Cloning and functional expression of intracellular loop variants of the honey bee (*Apis mellifera*) RDL GABA receptor

Jennina Taylor-Wells[1], Joseph Hawkins, Claudia Colombo, Isabel Bermudez and Andrew K. Jones*

Department of Biological and Medical Sciences, Faculty of Health and Life Sciences, Oxford Brookes University, Headington, Oxford, OX3 0BP, UK.

*Corresponding author. Tel: +44 01865 483602. Email address: a.jones@brookes.ac.uk

[1]Present address: Department of Entomology and Nematology, Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610, USA

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Taylor-Wells et al. Highlights

- Honeybee GABA receptor (RDL) has three variants with different intracellular loops.
- Each variant has similar sensitivity to the agonist, GABA.
- The antagonist, fipronil, has similar potency on all three variants.
- The antagonist, imidacloprid, has similar potency on all three variants.

Abstract

The insect GABA receptor, RDL (resistance to dieldrin), plays central roles in neuronal signalling and is the target of several classes of insecticides. To study the GABA receptor from an important pollinator species, we cloned *Rdl* cDNA from the honey bee, *Apis mellifera*. Three *Rdl* variants were identified, arising from differential use of splice acceptor sites in the large intracellular loop between transmembrane regions 3 and 4. These variants were renamed from previously, as Amel_RDLvar1, Amel_RDLvar2 and Amel_RDLvar3. When expressed in *Xenopus laevis* oocytes, the three variants showed no difference in sensitivity to the agonist, GABA, with EC_{50}s of 29 μM, 20 μM and 29 μM respectively. Also, the potencies of the antagonists, fipronil and imidacloprid, were similar on all three variants. Fipronil IC_{50} values were 0.18 μM, 0.31 μM and 0.20 μM whereas 100 μM imidacloprid reduced the GABA response by 17%, 24% and 31%. The possibility that differential splicing of the RDL intracellular loop may represent a species-specific mechanism leading to insensitivity to insecticides is discussed.

Keywords

Apis mellifera, fipronil, GABA receptor, imidacloprid, splice variant, Xenopus oocytes

Introduction

This article is dedicated to Professor Toshio Narahashi (1927-2013), whose pioneering and comprehensive research has provided the groundwork for much of our current understanding of the pharmacology of ion channels and insecticide toxicology (Cranmer, 2013). His work included the application of electrophysiology to study insecticide action on γ-aminobutyric acid (GABA) receptors in insect neurons (Zhao *et al.*, 2003). The insect GABA receptor, RDL (resistance to dieldrin), plays a central role in neuronal signalling and is involved in various processes, including regulation of sleep (Liu *et al.*, 2014), aggression (Yuan *et al.*, 2014) and olfaction (Choudhary *et al.*, 2012; Dupuis *et al.*, 2010). It is a member of the cys-loop ligand-gated ion channel (cysLGICs) superfamily and thus contains: an N-terminal extracellular domain, where GABA binding occurs; four transmembrane (TM) domains, the second of which lines the ion channel; and a large intracellular loop between TM3 and TM4 (Nys *et al.*, 2013). Work on vertebrate receptors has shown that the TM3-TM4 intracellular loop is involved in several aspects of cysLGIC function, such as receptor maturation, protein interaction, receptor localization and ion channel function (Stokes *et al.*, 2015; Jin *et al.*, 2014; Li *et al.*, 2012; Wu *et al.*, 2012). In comparison, little information is available concerning intracellular loop function in invertebrate cysLGICS.

RDL is of interest as it is the target of highly effective insecticides (Buckingham *et al.*, 2005; Raymond-Delpech *et al.*, 2005). Point mutations, particularly an alanine to serine or glycine mutation in TM2, underlie resistance to several insecticides, including dieldrin, picrotoxinin and fipronil (Ffrench-Constant *et al.*, 1993; Hosie *et al.*, 1995). This substitution of alanine is often used as a diagnostic marker for resistance and has been detected in varying species, ranging from crop pests (e.g. the planthopper *Laodelphax striatellus* (Nakao *et al.*, 2011)), pests afflicting livestock (the horn fly *Haematobia irritans* (Domingues *et al.*, 2013)), domesticated animals (the cat flea *Ctenocephalides felis* (Bass *et al.*, 2004)) to disease vectors (the malaria vector mosquito *Anopheles gambiae* (Du *et al.*, 2005)). Knowledge of mechanisms leading to insecticide insensitivity is crucial to detecting resistance in insect populations, which can then inform the use of alternative insecticide classes, as part of an effective resistance management program (Feyereisen *et al.*, 2015).

