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A single amino acid determines the toxicity of *Ginkgo biloba* extracts

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ABSTRACT **Ginkgo biloba extracts are currently used for a wide range of health-related conditions. Some of the medical benefits of these extracts are controversial, but their lack of toxicity in humans is not in doubt. These extracts are, however, highly toxic to insects. Their active components (ginkgolides and bilobalide) have structures similar to the convulsant picrotoxin, a $GABA_A$ receptor antagonist, so their lack of toxicity in mammals is puzzling. Here, we show that the different compositions of insect and vertebrate GABA receptor pores are responsible for the differing toxicities. Insect GABA receptors contain Ala at their 2′ position in the pore. Substitution with Val, which is the equivalent residue in vertebrate $GABA_A$ receptor α-subunits, decreases ginkgolide potency by up to 10,000-fold. The reverse mutation in vertebrate $GABA_A$ α1 subunits increased the sensitivity of α1β2 and α1β2γ2 receptors to ginkgolides. Mutant cycle analysis demonstrates a strong interaction between the ginkgolides and the 2′ residue, a result supported by *in silico* docking of compounds into a model of the pore. We conclude that the insecticidal activity of *G. biloba* extracts can be attributed to their effects at insect GABA receptors, and the presence of a Val at the 2′ position in vertebrate $GABA_A$ receptors explains why these compounds are not similarly toxic to humans.—Thompson, A. J., McGonigle, I., Duke, R., Johnston, G. A. R., Lummis, S. C. R. A single amino acid determines the toxicity of *Ginkgo biloba* extracts. *FASEB J.* 26, 1884–1891 (2012). www.fasebj.org**

Key Words: Cys-loop receptor · picrotoxin · antagonist

GINKGO BILOBA HAS BEEN USED as a traditional medicine for >2500 yr, and its leaf extract (EGb761) is currently used for a range of health-related conditions (1–3). EGb761 contains a range of compounds, the best studied of which are bilobalide (BB) and ginkgolides A and B (GA and GB); hereafter collectively referred to as the ginkgolides. The structures of these compounds (**Fig. 1*A***) are similar to that of picrotoxin (PTX), a classic γ-aminobutyric acid A ($GABA_A$) receptor antagonist that is composed of 2 closely related compounds, picrotoxinin and picrotin. $GABA_A$ receptors are members of the Cys-loop family of ligand-gated ion channels, which also includes nicotinic acetylcholine (nACh), 5-hydroxytryptamine 3 ($5\text{-}HT_3$), and glycine receptors (4–6). These important neuronal receptors are implicated in a range of neurological disorders, and they are also the site of action of some of the most widely used drugs, such as benzodiazepines and general anesthetics. All share a common structure, and binding of agonist to their extracellular domains opens an integral transmembrane channel that enables ions to cross the cell membrane. The channels of these receptors are lined by α-helices, and to simplify comparisons between receptors of this family, the amino acid residues of these α-helices are given an index number, with 0′ representing a conserved charged residue on the cytoplasmic side of the membrane, and a conserved Leu at 9′ forming the gate (Fig. 1*B*, Supplemental Fig. S1).

G. biloba also has a long history of use as an insecticide. Dry ginkgo leaves were traditionally used as bookmarks to protect against booklice and silverfish, ginkgo leaf extracts were used to control pests in paddy fields, and ginkgo wood was used for insect-proof cabinets in Japan hundreds of years ago (3). More recent work, seeking to understand these traditional uses, has shown that extracts have insecticidal activity on a range of insect species (7). Inhibition of GABA receptors provides a possible explanation for this property, as invertebrate GABA receptors are the target of a number of insecticides, including cyclodienes (such as dieldrin), lindane, and the leading pesticide, fipronil (8–10). These insecticides, however, are toxic to humans (fipronil, for example, has a U.S. Environmental Protection Agency ranking of moderately toxic; ref. 11), while *G. biloba* extracts are not, and instead may have neuroprotective, anxiolytic, and other beneficial properties (1–3).

Abbreviations: BB, bilobalide; GA, ginkgolide A; GABA, γ-aminobutyric acid; GB, ginkgolide B; nACh, nicotinic acetylcholine; PTX, picrotoxin; RDL, resistant to dieldrin.

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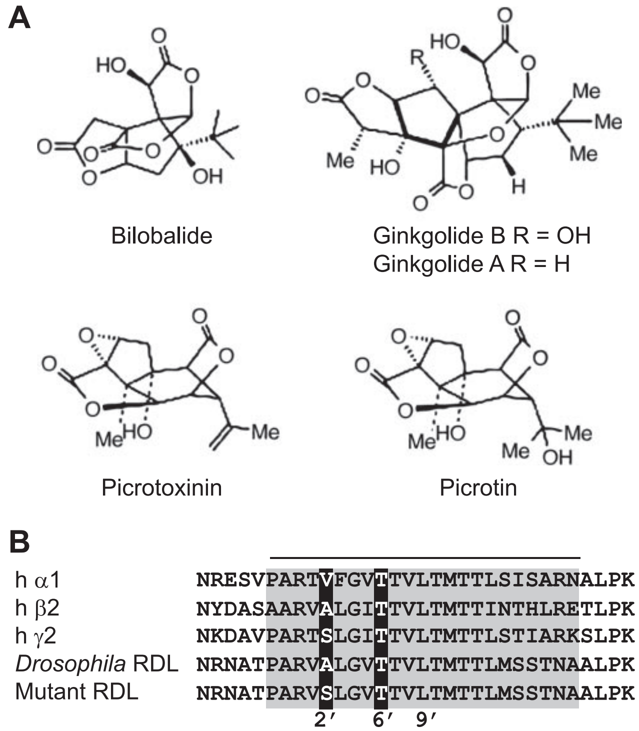

Figure 1. Compounds (*A*) and amino acid alignment of the M2 region (shaded) of the subunits (*B*) used in this study. Residues important for binding channel blockers are highlighted. Subunit accession numbers: human GABA α1, P14867; human GABA β2, P47870; human GABA γ2, P18507; *Drosophila* RDL, P25123.

