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Allosteric modulation of an expressed homo-oligomeric GABA-gated chloride channel of *Drosophila melanogaster*

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1 Functional GABA-gated chloride channels are formed when cRNA encoding the *Drosophila melanogaster* GABA receptor subunit RDL is injected into the cytoplasm of *Xenopus* oocytes. Two-electrode voltage-clamp was used to investigate allosteric modulation of GABA-induced currents recorded from the expressed, bicuculline-insensitive, RDL homo-oligomers.

2 Flunitrazepam (0.1 μM to 100 μM) had no effect on the amplitude of responses to 10 μM GABA (approximately EC_{10}), whereas 4'chlorodiazepam (100 μM) enhanced the amplitude of submaximal responses to GABA. 3-Hydroxymethyl-β-carboline (1 μM) and ethyl-β-carboline-3-carboxylate (both 1 and 100 μM) had no effect on currents induced by 30 μM (approximately EC_{50}) GABA. However 100 μM 3-hydroxymethyl-β-carboline reduced potentiation by 4'chlorodiazepam.

3 The sodium salts of pentobarbitone (10 μM to 1 mM) and phenobarbitone (50 μM to 1 mM) dose-dependently enhanced submaximal GABA responses. Neither barbiturate activated currents in the absence of GABA.

4 At 10 μM, the steroids 5α-pregnan-3α-ol-20-one and alphaxalone (5α-pregnan-3α-ol-11,20-dione), potentiated submaximal GABA responses. The stereoselectivity of steroid action seen on vertebrate $GABA_A$ receptors was observed on RDL homo-oligomers as 5α-pregnan-3β-ol-20-one (10 μM) was without effect. None of the three steroids tested activated currents in the absence of GABA.

5 The novel anticonvulsant, loreclezole (100 μM), potentiated the response to 10 μM GABA, but not that of saturating concentrations of GABA. δ-Hexachlorocyclohexane (0.1 μM to 30 μM) was a potent enhancer of submaximal responses to GABA of RDL.

6 The potencies of barbiturates and steroids on RDL homo-oligomers resemble those observed for several *in situ* insect GABA receptors, whereas those of benzodiazepine binding-site ligands are considerably reduced. The differences in the benzodiazepine pharmacology of RDL homo-oligomers and native GABA receptors, may reflect roles of other subunits in native insect receptors.

Keywords: Insect GABA receptor; Rdl; barbiturates, steroids; benzodiazepines; hexachlorocyclohexane; loreclezole

Introduction

Ionotropic γ-aminobutyric acid (GABA) receptors, which are widespread in the nervous system of vertebrates and invertebrates, are believed to be oligomeric proteins, composed of five subunits arranged pseudosymetrically around a central anion-selective, ion-channel. The binding of GABA elicits bursts of channel opening. Depending upon the subunit structure of the receptors, the frequency and duration of channel opening is, in some cases, subject to allosteric modulation.

Vertebrate ionotropic GABA receptors can be divided into two classes on the basis of their sensitivity to allosteric modulation. The activity of $GABA_A$ receptors, which are antagonized by bicuculline, can be either enhanced or suppressed by a range of allosteric modulators which bind to distinct sites on the receptor (Haefely, 1992; Sieghart, 1992). Thus, certain benzodiazepines (eg. flunitrazepam), sedative barbiturates (eg. pentobarbitone), pregnane steroids (eg. 5α-pregnan-3α-ol-20-one), and anaesthetics enhance GABA-induced currents, whereas, inverse agonists of the integral benzodiazepine binding site(s) (eg. β-carbolines) and zinc ions reduce the agonist response of certain $GABA_A$ receptor subtypes. By contrast, ionotropic, bicuculline-insensitive GABA receptors, sometimes referred to as $GABA_C$ receptors, and expressed homo-oligomers of the ρ subunits which these receptors are presumed to contain, appear insensitive to all tested allosteric modulators of $GABA_A$ receptors (Polenzani *et al.*, 1991; Feigenspan *et al.*, 1993; Qian & Dowling, 1993, 1994; Wang *et al.*, 1994).

Pharmacological subtypes of $GABA_A$ receptors exist, which can be mimicked by recombinant receptors of different subunit composition, and there is considerable evidence that native $GABA_A$ receptors are hetero-oligomers composed of differing combinations of α, β, γ and δ subunits (Wisden & Seeburg, 1992). Although some allosteric modulators (barbiturates, pregnane steroids, and convulsant ligands such as picrotoxinin) show little subunit specificity when tested on various heterologously expressed $GABA_A$ receptors, the activity of benzodiazepines and zinc is strongly dependent upon the subunit type and isoform present (Pritchett *et al.*, 1989; Draguhn *et al.*, 1990; Puia *et al.*, 1990a, b; Sigel *et al.*, 1990).

A class of insect GABA receptors exists that is insensitive to bicuculline, but which is subject to allosteric modulation (Sattelle *et al.*, 1990; Taylor *et al.*, 1993). Radioligand binding data and electrophysiological studies show that the binding sites on insect GABA receptors for benzodiazepines, steroids, barbiturates, and convulsant antagonists differ from those on vertebrate $GABA_A$ receptors (Lees *et al.*, 1987; Sattelle, 1990; Sattelle *et al.*, 1991; Rauh *et al.*, 1993). Such differences could be explored with the aim of locating modulator binding sites, given access to a recombinant insect GABA receptor of known composition.

