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Bilobalide, a sesquiterpene trilactone from *Ginkgo biloba*, is an antagonist at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

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Abstract

The sesquiterpene trilactone bilobalide is one of the active constituents of the 50:1 *Ginkgo biloba* leaf extract widely used to enhance memory and learning. Bilobalide was found to antagonise the direct action of γ-aminobutyric acid (GABA) on recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The effect of bilobalide on the direct action of GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp method was evaluated and compared with the effects of the classical $GABA_A$ receptor competitive antagonist bicuculline and noncompetitive antagonist picrotoxinin. Bilobalide ($IC_{50} = 4.6 \pm 0.5$ μM) was almost as potent as bicuculline and pictrotoxinin ($IC_{50} = 2.0 \pm 0.1$ and 2.4 ± 0.5 μM, respectively) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors against 40 μM GABA (GABA EC_{50}). While bilobalide and picrotoxinin were clearly noncompetitive antagonists, the potency of bilobalide decreased at high GABA concentrations suggesting a component of competitive antagonism.

Keywords: *Ginkgo biloba*; Bilobalide; Picrotoxinin; Bicuculline; $GABA_A$ receptor; Voltage clamp, two-electrode; *Xenopus* oocyte

1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system acting through $GABA_A$ receptors involved in many neurological states such as vigilance, anxiety, wakefulness and seizures. The native $GABA_A$ receptors are heterooligomeric protein complexes made up of five protein subunits from a collection of α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, π and θ subunits with the $\alpha_1\beta_2\gamma_2$ subunit combination representing the major receptor subtype in the brain (Upton and Blackburn, 1997; Woodward et al., 1992; Chebib and Johnston, 2000). The five protein subunits of $GABA_A$ receptors are pseudosymmetrically arranged around a central axis forming a channel that is directly gated by GABA. The action of GABA at $GABA_A$ receptors is competitively antagonised by bicuculline. The $GABA_A$ receptor channel is selectively permeable to Cl^- ions and is blocked by the widely used Cl^- channel blocker picrotoxinin (Fig. 1). Picrotoxinin is a potent convulsant, an insecticide and a noncompetitive antagonist of $GABA_A$ receptors (Casida, 1993). Other insecticides including organochlorine, cyclodiene and pyrethroid insecticides are also noncompetitive antagonists of $GABA_A$ receptors (Casida, 1993).

Bilobalide (Fig. 1) is a sesquiterpene trilactone isolated from the leaves of *Ginkgo biloba*. The ginkgo leaf was used traditionally in Japan to protect books against harmful worms and insects before the introduction of modern insecticides (Honda, 1997). Bilobalide is a potent insecticide (Ahn et al., 1997) but is better known as one of the active constituents in the 50:1 ginkgo leaf extract. The extract is widely employed to treat symptoms associated with mild-to-moderate dementia, impairment of other cognitive functions associated with ageing and senility and

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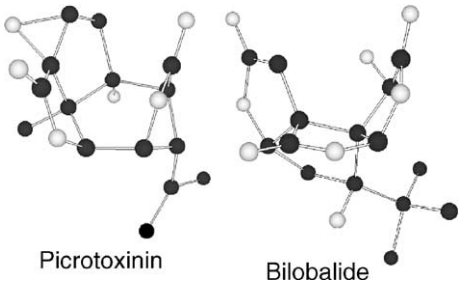

Fig. 1. Three-dimensional structures generated from the X-ray data of picrotoxinin and bilobalide (Weinges et al., 1987; Mackay and Sadek, 1983) using Chem3D, CS ChemDraw Ultra(R) version 6.0 (Cambridge-Soft, Cambridge, MA, USA) depicting the oxygen atoms in white and the carbon atoms in black illustrating structural similarities in their hydrophilic cage-like structures and lipophilic side chains, and the differences between the sizes of the cages and the location of the oxygen atoms with respect to the cage and the side chain. The side chain of bilobalide lies close to the oxygen atom and thus is in a more polar environment than the side chain of picrotoxinin. Bilobalide (Weinges et al., 1987) is a little wider and shallower than picrotoxinin (Mackay and Sadek, 1983). These structural differences may impose some constraints on how bilobalide and picrotoxinin can interact with $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

related neurosensory problems (Blumenthal et al., 2000). A study has indicated that the cognition-enhancing effects of the ginkgo leaf extract may be partly mediated by bilobalide via GABA receptors (Sasaki et al., 1999b). Enhanced hippocampal pyramidal neuronal excitability has been shown to correlate with learning and memory (Power et al., 1997), and bilobalide has been shown to enhance this excitability in rat hippocampal slices (Sasaki et al., 1999b). This action of bilobalide was proposed to involve blockade of GABAergic neurotransmission (Sasaki et al., 1999b).