To study the effects of gingkolides on insect GABA receptors, we used GABA-activated RDL (resistant to dieldrin) receptors. The RDL subunit (cloned from a dieldrin-resistant mutant; hence its name) is the best-studied insect GABA receptor subunit and is widely distributed throughout the *Drosophila* CNS (8, 9, 12, 13). By comparing the effects of the gingkolides at RDL with those at human $GABA_A$ receptors, we describe how the contrasting toxicities of these compounds in insects and humans can be explained by a single GABA receptor pore residue.

MATERIALS AND METHODS

Mutagenesis and preparation of mRNA and oocytes

$GABA_A$ (accession numbers α1 P14867, β2 P47870, and γ2 P18507) and RDL (P25123) receptor subunit cDNAs were subcloned into pGEMHE for oocyte expression, as described previously (14, 15). Site-directed mutagenesis was performed with the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). cRNA was *in vitro* transcribed from linearized plasmid cDNA template using the mMessage mMachine T7 Transcription kit (Ambion, Austin, TX, USA). Stage V and VI oocytes from *Xenopus laevis* (NASCO, Fort Atkinson, WI, USA) were injected with 50 nl of 100 ng/μl cRNA (5 ng injected) and incubated at 16°C; currents were recorded at 1–3 d postinjection. Vertebrate $GABA_A$ receptor subunits were expressed in the ratio 1:1 (α1:β2) or 1:1:10 (α1:β2:γ2).

Characterization of receptors

Two-electrode voltage clamping of *Xenopus* oocytes was performed as described previously (15). Concentration–response data were measured at a holding potential of −60 mV, and responses for each oocyte were normalized to the maximum current for that oocyte. The mean response was iteratively fitted to the equation $I = I_{min} + (I_{max} - I_{min})/(1 + 10^{(\log(EC50-[A])n_H)})$, where I_{max} is maximum response, I_{min} is minimum response, [A] is concentration of agonist, and n_H is the Hill coefficient.

Homology modeling and docking

RDL was aligned with GLIC using FUGUE (16), and the RDL receptor pore homology model was generated using Modeller 9.8 (17), based on the crystal structure of GLIC [Protein Data Bank (PDB) ID: 3EAM]. The best model was selected after Ramachandran plot analysis of all the generated models. Docking of compounds into the homology model was performed using GOLD 3.0 (Cambridge Crystallographic Data Centre, Cambridge, UK). The binding site was defined as a 15-Å-radius docking sphere surrounding the C_α of the 6′ residues in chains A and C. Ten genetic algorithm runs were performed on each docking exercise using default parameters. The structures were visualized and hydrogen bonds were identified using PyMOL 1.3 (DeLano Scientific LLC, South San Francisco, CA, USA).

Fly rearing and coated vial bioassays

Lab strains of resistant (Rdl^{MDRR}) *Drosophila* were kindly provided by Brandeis University (Boston, MA, USA) and susceptible (*CantonS*) *Drosophila* by the University of Cambridge. Fly cultures were kept at 25°C with a 12-h light-dark cycle on corn meal, yeast, sucrose, and agar food. Flies had similar genetic backgrounds and were originally sourced from the Bloomington Stock Center (Bloomington, IN, USA); they are described in FlyBase (http://flybase.org/). Coated vial bioassays were developed from method 011 v3 as recommended by the Insecticide Resistance Action Committee (http://www.irac-online.org). BB, GA, GB, PTX, and dieldrin were diluted in ethanol at 25 mM and serially diluted with ethanol to working concentrations. These solutions (100 μl) were added to a 22-cm^2 surface of a glass vial and rolled in a fume cupboard until the ethanol had evaporated. Flies were knocked down with CO_2, and 10–15 individuals were added to each vial with a vented lid. Moist agar food (1 ml) was added to each vial. Flies were checked for recovery before being placed at 20°C under a 16:8-h light-dark regime. The endpoint scoring (dead/alive) was at 24, 48, and 72 h, and data were iteratively fitted to a 4-parameter logistic equation, as described above.

Statistical analysis

Significance was calculated using a Student's *t* test in Prism 4.03 (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com); values of $P < 0.05$ were considered significant.

RESULTS

Application of GABA to RDL receptors expressed in *Xenopus* oocytes elicits concentration-dependent inward currents, with an EC_{50} of 19 μM and Hill slope of 1.8 (**Table 1**), similar to previously published results (13).

TABLE 1. *Properties of wild-type and mutant RDL, α1β2, and α1β2γ2 receptors*

Receptor	pEC_{50} (M)	EC_{50} (μM)	n_H	n
RDL	4.72 ± 0.03	19.0	1.80 ± 0.20	5
$RDL_{A2'V}$	4.70 ± 0.02	19.9	2.20 ± 0.02	5
α1β2	5.16 ± 0.04	6.92	1.43 ± 0.18	4
$α1_{V2'A}β2$	6.85 ± 0.03*	0.14	1.14 ± 0.12	3
α1β2γ2	4.13 ± 0.03	74.1	1.64 ± 0.16	3
$α1_{V2'A}β2γ2$	5.51 ± 0.04*	3.09	1.10 ± 0.11	3

*$P < 0.05$ *vs.* wild type; Student's *t* test.