Recently, the cloning of *Rdl* from the miridbug, *Cyrtorhinus lividipennis*, revealed two isoforms, differing in subunit length by 31 amino acid residues, arising from differential splicing of the TM3-TM4 intracellular loop (Jiang *et al.*, 2015). Two-electrode voltage-clamp electrophysiology applied to *C. lividipennis* RDL expressed in *Xenopus laevis* oocytes showed these two isoforms differed in their sensitivity to the antagonistic effects of fipronil, with the longer variant having greater insensitivity to this insecticide. It was thus suggested that *C. lividipennis* may take advantage of its RDL diversity to enhance tolerance to fipronil or other insecticides. Interestingly, the 31 amino acid insertion has so far been observed only

in *C. lividipennis*, highlighting the presence of such TM3-TM4 splice variants as a possible species-specific mechanism for insecticide insensitivity. Since *C. lividipennis* plays an important predatory role in controlling the rice pest, *Nilaparvata lugens*, this is a useful example of a study assessing the effects of fipronil on its molecular target from a beneficial insect (Jiang et al., 2015).

The honey bee, *Apis mellifera*, is another example of a beneficial insect, whose role in pollinating crop species is valued at over $200 billion worldwide (Fairbrother *et al.*, 2014). In 2013, the European Union placed a moratorium of at least two years on the use of neonicotinoids, which target nicotinic acetylcholine receptors (nAChRs) (Matsuda *et al.*, 2001), amidst fears that these insecticides are a contributing factor towards the alarming decline in bee numbers (Fairbrother et al., 2014). The use of fipronil was also restricted for the same reason (Official Journal of the European Union, 2013).

To understand further the molecular target of fipronil in *A. mellifera*, we report here the cloning of the honey bee *Rdl* as well as the identification of variants arising from differential splicing of the TM3-TM4 intracellular loop. In addition, we present the first heterologous expression studies of an *Apis* RDL to assess the sensitivity of the different variants to fipronil. Previously, we showed that the neonicotinoid, imidacloprid, acts as an antagonist on heterologously expressed RDL of the mosquito, *Anopheles gambiae* (Taylor-Wells *et al.*, 2015). We therefore used the *X. laevis* oocyte expression system to determine whether imidacloprid also acts directly on the honey bee GABA receptor.

Materials and methods

Isolation of *Rdl* from *A. mellifera* honey bees

The sequence of the honey bee RDL (Amel_RDL) identified from the *A. mellifera* genome has been previously reported (Jones and Sattelle, 2006). However, this sequence lacks the highly variable N-terminal signal leader peptide. In order to clone the full length Amel_*Rdl* subunit, the TBLASTN program was used to search *A. mellifera* RefSeq data at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the previously published Amel_RDL peptide (Jones and Sattelle, 2006) as the query sequence. This identified the predicted '*Apis mellifera* gamma-aminobutyric acid receptor subunit' (Accession XM_006565106) containing the N-terminus and signal peptide sequence.

The synthesis of first-strand cDNA from total RNA extracted from adult *A. mellifera* mushroom bodies has been previously described (Jones *et al.*, 2006). The coding sequence of Amel_*Rdl* was amplified from this cDNA by a nested PCR approach using Pfu polymerase (Promega). The first PCR reaction used the following primers: N-terminal 5'-CGTCCCATAAGGATATTACC -3', C-terminal 5'- TTAACCGCACTGTCTCTCG -3'. The first PCR was used at a final dilution of 1 in 5000 as template for the second nested PCR reaction which used the following primers: N-terminal 5'-CGATCGGAATTCATGTCCTTCCACGCCGCC -3', C-terminal 5'-CGATCGTCTAGATTATTTCGCCTCCTCGAGAAG -3'. Underlined sequences are *EcoR*I and *Xba*I restriction sites, respectively, which were used to clone the Amel_*Rdl* coding sequence into the pCI vector (Promega). Amel_*Rdl* clones were sequenced at Source BioScience (http://www.sourcebioscience.com/).

