Drug-resistant *Drosophila* indicate glutamate-gated chloride channels are targets for the antiparasitics nodulisporic acid and ivermectin

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The fruit fly *Drosophila melanogaster* was used to examine the mode of action of the novel insecticide and acaricide nodulisporic acid. Flies resistant to nodulisporic acid were selected by stepwise increasing the dose of drug in the culture media. The resistant strain, *glc¹*, is at least 20-fold resistant to nodulisporic acid and 3-fold cross-resistant to the parasiticide ivermectin, and exhibited decreased brood size, decreased locomotion, and bang sensitivity. Binding assays using *glc¹* head membranes showed a marked decrease in the affinity for nodulisporic acid and ivermectin. A combination of genetics and sequencing identified a proline to serine mutation (P299S) in the gene coding for the glutamate-gated chloride channel subunit DmGluClα. To examine the effect of this mutation on the biophysical properties of DmGluClα channels, it was introduced into a recombinant DmGluClα, and RNA encoding wild-type and mutant subunits was injected into *Xenopus* oocytes. Nodulisporic acid directly activated wild-type and mutant DmGluClα channels. However, mutant channels were ≈10-fold less sensitive to activation by nodulisporic acid, as well as ivermectin and the endogenous ligand glutamate, providing direct evidence that nodulisporic acid and ivermectin act on DmGluClα channels.

Because of the development of resistance and the search for less toxic drugs, there is a continuous need for novel antiparasitic compounds. A blowfly (*Lucilia seracata*) killing assay identified the natural product nodulisporic acid as a potent insecticide (1). Isolated from *Nodulisporium* sp., an endophytic fungus, nodulisporic acid is an indole terpene that effectively kills not only blowflies, but also mosquitoes (*Aedes aegypti*) and fruit flies (*Drosophila melanogaster*) (1, 2). In the blowfly and mosquito assays, nodulisporic acid was more potent than paraherquamide, malathion, and DDT, but less potent than ivermectin (1, 2).

Although the basis of the drug's insecticidal activity was not known, recent studies indicate that nodulisporic acid acts by a mechanism similar to that of ivermectin (3). In grasshopper metathoracic ganglia, nodulisporic acid, like ivermectin, activates a chloride conductance. No additional current is seen when nodulisporic acid is added after application of ivermectin. Furthermore, binding of radiolabeled nodulisporic acid to *Drosophila* head membranes is inhibited completely in the presence of saturating concentrations of ivermectin. Ivermectin, a macrocyclic lactone, is known to activate recombinant glutamate-gated chloride (GluCl) channels (4, 5). At least in *Caenorhabditis elegans*, the effect on GluCl channels underlies the anthelmintic activity of ivermectin and its homologues. A *C. elegans* strain, *avr-14;avr-15;glc-1*, carrying simultaneous mutations of the three GluCl channel α-type subunits α1, α2, and α3, shows no ivermectin binding and is ≈4,000-fold resistant (6). Ivermectin also activates insect GluCl channels, and it is assumed that the effect on GluCl channels is at least partly responsible for ivermectin's insecticidal activity (5). However, effects of ivermectin on other ion channels are known and may contribute to the drug's

efficacy. Most noticeably, ivermectin binds to and interacts with insect γ-aminobutyric acid (GABA)-gated chloride channels (7, 8). Together with glycine-gated channels, GABA-gated channels are the closest homologues of GluCl channels and are likely to be sites of ivermectin toxicity.

Because of its genetic accessibility, *Drosophila* is a valuable model system. In this study, *Drosophila* was used to examine the mode of action of the novel insecticide nodulisporic acid. A *Drosophila* strain resistant to nodulisporic acid was selected in the laboratory and examined for the molecular mechanism of resistance. Head membranes prepared from the resistant strain had lower affinity for nodulisporic acid and ivermectin than wild-type head membranes. A combination of genetics and sequencing identified a point mutation in the gene coding for the glutamate-gated chloride channel subunit DmGluClα. Electrophysiological studies of wild-type and mutant channels expressed in *Xenopus* oocytes revealed that nodulisporic acid directly activates DmGluClα and that the mutation (P299S) renders the channel less sensitive to activation by nodulisporic acid, ivermectin, and the endogenous ligand glutamate.

Materials and Methods

Fly Culture and Maintenance. Flies were cultured as described (9, 10) at 25°C, 65% humidity, and 12-h light:12-h dark cycle. Unless insecticides were added to the culture, flies were raised on standard cornmeal, yeast, sucrose, glucose, and agar medium. Fly food containing insecticides was made with Instant *Drosophila* medium, formula 4-24 (Carolina Biological Supply), to which a mixture of water and insecticide dissolved in DMSO was added. The following *Drosophila* strains were used: wild-type *Oregon-R, Or-R*; white eyed wild-type, *w*; *Resistance to dieldrin, Rdl^MD-RR* (A302S); a second and third chromosome balancer, *SM6aCyO; TM6B, Tb/ap^xa*; a second chromosome balancer, *SM6b, CyO/Sp J L Pin*; a third chromosome balancer, *TM3,Ser e/TM6B,Tb e* (all provided by the Bloomington *Drosophila* Stock Center, Bloomington, IN); a *glc* deficiency line *Df(3R)ora^12/TM6, Ubx*, referred to as *Df12*, which contains breakpoints 92A6:92D2; a deficiency line *Df(3R) ora^B16* adjacent to *Df12*, referred to as *DfB16*, which contains breakpoints 92A12–92B1; and 92E10–92E15 (11, 12).

