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Characterization and comparative pharmacological studies of a functional γ-aminobutyric acid (GABA) receptor cloned from the tobacco budworm, *Heliothis virescens* (Noctuidae:Lepidoptera)

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ABSTRACT This paper reports the functional expression and pharmacological characterization of a full length complementary deoxyribonucleic acid (cDNA) (pIVY12) cloned from a *Heliothis virescens* fertilized egg cDNA library that encodes for a γ-aminobutyric acid (GABA) receptor subunit (HVRDL-Ser 285). Two electrode voltage clamp recordings of *Xenopus* oocytes expressing the HVRDL GABA-gated chloride channel revealed robust chloride ion conductance in response to GABA and the $GABA_A$ receptor agonist, muscimol. Baclofen, a $GABA_B$ agonist had no effect. Phenobarbital showed a positive dose-dependent allosteric modulatory effect, whereas the benzodiazepine, flunitrazepam, had no effect. Chloride conductance was depressed by the novel insecticide, fipronil ((±)-5-amino-1-(2,6 dichloro-α, α, α-trifluoro-p-tolyl)-4-trifluoromethyl-sulfinylpyrazole-3-carbonitrile) and the $GABA_A$ antagonist, picrotoxinin. The HVRDL GABA receptor was insensitive to blockage by dieldrin and the $GABA_A$ antagonist, bicuculline. The comparative actions of fipronil, picrotoxinin and dieldrin were examined on oocytes expressing the *H. virescens* wild-type (HVRDL-Ser 285), the site-directed mutant (HVRDL-Ala 285), the *Drosophila melanogaster Rdl* wild-type (DMRDL-Ala 302) and the *Rdl* dieldrin resistant (DMRDL-Ser 302) homo-oligomeric GABA receptors. HVRDL-Ala 285 was 15-fold more sensitive to blockage by fipronil than HVRDL-Ser 285. DMRDL-Ala 302 and DMRDL-Ser-302 showed a similar level of sensitivity to blockage by fipronil. HVRDL-Ser 285 and DMRDL-Ser 302 exhibited a similar level of insensitivity to picrotoxinin. HVRDL-Ala 285 and DMRDL-Ala 302 showed a similar range of picrotoxinin sensitivity. DMRDL-Ala 302 and HVRDL-Ala 285 showed some sensitivity to blockage by dieldrin. Fipronil sensitivity was significantly altered by the serine to alanine mutation at position 285 in the M2 region of the HVRDL subunit, whereas no difference was observed between the DMRDL-Ser 302 and DMRDL-Ala 302 receptors.

KEY WORDS: GABA receptor; *H. virescens*; *Xenopus* oocyte; fipronil

Introduction

γ-amino butyric acid (GABA)-gated chloride channels play a critical role in the inhibitory modulation of neurotransmission in the vertebrate central nervous system (Olsen and Tobin, 1990). GABA-gated chloride channels are equally important in the insect, where GABA acts as the major inhibitory neurotransmitter both in the central nervous system (CNS) and at the neuromuscular junction. Chemicals interfering with this inhibition have proved to be effective insecticides (Anthony *et al.*, 1993; Casida, 1993; Bloomquist, 1993).

The binding of GABA to $GABA_A$ receptors results in the opening of chloride-selective channels that are intrinsic to $GABA_A$ receptors. In vertebrates, at least six different $GABA_A$ subunit type complementary deoxyribonucleic acids (cDNAs) have been cloned (α, β, γ, δ, ε, π). For the most part, functional vertebrate $GABA_A$ receptors are hetero-oligomeric (Olsen and Tobin, 1990).

In contrast to the extensive knowledge of vertebrate $GABA_A$ receptors, less is known about the invertebrate system. ffrench-Constant and co-workers used a field-isolated *Drosophila* mutant *Rdl* (insensitive to picrotoxinin and dieldrin) to clone the *Rdl* GABA receptor (ffrench-Constant *et al.*, 1990, 1991). The *Drosophila Rdl* subunit was shown to form functional homo-oligomeric channels when expressed in *Xenopus laevis* oocytes. The insensitivity to picrotoxinin and dieldrin was associated with a point mutation, resulting in an amino acid substitution of an alanine to serine in the second transmembrane domain, which is thought to line the pore of the chloride ion channel. This point mutation was found to be responsible for conferring insensitivity to GABA-gated chloride channel blockage by picrotoxinin and dieldrin in resistant *Drosophila* (ffrench-Constant *et al.*, 1993).

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GABA receptor subunits cloned from *D. melanogaster* include: *Rdl* (ffrench-Constant *et al.*, 1991); LCCH3 (Henderson *et al.*, 1993); GRD (Harvey *et al.*, 1994); and a *Rdl D. melanogaster* homolog with 90.2% nucleotide identity to *Rdl* (Chen *et al.*, 1994). *Rdl* homologs have also been identified from *Aedes aegypti* (Thompson *et al.*, 1993). Until the present time, only the *Rdl* subunit cloned from both dieldrin resistant and dieldrin susceptible *D. melanogaster* has been shown to form functional homo-oligomeric GABA-gated chloride channels.

The recent success in cloning of insect GABA receptor subunits and their functional heterologous expression as homo-oligomeric GABA-gated chloride channels has provided a unique opportunity to study the detailed pharmacology of pesticides at their site of action (Harvey *et al.*, 1991; ffrench-Constant *et al.*, 1991, 1993; Thompson *et al.*, 1993; Henderson *et al.*, 1993; Casida, 1993; Deng *et al.*, 1993; Chen *et al.*, 1994). The insect GABA receptor is the site of action for several pesticides (Lummis, 1990; von Keyserlingk and Willis, 1992; Bloomquist, 1993; Casida, 1993). Binding studies with novel synthetic radioligands have confirmed the nature of cyclodienes as GABA-gated chloride channel blockers (Lawrence and Casida, 1984). Ultimately, electrophysiological data have confirmed the particular physiological effects of cyclodienes on the insect GABAergic system (Wafford *et al.*, 1989).

Recently, the insect GABAergic system and the GABA-gated chloride channel have been implicated as the site of action for a new class of phenylpyrazole chemistry. The mode of action of the first commercial phenylpyrazole insecticide, fipronil ((±)-5-amino-1-(2,6 dichloro-α, α, α-trifluoro-p-tolyl)-4-trifluoromethyl-sulfinylpyrazole-3-carbonitrile)), was ascertained by a combination of electrophysiological tests, binding assays and bioassay (Gant *et al.*, 1990; ffrench-Constant *et al.*, 1993; Cole *et al.*, 1993; Millar *et al.*, 1994). These experiments indicate that fipronil acts as an antagonist at the GABA-gated chloride channel.

