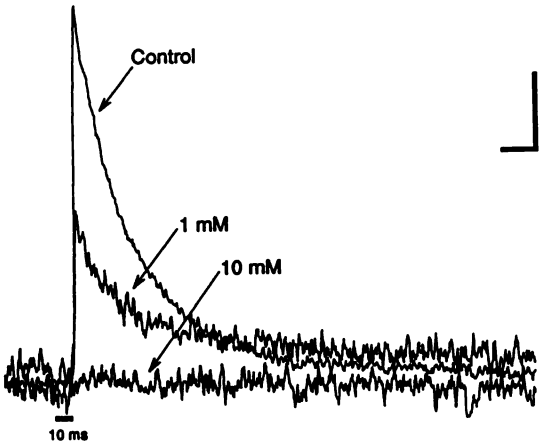

Fig. 2. Effect of α-DIGBL on IPSCs. At 1 mM, α-DIGBL diminished the IPSC peak but prolonged the decay; 10 mM α-DIGBL completely blocked the IPSC. *Line below the IPSC*, duration of stimulation of the presynaptic neuron. *Calibration bars*, 50 pA and 25 msec.

We then examined the effect of α-DIGBL on currents produced by application of GABA. One-second applications of 3 μM GABA spaced 15–30 sec apart produced currents of similar magnitude. When the magnitudes of the currents produced by varying GABA concentrations were normalized to the response produced by 30 μM GABA, a sigmoidal dose-response curve was obtained. The GABA EC_{50} was 9 μM and the response to GABA saturated at 100 μM, with a Hill slope of 1.5. The measured E_{GABA} of 1 ± 1 mV ($n = 3$) closely matched the calculated chloride equlibrium potential of −3 mV.

Coapplication of α-DIGBL with GABA increased the amplitude of GABA responses (Fig. 3A). This was a surprise, because lactones that antagonized IPSCs usually blocked GABA-induced currents (4). This potentiation was dependent on both GABA and α-DIGBL concentrations. The most consistent potentiation occurred at α-DIGBL concentrations of 1–10 mM and at GABA concentrations below its EC_{50}. At 1 mM, α-DIGBL increased responses to 1 μM GABA to 149 ± 7% of control ($n = 5$) and response to 3 μM GABA to 138 ± 16% of control ($n = 7$). At 10 mM, α-DIGBL produced even greater

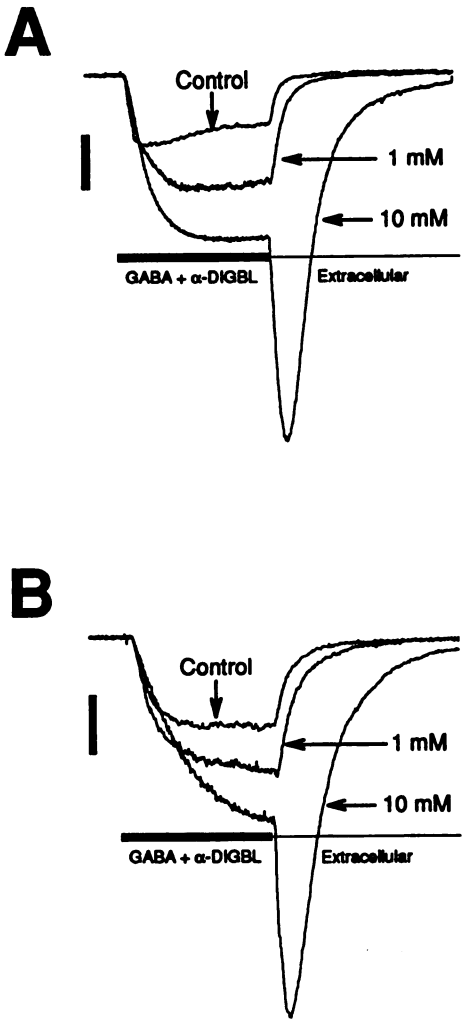

Fig. 3. Comparison of the potentiation of GABA-induced currents by α-DIGBL in hippocampal neurons (A) and HEK 293 cells transfected with $\alpha1\beta2\gamma2$ $GABA_A$ receptor subunits (B). The control traces represent responses to 3 μM GABA alone. *Thick lines*, duration of GABA or GABA plus α-DIGBL application, which was 2 sec (A) or 1 sec (B). *Current calibration bars*, 750 pA (A) and 1000 pA (B).

potentiation of GABA-induced currents, increasing the responses to 1 and 3 μM GABA to 312 ± 51% (n = 6) and 193 ± 33% (n = 6) of control, respectively.