These currents were inhibited by ginkgolides; inhibition of RDL receptors was slow to develop, and a subsequent application of agonist alone resulted in slowed receptor activation that was not dependent on the length of washout time, consistent with an inhibitor slowly leaving the open channel (**Fig. 2**). Ginkgolides had no effect when applied alone (data not shown).

Potent inhibition of GABA-evoked RDL currents was seen for GA (IC_{50}=3.1 nM) and GB (IC_{50}=18 nM), while BB (IC_{50}=320 nM) had a similar potency to PTX (IC_{50}=220 nM) (**Table 2** and **Fig. 3*A***). Association rates (k_{on}) determined at IC_{50} concentrations showed that those for GA and GB were higher than BB and PTX, while dissociation rates (k_{off}) were similar for all the compounds (Supplemental Fig S1). These yielded K_d values (k_{off}/k_{on}) that were similar to IC_{50} values.

PTX is known to bind to the channel of $GABA_A$ receptors, and there is evidence from other receptors in the family that ginkgolide binding sites may be similarly located (18–21). Therefore, we focused on GABA receptor pore-lining residues as potential binding sites. The 2′ and 6′ residues are the most likely candidates, as these residues form the binding site for PTX in $GABA_A$ receptors (18, 22, 23), and the amino acids at these locations in insect GABA receptors confer sensitivity or resistance to insecticides that target GABA-activated channels, such as dieldrin (8, 9). An alignment of the transmembrane regions of insect and human GABA receptors shows that the 6′ residue is identical in RDL and $GABA_A$ receptor

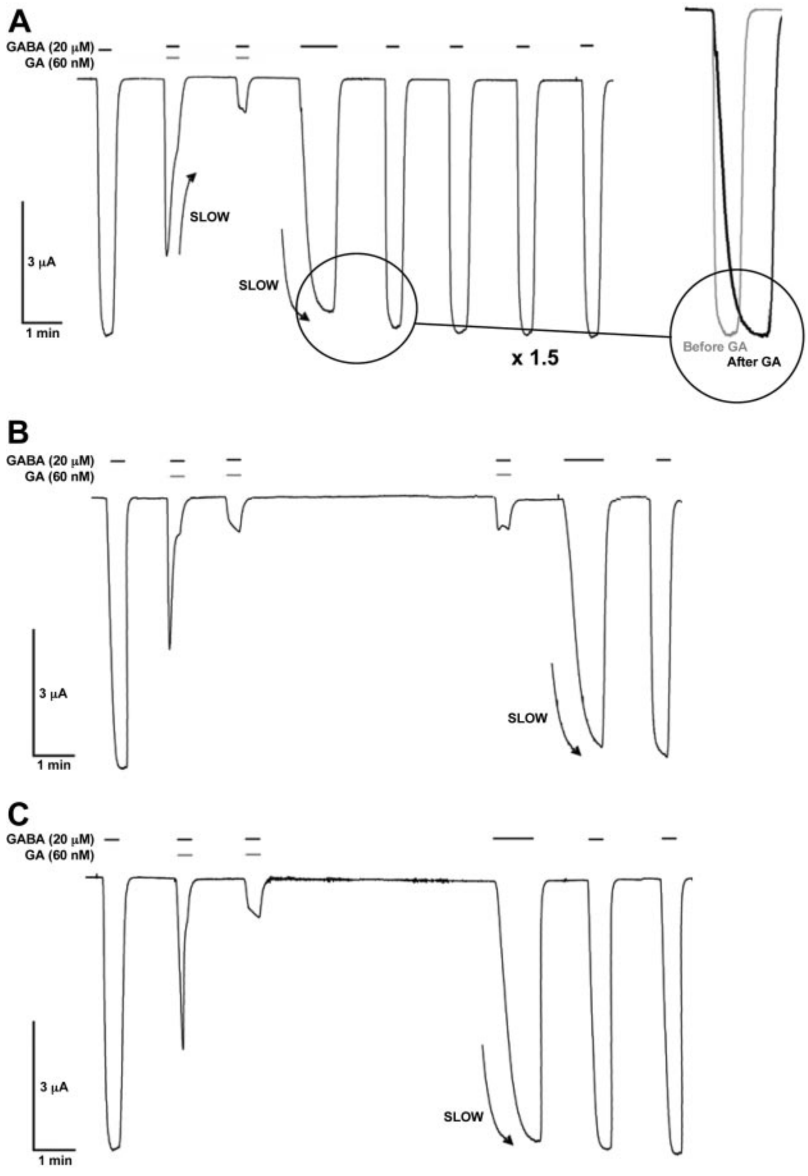

Figure 2. Typical experiments showing that the inhibition of GABA responses in RDL receptors is slow to develop and wash out. *A*) Following an application of GABA alone, coapplication of GA with GABA shows that the response reaches 60% of maximal before it is slowly inhibited. A stable level of inhibition is only reached with a subsequent coapplication of GA and GABA. *B*) The level of inhibition is unchanged even after a long washout. *C*) Following inhibition, activation is slow but is independent of the wash time between agonist applications. This suggests that GA becomes trapped within the channel, and only slowly leaves the open channel on agonist application. Preapplication did not change ginkgolide potency, consistent with access also requiring an open channel. In this work, all concentration-inhibition curves and the derived parameters were calculated from measurements taken during a second, stable, level of inhibition. Ginkgolides were obtained as previously described (19).

