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Ginkgolides, diterpene trilactones of *Ginkgo biloba*, as antagonists at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

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Abstract

Ginkgolides A, B, and C are diterpene trilactones and active constituents of the 50:1 *Ginkgo biloba* leaf extract widely used in the symptomatic treatment of mild to moderate dementia. Using the two-electrode voltage clamp methodology, these ginkgolides were found to be moderately potent antagonists at recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Ginkgolides A, B, and C inhibited the direct action of γ-aminobutyric acid (GABA) with K_i values of 14.5 ± 1.0, 12.7 ± 1.7, and 16.3 ± 2.4 μM respectively. Antagonism by these ginkgolides at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors appears to be noncompetitive as indicated by the nonparallel right shift and reduced maximal GABA response in their GABA concentration–effect curves.

Keywords: $GABA_A$ receptor; Noncompetitive; Ginkgolide; Picrotoxinin; Two-electrode voltage clamp; *Xenopus* oocyte

1. Introduction

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system mediating fast neurotransmission predominantly via the $GABA_A$ receptor. $GABA_A$ receptors belong to the family of inhibitory transmitter-gated channels that also include $GABA_C$ receptors and glycine receptors. The channel of $GABA_A$, $GABA_C$, and glycine receptors opens in response to binding of the respective transmitter, GABA, or glycine enabling Cl^- ions to follow its electrochemical gradient into the cells, balancing the effect of neuronal excitation (Jentsch, 2002). The channel of $GABA_A$, $GABA_C$, and glycine receptors is blocked by the plant convulsant picrotoxinin (Fig. 1; Zhorov and Bregestovski, 2000).

Picrotoxinin is a noncompetitive antagonist of $GABA_A$ receptors (Akaike et al., 1985). [^{35}S]*t*-butylphosphorothionate ([^{35}S]TBPS) is a radioligand for picrotoxinin binding sites at $GABA_A$ receptors (Olsen et al., 1989). Picrotoxinin and TBPS binding sites have been shown to reside within the channel of $GABA_A$ receptors (Jursky et al., 2000; Perret et al., 1999). $GABA_A$ receptors also incorporate the binding sites for GABA, barbiturates, benzodiazepines, and steroids. $GABA_A$ receptors are heterooligomeric pentamers assembled from several possible combinations of protein subunits. To date, at least 16 different subunits for human $GABA_A$ receptors have been identified, namely, α_{1-6}, β_{1-3}, γ_{1-3}, δ, π, and θ (Chebib and Johnston, 2000). Most $GABA_A$ receptors are formed by the coexpression of α, β, and γ subunits with the subunit combination of $\alpha_1\beta_2\gamma_{2L}$ being the major $GABA_A$ receptor subtype in the brain (Upton and Blackburn, 1997).

Ginkgolides (Fig. 1) are diterpene trilactones of the maiden hair tree, *Ginkgo biloba*. Ginkgo leaves have long been used in traditional Chinese medicine. Modern usage utilises the extract from the leaves for the treatment of

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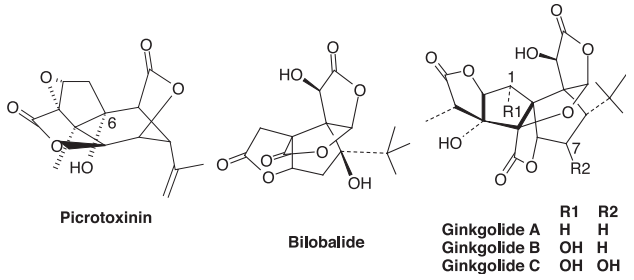

Fig. 1. Structures of picrotoxinin, bilobalide, and ginkgolides A, B, and C illustrating structural similarities in the hydrophilic cavity and lipophilic side chain. Picrotoxinin is a sesquiterpene epoxydilactone; bilobalide, a sesquiterpene trilactone; and the ginkgolides, diterpene trilactone. These compounds have cavity-like structures made up of a highly oxygenated carbon skeleton, including two lactone rings and an epoxy group in picrotoxinin, and three lactone rings in bilobalide and the ginkgolides. Bilobalide has only one carbocycle, making it smaller and more compact than the ginkgolides. The lipophilic side chain, the isopropenyl group in picrotoxinin and *t*-butyl group in bilobalide and ginkgolides, is attached to the underside of the cavity.