Another aspect of the α-DIGBL enhancement of GABA-induced currents was initially puzzling. At the termination of coapplication of 10 mM α-DIGBL and GABA, we observed a large inward "off response" (Fig. 3A). At 1 μM GABA, the off current was 513 ± 106% (n = 6) of the response to 1 μM GABA alone. The onset of this current coincided with the initiation of perfusion with normal extracellular solution. The off current was only variably present at α-DIGBL concentrations of 1 mM and below. E_{GABA} was unaffected by α-DIGBL, during either steady state (E_{GABA} = 1 ± 3 mV; n = 3) or the off responses (E_{GABA} = 0 ± 3 mV; n = 3). Using another lactone, α-EIGBL, which produces robust off responses, it is easy to see that continuing lactone perfusion after GABA has been stopped eliminates these responses (Fig. 4A).

Similar results were obtained in HEK 293 cells transiently transfected with α1β2γ2 GABA receptor subunits (Fig. 3B). These cells have an EC_{50} for GABA of 14 μM, with a Hill slope of 1.7, close to the values for the hippocampal neurons. The response to 3 μM GABA was increased to 150 ± 8% of control in the presence of 1 mM α-DIGBL and to 194 ± 13% in the presence of 10 mM α-DIGBL (n = 3). An off response to 350 ± 11% of the control GABA response, coinciding with the initiation of perfusion with the extracellular solution, was also present with 10 mM but not 1 mM α-DIGBL. The α-DIGBL enhancement of GABA current had one other peculiar feature; the onset of current slowed with increasing α-DIGBL concentration. This is very readily seen in the HEK 293 cells (Fig. 3B).

The off responses were not unique to α-DIGBL, because they could be seen with the related compounds α-tBGBL (data not shown) and α-EIGBL (Fig. 4A). They were not produced by all lactones, however. Coapplication of α-EMTBL (3 mM, a nearly saturated solution) with 3 μM GABA produced minimal current rebound and did not diminish the initial onset of inward current (Fig. 4B).

We initially found the conflicting results with GABA and IPSCs and the α-DIGBL off responses very confusing. We eventually realized that all of our results were consistent if α-DIGBL both blocked and potentiated GABA-induced currents, but with different time courses. We hypothesized that α-DIGBL might rapidly block opening of the $GABA_A$ receptor/ionophore but stabilize the open channel at steady state. In this way, currents produced by very short GABA applications would be reduced but currents from longer applications would be potentiated. Rapid exit from the blocked states with lactone removal could explain the off responses. It is possible to construct many models that incorporate these properties. We have developed a simplified model of GABA-receptor interactions that reproduces the most important observations from our intracellular recordings (Fig. 5A). Our model allows two GABA molecules to bind and open the receptor/ionophore (15, 16). If lactone binds to the liganded but closed ionophore, the pore remains closed. However, binding to the open pore stabilizes this state. The binding of a second lactone to the open pore results in closing. We have assumed that the conductance of the two open states is identical.

