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Agonist pharmacology of two *Drosophila* GABA receptor splice variants

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1 The *Drosophila melanogaster* γ-aminobutyric acid (GABA) receptor subunits, RDL_{ac} and DRC 17-1-2, form functional homo-oligomeric receptors when heterologously expressed in *Xenopus laevis* oocytes. The subunits differ in only 17 amino acids, principally in regions of the N-terminal domain which determine agonist pharmacology in vertebrate ionotropic neurotransmitter receptors. A range of conformationally restricted GABA analogues were tested on the two homo-oligomers and their agonist pharmacology compared with that of insect and vertebrate iontropic GABA receptors.

2 The actions of GABA, isoguvacine and isonipecotic acid on RDL_{ac} and DRC 17-1-2 homo-oligomers were compared, by use of two-electrode voltage-clamp. All three compounds were full agonists of both receptors, but were 4–6 fold less potent agonists of DRC 17-1-2 homo-oligomers than of RDL_{ac}. However, the relative potencies of these agonists on each receptor were very similar.

3 A more complete agonist profile was established for RDL_{ac} homo-oligomers. The most potent agonists of these receptors were GABA, muscimol and *trans*-aminocrotonic acid (TACA), which were approximately equipotent. RDL_{ac} homo-oligomers were fully activated by a range of GABA analogues, with the order of potency: GABA > ZAPA ((Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid) > isoguvacine > imidazole-4-acetic acid ≥ isonipecotic acid ≥ *cis*-aminocrotonic acid (CACA) > β-alanine. 3-Aminopropane sulphonic acid (3-APS), a partial agonist of RDL_{ac} homo-oligomers, was the weakest agonist tested and 100 fold less potent than GABA.

4 SR95531, an antagonist of vertebrate $GABA_A$ receptors, competitively inhibited the GABA responses of RDL_{ac} homo-oligomers, which have previously been found to be insensitive to bicuculline. However, its potency (IC_{50} 500 μM) was much reduced when compared to $GABA_A$ receptors.

5 The agonist pharmacology of *Drosophila* RDL_{ac} homo-oligomers exhibits aspects of the characteristic pharmacology of certain native insect GABA receptors which distinguish them from vertebrate GABA receptors. The high potency and efficacy of isoguvacine and ZAPA distinguishes RDL_{ac} homo-oligomers from bicuculline-insensitive vertebrate $GABA_C$ receptors, while the low potency of SR95531 and 3-APS distinguishes them from $GABA_A$ receptors. The differences in the potency of agonists on RDL_{ac} and DRC 17-1-2 homo-oligomers observed in the present study may assist in identification of further molecular determinants of GABA receptor function.

Keywords: GABA receptor; *Drosophila melanogaster*; RDL_{ac}; DRC-17-1-2; isoguvacine; ZAPA

Introduction

Ionotropic γ-aminobutyric acid (GABA) receptors mediate rapid neurotransmission in the nervous systems of vertebrates (Mody *et al.*, 1994) and invertebrates (Sattelle, 1990). The vertebrate receptors may be divided into two distinct pharmacological classes: (1) $GABA_A$ receptors which are sensitive to bicuculline and are regulated by numerous allosteric modulators (Sieghart, 1995); (2) bicuculline-insensitive $GABA_C$ receptors which are also insensitive to allosteric modulators of $GABA_A$ receptors (Polenzani *et al.*, 1991; Feigenspan *et al.*, 1993; Qian & Dowling, 1993; 1994). $GABA_A$ and $GABA_C$ receptors have distinct agonist profiles which point to differences in their agonist binding sites, and this has led to the suggestion that GABA assumes different conformations when activating these two classes of receptor (Kusama *et al.*, 1993; Woodward *et al.*, 1993; Qian & Dowling, 1994).

Insect ionotropic GABA receptors do not fit readily into either category of vertebrate GABA receptor. Unlike $GABA_A$ receptors, the majority of insect GABA receptors are insensitive to bicuculline (Sattelle, 1990), yet their activation by GABA analogues such as isoguvacine and ZAPA (Sattelle *et al.*, 1988; Taylor *et al.*, 1993) also distinguishes them from vertebrate $GABA_C$ receptors, where these compounds have little or no agonist efficacy (Woodward *et al.*, 1993; Qian & Dowling, 1994).

At present the structural basis of the distinct pharmacology of insect GABA receptors is unknown, but the recent cloning of two *Drosophila melanogaster* GABA receptor subunits, RDL_{ac} and DRC 17-1-2 (ffrench-Constant *et al.*, 1991; Chen *et al.*, 1994), may aid its elucidation. These subunits appear to arise from the alternative splicing of the *Rdl* gene (ffrench-Constant & Rocheleau, 1993) and are widely distributed throughout the central nervous system of *Drosophila* (Aronstein & ffrench-Constant, 1995; Harrison *et al.*, 1996). Their homologues have been identified in three orders of insects, in which mutant forms of these subunits engender resistance to the naturally occurring antagonist picrotoxin (ffrench-Constant *et al.*, 1993b; Thompson *et al.*, 1993; Kaku & Matsumura, 1994; Miyazaki *et al.*, 1995), suggesting that RDL-like subunits may be constituents of many insect GABA receptors. To date, no homologues of these subunits have been identified in vertebrates. As the *Drosophila* subunits readily form functional homo-oligomers, which closely mimic several distinctive aspects of native insect GABA receptor pharmacology (ffrench-Constant *et al.*, 1993a; Buckingham *et al.*, 1994; Chen *et al.*, 1994; Shirai *et al.*, 1995; Hosie & Sattelle, 1996), they provide models with which to address aspects of the distinct pharmacology of insect and vertebrate GABA receptors.