Recently, a cloned *Drosophila melanogaster* GABA receptor subunit, RDL (ffrench-Constant *et al.*, 1991), has been shown to form robust, functional, presumably homo-oligomeric,

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GABA receptors in transient (ffrench-Constant *et al.*,1993; Lee *et al.*, 1993; Buckingham *et al.*, 1994) and stable (Miller *et al.*, 1994) expression systems. The known agonist and convulsant antagonist pharmacology of these expressed, bicuculline-insensitive, homo-oligomers (Buckingham *et al.*, 1994; Shirai *et al.*, 1995) resembles that of several *in situ* insect GABA receptors (Sattelle, 1990; Anthony *et al.*, 1993). However, the extent to which the RDL homo-oligomer resembles *in situ* insect GABA receptors with respect to the actions of a range of allosteric modulators is unknown. When stably expressed in a *Drosophila* cell line, the GABA response of RDL homo-oligomers was enhanced by the pregnane steroid, 5α-pregnan-3α-ol-20-one (Millar *et al.*, 1994) yet, in the same study, 1–10 μM concentrations of flunitrazepam and 4′-chlorodiazepam did not affect the magnitude or duration of the maximum response to GABA. The latter results distinguish RDL homo-oligomers from native insect GABA receptors, some of which are sensitive to nanomolar concentrations of these benzodiazepines (Lees *et al.*, 1987; Sattelle *et al.*, 1988). In the present study we report the effects of compounds representative of major classes of allosteric modulators of ionotropic GABA receptors of RDL homo-oligomers expressed in *Xenopus* oocytes.

Note on nomenclature

In this paper, RDL refers to the subunit encoded by the cDNA described by ffrench-Constant *et al.* (1991), whereas, *Rdl*, refers to the gene at position 66F on chromosome III of *Drosophila melanogaster* which encodes this subunit. Although this terminology contradicts that used in some early papers, where both gene and subunit were referred to as *Rdl* (e.g. ffrench-Constant *et al.*, 1991; 1993; Buckingham *et al.*, 1994), it follows recently described conventions for nomenclature (Flybase Consortium, 1995). Furthermore, as it appears that the *Rdl* gene may encode, by alternate splicing, four subunits (RDL, DRC 17-1-2, and two unnamed cDNAs; Chen *et al.*, 1994; ffrench-Constant & Rocheleau, 1994) adoption of this convention will help clarify genes and gene products under investigation.

Methods

cRNA synthesis

The cloning of the cDNA encoding the wild-type (dieldrin-sensitive) form of RDL has been described elsewhere (ffrench-Constant *et al.*, 1991). Plasmid pNB 14.1, a gift of Dr R. T. Roush (Cornell), containing the wild-type cDNA was linearized with the restriction endonuclease *Not*I. $m^7G(5')ppp(5')G$ capped cRNA was synthesized with SP6 RNA-polymerase (Promega) using a standard protocol (Sambrook *et al.*, 1989).

Oocyte preparation and cRNA injection

Stage V and VI oocytes were removed from mature *Xenopus laevis* and defolliculated manually after a 40 min incubation with collagenase type IA (2 mg ml^{-1}) in a calcium-free version of oocyte saline (normal saline composition (μM): NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.6). Each oocyte was injected with 40–50 ng of cRNA in ≤ 50 nl and incubated at 17–18°C in normal saline supplemented with penicillin (100 units ml^{-1}), steptomycin (100 μg ml^{-1}), gentamycin (20 μg ml^{-1}) and 2.5 μM sodium pyruvate. Electrophysiology was performed 18–72 h after cRNA injection.

Electrophysiology

Oocytes were secured in a 90 μl Perspex recording chamber and perfused continuously with saline (5 ml min^{-1}). Drugs were applied in the perfusate. Compounds with low aqueous solubility were dissolved initially in dimethyl sulphoxide (4′-chlorodiazepam, loreclezole, pregnane steroids, flunitrazepam, ethyl-β-carboline-3-carboxylate, δ-hexachloro cyclohexane), ethanol (flunitrazepam, loreclezole) or methanol (3-hydroxymethyl-β-carboline, alphaxalone) at concentrations which ensured that final solvent concentration in the perfusate never exceeded 1% v/v. Such solvent concentrations did not affect the current required to clamp the membrane at −60 mV, nor the amplitude of currents induced by 10 μM and 30 μM GABA. Membrane currents were monitored under two-electrode voltage-clamp using 1M KCl-filled electrodes (3–10 MΩ) and an Axoclamp 2A amplifier (Axon Instruments). Signals were displayed on an oscilloscope (Trio) and recorded on a chart recorder (Gould). All experiments were performed at room temperature (approximately 20°C) with the membrane voltage-clamp at −60 mV.

Only oocytes which yielded stable responses to at least 3 successive control applications of GABA (2 min intervals) were used to determine the dose-dependency of putative allosteric modulators. Following recovery from the control applications, each oocyte was incubated for 30–60 s in saline containing the putative modulatory compound prior to co-application of GABA and the modulator. The process was repeated with increasing concentrations of the test compound. Where the effects of the modulatory compounds on the GABA dose-response curve were investigated, the GABA dose-response relationship of each oocyte was determined in the absence and then in the presence of the test compound. Again, the oocyte was incubated for 30–60 s in the presence of the candidate allosteric modulator before GABA was added. Data from these experiments were normalized to the maximum response observed in each cell, with GABA alone. In all experiments the peak amplitude of each GABA response was measured, and not the amplitude of the post-desensitization plateau. Numerical data are presented as the mean ± one standard error of the mean. GraphPad Prism (GraphPad Software) was used to analyse dose-response data. A four parameter logistic equation (1) which describes a sigmoid curve of variable slope was used to fit curves to the averaged, normalised, data.

$$\frac{I}{I_{max}} = \frac{I_{min}}{I_{max}} + \frac{I_{max} - I_{min}}{[1 + 10^{((\log EC_{50} - [GABA])n_H)}]I_{max}} \quad (1)$$

where I is the current induced by a given concentration of GABA ([GABA]), I_{max} is the amplitude of the maximal GABA response, EC_{50} is the concentration of GABA predicted to elicit half the maximal response and n_H is the slope (Hill) coefficient. Statistical analysis was performed with Students t test (2 tailed).

Chemicals

Reagents for cRNA synthesis were purchased from Promega (U.K.) except for $m^7G(5')ppp(5')G$ cap analogue, which was obtained from New England Biolabs (U.K.). GABA, alphaxalone (5α-pregnan-3α-ol-11,20-dione), 4′-chlorodiazepam (Ro5-4864), flunitrazepam, δ-hexachlorocyclohexane, phenobarbitone, pentobarbitone, 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, and collagenase type IA were obtained from Sigma (U.K.). 3-Hydroxymethyl-β-carboline and ethyl-β-carboline-3-carboxylate and were obtained from Research Biochemicals International. Loreclezole ((Z)-1-[2-chloro-2-(2,4-dichlorophenyl) ethenyl]1,2,4-triazole) was a gift of Dr N.C. Lan (Co-Censys Inc.).