cerebrovascular and peripheral vascular insufficiency, symptoms associated with cognitive decline and neurosensory impairments that may be associated with dementia, aging, and senility (Blumenthal et al., 2000). The extract is prepared to a 35–67:1 (average 50:1) ratio of dried leaves to final extract. The extract contains 5–7% terpene lactones, of which approximately 2.8–3.4% consist of ginkgolides A, B, and C, and approximately 2.6–3.2%, of bilobalide.

Ginkgolides are specific inhibitors of the lipid-mediator platelet aggregating factor (Braquet and Hosford, 1991; Hu et al., 1999). Recently, the ginkgolides have been reported to be selective and potent blockers of glycine receptors (IC_{50} = 0.27–2.0 μM) (Chatterjee et al., 2003; Ivic et al., 2003; Kondratskaya et al., 2002). There are reports in the literature suggesting the interaction of the ginkgolides with GABA receptors. Ginkgolides A and B shortened the sleeping time induced by barbiturates in mice (Brochet et al., 1999; Wada et al., 1993). Muscimol-stimulated Cl^- ion uptake in cortical synaptosomes was potentiated by ginkgolide B, and this effect was blocked by the benzodiazepine antagonist flumazenil (Miller et al., 1991). The ginkgolides have been shown to displace TBPS from its binding site (Chatterjee et al., 2002, 2003), suggesting interaction with the picrotoxinin site of $GABA_A$ receptors. A recent report showed that ginkgolide B was a blocker of $GABA_A$ receptors on rat cortical slices (Ivic et al., 2003).

We have previously reported that bilobalide is a non-competitive antagonist of the recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Huang et al., 2003). We wish to report that ginkgolides A, B, and C were also found to be noncompetitive antagonists at the same receptors but less potent than bilobalide. This paper reports the effect and possible mechanism of the action of ginkgolides A, B, and C on GABA-activated currents from recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes using two-electrode voltage clamp methods.

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, diazepam, zinc sulphate, and dimethyl sulphoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Ginkgolides A, B, and C were 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). Drug solutions were prepared by diluting 100 mM aqueous stock solutions of GABA and zinc sulphate and 100 mM DMSO stock solutions of ginkgolides A, B, and C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_2O$, 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 was 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 *NOT*I. Linearised plasmids containing α_1, β_2, and γ_{2L} cDNAs were transcribed using T7 RNA polymerase and capped with 5,7-methylguanosine using a "mMESSAGE mMACHINE" kit (Ambion, Austin, TX, USA). Ten nanograms per 50 nl of a 1:1:2 mixture of α_1, β_2, and γ_{2L} cRNAs were injected using a 15 to 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 a 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 standardised 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 $\%I_{max}$ ± 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. ($n = 5–15$ oocytes). The statistical significance of differences between GABA responses with and without antagonists was determined by two-way ANOVA method, whereas the differences between IC_{50} values at different GABA concentrations, by student t-test at the 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 magnitude of inward whole-cell currents of 300 to 3000 nA recorded 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 pharmacological profiles of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors were in line with reports previously described for these heterooligomeric receptors exhibiting GABA EC_{50} values of between ~35 and 60 μM and Hill coefficient (n_H) values of between 1.2 and 2.5 (Duke et al., 2000; Huang et al., 2003; Scheller and Forman, 2001). The incorporation of the γ_{2L} subunit was established by the observation of biphasic (low and high affinity) diazepam potentiation (Mihic et al., 1994; Walters et al., 2000) and insensitivity to Zn^{2+} ions (Draguhn et al., 1990).

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

Ginkgolides A, B, and C dose-dependently inhibited the chloride conductance generated by 40 μM GABA (Fig. 2A–C). No effects were observed when these compounds were tested on their own at 100 μM. The potency of the ginkgolides was calculated from the inhibition dose–response curves representing the effects of a range of antagonist concentrations on a fixed concentration of GABA (Fig. 3). The inhibition dose–response curves of ginkgolides A, B, and C at 10, 40, 100, 300 μM, and 1 mM GABA are shown in Fig. 3A–E, respectively. The IC_{50} and n_H values for each compound are tabulated in Table 1.