RDL_{ac} and DRC-17-1-2 differ at only 17 residues, principally in regions of the N-terminal domain that influence agonist potency in vertebrate $GABA_A$ receptors and closely related nicotinic acetylcholine and glycine receptors (cf. Karlin & Akabas, 1995; Kuhse *et al.*, 1995; Smith & Olsen, 1995). The

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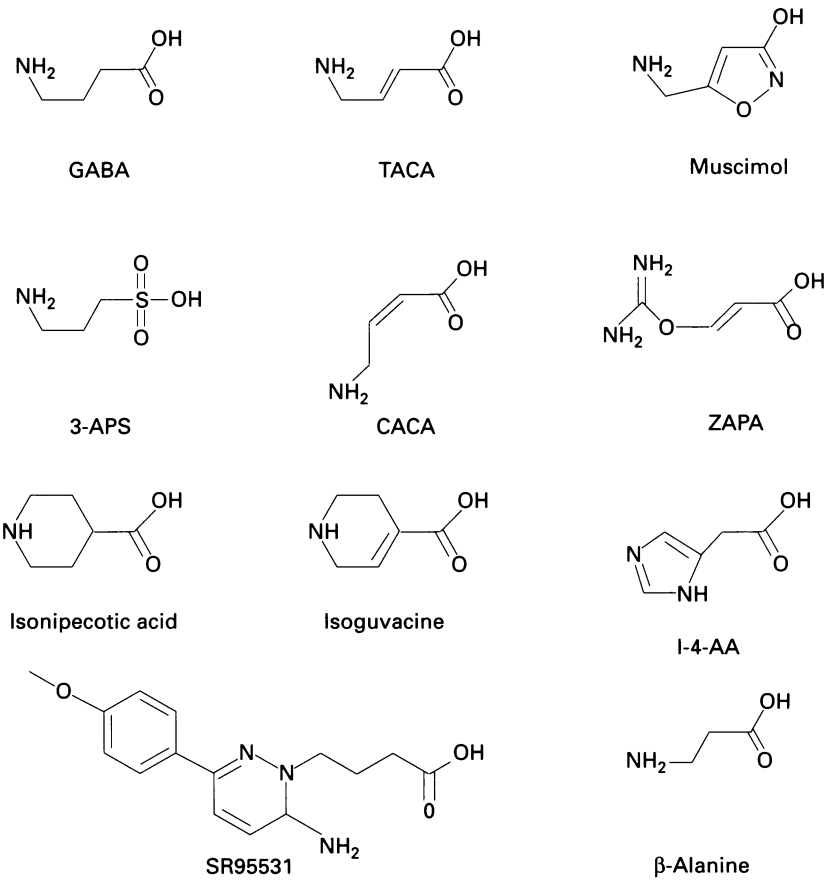

Figure 1 Structures of the ligands used in the present study. GABA, γ-aminobutyric acid; TACA, *trans*-aminocrotonic acid; 3-APS, 3-aminopropane sulphonic acid; CACA, *cis*-aminocrotonic acid; ZAPA, (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid; I-4-AA, imidazole-4-acetic acid.

EC_{50}s of GABA and muscimol differ for RDL_{ac} and DRC 17-1-2 homo-oligomers (cf. Buckingham *et al.*, 1994; Chen *et al.*, 1994). However, it is not known how the different primary amino acid structures of these subunits affect the relative potency of other GABA-mimetics, nor how closely the expressed receptors mimic the characteristic agonist profiles of native insect ionotropic GABA receptors. In this study we compared the actions of a range of conformationally restricted GABA-analogues on RDL_{ac} and DRC 17-1-2 homo-oligomers with those observed on vertebrate and insect ionotropic GABA receptors.

A note on terminology

The alternative transcripts of *Rdl* exon3, termed 'a' and 'b' encode two variant residues, while the variants of exon 6 encode a further ten amino acid changes and are termed 'c' and 'd' (ffrench-Constant & Rocheleau, 1993). RDL_{ac} contains variants 'a' and 'c' of exons 3 and 6, respectively, while DRC 17-1-2 bears splice variants 'b' and 'd' of these exons. However, DRC 17-1-2 differs from RDL_{ac} at an additional five residues which are encoded by exons 1, 2, 4 and 8. The source of this variation is as yet unknown but as RDL_{ac} and DRC 17-1-2 were cloned from different cDNA libraries (cf ffrench-Constant *et al.*, 1991; Chen *et al.*, 1994) they may reflect different alleles of the *Rdl* gene. As the effects of these five additional variations in primary amino acid sequence have not been addressed individually, for example by site-directed mutagenesis, we have elected to refer to DRC 17-1-2 as such rather than RDL_{bd}.

cRNA preparation

The cloning of cDNAs encoding wild-type RDL_{ac} (in pNB14.1) and DRC 17-1-2 (in pcDNA-1) has been described previously (ffrench-Constant *et al.*, 1991; Chen *et al.*, 1994). Both plasmids were linearized with the restriction endonuclease *Not*I and $m^7G(5')ppp(5')G$ capped cRNA was synthesized with either SP6 (RDL_{ac}), or T7 (DRC-17-1-2) RNA-polymerase (Promega), by use of standard protocol (Sambrook *et al.*, 1989).

Oocyte preparation and cRNA injection

Oocytes (stages V and VI) 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 cRNA, with a 1 ng nl^{-1} solution in distilled water, and incubated at 17–18°C in normal saline supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (100 μg ml^{-1}) and 2.5 mM 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 by a gravity fed system (5 ml min^{-1}). All drugs were readily soluble in saline and were applied dissolved in the perfusate. In all experiments, the membrane potential was clamped at −60 mV and membrane currents were monitored under two-electrode voltage-clamp with 3 M KCl filled electrodes (1–10 MΩ) and an Oocyte Clamp OC-725C amplifier (Warner Instruments). Signals were displayed on an osilloscope (Trio) and recorded onto computer by a TL-1 interface and Axotape software (both from Axon Instruments). Only those oocytes which gave stable responses

to at least 3 applications of 3 mM GABA were used. All dose-response curves were obtained by applying ever increasing concentrations of agonist to the oocyte. Agonist challenges were made at intervals of 3–5 min to allow full recovery between each application. Often dose-response relationships were determined for more than one compound on a single oocyte. In such cases EC_{50} and EC_{100} concentrations of GABA were applied after tests of each compound had been completed, to confirm the stability of the preparation. The data for muscimol, TACA and CACA on RDL_{ac} have been pooled with that presented elsewhere (Buckingham *et al.*, 1994) which had not previously been fitted with the logistic equation below. These earlier data were gathered under the conditions described here. When investigating the antagonist actions of the antagonist SR95531 the control responses to GABA were first determined. The oocyte was then pre-incubated for 30–60 s in the antagonist before each co-application of GABA and the antagonist. GABA responses were washed out with normal saline.