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glc¹, derived from *Or-R* by nodulisporic acid selection pressure, refers to nodulisporic acid- and ivermectin-resistant *glc¹/glc¹*.

Toxicity Assays. For larval lethal assays, approximately 40 egg-laying adults were placed in a vial of food containing various doses of drug and allowed to lay eggs for 5 days, after which adults were removed. The vials were monitored for 3 wk after adult removal for the development of larvae, pupae, and adults. If one of the parental genotypes was not able to survive on drugged food for 5 days, eggs and first instar larvae were collected in a drug-free vial and then transferred to the drugged vial. The dose of drug at which no adults emerged was considered the larval LD_{100}.

Binding Assays. *Drosophila* head membranes were prepared, and binding of radiolabeled nodulisporic acid and ivermectin was measured as described (3), with the modification that both tritiated and ^{35}S-labeled compounds were used. All radiolabeled compounds were synthesized at Merck Research Laboratories. [^{3}H]Nodulisporic acid (22 Ci/mmol) refers to the tritiated amide derivative *N*-(2-hydroxyethyl-2,2-^{3}H) nodulisporamide (3); [^{35}S]nodulisporic acid (1,000 Ci/mmol) is a disubstituted amide derivative, *N*-1-[(4-^{35}SO$_2$Me)piperizinyl] nodulisporamide (13); and [^{3}H]ivermectin (44 Ci/mmol) and [^{35}S]ivermectin (1,000 Ci/mmol) were synthesized as described (14, 15). Binding data were fit to the equation $B = (B_{max} \cdot L)/(K_d + L)$, where B is the concentration of specifically bound ligand, B_{max} is the maximal number of binding sites, L is the free ligand concentration, and K_d is the equilibrium dissociation constant. All assays were performed in triplicate and usually varied by <3%. Results obtained with tritiated and ^{35}S-labeled compounds were comparable, and average data include experiments with both ligands.

Genetic Mapping of Nodulisporic Acid Resistance. Chromosomes X, II, and III from *glc¹* flies were tested for the presence of the nodulisporic acid resistance gene by a series of outcrosses (10–15 lines for each chromosome) with balancer stocks (15, 16). Once nodulisporic acid resistance had been mapped to chromosome III, deficiency mapping was performed with the line *Df12,* known to contain a deletion at the *glc* locus. Male *glc¹* were crossed to *Df12* virgin females, and the progeny were tested in toxicity assays.

In Situ Hybridization. *In situ* hybridization of larval salivary polytene chromosomes from *Or-R*, *Df12*, and *DfB16* was performed as described previously (17) by using a DmGluClα cDNA probe (GenBank accession no. U58776) (5). DmGluClα in pBluescript (Stratagene) was linearized by *Bam*HI, and random-prime labeled with digoxigenin (DIG), by using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim). The labeled DNA was diluted to a concentration of 2 ng/μl in 5 × SSC/0.1% SDS, and stored at −20°C before use. Chromosome squashes were prepared by standard procedures (17). The chromosome slides were heated to 80°C in 2× SSC for 40 min. The chromosomes were denatured in 0.1 M NaOH for 2 min, rinsed in 2× SSC for 2 min, dehydrated through ethanol washes: twice for 2 min in 70% ethanol, once for 5 min in 95% ethanol, and air dried. The DIG-labeled probe was denatured, and 30 μl/slide was applied. The slides were sealed under coverslips with rubber cement. After overnight incubation at 65°C, the coverslips were washed in 2× SSC for 3 min. The slides were further washed three times for 2 min in PBT (PBS/0.1% Tween 20). Anti-DIG-alkaline phosphatase antibody of the DNA Detection kit was diluted 1:200 in PBT and incubated with the chromosomes (50 μl/slide) for 1.5 h at room temperature. The slides were then washed three times for 1 min in PBT and three times for 1 min in X-phosphate buffer (100 mM NaCl/50 mM MgCl2/100 mM Tris·HCl, pH 9.0/0.1% Tween 20), and the hybridization signal was developed

by using the DNA detection kit. The chromosomes were counterstained in 1:20 dilution of Giemsa (Sigma) for 10 min, destained in water, air dried, and mounted with Permount (Sigma).

Behavioral Tests. For spontaneous locomotion tests, individual flies were placed in a cylindrical chamber (8 mm diameter × 3 mm high), and the number of times each fly crossed the midline in a given amount of time was recorded. At least 20 7-day-old males of each genotype were tested at the same time of day for a 4-min period. Locomotion data were reported as quantiles and medians, and nonparametric statistical tests (Wilcoxon/ Kruskal-Wallis rank sums) were performed by using JMP 3.2.2 (SAS Institute, Cary, NC) software. *P* values of <0.01 were considered significant (9). Bang sensitivity was measured by placing flies in a vial, vortexing for 5 s, and timing the rate of recovery from paralysis (18, 19). Temperature sensitivity was determined by placing 10 flies in a humidified glass vial immersed in a 37°C water bath for 30 min. For all behavioral tests, flies were transferred by aspiration rather than CO$_2$ anesthetization and allowed to recover from aspiration for 10 min before testing.