Sequence analysis

The multiple protein sequence alignment was constructed with ClustalX (Thompson *et al.*, 1997). Signal peptide cleavage sites were predicted using the SignalP 4.1 server (Petersen *et al.*, 2011) and membrane-spanning regions were identified using the TMpred program (available at http://www.ch.embnet.org/software/TMPRED_form.html). The PROSITE database (Sigrist *et al.*, 2013) was used to identify potential phosphorylation sites.

Preparation and expression of RDL in *X. laevis* oocytes and two-electrode voltage clamp electrophysiology

The functional effects of the RDL intracellular loop splice variants in *A. mellifera* were evaluated using the *X. laevis* expression system and two-electrode voltage clamp electrophysiology. *Xenopus laevis* were purchased from Xenopus 1, Dexter, Michigan, USA and were handled strictly adhering to the guidelines of the Scientific Procedures Act, 1986, of the United Kingdom. Stage V and VI *X. laevis* oocytes were harvested and rinsed with

Ca^{2+} free solution (82 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.6), before defolliculating with 1 mg/ml type IA collagenase (Sigma, St. Louis, MO, USA) in Ca^{2+} free solution. Defolliculated oocytes were injected with 2.3 ng (23 nl) Amel_*Rdl* plasmid DNA into the nucleus of the oocyte and stored in standard Barth's solution (supplemented with 5% horse serum, 50 µg/ml neomycin and 10 µg/ml penicillin/streptomycin) at 17.5ºC.

Oocytes 1-7 days post-injection were placed in a recording chamber and clamped at -20 to -80 mV with two 3 M KCl filled borosilicate glass electrodes (resistance 0.5-5 MΩ) and an Oocyte Clamp OC-725C amplifier (Warner Instruments, CT, USA). Responses were recorded on a flatbed chart recorder (Kipp & Zonen BD-11E, Delft, Netherlands). Oocytes were perfused with standard oocyte saline (SOS; 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) at a flow rate of 10 ml/min. Oocytes were selected for experiments if stable after three or more consecutive challenges of 1 mM GABA at 3 min intervals.

GABA concentration response curves were generated by challenging oocytes with increasing concentrations of GABA in SOS, with 3 min between challenges. Curves were calculated by normalising the GABA current responses to the mean of control responses induced by 1 mM GABA before and after application.

Insecticide inhibition curves were generated by inhibiting responses to 1 mM GABA in SOS with 0.0001 - 500 µM fipronil or imidacloprid. The insecticides were initially diluted in dimethylsulphoxide (DMSO), before diluting to final concentrations in SOS. Final concentrations of 1% DMSO did not affect electrophysiological readings. Oocytes were initially incubated with a perfusion of the insecticide in SOS for 3 min (fipronil) or 6 min (imidacloprid) before challenging with 1 mM GABA plus insecticide, at the required concentrations. Inhibition curves were calculated by normalising the responses to the previous control response induced by 1 mM GABA.

Data analysis

Data are presented as mean ± SEM of individual oocytes from at least 3 separate frogs. The concentration of GABA required to evoke 50% of the maximum response (EC_{50}), the concentration of insecticide required to inhibit 50% of the maximal GABA response (IC_{50}) and the Hill coefficient (nH) were determined by non-linear regression using Graphpad Prism 5 (Graphpad Software, CA, USA). Statistical significance was determined as $P < 0.05$, performed using one-way ANOVA (Graphpad Software, CA, USA).