All data were normalized to the maximum GABA response seen in each oocyte, and are presented ± one s.e.mean (±s.e.mean) of n experiments. GraphPad Prism (GraphPad Software) was used to fit the following, four parameter, logistic equation (1), which describes a sigmoid curve of variable slope, to the averaged, normalized data.

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

where $\%I_{max}$ is the current induced by a given concentration of agonist ([ag]) expressed as a percentage of I_{max}, the amplitude of the maximal GABA response. I_{min} is the minimal agonist response, EC_{50} is the concentration of agonist predicted to elicit half the maximal response and n_H is the slope (Hill) coefficient. The estimated EC_{50} values are shown with their 95% confidence intervals (95% CI).

Chemicals

All reagents used in cRNA synthesis were obtained from Promega (U.K.) except for $m^7G(5')ppp(5')G$ cap analogue (New England Biolabs, U.K.). GABA, collagenase type IA, muscimol, 3-aminopropane sulphonic acid (3-APS), β-alanine and imidazole-4-acetic acid (I-4-AA) were obtained from Sigma (U.K.). The *cis*- and *trans*-forms of aminocrotonic acid (CACA and TACA, respectively), isoguvacine and (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA) were obtained from Tocris Cookson (U.K.),while isonipecotic acid, and 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl) pyridazinium bromide (SR95531) were supplied by Research Biochemicals International (via Semat, U.K.). The ligands used in this study are shown in Figure 1.

RDL_{ac} in pNB14.1 was a gift of R.T. Roush (Cornell University, U.S.A.). DRC 17-1-2 in pcDNA1 was a gift of N.C. Lan (Co-Censys Inc., U.S.A.).

Results

Actions of GABA on RDL_{ac} and DRC-17-1-2 homo-oligomers

Oocytes expressing RDL_{ac} and DRC-17-1-2 homo-oligomers responded to bath-applied GABA with dose-dependent inward currents, when voltage clamped at −60 mV. The estimated EC_{50}s for GABA on RDL_{ac} and DRC 17-1-2 were 27 μM (95% CI: 24–31 μM) ($n>20$) and 103 μM (95% CI: 91–115 μM) ($n=5$) respectively, and the dose-response curves reached their peaks at 1–3 mM for RDL_{ac}, and 3–10 mM for DRC 17-1-2. Similar findings have been published elsewhere (ffrench-Constant *et al.*, 1993a; Buckingham *et al.*, 1994; Chen *et al.*, 1994). However, the amplitude of the maximum GABA-induced current and the percentage of oocytes expressing functional receptors differed markedly with the cRNA injected. Thus, around 95% of oocytes injected with cRNA encoding RDL_{ac} expressed function receptors, which responded to 1 mM GABA with currents over 1 μA. By contrast, six batches of oocytes (approximately 100 oocytes total) were injected with cRNA encoding DRC 17-1-2, of which only 5 responded to 1 mM GABA with currents over 10 nA, although oocytes from the same batches readily assembled functional RDL_{ac} homo-oligomers. The mean, maximum GABA-induced current mediated by DRC 17-1-2 homo-oligomers was 244±69 nA.

As the apparent agonist affinity of heterologously expressed homo-oligomers has been observed to vary with levels of subunit expression (Taleb & Betz, 1994), we compared the amplitude of currents induced by 30 μM and 3 mM GABA in oocytes expressing high and low levels of RDL_{ac} homo-oligomers. In oocytes where the maximum GABA response was over 1000 nA, 30 μM GABA elicited currents that were 54±2% of the maximum ($n<20$). The mean amplitude of currents induced by 30 μM GABA in oocytes expressing low levels of RDL_{ac} homo-oligomers was similarly 49±2% of that induced by 3 mM GABA ($n=6$). In these oocytes, the mean amplitude of the response to 3 mM GABA was 300±64 nA, similar to that observed with DRC 17-1-2. Thus, the GABA dose-response curve of RDL_{ac} homo-oligomers was unaffected by the levels of receptor expression observed in this study, and it is therefore unlikely that the differences in the levels of expression of functional homo-oligomers composed of RDL_{ac} and DRC-17-1-2 were responsible for the observed differences in the EC_{50} for GABA on homo-oligomers.

Table 1 Comparison of agonist profiles of RDL_{ac} and DRC 17-1-2 homo-oligomers expressed in *Xenopus laevis* oocytes

	RDL_{ac}		*DRC 17-1-2*	
Agonist	*EC_{50}* (μM)	*n_H*	*EC_{50}* (μM)	*n_H*
GABA	27 (24–31)	1.8±0.2	103 (91–115)	2.2±0.2
Isoguvacine	161 (145–178)	2.6±0.1	611 (541–690)	2.0±0.1
Isonipecotic acid	688 (638–742)	2.1±0.1	3962 (3103–5108)	1.7±0.2
Muscimol	25 (16–40)	1.8±0.5		
TACA	34 (29–39)	2.0±0.2		
ZAPA	62 (47–81)	2.1±0.3		
I-4-AA	473 (414–540)	1.7±0.2		
CACA	870 (354–2141)	1.6±0.4		
β-Alanine	1373 (1149–1641)	2.2±0.3		
3-APS	3436 (2689–43391)	3.1±0.5		

EC_{50} and n_H values were calculated according to equation 1 by use of GraphPad Software (Prism). The mean EC_{50} values are shown with their 95% confidence interval and Hill coefficients are shown as the mean±s.e.mean of 4–6 observations (with the exception of GABA on RDL_{ac} where $n>20$). For key to abbreviations used see legend of Figure 1.

Actions of conformationally restricted GABA-analogues on the Drosophila *homo-oligomers*

In order to compare the agonist profile of the *Drosophila* homo-oligomers with those of other GABA receptors, the actions of conformationally restricted analogues of GABA and muscimol were observed. GABA is a flexible molecule which is considered to activate $GABA_A$ receptors when in a partially folded conformation, as a number of conformationally restricted molecules which mimic partially folded forms of GABA are potent agonists of these receptors (Krogsgaard-Larsen *et al.*, 1977; Andrews & Johnston, 1979; Woodward *et al.*, 1992; Kusama *et al.*, 1993). However, such partially-folded analogues behave as weak partial agonists or antagonists of native bicuculline-insensitive vertebrate $GABA_C$ receptors and expressed ρ homo-oligomers (which closely mimic the pharmacology of native $GABA_C$ receptors). This, and the high activity of CACA and CAMP (*cis*-2-aminomethylcyclopropane carboxylic acid) have been taken as evidence that $GABA_C$ receptors are selectively activated by an extended conformation of GABA (Kusama *et al*, 1993; Woodward *et al.*, 1993; Qian & Dowling 1994). Table 1 presents a summary of the estimated EC_{50}s and Hill coefficients of these compounds on RDL_{ac} and DRC 17-1-2 homo-oligomers. All the agonists had Hill coefficients greater than one.

