0026-895X/99/030584-10$3.00/0

MOLECULAR PHARMACOLOGY, **55:**584–593 (1999).



# Altered Properties of Neuronal Sodium Channels Associated with Genetic Resistance to Pyrethroids

DAEWOO LEE, YOONSEONG PARK, THOMAS M. BROWN, and MICHAEL E. ADAMS

*Departments of Entomology and Neuroscience, University of California, Riverside, California (D.L., M.E.A.); Department of Entomology, University of Arizona Tucson, Arizona (Y.P.); and Department of Entomology, Clemson University, Clemson, South Carolina (T.M.B.)*

Received September 17, 1998; accepted November 25, 1998

This paper is available online at http://www.molpharm.org

## ABSTRACT

Genetic resistance to pyrethroid insecticides involves nervous system insensitivity linked to regulatory and structural genes of voltage-sensitive sodium channels. We examined the properties and relative density of sodium channels in central neurons of susceptible and pyrethroid-resistant (Pyr-R) insects that were homozygous for the amino acid substitution V421M in the I-S6 transmembrane segment. Pyr-R sodium channels show ~21-fold lower sensitivity to the synthetic pyrethroid permethrin and a ~2-fold increased sensitivity to the $\alpha$-scorpion toxin Lqh$\alpha$IT. Pyr-R channels also exhibit altered gating properties, including a ~13 mV positive shift in voltage-dependent activation and ~7 mV positive shift in steady-state inactivation. Consistent with these changes in gating behavior, Pyr-R central neurons are less excitable, as evidenced by an ~11 mV elevation of action potential threshold. No differences in sodium channel density are evident. The altered properties of Pyr-R sodium channels provide a plausible molecular basis for nervous system insensitivity associated with pyrethroid resistance.





The prevalence of drug and pesticide use in modern society has led to numerous instances of genetic resistance in target organisms. This poses significant risks and challenges for control of infectious disease and agricultural pests (Collins and Paskewitz, 1995; Taylor and Feyereisen, 1996). Resistance development is an evolutionary process, arising through selection of low-frequency genes in the population that confer survival advantage. Of major importance are loci that promote decreased sensitivity to the drug/toxicant at the molecular site of action (Taylor and Feyereisen, 1996). Understanding the molecular bases for resistance can assist in devising effective strategies for mitigation and in the choice of suitable alternative chemicals.

Resistance to synthetic organic insecticides emerged soon after their introduction in the 1940s. However, elucidation of underlying molecular mechanisms has occurred only recently, aided by the revolution in molecular biology. Most insecticides are neurotoxins that modify the properties of ion channels, receptors, or enzymes involved in signaling within the nervous system. Variability in structural or regulatory genes for these targets provides a basis for selection of resistant individuals with target-site insensitivity (Taylor and Feyereisen, 1996). For example, resistance to cyclodiene insecticides arose from a single amino acid substitution in the neuronal $\gamma$-aminobutyric acid receptor channel pore (ffrench-Constant et al., 1993). Organophosphate resistance can result either from overproduction of acetylcholinesterase or from selection of structural isoforms with altered catalytic properties (Fournier et al., 1993). One form of pyrethroid resistance in temperature-sensitive *Drosophila melanogaster* mutants has been traced to the *nap* locus, a regulatory gene that controls sodium channel density (Kasbeker and Hall, 1988).

In one of earliest reports of insecticide resistance, insects previously exposed to dichlorodiphenyltrichloroethane (DDT) also exhibited resistance to pyrethroids (Busvine, 1951). The signature of this surprising cross-resistance was delayed paralysis attributed to nervous system insensitivity (Miller et al., 1979). The trait came to be known as "knockdown resistance", and two alleles were isolated: *kdr* and *superkdr* (Farnham, 1977; Sawicki, 1978). Both forms of resistance are linked to the *para*-homologous sodium-channel locus in houseflies (Williamson et al., 1993), and cross-resistance traits with similar linkage patterns occur in other insect species (Dong and Scott, 1994), including the tobacco budworm *Heliothis virescens* (Payne et al., 1988; Taylor et al., 1993). Sodium channels homologous to *para* of several resis-

This work was supported by Binational Agricultural Research and Development Grant IS-2486-94C (to M.E.A.) and United States Department of Agriculture Grant SR95 to 07-E-SC (to T.M.B.).