Cloning, expression and pharmacological characterization of a lepidopteran GABA subunit (HVRDL) from the tobacco budworm *H. virescens* has revealed an insect GABA receptor with important differences in sequence and pharmacology from previously cloned and expressed homo-oligomeric insect GABA-gated chloride channels. In particular, the second membrane spanning region of the HVRDL subunit contains a serine residue at the analogous position thought to confer cyclodiene insensitivity in *Drosophila* (ffrench-Constant *et al.*, 1993).

The present study examines the comparative antagonist pharmacology of fipronil, a novel phenylpyrazole insecticide, picrotoxinin, a $GABA_A$ receptor antagonist and noncompetitive channel-blocking convulsant, and dieldrin, a cyclodiene insecticide, on the wild-type *H. virescens* GABA receptor subunit, HVRDL -Ser 285, the site-directed mutant of HVRDL-Ala 285, the *D. melanogaster Rdl* dieldrin resistant mutant, DMRDL-Ser 302, and the wild-type DMRDL-Ala 302 subunit. These subunits have been expressed as functional homo-oligomeric GABA-gated chloride channels in *Xenopus* oocytes.

Materials and Methods

Animals

The *H. virescens* strain used was obtained from North Carolina State University and subsequently cultured in-house. *H. virescens* fertilized eggs (embryos) at various stages of development were harvested, snap frozen in liquid nitrogen and used to make messenger ribonucleic acid (mRNA).

Isolation of a full length *H. virescens Rdl* cDNA clone

Genomic DNA was obtained from second instar *H. virescens* larvae by the modified cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1988). The genomic DNA was amplified by the polymerase chain reaction (PCR) using degenerate primers vw121403 (5′-GGTCTAGAACIAC IGTICTTACI-ATGAC) and vw123002 (5′-GGCTCGAGGC (A/G)AAIACCATIAC (A/G)AA(A/G)CA) and Amplitaq polymerase (Perkin Elmer, Norwalk, CT, USA). The region amplified was a 70 bp DNA fragment encoding amino acids between membrane spanning domains 1 and 2 of HVRDL.

Total cellular RNA was isolated directly from fertilized eggs in a phenol, 0.1 M Tris-HCl emulsion, pH 9.0, and was purified as described previously (Lawton *et al.*, 1983). PolyA^+ mRNA was isolated from the total cellular RNA using the PolyATtract mRNA isolation system and following the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

Rapid amplification of cDNA ends (RACE) was used to obtain the 3′ end of the HVRDL cDNA (Frohman *et al.*, 1988). One microgram of polyA^+ mRNA was used with the 3′ RACE kit (GIBCO-BRL, Grand Island, NY, USA) utilizing as a specific primer vw05802 (5′-AGGTCCATCGATGTCTATCT-GGGAA), derived from the 70 bp fragment. The amplified DNA fragment was cloned into the plasmid pKS$^+$ (Stratagene, La Jolla, CA, USA) to produce a plasmid, designated pIVY7. Because the anchor primer had annealed to an A-rich region in the *H. virescens*

coding region, the 3′ coding region of the HVRDL cDNA was not complete. Therefore, a second 3′-RACE was performed, utilizing as the specific primer vw112293-301 (5′-GTTCACGATCCGAAG-GCATATTCT). The amplified product was cloned into plasmid pKS+ to generate a plasmid, designated pIVY10.

A specific primer, vw040401 (5′-AACTTGCTC-GAGACTTGATGGAT), was synthesized based upon the non-translated 3′ end of the HVRDL mRNA previously identified by 3′ RACE. Primer vw040401 was engineered to contain a XhoI site and was substituted for the first strand primer in the lambda Zap cDNA synthesis system (Stratagene). Five micrograms of polyA$^+$ mRNA isolated from fertilized eggs was used to make a cDNA library in the lambda ZAP expression cloning system (Stratagene), and was packaged with the Gigapack II packaging system (Stratagene) following the manufacturer's instructions. A non-amplified library of 5×10^5 recombinants was made and then amplified.

The amplified library was screened with ^{32}P random primer labelled probes of the 3′ RACE inserts of plasmids pIVY7 and pIVY10, using standard techniques (Maniatis *et al.*, 1982). One positive clone was identified and plaque purified. The phagemid, pBK-CMV, containing the insert was excised from the phage. Double-stranded DNA sequencing of the insert was accomplished by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase system (Amersham, Arlington Heights, IL, USA).

Site-directed mutagenesis

A mutated *H. virescens Rdl* protein was obtained, in which the serine residue 285 (encoded by nucleotides TCA) was mutated to an alanine (encoded by nucleotides GCA) following the method of Ho *et al.* (1989). Two oligonucleotides with the following sequences were used as primers for the PCR: primer vw091201-94, 5′-TGCAAGGGTA GCACTAG-GTGT; and primer vw091202-94, 5′-ACACCTAGT-GCTA CCCTTGCA. Two other primers used were the T7 and T3 promoters. Plasmid pIVY12 was amplified by PCR using Pfu polymerase (Stratagene) in two separate PCRs; one with primers vw091201-94 and T7 and the other with primers vw091201-94 and T3.

The two DNA fragments were purified from agarose gels using the QIAEX DNA gel extraction procedure (Qiagen, Chatsworth, CA, USA), and 0.3 μg of each DNA fragment was amplified by PCR using the T3 and T7 primers. The resulting 2 kbp DNA fragment (encompassing the coding region of *H. virescens Rdl*) was digested with BamH1 and XhoI and then ligated into pBluescript II SK+vector (Stratagene) to yield pIVY16. The DNA sequence of the mutation in pIVY16 was verified by double-stranded DNA sequencing, using the dideoxy chain termination method (Sanger *et al.*, 1977) with the Sequenase system (Amersham).