Sequencing. The DmGluClα cDNA was obtained from nodulisporic acid-resistant *glc¹* flies by reverse transcriptase PCR (RT-PCR). Total RNA was prepared from 10 glc¹ flies (Ultraspec RNA; Biotecx Laboratories, Houston), and first strand synthesis of cDNA was performed by using oligo(dT) (GIBCO/ BRL Superscript Preamplification System). The total first strand cDNA was used as template in six parallel PCR reactions, with primers based on wild-type DmGluClα: 5′-ATG GGC AGC GGA CAC TAT TTC and 3′-TTA CTC ATC CTC CTC CTC CCT GAAG or 3′-TTC GAA CCT GAT CGG GAT. The resulting 1,370- or 1,009-bp PCR products were purified (Gene Clean II; Bio 101), cloned into 2.1-Topo TA vector (Invitrogen) and transformed into DH5α electrocompetent *Escherichia coli* (GIBCO/BRL). The DNA sequence of 1–2 clones originating from each of six PCR reactions was determined. The GenBank/ EBI Data Bank accession numbers for these cDNAs are AF297500 and AF297501.

Mutagenesis and *in Vitro* RNA Synthesis. RNA coding for DmGluClα wild-type and P299S (C1299T) mutant channels was synthesized for injection into *Xenopus* oocytes. Wild-type DmGluClα (GenBank/EBI Data Bank U58776) was subcloned into pBluescript (Stratagene) to generate single-stranded DNA template, and site-directed mutagenesis was performed by using the Sculptor *in vitro* mutagenesis system (Amersham) and custom-designed mutagenic primers (GIBCO/BRL, 5′-GTC GGG CAT AAA CGC CTC CCT GTC GCC CGT TTC CTA TAC GAAG) according to the manufacturer's protocol. A T7 promoter and a poly(A^{+}) tail were added to wild-type and mutant DmGluClα by PCR, and the resulting PCR products were used as templates in the *in vitro* transcription reaction (mMessage mMachine; Ambion, Austin, TX).

Electrophysiology in *Xenopus laevis* Oocytes. Wild-type and P299S RNAs were injected into *X. laevis* oocytes for electrophysiological analysis. Oocytes were prepared as previously described (20) and studied by two-electrode voltage clamp 1–4 days after injection with 50 nl of 100–200 ng/μl RNA. Whole cell currents were measured by using a Dagan (Minneapolis) CA-1 amplifier interfaced to a Macintosh 7100/80 computer. Data were acquired at 100 Hz by using Pulse software (HEKA Electronics, Lambrecht/Pfalz, Germany). During recording, oocytes were continuously superfused with a solution containing 96 mM NaCl, 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, 3.5 mM BaCl$_2$, and 5 mM Hepes (pH 7.5). Drugs were dissolved in DMSO and applied in the recording solution at ≤0.1% DMSO. All experiments were

Table 1. Larval LD_{100} for the insecticides nodulisporic acid, ivermectin, and fipronil on *Drosophila* strains *Or-R*, *Rdl^MD-RR*, *glc^1*, *glc^1/+*, *glc^1/Df12*, and *Df12/+*

|	Nodulisporic acid, μM	Ivermectin, μM	Fipronil, μM
Or-R	0.44	0.03	0.57
	$n = 6$	$n = 4$	$n = 2$
Rdl^MD-RR	2.20	0.12	3.43
	$n = 4$	$n = 3$	$n = 3$
glc^1	>10.3*	0.12	0.57
	$n = 3$	$n = 2$	$n = 2$
glc^1/+	1.50		
	$n = 3$		
glc^1/Df12	>5.9*		
	$n = 5$		
Df12/+	0.44		
	$n = 3$		

Numbers represent the larval LD_{100}; n equals the number of trials.
*Highest concentration of nodulisporic acid tested.

performed at room temperature from a holding potential of 0 mV. Currents activated by ivermectin or nodulisporic acid were normalized to the maximal glutamate-induced current in the same oocyte, and dose-response curves were fit to the Hill equation with a Hill coefficient of 2: $I = I_{max}/(1 + (EC_{50}/L)^2)$, where L is the concentration of drug and I_{max} is the current activated by saturating drug concentrations.

Results

Toxicity Assays. Nodulisporic acid-resistant flies were generated from *Or-R* by growth for three generations on 0.29 μM nodulisporic acid, where the larval $LD_{100} = 0.44$ μM, followed by stepwise increases in the selection pressure over approximately 20 generations to a final concentration of 10.3 μM nodulisporic acid (Table 1). The resulting strain of flies, called *glc^1*, was >23-fold resistant to nodulisporic acid. The heterozygote *glc^1/+* shows intermediate resistance with an LD_{100} of 1.5 μM. In addition, *glc^1* flies are at least 3.3-fold cross-resistant to ivermectin with larval LD_{100} of 0.12 μM for *glc^1* and 0.035 μM for *Or-R*. In contrast, *glc^1* flies showed no differences in sensitivity to fipronil; the fipronil larval LD_{100} for both *Or-R* and *glc^1* was 0.57 μM. As with other drugs, adult flies were less sensitive to nodulisporic acid, ivermectin, and fipronil than larvae (data not shown). Because ivermectin is known to interact with GABA receptors in vertebrates and nematodes (for examples, see refs. 21 and 22), *Drosophila* carrying mutations in a GABA-gated channel were tested for drug sensitivities. *Rdl^MD-RR* flies contain an alanine to serine mutation in the second transmembrane domain of the *Rdl* gene coding for a GABA-gated channel and are >4000-fold resistant to the insecticide dieldrin (23). *Rdl^MD-RR* flies are approximately 3.3-fold resistant to ivermectin, 5-fold resistant to nodulisporic acid, and 6-fold resistant to fipronil.