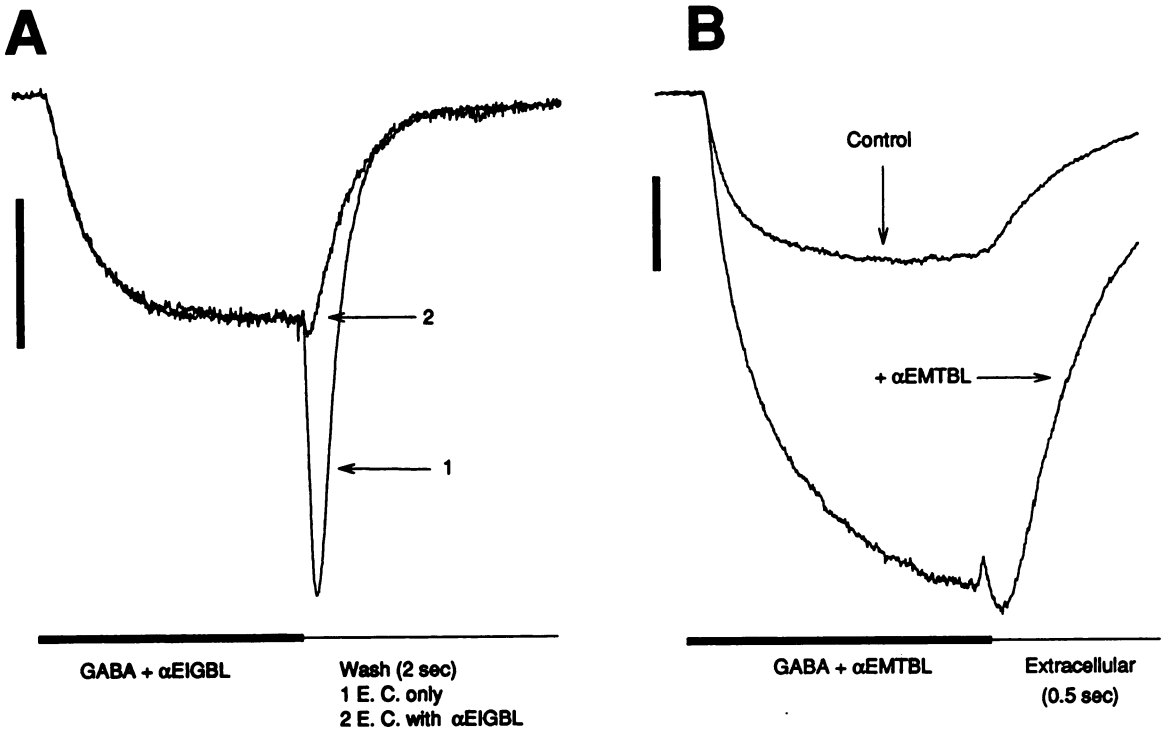

Fig. 4. A, The continuous presence of lactone prevents the appearance of off responses. Traces show the effect of 10 mM α-EIGBL on currents produced by 1 μM GABA. When α-EIGBL remained after the GABA application was stopped (*trace 2*), there was no off response. *Thick line*, 2-sec duration of GABA plus α-EIGBL application. *Current calibration bar*, 1000 pA. B, Off responses are much smaller with α-EMTBL. Traces show enormous enhancement of 3 μM GABA current by 3 mM α-EMTBL, with a minimal rebound when the drug application is stopped. *Thick line*, 1-sec duration of GABA or GABA plus α-EMTBL application. *Calibration bar*, 1000 pA, *E. C.*, extracellular solution.