Preparation of messenger RNA

The cDNA clone, pIVY12 (HVRDL-Ser 285), was linearized with the restriction endonuclease Not I and RNA transcripts were synthesized using the SP6 polymerase. The cDNA clone, pIVY16 (HVRDL-Ala 285), was linearized with XhoI and RNA transcripts were synthesized using T3 polymerase. The cDNA clones, pNB14.1 (DMRDL-Ala 302) and pNB14.1M (DMRDL-Ser 302), were linearized with the Not I and RNA transcripts were synthesized using SP6 polymerase. The linearized plasmids were diluted to a final concentration of 1 μg/ul with RNAse-free water. The *D. melanogaster* cDNAs (pNB14.1 GenBank accession number M69057) were obtained from Richard ffrench-Constant (University of Wisconsin at Madison) and Rick Roush (Cornell University). Restriction endonucleases were obtained from Gibco BRL (Grand Island, NY, USA) and all RNA transcripts were synthesized using the mMessage mMachine T3 or SP6 *In Vitro* transcription kit (Ambion, Austin, TX, USA).

Oocyte preparation and injection

Oocytes were removed from anaesthetized (0.2% solution of 3-aminobenzoic acid ethyl ester, methane sulphonate salt; Sigma Chemical Co., St. Louis, MO, USA) *Xenopus laevis* females (Nasco, Atkinson, WI, USA) and were treated with collagenase (Sigma type IA, 2 mg/ml in calcium-free OR-2 saline consisting of NaCl 82.5 mM, KCl 2 mM, $MgCl_2$ 1 mM, hydroxyethylpiperazine ethanesulphonic acid (HEPES) 5 mM, pH 7.5). Following collagenase treatment, oocytes were transferred to ND-96 saline (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.5) supplemented with 2.5 mM sodium pyruvate, streptomycin (1 mg/ml) and penicillin (1000 U/ml; Sigma Chemical Co.).

Oocytes were each injected (Drummond Series 500 Microinjector, Broomhall, PA, USA) with 50 nl of mRNA (1 ng/nl), placed on a rotator/shaker set to 35 rpm at room temperature and allowed to incubate for 24–48 h, with replacement of saline at 12 h intervals.

Electrophysiological recordings

Measurements of membrane current were made using a two electrode voltage clamp amplifier (TEV 200, Dagan Corp., Minneapolis, MN, USA). Glass electrodes (A-M Systems, Inc. Glass, Cat. # 6030, Everett,

WA, USA) were filled with 3M KCl. Electrodes with a resistance between 0.5 and 3.0 MW were used for recording. Oocytes were held at a resting potential between -70 and -50 mV under constant perfusion with ND-96 saline (Razel syringe perfusion pump, Stanford, CT, USA; model A99-FY at 93.9 cc/h). Recordings were made using the MacLab Digital Interface Module and Chart software (ADI Instruments, Inc. Milford, MA, USA) 24–48 h after injection of mRNA.

Control responses of GABA were obtained by perfusing the oocytes with a known GABA (Sigma Chemical Co., USA) concentration in ND-96 until maximal response was observed. The average of several GABA applications was taken as the maximal current.

10 μM stock solutions of flunitrazepam, pregnenolone, phenobarbital (Research Biochemicals International, Natick, MA, USA), picrotoxinin (Sigma Chemical Co., USA) and fipronil (99.9% pure) (Rhône-Poulenc Ag Co., RTP, NC, USA) were prepared in dimethylsulphoxide (DMSO) (EM Science, Gibbstown, NJ, USA). Perfusion solutions of the compounds were made with the stock solutions dissolved in ND-96 saline (net 0.1% DMSO).

Following two to three applications of control GABA, the oocyte was perfused with the compound of interest for 2–3 min. This was followed by co-perfusion with a solution of the appropriate compound and GABA. The oocyte was then perfused with a saline until recovery. Chloride currents obtained in the presence of an inhibitor were compared to the control GABA-evoked currents in the absence of an inhibitor or modulator and represented as a percentage of maximal response.

Quantitative data are reported as the mean±standard error of the mean (SEM). Values of median inhibitory concentration (IC_{50}) and the concentration producing half the maximal effect (EC_{50}) were calculated using a nonlinear curve-fitting program (Microcal Origin, Northhampton, MA, USA). Hill coefficients were calculated using least-squares fit linear regression. The EC_{50} was calculated from a Boltzman model nonlinear regression analysis ($Y=A2+(A1-A2)/(1+EXP((X-X_0)/dx))$ where A1= the upper asymatope and A2 = the lower asymatope of the Boltzman function. The error interval represents two times the error associated with the nonlinear best fit analysis, as described above.

The Boltzman model provides a robust curve-fitting equation for dose-response experiments, as it does not assume normal and independent distribution of error and a common variance associated with the mean across the dose-response curve.

Results

HVRDL cloning

One phagemid clone, designated pIVY12, that contained an insert of approximately 2.1 kbp cloned into the EcoRI/XhoI site of the polylinker of phagemid pBK-CMV was isolated. DNA sequencing indicated that pIVY12 encodes a full length HVRDL clone (GenBank accession number AJ224513). The coding sequence starts at 115 bp from the 5′ end of the cDNA and ends at 1596 bp, and encodes a polypeptide of 494 amino acids (Fig. 1A). The pIVY12 HVRDL nucleic acid and amino acid sequence is homologous to an unpublished *H. virescens* cDNA (GenBank accession number AF006189), with the exception of an insertion of a proline at positions 387 and 419 of the AF006189 sequence (positions 455 and 504 in Fig. 1A). Secondly, in the AF006189 amino acid sequence there is an alanine in position 327 (position 343 in Fig. 1A), whereas in pIVY12 there is a threonine.

The pIVY12 HVRDL amino acid sequence is more homologous to the *D. melanogaster* splice variant, DRC17-1-2, than the *D. melanogaster* splice variant, NB14.1, in the regions of exon 3 (positions 80 to 105) and exon 6 (positions 207 to 255) (Chen *et al.*, 1994). The intracellular loop between membrane spanning domains 3 and 4 is different both in sequence composition and length between the *D. melanogaster Rdl* sequence and the *H. virescens Rdl* sequence.

Site-directed mutagenesis

Mutation of the HVRDL protein resulted in the substitution of the serine residue 285 (encoded by nucleotides TCA) to an alanine (encoded by nucleotides GCA), with the mutant pIVY16 construct designated as HVRDL-Ala 285. Figure 1B illustrates the alignment of GABA receptor amino acid sequences (single-letter code) around domain one and two of insect GABA receptor homologs from *H. virescens* and *D. melanogaster*.