TABLE 2. *Effects of BB, GA, GB, and PTX on wild-type and mutant receptors*

Receptor	pIC_{50} (M)	IC_{50} (μM)	n_H	n
BB				
RDL	6.50 ± 0.09	0.32	0.89 ± 0.09	4
$RDL_{A2'V}$	3.65 ± 0.03*	224	1.85 ± 0.21	4
α1β2	5.03 ± 0.07	9.33	0.85 ± 0.10	5
$\alpha 1_{V2'A}\beta 2$	5.69 ± 0.04*	2.04	1.06 ± 0.10	3
α1β2γ2	4.64 ± 0.09	22.9	0.68 ± 0.10	5
$\alpha 1_{V2'A}\beta 2\gamma 2$	5.45 ± 0.07*	3.55	1.16 ± 0.20	4
GA				
RDL	8.49 ± 0.07	0.003	1.13 ± 0.18	6
$RDL_{A2'V}$	3.44 ± 0.04*	360	1.48 ± 0.19	6
α1β2	4.80 ± 0.07	15.8	0.65 ± 0.08	5
$\alpha 1_{V2'A}\beta 2$	4.69 ± 0.09	20.4	0.87 ± 0.14	5
α1β2γ2	4.41 ± 0.11	38.9	0.49 ± 0.07	3
$\alpha 1_{V2'A}\beta 2\gamma 2$	5.33 ± 0.08*	4.68	0.86 ± 0.10	6
GB				
RDL	7.74 ± 0.03	0.018	1.43 ± 0.14	5
$RDL_{A2'V}$	3.51 ± 0.07*	310	1.02 ± 0.20	3
α1β2	4.82 ± 0.04	15.1	0.94 ± 0.06	4
$\alpha 1_{V2'A}\beta 2$	5.33 ± 0.04*	4.68	1.13 ± 0.09	4
α1β2γ2	3.94 ± 0.05	115	0.61 ± 0.06	4
$\alpha 1_{V2'A}\beta 2\gamma 2$	4.83 ± 0.05*	14.8	1.43 ± 0.29	4
PTX				
RDL	6.66 ± 0.12	0.22	0.73 ± 0.13	6
$RDL_{A2'V}$	3.75 ± 0.05*	178	1.32 ± 0.23	4
α1β2	5.65 ± 0.05	2.23	1.00 ± 0.09	5
$\alpha 1_{V2'A}\beta 2$	4.61 ± 0.02*	24.5	1.34 ± 0.06	5
α1β2γ2	5.35 ± 0.06	4.47	0.80 ± 0.11	4
$\alpha 1_{V2'A}\beta 2\gamma 2$	5.45 ± 0.06	3.55	0.95 ± 0.09	3

*$P < 0.05$ *vs.* wild type; Student's *t* test.

subunits, while the 2′ residue differs between subunits: RDL subunits have an Ala, while human $GABA_A$ receptor α subunits have Val, and β, γ, δ, ε Ala, Ser, or Thr (Fig. 1*B* and Supplemental Fig. S1). To probe the role of the 2′ residue in ginkgolide sensitivity, we substituted Val for Ala at the 2′ location in RDL subunits. This had a dramatic effect on ginkgolide potency (up to a 10,000-fold increase in IC_{50}), while GABA sensitivity was unaltered (**Fig. 4** and Tables 1 and 2). We also examined the reverse substitution: replacement of Val with Ala in the human α1 subunit. This caused decreases in almost all ginkgolide IC_{50} values in both $\alpha 1_{V2'A}\beta 2$ and $\alpha 1_{V2'A}\beta 2\gamma 2$ mutant receptors (up to 8-fold), and the vertebrate mutant receptors also had increased GABA sensitivity (**Fig. 5** and Tables 1 and 2). PTX behaved differently to the ginkgolides in the human receptor (its IC_{50} was increased and not decreased in $\alpha 1_{V2'A}\beta 2$ mutant receptors and unchanged in $\alpha 1_{V2'A}\beta 2\gamma 2$ receptors; Table 2).

The subtle difference between GA and GB (which only differ by a single hydroxyl group at position 1; Fig. 1*A*) allowed us to use mutant cycle analysis to probe for direct interactions with the 2′ residue (24). This analysis identifies whether there is a specific interaction between a ligand atom and protein residue. A coupling parameter (Ω), calculated from IC_{50} values, will be 1 if the residue has no structural or energetic effect on the ligand binding group. However, if the residue and the group interact, the simultaneous change at both sites has an effect that is either greater or less than the product of their individual effects, producing a Ω value that differs from 1. Here the Ω values were significantly >1 (P<0.05) for both human and insect receptors, demonstrating an interaction between the ginkgolides and the 2′ residue (**Fig. 6**).

To determine whether the 2′ residue could be responsible for ginkgolide sensitivity *in vivo*, we used a naturally occurring A2′S mutant strain of dieldrin-resistant *Drosophila*. Dieldrin is a widely used insecticide whose mechanism of action is *via* insect GABA receptors. Our data demonstrate that it is highly effective in killing wild-type (susceptible) flies but has little effect in flies with an A2′S mutation (**Fig. 7**). To explore whether ginkgolide action *in vivo* was also mediated *via* GABA receptors, we tested gingkolide toxicity in wild-type and mutant flies. In coated vial bioassays, no mortality could be attributed to the compounds in flies containing the A2′S mutation (P>0.05 at all concentrations), even at maximal concentration (5000 ppm, limited by solubility). In contrast, the wild-type strain showed significant mortality at 24 h, with values of LD_{50} ~ 100-fold lower than this maximal concentration at 48 h (Fig. 7 and **Table 3**).