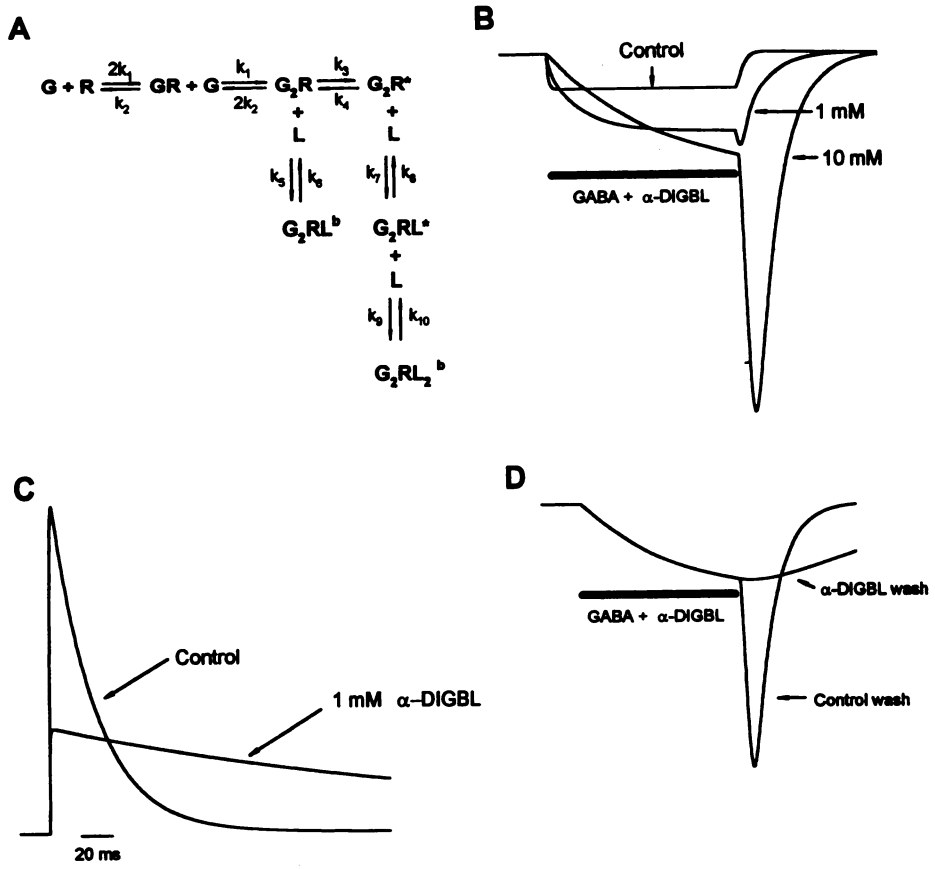

Fig. 5. Simulation of α-DIGBL experimental results using a simplified model of GABA-receptor-ionophore interaction. A, Kinetic scheme for binding of GABA (G) to receptor (R) leading to channel opening (*). One or two lactone molecules (L) can bind to the G_2R complex, either stabilizing an open state or producing block (b). This model assumes that both GABA binding sites have the same affinity and that the conductances of the two open states (G_2R^* and G_2RL^*) are identical. Values for the rate constants are as follows: k_1, 9×10^6 M^{-1} sec^{-1}; k_5, 6×10^6 M^{-1} sec^{-1}; k_7, 10^4 M^{-1} sec^{-1}; k_9, 10^5 M^{-1} sec^{-1}; k_2, 35 sec^{-1}; k_3, 50 sec^{-1}; k_4, 2000 sec^{-1}; k_6, 2500 sec^{-1}; k_8, 4 sec^{-1}; k_{10}, 250 sec^{-1}. We picked k_1 and k_2 to give a GABA EC_{50} of 10 μM, close to the experimental EC_{50}. B, Effect of α-DIGBL on the current produced by exogenous GABA application (3 μM), using this model. We assume that onset of GABA plus α-DIGBL is a step function and that both come off with identical, single-exponential time courses (time constant = 20 sec^{-1}). Note that, whereas GABA-induced currents are enhanced by α-DIGBL, the rise time decreases, consistent with an initial slow block (compare with Fig. 3). C, Effect of α-DIGBL on a simulated IPSC. We have assumed that GABA (200 μM) is present as a 0.5-msec step and α-DIGBL is present for the entire duration of the trace. Note that the model predicts that the duration of the IPSC will be increased by α-DIGBL, although the peak is diminished. Compare with Fig. 2. D, Effect of continuing lactone perfusion after GABA application has been stopped. Using the rate constants from A, we found that there is no off response if lactone is present after GABA has been allowed to exponentially disappear. The trace with the off response is identical to B and provided for comparison. This result is in agreement with the experimental data on α-EIGBL provided in Fig. 4A.

When a 2-sec application of 3 μM GABA is simulated, there is dose-dependent potentiation of the final GABA-induced current (Fig. 5B). Onset of potentiation lags at the higher α-DIGBL concentration and an off response is prominent, just as with the real data (Fig. 3). When we simulate IPSCs with a 0.5-msec pulse of 200 μM GABA, α-DIGBL reduces the peak current but prolongs the IPSC decay (Fig. 5C). This is qualitatively similar to the α-DIGBL effect on real IPSCs (Fig. 2). Finally, if α-DIGBL remains after the GABA is removed in the simulation, no off response appears (Fig. 5D). We made identical observations with α-EIGBL (Fig. 4A).

When we re-examined some of our old data, we realized that there were other GBLs that might also have dual effects on GABA-induced currents. For example, β-EMTBL behaves as an allosteric modulator of the picrotoxin receptor at high concentrations and a competitive inhibitor at low concentrations in ligand binding assays (7). Therefore, we carefully reinvestigated the dose-response relationship for this compound at low and high concentrations (Fig. 6). As has been previously demonstrated (4), β-EMTBL produced a dose-dependent, partial inhibition of GABA responses between 10 μM and 2 mM. However, at concentrations above 2 mM, this compound began to increase GABA currents. Maximal potentiation was reached at 6 mM. As with α-DIGBL, β-EMTBL produced off responses at the concentrations where it potentiated the GABA-induced current (Fig. 6A).