Results

Cloning of the full length *Rdl* coding sequencing from *A. mellifera*

The full coding region of the *A. mellifera Rdl* subunit was amplified by reverse-transcriptase PCR and cloned into the pCI plasmid. Sequencing of twelve clones revealed them all to be the bd splice variant (ffrench-Constant and Rocheleau, 1993; Jones and Sattelle, 2006), which is consistent with findings that this is the most predominant variant in *Drosophila melanogaster* (Jones *et al.*, 2009). However, the nucleotide sequences encoded for open reading frames with three different lengths of 476 (3 clones), 477 (4 clones) and 483 amino acids (5 clones) (Fig. 1). Comparison of these sequences with that of the *A. mellifera* genome (Honeybee Genome Sequencing Consortium, 2006) revealed that the use of the ag splice acceptor site (Sahebi *et al.*, 2016) at different positions for exon 9 resulted in varying lengths of the TM3-TM4 intracellular loop (Fig. 2). Two of these variants had been previously identified and were denoted Amel_RDL short and Amel_RDL long (Jones and Sattelle, 2006). Here, they have been renamed to Amel_RDLvar1 and Amel_RDLvar2, respectively. The third, and longest, variant is novel and is denoted Amel_RDLvar3.

Sensitivity of *X. laevis* expressed RDL splice variant receptors to GABA and fipronil

The three RDL receptor variants were generated for expression in *X. laevis* oocytes; Amel_RDLvar1, Amel_RDLvar2, and Amel_RDLvar3. The potential association between receptor splice variant and maximum current (*I*max) was evaluated by recording the 1 mM GABA response obtained immediately before the start of an experiment, on oocytes clamped at -60 mV (Table 1). The *I*max values recorded were not significantly different. GABA concentration response curves were generated with concentrations ranging from 0.1 µM – 2 mM for each variant (Fig. 3). The EC_{50} values for the three receptor variants were not significantly different from each other (Table 1).

The contribution of the splice variant to insecticide sensitivity was evaluated with fipronil, which is known to act on RDL (Raymond-Delpech et al., 2005). Inhibition curves were generated using 0.0001 – 10 µM fipronil (Fig. 4). IC_{50} values for the three receptor variants were not significantly different from each other (Table 1).

Sensitivity of *X. laevis* expressed RDL splice variant receptors to imidacloprid

Neonicotinoids, such as imidacloprid, act as agonists on their primary molecular targets, nicotinic acetylcholine receptors (Millar and Denholm, 2007). However there is also evidence that imidacloprid acts as an antagonist on the GABA receptor, shown by a reduction in GABA-induced responses in cultured honey bee Kenyon cells (Deglise *et al.*, 2002) and

directly on the *An. gambiae* RDL expressed in *Xenopus* oocytes (Taylor-Wells et al., 2015). The sensitivity of *A. mellifera* RDL and the effects of the intracellular loop variants to imidacloprid were investigated (Fig. 5). Imidacloprid was found to be an antagonist of *A. mellifera* RDL. However, in the range of 0.001 – 500 μM imidacloprid inhibition was not sufficient to generate inhibition curves or IC_{50} values (data not shown). Therefore inhibition was recorded at 100 μM for comparison. The *A. mellifera* variants were not significantly different to one another at this concentration. However in a comparison with *An. gambiae* RDL, the sensitivity of Amel_RDLvar1 and Amel_RDLvar2 to 100 μM imidacloprid was significantly reduced from that of *An. gambiae* at the same concentration (Fig. 5B).