The order of potency of the ginkgolides in the *in vivo* assay differed from that of the *in vitro* work in that GA was the least and not the most potent. We consider this is likely to be due to small variations between the mutant and wild-type fly populations, perhaps involving heterogeneous receptors or other proteins, such as those involved in metabolic or penetration mechanisms. In addition, as RDL subunits have been found to coexpress with GluCl subunits (25), a range of heteromeric receptors with different ginkgolide sensitivities may exist in the different flies. Overall, however, the *in vivo* data strongly support the *in vitro* studies in that *in vivo* mutations at the 2′ position, and *in vitro* mutations at the 2′ position, dramatically decrease organism sensitivity and receptor sensitivity, respectively, to ginkgolide effects.

To further examine how the ginkgolides might bind in the pore, we generated a homology model of the RDL receptor channel. As our data suggested that ginkgolides act in the channel in the open state, we used a bacterial

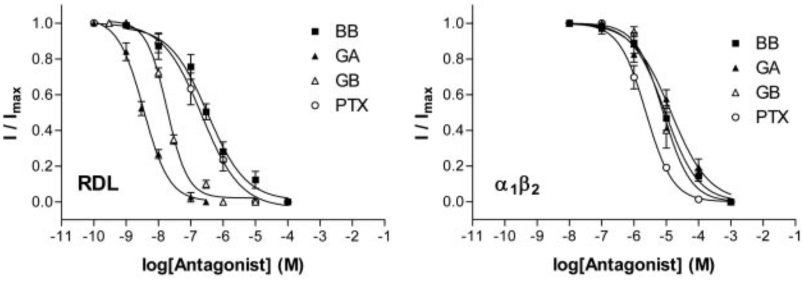

Figure 3. Concentration-inhibition curves show that ginkgolides are more potent at insect RDL receptors than at human α1β2 $GABA_A$ receptors. Inhibition was measured at the respective GABA EC_{50} values for each receptor. Values are expressed as means ± SE, with sample size and other parameters shown in Table 2.

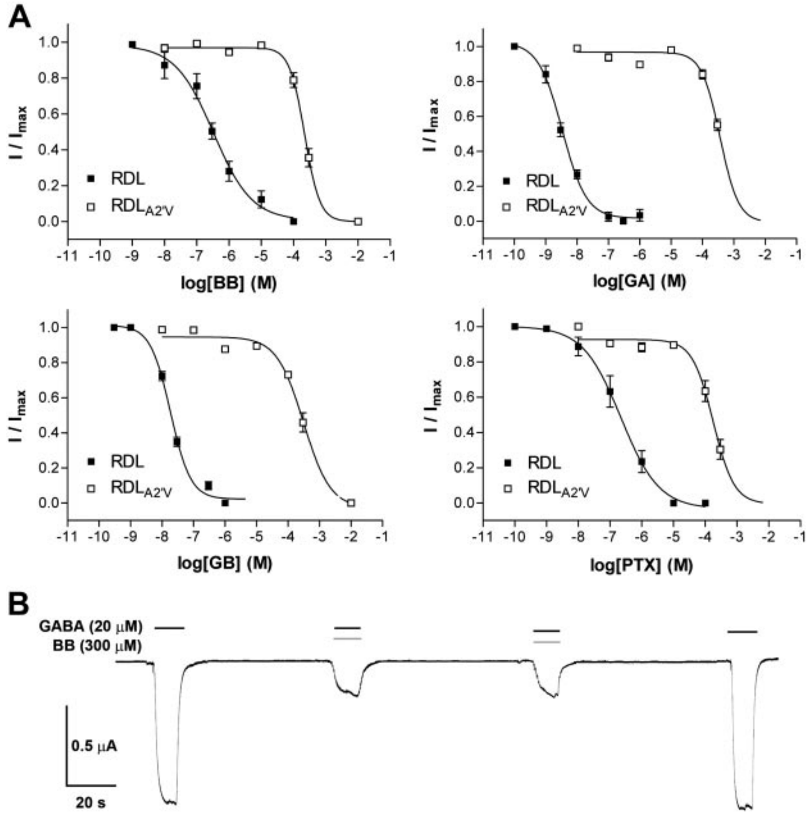

Figure 4. Ginkgolide potency at $RDL_{A2'V}$ mutant receptors. *A*) Concentration-inhibition curves for $RDL_{A2'V}$ mutant receptors show that ginkgolide potency is significantly decreased compared to wild-type receptors. Values are expressed as means ± SE, with sample size and other parameters shown in Table 2. *B*) Typical responses from an $RDL_{A2'V}$ mutant receptor show that onset and recovery from inhibition are faster in these receptors.

channel in the same conformation (Supplemental Fig S2). Docking of the ginkgolides into the channel revealed a binding location between the 2′ and 6′ residues (**Fig. 8**), supporting our functional data. The compounds were coordinated by hydrogen bonds with several of the hydroxyl side chains of 6′Thr residues and had hydrophobic interactions with the 2′Ala. To further probe the accuracy of our model, we removed the hydrogen bonding ability at the 6′ position by creating a T6′V mutant RDL receptor, and tested the consequences of this mutation both functionally and *in silico*. The substitution had a major effect on ginkgolide potency (>1000-fold increase in IC_{50}; **Table 4** and **Fig. 9**) at expressed receptors, and *in silico* docking revealed that the ginkgolides docked at a new location toward the extracellular end of the pore (**Fig. 10**). The docked poses generated from docking into this mutant also had a significantly increased RMSD variation compared to wild type, suggesting that the ginkgolides were located in a less energetically favorable position.

