Glycine and γ-aminobutyric acid, type A (GABA$_A$) receptors are members of the ligand-gated ion channel superfamily that mediate inhibitory synaptic transmission in the adult central nervous system. During development, the activation of these receptors leads to membrane depolarization. Ligands for the two receptors have important implications both in disease therapy and as pharmacological tools. Terpene trilactones (ginkgolides and bilobalide) are unique constituents of *Ginkgo biloba* extracts that have various effects on the central nervous system. We have investigated the relative potency of these compounds on glycine and GABA$_A$ receptors. We find that most of the ginkgolides are selective and potent antagonists of the glycine receptor. Bilobalide, the single major component in *G. biloba* extracts, also reduces glycine-induced currents, although to a lesser extent. Both ginkgolides and bilobalide inhibit GABA$_A$ receptors, with bilobalide demonstrating a more potent effect. Additionally, we provide evidence that open channels are required for glycine receptor inhibition by ginkgolides. Finally, we employ molecular modeling to elucidate the similarities and differences in the structure of the terpene trilactones to account for the pharmacological properties of these compounds and demonstrate a striking similarity between ginkgolides and picrotoxinin, a GABA$_A$ and recombinant glycine α-homomeric receptor antagonist.

Glycine and γ-aminobutyric acid receptors (GlyRs$^1$ and GABA$_A$Rs) are anion-selective ligand-gated ion channels, which together with the cation-selective nicotinic acetylcholine and serotonin receptors constitute a superfamily of membrane receptors that mediate fast chemical synaptic transmission in the nervous system. These receptors share several structural similarities, i.e. a pentameric arrangement of subunits, each composed of four transmembrane domains (M1–M4) and an extracellular 15-residue Cys-loop motif-bearing N terminus (1, 2).

In the adult, GlyRs consisting of α1–α4 and β subunits are found primarily in spinal cord and brain stem but are also present in higher brain regions such as hippocampus. During embryonic development, functional GlyRs are also expressed in the neocortex (3) with α2 as the predominant subunit (4). Few antagonists for GlyRs are known; the classical example is strychnine, which is a competitive antagonist. Also, the plant alkaloids picrotin and picrotoxinin (components of picrotoxin) are non-use-dependent antagonists that are equally efficacious in blocking recombinant homo-oligomeric GlyRs containing α subunits (5) but not the native α2/β heteromers. GABA$_A$Rs are functionally expressed very early in cortical development by proliferating precursor cells (6), now known to be radial glial cells (7, 8), and by immature cortical neurons. The subunit composition of GABA$_A$Rs changes significantly during development, and this is reflected in the pharmacological properties of the GABA-induced responses (9). GABA$_A$Rs have been implicated not only in synaptic signaling but also in a number of developmental events including proliferation and migration (10). It is also likely that GlyRs and GABA$_A$Rs have overlapping pathways during development because both receptors activate chloride channels and indirectly induce an increase in intracellular calcium in embryonic cortical neurons, thereby mediating a variety of cellular processes.

Plant and animal products traditionally provide a rich source of drug candidates and pharmacological tools. The tree *Ginkgo biloba* has long been believed to have medicinal properties, and its extracts are among the most widely sold herbal supplements in the world. *G. biloba* extracts are standardized according to their content of flavonoids (22–24%) and terpene triacontes (6–8%), which are believed to be the active components. Although the bioavailability of flavonoids is limited, terpene triacontes, in particular ginkgolides A and B (GA and GB), and bilobalide (BB) are highly bioavailable (11).

Extracts of *G. biloba* have been shown to be effective in symptomatic treatment of mild to moderate dementia of Alzheimer disease type as well as dementia of cerebrovascular origin (11) and have been reported to improve memory, although the latter claim remains controversial (12). Although a number of studies have shown various effects of *G. biloba* extract, very little is known about the direct effect of the individual components on the central nervous system. Recent work has indicated that ginkgolides and BB might interact with inhibitory signaling in the brain. A recent report suggested that GB inhibits glycine receptors in the hippocampus (13), and several reports have shown that BB interferes with the GABAergic system, although both enhancement and inhibition have been reported (14–16).

For these reasons we have addressed exactly how the unique components of *G. biloba*, ginkgolides and bilobalide, may exert...
their effect in the central nervous system. We show that the ginkgolides and bilobalide are selective antagonists of glycine and GABA_A receptors, we demonstrate that GC inhibition of embryonic cortical GlyRs requires open channels, and that GB has several structural similarities to the GABA_A receptor antagonist, picrotxinin.