Table 1 shows that ginkgolides A, B, and C displayed the greatest potency in inhibiting 10 μM GABA compared to their potencies obtained at higher GABA concentrations (40 μM–1 mM). The potency of ginkgolide A at 10 μM GABA ($IC_{50} = 2.6 \pm 0.6$ μM) was approximately five times higher than its potency at higher concentrations of GABA (40, 100, 300 μM, and 1mM GABA, $IC_{50} = 13.0 \pm 3.4$, 13.7 ± 1.1, 11.9 ± 1.7, and 12.2 ± 2.4 μM, respectively). Using the IC_{50} value at 40 μM as the standard point for comparison (the same comparison measure was also used for ginkgolides B and C), the potency of ginkgolide A determined at 10 μM was significantly different ($P = 0.0046$) whereas the variation in the potencies determined over 40 μM–1mM GABA was not significant ($P = 0.8428$, 0.5726, and 0.8498, re-

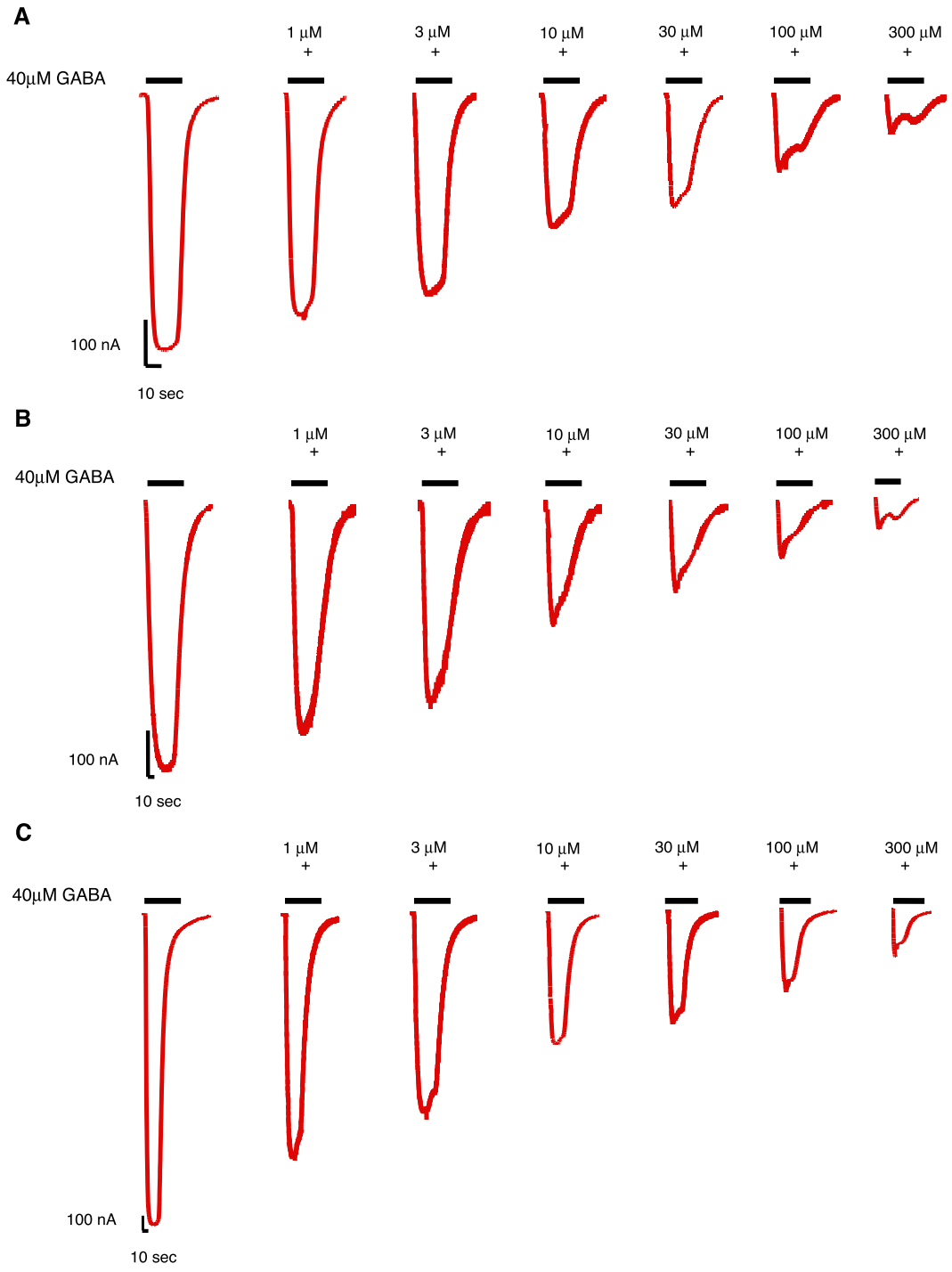

Fig. 2. Current traces produced by 40 μM GABA (solid bar) in the presence of (A) ginkgolide A, (B) ginkgolide B, and (C) ginkgolide C at various concentrations from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. The bars indicate duration of drug application. The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

spectively). Thus, the potency of ginkgolide A appears to be independent of the GABA concentration at 40 μM GABA and above, indicating that it largely exerts noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

The potency of ginkgolide B at 10 μM GABA ($IC_{50} = 4.8 \pm 0.8$ μM) was approximately two times higher than its potency at GABA concentrations between 40 μM and 1mM ($IC_{50} = 9.6 \pm 1.5$, 9.5 ± 1.5, 10.1 ± 2.9, and 11.4 ± 2.9 μM, respectively). Similar to ginkgolide A, the IC_{50} value of ginkgolide B at 10 μM GABA was significantly different ($P = 0.0210$) from that at 40 μM GABA, but there was no significant difference across the values obtained for 40 μM–1mM GABA ($P = 0.9693$, 0.8654, and 0.5676, respectively). The potency of ginkgo-