This paper reports and compares the effects of bilobalide, picrotoxinin and bicuculline on GABA-mediated currents from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes using two-electrode voltage-clamp electrophysiology.

2. Material and methods

2.1. Materials

Human α_1, β_2 and γ_{2L} cDNAs subcloned in pcDM8 (Stratogene, La Jolla, CA, USA) were kindly provided by Dr. Paul Whiting (Department of Biochemistry and Molecular Biology, Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Harlow, Essex, UK). GABA, bicuculline, diazepam, zinc sulphate, and dimethyl sulphoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Bilobalide was isolated from the extract of *G. biloba* leaves provided by Japan Greenwave (Tokyo) and purified following the method described previously by Wada et al. (1993). Picrotoxinin was purified by chromatographic separation from picrotoxin following the method described by Jarboe et al. (1968). ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra in $CDCl_3$ and ^{13}C-NMR spectra in $CDCl_3/CD_3OD$ of picrotoxinin were consistent with the published spectra in $(CD_3)_2CO$ (Perry et al., 2001). Drug solutions were prepared by diluting 100 mM aqueous stock solutions of GABA and zinc sulphate and 100 mM DMSO stock solutions of bicuculline, bilobalide and picrotoxinin in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). The highest concentration of DMSO superfusing the oocytes was 0.8%, at which concentration DMSO had no effects.

2.2. Expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors in Xenopus laevis oocytes

The procedures involved in the use of *X. laevis* were approved by the Animal Ethics Committee of the University of Sydney. Female *X. laevis* were anaesthetised with 0.17% ethyl 3-aminobenzoate in saline and a lobe of the ovaries surgically removed. The lobe of ovaries was rinsed with OR-2 buffer that contained 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 5 mM HEPES, pH 7.4, and suspended in a solution of collagenase A (2 mg/ml in OR-2, Boehringer Mannheim, Germany) for 2 h to separate oocytes from connective tissues and follicular cells. Released oocytes were then thoroughly rinsed in ND96 buffer supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline and 50 μg/ml gentamycin, and stage V to VI oocytes were collected.

Human α_1, β_2 and γ_{2L} cDNAs subcloned in pcDM8 were linearised using the restriction enzyme *NOT1*. Linearised plasmids containing α_1, β_2 and γ_{2L} cDNAs were transcribed using T7 RNA Polymerase and capped with 5,7-methylguanosine using the "mMESSAGE mMACHINE" kit (Ambion, Austin, TX, USA). Ten nanograms per 50 nl of a 1:1:1 mixture of α_1, β_2 and γ_{2L} cRNAs were injected using a 15–20 μm diameter tip micropipette (micropipette puller, Sutter Instruments, USA) into the cytoplasm of individual defolliculated oocytes by using a Nanoject injector (Drummond Scientific, Broomali, PA, USA). The oocytes were incubated in ND96 buffer at 16 °C in an orbital shaker with a twice-daily change of buffer.

2.3. Electrophysiogical recording

Receptor activity was measured with two-electrode voltage-clamp techniques 2–8 days after injection. Recording microelectrodes were fabricated with a micropipette puller (Narishige Scientific Instrument Lab., Tokyo, Japan) and filled with 3 M KCl solution. Oocytes were placed in a cell bath and voltage clamped at −60 mV. Cells were continuously superfused with ND96 buffer. The currents elicited in response to the application of drugs were recorded using a Geneclamp 500 amplifier (Axon Instrument, Foster City, CA, USA), a Mac Lab 2e recorder (AD Instruments, Sydney, NSW, Australia), and Chart version 3.5.2 program

on a Macintosh Quadra 605 computer. Drugs were tested for direct activation of GABA at $GABA_A$ receptors. For measurements of inhibitory action of drugs on receptor activation, drugs were added to the buffer solution containing GABA at the concentration producing 10%, 50%, 75%, 90% and 100% of the effect (GABA EC_{10}, EC_{50}, EC_{75}, EC_{90} and EC_{100}) at the receptors for constructing GABA inhibition dose–response curves. The same procedure, but with a fixed concentration of antagonists and increasing concentrations of GABA, was applied to construct GABA dose–response curves. A washout period of 3–5 min was allowed between each drug application to prevent receptor desensitisation.