Some of the work described here was presented at the Annual Society for Neuroscience meeting held in November, 1996, in Washington, DC [*Soc Neurosci Abstr* **22:**60 (1996)].

**ABBREVIATIONS:** Pyr-R, pyrethroid-resistant; TTX, tetrodotoxin; *hscp*, *H. virescens* sodium channel *para* homolog; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism analysis; $LC_{50}$, median lethal concentration; UCR-S, UCR-susceptible; Ace-R, acetylcholinesterase-resistant.

tant strains have been cloned, and a pattern of amino acid substitutions has emerged in two different S6 transmembrane segments and a cytoplasmic loop (Dong and Scott, 1994; Williamson et al., 1996; Park et al., 1997; Park and Taylor, 1997). These findings clearly indicate that the mechanism of knockdown resistance results from target-site insensitivity, specifically changes in sodium channel structure. They also confirm that sodium channels are a primary site of action for pyrethroids.

At least five mutations have now been associated with knockdown resistance originating in field populations of insects, but no information is yet available on the properties of sodium channels in neurons of resistant insects. In a preliminary report (Lee et al., 1996), we demonstrated changes in both pharmacological and biophysical properties of sodium channels in pyrethroid-resistant (Pyr-R) *H. virescens*. Since then, the resistance trait has been correlated with the amino acid substitution V421M in the I-S6 transmembrane segment of the *hscp* sodium channel (*H. virescens* sodium channel *para* homolog) (Park et al., 1997). This is the first association of a I-S6 mutation with altered sodium channel properties and pyrethroid resistance. Interestingly, this region of the channel also is at or near binding domains for site 2 and site 3 neurotoxins known to be allosterically coupled to pyrethroid binding (Trainer et al., 1996, 1997). Our evidence shows that sodium channels of Pyr-R insects have reduced sensitivity to pyrethroids as well as altered voltage-dependent gating properties, providing a compelling rationale for reduced nervous system sensitivity to pyrethroids.

## Materials and Methods

### Neurotoxins

The synthetic pyrethroid permethrin (79% *cis* and 21% *trans* isomers; Chem Service Inc., West Chester, PA) was dissolved in dimethyl sulfoxide to make stock solutions (which were kept frozen) at concentrations of 10 and 100 mM. An aliquot of the stock solution was diluted in external recording solution (see below) to give secondary stock solutions of 10 or 100 $\mu$M. Working solutions were prepared by dilution of secondary stocks in external recording solution. Dimethyl sulfoxide was present in control and test solution at concentrations lower than 0.1% (v/v), which had no effect on sodium current. Tetrodotoxin (TTX) was purchased from Sigma Chemical Company (St. Louis, MO). Lqh$\alpha$IT was kindly supplied by Dr. Eliahu Zlotkin (Hebrew University of Jerusalem, Jerusalem, Israel) and Dr. Michael Gurevitz (Tel Aviv University, Tel Aviv, Israel).