Agonist pharmacology

Uninjected oocytes showed no response to application of GABA solutions as high as 1 mM (data not shown). All oocytes injected with HVRDL and DMRDL mRNA responded to GABA. Only oocytes responding to 10 and 100 μM GABA with a resultant chloride current of >100 nA at 24–48 h after injection were used for recordings.

Figure 2 illustrates the dose-response relationship of GABA-activated chloride currents from a single oocyte expressing the HVRDL subunit, in response to increasing concentration of the agonist GABA. Bath application of the $GABA_B$ receptor agonist, baclofen (100 μM)

A.

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           1            15 16           30 31           45 46           60 61           75 76           90
NB14.1     MSDSKMDKLARMAPL PRTPLLTIWLAINMA LIAQETGHKRIHTVQ AATGGGSMLGDVNIS AILDSFSVSYDKRVR PNYGGPPVEVGVTMY
DRC17-1-2 MSDSKMDKLARMAPL PRTPLLTIWLAINMA LIAQETGNKRIHTVQ AATGGGSMLGDVNIS AILDSFSISYDKRVR PNYGGPPVEVGVTMY
IVY12      MHTS----------R PRG-VHSIALVLSLA IAWLPHAD-H----A AGAGGGGMFGDVNIS AILDSLSVSYDKRVR PNYGGPPVDVGVTMY
AF006189   MHTS----------R PRG-VHSIALVLSLA IAWLPHAD-H----A AGAGGGGMFGDVNIS AILDSLSVSYDKRVR PNYGGPPVDVGVTMY

           91          105 106         120 121         135 136         150 151         165 166         180
NB14.1     VLSISSVSEVLMDFT LDFYFRQFWTDPRLA YRKRPGVETLSVGSE FIKNIWVPDTFFVNE KQSYFHIATTSNEFI RVHHSGSITRSIRLT
DRC17-1-2 VLSISSLSEVKMDFT LDFYFRQFWTDPRLA YGKRPGVETLSVGSE FIKNIWVPDTFFVNE KQSYFHIATTSNEFI RVHHSGSITRSIRLT
IVY12      VLSISSLSEVKMDFT LDFYFRQFWTDPRLA YKKRTGVETLSVGSE FIRNIWVPDTFFVNE KQSYFHIATTSNEFI RIHHSGSITRSIRLT
AF006189   VLSISSLSEVKMDFT LDFYFRQFWTDPRLA YKKRTGVETLSVGSE FIRNIWVPDTFFVNE KQSYFHIATTSNEFI RIHHSGSITRSIRLT

           181         195 196         210 211         225 226         240 241         255 256         270
NB14.1     ITASCPMNLQYFPMD RQLCHIEIESFGYTM RDIRYFWRDGLSSVG MSSEVELPQFRVLGH RQRATEINLTTGNYS RLACEIQFVRSMGYY
DRC17-1-2 ITASCPMNLQYFPMD RQLCHIEIESFGYTM RDIRYKWNEGPNSVG VSSEVSLPQFKVLGH RQRAMEISLTTGNYS RLACEIQFVRSMGYY
IVY12      ITASCPMDLQYFPMD RQLCNIEIESFGYTM RDIRYKWNEGPNSVG VSSEVSLPQFKVLGH RQRAMEISLTTGNYS RLACEIQFVRSMGYY
AF006189   ITASCPMDLQYFPMD RQLCNIEIESFGYTM RDIRYKWNEGPNSVG VSSEVSLPQFKVLGH RQRAMEISLTTGNYS RLACEIQFVRSMGYY

           271         285 286         300 301         315 316         330 331         345 346         360
NB14.1     LIQIYIPSGLIVVIS WVSFWLNRNATPARV ALGVTTVLTMTTLMS STNAALPKISYVKSI DVYLGTCFVMVFASL LEYATVGYMAKRIQM
DRC17-1-2 LIQIYIPSGLIVVIS WVSFWLNRNATPARV ALGVTTVLTMTTLMS STNAALPKISYVKSI DVYLGTCFVMVFASL LEYATVGYMAKRIQV
IVY12      LIQIYIPSGLIVIIS WVSFWLNRNATPARV SLGVTTVLTMTTLMS STNAALPKISYVKSI DVYLGTCFVMVF  TSL LEYATVGYMAKRIQM
AF006189   LIQIYIPSGLIVIIS WVSFWLNRNATPARV SLGVTTVLTMTTLMS STNAALPKISYVKSI DVYLGTCFVMVF  ASL LEYATVGYMAKRIQM

           361         375 376         390 391         405 406         420 421         435 436         450
NB14.1     RKQRFMAIQKIAEQK KQQLDGANQQQANPN PNANVGGPGGVGVGP GGPGGPGGGVNVGVG MGMGPEHGHGHGHHA HSHGHPHAPKQTVSN
DRC17-1-2 RKQRFMAIQKIAEQK KQQLDGANQQQANPN PNANVGGPGGVGVGP GGPGGPGGGVNVGVG MGMGPEHRHGHGHHA HSHGHPHAPKQTVSN
IVY12      RKQRFTAVQKMAAEK KMQIDGP-PGSAEPI P------P------- --------------- --------------- --------PRTSTLS
AF006189   RKQRFTAVQKMAAEK KMQIDGP-PGSAEPI P------P------- --------------- --------------- --------PRTSTLS

           451         465 466         480 481         495 496         510 511         525 526         540
NB14.1     RPIGFSNIQQNVGTR GCSIVGPLFQEVRFK VHDPKAHSKGGTLEN TVNGGRGGPQSHGPG PGQGGGPPGGGGGGG GGGGPPEGGGDPEAA
DRC17-1-2 RPIGFSNIQQNVGTR GCSIVGPLFQEVRFK VHDPKAHSKGGTLEN TVNGGRGGPQSHGPG PGQGGGPPGGGGGGG GGGGPPEGGGDPEAA
IVY12      RPPP----------- ------SRLSEVRFK VHDPKAYSKGGTLEN TINGARG -----QPG P----APPADEEAG- ----PP-----P---
AF006189   RPPPP---------- ------SRLSEVRFK VHDPKAYSKGGTLEN TINGARG P----APG P----APPADEEAG- ----PP-----P---

           541         555 556         570 571         585 586         600 601
NB14.1     VPAHLLHPGKVKKDI NKLLGITPSDIDKYS RIVFPVCFVCFNLMY WIIYLHVSDVVADDL VLLGEE-
DRC17-1-2 VPAHLLHPGKVKKDI NKLLGITPSDIDKYS RIVFPVCFVCFNLMY WIIYLHVSDVVADDL VLLGEE-
IVY12      ---HLVH---ASKGI NKLLGTTPSDIDKYS RIVFPVCFVCFNLMY WIIYLHVSDVVADDL VLLGEEN
AF006189   ---HLVH---ASKGI NKLLGTTPSDIDKYS RIVFPVCFVCFNLMY WIIYLHVSDVVADDL VLLGEEN
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B.