2.4. Analysis of data

The peak amplitude of current in response to each concentration of drug was recorded and standardized by calculating the ratio $\%I_{max} = I/I_{max} \times 100$, where I is the peak amplitude of current at a given dose of agonist or agonist/antagonist, and I_{max} is the maximal current generated by GABA for each individual cell. Data were expressed as the averaged $\%I_{max} \pm$ standard error of the mean (S.E.M.). The effective doses that evoked 50% of I_{max} (EC_{50}) were calculated from dose–response data constructed with $\%I_{max}$ as a function of agonist concentration ([A]) by least square method to the Hill equation $I = I_{max}[A]^{n_H}/(EC_{50}^{n_H}+[A]^{n_H})$, where n_H is the Hill coefficient. The effective doses that inhibited 50% of I_{max} (IC_{50}) were calculated in a similar manner to EC_{50} values from the inverse Hill equation $I = I_{max} - \{I_{max}[Ant]^{n_H}/(IC_{50}^{n_H}+[Ant]^{n_H})\}$, where [Ant] is the concentration of the antagonist.

EC_{50}, IC_{50}, maximal efficacy and Hill coefficient numbers were estimated by fitting the concentration–response relationships to the logistic equation using GraphPad Prism v3.02 (GraphPad Software). Unless otherwise noted, parameters were calculated for individual cells and then averaged. These parameters are reported as mean ± S.E.M. ($n = 3–5$ oocytes). The statistical significance of differences between mean maximal GABA response with and without antagonists is determined by Student's t-test at significance level of $P < 0.05$.

3. Results

3.1. Functional property of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

In *X. laevis* oocytes, human wild-type α_1, β_2, and γ_{2L} cRNAs generated GABA-gated channels with the amplitude of inward whole-cell currents recorded ranging from 300 to 3000 nA at −60 mV. GABA-mediated currents were not detectable when an α_1, β_2 or γ_{2L} subunit alone was expressed in the oocytes under the same conditions used for the expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors exhibited similar pharmacological profiles as previously described for heterooligomeric receptors exhibiting GABA EC_{50} (~ 35–60 μM) and Hill coefficient (n_H) values (1.1–1.3) similar to those reports for these receptors (Mihic et al., 1994; Duke et al., 2000; Scheller and Forman, 2001). These receptors also exhibited biphasic (low and high affinity) diazepam potentiation and were insensitive to Zn^{2+} ions, thus establishing the incorporation of the γ_{2L} subunit.

3.2. Inhibition of direct GABA-mediated currents at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

Bicuculline, picrotoxinin and bilobalide dose-dependently inhibited the Cl^- conductance generated by 40 μM GABA (Fig. 2A–C). No effects were observed when these