Drug Binding to *glc^1* *Drosophila* Membranes. The nodulisporic acid- and ivermectin-resistant *glc^1* flies were tested for changes in the binding affinities of radiolabeled drug to head membrane receptors. [^{35}S]Nodulisporic acid binds with high affinity and in a saturable manner to *Drosophila Or-R* head membranes (Fig. 1A). Fitting of the data to a simple mass action binding isotherm yielded $K_d = 33$ pM and $B_{max} = 1.1$ pmol/mg protein. [^{35}S]Nodulisporic acid binding to head membranes of *glc^1* flies was of considerably lower affinity and was near saturation at 400 pM, the highest concentration tested. Least squares fitting of the data according to the law of mass action yielded $K_d = 140$ pM and
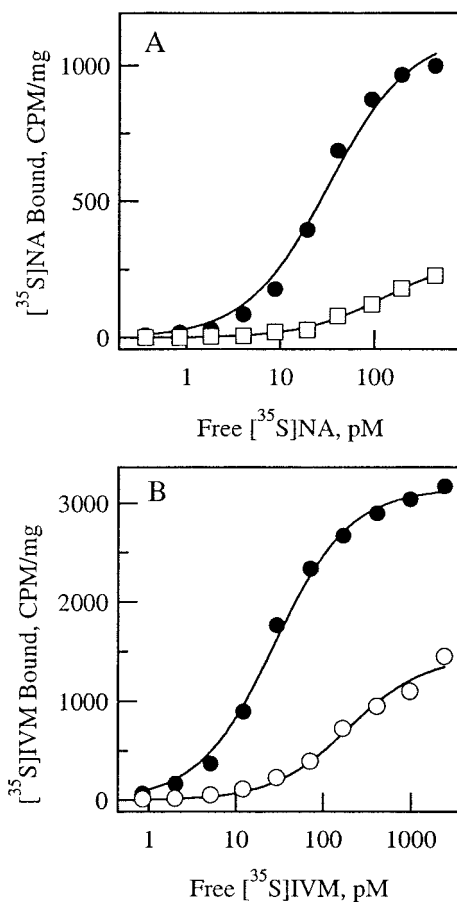
Fig. 1. Nodulisporic acid and ivermectin binding to wild-type and *glc^1 Drosophila* head membranes. (A) Specific binding of [^{35}S]nodulisporic acid to wild-type (filled symbols) and *glc^1* (open symbols, two separate experiments) *Drosophila* head membranes. Specific binding was defined as total binding, measured in triplicate, minus binding in the presence of 100 nM unlabeled nodulisporic acid. Data were fit to the binding isotherm of the form: $B = (B_{max} \cdot L)/(K_d + L)$, where B is the concentration of specifically bound ligand, B_{max} is the maximal number of binding sites, L is the free ligand concentration, and K_d is the equilibrium dissociation constant. (B) Specific binding of [^{35}S]ivermectin to wild-type (filled symbols) and *glc^1* (open symbols, two separate experiments) *Drosophila* head membranes.

$B_{max} = 0.27$ pmol/mg protein. In four experiments, [^{35}S]nodulisporic acid binding affinity was shifted a geometric mean of 7-fold in *glc^1* flies compared with wild-type flies. Because *glc^1* flies showed 3.3-fold cross-resistance to ivermectin (Table 1), [^{35}S]ivermectin binding was examined in head membranes of wild-type and *glc^1* flies (Fig. 1B). [^{35}S]Ivermectin binding to wild-type membranes was saturable, with $K_d = 26$ pM and $B_{max} = 2.7$ pmol/mg. For the *glc^1* flies, fitting of the data according to the law of mass action was in agreement with binding to a single site, with $K_d = 190$ pM and $B_{max} = 1.4$ pmol/mg. Although the existence of multiple sites cannot be ruled out, no population of sites was seen at the K_d for [^{35}S]ivermectin binding to wild-type membranes. Therefore, all ivermectin binding sites of head membranes appear to be affected in the resistant strain, and the average reduction in binding affinity was 7-fold ($n = 7$), the same as for nodulisporic acid.

Genetic Mapping. The DmGluClα gene location was examined by using *in situ* hybridization. A single hybridization band was detected at cytological position 92A-B on chromosome 3 of wild-type *Or-R* third instar larvae (Fig. 2A). This site was
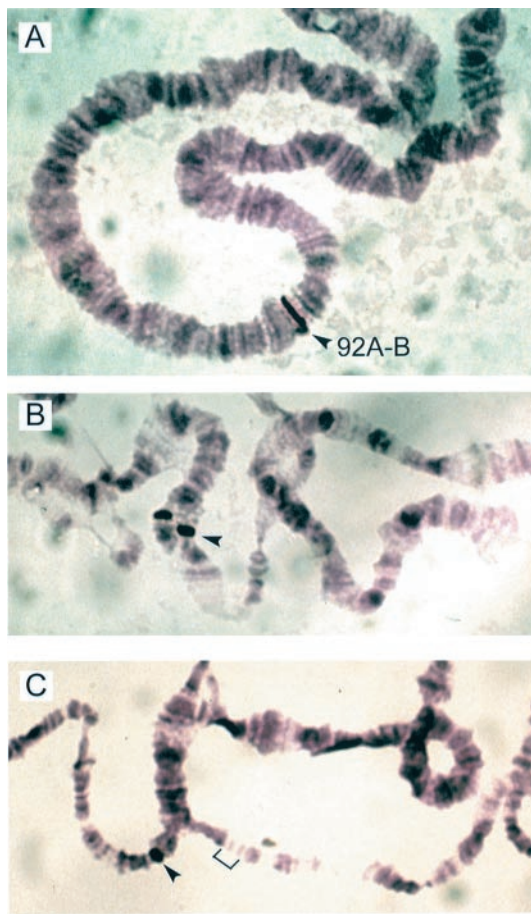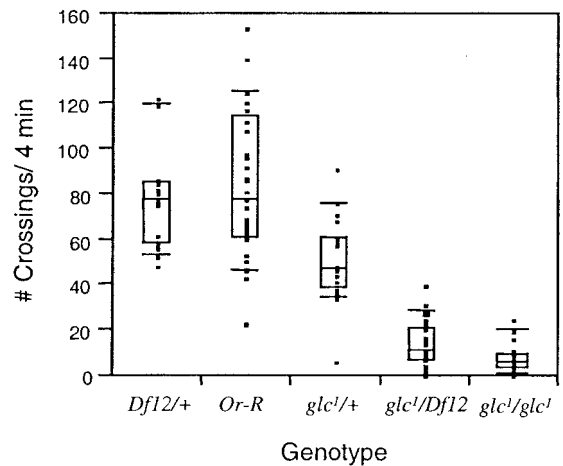
Fig. 2. *In situ* hybridization of DmGluClα probe to cytological position 92A-B on *Or-R* polytene chromosome 3 (*A*). The DmGluClα probe hybridizes to heterozygous deficiency chromosome *DfB16* (*B*) but not *Df12* (*C*).