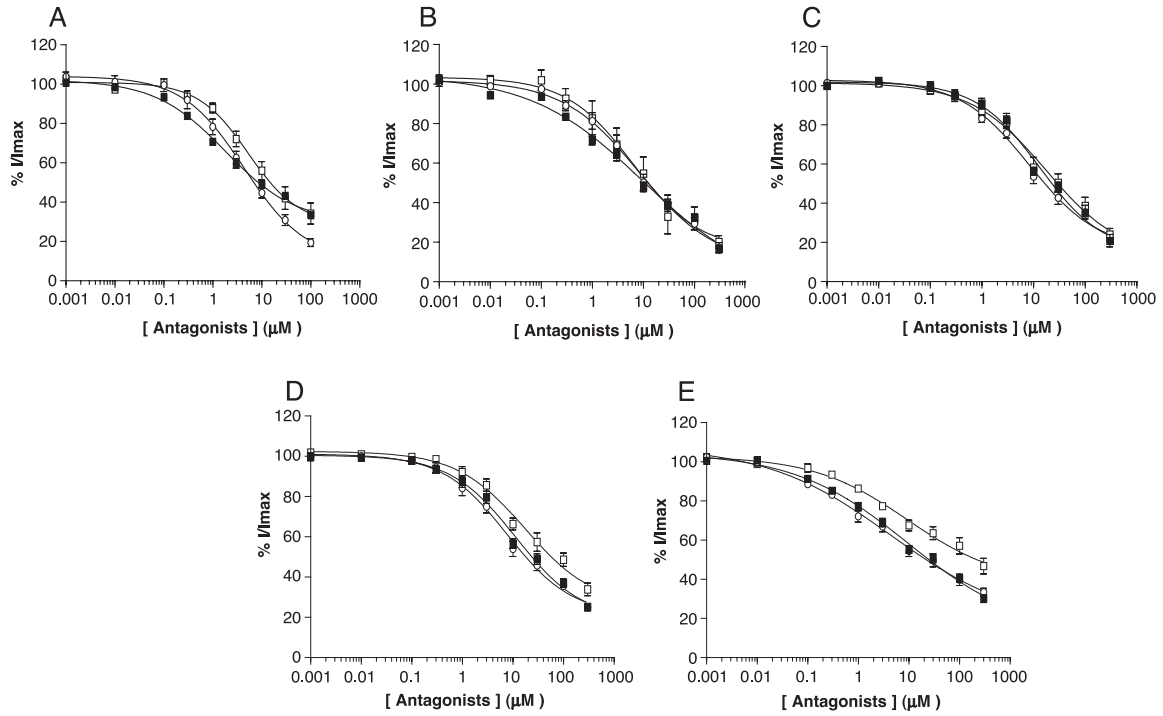

Fig. 3. Inhibition dose–response curves of (A) 10 μM, (B) 40 μM, (C) 100 μM, (D) 300 μM, and (E) 1 mM GABA in the presence of ginkgolide A (■), ginkgolide B (○), and ginkgolide C (□) from recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. ($n = 5–15$ oocytes).

lide B at 40 μM GABA and above appears to be independent on GABA concentrations, indicating that it also largely exerts noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

Similar to ginkgolides A and B, ginkgolide C was more potent at inhibiting the lowest GABA concentration tested (10 μM, $IC_{50} = 5.6 \pm 0.6$ μM). The potency of ginkgolide C at 10 μM was approximately two times higher than its potency determined over 40 μM–1 mM GABA ($IC_{50} = 9.5 \pm 1.8$, 12.8 ± 1.4, 12.0 ± 2.2, and 10.3 ± 2.7 μM, respectively). The IC_{50} value determined at 10 μM ($P = 0.0288$) was significantly different from that at 40 μM, and again, there was no significant difference between values determined for 40, 100, 300 μM, and 1 mM GABA ($P = 0.8161$, 0.6182, and 0.9951, respectively). The results indicate that antagonism by ginkgolide C at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors is largely noncompetitive. Overall, ginkgolides A, B, and C are largely equipotent at inhibiting the direct action of GABA at 40 μM and above.