### Experimental Animals

The Pyr-R *H. virescens* strain, maintained at the Department of Entomology, Clemson University, originated from ICI-82 and is descended from the resistant HSB strain, in which pyrethroid resistance was inherited as a partial dominant trait (Payne et al., 1988). Pyr-R was founded in 1987 by outcrossing HSB to the pyrethroid-susceptible strain Woodrow83 (Brown et al., 1996) and selecting hybrid larvae with permethrin applied topically at 14 $\mu$g/g. This was followed by six additional rounds of outcrossing Pyr-R to Woodrow83 and selecting F2 progeny with permethrin at 143 $\mu$g/g, a discriminating dose that sacrificed most hybrids. Pyr-R larvae were selected with permethrin at 714 $\mu$g/g in generations 24, 25, 29, 33, 34, 38, 39, 41, 44, 50, 56, 59, 65, 72, 78, 83, and 90. The average mortality in the 17 selections was 43 $\pm$ 13%. The higher selecting dose was sufficient to kill >99% of Pyr-R $\times$ Woodrow83 hybrids. In its current form, Pyr-R exhibits pyrethroid resistance linked to *hscp* in a Woodrow83 background (Payne et al., 1988; Taylor et al., 1993). Pupae of generations 82 through 88 and 90 were used for sodium channel analysis. In this study, we used adult moths (2–9 days after emergence) for all experiments.

Two control strains of *H. virescens* were used for comparison to Pyr-R: UCR-susceptible (UCR-S) and acetylcholinesterase-resistant (Ace-R). UCR-S is a pyrethroid-susceptible tobacco budworm *H. virescens* (UCR-S) that has been maintained at the University of California-Riverside for the past 15 years. The Woodrow83 strain could not be used for comparison because it was lost before this study, but in some experiments, Pyr-R was compared with the pyrethroid-susceptible strain Ace-R, which was produced by crossing Woodrow83 with Florence87 for seven generations (Gilbert et al., 1996).

### Bioassay of Permethrin

The vial technique was used to quantify the in vivo toxicity of permethrin against adult (3–9 days postemergence) *H. virescens*. Permethrin was dissolved in pure acetone and deposited uniformly on the inner walls of 20-ml glass scintillation vials. Two insects were tested per vial. Susceptible (UCR-S and Ace-R) *H. virescens* were exposed to six dosages (0.06, 0.2, 0.6, 2, 6, and 10 $\mu$g/vial. Pyr-R moths were exposed to six dosages (2, 6, 10, 20, 60, and 200 $\mu$g/vial); 22 to 24 moths were tested at four dosages (6, 10, 20, and 60 $\mu$g/vial), and 10 moths were tested at the lowest (2 $\mu$g/vial) and highest (200 $\mu$g/vial) dosages. After a 24-h holding period at room temperature, percentage mortality was determined and corrected for control mortality. Animals were scored as positive for "mortality" if uncontrolled movement and/or loss of righting response were observed. Probit analysis was performed as described previously (Raymond, 1985).

### Molecular Analysis of *hscp* Sodium Channel

Each of four individual specimens from the Pyr-R and UCR-S strains were treated with 5 $\mu$g of permethrin (Chem Service) using the vial technique. All susceptible specimens were sacrificed, whereas none of the resistant individuals were affected. DNA from each individual was extracted by the standard phenol-chloroform method. Two independent polymerase chain reactions (PCR) were performed for each individual specimen to obtain the nucleotide sequence in the region where mutations associated with pyrethroid resistance were identified (Park et al., 1997; Park and Taylor, 1997) (Fig. 2A). The PCR reactions were conducted with primers Nhp1375+ (5′-CCGAACCCTAACTACGGNTA-3′) and IS6r2 (5′-CTGTTCCTCTTCTTCGGCTT-3′) for determination of the polymorphism at V421M in I-S6, and primers Nhp3304+ (5′ ATGTGGGACTGIATGTTGGT-3′) and Nhp3448- (5′-CTGTTGAAGGCCTCTGCTAT-3′) for determination of the polymorphism at L1029H in II-S6 according to reaction conditions described previously (Park et al., 1997; Park and Taylor, 1997). Controls for homozygotes, *His*/*His*, *Leu*/*Leu*, *Met*/*Met*, and *Val*/*Val* shown in Fig. 1 are *RcaI* or *Hsp92II* restrictions of the PCR product using a template of cloned DNA for respective sequences that were published elsewhere (Park et al. 1997). Controls for heterozygotes, *His*/*Leu* and *Met*/*Val*, are restrictions of PCR product using a template of gDNA of heterozygous individuals that were determined previously (Y. Park, unpublished observations). The *hscp* sequence was aligned with the *D. melanogaster para* sodium channel sequence, GenBank accession number M32078 (Loughney et al., 1989); numbering of amino acid positions followed the scheme used for the *para* channel.