Fig. 3. Spontaneous Locomotion. Twenty to thirty-three flies of specified genotypes were tested in spontaneous locomotion assays. Data are presented as medians and quantiles, where the center lines indicate medians, boxes represent the 25% and 75% quantiles, bars the 10% and 90% quantiles, and black squares are the individual data points.

mapped further by *in situ* hybridization to deficiency chromosomes *DfB16* and *Df12* (11). The DmGluClα probe hybridized to *DfB16* (Fig. 2*B*) but not to *Df12* (Fig. 2*C*); therefore, the DmGluClα gene is located on chromosome 3, between the breakpoints of *Df12* and *DfB16* (92A4–92B2).

Standard linkage analysis techniques mapped the nodulisporic acid resistance in *glc¹* flies to chromosome 3. Like homozygous *glc¹/glc¹* flies, *Df12/glc¹* flies were more than 10-fold resistant to nodulisporic acid, whereas *Df12/+* exhibited the same LD_{100} as *Or-R* (Table 1). These data suggest that the *Df12* deletion, spanning the region 92A6–92D2 and containing DmGluClα, includes the site of the resistance gene.

Behavioral Phenotype of Drug-Resistant *Drosophila*. Compared with wild type, *glc¹* flies are less vigorous, as evidenced by impaired locomotion and flight ability and a greatly reduced brood size. A quantitative measure of reduced locomotion is shown in Fig. 3. *glc¹* and *glc¹/Df12* flies exhibited significantly reduced locomotion, compared with *Or-R* and *Df12/+* ($P < 0.0001$) and were not significantly different from each other ($P > 0.02$). During a period of 4 min, *glc¹* and *glc¹/Df12* flies crossed the center line a median of 5.5 times and 11 times, respectively, compared with 78 times for *Or-R* and *Df12/+*. The heterozygotes, *glc¹/+*, exhibited an intermediate level of locomotion of 47 crosses/4 min, significantly different from all other genotypes ($P < 0.0001$). These phenotypes combined with the moderate resistance of the heterozygotes to nodulisporic acid (Table 1) suggest that the *glc¹* mutation is semidominant. None of the genotypes

tested were temperature sensitive, and only *glc¹* and *glc¹/Df12* were bang sensitive, exhibiting significantly more paralysis than *Or-R* after 5 s of vortexing.

Molecular Analysis. The molecular nature of nodulisporic acid resistance in *glc¹* flies was further examined by direct analysis of DmGluClα cDNAs. Several DmGluClα cDNAs were obtained from *glc¹* flies by reverse transcription, followed by six separate PCR reactions to control for artifacts. Nine DmGluClα cDNA clones from *glc¹* flies were analyzed, and all contained a point mutation resulting in a proline to serine change (P299S). The proline residue P299 is ubiquitous in all known glutamate-, GABA-, and glycine-gated chloride channels and is located immediately C-terminal to the M2 domain. In addition to the P299S mutation, sequencing revealed alternative splicing and RNA editing in DmGluClα, also documented by Semenov and Pak (24). Therefore, in *glc¹* flies, a mixture of alternatively spliced and edited DmGluClα mRNAs appeared to be expressed, all containing the P299S mutation.

Nodulisporic Acid Activation of DmGluClα. The effects of nodulisporic acid on recombinant DmGluClα channels were examined in *Xenopus* oocytes. Fig. 4 shows representative glutamate- and nodulisporic acid-activated currents from an oocyte expressing DmGluClα. The nodulisporic acid-sensitive current had slower activation kinetics than the glutamate-sensitive current and was essentially irreversible on wash-out of the drug. In the experiment shown, saturating concentrations of nodulisporic acid activated approximately 60% of the maximum glutamate-activated current. The relative current amplitudes varied somewhat between experiments, and values between 30% and 60% were observed, with average value of $43 \pm 12\%$ ($n = 5$).