Fig. 4A, B and C (Dixon plots) shows the reciprocal plots of the percentage response of 10 μM–1mM GABA to 0.1–10 μM ginkgolides A, B, and C, respectively. Linear regression analysis of the data showed that linearity of Dixon plots is significant ($P < 0.0001$) with the goodness of fit (r^2) values of 0.8671 to 0.9969. The inhibitory constant values (K_i) determined from the Dixon plots were 14.5 ± 1.0, 12.7 ± 1.7, and 16.3 ± 2.4 μM for ginkgolides A, B, and C, respectively. The K_i values between ginkgolides A and B, ginkgolides A and C, and ginkgolides B and C were not significantly different ($P = 0.3918$, 0.4990, and 0.2511, respectively).

3.3. Antagonism of ginkgolides A, B and C at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

The mechanism of the action of ginkgolides was determined by comparison of GABA dose–response curves (GABA concentration–effect curves) in the absence and

Table 1
IC_{50} and Hill coefficient values for ginkgolides A, B, and C in the presence of 10, 40, 100, 300 μM, and 1 mM GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

Compounds	10 μM GABA		40 μM GABA		100 μM GABA		300 μM GABA		1 mM GABA	
	IC_{50}[a] (μM)	n_H[b]	IC_{50}[a] (μM)	n_H[b]	IC_{50}[a] (μM)	n_H[b]	IC_{50}[a] (μM)	n_H[b]	IC_{50}[a] (μM)	n_H[b]
Ginkgolide A	2.6 ± 0.6	-0.6 ± 0.1	13.0 ± 3.4	-0.5 ± 0.1	13.7 ± 1.1	-0.7 ± 0.1	11.9 ± 1.7	-0.8 ± 0.1	12.2 ± 2.4	-0.5 ± 0.1
Ginkgolide B	4.8 ± 0.8	-0.7 ± 0.1	9.6 ± 1.5	-1.4 ± 0.3	9.5 ± 1.5	-0.7 ± 0.1	10.1 ± 2.9	-0.7 ± 0.1	11.4 ± 2.9	-0.5 ± 0.1
Ginkgolide C	5.6 ± 0.6	-0.9 ± 0.1	9.5 ± 1.8	-0.7 ± 0.1	12.8 ± 1.4	-0.9 ± 0.1	12.0 ± 2.2	-0.8 ± 0.1	10.3 ± 2.7	-0.5 ± 0.1

[a] IC_{50} is the concentration that inhibits 50% of responses. Data are the mean ± S.E.M. ($n = 5–15$ oocytes).
[b] n_H is the Hill coefficient. Data are the mean ± S.E.M. ($n = 5–15$ oocytes).