EXPERIMENTAL PROCEDURES

All experiments followed National Institutes of Health guidelines and were done in full compliance with the Columbia University Institutional Animal Care and Use Committee.

Brain Slice Preparation—Timed pregnant Sprague-Dawley rats (Taconic, New York) at embryonic day E20 were used for all experiments. Rats were anesthetized (ketamine, 90 mg/kg, and xylazine, 10 mg/kg), and embryos were removed for further dissection. Following rapid decapitation, the brain was removed in chilled artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM MgSO_4, 2 mM CaCl_2, 25 mM NaHCO_3, and 25 mM glucose, pH 7.4, 310 mosmol/liter). The dissected brain was placed in 4% low melting agarose (Fisher Scientific) in ACSF. Agarose was cooled on ice and allowed to solidify, and the embedded brain was sliced into coronal sections (400 μm) in ice-cold ACSF using a Leica VT100S vibrating blade microtome (Nussloch, Germany). Slices were allowed to recover at room temperature in oxygenated (95% O_2 and 5% CO_2) ACSF. They were subsequently used for electrophysiological recording or loaded for calcium imaging.

Calcium Imaging—Brain slices were bath-loaded with the acetoxyethyl ester form of the calcium indicator dye fluo-3 (fluor-3 AM, 10–15 μM, Molecular Probes, Eugene, OR). Loading was performed in the dark, at room temperature for 1–3 h. Loaded slices were placed in an imaging chamber on the stage of an upright compound microscope (Olympus BX50-WI, Tokyo, Japan) and continuously perfused with oxygenated ACSF. Epi-fluorescence imaging of fluo-3 was performed using a 100-watt mercury light source and a low light charge-coupled device camera (Dage-MTI 300-T, Michigan City, IN). For fluo-3 imaging, we used following fluorescence filters (Chroma Technology, Brattleboro, VT): excitation filter, 480 ± 20 nm long-pass; and emission filter, 535 ± 25 nm. Cells were imaged using 10× water immersion objective, and the photobleaching was minimized by controlling a shutter positioned in the excitation light path (Uniblitz S25, Vincent Associates, Rochester, NY). Time-lapse images were acquired every 2 s using the Scion Image program on a Macintosh G3 computer equipped with video frame grabber (Scion LG-3, Scion Corp., Frederick, MD). Fluorescence changes were measured in selected cells using Scion Image.

Pharmacological Agents—Ginkgolides A, B, C, and J (GA, GB, GC, and GJ) and bilobalide were obtained by extraction of leaves from G. biloba, purification by column chromatography, and recrystallization of the previously described (17, 18). Ginkgolide M (GM) was obtained from previous studies (19). The purity of these compounds was >99%, as determined by 1H NMR. Stock solutions of ginkgolides, bilobalide, and picrotoxinin (RBI, Natick, MA) were prepared by dissolving in Me_SO to 10 mM and were stored at –20°C until use. Glycine, taurine (Sigma), and γ-aminobutyric acid (RBI, Natick, MA) were prepared as stock solutions in distilled deionized H_2O and diluted in ACSF to final concentration. All drugs were applied focally by DAD-12 Superfusion System (ALA Scientific Instruments, Westbury, NY).

Electrophysiology—Whole-cell patch-clamp recordings were performed with an EPC-9 patch-clamp amplifier (HEKA Electronic, Lambrcht, Germany). Data were acquired with HEKA Pulse v. 8.0 software (HEKA Electronic, Lambrcht, Germany) or Borisicate software (Warner Instrument Corp., Hamden, CT). Electrode pipettes (5–8 megohms) were filled with 130 mM CsCl, 2 mM CaCl_2, 10 mM HEPES, and 11 mM EGTA (pH 7.4 at 25°C, 265–275 mosmol/liter). Unless otherwise indicated, neurons were voltage-clamped at −60 mV.

Data Analysis—All calcium imaging data were presented as a change in fluorescence over base-line fluorescence, ΔF/F_0 = (F_0 − F/F_0), and plotted over time. Analysis was done by averaging 10–15 cells for each condition, and data were plotted as the mean ± S.E. Statistical significance between two groups was evaluated with a Student’s t test. Probability (p) values of less than 0.01 were considered to be statistically significant.