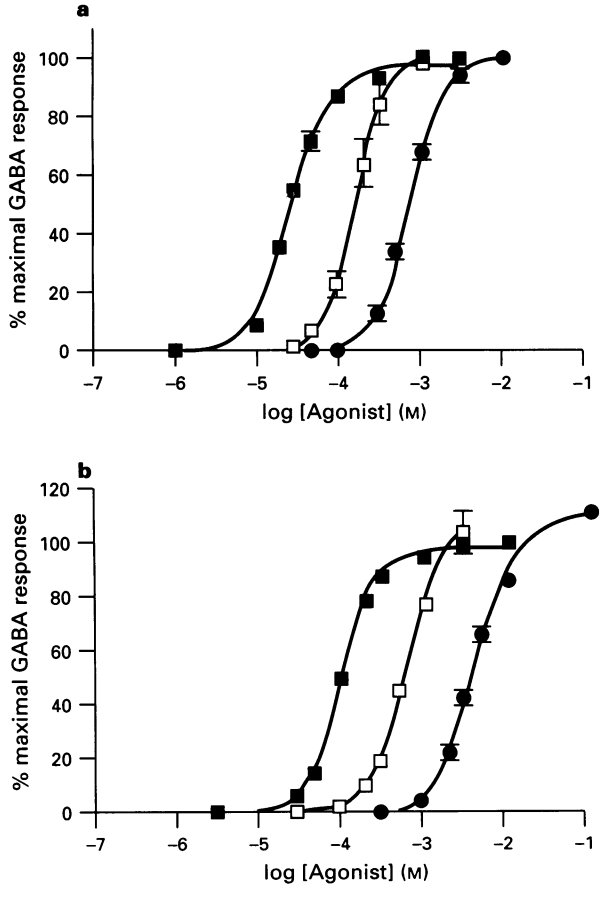

Figure 2 Agonist profiles of *Xenopus* oocytes injected with cRNA encoding RDL_{ac} or DRC 17-1-2 subunits, voltage clamped at −60 mV. GABA (■) was a more potent agonist of (a) RDL_{ac} homo-oligomers than of receptors composed of (b) DRC 17-1-2 subunits. Isoguvacine (□) and isonipecotic acid (●) were full agonists of both RDL_{ac} and DRC 17-1-2 homo-oligomers. The order of potency of the three agonists was the same on RDL_{ac} and DRC 17-1-2 homo-oligomers. Data were normalized to the maximum GABA response seen in each oocyte (3 mM for RDL_{ac}; 3 or 10 mM for DRC 17-1-2). Each point represents the mean of data from 4–6 separate oocytes, with the exception of GABA on RDL_{ac} where $n > 20$; vertical lines show twice the s.e.mean.

Relative potencies of heterocyclic GABA agonists on RDL_{ac} and DRC-17-1-2 homo-oligomers

Two heterocyclic GABA analogues were tested on both DRC 17-1-2 and RDL_{ac} homo-oligomers and their actions compared to those of GABA. Isoguvacine and its saturated analogue, isonipecotic acid, induced dose-dependent inward currents in oocytes expressing either homo-oligomer (Figure 2a and b). In both preparations, isoguvacine was the more potent of the two agonists, being 6 fold less potent than GABA and approximately 3 fold more potent than isonipecotic acid. Both compounds were full agonists of both RDL_{ac} and DRC 17-1-2. Thus, there was little difference in the relative potencies or efficacies of these compounds on either receptor (see Table 1), as all the agonists tested were approximately 4-6 fold more potent on RDL_{ac} homo-oligomers than on DRC 17-1-2. The differences in the derived primary amino acid sequences of the two subunits do not appear to discriminate between the agonists tested here.

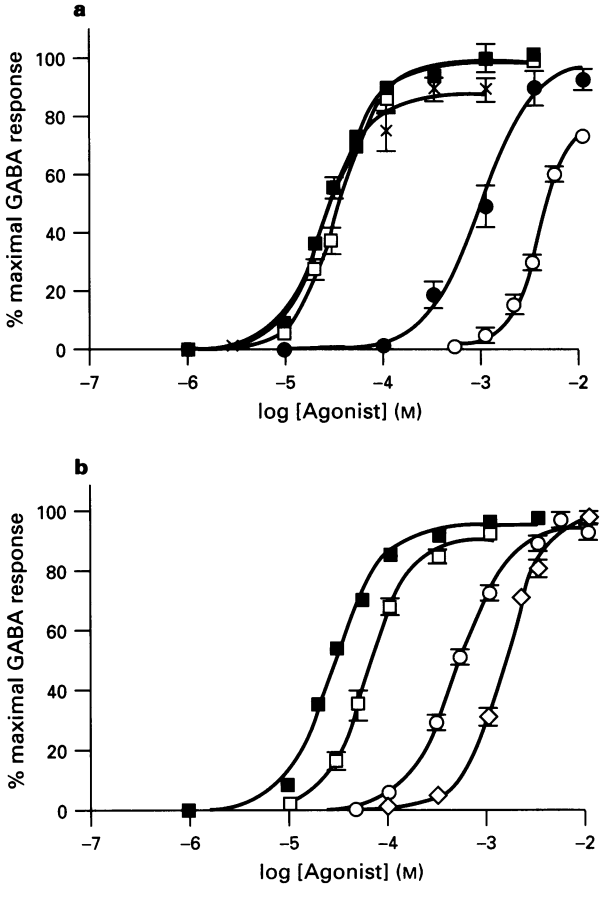

Figure 3 Dose-response relationships of conformationally restricted analogues on RDL_{ac} homo-oligomers. (a) *Trans*-aminocrotonic acid (TACA; □) and muscimol (X), which are locked in an extended conformation in which rotational freedom is restricted to the aminomethyl group, were full agonists of RDL_{ac} and equipotent with GABA (■). The folded GABA analogue, *cis*-aminocrotonic acid (CACA; ●), though a full agonist, was approximately 30 fold less potent that the its *trans*-isomer, TACA. The anionic centres of TACA, CACA and muscimol are approximately planar whereas that of 3-aminopropane sulphonic acid (3-APS, ○), a sulphonic acid analogue of GABA, is not. 3-APS was the least potent agonist tested, and was a partial agonist. (b) (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA; □) was a potent agonist of RDL_{ac} homo-oligomers. Imidazole-4-acetic acid (I-4-AA; ○), an extended GABA analogue and β-alanine (◇) were less potent full agonists. The GABA (■) dose-response curve is shown for reference. Data were normalized to the maximal GABA response observed in each oocyte, and each point is the mean of observations from at 4–6 oocytes, except for those representing GABA, where $n > 20$; vertical lines show twice the s.e.mean.