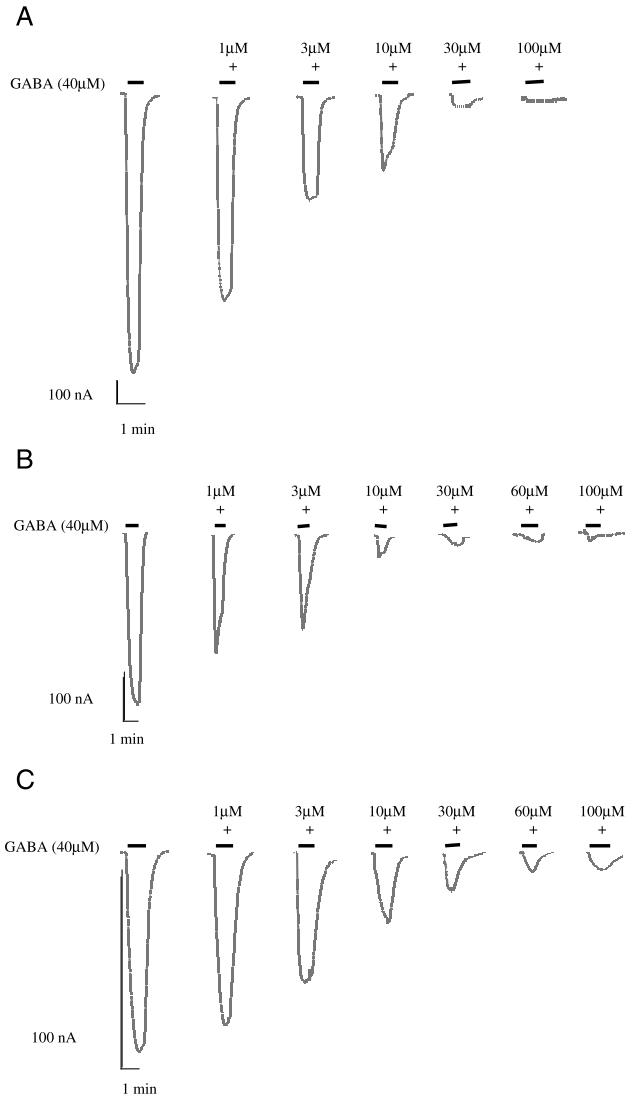

Fig. 2. Current traces produced by 40 μM GABA (solid bar) in the presence of (A) bicuculline, (B) picrotoxinin and (C) bilobalide at various concentrations from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. The bars indicate duration of drug application. Bilobalide did not have any effect on its own when tested at 100 μM.

compounds were applied on their own at 100 μM. The inhibition dose–response curves of picrotoxinin and bilobalide on 10 μM GABA (EC_{10}), 40 μM GABA (EC_{50}), 100 μM GABA (EC_{75}), 300 μM GABA (EC_{90}) and 1 mM GABA (EC_{100}) are shown in Fig. 3A–E, respectively. Fig. 3B also includes the inhibition dose–response curve of bicuculline on 40 μM GABA (EC_{50}). The IC_{50} and n_H values for each compound are tabulated in Table 1.

Bicuculline ($IC_{50} = 2.0 \pm 0.1$ μM) was approximately equipotent to picrotoxinin ($IC_{50} = 2.4 \pm 0.5$ μM) and about two times more potent than bilobalide ($IC_{50} = 4.6 \pm 0.5$ μM) at inhibiting the agonist action of GABA at its EC_{50} value (40 μM) at $\alpha_1\beta_2\gamma_{2L}$ receptors.

Table 1 shows that the potency of picrotoxinin was largely unaffected by different concentrations of GABA. The variation in the potency of picrotoxinin determined over 10 μM–1 mM concentrations of GABA was not significant ($P = 0.1258$–1.0000 compared with the potency of picrotoxinin at 40 μM GABA (GABA EC_{50})). Thus, the potency of picrotoxinin appeared to be independent of the GABA concentration, indicating that it largely exerts noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

On the other hand, the potency of bilobalide was clearly dependent on the GABA concentrations. Bilobalide became significantly less potent at higher GABA concentrations. At 100 μM, 300 μM and 1 mM GABA, the potency of bilobalide ($IC_{50} = 7.9$, 10.6 and 6.9 μM, respectively) were approximately half of that determined at 40 μM GABA ($IC_{50} = 4.6$ μM, $P = 0.0004$, $P = 0.0217$ and $P = 0.0098$) and 10 μM GABA ($IC_{50} = 4.9$ μM, $P = 0.0001$, $P = 0.0248$ and $P = 0.0104$). The result indicates that the action of bilobalide at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors may also involve competitive antagonism.

Bilobalide was less potent than picrotoxinin at all five concentrations of GABA examined. It was especially less potent than picrotoxinin at high concentrations of GABA due to its lower potency at high GABA concentrations. At lower GABA concentrations (10 and 40 μM), bilobalide ($IC_{50} = 4.9 \pm 0.3$ and 4.6 ± 0.5 μM) was approximately two times less potent than picrotoxinin ($IC_{50} = 2.3 \pm 0.5$ and 2.4 ± 0.5 μM). At higher GABA concentrations (100 μM, 300 μM and 1 mM), bilobalide ($IC_{50} = 7.9 \pm 0.2$, 10.6 ± 2.8 and 6.9 ± 0.7 μM) was approximately four times less potent than picrotoxinin ($IC_{50} = 1.7 \pm 0.2$, 2.5 ± 0.3 and 2.3 ± 0.3 μM) (Table 1).

Fig. 4A and B (Dixon plots) shows the reciprocal plots of the percentage response of 10 μM–1 mM GABA to 1–30 μM picrotoxinin and to 1–30 μM bilobalide, respectively. The inhibitory constant value (K_i) of picrotoxinin determined from (Fig. 4A) was 9.7 ± 0.7 μM and that of bilobalide (Fig. 4B) was 14.8 ± 0.6 μM.