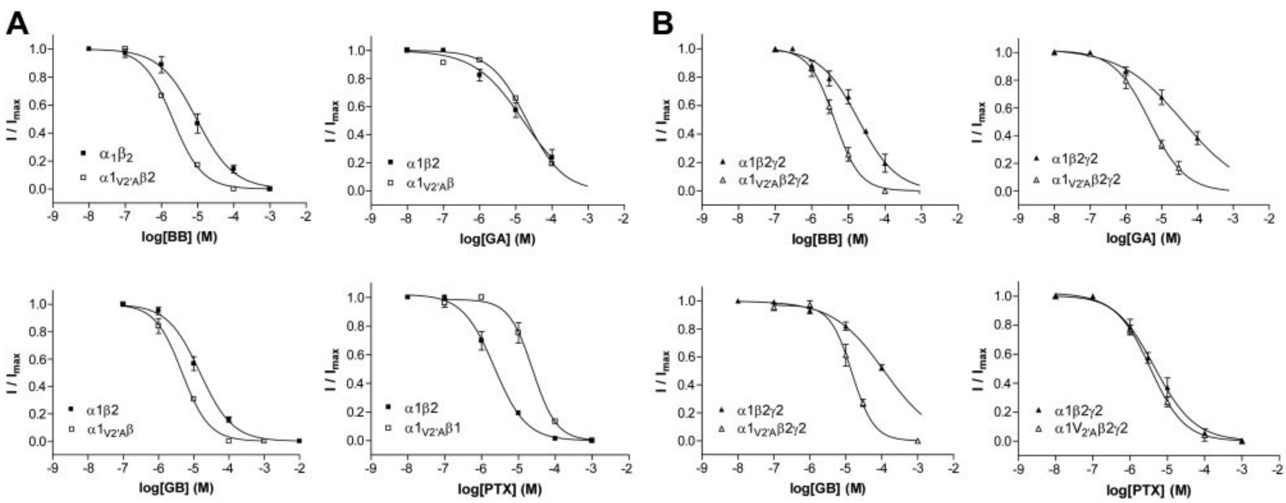

Figure 5. Ginkgolide potency at wild-type and mutant α1β2 (*A*) and α1β2γ2 (*B*) $GABA_A$ receptors. Concentration-inhibition curves show that ginkgolide potency is mostly increased compared to wild-type receptors, although PTX potency is either decreased ($\alpha 1_{V2'A}\beta 2$ receptors; *A*) or unchanged ($\alpha 1_{V2'A}\beta 2\gamma 2$ receptors; *B*). Values are expressed as means ± SE, with sample size and other parameters shown in Table 2.

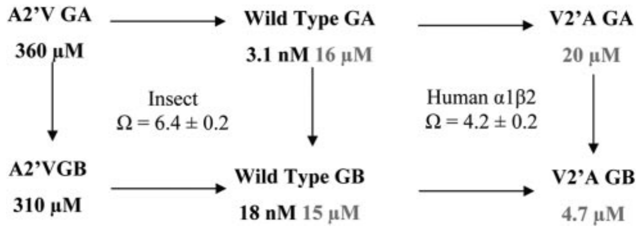

Figure 6. Mutant cycle analysis of interactions between ginkgolides and the 2′ residue. IC_{50} values from RDL receptors are at left; those from human $GABA_A$ receptors are at right. A coupling parameter (Ω) was calculated from IC_{50} values (Table 2) using the standard equation $\Omega = IC_{50\ \mathrm{wtL1}} \times IC_{50\ \mathrm{mutL2}}/IC_{50\ \mathrm{wtL2}} \times IC_{50\ \mathrm{mutL1}}$, where wt = wild type, mut = mutant, and L1 and L2 are the ligands GA and GB, respectively.

TABLE 3. *LD_{50} in* Canton-S *adult* D. melanogaster

	LD_{50} (mg/ml)		
Compound	24 h	48 h	72 h
BB	0.331	0.009	0.006
GA	0.345	0.063	0.019
GB	0.171	0.031	0.010

DISCUSSION

Ginkgolides have structures similar to the well-studied $GABA_A$ receptor antagonist picrotoxin and have a similar mechanism of action at both this and a range of other Cys-loop receptors. Picrotoxin, however, is a potent and toxic convulsant in humans, whereas ginkgolides are widely used as herbal medicines (1–5). Nevertheless, ginkgolides are toxic in insects, where they are highly effective insecticidal agents (7). Here, we reveal a possible explanation for this discrepancy: ginkgolides potently inhibit insect but not human GABA receptors because there is no 2′Val in insect receptor pores.

Many $GABA_A$ receptor channel blockers act at the 2′ and/or 6′ channel lining residues. An amino acid alignment of insect and human $GABA_A$ receptor subunits shows that the 6′ residue of all these subunits is a conserved Thr, while the 2′ residue varies between subunits; this residue is, however, conserved as Val in all $GABA_A$ α subunits (Fig. 1 and Supplemental Fig. S1). Our results demonstrate the importance of this residue, as substitution of Val at the 2′ position into the RDL receptor pore caused dramatic (up to 10,000-fold) decreases of ginkgolide potency. $GABA_A$ receptors with the reverse mutation (V2′A) in the α1 subunit had predominantly the opposite effect, although the changes in potencies were less pronounced (up to 8-fold) and were accompanied by an increase in GABA sensitivity (EC_{50}: α1β2, 6.9 μM; $\alpha_{V2'A}1\beta2$, 0.14 μM; α1β2γ2, 74 μM; $\alpha1_{V2'A}\beta2\gamma2$, 3.1 μM; Table 1). All RDL receptors identified to date have an Ala at the 2′ position, unless they are dieldrin resistant (Fig. 1 and Supplemental Fig. S1), emphasizing the importance of this position.

Our data also show the importance of the 6′ residues: ginkgolide inhibition was eliminated in the RDL T6′V mutant, showing the hydrogen bonding ability of this residue is critical, as indicated in our models of ginkgolide binding in the RDL receptor pore (Fig. 8). Introduction of a 6′ residue that cannot hydrogen bond results in ginkgolides binding at quite different locations, toward the extracellular end of the pore (Fig. 10). Taken together, these data show both 2′ and 6′ residues are critical in defining ginkgolide potency.