Agonist profile of RDL_{ac} homo-oligomers

The carbon backbones of muscimol and TACA are held in extended, near planar conformations by the isoxazolol ring of muscimol and the double bond between carbons 2 and 3 of TACA, respectively (Johnston *et al.*, 1975; Andrews & Johnston, 1979). Muscimol and TACA were the most potent agonists of RDL_{ac} homo-oligomers and were full agonists, equipotent with GABA (Figure 3a). By contrast, CACA which is locked in a planar, folded conformation (Bowery & Jones, 1976), was approximately 30 fold less potent than its *trans*-isomer (TACA). However, CACA was also a full agonist of RDL_{ac} homo-oligomers.

I-4-AA is considered to be an analogue of a fully extended GABA molecule (Bowery & Jones, 1976) and exhibits both low affinity and efficacy for a variety of $GABA_A$ receptor subtypes (Bowery & Jones, 1976; Karobath *et al.*, 1979; Barker & Mathers, 1981; Kusama *et al.*, 1993). I-4-AA activated RDL_{ac} homo-oligomers, eliciting maximal amplitude responses and was approximately equipotent with CACA and isonipecotic acid. ZAPA was a full agonist of RDL_{ac} homo-oligomers, only slightly less potent than GABA itself (Figure 3b). β-Alanine, which has been observed to activate both GABA and glycine receptors of vertebrates (Parker *et al.*, 1988; Schmieden *et al.*, 1993), was a less potent full agonist of RDL_{ac} homo-oligomers than either the partially folded or extended GABA analogues.

3-APS, the sulphonic acid analogue of GABA, is a potent agonist of $GABA_A$ receptors (Kemp *et al.*, 1986; Woodward *et al.*, 1993), but was the weakest agonist tested in this study, being 100 fold less potent than GABA and a partial agonist (Figure 3a).

For RDL_{ac} homo-oligomers, the relative order of agonist potency was as follows: GABA ≅ muscimol ≅ TACA > ZAPA > isoguvacine > I - 4-AA ⩾ CACA ⩾ isonipecotic acid > β-alanine > 3-APS.

Competitive antagonism of RDL_{ac} homo-oligomers

Like many native insect GABA receptors, heterologously expressed RDL_{ac} and DRC 17-1-2 homo-oligomers have previously been found to be insensitive to bicuculline at concentrations up to 100 μM (ffrench-Constant *et al.*, 1993a; Buckingham *et al.*, 1994; Chen *et al.*, 1994). However, SR95531 (50 μM – 1 mM), a potent competitive antagonist of vertebrate $GABA_A$ receptors, reduced the GABA response of RDL_{ac} homo-oligomers. Antagonism by SR95531 was reversible and dose-dependent (Figure 4a). The amplitudes of currents induced by 30 μM GABA alone were 54 ± 4% ($n = 20$) of the maximum, and 25 ± 4% ($n = 6$) of the maximum when GABA and 500 μM SR95531 were co-applied following a 30–60 s pre-incubation in the antagonist. SR95531 (500 μM) elicited a parallel shift to the right in the GABA dose-response curve, but did not suppress the amplitude of the maximum GABA response (Figure 4b). Thus, SR95531 is a competitive antagonist of GABA on RDL_{ac} homo-oligomers.

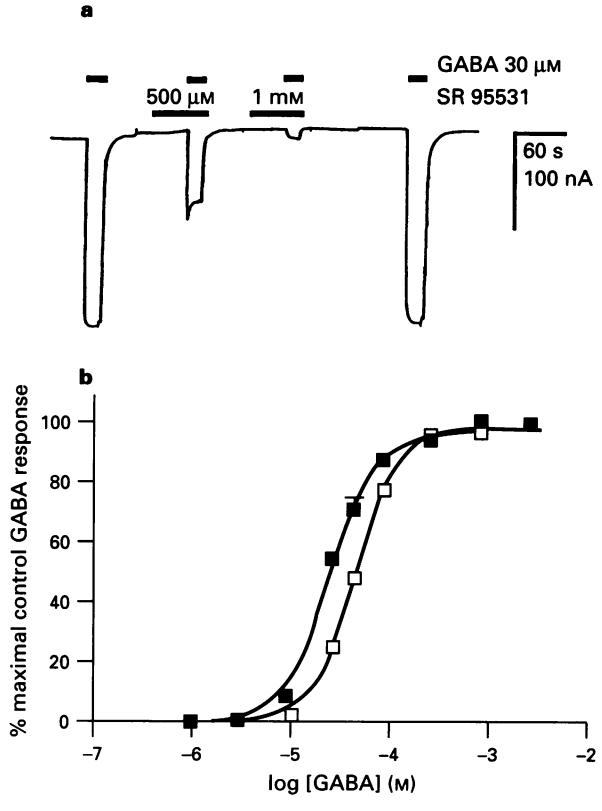

Figure 4 Effects of SR95531 on GABA-induced currents mediated by RDL_{ac} homo-oligomers expressed in *Xenopus* oocytes. (a) Consecutive responses to 30 μM GABA recorded from an oocyte expressing RDL_{ac} homo-oligomers show the dose-dependency and reversibility of SR95531 antagonism. GABA was applied at intervals of 2–3 min. The horizontal bars represent the duration of GABA and SR95531 application. (b) GABA dose-response curves obtained in the absence (■) and after 30–60 s incubation in 500 μM SR95531 (□). The Hill coefficient was little changed by SR95531 (1.8 ± 0.2 to 2.0 ± 0.1 in the presence of the antagonist) which effected a parallel shift in the GABA dose-response curve, reducing the GABA EC_{50} from 27 μM (95% CI: 24–31 μM) to 51 μM (95% CI: 49–53 μM) but did not reduce the amplitude of the maximum responses of the oocytes to GABA. Data were normalized to the maximum response seen with GABA alone. Each point on the antagonized dose-response curve is the mean of 6 experiments, whereas points on the control curve represent the mean of data from over 20 oocytes; Vertical lines show twice the s.e.mean.

