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Effects of bilobalide, ginkgolide B and picrotoxinin on GABA_A receptor modulation by structurally diverse positive modulators

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ABSTRACT

Anxiolytics and anticonvulsants generally positively modulate the action of GABA, whereas many convulsants (including the chloride channel blocker picrotoxinin) negatively modulate the action of GABA on GABA_A receptors. Like picrotoxinin, bilobalide and ginkgolide B, active constituents of *Ginkgo biloba*, have been shown to negatively modulate the action of GABA at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors. However, unlike picrotoxinin, bilobalide and ginkgolide B are not known to cause convulsions. We have assessed the action of bilobalide, ginkgolide B and picrotoxinin on a range of GABA_A modulators (etomidate, loreclezole, propofol, thiopentone sodium, diazepam, and allopregnanolone), using two-electrode voltage clamp electrophysiology at recombinant $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors expressed in *Xenopus* oocytes. The results indicate that bilobalide and ginkgolide B differ from picrotoxinin in their ability to inhibit the actions of a range of these structurally diverse GABA_A positive modulators consistent with these modulators acting on a multiplicity of active sites associated with GABA_A receptors. In the presence GABA, ginkgolide B was more potent than bilobalide in inhibiting the GABA-potentiating effect of propofol, equipotent against loreclezole and allopregnanolone, and less potent against etomidate, diazepam, and thiopentone sodium. This indicates that in comparison to picrotoxinin, bilobalide and ginkgolide B differ in their effects on the different modulators.

1. Introduction

γ -Aminobutyric acid (GABA) is a major neurotransmitter, regulating the overall balance between neuronal excitation and inhibition in the central nervous system (Chebib and Johnston, 2000). GABA_A receptors are classified as members of the cys-loop ligand-gated ion channel (LGIC) superfamily. They mediate fast synaptic neurotransmission via the gating of chloride ion movement through the channel by GABA.

GABA_A receptors are made up of five subunits arranged pseudo-symmetrically, around a central conducting chloride ion channel. Each subunit comprises four transmembrane domains, M1, M2, M3 and M4. The helical M2 domain surrounds the ion channel. The ion channel of these receptors is activated (opened) following the binding of GABA to its recognition site to permit chloride permeability. The channel helical structure is extended to the M2-M3 loop residues (Bera et al., 2002). The extracellular M2-M3 loop is required for ion channel activation through the rotation of M2 domains while the intracellular M3-M4 loop is responsible for the attachment to the cytoskeleton and modulating protein phosphorylation (Absalom et al., 2004). In addition, the extracellular N-terminal domain forms most, if not all, of the

agonist and antagonist binding residues. The intracellular loop linking to both M1 and M2 domains is involved in ion selectivity (Jensen et al., 2005).

Many current therapeutic agents, including general anaesthetics, benzodiazepines, barbiturates, neurosteroids and loreclezole, act via positive modulation of GABA_A receptors (Hales and Lambert, 1991; Krasowski and Harrison, 1999; Study and Barker, 1981; Tomlin et al., 1998; Wafford et al., 1994). These positive modulators enhance the action of GABA, by increasing the probability or duration of chloride channel opening, resulting in anti-anxiety and anticonvulsant effects. In contrast, negative modulators, including the chloride channel blocker picrotoxinin (Inoue and Akaike, 1988; Zhang et al., 1995), keep the channels in inactive/closed states, resulting in over-excitation of the neurons and convulsions.

Ginkgo biloba extract EGb 761 contains 24% flavonoids and 6% terpenoids (bilobalide, and ginkgolides A, B, C and J), and has been shown in clinical studies to reduce symptoms of anxiety without causing sedation with minimal unwanted side effects (Scripnikov et al., 2007; Van Beek, 2002; Woelk et al., 2007). This is in contrast to currently used benzodiazepine anxiolytics that also produce sedation and cognitive impairment. Bilobalide, ginkgolide B and picrotoxinin

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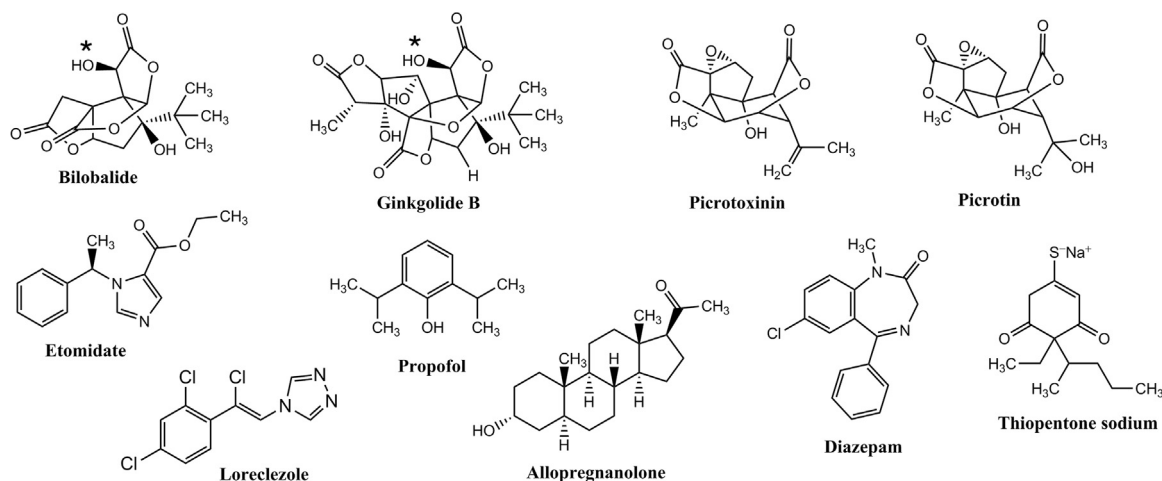


Fig. 1. Chemical structures of the negative modulators bilobalide, ginkgolide B, picrotoxinin and picrotin, and the positive modulators etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone. Key hydroxyl groups in bilobalide and ginkgolide B are marked with an asterisk.