HvRdl (wild type)	WVSFWLNRNATPARV**S**LGVTTVLTMTT
HvRdl (Ala mutant)	WVSFWLNRNATPARV**A**LGVTTVLTMTT
DmRdl (resistant mutant)	WVSFWLNRNATPARV**S**LGVTTVLTMTT
DmRdl (wild type)	WVSFWLNRNATPARV**A**LGVTTVLTMTT
	domain 1 domain2

Fig. 1. A. The complete amino acid sequence of *H. virescens Rdl* (HVRDL) derived from the DNA sequence of plasmid pIVY12 is compared to the sequences of *H. virescens* cDNA AF006189, and *D. melanogaster* cDNA sequences derived from plasmids NB14.1 and DRC17-1-2. *The differences in sequence between pIVY12 and AF006189 are indicated in bold.* **B. Alignment of GABA receptor amino acid sequences (single-letter code) around domain one and two of insect GABA receptor homologs from *H. virescens* and *D. melanogaster*.** *Sequence differences are indicated in bold. Partial sequences of transmembrane domains are underlined. Abbreviations: DNA, deoxyribonucleic acid; cDNA, complementary deoxyribonucleic acid; GABA, γ-aminobutyric acid.*

(Research Biochemicals International), had no effect on oocytes expressing the HVRDL subunit (n=3).

Agonist dose-response relationships for GABA and muscimol, expressed as a percentage of maximum current, are shown in Fig. 3A. Chloride currents were measured in response to increasing doses of agonist, normalized to the maximum activated current and plotted against the log molar dose. Analysis of GABA and muscimol dose-response curves revealed a GABA EC_{50} of 19.1 μM (18.0–20.2) (n=6) and a muscimol EC_{50} of 14.9 μM (12.8–17.4) (n=5), indicating that muscimol is about 30% more potent in activating chloride currents than GABA. This difference was also observed in the maximal current produced by each agonist.

Figure 3B illustrates a Hill plot of agonist action. Hill coefficients calculated by linear regression were 1.98 (r^2=0.99) for GABA and 2.2 (r^2=0.98) for muscimol. Comparison of Hill coefficients indicates the homo-oligomeric GABA-gated chloride channels formed by HVRDL expressed in *Xenopus* oocytes require at least two molecules of agonist to open the channel.

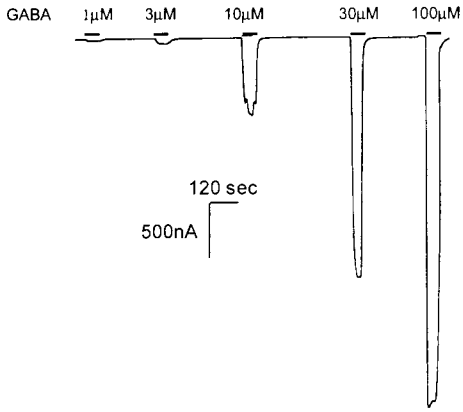

Fig. 2. GABA-activated chloride currents recorded under two-electrode voltage clamp conditions from a single oocyte, expressing the HVRDL subunit, in response to increasing concentrations of bath-applied agonist. *Abbreviations: GABA, γ-aminobutyric acid.*

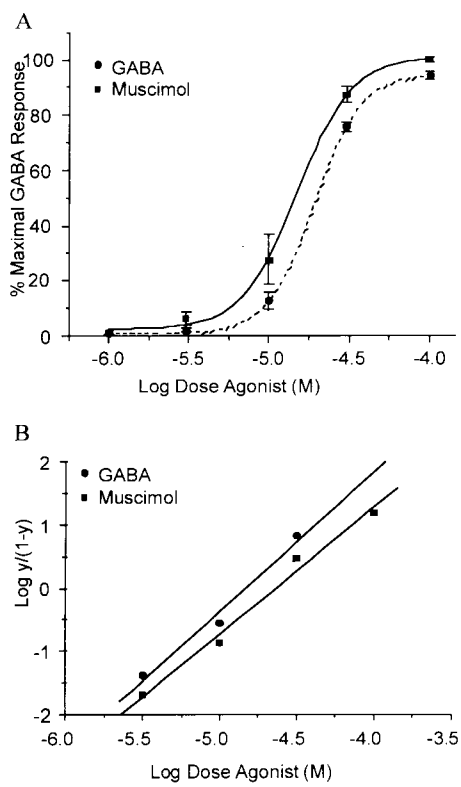

Fig. 3. A. Agonist dose-response relationship for GABA and muscimol expressed as a percentage of maximum current. *Error bars represent the standard error of the mean (SEM) of at least three oocytes.* **B. Hill plot of GABA and muscimol dose-response relationships.** *Line fitted to data points by least squares regression analysis for GABA (closed circle ●) and for muscimol (closed square ■). Abbreviations: GABA, γ-aminobutyric acid.*

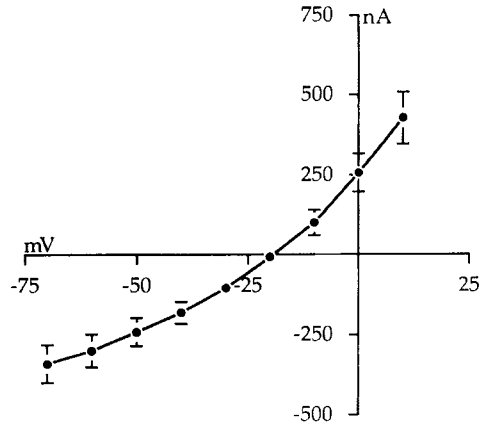

Fig. 4. Current-voltage relationship for homo-oligomeric GABA-gated chloride channels heterologously expressed in *Xenopus laevis* oocytes. *Recordings were made 24 h after injection with mRNA coding for the HVRDL GABA receptor subunit. The oocyte membrane potential was ramped from -70 to +15 mV in the absence of GABA and in the presence of 10 μM GABA. To resolve the GABA-activated current, the ramp-induced current under standard recording conditions was subtracted from that obtained in the presence of 10 μM GABA. Error bars represent the standard error of the mean (SEM). Each data point represents at least three oocytes. Abbreviations: GABA, γ-aminobutyric acid; mRNA, messenger ribonucleic acid.*

GABA-induced currents reversed at -24 mV (Fig. 4). This value corresponded to the expected Cl^- equilibrium potential (E_{Cl} = -24 mV) as derived from the Nernst equation (Blair *et al.*, 1988), and suggests that the measured current is probably carried by chloride.