Discussion

Comparison of the agonist pharmacology of RDL_{ac} and DRC 17-1-2 homo-oligomers with that of native insect GABA receptors

One of the principal aims of this project was to ascertain whether or not RDL_{ac} homo-oligomers exhibited the characteristic pharmacology of native insect ionotropic GABA receptors. It was reasoned that if they do, RDL_{ac} homo-oligomers would be a suitable model with which to investigate the molecular determinants of insect GABA receptor pharmacology by site-directed mutagenesis. Unfortunately, little electrophysiological data on the detailed pharmacology of native *Drosophila* receptors are available at present. However, data gathered in electrophysiological studies and radioligand binding experiments suggest that the majority of native insect ionotropic GABA receptors have a common pharmacology, as do, for example, $GABA_A$ receptors from a variety of vertebrate species. The common pharmacology of many native insect receptors differs in several repects from that of vertebrate ionotropic GABA receptors (for review see Sattelle 1990; Sattelle *et al.*, 1991). Aspects of this distinct pharmacology appear to be well mimicked by the heterologously expressed *Drosophila* homo-oligomers (Hosie & Sattelle, 1996).

Insensitivity to bicuculline, a definitive competitive antagonist of $GABA_A$ receptors (Curtis *et al.*, 1970; Andrews & Johnston, 1979; Sieghart, 1995), is a characteristic of the majority of native insect ionotropic GABA receptors (Scott & Duce, 1987; Lees *et al.*, 1987; Benson, 1988; Sattelle *et al.*, 1988; 1991; Sattelle, 1990) including those of *Drosophila* (Zhang *et al.*, 1995). However, muscimol, isoguvacine and ZAPA are potent, full agonists of such native insect GABA receptor (Sattelle *et al.*, 1988; Taylor *et al.*, 1993; Bai, 1994) whereas they are partial agonists or competitive antagonists of bicuculline-insensitive $GABA_C$ receptors and ρ homo-oligomers (Fiegenspan *et al.*, 1993; Kusuma *et al.*, 1993; Qian & Dowling, 1993, 1994; Lukasiewicz *et al.*, 1994). RDL_{ac} and

DRC 17-1-2 homo-oligomers have previously been shown to be insensitive to 100 μM bicuculline and fully activated by muscimol (ffrench-Constant *et al.*, 1993a; Buckingham *et al.*, 1994; Chen *et al.*, 1994; Zhang *et al.*, 1995). The present study demonstrates that the agonist profile of the *Drosophila* homo-oligomers is quite distinct from that of bicuculline-insensitive ionotropic GABA receptors of vertebrates, but similar to those of native insect receptors, as both RDL_{ac} and DRC 17-1-2 receptors are fully activated by the partially-folded GABA analogues isoguvacine and isonipecotic acid, and in the case of RDL_{ac} by ZAPA. Indeed ZAPA is approximately equipotent with GABA and muscimol as an agonist of GABA receptors on certain unidentified locust and cockroach neurones (Taylor *et al.*, 1993) as it is on RDL_{ac} homo-oligomers. Another characteristic of insect GABA receptors is the low potency of 3-APS relative to GABA (Abalis *et al.*, 1986; Lummis & Sattelle, 1986; Murphy & Wann, 1988; Sattelle *et al.*, 1988; Taylor *et al.*, 1993) as compared to vertebrate $GABA_A$ receptors where it is a relatively potent agonist (Kemp *et al.*, 1986; Woodward *et al.*, 1993). 3-APS was the least potent agonist of RDL_{ac} tested in the present study and was 100 fold less potent than GABA.

Finally, the relative potencies of muscimol, GABA, isonipecotic acid, I-4-AA, β-alanine and bicuculline on RDL_{ac} homo-oligomers resemble those observed in radioligand binding studies on native *Drosophila* GABA receptors (Rosario *et al.*, 1989). It therefore appears that the agonist site pharmacology of RDL_{ac} and DRC 17-1-2 homo-oligomers is characteristic of that of native, bicuculline-insensitive GABA receptors from a variety of insect species.

Comparison of the agonist pharmacophores of Drosophila *and vertebrate ionotropic GABA receptors*

Vertebrate $GABA_A$ and $GABA_C$ receptors show markedly different sensitivities to conformationally restricted GABA analogues, and although structure-activity relationships should be interpreted with care, particularly as it has not been determined if all GABA-analogues interact with the same residues on the receptor, such studies suggest that $GABA_A$ receptors are activated by partially-folded forms of GABA, whereas $GABA_C$ receptors are activated by GABA in an extended conformation (Kusama *et al.*, 1993; Qian & Dowling, 1994). An alternative explanation of these data is that the agonist binding sites of $GABA_C$ receptors are less tolerant of the bulky ring structures which restrict analogues such as isoguvacine and isonipecotic acid to partially-folded conformations than are $GABA_A$ receptors (Woodward *et al.*, 1993). The high potency and efficacy of partially folded GABA analogues (isoguvacine and isonipecotic acid), and of ZAPA and I-4-AA, distinguishes the RDL_{ac} homo-oligomers from bicuculline-insensitive GABA receptors of vertebrates. However, the agonist profile of the *Drosophila* homo-oligomers reflects that the $GABA_A$ receptors where the above compounds act as agonists and have a similar order of potency (Barker & Mathers, 1978; Kusama *et al.*, 1993; Woodward *et al.*, 1993). For example, ZAPA is a potent agonist of both RDL_{ac} and $GABA_A$ receptors (Allan *et al.*, 1986). Similarly, isoguvacine is a more potent agonist of RDL_{ac} homo-oligomers and $GABA_A$ receptors (Woodward *et al.*, 1993) than is isonipecotic acid, indicating that a decrease in the planarity of the piperidine ring reduces agonist activity on both preparations.