Discussion

These observations demonstrate a previously unappreciated level of complexity for the $GABA_A$ receptor/ionophore, and they identify a type of ligand-receptor interaction that is unusual in pharmacology. We had previously believed that the GBLs all interacted competitively at the picrotoxin site on the $GABA_A$ receptor/ionophore. This interaction was evaluated by measurement of TBPS displacement in binding experiments (9, 17). There was a previous suggestion that this was an oversimplification, when we found that several

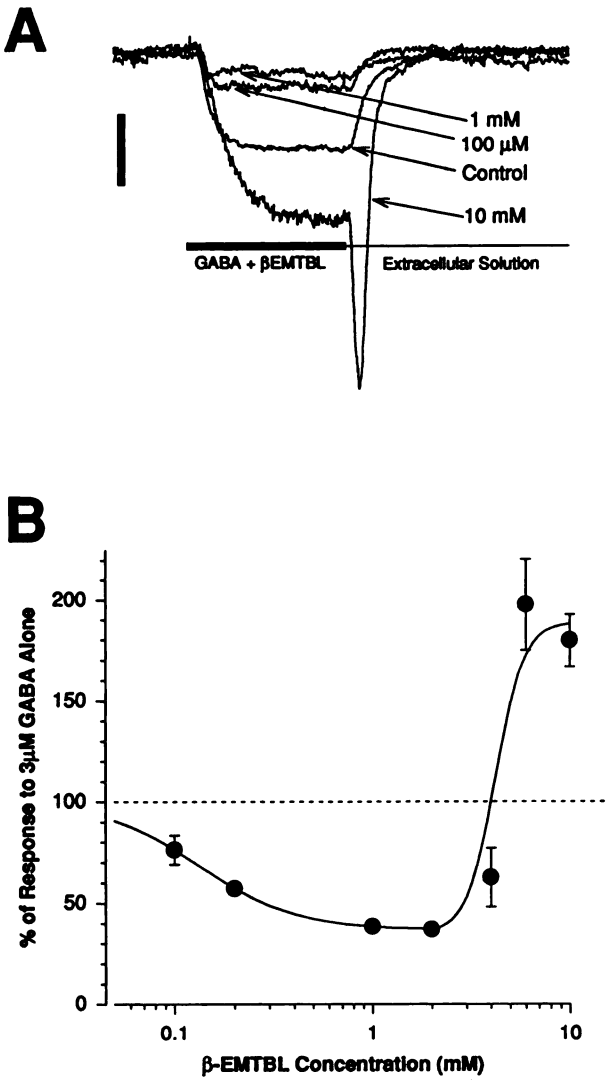

Fig. 6. Comparison of the effects of various β-EMTBL concentrations on GABA responses. A, Inhibition of 3 µM GABA-induced current by 100 µM and 1 mM β-EMTBL and potentiation by 10 mM β-EMTBL in hippocampal neurons. *Thick line*, 1-sec duration of GABA or GABA plus β-EMTBL application. *Calibration bar*, 500 pA. B, Concentration-response curve for β-EMTBL, showing block and enhancement of 3 µM GABA currents at low and high β-EMTBL concentrations, respectively. Each data point represents the mean of four to nine cells; *error bars*, standard error.

GBLs, including β-EMTBL, increased the dissociation rate for TBPS (7). For β-EMTBL, this occurred at concentrations much higher than the IC_{50} for TBPS binding. This is not the anticipated finding for a competitive interaction, where the association rate should be decreased. This should have suggested two different sites for GBL effects on $GABA_A$ receptor/ionophores. However, at the time these experiments were done, we had no physiological evidence for two distinct sites. The results described above provide that evidence.

We currently believe that there are two sites for lactone interaction; one is identical to the picrotoxin or TBPS site and inhibits GABA-induced current, and the other ("lactone site") potentiates currents. The effect of any single GBL is then determined by its affinity (K_d) for each of these sites and the values of the forward and reverse rate constants (k_{+1} and k_{-1}) for binding to the receptor/ionophore. Compounds like β-EMTBL block GABA-induced currents until the concentration exceeds 2 mM, because they have a much higher affinity for the TBPS site. Potentiation of GABA-induced currents dominates for other lactones with low affinity for the TBPS site, such as α-EMTBL. There were compounds that we previously identified as "antagonists" at the TBPS site (4, 18). These substances had little or no effect on GABA-induced currents but prevented other GBLs from either blocking or potentiating GABA-induced currents. It now seems most economical to suggest that these componds have approximately equal affinities for the TBPS and "lactone" sites. We believe that this model is consistent with most of the physiological data we have collected over the past decade. It qualitatively explains the seemingly contradictory effects of α-DIGBL on IPSCs and GABA currents and the confusing off responses.