(Fig. 1) have been shown to negatively modulate the action of GABA at GABA_A receptors (Huang et al., 2003, 2004). However, unlike picrotoxinin, bilobalide and ginkgolide B have not been reported to cause convulsions. Moreover, animal studies have demonstrated that bilobalide has an anticonvulsant action (Sasaki et al., 1995) and ginkgolide A reduces anxiety (Kuribara et al., 2003).

Bilobalide (IC₅₀ 3.9 μM) and ginkgolide B (IC₅₀ 19 μM) have been shown to potentially displace binding of the radioligand for picrotoxinin binding site [³⁵S]-*t*-butylbicyclopophosphorothionate ([³⁵S]TBPS) (Chatterjee et al., 2002, 2003). These findings suggest that bilobalide and ginkgolide B may act at overlapping and/or coupled binding sites to picrotoxinin at GABA_A receptors. We have recently shown in a study of cysteinyl mutants of α₁β₂γ_{2L} GABA_A receptors that bilobalide and ginkgolide B differ from picrotoxinin in their binding within the chloride channel (Ng et al., 2016). The present study aimed to investigate the action of bilobalide and ginkgolide B in comparison to picrotoxinin on the actions of a range of structurally diverse positive GABA_A modulators (etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone) at α₁β₂γ_{2L} GABA_A receptors using two-electrode voltage clamp electrophysiology. The α₁β₂γ_{2L} subunit combination was employed as it constitutes the major GABA_A receptor subtype in the mammalian brain (McKernan and Whiting, 1996).

2. Material and methods

The materials and methods used were essentially as described by Ng et al. (2016). The procedures involving the use of *Xenopus laevis* were approved by the Animal Ethics Committee of the University of Sydney.

2.1. Materials

Human α₁, β₂ and γ_{2L} deoxyribonucleic acid (DNA) subcloned in pCDM8 were provided by Dr. Paul Whiting (then at Merck, Sharp and Dohme Research Labs, Harlow, UK). Picrotoxinin, bilobalide and ginkgolide B were kind gifts of Dr. Rujee Duke (The University of Sydney, NSW, Australia). GABA, dimethyl sulfoxide (DMSO), propofol and allopregnanolone were purchased from Sigma Aldrich (St Louise, MO, USA). Zinc sulphate (ZnSO₄), thiopentone sodium and diazepam were obtained from Ajax Finechem (Seven Hills, NSW, Australia), Jurox (Rutherford, NSW, Australia) and APIN Chemicals (Abingdon, Oxon, UK), respectively. Etomidate and loreclezole were purchased from Tocris Bioscience (Bristol, UK). GABA, ZnSO₄ and thiopentone sodium were prepared from 50 or 100 mM milli-Q water stock

solutions. Picrotoxinin, bilobalide, ginkgolide B, etomidate, propofol, diazepam, loreclezole and allopregnanolone were prepared from 100 or 200 mM DMSO stock solutions. The highest DMSO concentration used was 0.6%, which was shown to have no significant effect on the oocytes.

2.2. Expression of α₁β₂γ_{2L} GABA_A receptors in *Xenopus laevis* oocytes

Female *Xenopus laevis* were anaesthetised with 0.17% ethyl 3-aminobenzoate in saline for 10–15 min, and ovarian lobes were surgically removed. The lobes were rinsed with OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂·6H₂O, 5 mM HEPES, pH 7.5) and treated with 2 mg/ml collagenase A in OR-2 buffer for 2 h to separate oocytes from follicle cells and connective tissues. Released stage V to VI oocytes were collected and rinsed in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂·6H₂O, 1.8 mM CaCl₂, 5 mM HEPES, 2.5 mM sodium pyruvate, 0.5 mM theophylline, pH 7.5). The oocytes were then stored at 15–16 °C in ND96 storage solution (ND96 solution supplemented with 50 μg/ml gentamycin) in an orbital shaker (Ratex Instruments, Victoria, Australia).

Human DNA plasmids of α₁, β₂ and γ_{2L} subunits were linearised with Not 1 restriction enzyme and then transcribed using T7 mMessage mMachine kit from Ambion (Austin, TX, USA). A Nanoject injector (Drummond Scientific, Broomall, PA, USA) was used to inject 10 ng per 50 nl of a 1:1:2 mixture of α₁, β₂ and γ_{2L} RNAs into the cytoplasm of each oocyte. The purpose of injecting a higher concentration of γ_{2L} subunit was to prevent the formation of α₁β₂ subtype GABA_A receptors. The oocytes were kept in ND96 storage solution at 15–16 °C in an orbital shaker with a twice-daily change of buffer.

2.3. Electrophysiological recording

Receptor activity was measured using two-electrode voltage clamp recording 2–7 days after RNA injection. Two glass microelectrodes (Harvard Apparatus, Edenbridge, Kent, UK) were made using a micropipette puller and filled with 3 M KCl solution. Both microelectrodes were inserted into the membrane of an oocyte placed in a cell bath continuously superfused with ND96 recording solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂·6H₂O, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5). The membrane potential was clamped at −60 mV using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, USA). The current traces (nA) were detected by a MacLab2e recorder (ADInstruments, Sydney, NSW, Australia) and recorded using LabChart 3.6.3 (ADInstruments, Sydney, NSW, Australia). The sampling frequency used was 10 kHz. The GABA-modulatory and GABA-mimetic effects of etomidate (0.01 μM to 1 mM), propofol (0.01 μM to