3.3. Antagonism of bicuculline, picrotoxinin and bilobalide at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

To determine the mechanism of action, bicuculline, picrotoxinin and bilobalide were co-administered with increasing concentrations of GABA, and the responses

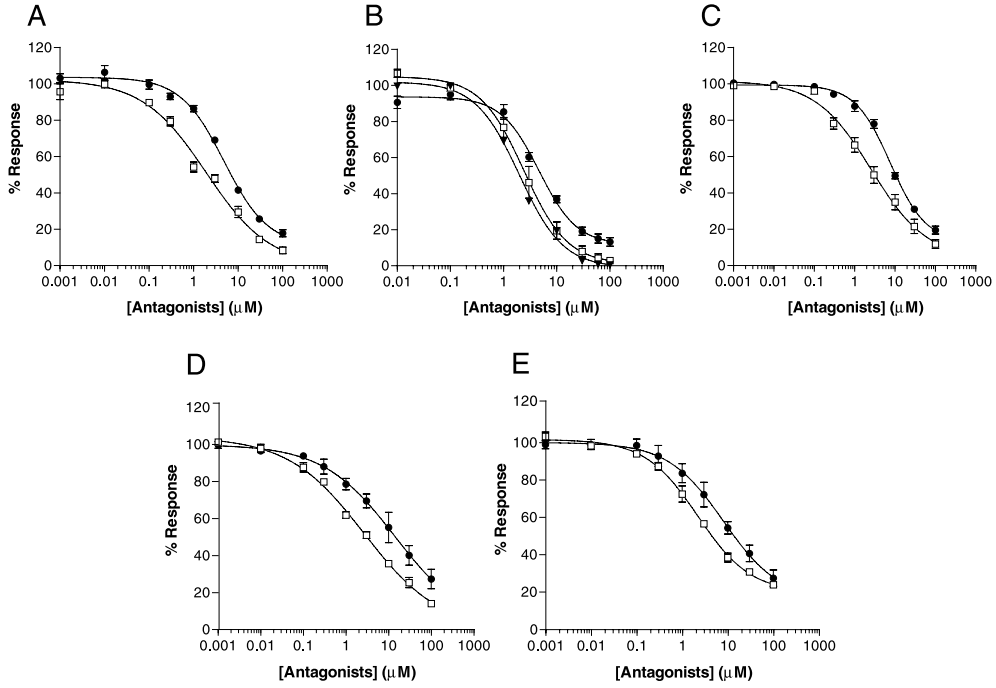

Fig. 3. Inhibition dose–response curves of (A) 10 μM GABA in the presence of bilobalide (●) and picrotoxinin(□); (B) 40 μM GABA in the presence of bilobalide (●), bicuculline (▼) and picrotoxinin(□); (C) 100 μM GABA in the presence of bilobalide (●) and picrotoxinin(□); (D) 300 μM GABA in the presence of bilobalide (●) and picrotoxinin(□); and (E) 1 mM GABA in the presence of bilobalide (●) and picrotoxinin(□) from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. ($n = 4$–7 oocytes).

Table 1
IC_{50} and Hill coefficient values for bicuculline, picrotoxinin and bilobalide in the presence of 10, 40, 100, 300 and 1000 μM GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

Compounds	10 μM GABA		40 μM GABA		100 μM GABA		300 μM GABA		1000 μM GABA	
	IC_{50} (μM)[a]	n_H^b	IC_{50} (μM)[a]	n_H^b	IC_{50} (μM)[a]	n_H^b	IC_{50} (μM)[a]	n_H^b	IC_{50} (μM)[a]	n_H^b
Bicuculline	–	–	2.0 ± 0.1	− 1.1 ± 0.1	–	–	–	–	–	–
Picrotoxinin	2.3 ± 0.5	− 0.6 ± 0.1	2.4 ± 0.5	− 1.3 ± 0.2	1.7 ± 0.2	− 0.7 ± 0.1	2.5 ± 0.3	− 0.5 ± 0.1	2.3 ± 0.3	− 0.8 ± 0.1
Bilobalide	4.9 ± 0.3	− 0.9 ± 0.1	4.6 ± 0.5	− 1.2 ± 0.2	7.9 ± 0.2	− 1.0 ± 0.1	10.6 ± 2.8	− 0.6 ± 0.1	6.9 ± 0.7	− 0.7 ± 0.1

[a] IC_{50} is the concentration that inhibits 50% of receptors. Data are the mean ± S.E.M. (n = 4–7 oocytes).
[b] n_H is the Hill coefficient. Data are the mean ± S.E.M. (n = 4–7 oocytes).

obtained were compared with those elicited by GABA alone. GABA concentration–effect curves of GABA alone and GABA in the presence of antagonists at different fixed concentrations were constructed.