Molecular Modeling—Molecular mechanics calculations with MM2* force field and Monte Carlo conformational searches were executed with MacroModel 6.0 (Schrödinger, Inc., Portland, OR). Non-empirical molecular modeling calculations were executed with Jaguar 4.1 (Schrödinger, Inc., Portland, OR) at the B3LYP level with 6-31G G basis set (20) including the geometry optimization. GB and picrotoxinin (PTX) were overlaid using a rigid body fit between C-11, C-15, O-6, and O-10 of GB and C-15, C-14, O-5, and O-3 of PTX as seen in Fig. 5a. Monte Carlo simulations revealed nine conformers for GA within 10 kcal/mol of the global minimum, whereas GB had 32 unique conformations in the 10 kcal/mol range. X-ray crystallographic structures were obtained from Cambridge structural data base using ConQuest 1.1 (CCDC, Cambridge, UK) and compared with the minimized conformations. The comparison showed that root mean square values were 0.4 Å for GB and 0.6 Å for PTX.

RESULTS

Ginkgolides and bilobalide, compounds classified as terpene trilactones, share several structural similarities, in particular three lactones and a tert-butyl group. The five ginkgolides differ only by the number and placement of hydroxyl groups (Fig. 1a) (19). GA, GB, and GC are found in both leaves and root bark of G. biloba, whereas GJ and GM are found only in the leaves and root bark, respectively. BB is chemically related to the ginkgolides, having three lactones but only one carboboro, and is found in the leaves (Fig. 1a) (21).

Ginkgolides Antagonize Glycine Receptors—GlyR activation opens a chloride ion channel and induces a membrane depolarization in embryonic cortical neurons because of a high intracellular chloride concentration in immature neurons (3). The resulting depolarization leads to the activation of voltage-gated calcium channels and a subsequent influx of calcium ions. We took advantage of this mechanism and used calcium imaging to compare the potency of various ginkgolides on glycine-induced responses in embryonic cortical neurons.

Application of glycine (200 μM) resulted in an increase in intracellular calcium, which was reduced by co-application with GB (10 μM) (Fig. 1b). The effect was highly use-dependent as repeated applications of glycine and GB gradually decreased the glycine response, which by the third application was reduced to 30% (Fig. 1b). The effect was reversible, as seen by the recovery of glycine responses after a 20-min wash with artificial cerebrospinal fluid (Fig. 1c). The glycine responses were reduced to 67, 48, and 30%, respectively, by three consecutive co-applications of glycine and GB (Fig. 1c). These findings are in agreement with an initial report on the effect of GB on glycine receptors in hippocampal pyramidal neurons isolated from adult rats (13).

We wondered whether the other ginkgolides and bilobalide also antagonized glycine responses because they share a similar chemical structure, and their effect has not yet been reported. We tested GA, GB, GC, GJ, and GM, together with BB and picrotoxinin at the same low concentration (10 μM), to determine the relative inhibiting potency of these compounds. GB, GC, and GM were the most potent antagonists and by the third application decreased glycine responses to 30–40% of control, whereas GA and GJ reduced the response to a lesser extent (70–80% of control, Fig. 1d). The sesquiterpenoid BB had no effect on glycine-induced calcium increases in this assay at the concentration tested. Picrotoxin had no effect on glycine responses (Fig. 1d).

Changes in intracellular calcium only indirectly reflect activation of GlyRs and depend on the kinetics of the voltage-gated calcium channels. In addition, restoration of base-line calcium levels depends both on the GlyR gating and on the recovery of the resting intracellular calcium concentration. To directly observe the effect of BB and the most potent ginkgolides, GB and GC, on glycine-induced currents, we used whole-cell patch-clamp recordings.

A short (1-min) preincubation in GB (10 μM), followed by its co-application with glycine (500 μM) resulted in potent inhibition of the glycine response in cortical neurons (Fig. 2a). Because taurine has been suggested as an endogenous ligand for GlyRs, we demonstrate that GC inhibition of embryonic cortical GlyRs requires open channels, and that GB has several structural similarities to the GABA_A receptor antagonist, picrotxinin.
GlyR (3, 22), we also tested the effect of GB on taurine (5 mM)-induced responses (Fig. 2b). The effect was consistent with GlyR inhibition and was use-dependent and reversible.

Because BB represents about 50% of the terpene trilactones, being the major single component in *G. biloba* extracts, we wondered if it would reduce glycine-induced currents. BB (10 μM) was much less potent than GB (10 μM), but it still induced a significant decrease of glycine (200 μM) responses (Fig. 2c). Contrary to the pattern of GB inhibition, BB antagonism did not change significantly with repeated application. Interestingly, both BB and GB showed a more pronounced effect on glycine-induced currents, measured in whole-cell patch-clamp recordings, compared with their effect on intracellular calcium increases (compare Figs. 1d and 2c). Therefore, the fact that we
did not observe a significant decrease in the glycine-induced calcium response in the presence of BB most likely results from an incomplete block of glycine-induced currents by BB that was not sufficient to alter the intracellular calcium change.