1 mM), diazepam (0.001 μ M to 1 mM), thiopentone sodium (0.1 μ M to 300 μ M), loreclezole (0.01 μ M to 300 μ M) and allopregnanolone (0.0001 μ M to 10 μ M) were tested in the presence of EC₅₀ GABA (5 μ M) and/or absence of GABA at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors. Recombinant $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors were also tested with increasing concentrations of picrotoxinin, bilobalide and ginkgolide B (0.001 μ M to 1 mM) in the presence of EC₅₀ GABA. In addition, antagonists (picrotoxinin, bilobalide and ginkgolide B) were used to probe for receptor activation for both GABA-mimetic and GABA-modulatory actions of positive modulators at $\alpha_1\beta_2\gamma_{2L}$ GABA receptors. For GABA-modulatory inhibition curves, a range of antagonist concentrations was co-applied with 5 μ M GABA (~EC₅₀ GABA) and positive modulators at EC₅₀ concentrations. The co-application of 60 s was of sufficient duration to ensure the complete effect of picrotoxinin, bilobalide and ginkgolide B. For GABA-mimetic inhibition curves, a range of antagonist concentrations was co-applied with the positive modulators at EC₅₀ concentrations. The co-application of 90 s was of sufficient duration to ensure the complete effect of picrotoxinin, bilobalide and ginkgolide B. A washout period of 3–5 min was allowed between each application in order to prevent receptor desensitisation and to ensure that the baseline currents of oocytes were fully recovered.

2.4. Data analysis

Data obtained from LabChart were interpreted as the change in current from the baseline after application of a single dose of a test drug. The peak amplitude of current at the given dose was calculated and standardised using equation $I/I_{\max} (\%) = I/I_{\max} \times 100$, where I represents the amplitude of current at a given dose of GABA or drug and I_{\max} is the maximum amplitude of current generated at a given GABA concentration. Data were analysed using GraphPad Prism (GraphPad Software, San Francisco, CA, USA) and expressed as the averaged percentage of $I/I_{\max} \pm$ standard error of mean (S.E.M.). The GABA dose response curves of $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors and the inhibitory GABA dose response curves of picrotoxinin, bilobalide and ginkgolide B at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors were plotted using a non-linear sigmoidal dose response (variable slope) equation, where $I/I_{\max} (\%)$ is a function of GABA/antagonist concentration (X):

$$I/I_{\max} (\%) = \text{minimum} + (\text{maximum} - \text{minimum}) / (1 + 10^{((\log EC_{50} - X) * \text{Hill coefficient})})$$

For the GABA-modulatory and/or GABA-mimetic actions of positive GABA modulators at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors, a bell-shaped dose response equation, where $I/I_{\max} (\%)$ is a function of an agonist concentration (X) was used:

$$I/I_{\max} (\%) = \text{minimum} + ((\text{maximum} - \text{minimum})^{(-1 * ((X - (\log EC_{50} + \text{Hill coefficient} * \sqrt{(-\ln 0.5))}) / \text{Hill coefficient}^2))})$$

For the GABA-modulatory action of diazepam at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors, where the data displayed two phases enhancement, a biphasic dose response equation, where $I/I_{\max} (\%)$ is a function of diazepam concentration (X) was used:

$$I/I_{\max} (\%) = \text{minimum} + (\text{maximum} - \text{minimum}) * \frac{1}{1 + 10^{((\log EC_{50} - X) * \text{Hill coefficient} .1)}} + (\text{maximum} - \text{minimum}) * (1 - \frac{1}{1 + 10^{((\log EC_{50} - X) * \text{Hill coefficient} .2)}})$$

Log EC₅₀ and log IC₅₀ obtained from the equations were compared using one-way analysis of variance (ANOVA). Significant ANOVAs were further evaluated using post-hoc Newman-Keuls tests, where the significance level was taken at $P < 0.05$. The EC₅₀, IC₅₀ and Hill coefficient value are expressed as mean \pm S.E.M.

3. Results

3.1. Expression of functional $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors

When tested with a saturating GABA concentration of 3 mM, the $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors generated inward whole-cell currents ranging from 600 to 3000 nA at -60 mV. The mean EC₅₀ GABA value for the $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors was 51.17 ± 1.31 μ M with a Hill coefficient (n_H) value of 1.02 ± 0.11 . Thus, the EC₅₀ GABA and n_H values exhibited by $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors in the present study were found to be similar to those reported by Duke et al. (2000), Huang et al. (2004) and Hall et al. (2004). The $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors were insensitive to zinc inhibition and produced a biphasic curve for the diazepam potentiating action of EC₅₀ GABA, indicating that the γ_{2L} subunit was incorporated into the receptors.

3.2. Inhibitory effect of ginkgo terpenoid lactones at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors

Picrotoxinin, bilobalide and ginkgolide B dose-dependently inhibited the action of EC₅₀ GABA (50 μ M) at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors. All compounds produced no effect when tested alone at 300 μ M and 1 mM. Picrotoxinin (IC₅₀ 1.15 ± 0.16 μ M; n_H -1.03 ± 0.15) appeared to be the most potent compound, followed by bilobalide (IC₅₀ 4.88 ± 0.72 μ M; n_H -0.67 ± 0.07) then ginkgolide B (IC₅₀ 7.22 ± 1.32 μ M; n_H -0.60 ± 0.12) ($P < 0.001$). The calculated ratios of IC₅₀ value of bilobalide and ginkgolide B relative to the IC₅₀ value of picrotoxinin are 4.24 and 6.28, respectively.

3.3. Dose response curves of positive GABA_A receptor modulators

The calculated EC₅₀, n_H and maximal $I/I_{\max} (\%)$ values of the positive GABA_A receptor modulators are tabulated in Table 1.

Etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone dose-dependently enhanced the GABA action at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors, tested at EC₅₀ GABA (5 μ M). Except for diazepam, all compounds tested from a low concentration (0.001 μ M to 0.1 μ M) to high concentration (300 μ M to 1000 μ M) produced a bell-shaped concentration-response relationship. In contrast, diazepam (0.001 μ M to 300 μ M) produced two phases of enhancement (high and low affinity effects). Etomidate and propofol also elicited a bell-shaped dose-dependent inward current response in the absence of GABA (Table 2). Both compounds were found to be more potent with higher maximal $I/I_{\max} (\%)$ when tested in the presence of GABA.

The GABA-mimetic action of thiopentone sodium, loreclezole and

Table 1

EC₅₀, Hill coefficient (n_H) and maximal $I/I_{\max} (\%)$ of etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone in the presence of EC₅₀ GABA at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors.

Positive GABA _A modulator	With EC ₅₀ GABA		
	EC ₅₀ (μ M)	Hill coefficient (n_H)	Maximal $I/I_{\max} (\%)$
Etomidate	3.61 ± 0.76	1.68 ± 0.08	120.53 ± 3.19
Propofol	15.59 ± 1.12	1.55 ± 0.09	132.23 ± 2.81
Diazepam (high affinity)	0.16 ± 0.04	2.05 ± 0.88	93.63 ± 8.08
Diazepam (low affinity)	71.94 ± 1.34	3.98 ± 0.89	
Thiopentone sodium	34.67 ± 1.09	0.81 ± 0.06	93.68 ± 2.68
Loreclezole	10.43 ± 1.11	0.96 ± 0.08	55.42 ± 2.16
Allopregnanolone	0.36 ± 0.07	1.22 ± 0.10	40.82 ± 1.17

I denotes the maximum amplitude of current following the application of a given dose of positive GABA_A modulator in the presence of EC₅₀ GABA.

I_{\max} is the maximum amplitude of current at 3 mM GABA.

Data are mean \pm S.E.M. ($n=4-7$ oocytes).

Table 2:

EC₅₀, Hill coefficient (n_H) and maximal I/I_{max} (%) of etomidate and propofol tested alone at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors.

GABA-mimetic	Without GABA		
	EC ₅₀ (μ M)	Hill coefficient (n_H)	Maximal I/I _{max} (%)
Etomidate	36.44 \pm 1.24	1.08 \pm 0.12	54.70 \pm 2.78
Propofol	46.63 \pm 1.23	2.16 \pm 0.06	79.27 \pm 4.07

I denotes the maximum amplitude of current following the application of a given dose of GABA-mimetic in the absence of GABA.

I_{max} is the maximum amplitude of current at 3 mM GABA.

Data are mean \pm S.E.M. (n=4–7 oocytes).

allopregnanolone was not examined in this study due to either inconsistent response, limited amount of a compound or solubility problems. Diazepam only potentiates the action of GABA, it does not activate the receptors by itself (Rogers et al., 1994). The GABA-modulatory and/or GABA-mimetic actions of the positive GABA_A modulators at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors in the present study were found to be consistent to those reported by Hill-Venning et al. (1997), Hall et al. (2004), Walters et al. (2000), Cordato et al. (1999) and Boulineau et al. (2005).

3.4. Inhibitory effect of picrotoxinin, bilobalide and ginkgolide B on GABA-modulatory and GABA-mimetic actions of positive GABA_A receptor modulators

Picrotoxinin, bilobalide and ginkgolide B (0.001 μ M to 300 μ M) dose-dependently inhibited the potentiating actions of positive GABA_A receptor modulator EC₅₀'s in the presence of EC₅ GABA (5 μ M) and/or absence of GABA at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors (Tables 3, 4).

3.4.1. Etomidate

For the inhibition of the GABA-modulatory action of etomidate, picrotoxinin appeared to be the most potent compound, followed by bilobalide then ginkgolide B (Table 3, Fig. 4A). Newman-Keuls analysis showed that the IC₅₀ value of picrotoxinin was significantly different from that of bilobalide (q=6.55, P < 0.001) and ginkgolide B (q=7.70, P < 0.001).

Bilobalide and ginkgolide B were 2 and 5.7 fold less potent than picrotoxinin, respectively, at inhibiting the GABA-mimetic action of etomidate (Table 4, Fig. 4H). Picrotoxinin was found to be significantly more potent when compared to ginkgolide B (q=4.55, P < 0.05).

3.4.2. Propofol

Picrotoxinin appeared to be the most potent inhibitor of the GABA-modulatory action of propofol (Table 3, Figs. 2 and 4B), with an IC₅₀

value that was significantly different to bilobalide (q=15.53, P < 0.001) and ginkgolide B (q=11.99, P < 0.001). Bilobalide was found to be significantly less potent when compared to ginkgolide B (q=4.23, P < 0.05). Picrotoxinin (q=9.32, P < 0.001) and ginkgolide B (q=9.40, P < 0.001) were found to be 4–6 fold more potent than bilobalide in inhibiting the GABA-mimetic action of propofol (Table 4, Figs. 3 and 4I).

3.4.3. Diazepam

Both picrotoxinin (q=8.64, P < 0.001) and bilobalide (q=6.22, P < 0.01) were significantly more potent than ginkgolide B in inhibiting the high-affinity effect of diazepam (Table 3, Fig. 4C). Meanwhile, picrotoxinin was significantly more potent than bilobalide (q=3.69, P < 0.05) and ginkgolide B (q=5.56, P < 0.01) in inhibiting the low-affinity effect of diazepam (Table 3, Fig. 4D).

3.4.4. Thiopentone sodium

Picrotoxinin was significantly more potent than bilobalide (q=4.24, P < 0.01) and ginkgolide B (q=8.29, P < 0.001) in its inhibitory action on thiopentone sodium modulation (Table 3, Fig. 4E). The IC₅₀ value of bilobalide was also found to be significantly different from that of ginkgolide B (q=3.66, P < 0.05).