Discussion

We report here the cloning and functional expression of the *A. mellifera* RDL GABA receptor. We identified three splice variants of the honey bee RDL arising from the differential use of the ag splice acceptor site (Fig. 2). This results in sequence variation and length in the large intracellular loop. Recently, two variants of *C. lividipennis* RDL were reported, the longer variant having a 31 amino acid insertion in the intracellular loop (Jiang et al., 2015). Interestingly, the site of insertion disrupts a TVR motif conserved in both *A. mellifera* and *C. lividipennis* RDL (Fig. 1). The TVR sequence is a protein kinase C phosphorylation consensus site (Sigrist et al., 2013). Along with putative casein kinase II phosphorylation sites also commonly found in the intracellular loop of RDL (Jones and Sattelle, 2006), these sites are predicted based on a database of protein families and domains from which signature sequences have been derived (Sigrist et al., 2013). A similar situation is evident in *D. melanogaster* RDL where one variant has the TVR motif (Accession NP_729462), which is disrupted by a 29 amino acid insertion in another variant (NP_523991). The 31 and 29 amino acid insertions in *C. lividipennis* and *D. melanogaster* RDL, respectively, are not similar to each other nor are they conserved in the *A. mellifera* genome, highlighting that the insertion sequences are species specific. Since phosphorylation of the intracellular loop of cysLGICs can modulate turnover, assembly, subcellular localization, desensitization, insecticide sensitivity and interactions with other proteins (Stokes et al., 2015; Talwar and Lynch, 2014; Thany *et al.*, 2007; Bermudez and Moroni, 2006), the differential splicing has the potential to affect several receptor properties, assuming the TVR site is phosphorylated.

Similar to *C. lividipennis* RDL (Jiang et al., 2015), we found that the intracellular loop variants of *A. mellifera* RDL had no effect on GABA EC_{50} (Table 1). Also, as with *C. lividipennis*, there was no significant difference in maximal GABA responses (Table 1). However, whereas the 31 amino acid insertion in *C. lividipennis* RDL resulted in a decrease in fipronil sensitivity (Jiang et al., 2015), we observed that the three honey bee RDL variants had similar fipronil IC_{50} values (Table 1). We therefore speculate that the insertion sequence, as opposed to disruption of the putative TVR phosphorylation site, had an effect on insecticide sensitivity. These findings highlight an intriguing species-specific route to altering target site sensitivity to insecticides. Thus, even though the site of insertion is highly conserved, the actual sequence introduced may mediate certain effects dependant on the insect species. It will be of interest to see whether RDL of pest species also show a similar mechanism to diversify the *Rdl* transcriptome. If so, these variants can be monitored to determine whether the expression of certain isoforms is altered in association with insecticide exposure/resistance, thereby identifying novel routes to insecticide insensitivity in the field. Furthermore, perhaps these species-specific variants can be exploited to develop strategies to control pests whilst sparing non-target organisms. For example, sequences of

these variants in pest species could be targeted by RNA interference, a newly emerging technology for crop protection (Lombardo *et al.*, 2015).

It remains to be determined what the roles are for the RDL intracellular loop variants. Other insect cysLGICs also have intracellular loop variants, such as the long and short variants of the *A. mellifera* α3 nicotinic acetylcholine receptor subunit (Jones et al., 2006), the function of which is also unknown. In this regard, it is interesting to note that the vertebrate GABAA γ2 subunit has short (γ2S) and long (γ2L) variants differing by an 8 amino acid insertion in the intracellular loop, the long version of which has a potential protein kinase C phosphorylation site (Whiting *et al.*, 1990). γ2S and γ2L show different distribution patterns in the adult brain (Gutierrez *et al.*, 1994). Green fluorescent protein-tagging studies revealed that γ2L displayed a significantly higher capacity to accumulate at inhibitory synapses than γ2S, with phosphorylation of the protein kinase C site in γ2L playing a role in this postsynaptic clustering (Meier and Grantyn, 2004). Work on cell lines indicate that γ2S can act as an accessory protein, modulating functional properties of GABAA receptors without being incorporated in the ion channel-forming pentamer (Boileau *et al.*, 2010). Several studies have shown that specific sequence or structural motifs in the intracellular loop mediate interaction with other proteins. For example, the 14-3-3 adaptor protein appears to bind the α3 nAChR loop at an RSSSSES consensus sequence or interacts with a coiled-coil motif (Rosenberg *et al.*, 2008). Concerning RDL, further experiments are required to confirm whether the intracellular loop and its variants play similar important roles in insects. Also, future studies are required to determine which sites in the intracellular loop of insect cysLGICS are phosphorylated.