Allosteric modulators

The benzodiazepine, flunitrazepam, (10 μM) had no effect on GABA-evoked currents when co-perfused with 50 μM (n=3) or 10 μM (n=3) GABA. The steroid, pregnenolone (Research Biochemicals International, USA), (10 μM) also showed no modulation of GABA-evoked currents (10 μM) (n=3). The anticonvulsant, phenobarbital (Research Biochemicals International, USA), had no effect when applied in the absence of GABA but did have a dose-dependent positive allosteric effect when co-perfused with GABA (10 μM) (n=4) (Fig. 5), significantly enhancing GABA-evoked currents in excess of 160% at doses as high as 1 mM.

Antagonist pharmacology

Bicuculline, a classic competitive antagonist of vertebrate $GABA_A$ receptors, had no effect at 100 μM on GABA-evoked currents (10 μM)(n=4).

The IC_{50} of fipronil for HVRDL-Ser 285 was calculated to be 1.64 μM (1.60–1.68) (n=5). HVRDL-Ala 285 was nearly 15 times more sensitive to block by

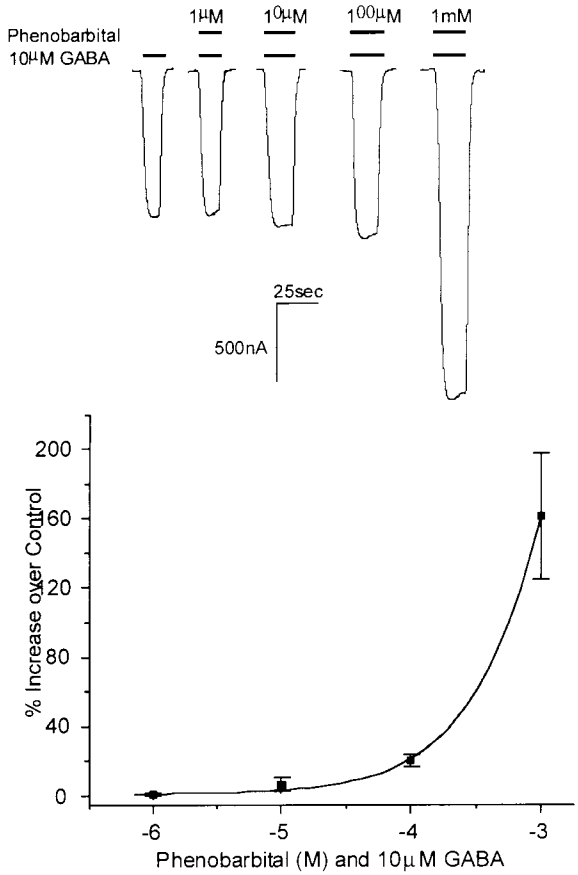

Fig. 5. Dose-response effect of phenobarbital in the presence of 10 μM GABA on homo-oligomeric GABA-gated chloride channels recorded from *Xenopus laevis* oocytes 24–36 h after injection with mRNA encoding the HVRDL subunit. *Error bars represent the standard error of the mean (SEM). Each data point represents three oocytes. Bars above recording represent duration of perfusion relative to time and amplitude scale below. Abbreviations: GABA, γ-aminobutyric acid.*

fipronil, with an IC_{50} of 0.11 μM (0.10–0.12) (n=4). Fipronil IC_{50} values for DMRDL-Ser 302 and DMRDL-Ala 302 were 0.22 μM (0.17–0.28) (n=5) and 0.25 μM (0.21–0.29) (n=6), respectively. There was no statistical difference between the two dose-response curves (Fig. 6).

The IC_{50} values calculated for picrotoxinin were HVRDL-Ser 285 = 13.20 μM (10.80–16.10) (n=8) and HVRDL-Ala 285 = 0.38 μM (0.37–0.39) (n=5), serine/alanine IC_{50} ratio = 34.7. For *D. melanogaster* the IC_{50} values were DMRDL-Ala 302 = 0.48 μM (0.36–0.58) (n=3) and DMRDL-Ser 302 = 3.24 μM (2.81–3.72) (n=4) (Fig. 7), serine/alanine IC_{50} ratio = 6.75.

The IC_{50} values for dieldrin could not be accurately calculated for HVRDL-Ala 285, HVRDL-Ser 285 and DMRDL-Ser 302. In all but one case, the highest dose of dieldrin did not elicit 50% or more block. The highest level of block was observed in DMRDL-Ala 302, where 10 μM dieldrin blocked just over 50% (Fig. 8).

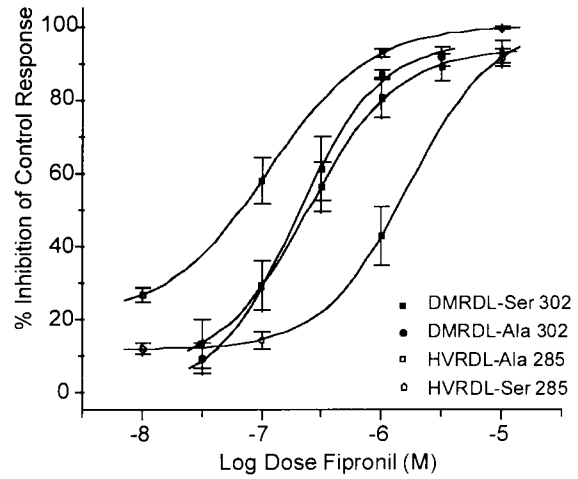

Fig. 6. Dose response inhibition curves for fipronil HVRDL (wild-type), Ser-285 (open circle O), HVRDL (Ala mutant), Ala 285 (open square □), DMRDL (wild-type), Ala 302 (closed circle ●), and DMRDL (resistant mutant), Ser 302 (closed square ■) homo-oligomeric GABA-gated chloride channels expressed in *Xenopus* oocytes. *Responses are expressed as percentage of maximal current produced by control dose of GABA alone. Error bars indicate ±SEM of at least three oocytes. Abbreviations: GABA, γ-aminobutyric acid; SEM, standard error of the mean.*