Results

Actions of GABA

In response to GABA (3 μM to 3 mM), dose-dependent inward currents were observed in oocytes injected with the RDL cRNA, voltage-clamped at −60 mV. As in previous studies

(ffrench-Constant *et al.*, 1993; Buckingham *et al.*, 1994), the GABA-induced current was found to reverse near the predicted oocyte chloride reversal potential (≈ -20 mV), and desensitization was observed at higher concentrations of GABA (data not shown). The normalized GABA dose-response curve for over twenty oocytes reached a plateau at 1 mM, with an estimated EC_{50} of 28.0 ± 1.1 μM and a Hill coefficient of 1.8 ± 0.2. At concentrations of 10 μM and 30 μM, GABA elicited currents $9.1 \pm 0.8\%$ ($n=21$) and $55 \pm 1.3\%$ ($n=20$) of the maximum peak response respectively.

Benzodiazepines and β-carbolines

Flunitrazepam (0.1 – 100 μM) had no effect on the amplitude of 10 μM GABA-induced currents ($100 \pm 3\%$ control, $n=6$ at 100 μM) (Figure 1a). Similarly, the amplitude of responses to 10 μM GABA were unaltered by 4'-chlorodiazepam (Ro5-4864) at concentrations between 1 μM and 50 μM, but, 100 μM 4'-chlorodiazepam enhanced the response to 10 μM GABA to $180 \pm 13\%$ control ($n=8$, Figure 1b). However, the peak amplitude of the maximum response, elicited by 1 mM GABA, was unaffected by 100 μM 4'-chlorodiazepam ($99 \pm 1\%$ control, $n=4$).

At concentrations between 1 μM and 100 μM, the β-carbolines, 3-hydroxymethyl-β-carboline (3-HMC) and ethyl-β-carboline-3-carboxylate (β-CCE), did not alter the amplitude of currents resulting from the application of 30 μM GABA. Peak currents were $107 \pm 5\%$ ($n=3$) and $98 + 4\%$ ($n=4$) of the control in the presence of 100 μM β-CCE and 100 μM 3-HMC respectively (Figure 1c). 3-HMC did, however, antagonize the effects of 4'-chlorodiazepam, demonstrating that this β-carboline can interact with RDL homo-oligomers. Co-application of 3-HMC and 4'-chlorodiazepam (each at 100 μM) potentiated the response to 10 μM GABA to $129 \pm 5\%$ control, ($n=6$), a significantly smaller enhancement than the approximate doubling seen with 4'-chlorodiazepam alone ($P=0.02$, Student's t test) (Figure 1b).

Barbiturates

When applied alone in the perfusate at 50 μM to 1 mM, neither sodium phenobarbitone or sodium pentobarbitone altered the current required to clamp the oocyte membrane at −60 mV. However, at concentrations greater than 10 μM the sodium salts of these barbiturates potentiated the response of RDL homo-oligomers to 10 μM GABA, in a dose-dependent manner. At 50 μM, sodium pentobarbitone enhanced the amplitude of currents induced by 10 μM GABA to $126 \pm 6\%$ control ($n=7$), and in the presence of 100 μM and 1 mM sodium pentobarbitone the amplitudes of this GABA response were respectively $175 \pm 14\%$ ($n=6$) and $456 \pm 32\%$ control ($n=3$). Phenobarbitone appeared to be less potent. The response to 10 μM GABA and 50 μM phenobarbitone was $112 \pm 8\%$ control ($n=3$) whereas with 1 mM of the barbiturate, the amplitude was $342 \pm 26\%$ control ($n=5$). Phenobarbitone (1 mM) shifted the GABA dose-response curve to the left reducing the EC_{50} for GABA from 26.2 ± 1.1 μM to 14.0 ± 1.0 μM, and increasing the estimated Hill coefficient from 1.8 ± 0.2 to 2.5 ± 0.2 (Figure 2). However, at 1 mM, neither barbitone affected the amplitude of the response to 3 mM GABA ($97 \pm 1\%$ control with sodium phenobarbitone, $n=3$; $98 \pm 2\%$ control with sodium pentobarbitone, $n=3$).

Pregnane steroids

When applied alone, 5α-pregnan-3α-ol-20-one (0.1 μM – 1 μM) had no effect on the current required to voltage clamp the oocyte at −60 mV, but at 5 μM to 10 μM this steroid reversibly enhanced submaximal GABA responses. In the presence of 10 μM 5α-pregnan-3α-ol-20-one the response to 6 μM GABA was $210 \pm 10\%$ control ($n=4$) (Figure 3a) and that to 10 μM GABA to $186 \pm 13\%$ control ($n=4$). 5α-Pregnan-3α-ol-20-one (10 μM) affected a slight shift to the left in the GABA dose-response curve reducing the EC_{50} for GABA to 20.7 ± 1.1μM, while increasing the Hill slope to 2.3 ± 0.4. The amplitude of the maximum GABA response was unchanged by 10 μM 5α-pregnan-3α-ol-20-one ($104 \pm 2\%$ control, $n=4$) (Figure 3a).