3.4.5. Loreclezole

As shown in Table 3 and Fig. 4F, picrotoxinin appeared to be the most potent inhibitor of loreclezole modulation, and significantly more potent than bilobalide (q=22.93, P < 0.001) and ginkgolide B (q=22.60, P < 0.001).

3.4.6. Allopregnanolone

Picrotoxinin was found to be significantly more potent than bilobalide (q=3.98, P < 0.05) and ginkgolide B (q=3.92, P < 0.05) in inhibiting allopregnanolone modulation (Table 3, Fig. 4G). There was no significant difference between the IC₅₀ values of bilobalide and ginkgolide B.

Compared to picrotoxinin, the potency difference in the inhibitory action of bilobalide and ginkgolide B on the GABA-modulatory action of propofol and loreclezole was much less than that of etomidate, diazepam, thiopentone sodium and allopregnanolone (Table 3). This trend is also clearly shown in Table 5 by the bolded ratios of IC₅₀ for bilobalide and ginkgolide B relative to IC₅₀ for picrotoxinin.

4. Discussion

The present study showed that the antagonists picrotoxinin, bilobalide and ginkgolide B inhibited GABA responses potentiated by the positive modulators etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone (Table 3) and the direct

Table 3

IC₅₀ and Hill coefficient (n_H) of picrotoxinin, bilobalide and ginkgolide B on the GABA-modulatory actions of etomidate, propofol, diazepam, thiopentone sodium, loreclezole and allopregnanolone in the presence of EC₅ GABA at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors.

Positive GABA _A modulator	With EC ₅ GABA					
	Picrotoxinin		Bilobalide		Ginkgolide B	
	IC ₅₀ (μ M)	Hill coefficient (n_H)	IC ₅₀ (μ M)	Hill coefficient (n_H)	IC ₅₀ (μ M)	Hill coefficient (n_H)
Etomidate	0.55 \pm 0.06	−0.93 \pm 0.11	1.98 \pm 0.04	−1.09 \pm 0.11	4.05 \pm 0.82	−0.46 \pm 0.11
Propofol	0.49 \pm 0.03	−0.68 \pm 0.06	21.02 \pm 1.28	−0.52 \pm 0.15	7.82 \pm 1.08	−0.47 \pm 0.05
Diazepam (high affinity)	0.35 \pm 0.06	−0.98 \pm 0.18	0.77 \pm 0.07	−0.83 \pm 0.12	3.42 \pm 0.53	−0.72 \pm 0.09
Diazepam (low affinity)	0.36 \pm 0.04	−1.06 \pm 0.10	0.99 \pm 0.05	−0.86 \pm 0.09	2.07 \pm 0.10	−0.66 \pm 0.09
Thiopentone sodium	0.50 \pm 0.08	−0.92 \pm 0.13	1.60 \pm 0.31	−0.86 \pm 0.15	3.72 \pm 1.14	−0.64 \pm 0.14
Loreclezole	0.14 \pm 0.02	−0.72 \pm 0.06	5.73 \pm 0.80	−1.14 \pm 0.13	5.49 \pm 0.74	−0.93 \pm 0.12
Allopregnanolone	0.44 \pm 0.03	−1.31 \pm 0.11	1.23 \pm 0.02	−0.77 \pm 0.08	1.26 \pm 0.02	−0.66 \pm 0.08

The IC₅₀ values of compounds found to be significantly different from that of picrotoxinin are bolded.

Data are mean \pm S.E.M. (n=3–8 oocytes).

Table 4

IC₅₀ and Hill coefficient (n_H) of picrotoxinin, bilobalide and ginkgolide B on the GABA-mimetic actions of etomidate and propofol at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors.

GABA-mimetic	Without GABA					
	Picrotoxinin		Bilobalide		Ginkgolide B	
	IC ₅₀ (μ M)	Hill coefficient (n_H)	IC ₅₀ (μ M)	Hill coefficient (n_H)	IC ₅₀ (μ M)	Hill coefficient (n_H)
Etomidate	0.06 \pm 0.01	-0.79 \pm 0.13	0.13 \pm 0.05	-0.79 \pm 0.13	0.34 \pm 0.08	-0.74 \pm 0.14
Propofol	0.07 \pm 0.01	-1.03 \pm 0.10	0.43 \pm 0.07	-0.63 \pm 0.07	0.09 \pm 0.01	-0.56 \pm 0.07

The IC₅₀ values of compounds found to be significantly different from that of picrotoxinin are bolded.

Data are mean \pm S.E.M. (n=3–8 oocytes).

action of etomidate and propofol (Table 4) at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors. The results strongly support various conclusions from receptor binding, electrophysiological and photolabeling studies that showed that binding sites of GABA_A receptor positive modulators are allosterically coupled to the picrotoxinin binding site (Concas et al., 1994; Jayakar et al., 2015; Nourmahmad et al., 2016; Sanna et al., 1996).

Positive GABA_A receptor modulators are known to enhance the binding of [³H]GABA and/or [³H]muscimol at GABA_A receptors by binding to allosteric sites independent of the GABA recognition site (Concas et al., 1991; Ghiani et al., 1996; Sieghart, 1995; Thyagarajan

et al., 1983). The actions of positive GABA_A receptor modulators are involved in the modulation of channel opening which requires rotation of the M2 domains (Horenstein et al., 2001) and electrostatic interactions between the 24' lysine residue (α_1 K279) in the M2-M3 loop and the aspartate residues (α_1 D57 and α_1 D149) in the N-terminal domain (Kash et al., 2003). The M2 residues at 15' position (α_1 S, $\beta_{2,3}$ N and γ_2 S) that are proposed to be involved in the action of positive GABA modulators (Cestari et al., 2000; Jonsson et al., 2010; Siegwart et al., 2002; Walters et al., 2000; Wingrove et al., 1994; Zeller et al., 2007).