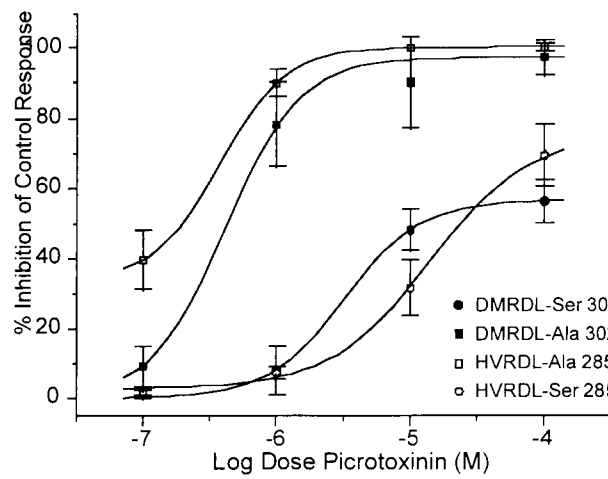

Fig. 7. Dose-response inhibition curves for picrotoxinin HVRDL (wild-type), Ser-285 (open circle O), HVRDL (Ala mutant), Ala 285 (open square □), DMRDL (wild-type), Ala 302 (closed square ■), and DMRDL (resistant mutant), Ser 302 (closed circle ●) homo-oligomeric GABA-gated chloride channels expressed in *Xenopus* oocytes. *Responses are expressed as percentage of maximal current produced by control dose of GABA alone. Error bars indicate ±SEM of at least three oocytes. Abbreviations: GABA, γ-aminobutyric acid; SEM, standard error of the mean.*

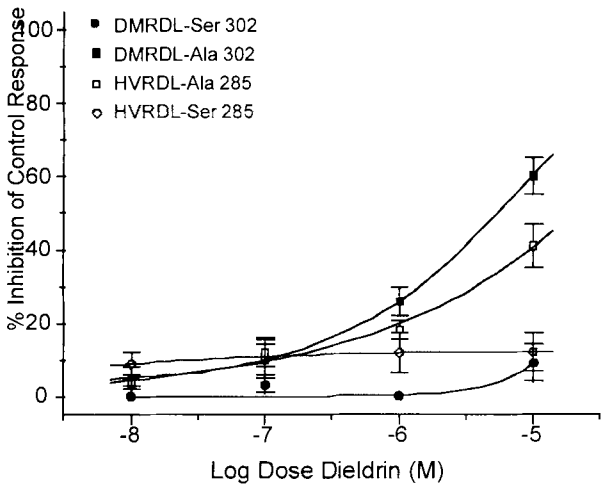

Fig. 8. Dose-response inhibition curves for dieldrin HVRDL (wild-type), Ser-285 (open circle O), HVRDL (Ala mutant), Ala 285 (open square □), DMRDL (wild-type), Ala 302 (closed square ■), and DMRDL (resistant mutant), Ser 302 (closed circle ●) homo-oligomeric GABA-gated chloride channels expressed in *Xenopus* oocytes. *Responses are expressed as percentage of maximal current produced by control dose of GABA alone. Error bars indicate ±SEM of at least three oocytes. Abbreviations: GABA, γ-aminobutyric acid; SEM, standard error of the mean.*

Discussion

In *Drosophila*, resistance to the cyclodiene insecticide, dieldrin, is associated with a point mutation resulting in a one amino acid substitution of alanine to serine in the second transmembrane domain of the *Rdl* GABA receptor subunit (ffrench-Constant *et al.*, 1993). Analysis of the cDNA clone, HVRDL, revealed complete sequence homology with the dieldrin resistant *Rdl* receptor from *Drosophila* across the M2 region.

PCR analysis of several laboratory-reared *H. virescens* was carried out. Initially no residue other than serine was found at position 302 (Wingate, unpublished data). Subsequent PCR analysis of a new in-house strain of *H. virescens* identified the presence of an alanine residue at this position, as well as a serine. Single insect PCR studies will be required in order to determine whether both genotypes are present in a single strain.

We believe each in-house strain of *H. virescens* to have no known resistance phenotypes. Fipronil bioassay data on in-house strains indicated that all were indeed 'susceptible', with no significant difference in median lethal dose (LD_{50}). Moreover, no resistant phenotypes were observed in bioassay against several cyclodienes and pyrethroids (Rhone-Poulenc Ag Co., unpublished data).

Analysis of dose-response effects of GABA and muscimol, and the calculation of Hill coefficients indicate that at least two molecules of agonist are necessary to open the HVRDL chloride channel. These data confirm previous reports from a functionally expressed GABA-gated chloride channel (*Rdl*) from *Drosophila* (ffrench-Constant *et al.*, 1993).

Allosteric modulators have been shown to potentiate GABA agonisim in vertebrate $GABA_A$ receptors by binding to discrete regions on the GABA receptor, which are not involved in either agonist or antagonist binding domains (Sieghart, 1992). Previous studies on invertebrate heterologously expressed GABA receptors and whole tissue preparations have yielded mixed results (Anthony *et al.*, 1993; Chen *et al.*, 1994; Lummis, 1990; Sigel *et al.*, 1990; Zaman *et al.*, 1992). In the present study, repeated perfusion of a representative benzodiazepine and steroid, and co-perfusion of each with GABA, had no effect in potentiating currents. Phenobarbital enhanced chloride currents in a dose-dependent fashion, with a significant increase in current (>160%) occurring at the highest dose of phenobarbital (1 mM). Studies by Chen and co-workers (1994) on the *Rdl* subunit expressed in oocytes revealed similar results, although potentiation by the anticonvulsant, pentobarbitone, produced even larger increases in chloride conductance, where peak potentiation (554%) occurred at 1 mM pentobarbitone.