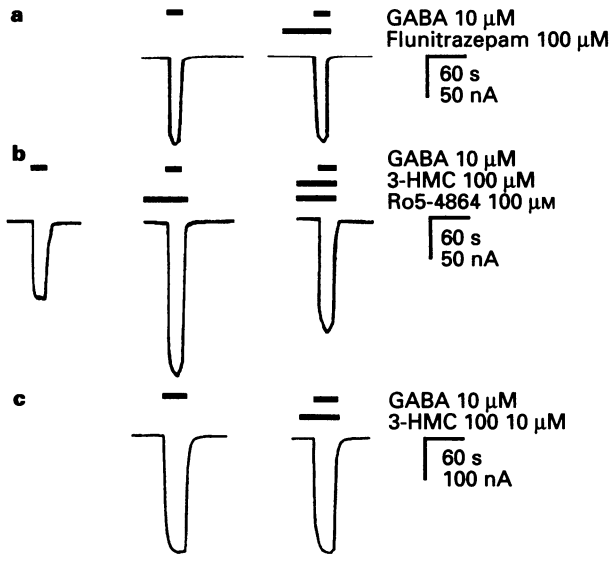

Figure 1 Effects of high concentrations of benzodiazepines on submaximal GABA responses of oocytes expressing RDL subunits, voltage clamped at −60 mV; 15 s applications of 10μM GABA (approximately EC_{10}) or 30μM GABA (approximately EC_{50}) at 2 min intervals induced control currents of a consistent amplitude. The oocytes were incubated for 30 – 60 s with each benzodiazepine before co-application of the benzodiazepine and GABA. (a) Flunitrazepam (100 μM) had no effect on the peak amplitude of the response to 10 μM GABA. (b) By contrast, 4'-chlorodiazepam (100 μM) enhanced the response to 10 μM GABA and this enhancement was reduced by 100 μM of the β-carboline, 3-HMC. The figure shows consecutive responses recorded at 2 min intervals. (c) However, 100 μM 3-HMC did not exhibit inverse agonist activity, as it did not reduce the response to 30 μM GABA.

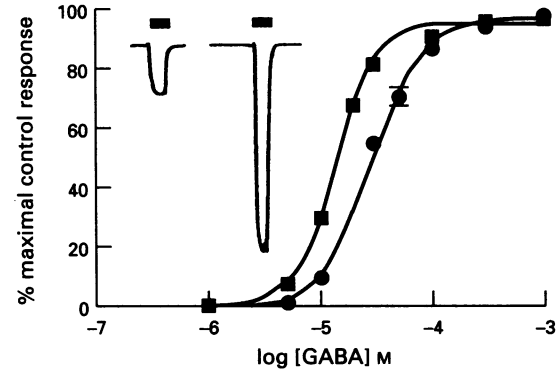

Figure 2 Effects of 1 mM phenobarbitone on RDL-mediated chloride currents voltage clamped at −60 mV. The GABA dose-response curves obtained in the absence (●) and presence (■) of 1 mM phenobarbitone show that phenobarbitone enhances the responses to submaximal concentrations of GABA. Data were normalized to the maximum response seen with GABA alone. Each point represents the mean ± s.e.mean of over 20 experiments for the control, and 5 or 6 experiments for the modulated curve. The inset shows the response to 10 μM GABA applied alone (left) and co-applied with 1 mM phenobarbitone (right), after a 30 s incubation in the barbiturate. Horizontal bars indicate the duration of GABA application.

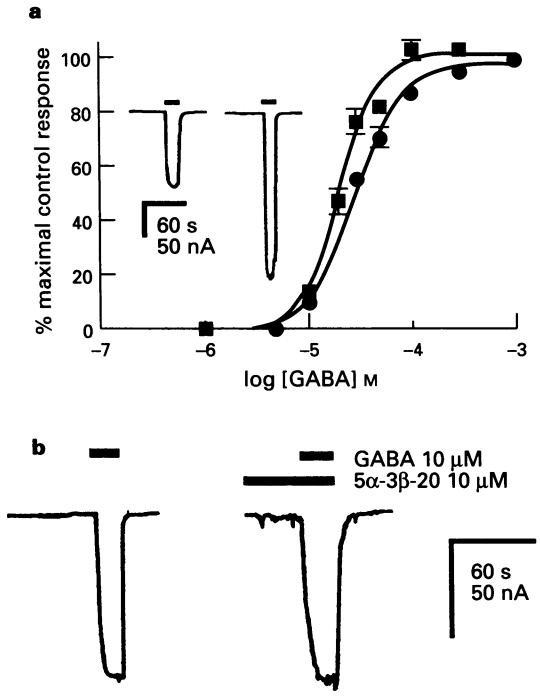

Figure 3 Pregnane steroids effect slight potentiation of the GABA-evoked currents of RDL homo-oligomers. (a) 5α-pregnan-3α-ol-20-one (10 μM) (■) effects a slight shift to the left in the GABA dose-response curve (●, control). Data were normalized to the maximum response seen with GABA alone. Each point on the modulated curve represents the mean of at least 4 experiments and is shown ±s.e.mean, whereas the curve for GABA alone is fitted to the mean data from over 20 oocytes. The inset shows that the response to 6 μM GABA was enhanced by 10 μM 5α-pregnan-3α-ol-20-one (5α-3α-20-one) whereas (b) it was unaffected by 10 μM 5α-pregnan-3β-ol-20-one (5α-3β-20-one).

The actions of pregnane steroids on RDL homo-oligomers are stereo-specific, as 5α-pregnan-3β-ol-20-one (10 μM) did not affect the amplitude of currents induced by 6 μM (101±4, $n=3$) (Figure 3b).

The chemically-related steroid anaesthetic alphaxalone (10 μM, 5α-pregnan-3α-ol-20-dione) also enhanced the response of RDL homo-oligomers to 10 μM GABA (152±16% control, $n=3$). Lower concentrations of alphaxalone were without effect (data not shown).

Zinc

The co-applications of zinc (1–100 μM) had no effect on the amplitude of currents induced by 30 μM GABA (e.g. 97±3% control with 100 μM zinc, $n=4$).

Loreclezole

Loreclezole (100 μM), an anticonvulsant allosteric enhancer of vertebrate $GABA_A$ receptors, enhanced the response to 10 μM GABA (180 ± 10% control, $n=8$) (Figure 4a), but did not increase the amplitude of the maximum GABA-induced current (99±1% control, $n=5$) (Figure 4b). Lower concentrations of loreclezole (0.1–10 μM) were without effect (data not shown).