We show that the neonicotinoid, imidacloprid, acts directly on *A. mellifera* RDL (Fig. 5) as an antagonist, consistent with findings that it reduced GABA-induced responses in cultured honey bee Kenyon cells (Deglise et al., 2002). All three Amel_RDL variants showed similar sensitivity to imidacloprid. We did, however, observe that the honey bee RDL was less sensitive to the antagonistic effects of imidacloprid than *An. gambiae* RDL (Fig. 5B). The A296G mutation in TM2 of *An. gambiae* RDL abolished imidacloprid action, suggesting the neonicotinoid is acting as a channel blocker (Taylor-Wells et al., 2015). The TM2 peptide sequences of *A. mellifera* and *An. gambiae* RDL are identical, indicating that residues other than those lining the ion channel are involved in the differential sensitivities of the two species. Since neonicotinoids interact with the agonist binding domain in nAChRs (Ihara *et al.*, 2015), perhaps the action of imidacloprid on RDL is influenced by residues in the N-terminal extracellular domain. The concentration of imidacloprid required to antagonise *A. mellifera* or *An. gambiae* RDL is notably high and it remains to be determined whether RDL plays any role in the insecticidal effects of neonicotinoids. Novel and selective chemistry that

both activates nAChRs and inhibits GABA receptors, or a combination of molecules with these activities, could be additive in the impact on neuronal excitability and represent a new generation of improved insecticides.

Single-cell reverse transcriptase-PCR on *A. mellifera* antennal lobe (AL) cells showed the expression of RDL as well as another GABA receptor like subunit, LCCH3 (Dupuis et al., 2010). Based on whole cell patch-clamp electrophysiology it was concluded that the predominant GABA receptors in AL cells are homomeric RDL complexes (Dupuis et al., 2010). In line with this, the GABA EC_{50} observed in AL cells (12.3 ± 0.33 μM) is similar to that of homomeric Amel_RDL expressed in *Xenopus* oocytes (Table 1). The GABA receptors in AL cells, however, appear to be slightly less sensitive to fipronil (IC_{50} 0.823 ± 0.19 μM) when compared to Amel_RDL in this study (Table 1). This difference could be due to the presence of RDL + LCCH3 heteromeric receptors in AL cells or perhaps the association of GABA receptors with accessory proteins found in native cells that may affect ion channel properties (Dupuis et al., 2010).

Conclusions

It is concluded that the diversity of the insect GABA receptor, RDL, is broadened by differential splicing in the TM3-TM4 intracellular loop. These variants do not appear to modulate GABA potency but can affect the actions of fipronil in a species-specific manner. When studying RDL from a certain species, several clones should be analysed to ascertain whether there are intracellular loop variants. A major challenge is to determine whether the intracellular loop variants are expressed in different tissues, at different development stages or co-assemble to form a heterogeneous population of ion channels. The heterologous expression of RDL variants found in *A. mellifera* may provide a useful counter-screen tool to assess the effects of agrochemical compounds on the GABA receptor of an important pollinator.

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Figure legends

Fig. 1 Amino acid sequence alignment of *A. mellifera* and *C. lividipennis* RDL intracellular loop variants. The whole subunit is shown with N-terminal signal leader peptides in lower case and the four transmembrane regions (TM1-4) indicated. Additional amino acid residues introduced by differential splicing are underlined. Potential protein kinase C and casein kinase II phosphorylation sites are highlighted in grey shading. The sequences used in the alignment are as follows: *A. mellifera* Amel_RDLvar1 (KU201321), Amel_RDLvar2 (KU201322), Amel_RDLvar3 (KU201323);*C. lividipennis* ClivRDL (KJ174466), ClivRDL-In (KJ174465).

Fig. 2 Use of the common ag splice acceptor site (Sahebi et al., 2016) at different positions of *A. mellifera* RDL generates TM3-TM4 intracellular loops with varying lengths. The splice acceptor sites are highlighted in black shading.

Fig. 3 Responses to GABA in *X. laevis* oocytes expressing three *A. mellifera* RDL receptor variants. A) Representative current trace of a GABA concentration response curve showing responses to GABA from 0.1 µM – 2 mM for Amel_RDLvar2. B) GABA concentration response curves obtained for Amel_RDLvar1, Amel_RDLvar2 and Amel_RDLvar3 receptors. Data were normalised to the maximal response (1 mM GABA). Data is the mean ± SEM from n = 4-6 oocytes from 3-4 different frogs.