Buckingham and co-workers (1994) reported on the actions of picrotoxinin, fipronil, t-butylcyclo-orthobenzoate (TBPS) and 4′-ethylnyl-4-n-propylbicycloorthobenzoate (EBOB) on the wild-type DMRDL (Ala isoform) homo-oligomer in *Xenopus* oocytes, using two concentrations of antagonist in the presence of 10 μM GABA. At 0.1 μM and 10 μM, picrotoxinin blocked 61.3% and 100%, respectively, and fipronil blocked 11.2% and 75.6%, respectively. In the present study it was found that the effects of picrotoxinin on the DMRDL-Ala 302 were similar to the results obtained by Buckingham and co-workers; however, the DMRDL-Ala 302 receptor was found to be somewhat more sensitive to the blocking action of fipronil than reported previously (Buckingham *et al.*, 1994).

The present study illustrates that the second transmembrane domain, in particular position 285, plays a critical role in the sensitivity of the *H. virescens* GABA receptor (HVRDL) to fipronil. The presence of either a serine (wild-type) or alanine (site-directed mutant) in the HVRDL receptor significantly alters the receptor's sensitivity to blockage by fipronil. In contrast, the presence of a serine (dieldrin resistant phenotype) or alanine (wild-type) at the homologous position (302) in the DMRDL receptor has no significant effect on the receptors sensitivity to blockage by

fipronil. However, the role of this domain in modulating picrotoxinin and dieldrin sensitivity in *H. virescens* seems to be very similar to that observed in *D. melanogaster*.

The alanine mutation in HVRDL increased sensitivity to blockage by fipronil. The HVRDL-Ser 285 was the least sensitive to blockage by fipronil of all four receptor types, and the HVRDL-Ala 285 mutant was the most sensitive to blockage by fipronil of all receptors tested so far. No significant difference was observed in the response of the cyclodiene resistant and susceptible *D. melanogaster* clones to the blocking action of fipronil. These data indicate that cyclodiene insensitivity in *D. melanogaster* seems to play little or no role in conferring insensitivity or cross-resistance to fipronil, as observed in heterologously expressed homo-oligomeric GABA receptors. These data indicate the need for a fipronil toxicity bioassay of the dieldrin susceptible and dieldrin resistant strains of *D. melanogaster*.

Picrotoxinin and dieldrin exhibited remarkably similar effects on HVRDL and DMRDL GABA-gated chloride channels and showed a similar pharmacology in response to the presence of either a serine or alanine at position 285 in HVRDL and position 302 in DMRDL, respectively. Although absolute sensitivities varied, the serine conferred relative insensitivity while the alanine conferred a relative increase in sensitivity to channel blocking by both compounds. Furthermore, blocking of GABA-gated chloride channels by picrotoxinin saturated at about 50% inhibition in the serine isoform of the *Rdl* receptor whereas a complete dose-response relationship was observed in the alanine isoform. Inhibition by fipronil resulted in full dose-response relationships in both receptor isoforms. These data may suggest that fipronil and picrotoxinin block at two different but perhaps closely-related sites within the heterologously expressed *Rdl* GABA receptor; further study may offer insights as to the specific sites within the M2 region of the heterologously expressed *Rdl* GABA receptor where both compounds interact.

ffrench-Constant and co-workers (1993) reported on the actions of picrotoxinin and dieldrin on the DMRDL-Ser 302 and DMRDL-Ala 302 GABA receptors expressed in *Xenopus* oocytes. In their study, chloride currents were recorded in response to 10 or 50 μM GABA. At the highest GABA dose (50 μM), the level of blocking by 10 μM of picrotoxinin and dieldrin in DMRDL-Ser 302 (dieldrin resistant mutant) was reduced from that observed at the 10 μM GABA dose. No such reduction in chloride channel blocking was observed with a similar increase in GABA concentration on the DMRDL-Ala 302 (wild-type) receptor. Similar levels of inhibition by picrotoxinin in response to 50 μM GABA-evoked currents in DMRDL-Ala 302 were also reported by Buckingham and co-workers (1994). Based on the GABA dose-response data presented in their study, a 50 μM dose of agonist would represent a concentration outside the linear portion of the GABA potency curve.

Hosie and co-workers (1995) reported dose-response effects of picrotoxinin and fipronil in the presence of 1 mM GABA on the DMRDL-Ser 302 and DMRDL-Ala 302 subunits expressed in *Xenopus* oocytes. GABA-evoked current (1 mM) was blocked less than 10% by 10 μM fipronil in DMRDL-Ser 302 and about 70% in DMRDL-Ala 302. These authors concluded that cross-resistance to fipronil exists, based on the insensitivity of the heterologously expressed homo-ologomer, DMRDL-Ser 302, to blockage by fipronil at doses of ≥1 μM, with complete insensitivity to blockage at 10 μM. This contradicts the results obtained in the present study. The discrepancy may be explained by the different protocols used in the experiments. Hosie and co-workers (1995) used high concentrations of GABA (1 mM), while other studies (including the work reported here) have tended to use GABA concentrations below 100 μM in an attempt to investigate antagonist pharmacology occurring in the linear portion of the dose-response curve of GABA. Previous studies with heterologously expressed GABA receptors from invertebrates and mammals have shown a decrease in the level of blocking by antagonists associated with an increase of agonist dose at or above the linear portion of the GABA potency curve or at GABA doses at or near saturation (Schofield *et al.*, 1987; van Renterghem *et al.*, 1987; Blair *et al.*, 1988; Malherbe *et al.*, 1990; Sigel *et al.*, 1990; Harvey *et al.*, 1991; ffrench Constant *et al.*, 1991, 1993; Amin and Weiss, 1994 ; Buckingham *et al.*, 1994; Chen *et al.*, 1994; Quian and Dowling, 1994; Hosie, *et al.*, 1995; Xu *et al.*, 1995).

Summary

The cloning and expression of a functional GABA receptor from an insect order other than Diptera has made possible a comparative pharmacological study of heterologously expressed GABA-gated chloride channels. In particular, the relevance of resistance associated mutations in one insect species was evaluated within the context of another insect species. Such differences may be further investigated through site-directed mutagenesis studies. Given the results presented in the present study, we could not demonstrate 'cross-resistance' to fipronil as a function of the cyclodiene resistance associated mutation in

D. melanogaster; however, we were able to demonstrate a significant difference in the sensitivity of the *H. virescens Rdl* subunit linked to the presence of either a serine or alanine at position 285.

The utility of functional pharmacological target based assays for the study of novel chemistry and their possible efficacy on specific target organisms is apparent. The ability to investigate specific physiologically relevant amino acid sequence substitutions via site-directed mutagenesis, which may also occur naturally as mutations, offers a tool for optimization of insecticidal action and proactive management of pesticide use against target organisms in the field.

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