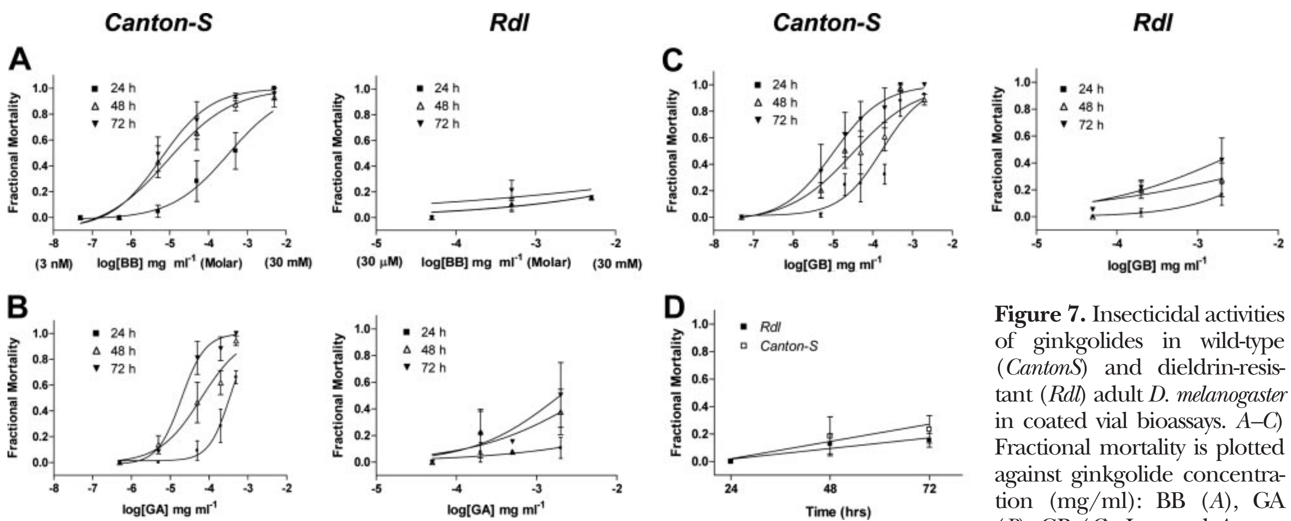

Figure 7. Insecticidal activities of ginkgolides in wild-type (*CantonS*) and dieldrin-resistant (*Rdl*) adult *D. melanogaster* in coated vial bioassays. *A–C*) Fractional mortality is plotted against ginkgolide concentration (mg/ml): BB (*A*), GA (*B*), GB (*C*). In panel *A*, molar equivalents are shown in parentheses. *D*) Mortality in the absence of compound. The resistant strain carries a target-site mutation (A2′S) in its *Rdl* gene that confers resistance (no significant mortality up to 72 h) to the cyclodiene insecticide dieldrin; in wild-type flies, LD_{50} was 2.0, 2.0 and 1.7 μg/ml at 24, 48, and 72 h, respectively. The resistant strain survived well at the highest concentrations of ginkgolides tested, showing that a mutation at the 2′ residue confers cross-resistance. For the wild-type strain, data are fitted with a 4-parameter logistic equation, yielding the LD_{50} values shown in Table 3. Comparable data could not be derived for *Rdl*, owing to its resistance to the compounds. Values are means ± SE for the sample size shown. Similar experiments with PTX revealed no toxicity in either batch of flies, probably due to access problems, as in previous studies (28).

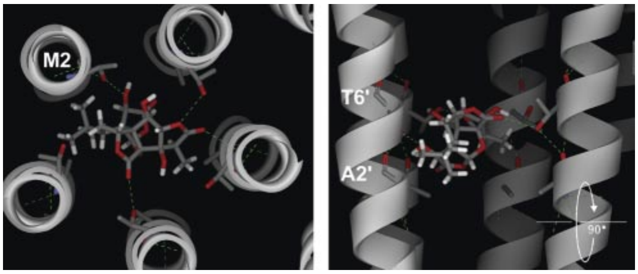

Figure 8. Docking of ginkgolides to wild-type RDL receptors. All ginkgolides and PTX docked at a similar location; here, GB is shown as a typical example. From above, it can be seen to fit snugly in the pore between the 5 M2 helices; from the side, it is located between the 2′ and 6′ residues, where it forms hydrogen bonds with 6′Thr residues.

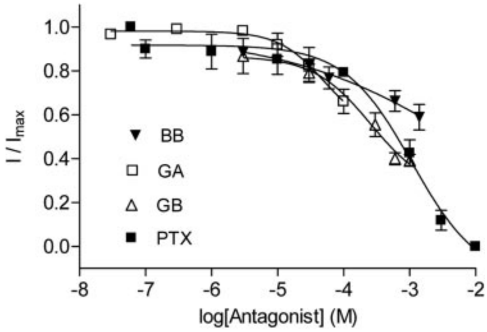

Figure 9. Ginkgolide concentration-inhibition curves at $RDL_{T6'V}$ mutant receptors. Gingkolides inhibited GABA-activated responses only at high concentrations, with values of $IC_{50} > 100$ μM. Values are means ± SE, with sample size and other parameters shown in Table 4.