3.3.1. Bicuculline

The GABA concentration–effect curve in the presence of 1 and 3 μM bicuculline (Fig. 5A and B) displayed a parallel right shift and attained the maximal response of GABA. GABA response in the presence of 1 and 3 μM bicuculline was 99.5% (P = 0.9415) and 101.0% (P = 0.0702) of GABA maximal response, respectively (Table 2). Bicuculline at 1 and 3 μM increased GABA EC_{50} values: 1.6 times (41.0–67.0 μM) and 3.6 times (36.1–129.0 μM), respectively (Table 2). Bicuculline appears to shift the dose–response curves of GABA in parallel to the right without decreasing GABA maximal response, suggesting that it is a competitive antagonist at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

3.3.2. Picrotoxinin

The GABA concentration–effect curves in the presence of 3, 10 and 30 μM picrotoxinin (Fig. 6A–C) displayed similar right shifts and did not reach the maximal response of GABA.

GABA response in the presence of 3, 10 and 30 μM picrotoxinin attained 85.8% (P = 0.0086), 50.6% (P < 0.0001) and 50.1% (P = 0.0003) of GABA maximal response, respectively (Table 2). Picrotoxinin at 3, 10 and 30 μM produced similar increases in the GABA EC_{50} values: 2.3 times (36.4–81.9 μM), 3 times (54.9–162.4 μM) and 2.5 times (56.1–139.3 μM), respectively

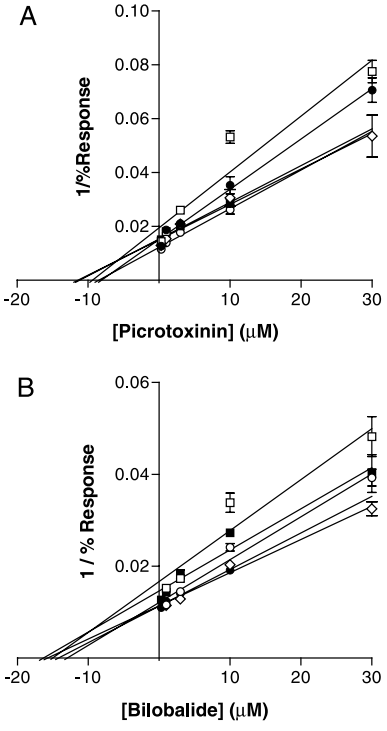

Fig. 4. Dixon plots constructed using various concentrations of (A) picrotoxinin and (B) bilobalide at fixed GABA concentrations (10 μM GABA, 40 μM GABA, 100 μM GABA, 300 μM GABA and 1 mM GABA) from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. (n = 4–7 oocytes).

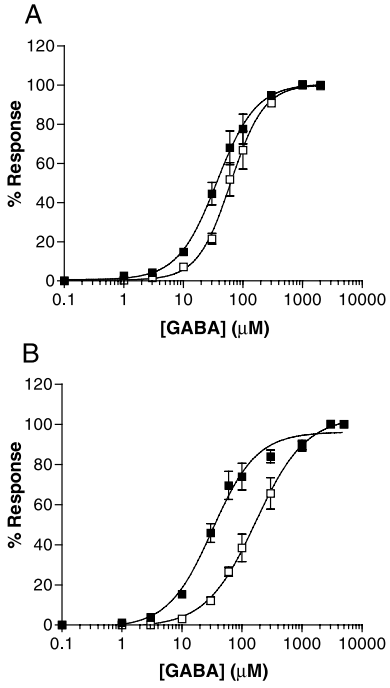

Fig. 5. Agonist dose–response curves of GABA alone (■) and GABA in the presence of bicuculline (□) (A) 1 μM and (B) 3 μM from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. (n = 4–7 oocytes).