Such findings suggest that *Drosophila* homo-oligomers and $GABA_A$ receptors are either activated by a partially-folded form of GABA or exhibit a similar tolerance for GABA-analogues with a bulky cationic centre (e.g. ZAPA, isoguvacine, isonipecotic acid and I-4-AA). However, there are differences in the efficacies of some of these compounds as agonists of RDL_{ac} and $GABA_A$ receptors. For example, I-4-AA is a partial agonist of certain recombinant (Kusama *et al.*, 1993) and native (Bowery & Jones, 1976) $GABA_A$ receptors, but was a full agonist of RDL_{ac}. More distinct differences in the agonist binding site pharmacology of RDL_{ac} and $GABA_A$ receptors are illustrated by the relative potencies of GABA, muscimol, 3-APS and CACA, and the actions of the competitive antagonists bicuculline and SR95531.

Muscimol is invariably a more potent agonist of $GABA_A$ receptors than GABA (Allen & Johnston, 1979; Karobath *et al.*, 1979; Barker & Mathers, 1978; Amin & Weiss, 1993; Woodward *et al.*, 1993), whereas muscimol and GABA are found to be equipotent agonists of RDL_{ac} homo-oligomers. Furthermore, muscimol is a slightly less potent agonist of DRC 17-1-2 homo-oligomers than is GABA (Chen *et al.*, 1994). The isoxazol ring of muscimol restricts the molecule to a near planar, extended conformation, and it has been suggested that this may be close to the optimal conformation for activation of $GABA_A$ receptors (Andrews & Johnston, 1979). By contrast, GABA, which is a considerably more flexible molecule, will be free to assume conformations of greater or lesser efficacy. Thus, the equipotency of GABA and muscimol on RDL_{ac} may reflect either, the latter molecule being locked in a sub-optimal conformation for promoting channel opening, or, adverse steric interactions between the isoxazol ring of muscimol and the binding site of the *Drosophila* homo-oligomer.

Differences in the binding site for the anionic centre of GABA analogues may also account for a second discrepancy between $GABA_A$ receptors and RDL_{ac} homo-oligomers, namely the reduced potency of 3-APS on RDL_{ac} homo-oligomers. 3-APS differs from GABA in the structure of its anionic centre, and contains a sulphonic acid group rather than a carboxyl group which results in differences in the charge distribution and steric bulkiness of the two agonists. RDL_{ac} also differs from $GABA_A$ receptors in its relatively high sensitivity to CACA, which has a higher potency on vertebrate $GABA_C$ receptors than $GABA_A$ receptors (Fiegenspan *et al.*, 1993; Qian & Dowling, 1993; Kusama *et al.*, 1993; Woodward *et al.*, 1993). Although restricted to a folded conformation, the charged centres of CACA better overly those of fully extended GABA molecules than those of partially folded GABA analogues, albeit when their carbon backbones are out of alignment (Kusama *et al.*, 1993). This has been taken as evidence that fully extended GABA molecules activate the bicuculline-insensitive vertebrate receptors (Kusama *et al.*, 1993). The high efficacy of CACA distinguishes RDL_{ac} from $GABA_C$ receptors, while the high potency contrasts with $GABA_A$ receptors. Similarly, I-4-AA, although a weak agonist of $GABA_A$ receptors (Kemp *et al.*, 1986; Kusama *et al.*, 1993), was a fully agonist of RDL_{ac} homo-oligomers. Thus, although RDL_{ac} homo-oligomers are potently and fully activated by partially folded ligands, their agonist selectivity is somewhat different to that of $GABA_A$ receptors.

The most obvious distinction between native insect GABA receptors and vertebrate $GABA_A$ receptors is their bicuculline sensitivity, which is mimicked by RDL_{ac} homo-oligomers. SR95531 is another competitive antagonist of $GABA_A$ receptors (Heaulme *et al.*, 1986), and it competitively blocks the GABA response of RDL_{ac} homo-oligomers. However, as the IC_{50} of SR95531 on RDL_{ac} is 500 μM, it is a markedly less potent antagonist of the *Drosophila* homo-oligomer than of recombinant $GABA_A$ receptors (IC_{50} approximately 0.1 μM; Sigel *et al.*, 1992).

The potency of SR95S31 and bicuculline on rat $GABA_A$ receptors is strongly dependent on a single residue on the α subunit (rat $\alpha1^{F64}$ or equivalent; Sigel *et al.*, 1992). The IC_{50} of SR95531 is increased approximately 50 fold by the $\alpha1^{F64L}$ substitution, while that of bicuculline increased 200 fold. A tyrosine (Y) residue is found at the equivalent position to $\alpha1^{F64}$ in RDL_{ac} and DRC 17-1-2 (Y109) and ρ subunits which are believed to contribute to bicuculline-insensitive, $GABA_C$ receptors. Whether or not this accounts for the low bicuculline and SR95531 sensitivity of *Drosophila* and ρ subunit homo-oligomers remains to be determined, but the substitution of tyrosine and phenylalanine residues can have a dramatic effect on agonist binding in $GABA_A$ and glycine receptors (Schmieden *et al.*, 1993; Amin & Weiss, 1993). However the $\alpha1^{F64L}$ substitution also reduced the potency of GABA more

than 200 fold, raising the EC_{50} above 1 mM, whereas, ρ homo-oligomers exhibit a high GABA affinity, and the GABA EC_{50} of RDL_{ac} is similar to that of $GABA_A$ receptors containing the wild-type $\alpha 1$ subunit.

GABA-like motifs have been identified in both bicuculline (Curtis *et al.*, 1970; Andrews & Johnston, 1979 for review) and SR95531 (Heaulme *et al.*, 1986). Similarly, glycine-like motifs have been identified in strychnine, the competitive antagonist of inhibitory glycine receptors (Aprison *et al.*, 1995). That mutations of $GABA_A$ receptor subunits which reduce the potency of these antagonists also reduce the potency of GABA (Sigel *et al.*, 1992; Amin & Weiss, 1993) suggests that agonists and antagonists do indeed share a common binding site. However, the antagonists are considerably larger molecules than GABA and its analogues, and so could be expected to interact with parts of the receptor not associated with agonist binding. Given the similarities between the agonist profiles of RDL_{ac} and $GABA_A$ receptors, and the similar potency of GABA on RDL_{ac} and vertebrate $GABA_A$ receptors, the possibility arises that the *Drosophila* and vertebrate ionotropic GABA receptors differ principally in a region which interacts exclusively with competitive antagonists.