δ-Hexachlorocyclohexane (δ-HCH)

When applied alone at concentrations between 1 μM and 30 μM, δ-HCH, an enantiomer of lindane, did not alter the current required to clamp the membrane at potentials between −70 and −50 mV. However, the same concentrations of δ-HCH did greatly enhance the response to submaximal concentrations of GABA. Brief incubation (30–60 s) in 30 μM δ-HCH elicited a pronounced shift to the left in the GABA dose-

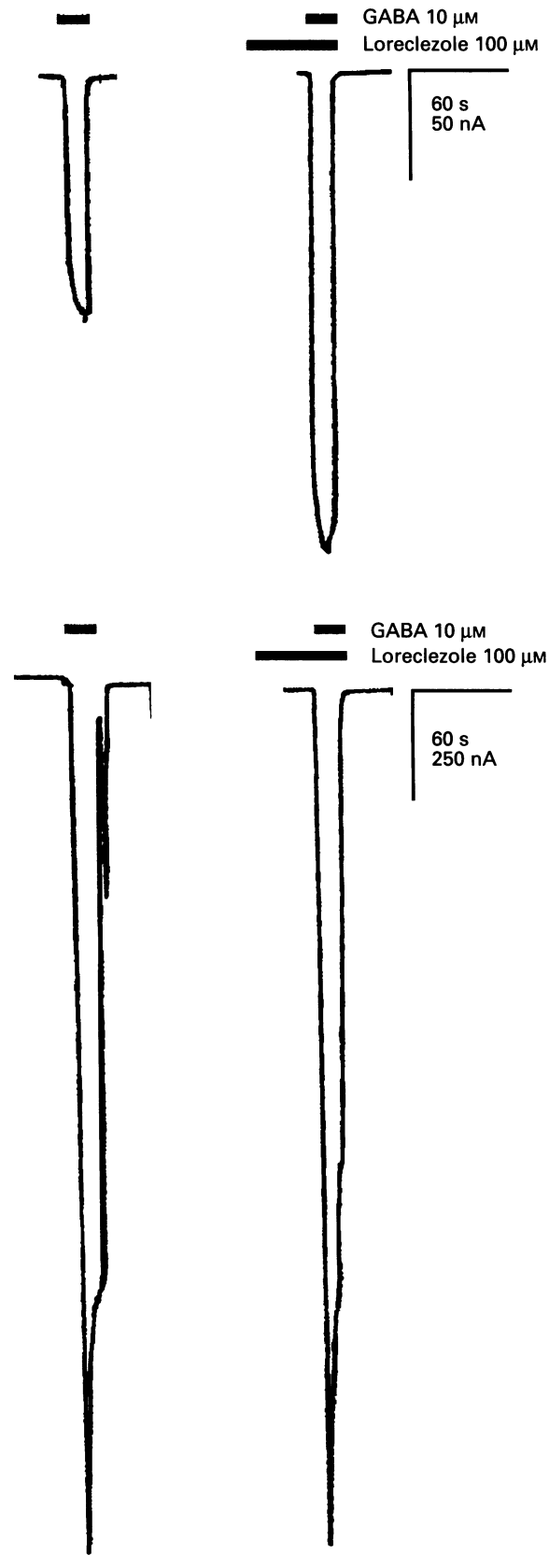

Figure 4 Effects of loreclezole on sub- and supra-maximal doses of GABA. After 30 to 60 s preincubation in 100 μM loreclezole, the response of oocytes expressing RDL to (a) 10 μM GABA was potentiated, whereas the amplitude of the maximum GABA response, induced by 1 mM GABA, was not (b).

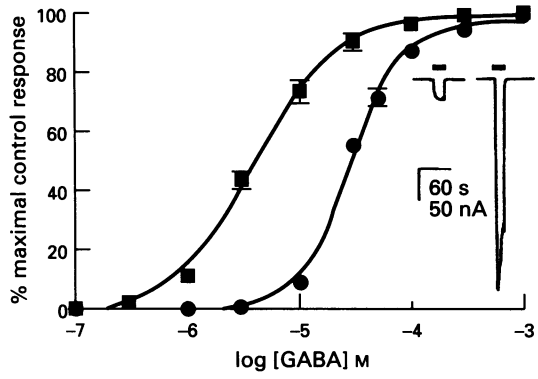

Figure 5 δ-HCH potentiation of GABA-induced currents mediated by RDL homo-oligomers expressed in *Xenopus* oocytes. GABA dose-response curves obtained in the absence (●) and after 30–60 s incubation in 30 μM δ-HCH (■) show that the isomer of lindane potentiates the amplitude of responses of the RDL homo-oligomer to submaximal doses of GABA. The Hill coefficient was decreased from 1.7±0.2 to 1.2±0.1 in the presence of δ-HCH. Data were normalized to the maximum response seen with GABA alone. Each point on the modulated dose-response curve is the mean of between 4 and 6 experiments, whereas points on the control curve represent the mean of data from over 20 oocytes. All points are shown ±s.e.mean. The inset shows the potentiation by 30 μM δ-HCH (right) of the control response to 10 μM GABA (left). Horizontal bars represent the duration of GABA application. The horizontal scale bar represents 60s, and the vertical bar 50 nA.

response curve; the EC_{50} for GABA being reduced from 26.2±1.1 μM to 3.8±1.1 μM (average of data from 6 oocytes, Figure 5). δ-HCH flattened the GABA dose-response curve and reduced the Hill slope to 1.2±0.1. As was the case for all the other positive allosteric modulators tested, δ-HCH did not increase the peak amplitude of the maximum response to GABA of these cells. The effects of δ-HCH were only partly reversed after a 10 min wash with saline.

Discussion

We have demonstrated that RDL homo-oligomers are sensitive to certain allosteric modulators. The activity of vertebrate $GABA_A$ and ionotropic invertebrate GABA receptors is subject to allosteric modulation and, from the data available, it is clear that there are differences in the effects of modulatory compounds on native vertebrate and invertebrate preparations (Sattelle *et al.*, 1991; Rauh *et al.*, 1993). Here, it has been shown that the activity of RDL homo-oligomers is regulated by several of these modulatory compounds, suggesting a role for this subunit in bicuculline-insensitive insect GABA receptors that are subject to allosteric modulation. However, while the potency of some modulators (e.g. barbiturates, steroids) was in accord with that observed on *in situ* GABA receptors, this was not the case for others (e.g. benzodiazepines and β-carbolines). It is possible that the differences in the benzodiazepine pharmacology of RDL homo-oligomers and native GABA receptors, reflects roles for additional subunits in hetero-oligomeric native insect receptors.