Electrophysiology of DmGluClα P299S. To further test the hypothesis that the activity of nodulisporic acid and ivermectin on glutamate-gated chloride channels was the basis for their insecticidal properties, DmGluClα P299S was constructed by site-directed mutagenesis. Wild-type and P299S DmGluClα were expressed in *Xenopus* oocytes and examined by the two-electrode voltage clamp method. Oocytes injected with either wild-type or P299S DmGluClα RNA produced similar currents in response to high concentrations of glutamate; however, glutamate dose-response curves revealed a reduced sensitivity to glutamate in
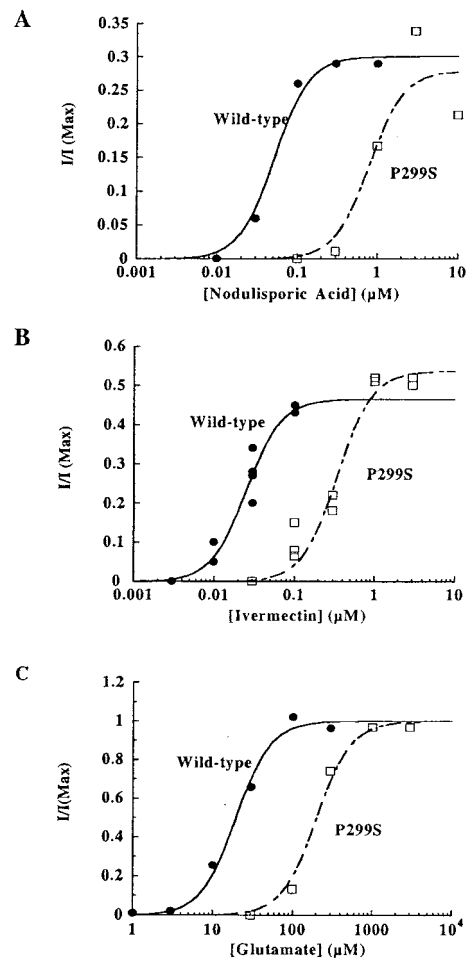
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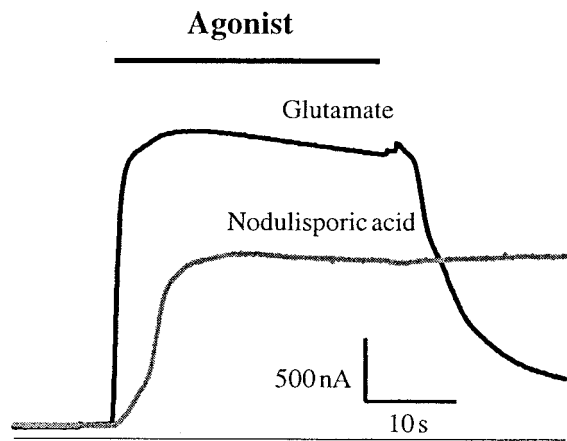
Fig. 4. Nodulisporic acid activates DmGluClα. An oocyte expressing Dm-GluClα exhibited a rapidly activating, reversible current in response to application of 100 μM glutamate. In the same oocyte, 1 μM nodulisporic acid activated a current that was essentially irreversible on wash-out of nodulisporic acid and measured 60% of the glutamate-activated current.

Fig. 5. Agonist sensitivity of DmGluClα P299S. Dose-response curves for nodulisporic acid (*A*), ivermectin (*B*), and glutamate (*C*) activation of Dm-GluClα wild-type (filled circles) and P299S (open squares) channels. Oocytes injected with DmGluClα wild-type or P299S RNA were superfused with varying drug concentrations, and currents were normalized to the current activated by saturating concentrations of glutamate. Data were fit to the Hill equation with $n_h = 2$.

P299S, as compared with wild-type (Fig. 5*C*). Fitting the data to the Hill equation ($n_h = 2$) yielded $EC_{50} = 19.5$ μM for wild-type and 201 μM for P299S channels. Nodulisporic acid and ivermectin activated wild-type and mutant channels irreversibly; therefore, dose-response curves were generated by normalizing nodulisporic acid- and ivermectin-activated currents to the current observed with saturating glutamate concentrations. The maximum current activated by nodulisporic acid was similar for wild-type and P299S channels and in separate experiments reached 30–60% of the maximal glutamate-activated current. However, DmGluClα P299S showed greatly reduced sensitivity to nodulisporic acid (Fig. 5*A*). EC_{50} were 51 nM for wild-type and 790 nM for P299S. This observed 15-fold reduction in nodulisporic acid sensitivity strengthens the hypothesis that, similar to ivermectin, nodulisporic acid acts by activating Dm-GluCl channels. Finally, responses of wild-type and P299S channels to ivermectin were examined. As for glutamate and nodulisporic acid, the dose-response curve for ivermectin was right-shifted in P299S, yielding an EC_{50} of 340 nM compared with 25 nM for DmGluClα wild-type (Fig. 5*B*). This approximately 14-fold reduction in ivermectin sensitivity on the level of the DmGluClα channel was greater than expected from the ivermectin cross-resistance seen in *glc^1* flies and was similar to the reduction in nodulisporic acid sensitivity.