The sequence for the Pyr-R strain was obtained in both directions from one individual specimen for the L1029H site and two specimens for the V421M site. The sequence for UCR-S was obtained as described previously (Park et al., 1997; Park and Taylor, 1997). PCRs coupled with restriction fragment length polymorphism analysis (RFLP) were conducted with *Hsp92*II and *Rca*I for L1029H and V421M sites, respectively, as described previously (Park et al., 1997; Park and Taylor, 1997). For the V421M site, *Rca*I cut at the TCATGA motif was counted as methionine and uncut as valine (Fig. 2B). For the L1029H site, *Hsp92*II cut at the motif CATG was counted as histidine and uncut as leucine (Fig. 2C).

### Preparation of Central Neurons

Central neurons of adult *H. virescens* were prepared from dissociated thoracic and abdominal ganglia and cultured for 1 to 3 days. Ganglia were removed and desheathed in sterile, ice-cold $Ca^{2+}$-free insect saline containing 100 mM NaCl, 4 mM KCl, 10 mM HEPES, 5 mM glucose, and 137 mM mannitol, pH 7.0 (modified from Hayashi and Levine, 1992). Desheathed ganglia were treated for 5 to 7 min (~37°C) with a mixture of 0.5 mg/ml collagenase (Type IA; Sigma) and 2 mg/ml dispase (Boehringer Mannheim, Indianapolis, IN) in $Ca^{++}$-free saline. After the ganglia were washed and transferred to a sterile hood, they were gently triturated in L-15 Leibovitz culture medium (GIBCO, Grand Island, NY) supplemented with 700 mg/liter glucose, 400 mg/liter fructose, 60 mg/liter succinate, 3000 mg/liter TC yeastolate, 2800 mg/liter lactalbumin hydrolysate, 60 mg/liter imidazole, 100 μg/ml streptomycin, 100 units/ml penicillin, and 1 μg/ml 20-hydroxyecdysone (modified from Hayashi and Levine, 1992). Dissociated cell bodies were plated onto poly-D-lysine-coated coverslips and incubated for 1 to 3 days at room temperature. Although peak sodium currents decreased over a period of 3 days in culture, no significant changes in channel-gating properties (voltage-dependent activation, steady-state inactivation, and kinetics of fast inactivation) were observed.

### Whole Cell Recording

Sodium channel currents were measured using the whole cell configuration of the patch clamp technique. Patch pipettes were made of borosilicate glass tubing (Drummond, Broomall, PA) using a Zeitz Universal micropipette puller (Augsburg, Germany), coated with Sylgard, and fire polished. Pipettes filled with the internal solution described below had tip resistances of 1 to 2 MΩ.

**Voltage Clamp.** Sodium currents were recorded with patch pipettes filled with an internal recording solution containing 100 mM CsF, 40 mM CsCl, 3 mM $MgCl_2$, 10 mM EGTA, and 5 mM HEPES, pH 7.0. The extracellular recording solution contained 100 mM NaCl, 50 mM choline-Cl, 4 mM KCl, 2 mM $CaCl_2$, 30 mM tetraethyl ammonium-Cl, 1 mM 4-aminopyridine, 10 mM HEPES, and 10 mM glucose, pH 7.0. Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and filtered at 2 kHz.