Fig. 4 Effects of fipronil on 1 mM GABA activated currents in *X. laevis* oocytes expressing three *A. mellifera* RDL receptor variants. A) Representative current traces showing the effect of 0.0001 - 10 µM fipronil on the 1 mM GABA response for Amel_RDLvar2. B) Fipronil inhibition curves for Amel_RDLvar1, Amel_RDLvar2 and Amel_RDLvar3 receptors generated with concentrations from 0.0001 µM – 10 µM. Each data point was normalised to the response to 1 mM GABA. Data are the mean ± SEM from n = 3-4 oocytes from 3 different frogs.

Fig. 5 Effects of 100 µM imidacloprid on 1 mM GABA activated currents in *X. laevis* oocytes expressing three *A. mellifera* RDL receptor variants. A) Representative current traces

showing the effect of 100 μM imidacloprid on the 1 mM GABA response for *An. gambiae* RDL and Amel_RDLvar1. B) Effects of 100 μM imidacloprid on the 1 mM GABA response for *An. gambiae* RDL (AgamRDL), Amel_RDLvar1, Amel_RDLvar2 and Amel_RDLvar3. Data are expressed as a percentage of the 1 mM GABA response. Data are the mean ± SEM from n = 5 oocytes from 3-4 different frogs. * indicates significant difference from *An. gambiae* RDL ($P < 0.05$). The reductions in response of the three *A. mellifera* RDL variants were not significantly different from each other.

Tables

Table. 1 Effects of GABA on membrane currents from *X. laevis* oocytes expressing *A. mellifera* RDL receptor splice variants, with maximum amplitude (*I*max), EC_{50} and hill coefficient (nH) displayed. The *I*max was obtained from the initial 1 mM GABA response obtained from eggs clamped at -60 mV. *I*max data are the mean ± SEM of n= 8-10 oocytes from ≥ 3 different frogs. Also shown are the effects of fipronil and imidacloprid on 1 mM GABA induced membrane currents, with IC_{50} values displayed for fipronil and 100 μM responses for imidacloprid. EC_{50}, IC_{50} and imidacloprid data are the mean ± SEM of n=3-6 oocytes from ≥ 3 different frogs. Values were not significantly different.

RDL splice variant	*I*max (μA)	GABA		Fipronil IC_{50} (μM)	% of GABA response with 100 μM Imidacloprid
		EC_{50} (μM)	nH		
Amel_RDLvar1	12.7 ± 2.5	29 ± 3	1.02 ± 0.08	0.18 ± 0.08	83 ± 1
Amel_RDLvar2	7.6 ± 1.2	20 ± 2	1.13 ± 0.06	0.31 ± 0.16	76 ± 9
Amel_RDLvar3	8.4 ± 1.5	29 ± 8	1.70 ± 0.15	0.20 ± 0.04	69 ± 3

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AmelRDL var1 : MSFHAASWSFALLAATVAL--LPATHRAPFAQAATGGGSMLNDVNISAILDSFSVSYDKR :  58
AmelRDL var2 : MSFHAASWSFALLAATVAL--LPATHRAPFAQAATGGGSMLNDVNISAILDSFSVSYDKR :  58
AmelRDL var3 : MSFHAASWSFALLAATVAL--LPATHRAPFAQAATGGGSMLNDVNISAILDSFSVSYDKR :  58
ClivRDL      : MRAALASWAFLTLAATLIHPRTPFAYAGEPERTVSGGGSMLGDVNISAILDSFSVSYDKR :  60
ClivRDL In   : MRAALASWAFLTLAATLIHPRTPFAYAGEPERTVSGGGSMLGDVNISAILDSFSVSYDKR :  60