Table 2
EC_{50} and Hill coefficient values and maximal responses of GABA for GABA alone and in the presence of bicuculline, picrotoxinin and bilobalide at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

Antagonists (Ant)	GABA EC_{50} (μM)[a]	GABA + Ant EC_{50} (μM)[a]	GABA n_H[b]	GABA + Ant n_H[b]	Maximal GABA response (%)
1 μM Bicuculline	41.0 ± 11.4	67.0 ± 16.8	1.4 ± 0.1	1.8 ± 0.1	99.5 ± 1.2
3 μM Bicuculline	36.1 ± 4.3	129.0 ± 29.9	1.3 ± 0.3	1.3 ± 0.1	101.0 ± 0.5
3 μM Picrotoxinin	36.4 ± 14.3	81.9 ± 15.9	1.5 ± 0.2	1.2 ± 0.3	85.8 ± 1.5
10 μM Picrotoxinin	54.9 ± 0.5	162.4 ± 30.5	1.5 ± 0.1	1.0 ± 0.1	50.6 ± 5.3
30 μM Picrotoxinin	56.1 ± 10.6	139.3 ± 25.7	2.0 ± 0.2	1.2 ± 0.2	50.1 ± 8.1
3 μM Bilobalide	56.0 ± 6.8	82.0 ± 11.8	1.3 ± 0.1	1.2 ± 0.1	72.0 ± 3.1
10 μM Bilobalide	54.1 ± 13.8	95.9 ± 48.7	1.1 ± 0.2	1.3 ± 0.2	66.6 ± 4.0
30 μM Bilobalide	67.7 ± 10.6	182.0 ± 48.4	1.1 ± 0.2	0.8 ± 0.1	62.4 ± 8.1

[a] EC_{50} is the concentration that activates 50% of receptors. Data are the mean ± S.E.M. (n = 4–7 oocytes).
[b] n_H is the Hill coefficient. Data are the mean ± S.E.M. (n = 4–7 oocytes).

(Table 2). Picrotoxinin reduced GABA maximal responses and caused nonparallel shifts in the GABA dose–response curves, indicating that it is a noncompetitive antagonist at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

3.3.3. Bilobalide

The GABA concentration–effect curves in the presence of 3, 10 and 30 μM bilobalide (Fig. 7A–C) displayed right shifts and did not reach the maximal response of GABA.

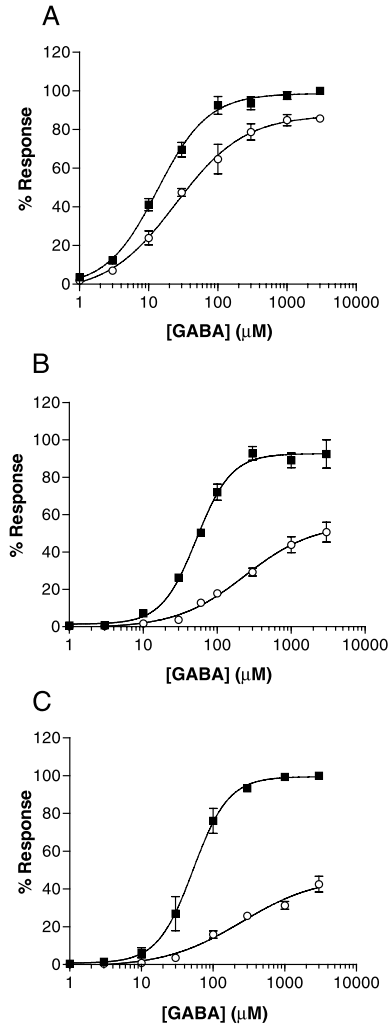

Fig. 6. Agonist dose–response curves of GABA alone (■) and of GABA in the presence of picrotoxinin (○) at (A) 3 μM, (B) 10 μM and (C) 30 μM from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. (n = 4–7 oocytes).