Results from the present and previous studies suggest that biloba-

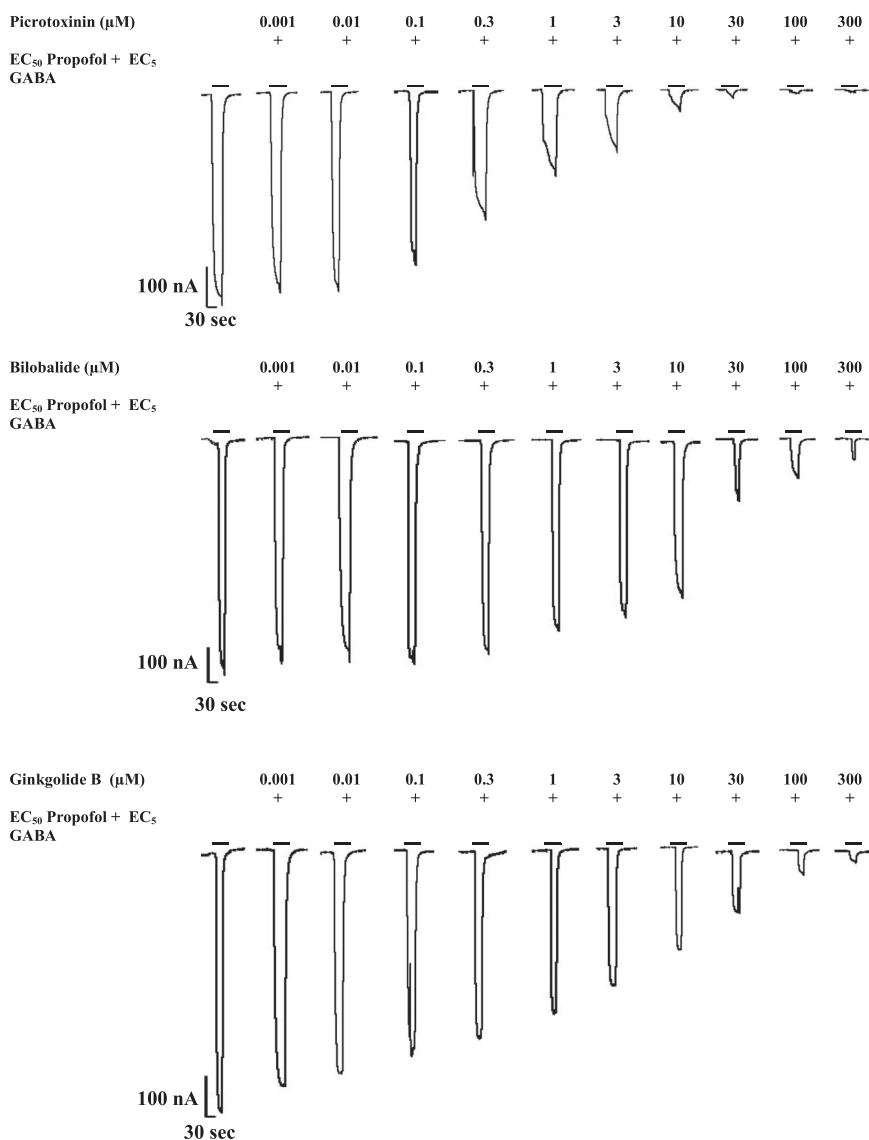


Fig. 2. Effect of picrotoxinin, bilobalide and ginkgolide B on the GABA-modulatory (with EC₅ GABA) action of EC₅₀ propofol at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors. (n=3–7 oocytes).