AmelRDL var1 : VRPNYGGPPVEVGVTMYVLSISSLSEVKMDFTLDFYFRQFWTDPRLAFKKRTGVETLSVG : 118
AmelRDL var2 : VRPNYGGPPVEVGVTMYVLSISSLSEVKMDFTLDFYFRQFWTDPRLAFKKRTGVETLSVG : 118
AmelRDL var3 : VRPNYGGPPVEVGVTMYVLSISSLSEVKMDFTLDFYFRQFWTDPRLAFKKRTGVETLSVG : 118
ClivRDL      : VRPNYGGPPVEVGVTMYVLSISSVSEVLMDFTLDFYFRQFWTDPRLAFRKRPGVETLSVG : 120
ClivRDL In   : VRPNYGGPPVEVGVTMYVLSISSVSEVLMDFTLDFYFRQFWTDPRLAFRKRPGVETLSVG : 120

AmelRDL var1 : SEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFP : 178
AmelRDL var2 : SEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFP : 178
AmelRDL var3 : SEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFP : 178
ClivRDL      : SEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFP : 180
ClivRDL In   : SEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFP : 180

AmelRDL_var1 : MDRQLCHIEIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGN : 238
AmelRDL var2 : MDRQLCHIEIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGN : 238
AmelRDL var3 : MDRQLCHIEIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGN : 238
ClivRDL      : MDRQLCHIEIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGN : 240
ClivRDL In   : MDRQLCHIEIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGN : 240

                           ---------TM1---------           ----------TM2-
AmelRDL var1 : YSRLACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTL : 298
AmelRDL var2 : YSRLACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTL : 298
AmelRDL var3 : YSRLACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTL : 298
ClivRDL      : YSRLACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTL : 300
ClivRDL In   : YSRLACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTL : 300

               ---------           --------TM3---------
AmelRDL var1 : MSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKNRFQKIAESMK : 358
AmelRDL var2 : MSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKNRFQKIAESMK : 358
AmelRDL var3 : MSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKNRFQKIAESMK : 358
ClivRDL      : MSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKNRFLAIQKLAE : 360
ClivRDL In   : MSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKNRFLAIQKLAE : 360

AmelRDL var1 : TARENPGPPGVPGDHGDHAPKQT------------------------------VRFKVH : 387
AmelRDL var2 : TARENPGPPGVPGDHGDHAPKQT-------------------------------EVRFKVH : 388
AmelRDL var3 : TARENPGPPGVPGDHGDHAPKQT-----------------------WSRVVQEVRFKVH : 394
ClivRDL      : QKQKSLESHAGPGDS-DHAPKQT------------------------------VRYKVH : 388
ClivRDL In   : QKQKSLESHAGPGDS-DHAPKQTRDPSIICGSYAATLPSKPVHPERRQQMQQTEVRYKVH : 419

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AmelRDL var1 : DPKAHSKGGTLENTINGRADEEAAPAPQHLIHPGKDINKLYGMTPSDIDKYSRIVFPVCF : 447
AmelRDL var2 : DPKAHSKGGTLENTINGRADEEAAPAPQHLIHPGKDINKLYGMTPSDIDKYSRIVFPVCF : 448
AmelRDL var3 : DPKAHSKGGTLENTINGRADEEAAPAPQHLIHPGKDINKLYGMTPSDIDKYSRIVFPVCF : 454
ClivRDL      : DPKAHFKGGTLENTINGRPEEEAIP--QHLIHPTKDYNKLFGLTSSDIDKYSRIVFPVCF : 446
ClivRDL In   : DPKAHFKGGTLENTINGRPEEEAIP--QHLIHPTKDYNKLFGLTSSDIDKYSRIVFPVCF : 477

               --TM4---------
AmelRDL var1 : VCFNLMYWIIYLHISDVVADDLVLLEEAK : 476
AmelRDL var2 : VCFNLMYWIIYLHISDVVADDLVLLEEAK : 477
AmelRDL var3 : VCFNLMYWIIYLHISDVVADDLVLLEEAK : 483
ClivRDL      : ICFNLMYWIIYLHISDVVADDLVLLEEGK : 475
ClivRDL In   : ICFNLMYWIIYLHISDVVADDLVLLEEGK : 506
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