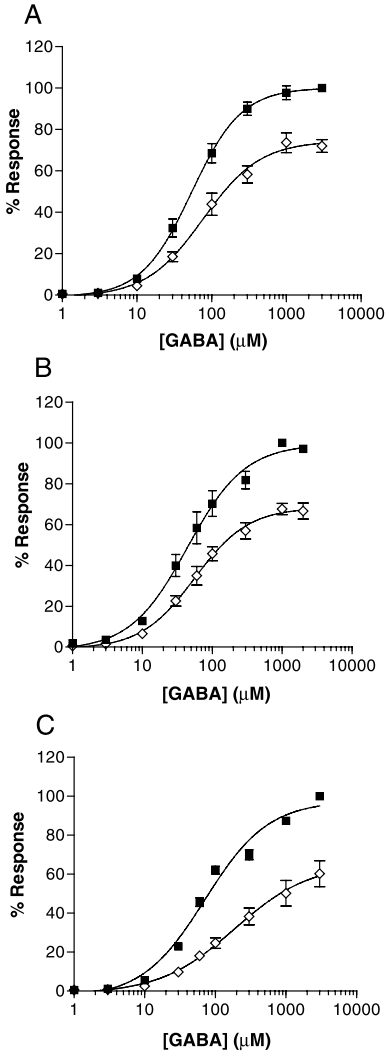

Fig. 7. Agonist dose–response curves of GABA alone (■) and of GABA in the presence of bilobalide (◇) at (A) 3 μM, (B) 10 μM and (C) 30 μM from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. (n = 4–7 oocytes).

GABA maximal response in the presence of 3, 10 and 30 μM bilobalide attained 83.3% ($P=0.0657$), 66.6% ($P=0.0009$) and 62.4% ($P=0.041$) of GABA maximal response, respectively (Table 2). Bilobalide at 3, 10 and 30 μM increased GABA EC_{50} values: 1.5 times (56.0–82.0 μM), 1.8 times (54.1–95.9 μM) and 2.7 times (67.7–182.0 μM), respectively (Table 2). Bilobalide caused right shifts of the GABA dose–response curves and reduced GABA maximal responses, indicating that it exhibits noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

4. Discussion

This study shows that bilobalide, the sesquiterpene trilactone of *G. biloba*, potently inhibits the direct GABA-mediated currents from recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The finding strongly supports the proposal by Sasaki et al. (1999b) that the observed enhanced neuronal excitability in hippocampal slices was due to its blockade of GABAergic neurotransmission and also suggests that GABA receptors may be involved in mediating its insecticidal activity.

Bicuculline is a competitive antagonist of $GABA_A$ receptors (Akaike et al., 1985). The competitive antagonism of bicuculline and noncompetitive antagonism of picrotoxinin at $GABA_A$ receptors are also exemplified at the human $\alpha_1\beta_2\gamma_{2L}$ subunit combination. At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, bicuculline displayed the general property of the competitive antagonist, producing a parallel shift of GABA concentration–effect curves and having no effect on the maximal response of GABA.

Picrotoxinin is a noncompetitive antagonist of $GABA_A$ receptors (Akaike et al., 1985). At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, picrotoxinin showed properties characteristic of the noncompetitive antagonist including the nonparallel right shift of GABA concentration–effect curves and decreased maximal responses of GABA. Bilobalide also showed properties characteristic of the noncompetitive antagonist in reducing GABA maximal responses and producing right shifts of the GABA concentration–effect curves, indicating noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

Bilobalide and picrotoxinin share common structural features including a hydrophilic cage and lipophilic side chain. The structural similarities and differences of their cages and side chains are depicted in Fig. 1. However, bilobalide and picrotoxinin have opposite actions on systemic administration to animals. Bilobalide is an anticonvulsant (Sasaki et al., 1995, 1997) whereas picrotoxinin is a potent convulsant (Jarboe et al., 1968). There are, however, only minor differences in their activities at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Bilobalide ($K_i=14.8\pm0.6$ μM) was less potent than picrotoxinin ($K_i=9.7\pm0.7$ μM) and exhibited a differing potency profile from that of picrotoxinin.

The potency of picrotoxinin appeared to be largely independent of GABA concentrations, consistent with its noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The potency of bilobalide, however, was clearly dependent on GABA concentrations (approximately two times less potent at high concentrations of GABA (>GABA EC_{50})), suggesting that competitive antagonism may also be involved in the action of bilobalide.

Bilobalide has been shown to increase GABA levels in the hippocampus and cerebral cortex of mice (Sasaki et al., 1999a). This increase may override the $GABA_A$ antagonist action of bilobalide, which is known to be anticonvulsant on systemic administration rather than a convulsant like other $GABA_A$ antagonists.

In summary, the present study demonstrates for the first time that bilobalide is an antagonist at recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The finding supports the proposal by Sasaki et al. (1999b) that enhanced neuronal excitability in hippocampal slices by bilobalide was due to its blockade of GABAergic neurotransmission and provides a basis for the direct effect of the 50:1 leaf extract of *G. biloba* on memory and learning.

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