**Ginkgolide C Requires an Open Channel for the Inhibition of Embryonic Glycine Receptors**—GC is one of the three most potent glycine receptor antagonists present in *G. biloba* extracts. To get a better understanding of the use-dependent effect characteristic of ginkgolides, we used a whole-cell patch-clamp recording and applied glycine (500 μM) every 60 s in the presence of 10 μM GC (Fig. 3a) following two initial control glycine applications. Even after a short 1-min GC preincubation, the glycine response was dramatically reduced (to 32.7 ± 4.5%) and quickly (after 3–4 applications) reached a plateau. The effect was partially reversible as demonstrated by a slow recovery of the glycine response after a washout period. Control glycine applications were done every minute in the absence of GC. The glycine responses showed a slight desensitization with repeated application. To determine whether open channels are necessary for the use-dependent effect, we examined the effect of prolonged preincubation on the degree of inhibition. We used a double application protocol, wherein the first glycine application was a control, and during the second, glycine and GC were co-applied following a 1- or 10-min-long GC preincubation (Fig. 3b). In control experiments, glycine was applied twice without GC. Results are presented as the ratio between the second and the first glycine responses recorded from the same cell. Our data show that the degree of inhibition did not depend on the length of preincubation. Moreover, when glycine and GC were co-applied without any preincubation, responses were not only reduced, but the response kinetics were dramatically changed reflecting an abrupt blockade of glycine channels even before the end of drug application (Fig. 3c). These data, taken together, suggest a requirement for open channels in glycine receptor inhibition.

**Bilobalide and Ginkgolide B Antagonize GABA<sub>A</sub> Receptors**—The GABA<sub>A</sub> receptor subtype is the only functional ionotropic GABA receptor type expressed in embryonic pyramidal neurons as demonstrated by a complete block of GABA-induced current with 50 μM bicuculline (9). Whole-cell recordings performed on cortical neurons demonstrated attenuation of GABA<sub>A</sub> responses by both GB and BB, with BB proving the more potent of the two compounds. A short (1-min) incubation in the drug was followed by co-application of the drug with GABA (30 μM). Fig. 4 illustrates the typical responses to 30 μM GABA that were reduced by co-application with 50 μM GB (Fig. 4a) or BB (Fig. 4b). At this concentration, GB and BB reduced GABA responses to 63.2 ± 0.3% (n = 5) and 46.8 ± 0.3% (n = 5), respectively. Interestingly, the effect of GB on GABA responses was not use-dependent, as tested by three successive applications in which the response was reduced no further (not shown). The dose-response relationships for the inhibitory action of GB and BB on GABA-induced (30 μM) responses are illustrated in Fig. 4c. Calculated values for the IC<sub>50</sub> (GB, IC<sub>50</sub> = 73 μM; BB, IC<sub>50</sub> = 46 μM) demonstrated that BB is about 1.6 times as potent as GB. The IC<sub>50</sub> value for BB was significantly smaller than the one obtained on recombinant α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> GABA<sub>A</sub>R expressed in *Xenopus* oocytes (23) most likely reflecting a different effect of BB on receptors with different subunit composition.

**Ginkgolide B and Picrotoxinin Show Structural Similarity**—These findings led us to investigate a structural rationalization for the effects of ginkgolides, particularly of GB, and to compare them with PTX. We analyzed their molecular properties through modeling (24) and validated our approach by comparison with the x-ray crystallographic structures of GB (25) and PTX (26). The minimized structures were found to be similar to those determined by x-ray (root mean square deviations: GB = 0.4 Å and PTX = 0.6 Å), although there were some differences in the extent of intramolecular hydrogen bonding.

The minimized structures of GB and PTX showed remarkable similarity in several respects. Each molecule forms a cave structure, where the bottom is composed of a five- or six-membered carbocycle, and the walls are a pair of lactones (Fig. 5). The caves are of similar size, the distance between the lactone carbonyl oxygen molecules being 5.8 and 5.2 Å for GB and PTX, respectively, with a root mean square deviation of 0.4 Å for the rigid body fit of the two lactone rings in both molecules (Fig. 5). Moreover, the orientation of the lactone pairs is of a similar magnitude, their projection angles being −26° in GB and −24° in PTX. In addition to this similarity in core structure, the two compounds have important moieties located in analogous positions. The tert-butyl group of GB is located in the same region as the isoprenyl group of PTX, although the tert-butyl group is bulkier, and the 1-OH of GB overlays very well with the 6-OH of PTX (Fig. 5).