Neurons were maintained at a holding potential of −108 mV and sodium currents were evoked by brief depolarizing steps to a test potential ($V_T$). Potentials were corrected for average liquid junctional potentials of about −8 mV, and leak currents were subtracted on-line using a P/4 (Bezanilla and Armstrong, 1977) procedure. Data were discarded if voltage errors caused by series resistance remaining after partial compensation were greater than 5 mV. Data acquisition and analysis were performed with the pCLAMP program (ver. 5.5.1; Axon Instruments) using a Dell 466/MX personal computer.

**Current Clamp.** Pipettes were filled with 140 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, and 0.5 mM $CaCl_2$, pH 7.0. The external solution contained 140 mM NaCl, 4 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.0. Neurons were maintained a resting potential of about −72 mV by injection of direct current. Square current pulses were injected through the patch pipette to determine the threshold of action potential generation.

## Results

**Resistance to Permethrin in Pyr-R *H. virescens*.** The Pyr-R line of resistant *H. virescens* resulted from repeated outcrossing of the resistant HSB strain (Payne et al., 1988) to the pyrethroid-susceptible Woodrow83 line and selection of hybrids with lethal doses of permethrin. This placed the Pyr-R gene in a Woodrow83 background and resulted in a resistance ratio of 22-fold using cypermethrin (Pimprale et al., 1997). Because the Woodrow83 line was unavailable for this study, we used two pyrethroid-susceptible strains for comparison to Pyr-R: UCR-S and Ace-R; the latter strain contained a substantial contribution from Woodrow 83 (see *Materials and Methods*). Insects were held in permethrin-coated glass vials or a vehicle control and scored after a 24-h exposure. The median effective concentration ($EC_{50}$) values for the UCR-S and Ace-R strains were 1.1 and 1.0 μg/vial, respectively, whereas the $LC_{50}$ value for the Pyr-R strain was 25.1 μg/vial (Table 1). The resistance ratio comparing Pyr-R to either susceptible stain was thus ~23- to 25-fold, which is