Using the kinetic model we have constructed, we can at least qualitatively simulate our physiological traces. We emphasize that this model is not unique and that there are probably many other models that would also provide an excellent "fit" to our data. Furthermore, we recognize that this model is an oversimplication. Monoliganded open states and desensitization are neglected (15, 16). It also does not take into account the possibility that lactones may interact with more than one of the subunits that come together to form the $GABA_A$ receptor/ionophore. Interactions with more than one subunit may explain the steep concentration-response curve for β-EMTBL (Fig. 6B). The main point is that there are some GBLs that both potentiate and block GABA-induced currents, so there must be at least two different functional sites for lactone interaction within the receptor/ionophore. Addition of the second blocked state ($G_2RL_2^b$) was necessary because we were unable to obtain potentiation of GABA currents and block of IPSCs with only two lactone-binding states (G_2RL^b and G_2RL^*).

We initially considered some alternative explanations for our data, but we have had to reject them. It seemed possible that there were multiple subtypes of $GABA_A$ receptor/ionophores on our cells that could differ in their lactone sensitivities. That possibility now seems very unlikely, because we obtained identical results with α-DIGBL in hippocampal neurons and HEK 293 cells transfected with $GABA_A$ receptor subunits. We also wondered whether the off responses might be attributed to unusual, voltage-dependent effects of the lactones. This also seems improbable, because the physiology experiments were all done under voltage-clamp conditions and the off responses disappeared when lactone perfusion was continued after the GABA had been stopped. We cannot attribute the behavior of α-DIGBL simply to high lactone concentrations, because it had opposite effects on IPSCs and GABA-induced current at 1 mM.

We considered and rejected the possibility that our results could be explained by impure lactone preparations containing a combination of potentiators and blockers. The synthetic methods used to prepare potentiating α-alkyl-substituted compounds preclude the possibility of contamination by blocking β-alkyl-substituted compounds (8). Hence, the results cannot be explained by minor contamination of an α-substituted compound with a β-substituted compound. Moreover, analysis by gas chromatography established that the compounds had a minimal purity of 99.5%.

There is also independent evidence supporting a model of the $GABA_A$ receptor/ionophore that contains two regions

where the individual GBLs interact. When the $GABA_A$ receptor α or β subunits are mutated to produce picrotoxin resistance (by changing the M2 region sequence from -TTVLTMTTL- to -FTVLTMATL-) (19), β-EMTBL loses its ability to block GABA currents in HEK 293 cells and *Xenopus* oocytes expressing either of the mutated subunits.[1] Potentiation by β-EMTBL and α-EMTBL is still robust. This strongly suggests two different regions for lactone interaction, one closely linked to picrotoxin and another yet to be mapped. The finding that specific receptor subunit mutations have dramatic effects on lactone properties also argues against nonspecific interactions of high concentrations of lactones with the cytoskeleton or membrane lipids. It does leave open the possibility of their affecting other ion channels with similar transmembrane domains.

There is one counterintuitive aspect of this scheme, namely the difficulty envisioning how an agent acting within a pore can stabilize an open channel or increase its probability of remaining open. There is no obvious explanation for this at present. However, it is not an obligatory aspect of our model. It is not inconceivable that a drug could influence the M2 region by acting within the membrane but outside of the pore. Even for picrotoxin, despite the compelling evidence for a site within the M2 region governing block, two recent papers suggest that this agent does not produce open channel block but instead stabilizes desensitized channels or allosterically modulates channel opening (20, 21). Determining the specific molecular requirements for GBL activity will be a major focus of this research effort for the next several years.