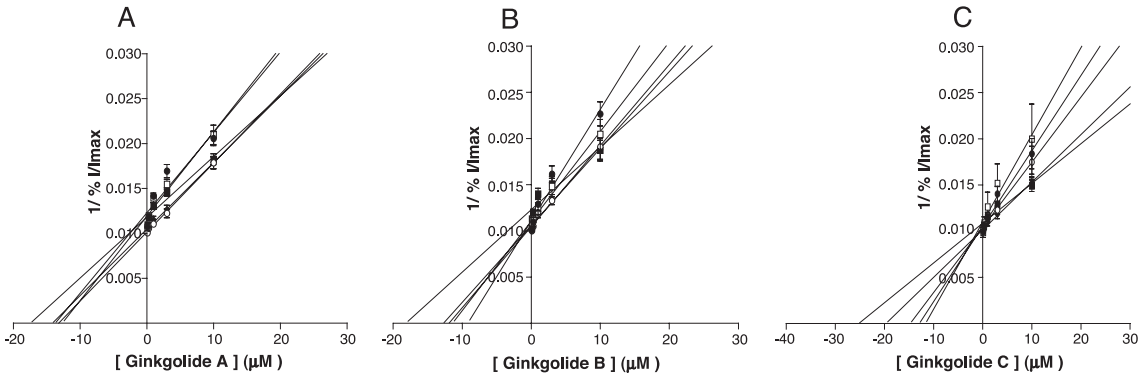

Fig. 4. Dixon plots constructed using various concentrations of (A) ginkgolide A, (B) ginkgolide B, and (C) ginkgolide C at fixed GABA concentrations (10, 40, 100, 300 μM, and 1 mM) from human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. ($n = 5–15$ oocytes).

the presence of the antagonist (representing the effect of a fixed concentration of each ginkgolide [30, 50, and 100 μM] on a range of GABA concentrations).

The GABA dose–response curves of GABA in the presence of 30, 50, and 100 μM ginkgolides A, B, and C (Fig. 5A–C) all showed right shifts without reaching the maximal response of GABA. GABA responses in the presence of 30, 50, and 100 μM ginkgolide A were 59.9% ($P < 0.0001$), 77.1% ($P < 0.0001$), and 52.6% ($P < 0.0001$); those of ginkgolide B were 71.1% ($P < 0.0001$), 53.2% ($P < 0.0001$), and 60.4% ($P < 0.0001$); and those of ginkgolide C were 77.3% ($P < 0.0001$), 70.9% ($P < 0.0001$), and 69.0% ($P < 0.0001$) of GABA maximal response, respectively (Table 2). At 30, 50, and 100 μM, ginkgolide A increased GABA EC_{50} values 1.5 times (39.0–58.1 μM), 2.6 times (41.8–77.1 μM), and 4.1 times (59.5–242.9 μM), respectively; ginkgolide B increased GABA EC_{50} values 3.8 times (49.2–188.2 μM), 5.2 times (38.6–201.4 μM), and 4.4 times (49.5–218.2 μM), respectively; and ginkgolide C increased GABA EC_{50} values 1.3 times (35.5–45.9 μM), 1.5 times (41.8–62.1 μM), and 3.2 times (50.2 –162.1 μM), respectively (Table 2). Ginkgolides A, B, and C reduced GABA maximal responses and caused nonparallel right shifts in the GABA concentration–effect curves, indicating that the ginkgolides exhibit noncompetitive antagonism at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

4. Discussion

This study shows that ginkgolides A, B, and C inhibit GABA-mediated currents from recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The result supports various indications that the ginkgolides possess activities at $GABA_A$ receptors such as shortened barbital-induced sleeping time (Brochet et al., 1999; Wada et al., 1993), potentiated muscimol-stimulated Cl^- ion uptake and blockade by flumazenil (Miller et al., 1991), and the displacement of [^{35}S]TBPS from its binding site (Chatterjee et al., 2002, 2003).