Barbiturates, steroids and benzodiazepines

The barbiturates, phenobarbitone and pentobarbitone, potentiated the responses of RDL homo-oligomers to submaximal concentrations of GABA. Although full dose-potentiation studies were not conducted, it nevertheless appears that the order of potency of these two compounds on RDL homo-oligomers and $GABA_A$ receptors (Peters *et al.*, 1988) is the same; namely pentobarbitone is more potent than phenobarbitone. However, these barbiturates were less potent modulators of RDL homo-oligomers than of $GABA_A$ receptors. In a variety of vertebrate preparations the greatest reduction in the EC_{50} for GABA (at least 7 fold) was affected by 100 μM to 300 μM pentobarbitone (Parker *et al.*, 1986; Akaike *et al.*, 1988; Polenzani *et al.*, 1991), with higher concentrations being less effective. By contrast, the greatest potentiation of RDL homo-oligomers by either barbiturate was observed at 1 mM. Studies on another *Drosophila* GABA receptor subunit (DRC 17-1-2, Chen *et al.*, 1994) have shown that this concentration of pentobarbitone effects maximal enhancement of GABA responses, with higher concentrations again being less effective. As the percentage potentiation of the pentobarbitone response to EC_{10}GABA of RDL and DRC 17-1-2 are very similar (see below), it appears that barbiturates have a greater potency on $GABA_A$ receptors than on the *Drosophila* homo-oligomers. High barbiturate concentrations (1–3 mM) are capable of activating $GABA_A$ receptor-mediated currents in the absence of GABA (Parker *et al.*, 1986; Akaike *et al.*, 1988; Peters *et al.*, 1988). However, no such effect was observed on RDL homo-oligomers. Thus, it appears that RDL homo-oligomers are intrinsically less sensitive to barbiturates than are $GABA_A$ receptors and, in this respect, RDL homo-oligomers resemble native insect GABA receptors. Sodium pentobarbitone (50–100 μM) enhanced, by at most 70%, the GABA responses of *Locusta migratoria* and *Schistocerca gregaria* neurones (Lees *et al.*, 1987), and it was even less effective on the GABA response of the cockroach D_f motoneurone (20% enhancement, D.B. Sattelle and D. Cordova, unpublished observations). Similarly, 100 μM pentobarbitone enhanced the response of RDL homo-oligomers to 10 μM GABA to 175±36% control.

The steroid pharmacology of RDL homo-oligomers also differs from that of vertebrate $GABA_A$ receptors. The response of RDL homo-oligomers to approximately EC_{10} GABA was potentiated by concentrations of 5α-pregnan-3α-ol-20-one and alphaxalone greater than 1 μM, whereas the activity of $GABA_A$ receptors is enhanced by nanomolar concentrations of the same compounds (Barker *et al.*, 1986; Majewska *et al.*, 1986; Callachan *et al.*, 1987; Lambert *et al.*, 1987; Peters *et al.*, 1988). This weak effect of steroids on RDL homo-oligomers prevented a detailed dose-response study but, for comparison, 10 μM 5α-pregnan-3α-ol-20-one potentiated the EC_{10} GABA responses of RDL homo-oligomers and recombinant $GABA_A$ receptors consisting of α3, β1 and $\gamma 2_L$ subunits to approximately 180% control (this study) and 520% control respectively (Belelli *et al.*, 1994). Furthermore, alphaxalone, and 5α-pregnan-3α-ol-20-one, at the concentrations used here, induced $GABA_A$ receptor-mediated currents in the absence of GABA (Callachan *et al.*, 1987; Lambert *et al.*, 1987; Peters *et al.*, 1988), however, no such effect was observed on RDL homo-oligomers. Steroids exhibit a marked stereoselectivity for $GABA_A$ receptors with the 3β-hydroxy isomers of pregnane steroids and alphaxalone (i.e. betaxalone) exhibiting a considerably lower efficacy than the 3α-hydroxy isomers (Lambert *et al.*, 1987; Peters *et al.*, 1988). Similarly, 5α-pregnan-3β-ol-20-one had no effect on RDL homo-oligomers at 10 μM. Pregnane steroids, including those tested here, are considerably less potent, displacers of [^{35}S]-TBPS from *Musca domestica* membranes than they are from rat brain membranes (Rauh *et al.*, 1993), although they exhibit the same stereospecificity observed in electrophysiological experiments. Thus, RDL homo-oligomers closely mimic the barbiturate responses of certain *in situ* insect GABA receptors, and the small, stereospecific, enhancement seen with pregnane steroids appears to reflect the low affinity observed for native insect GABA receptors in radio-ligand binding studies.

Insect benzodiazepine binding sites are pharmacologically distinct from those of vertebrate $GABA_A$ receptors. The radiolabelled-benzodiazepine binding profile of insect GABA receptors differs from that of $GABA_A$ receptor in that 4'-chlorodiazepam is a more potent displacer of [^{3}H]-flunitrazepam from insect membrane preparations, than is clonazepam (Lummis & Sattelle, 1986; Sattelle *et al.*, 1991). Although 4'-chlorodiazepam is a non-competitive antagonist

of $GABA_A$ receptors, and appears to act at a site distinct from that of other benzodiazepine agonists or inverse agonists (Simmonds, 1985; Puia *et al.*, 1990b), it potently enhances the agonist response of certain insect GABA receptors (von Keyseringk & Willis, 1992; Aydar *et al.*, 1995). The differences in the benzodiazepine pharmacology of insect and vertebrate GABA receptors extends to the actions of β-carboline inverse-agonists, which non-competitively antagonize GABA responses. Thus, while the GABA response of cockroach D_f neurones are inhibited by some β-carbolines (e.g. 3-HMC) others which antagonize $GABA_A$ receptors, are inactive on the cockroach (e.g. DMCM (methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate)) (Braestrup *et al.*, 1982; Bai, 1994).