Discussion

Using a drug-resistant strain of *Drosophila* and oocyte expression of recombinant channels, we have shown that glutamate-gated chloride channels are a target for the novel insecticide nodulisporic acid and substantiated this receptor as a target for ivermectin. *Glc^1* flies resistant to nodulisporic acid carry a single point mutation in DmGluClα. This change from a highly conserved proline, located distal to the second transmembrane domain, to a serine residue was found in all nine *glc^1* DmGluClα cDNAs sequenced. Although we cannot rule out the possibility that additional mutations within the region 92A6–92D2 of chromosome 3 may contribute to nodulisporic acid resistance, the properties of recombinant mutant and wild-type channels are consistent with this point mutation underlying the major site of nodulisporic acid resistance seen in *glc^1* flies. Expressed in oocytes, the P299S mutation rendered recombinant DmGluClα channels >10-fold less sensitive to activation by a number of ligands, including nodulisporic acid and ivermectin. That the mutant DmGluClα channel found in *glc^1* flies was significantly less sensitive to activation by the endogenous ligand glutamate may be the reason for the reduced viability and the sickly phenotype of *glc^1* flies. This hypothesis was supported by the fact that *glc^1* flies appeared healthier when kept on nodulisporic acid than in the absence of any drug (unpublished results). Like ivermectin, nodulisporic acid potentiated the response of Dm-GluClα channels to glutamate at concentrations less than needed for channel activation (data not shown); therefore, sublethal levels of nodulisporic acid may restore some of the glutamate-dependent neurotransmission in *glc^1* flies.

On a molecular level, the P299S mutation further highlights the significance of the M2-M3 linker region in ligand-gated chloride channels. Resistance of *Drosophila* to the insecticides dieldrin and fipronil is due to a point mutation in the M2 region of GABA-gated channels (23, 25). In human glycine-gated chloride channels, a point mutation in the extracellular terminus of the M2 segment results in hyperekplexia, known as startle disease, which is a dominant neurological disorder. People with hyperekplexia exhibit increased neural hyperexcitability, probably as a result of decreased agonist affinities (26). In addition, residues in the M2-M3 linker region are critical to volatile anesthetic response in both glycine- and GABA-gated chloride channels (27).

The physiological function of the DmGluClα gene remains unclear. The bang-sensitive and lethargic phenotype of the *glc¹* flies suggests neurologic defects. The map location of Dm-GluClα and *glc¹* drug resistance on chromosome 3;92A4–92B corresponds to a region known to play a role in the visual system. The *ora transientless* or *ort* gene maps to position 92B1, and mutations in this gene lead to visual behavior-defective phenotypes (11, 28). Electroretinograms obtained from *ort* mutants lack the membrane potential transients typically seen at the on- and offset of a light stimulus (11). Because these transients originate from cells in the lamina (29), it was suggested that the *ort* gene may play a role in photoreceptor signaling (11). Several lines of evidence point to histamine as the major neurotransmitter in *Drosophila* photoreceptors (30), and it has been postulated that the *ort* gene may encode a histamine-gated ion channel (W. L. Pak, personal communication). The DmGluClα homomultimer channel as expressed in oocytes does not respond to histamine (5); however, the *Drosophila* genome sequence now reveals another predicted ligand-gated channel at position 92B1, which may be the candidate for the *ort* gene (28).

Glutamate-gated chloride channels have been found only in invertebrates, making them a safe target for anthelmintic and insecticidal drugs. Ivermectin activates glutamate-gated chloride channels, and the ivermectin cross-resistance of *glc¹* flies carrying a mutation in the DmGluClα gene further suggests that this mode of action contributes to ivermectin's insecticidal activity. Ivermectin is widely used to control parasites in humans and animals; however, some cases of ivermectin toxicity have been observed in collies and CF-1 mice, suggesting that ivermectin can act on targets in mammalian brain (31, 32). Possible candidates are mammalian GABA-gated and glycine-gated ion channels, the closest mammalian homologues of invertebrate GluCl channels. In fact, there are a number of reports of ivermectin binding to GABA$_A$ and glycine receptors and activation of GABA$_A$ receptors in rodent brain (21, 33, 34). Binding studies on *Drosophila* head membranes suggest that nodulisporic acid binds only to a subset of ivermectin binding sites with high affinity (3). Hopefully, this greater specificity of nodulisporic acid will be associated with reduced toxicity.

The mutation found in *glc¹* flies confers greater resistance to nodulisporic acid than to ivermectin. There are two possible explanations for this phenomenon. It may be that the P299S mutation has differential effects on ivermectin and nodulisporic acid actions at the same receptor, which is not the case for the recombinant DmGluClα homomultimer expressed in oocytes but cannot be completely ruled out for the native receptor. Alternatively, nodulisporic acid-insensitive ivermectin receptors may be less affected than nodulisporic acid-sensitive ivermectin receptors. Considering the strong effect of the P299S mutation on ivermectin activation of DmGluClα homomultimers, one could postulate the presence of additional unidentified subunits in native nodulisporic acid-insensitive ivermectin receptors.

Recent immunoprecipitation studies suggest that *Drosophila* nodulisporic acid receptors contain both glutamate-gated (Dm-GluClα) and GABA-gated (Rdl) chloride channel subunits (S.W.L., unpublished results). In support of these results, flies carrying a mutation in the *Rdl* gene were 5-fold resistant to nodulisporic acid. In the same studies, all of the solubilized nodulisporic acid and ivermectin binding sites could be immunoprecipitated with antibodies to DmGluClα. A mutation in DmGluClα, as is found in the nodulisporic acid-resistant *Drosophila* strain *glc¹*, would therefore be expected to affect all nodulisporic acid and ivermectin binding sites, in agreement with the binding data presented in this study.

Indirect evidence from binding studies and electrophysiological recordings in grasshopper neurons has pointed to glutamate-gated chloride channels as a target for the novel insecticide nodulisporic acid. The direct activation of the DmGluClα channel and the DmGluClα P299S mutation found in nodulisporic acid-resistant *Drosophila* strongly support this mode of action. In addition, the P299S mutation is a useful tool in mapping sites on ligand-gated ion channels that play a role in channel activation.