Ginkgolide B was more potent at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors ($IC_{50} = 9.6$ μM at 40 μM GABA; Table 1) than at native $GABA_A$ receptors on rat cortical slices ($IC_{50} = 73$ μM at 30 μM GABA; Ivic et al., 2003). Ivic et al. (2003) also reported that the actions of ginkgolide B and bilobalide on $GABA_A$ receptors on rat cortical slices are not use-dependent. Interestingly, bilobalide was also more potent at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors ($IC_{50} = 4.6$ μM at 40 μM GABA; Huang et al., 2003) than at $GABA_A$ receptors on rat cortical slices ($IC_{50} = 46$ μM at 30 μM GABA; Ivic et al., 2003). These findings suggest that the activities of bilobalide and ginkgolides at $GABA_A$ receptors are dependent on subunit composition.

Ginkgolides A, B, and C inhibited GABA-mediated currents at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors with comparable

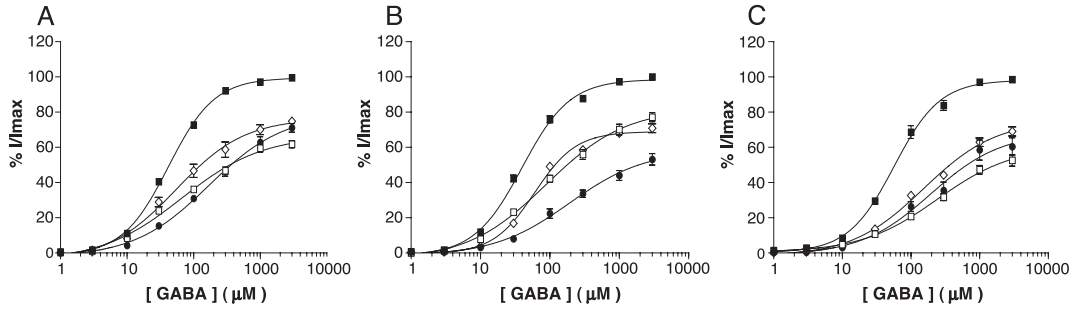

Fig. 5. Agonist dose–response curves of GABA alone (■) and GABA in the presence of (A) 30 μM, (B) 50 μM, and (C) 100 μM ginkgolide A (□), ginkgolide B (●), and ginkgolide C (◇) from recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are mean ± S.E.M. ($n = 5–15$ oocytes).

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

Antagonists (Ant)	GABA EC_{50}[a] (μM)	GABA + Ant EC_{50}[a] (μM)	GABA n_H[b]	GABA + Ant n_H[b]	Maximal GABA response (%)
30 μM Ginkgolide A	39.0 ± 2.6	58.1 ± 8.6	1.3 ± 0.1	0.8 ± 0.1	59.9 ± 1.2
50 μM Ginkgolide A	41.8 ± 3.4	107.4 ± 18.4	1.3 ± 0.1	0.8 ± 0.1	77.1 ± 2.6
100 μM Ginkgolide A	59.5 ± 3.7	242.9 ± 34.0	1.4 ± 0.2	0.8 ± 0.1	52.6 ± 3.3
30 μM Ginkgolide B	49.2 ± 3.3	188.2 ± 19.1	1.4 ± 0.1	0.8 ± 0.1	71.1 ± 2.4
50 μM Ginkgolide B	38.6 ± 1.3	201.4 ± 23.7	1.2 ± 0.1	0.8 ± 0.1	53.2 ± 3.2
100 μM Ginkgolide B	49.5 ± 1.3	218.2 ± 31.1	1.6 ± 0.1	0.8 ± 0.1	60.4 ± 5.1
30 μM Ginkgolide C	35.5 ± 1.1	45.9 ± 1.4	1.3 ± 0.1	1.1 ± 0.1	77.3 ± 1.8
50 μM Ginkgolide C	41.8 ± 0.7	62.1 ± 3.0	2.5 ± 0.1	1.4 ± 0.1	70.9 ± 2.5
100 μM Ginkgolide C	50.2 ± 2.6	162.1 ± 14.5	1.2 ± 0.1	0.8 ± 0.1	69.0 ± 2.6