The present study has shown that the potency of benzodiazepines on RDL homo-oligomers is reduced relative to that of native insect GABA receptors. At concentrations in the range 10 nM – 10 μM, 4′-chlorodiazepam potentiates the GABA response of locust neurones (von Keyserlingk & Willis, 1992) and *Periplaneta americana* D_f neurones (Y. Higashino & D. B. Sattelle, unpublished data). Peak enhancement of the GABA response of the cockroach neurones was observed with 300 nM 4′-chlorodiazepam with higher concentrations being less active. Potentiation of the GABA response of RDL homo-oligomers was observed with 100 μM 4′-chlorodiazepam, but as concentrations as high as 50 μM were ineffective, this represents an increase of over 1000 fold in the threshold for 4′-chlorodiazepam enhancement relative to the D_f neurone.

Flunitrazepam, which was without effect on RDL homo-oligomers, markedly potentiated the GABA responses of cockroach D_f neurones (Sattelle *et al.*, 1988) and some cultured locust neurones (Lees *et al.*, 1987; Taylor *et al.*, 1993). Flunitrazepam (1 μM) shifted the GABA dose-response curve of cockroach and locust neurones to the left, and in contrast to its effects on $GABA_A$ receptors, enhanced the maximum GABA response. However, it appears that a mixed population of GABA-benzodiazepine receptors may exist in insects, as flunitrazepam-insensitive cockroach (Aydar *et al.*, 1995) and locust (Taylor *et al.*, 1993) GABA receptors have been observed. Some of these GABA receptors were, however, potentiated by 4′-chlorodiazepam (Aydar *et al.*, 1995). RDL homo-oligomers could therefore contribute to flunitrazepam-insensitive, 4′-chlorodiazepam-sensitive GABA receptors *in situ*. Alternatively, the lack of activity of flunitrazepam may reflect its lower affinity (than 4′-chlorodiazepam) in ligand binding studies (Lummis & Sattelle, 1986), and it is possible that, at concentrations higher than 100 μM flunitrazepam will potentiate the activity of RDL homo-oligomers.

The actions β-carbolines also distinguish RDL homo-oligomers from native insect GABA receptors. The GABA response of the cockroach D_f motoneurone was partially blocked by 3-HMC (Bai, 1994) yet, neither 3-HMC nor β-CCE (both at 0.1 μM to 100 μM) affected the response of RDL homo-oligomers to 30 μM GABA. Problems of insolubility prevented the testing of higher concentrations of the β-carbolines so it is not possible to say whether these observations represent a decrease in potency and/or efficacy of 3-HMC on RDL homo-oligomers. Although 100 μM showed no inverse agonist activity on RDL homo-oligomers it did antagonize the effects of 4′-chlorodiazepam. This suggests that the effects of 4′-chlorodiazepam do result from a specific interaction with a benzodiazepine binding site on RDL homo-oligomers, and that this binding site is functionally coupled to one for benzodiazepine inverse agonists, as in $GABA_A$ receptors. However, the reduced potency of 4′-chlorodiazepam, relative to native insect receptors, and the lack of inverse agonist activity exhibited by 3-HMC, means that the binding site(s) on RDL homo-oligomers for these modulators, differs from those of known native insect GABA receptors.

In vertebrates barbiturates and steroids have similar effects on *in situ* and heterologously expressed $GABA_A$ receptors of many tested subunit combinations, although subunit isoforms do affect their potency to some extent (Pritchett *et al.*, 1988; Puia *et al.*, 1990a; Sigel *et al.*, 1990; Lan *et al.*, 1991; Puia *et al.*, 1993). In contrast, the co-expression of specific subunits is a prerequisite for pharmacological actions of benzodiazepines, β-carbolines and zinc typical of subtypes of native $GABA_A$ receptors (Pritchett *et al.*, 1989; Sigel *et al.*, 1990; Draguhn *et al.*, 1990; Saxena & MacDonald, 1994). Interestingly, the classes of modulator whose potency on RDL homo-oligomers is in accord with that seen on native insect receptors are the same as those which do not show a strict subunit specificity on $GABA_A$ receptors. The possibility therefore arises that benzodiazepine-sensitive insect GABA receptors are hetero-oligomeric, like vertebrate $GABA_A$ receptors. Recently, cDNAs encoding two *Drosophila* ligand gated ion-channel subunits, LCCH3 and GRD, were isolated which have amino-acid sequences characteristic of GABA receptor subunits, but unlike RDL, these subunits do not form functional homo-oligomers (Henderson *et al.*, 1993; Harvey *et al.*, 1994). A similar situation occurs with vertebrate glycine receptors, the α subunits of which form functional homo-oligomers, whereas the β subunits do not. However, the co-expression of α and β subunits is required for a picrotoxin pharmacology typical of some native receptors (Pribilla *et al.*, 1992). Given that there are both functional and sequence (Darlison, 1992; Ortells & Lunt, 1995) similarities between RDL and glycine receptor α subunits, it may be that the co-expression of RDL with LCCH3, GRD, or as yet unknown subunits will result in the formation of GABA receptors with the benzodiazepine pharmacology seen in native insect preparations.

Zinc

In vertebrates, zinc antagonizes some $GABA_A$ receptor subtypes, as well as the ρ subunit homo-oligomers and metabotropic $GABA_A$ receptors (Xie & Smart, 1991; Wang *et al.*, 1994). Expressed vertebrate α, β, and α, β, and δ hetero-oligomers are blocked by zinc, whereas the co-expression of α, β, and γ subunits yields receptors which are insensitive to this divalent cation (Draguhn *et al.*, 1990; Saxena & MacDonald, 1994). Zinc (1 – 100 μM) had no effect on 30 μM GABA-induced currents of RDL homo-oligomers, nor on GABA receptors of the cockroach D_f neurone (Bai, 1994). This may reflect differences in the structure of ionotropic vertebrate $GABA_A$ receptors and certain insect GABA receptors. One should note however, that not all invertebrate GABA receptors are insensitive to zinc (Smart & Constanti, 1982).