1. Ostlind, D. A., Felcetto, T., Misura, A., Ondeyka, J., Smith, S., Goetz, M., Shoop, W. & Mickle, W. (1997) *Med. Vet. Entomol.* **11,** 407–408.
2. Ondeyka, J. G., Helms, G. L., Hensens, O. D., Goetz, M. A., Zink, D. L., Tsipouras, A., Shoop, W. L., Slayton, L., Dombrowski, A. W., Polishook, J. D., *et al.* (1997) *J. Am. Chem. Soc.* **119,** 8809–8816.
3. Smith, M. M., Warren, V. A., Thomas, B. S., Brochu, R. M., Ertel, E. A., Rohrer, S., Schaeffer, J., Schmatz, D., Petuch, B. R., Tang, Y. S., *et al.* (2000) *Biochemistry* **39,** 5543–5554.
4. Cully, D. F., Vassilatis, D. K., Liu, K. K., Paress, P. S., Van der Ploeg, L. H., Schaeffer, J. M. & Arena, J. P. (1994) *Nature (London)* **371,** 707–711.
5. Cully, D. F., Paress, P. S., Liu, K. K., Schaeffer, J. M. & Arena, J. P. (1996) *J. Biol. Chem.* **271,** 20187–20191.
6. Dent, J. A., Smith, M. M., Vassilatis, D. K. & Avery, L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2674–2679.
7. Abalis, I. M. & Eldefrawi, A. T. (1986) *Pestic. Biochem. Physiol* **25,** 279–287.
8. Bermudez, I., Hawkins, C. A., Taylor, A. M. & Beadle, D. J. (1991) *J. Recept. Res.* **11,** 221–232.
9. Kane, N. S., Robichon, A., Dickinson, J. A. & Greenspan, R. J. (1997) *Neuron* **18,** 307–314.
10. Griffith, L. C., Verselis, L. M., Aitkin, K. M., Kyriacou, C. P., Danho, W. & Greenspan, R. J. (1993) *Neuron* **10,** 501–509.
11. O'Tousa, J. E., Leonard, D. S. & Pak, W. L. (1989) *J. Neurogenet.* **6,** 41–52.
12. Wilson, J. E., Connell, J. E., Schlenker, J. D. & Macdonald, P. M. (1996) *Dev. Genet.* **19,** 199–209.
13. Meinke, P. T., Ayer, M. B., Coletti, S. L., Li, C., Lim, J., Ok, D., Salva, S., Schmatz, D. M., Shih, T. L., Shoop, W. L., *et al.* (2000) *Bioorg. Med. Chem. Lett.* **10,** 2371–2374.
14. Schaeffer, J. M. & Haines, H. W. (1989) *Biochem. Pharmacol.* **38,** 2329–2338.
15. Meinke, P. T., Rohrer, S. P., Hayes, E. C., Schaeffer, J. M., Fisher, M. H. & Mrozik, H. (1992) *J. Med. Chem.* **35,** 3879–3884.
16. Roberts, D. B. (1998) in *The Practical Approach*, ed. Hames, B. D. (IRL, Oxford), p. 389.
17. Greenspan, R. J. (1997) *Fly Pushing: The Theory and Practice of Drosophila Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
18. Ashburner, M. (1989) *Drosophila: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
19. Jan, Y. N. & Jan, L. Y. (1978) *Proc. Natl. Acad. Sci. USA* **73,** 515–519.
20. Ganetzky, B. & Wu, C. (1982) *Genetics* **100,** 597–614.
21. Huang, J. & Casida, J. E. (1997) *J. Pharmacol. Exp. Ther.* **281,** 261–266.
22. Martin, R. J. & Pennington, A. J. (1989) *Br. J. Pharmacol.* **98,** 747–756.
23. Ffrench-Constant, R. H., Rocheleau, T. A., Steichen, J. C. & Chalmers, A. E. (1993) *Nature (London)* **363,** 449–451.
24. Semenov, E. P. & Pak, W. L. (1999) *J. Neurochem.* **72,** 66–72.
25. Hosie, A. M., Baylis, H. A., Buckingham, S. D. & Sattelle, D. B. (1995) *Br. J. Pharmacol.* **115,** 909–912.
26. Langosch, D., Laube, B., Rundstrom, N., Schmieden, V., Bormann, J. & Betz, H. (1994) *EMBO J.* **13,** 4223–4228.
27. Mihic, S. J., Ye, Q., Wick, M. J., Kolchine, V. V., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., *et al.* (1997) *Nature (London)* **389,** 385–389.
28. The FlyBase Consortium (1999) *Nucleic Acids Res.* **27,** 85–88.
29. Coombe, P. E. (1986) *J. Comp. Physiol.* **159,** 655–665.
30. Stuart, A. E. (1999) *Neuron* **22,** 431–433.
31. Paul, A. J., Tranquilli, W. J., Seward, R. L., Todd, K. S. J. & DiPietro, J. A. (1987) *Am. J. Vet. Res.* **48,** 684–685.
32. Lankas, G. R., Cartwright, M. E. & Umbenhauer, D. (1997) *Toxicol. Appl. Pharmacol.* **143,** 357–365.
33. Kamijima, M. & Casida, J. (2000) *Appl. Pharmacol.* **163,** 188–194.
34. Krusek, J. & Zemkova, H. (1994) *Eur. J. Pharmacol.* **259,** 121–128.