[a] EC_{50} is the concentration that evokes 50% of responses. Data are the mean ± S.E.M. ($n = 5–15$ oocytes).
[b] n_H is the Hill coefficient. Data are the mean ± S.E.M. ($n = 5–15$ oocytes).

potencies ($K_i = 14.5 \pm 1.0$, 12.7 ± 1.7, and 16.3 ± 2.4 μM, respectively) and are similar to bilobalide ($K_i = 14.8 \pm 0.6$ μM, Huang et al., 2003). Antagonism of bilobalide at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was noncompetitive with a possibility of a small degree of competitive antagonism at lower concentrations of GABA. The potency of the classic noncompetitive antagonist of $GABA_A$ receptors, picrotoxinin, determined under the same condition was independent of GABA concentrations.

At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, ginkgolides A, B, and C exhibited features characteristic of noncompetitive antagonists including nonparallel right shift of GABA concentration–effect curves and decreased maximal response of GABA. Ginkgolides A, B, and C displayed the highest potency at 10 μM GABA and became approximately two to five times less potent at inhibiting the direct action of GABA at 40 μM and above. There is no significant difference in the potency of the individual ginkgolide determined over 40 μM–1mM GABA. In comparison, bilobalide displayed the highest potency at 10 and 40 μM GABA and became approximately two times less potent at inhibiting the direct action of GABA at 100 μM and above (Huang et al., 2003).

Bilobalide and ginkgolides A, B, and C have been shown to inhibit [^{35}S]TBPS binding ($IC_{50} = 4.9$, >50, 14.9, and >50 μM, respectively; Chaterjee et al., 2002, 2003), suggesting that they bind to the picrotoxinin binding site of $GABA_A$ receptors. In view of their structural similarity to picrotoxinin (Fig. 1), this finding is not entirely unexpected. Similarities of the structural features include the hydrophilic cavity and hydrophobic side chain which may be important for their antagonism at $GABA_A$ receptors.

Molecular modeling study (Ivic et al., 2003) has shown that ginkgolide B and picrotoxinin form a cavity of similar size. The absolute and relative positions of the two lactone groups that made up part of the cavity structure are also very similar. The projected position of the more bulky *t*-butyl group of ginkgolide B is also similar to the isoproprenyl group of picrotoxinin. Ivic et al. (2003) suggested that the *t*-butyl group of ginkgolide B fits in the lipophilic pocket of glycine receptors as does the isoproprenyl group of picrotoxinin in $GABA_A$ receptors.

Ivic et al. (2003) also showed that the 1-OH of ginkgolide B overlaid very well with the 6-OH of picrotoxinin. Acetylation of 6-OH leads to the reduced activity of picrotoxinin at $GABA_A$ receptors (Anthony et al., 1993). Zhorov and Bregestovski (2000) suggested from the results of docking studies that the 6-OH of picrotoxinin forms hydrogen bonds with threonine residues in the M2 segment lining the pore of $GABA_A$ receptors. Taken together, these observations suggest that 1-OH may also be important for the activity of the ginkgolides at $GABA_A$ receptors. However, at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, the potencies of ginkgolide A ($K_i = 14.5 \pm 1.0$ μM) and ginkgolide B ($K_i = 12.7 \pm 1.7$ μM) are not significantly different ($P = 0.3918$). The potencies of ginkgolide B and ginkgolide C ($K_i = 16.3 \pm 2.4$) are also not significantly different ($P = 0.2511$). The results suggest that the hydroxyl substitution at positions 1 and 7 bears little effect if any on the activity of the ginkgolides at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

In summary, our study demonstrates that diterpene trilactones of *G. biloba* ginkgolides A, B, and C are noncompetitive antagonists of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors with similar antagonism profiles to, but less potent than the previously reported, *G. biloba* sequiterpene trilactone bilobalide.

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