Actions of δ-hexachlorocyclohexane

In the present study, δ-HCH was found to be a potent positive allosteric modulator of RDL homo-oligomers and effected a marked, dose-dependent, shift to the left in the GABA dose-response curve. The Hill coefficient was reduced to 1.2 ± 0.1 in the presence of δ-HCH; thus, it may be that in the presence of δ-HCH the binding of a single molecule of GABA is sufficient to activate the channel, whereas two or more GABA molecules are required by the unmodulated receptor. The different enantiomers of HCH have differing effects on ionotropic vertebrate GABA receptors, although their site(s) of action is as yet undetermined. δ-HCH enhanced the GABA dose-response curve of expressed $GABA_A$ receptors shifting the dose-response to the left, but depressing the amplitude of the maximal response (Woodward *et al.*, 1992). Interestingly, in the same study, δ-HCH had little effect on the activity of bicuculline-insensitive, ionotropic GABA receptors of vertebrates. On the other hand, γ-HCH (lindane) is a non-competitive antagonist of native cockroach (Wafford *et al.*, 1989) and *D. melanogaster* (Zhang *et al.*, 1994) GABA receptors as well as vertebrate $GABA_A$ receptors (Woodward *et al.*, 1992). It has been suggested that HCH enantiomers may exert their opposing modulatory effects through the picrotoxinin binding site as the actions of δ-HCH on $GABA_A$ receptors was unaffected by the benzodiazepine antagonist Ro-15-1788 (Woodward *et al.*, 1992) and the α, δ, and γ enantiomers of HCH inhibit binding

of [^{35}S]-TBPS to invertebrate membranes (Olsen *et al.*, 1989). Although γ-HCH, like picrotoxinin, is a competitive inhibitor of [^{3}H]-EBOB binding to the house fly head membranes (Deng *et al.*, 1993), it should be noted that steroids, sedative and convulsant barbiturates also inhibit [^{35}S]-TBPS binding to $GABA_A$ receptors (Ticku *et al.*, 1985; Siegart, 1992; Haefely, 1994), but are thought to bind at sites distinct from that for TBPS and picrotoxinin. Whether interaction with the picrotoxinin site also underlies the enhancement observed with δ-HCH remains to be determined.

Actions of loreclezole

The degree of enhancement of RDL homo-oligomers effected by loreclezole is similar to that seen on heterologously expressed $GABA_A$ receptors containing the β_1 subunit (Wafford *et al.*, 1994). Loreclezole (100 μM) enhanced the responses, to EC_{10} GABA, or RDL homo-oligomers and mammalian receptors containing the β_1 subunit by approximately 80%. The efficacy of loreclezole on recombinant $GABA_A$ receptors is greatly increased in receptors expressing human β_2 or β_3 subunits, relative to those containing the β_1 subunit, by the presence of an asparagine residue at position 289, near the presumed extracellular mouth of the ion-channel (Wingrove *et al.*, 1994). Serine and a methionine residues are found at the homologous location on β_1 and RDL subunits respectively. Thus, RDL homo-oligomers form a loreclezole site similar to that seen in recombinant human receptors expressing the β_1 subunit. However, loreclezole has no effect on the amplitude of the maximum GABA response of RDL, although it reduced the peak response of recombinant $GABA_A$ receptors expressing both the β_1 and β_2 subunit (Wingrove *et al.*, 1994; Wafford *et al.*, 1994). Presumably, the differences in the effect of δ-HCH and loreclezole on the maximum response of vertebrate $GABA_A$ and homo-oligomeric RDL receptors reflect differences in the interactions between the binding sites for these compounds and the determinants of ion-channel function.

Comparison of RDL homo-oligomers in two expression systems

When RDL was stably expressed in a *Drosophila* S2 cell line, no potentiation was observed with flunitrazepam or 4′-chlorodiazepam (0.1 – 1 μM), whereas 100 μM 4′-chlorodiazepam, effected a similar enhancement of the response to EC_{10} GABA as observed in *Xenopus* oocytes (Millar *et al.*, 1994; S.D. Buckingham, unpublished data). Pentobarbitone and 5α-pregnan-3α-ol-20-one exhibited a similar potency on RDL homo-oligomers in both systems, and enhanced submaximal GABA responses, while 10 μM 5α-pregnan-3β-ol-20-one had no effect on RDL homo-oligomers in S2 cells (Millar *et al.*, 1994; S.D. Buckingham, unpublished data).

Comparison of allosteric modulation of two putative Rdl *splice variants*

The *Rdl* gene can undergo extensive alternate splicing, yielding four possible subunit isoforms (ffrench-Constant & Rocheleau, 1994) of which two have been cloned; RDL (ffrench-Constant *et al.*, 1991) and DRC 17-1-2 (Chen *et al.*, 1994). The predicted amino acid sequence of these two subunits differs by only 17 amino acids, principally in a region of the N-terminal which influences the agonist binding site of other members of the $GABA_A$ and nicotinic acetylcholine receptor superfamily. Both subunits form GABA-gated, bicuculline-insensitive homo-oligomers when expressed in *Xenopus* oocytes and there appears to be little or no difference in the potentiation by pentobarbitone or 5α-pregnan-3α-ol-20-one, or δ-HCH of response of either homo-oligomer to approximately EC_{10} GABA (this study; Belelli *et al.*, 1994; Chen *et al.*, 1994). Similarly, both receptors were insensitive to flunitrazepam (3 μM) and to 100 μM zinc. Unlike mammalian $GABA_A$ receptors, neither homo-oligomer responded to high concentrations of 5α-pregnan-3α-ol-20-one or pentobarbitone with picrotoxin-sensitive currents in the absence of GABA. Thus, it appears that the differences in the amino acid structure of these two subunits, do not underlie major differences in modulator pharmacology of $GABA_A$ receptors and *Drosophila* homo-oligomers.

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