The 2′ position acts as a secondary binding site for PTX in $GABA_A$ receptors, with the 6′ residue being the main interacting residue (22). Our results show that the 6′ residue is also a major determinant of PTX binding in insect GABA receptors. Inhibition curves in T6′V mutant RDL receptors reveal an IC_{50} for PTX of 1.1 mM, which is 5000-fold higher than in wild-type receptors (0.22 μM). This change in PTX IC_{50} was more pronounced than for the human $GABA_A$ receptor, where studies have shown substitution causes only a 48-fold shift (23). In insect GABA receptors, however, the 2′ position plays a more important role in PTX inhibition (an ~1000-fold increase in IC_{50} in $RDL_{V2'A}$ receptors compares to small or no change in $\alpha_{V2'A}1\beta2$ or $\alpha1_{V2'A}\beta2\gamma2$ receptors), suggesting that the structures of the vertebrate and insect pore regions may be subtly different.

An important question is whether we can explain the toxicity of ginkgolides in insects, and the lack of toxicity in humans. In insects, GA and GB are highly effective at inhibiting GABA receptors at nanomolar concentrations, providing an explanation for their insecticidal activity. In humans, plasma concentrations of ginkgolides are between 4 and 70 nM, depending on the treatment, with values as high as 0.5 μM being reported (26, 27). At these concentrations, ginkgolides would not significantly inhibit human $GABA_A$ receptors, and they would therefore not cause GABA-related toxic effects. There is the potential for such effects if there is a naturally occurring $GABA_A$ α1 A2′V mutation, but we consider this unlikely, as the resulting increase in GABA sensitivity (20–50-fold) would have dramatic effects on GABA-activated responses *in vivo* that would probably render the fetus nonviable.

In summary, we have found the major active ingredients of *G. biloba* extract (BB, GA, and GB) to be potent antagonists of insect RDL receptors, but only weak inhibitors of human $GABA_A$ receptors. Both 2′ and 6′ residues

TABLE 4. *Properties of $RDL_{T6'V}$*

Compound	pEC_{50}/pIC_{50}	EC_{50}/IC_{50} (μM)	n_H	n
GABA	5.13 ± 0.06	7.41	1.44 ± 0.29	9
BB	NI	–	–	4
GA	NI	–	–	3
GB	3.59 ± 0.36*	257	1.00 ± 0.70	4
PTX	2.96 ± 0.19*	1096	0.85 ± 0.18	5

NI, not inhibited (50% inhibition was not reached at 1 mM). *$P < 0.05$ *vs.* wild type; Student's *t* test.

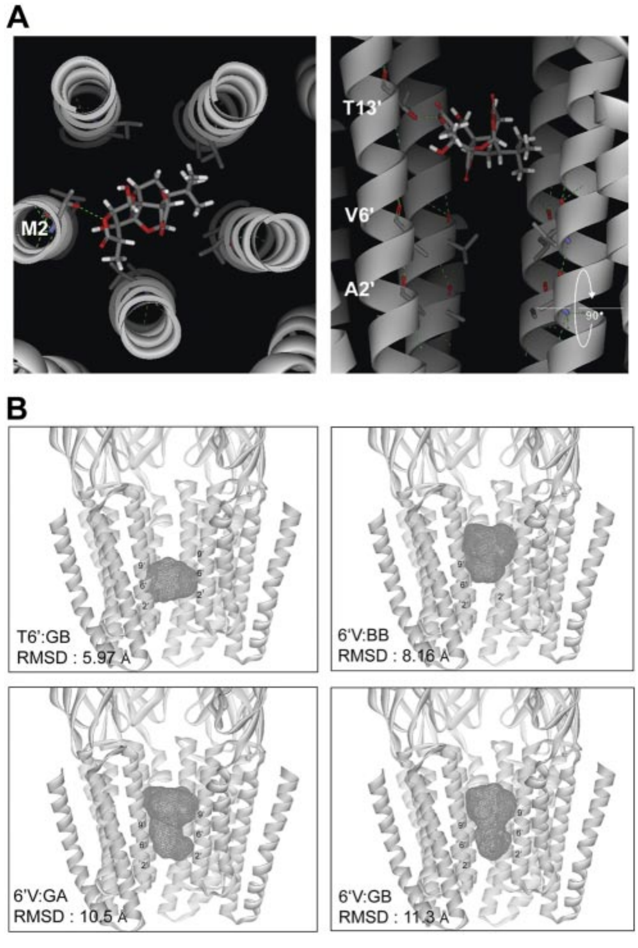

Figure 10. Docking at $RDL_{T6'V}$ mutant receptors. *A*) Docked pose for GB using the same template as in Fig. 8. *B*) Locations of 10 docked poses are superimposed to show the distributions of ginkgolides in the channel. These data show that all ligands docked ~10 Å higher in the channel compared with wild-type receptors. In addition, the poses of GB in the wild-type (T6′) receptor are closely clustered, while in $RDL_{T6'V}$ (6′V) receptors, they are more widely distributed. RMSD for clusters is shown at bottom left in each panel.

affect their potency at RDL receptors, but only the 2′ residue differs between insects and humans. The introduction of the human α1 2′Val residue into RDL subunits hugely decreases the potency of the compounds, and *Drosophila* carrying a 2′ mutation are resistant to ginkgolides, while the introduction of the insect 2′Ala residue into the human α1 subunit results in increased susceptibility. These data can explain why the ginkgolides display potent insecticidal activity but do not have similar GABA-related toxicities in humans. As such, these natural and nontoxic compounds could provide a suitable pest-management approach for the control of range of species, such as bedbugs, where the likelihood of human contact is high. Given the wide range of insect GABA receptor subunits and the variety of ginkgolides, there is also the exciting possibility of discovering ginkgolides that have actions that target specific insect species. This is potentially a route to solve serious agricultural problems, such as bee colony collapse disorder; ginkgolides could provide an insecticide that is harmless to bees, but highly toxic to the pests that may contribute to this disorder. FJ

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