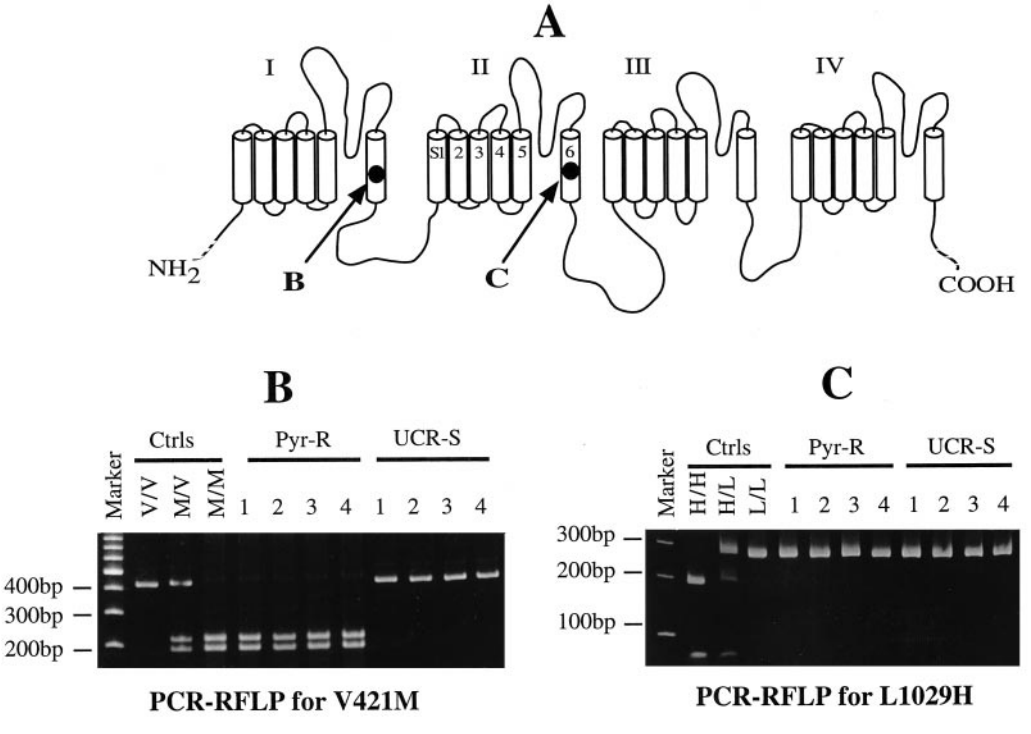


**Fig. 1.** A, diagram showing the predicted *hscp* sodium channel structure. Sequence comparisons made between the Pyr-R and UCR-S strains (except for dashed areas at the amino and carboxyl termini) showed only one sequence polymorphism: V421M (arrow, B) in the I-S6 transmembrane segment (Park et al., 1997). Analysis of a different *H. virescens* collected from Louisiana revealed the L1029H mutation (arrow, C) in the II-S6 segment (Park and Taylor, 1997). B, PCR-RFLP using the restriction enzyme *RcaI* that cuts a methionine motif. Controls (Ctrls) for *Met/Met* homozygotes, *Met/Val* heterozygotes, and Val/Val homozygotes, and each of four individual specimens from Pyr-R and UCR-susceptible (UCR-S) strains are shown. C, PCR-RFLP using the restriction enzyme *Hsp92*II that cuts a histidine motif. Controls (Ctrls) for *His/His* homozygotes, *His/Leu* heterozygotes, and *Leu/Leu* homozygotes, and each four individuals of Pyr-R and UCR-S strains are shown.

virtually identical with the value of 22-fold obtained from previous comparisons of Pyr-R with Woodrow83.

**A Single Amino Acid Substitution in *hscp* Sodium Channel.** Recently, sequence analysis of the *hscp* sodium channel identified two coding polymorphisms associated with pyrethroid resistance in different resistant strains of *H. virescens* (Park et al., 1997; Park and Taylor, 1997). One polymorphism, detected in individual specimens ancestral to Pyr-R (RR; see Park et al., 1997), involves a deduced amino acid change from valine to methionine at position 421 (V421M) located in the IS-6 transmembrane segment (I-S6 nomenclature of Noda et al., 1986; see Fig. 1 for further details). The second polymorphism is a leucine-to-methionine change at amino acid 1029 (L1029H) in transmembrane segment II-S6 (Fig. 1A), found in Louisiana populations of *H. virescens* (Park and Taylor, 1997)

Pyr-R individuals were examined for the occurrence of mutations V421M and L1029H. Before this, each of four UCR-S and 4 Pyr-R individual specimens were exposed to a discriminating dosage of permethrin (5 $\mu$g) to ensure that they carried the resistance phenotype. All UCR-S individuals were sacrificed, whereas all Pyr-R specimens survived. PCR-RFLP analysis was performed on these specimens in two regions of the sodium channel corresponding to reported point mutations in resistant strains (Park et al., 1997; Park and Taylor, 1997). Restriction with *Rca*I in the region corresponding to the mutation V421M in I-S6 was positive at the motif TCATGA in all four Pyr-R specimens tested, which indicates that this genotype is fixed in the Pyr-R strain and that all four individual specimens were homozygous for the V421M mutation (Fig. 1B). Similar analysis of UCR-S specimens confirmed the absence of the V421M mutation. Restriction with *Hsp92*II in the region L1029H of II-S6 (Park and Taylor, 1997) failed in both Pyr-R and UCR-S individuals, which indicates the absence of the L1029H mutation in the specimens tested (Fig. 1C).

Nucleotide sequencing of the 421 and 1029 regions from one Pyr-R specimen yielded results identical with those reported previously for the RR strain of resistant *H. virescens* that is ancestral to Pyr-R (Park et al., 1997). The deduced amino acid sequence in the I-S6 segment reflecting the V421M mutation is aligned with related sodium channel homologs (Fig. 2). Interestingly, the sequence for the squid sodium channel also has methionine at position 421. Comprehensive sequence comparison of UCR-S and RR strains encompassing almost the entire coding region of the channel (>80%, except amino and carboxyl termini) showed that the V421M mutation is the only coding polymorphism (Park et al., 1997).