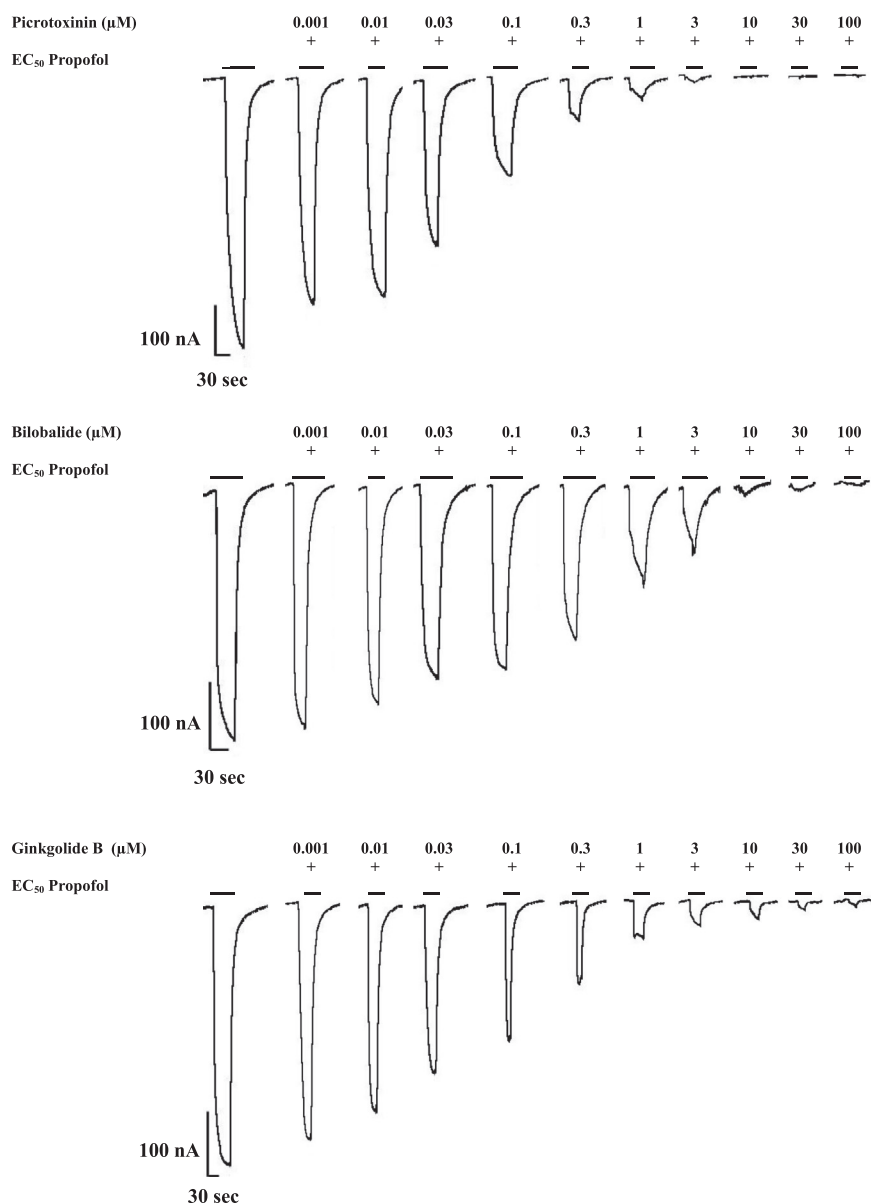


Fig. 3. Effect of picrotoxinin, bilobalide and ginkgolide B on the GABA-mimetic action of EC₅₀ propofol at $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors. (n=3–7 oocytes).

lide and ginkgolide B do not share the same binding sites with picrotoxinin at the cysteinyl mutants of $\alpha_1\beta_2\gamma_{2L}$ GABA_A receptors (Ng et al., 2016). The proposed intracellular pore binding sites for picrotoxinin, bilobalide and ginkgolide B are 2'β–6'β6'γ, 2'α2'β2'γ–6'β6'γ and 2'β2'γ–6'β, respectively (Ng et al., 2016). The findings that intracellular M2 residues at 2' and 6' positions are important for the binding of picrotoxinin are consistent to the reports from other mutational studies (Chen et al., 2006; Erkkila et al., 2008; Zhorov and Bregestovski, 2000). Unlike bilobalide and ginkgolide B, there was no significant change in picrotoxinin sensitivity with mutations at 15' position (Ng et al., 2016). It is proposed that 15'α15'β forms the extracellular pore binding site for bilobalide and ginkgolide B.

The present study suggests that picrotoxinin, bilobalide and ginkgolide B differ in their mechanisms in inhibiting the actions of a range of structurally diverse GABA_A positive modulators. Picrotoxinin was the most potent inhibitor of both the GABA-potentiated and direct action of all positive modulators tested (Tables 3–5). In the presence of EC₅ GABA, ginkgolide B was more potent than bilobalide in inhibiting the GABA-potentiating effect of propofol, equipotent against loreclezole and allopregnanolone, and less potent against etomidate, diaze-

pam, and thiopentone sodium (Table 3). In the absence of GABA, bilobalide was significantly more potent than ginkgolide B in inhibiting the GABA-mimetic action of etomidate, whereas ginkgolide B was significantly more potent than bilobalide in inhibiting the action of propofol (Table 4).

A study by Carpenter et al. (2013) has identified a possible secondary picrotoxinin binding site comprising extracellular sections of the M2 transmembrane domain (15', 17', 19'–21' residues), M2-M3 helix loop and Cys loop. The narrowest potency range exhibited by picrotoxinin could be explained by binding to its secondary site and allosterically inhibiting the 15' M2 residues in influencing the binding of positive modulators at GABA_A receptors. The finding that the benzodiazepine inverse agonist, β-carboline modulated GABA *via* the benzodiazepine site as well as the loreclezole binding site (Stevenson et al., 1995), helps to explain picrotoxinin's equipotency (Table 3) in inhibiting GABA responses potentiated by diazepam at both high and low affinity binding sites.

Furthermore, bilobalide and ginkgolide B have been shown to displaced the binding of [³⁵S]TBPS, a well-known radioligand for picrotoxinin binding site (Chatterjee et al., 2002, 2003). Taken together, our results suggest that bilobalide and ginkgolide B binding