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References

1. Macdonald, R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17**:569–602 (1994).
2. Baker, K., J. Yang, D. F. Covey, D. B. Clifford, and C. F. Zorumski. α-Substituted thiobutyrolactones potentiate GABA currents in voltage-clamped chick spinal cord neurons. *Neurosci. Lett.* **87**:133–138 (1988).
3. Zorumski, C. F., J. Yang, K. Baker, D. F. Covey, and D. B. Clifford. Convulsant γ-butyrolactones block GABA currents in cultured chick spinal cord neurons. *Brain Res.* **484**:102–110 (1989).
4. Holland, K. D., J. A. Ferrendelli, D. F. Covey, and S. M. Rothman. Physiological regulation of the picrotoxin receptor by γ-butyrolactones and γ-thiobutyrolactones in cultured hippocampal neurons. *J. Neurosci.* **10**: 1719–1727 (1990).
5. Ferrendelli, J. A., K. D. Holland, A. C. McKeon, and D. F. Covey. Comparison of the anticonvulsant activities of ethosuximide, valproate, and a new anticonvulsant, thiobutyrolactone. *Epilepsia* **30**:617–622 (1989).
6. McKeon, A. C., D. J. Canney, D. F. Covey, and J. A. Ferrendelli. Relative anticonvulsant effects of GABA-mimetic and GABA modulatory agents. *Epilepsia* **33**:981–986 (1992).
7. Holland, K. D., M. G. Bouley, D. F. Covey, and J. A. Ferrendelli. Alkyl-substituted γ-butyrolactones act at a distinct site allosterically linked to the TBPS/picrotoxinin site on the $GABA_A$ receptor complex. *Brain Res.* **615**:170–174 (1993).
8. Canney, D. J., K. D. Holland, J. A. Levine, A. C. McKeon, J. A. Ferrendelli, and D. F. Covey. Synthesis and structure-activity studies of alkyl-substituted γ-butyrolactones and γ-thiobutyrolactones: ligands for the picrotoxin receptor. *J. Med. Chem.* **34**:1460–1467 (1991).
9. Peterson, E. M., K. Xu, K. D. Holland, A. C. McKeon, S. M. Rothman, J. A. Ferrendelli, and D. F. Covey. α-Spirocyclopentyl- and α-spirocyclopropyl-γ-butyrolactones: conformationally constrained derivatives of anticonvulsant and convulsant α,α-disubstituted γ-butyrolactones. *J. Med. Chem.* **37**:275–286 (1993).
10. Levine, J. A., J. A. Ferrendelli, and D. F. Covey. Alkyl-substituted thiolo-, thiono-, and dithio-γ-butyrolactones: new classes of convulsant and anticonvulsant agents. *J. Med. Chem.* **29**:1996–1999 (1986).
11. Yamada, K. A., and S. M. Rothman. Diazoxide blocks glutamate desensitization and prolongs excitatory postsynaptic currents in rat hippocampal neurons. *J. Physiol. (Lond.)* **458**:409–423 (1992).
12. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
13. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers. Arch.* **391**:85–100 (1981).
14. Andrade, R. Blockade of neurotransmitter-activated K^+ conductance by QX-314 in the rat hippocampus. *Eur. J. Pharmacol.* **199**:259–262 (1991).
15. Weiss, D. S., and K. L. Magleby. Gating scheme for single GABA-activated Cl^- channels determined from stability plots, dwell-time distributions, and adjacent-interval durations. *J. Neurosci.* **9**:1314–1324 (1989).
16. Macdonald, R. L., C. J. Rogers, and R. E. Twyman. Kinetic properties of the $GABA_A$ receptor main conductance state of mouse spinal cord neurones in culture. *J. Physiol. (Lond.)* **410**:479–499 (1989).
17. Holland, K. D., A. C. McKeon, D. F. Covey, and J. A. Ferrendelli. Binding interactions of convulsant and anticonvulsant γ-butyrolactones and γ-thiobutyrolactones with the picrotoxin receptor. *J. Pharmacol. Exp. Ther.* **254**:578–583 (1990).
18. Holland, K. D., K. W. Yoon, J. A. Ferrendelli, D. F. Covey, and S. M. Rothman. γ-Butyrolactone antagonism of the picrotoxin receptor: comparison of a pure antagonist and a mixed antagonist/inverse agonist. *Mol. Pharmacol* **39**:79–84 (1991).
19. Gurley, D. A., J. Amin, P. Ross, D. Weiss, and G. White. Point mutations in the M2 region of the $GABA_A$ receptor/ionophore complex abolish sensitivity to picrotoxin. *Soc. Neurosci. Abstr.* **20**:509 (1994).
20. Zhang, H.-G., R. H. ffrench-Constant, and M. B. Jackson. A unique amino acid sequence of the *Drosophila* GABA receptor influences drug sensitivity by two mechanisms. *J. Physiol. (Lond.)* **479**:65–75 (1994).
21. Newland, C. F., and S. G. Cull-Candy. On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. *J. Physiol. (Lond.)* **477**:191–213 (1992).

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