Comparison of RDL^{ac} and DRC 17-1-2 homo-oligomers

The *Rdl*-encoded subunits belong to a superfamily of gene products which have been termed cys-loop receptor subunits that includes the polypeptides which contribute to $GABA_A$, $GABA_C$, nicotinic acetylcholine (nACh) and inhibitory glycine receptors (Karlin & Akabas, 1995). Photoaffinity labelling and mutagenesis studies have indicated that a number of discrete, homologous regions in the N-terminal domain of cys-loop receptor subunits are the principal constituents of the binding sites for their agonists, and that there is a remarkable conservation in the location of these domains (for reviews see Karlin & Akabas, 1995; Bertrand & Changeux, 1995; Kuhse *et al.*, 1995; Smith & Olsen, 1995). The *Rdl*-gene is unusual in that can be alternatively spliced at 2 exons which encode regions of the extracellular domain, as most splice variants of vertebrate GABA receptor subunits differ in their large intracellular loops (ffrench-Constant & Rocheleau, 1993). These two alternatively spliced exons account for 12 of the amino acid differences between RDL_{ac} and DRC 17-1-2 which align closely with determinants of agonist potency in vertebrate cys-loop receptors.

The two amino acid differences encoded by exon 3 of *Rdl* lie close to the equivalent of rat $\alpha 1^{F64}$ (cf. Sigel *et al.*, 1992) which in bovine α subunits is photoaffinity labelled by [^{3}H]-muscimol (Smith & Olsen, 1994). The remaining 10 differences, which are encoded by *Rdl* exon 6, align with a region which lies between two domains that determine agonist potency in vertebrate GABA receptor β and ρ subunits (Amin & Weiss, 1993; 1994). Further, this region corresponds to agonist binding domain E of nACh receptors (Bertrand & Changeux, 1995; Karlin & Akabas, 1995). As yet the effects of substituting residues in this region of GABA receptor subunits remains undetermined, but it is interesting that this region is poorly conserved in vertebrate $GABA_A$ receptor subunit isoforms, and that recombinant $GABA_A$ receptors which differ in their isoform composition also differ in their agonist affinity and efficacy (Levitan *et al.*, 1988; Ebert *et al.*, 1994; White *et al.*, 1995). It is therefore possible that the alternative splicing of *Rdl* exon 6 underlies, at least in part, the 4-6 fold decrease in the potency of all agonists on DRC 17-1-2 relative to RDL_{ac}, which could serve a physiological role in *Drosophila* GABA receptors composed of different forms of *Rdl*-encoded subunits. As RNA encoding all four possible splice variants of the *Rdl* gene have been identified in *Drosophila* embryos (ffrench-Constant & Rocheleau, 1993), it will be of interest to see how the alternate splicing of exon 3 or exon 6 alone affects the GABA responses of *Drosophila* homo-oligomers.

There appears to be little difference in the relative potencies of GABA isoguvacine and isonipecotic acid on either homo-oligomer. Thus, the variant amino acids did not appear to discriminate between the agonists tested here, rather they alter the potency of them all. Yet, in a previous study of DRC 17-1-2, muscimol was found to be a slightly less potent agonist than GABA (Chen *et al.*, 1994), in contrast to the data for RDL_{ac} presented here. It is therefore possible that the reduced potency of muscimol observed by Chen *et al.* (1994) may reflect slight differences in the optimal orientation or structure of the anionic centre of agonists on the two homo-oligomers.

Apart from the differences in agonist potency, RDL_{ac} and DRC 17-1-2 mediated currents differed markedly in their maximal amplitude. Different amplitudes of maximal GABA responses have also been observed in oocytes expressing vertebrate $\rho 1$ and $\rho 2$ homo-oligomers (Wang *et al.*, 1994). Unfortunately, the DNAs encoding RDL_{ac} and DRC 17-1-2 differ in the 5′ untranslated region (UTR) which lies between the RNA polymerase promoter and the start codon of each subunit cDNA. As yet, we have been unable to standardize the 5′ UTR of both clones and so it is not possible, at present, to say whether or not differences in the maximal currents obtained from RDL_{ac} and DRC 17-1-2 homo-oligomers reflect differences in the level of expression of each subunit or differences in the coupling of the agonist binding site to the ion-channel. Although we suspect that the 5′ UTR of the DRC 17-1-2 clone, which is considerably longer than that of RDL_{ac}, may reduce the efficiency of expression and thus the maximum response of a DRC 17-1-2 injected oocyte, it is also possible that the probability of entering a high conductance conformation is reduced in DRC 17-1-2. Such questions are best addressed by patch-clamp studies. However, the response of oocytes to 30 μM GABA was independent of the amplitude of the GABA-induced current and we therefore consider the differences in the EC_{50}s for the two homo-oligomers to be genuine.

RDL-like subunits have been identified in three orders of insects (ffrench-Constant *et al.*, 1993b; Thompson *et al.*, 1993; Kaku & Matsumura, 1994; Miyazaki *et al.*, 1995), but so far not in vertebrates. The present study demonstrates that *Rdl*-encoded subunits of *Drosophila* exhibit the characteristic agonist-site pharmacology of many native insect GABA receptors. Furthermore, the pharmacology of convulsant antagonists (Buckingham *et al.*, 1994; Shirai *et al.*, 1995) and certain allosteric modulators (Chen *et al.*, 1994; Hosie & Sattelle, 1996) on the heterologously expressed *Drosophila* RDL_{ac} and DRC 17-1-2 homo-oligomers is characteristic of native insect GABA receptors. A single amino acid substitution (A302S) in *Drosophila Rdl*-encoded subunits underlies resistance to naturally occurring (e.g. picrotoxinin) and synthetic insecticides e.g. cyclodienes (ffrench-Constant *et al.*, 1992) in living *Drosophila*. The same substitution has since been observed in RDL homologues of insecticide-resistant strains of insects from three orders (Thompson *et al.*, 1993; Kaku & Matsamura, 1994; Miyazaki *et al.*, 1995). These data suggest that RDL-like subunits are likely to be constituents of many native insect GABA receptors and are important determinants of the differential pharmacology of insect and vertebrate ionotropic GABA receptors.

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