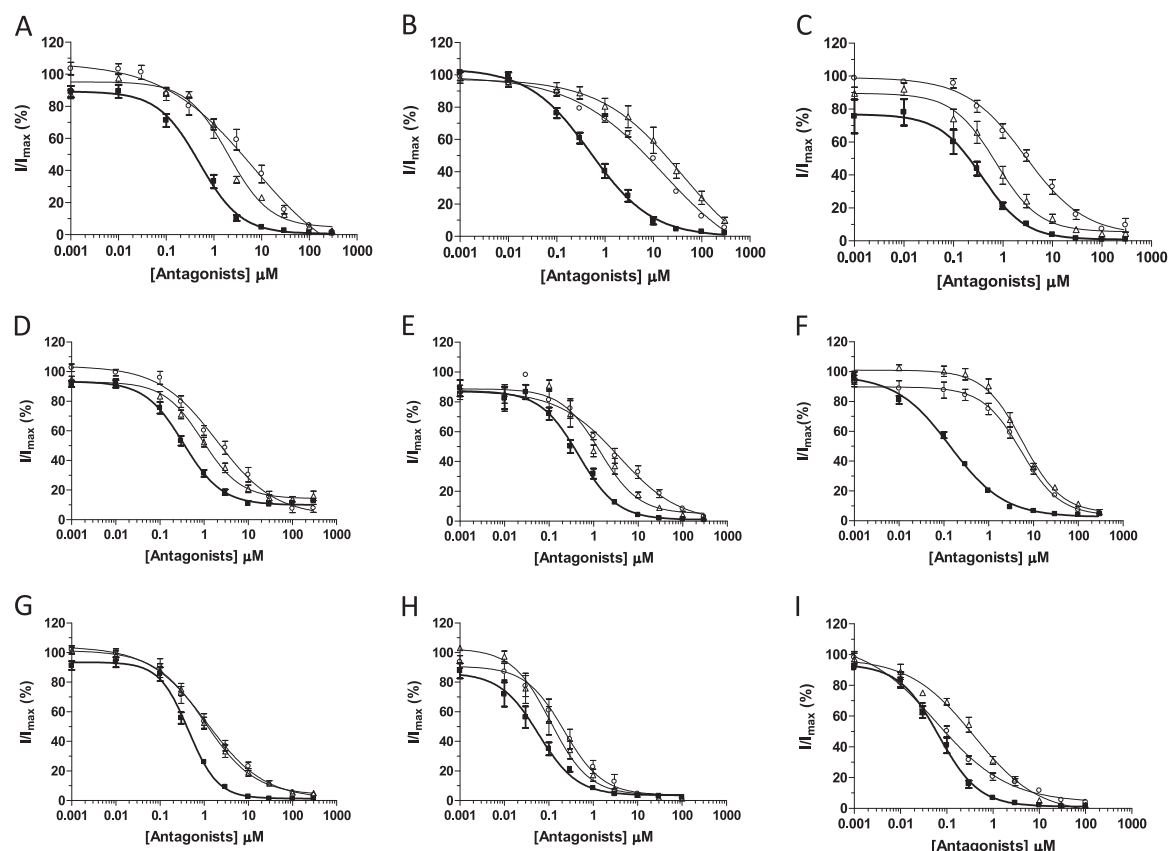


Fig. 4. Inhibition dose response curves of picrotoxinin (■), bilobalide (Δ) and ginkgolide B (○) on the GABA-modulatory (with EC₅₀ GABA) action of EC₅₀ of (A) etomidate, (B) propofol, (C) high affinity effect of diazepam, (D) low-affinity effect of diazepam, (E) thiopentone sodium, (F) loreclezole and (G) allopregnanolone, and GABA-mimetic action of EC₅₀ of (H) etomidate and (I) propofol at $\alpha_1\beta_2\gamma_2\text{L}$ GABA_A receptors. Data are mean \pm S.E.M. (n=3–8 oocytes).

Table 5

The calculated ratios of IC₅₀ value of bilobalide and ginkgolide B relative to the IC₅₀ value of picrotoxinin from Tables 3, 4.

Positive GABA _A modulator	Ratio IC ₅₀ compound /IC ₅₀ picrotoxinin			
	With EC ₅ GABA		Without GABA	
	Bilobalide	Ginkgolide B	Bilobalide	Ginkgolide B
Etomidate	3.60	7.36	2.17	5.67
Propofol	42.90	15.96	6.14	1.29
Diazepam (high affinity)	2.20	9.77	–	–
Diazepam (low affinity)	2.75	5.75	–	–
Thiopentone sodium	2.80	2.86	–	–
Loreclezole	40.93	39.21	–	–
Allopregnanolone	2.80	2.83	–	–

Bolded ratios (> 10) denote significant difference in IC₅₀ ratio.

– Denotes data are not available. (n=3–8 oocytes).

sites may overlap with both intracellular and secondary picrotoxinin binding sites.

Although picrotoxinin, bilobalide and ginkgolide B possess some structural similarities there are significant differences, notably the presence of a hydroxyl group adjacent to the lipophilic *t*-butyl group and an hydroxy-lactone group in bilobalide and ginkgolide B that are absent in picrotoxinin (Fig. 1). The presence of the potentially strong binding hydroxyl groups in bilobalide and ginkgolide B may represent a key structural feature for their lack of convulsant activity.

A range of terpenoids in addition to bilobalide and ginkgolide B that have been described as negative modulators of GABA_A receptors *in*

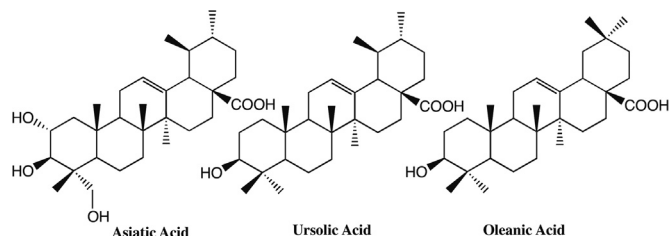


Fig. 5. Structures naturally occurring terpenoids without overt convulsant activity *in vivo* that potentiate the action of GABA on $\alpha_1\beta_2\gamma_2\text{L}$ GABA_A receptors *in vitro*.

vitro, yet act as anxiolytics, anticonvulsants and depressants *in vivo* have been described (Abdelhalim et al., 2014; Hamid et al., 2016). These include asiatic, ursolic and oleanic acids (Fig. 5). It seems likely that effects on other than GABA_A receptors contribute to their *in vivo* actions. Bilobalide has been reported to possess anticonvulsant activity (Sasaki et al., 1995). Consistent with its anticonvulsant activity, bilobalide has been shown to increase GABA levels in the hippocampus and cerebral cortex of mice (Sasaki et al., 1999) and to inhibit glutamate and aspartate release (Davies et al., 2003). However, the apparent paradox remains of these five terpenoids blocking GABA_A receptors *in vitro* and not acting as convulsants *in vivo*.

In summary, the present study shows that bilobalide and ginkgolide B inhibited the receptor modulation by positive GABA_A modulators differently to picrotoxinin. The lack of convulsant effects of bilobalide and ginkgolide B may be associated, at least in part, with their structural differences and overlapping binding locations to picrotoxinin at $\alpha_1\beta_2\gamma_2\text{L}$ GABA_A receptors. Further investigation into the binding sites for ginkgo terpenoid lactones outside of the channel remains crucial. This will provide a better understanding of the molecular actions of convulsants and anticonvulsants at GABA_A receptors.

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