**Structural Investigation of the Relative Activities of Ginkgolides**—We also investigated differences in activity among the ginkgolides from a structural perspective. GB, GC, and GM, the more potent glycine antagonists, share a 1-OH, whereas the less potent GA and GJ do not (Fig. 1a). The importance of the 1-OH for the core structure was investigated via a Monte Carlo simulation (27) to predict the most probable orientation of the 1-OH with respect to the lactones. The concept of the hydrophobic pocket was generated with a Monte Carlo algorithm that allowed us to obtain the most probable orientation of the 1-OH. The resulting pocket was the most active among the four compounds, illustrating the importance of the 1-OH for the core structure.
Carlo conformational search performed on GA and GB as representative compounds. All conformers of GA were found to have larger distance-spanning lactone rings C and F compared with GB. Moreover, very little flexibility in either lactone ring of GB was seen, suggesting that the larger lactone distance in GA is significant. The 1-OH in GB may form a hydrogen bond with the 10-OH to hold the two lactones together.

Comparison of Bilobalide with Ginkgolide B and Picrotoxin—BB is structurally related to GB and PTX in that it contains three lactone groups but unlike GB has only one carbocycle. BB was overlaid with GB and PTX, respectively (data not shown). A comparison of BB with GB showed that the proposed important functional groups of GB and BB can be overlaid individually but not at the same time, underlining important differences in GB and BB. Comparing the minimized structures of BB and PTX showed much less similarity. In neither of those cases is there a striking overlap of lactones, tert-butyl/isoprenyl groups, or hydroxyl functionalities as was observed for GB and PTX (Fig. 5).
C-5 is essential for activity (31). In addition, the projected structure of PTX have revealed that the lactone fused between C-3 and C-3 is functional, as structure-activity relationship studies of the two lactone groups are very similar. This is likely to be because of a direct interaction of 1-OH with the receptor, or it could be that 1-OH, by hydrogen-bonding, keeps the lactone groups in GB in a position that is more favorable for receptor interaction.

Additionally, we studied BB, the major single component in G. biloba extract constituting 3–4%. BB is structurally related to ginkgolides and shares several functional groups, in particular three lactone groups and a tert-butyloxyl group, and has been postulated to have anxiolytic properties and possibly to be useful as a neuroprotective agent (27). Recent studies indicated that BB might modulate GABAergic neurotransmission, as BB has been reported to elevate the GABA levels, probably by enhancing glutamic acid decarboxylase activity (14, 15), reduce muscimol responses (16), and decrease the frequency of GABA uptake inhibitor-induced depolarizations (28). BB has also been reported to antagonize recombinant 

![Image](image.png)

**Fig. 5. Comparison of GB with PTX by molecular modeling.** a, the structures of GB (blue) and PTX (orange) were minimized in MacroModel using density functional theory parameters and overlaid. GB and PTX are both cave molecules and have a highly similar position of important functional groups that all can be overlaid. Red, carbonyl. b, structures of GB and PTX indicating the similar distances between the carbonyl oxygen and similar position of the lipophilic tert-butyl and isoprenyl groups, respectively.
seizures within 2 weeks of commencing G. biloba extract that ceased after discontinuation of the supplement (33).

In considering the role of GABA\textsubscript{\alpha} and Gly receptors in cortical development it is also important to evaluate the potential teratogenic effects of G. biloba extract. Further studies are necessary to establish not only the effect of terpene trilactones on the developing and adult nervous system but also the combinatorial effect of all extract constituents.

In conclusion, we have shown that the ginkgolides and bilobalide are antagonists of both Gly and GABA\textsubscript{\alpha} receptors. In particular, GB and GC are selective and potent antagonists of the GlyRs thus being highly promising pharmacological tools for the study of this receptor. Molecular modeling reveals several structural similarities between GB and PTX, suggesting a similar mode of interaction with the GlyR and GABA\textsubscript{\alpha}R, respectively. Finally, the inhibitory activity of these components of G. biloba extract, especially the action of the major constituent, BB, on the GABA\textsubscript{\alpha}R may have important implications for people taking this supplement.

Acknowledgments—We thank Drs. Abhay Kini and David Owens for valuable suggestions on the manuscript. We thank Dr. Stephen Noctor for help with preparing figures.

REFERENCES