***H. virescens* Sodium Channels Exhibit Fast Kinetics and TTX Sensitivity.** Because sodium channels in central neurons of *H. virescens* have not been characterized previously, we examined their activation and inactivation kinetics and sensitivity to TTX. Short-term cultured *H. virescens* central neurons show robust voltage-activated sodium currents that display TTX sensitivity and rapid gating kinetics. Low concentrations (0.1–60 nM) of TTX completely abolished sodium currents of both UCR-S and Pyr-R neurons with $K_d$ ~2 nM (Fig. 3, A-C). No difference in TTX sensitivity was observed for the two strains.

We plotted the kinetics of channel activation and inactivation as latency to peak and fast decay of current, respectively. Latency to peak current, expressed as a function of test potential applied, decreased with increasing depolarization (Fig. 3D). The voltage-dependent time to peak was similar in both UCR-S and Pyr-R strains, although the curve was shifted slightly (~5 mV) to more positive potentials in the latter. To examine the kinetics of channel inactivation, the fast decay of the current was fitted with a single exponential (Fig. 3, E and F). Average time constants ($\tau$) of decay were 0.27 ± 0.05 ms (mean ± S.D.; $n$ = 9) for UCR-S and 0.29 ± 0.05 ms ($n$ = 8) for Pyr-R neurons, respectively. These data indicate that sodium channels of UCR-S and Pyr-R strains have similar kinetics of activation and inactivation.

**Sodium Channels of Pyr-R Neurons Have Altered Gating Properties.** We observed marked differences in current-voltage relationships of sodium currents in UCR-S and Pyr-R neurons. Voltage-dependent activation of UCR-S sodium channels began at approximately −48 mV and reached peak values at approximately −23 mV (Fig. 4A). In contrast, Pyr-R channels activated at approximately −38 mV and reached peak near −10 mV (Fig. 4B). Differences in average sodium conductance as a function of test potential were analyzed from a sample population of 18 UCR-S and 13 Pyr-R neurons (Fig. 4C). For UCR-S channels, 50% activation was achieved at −29.1 ± 0.4 mV, whereas for Pyr-R channels, 50% activation occurred at −16.1 ± 0.3 mV. The slope factors describing voltage-dependent activation for UCR-S and Pyr-R were very similar: 4.4 ± 0.2 and 4.5 ± 0.1 for susceptible and resistant neurons, respectively. Our findings thus indicate that voltage-dependent activation of Pyr-R sodium channels is shifted ~13 mV in the positive direction.

Sodium channels of Pyr-R neurons also showed a shift of steady-state inactivation to more positive potentials (Fig.

| | | |
|---|---|---|
| Pyr-R | VLFFVVIIFLGSFYL**M**NLILAIV | |
| UCR-S | VLFFVVIIFLGSFYL**V**NLILAIV | AF025906 |
| B.germanica | MLFFIVIIFLGSFYL**V**NLILAIV | U73584 |
| M.domestica | MLFFIVIIFLGSFYL**V**NLILAIV | X96668 |
| Para | MLFFIVIIFLGSFYL**V**NLILAIV | M32078 |
| Fugu | MIFFVLVIFLGSFYL**I**NLILAVV | D37977 |
| Ratbr1 | MIFFVLVIFLGSFYL**I**NLILAVV | X03638 |
| Ratbr2 | MIFFVLVIFLGSFYL**I**NLILAVV | X03639 |
| Ratbr3 | MIFFVLVIFLGSFYL**V**NLILAVV | Y00766 |
| Rat µ1 | MIFFVVIIFLGSFYL**I**NLILAVV | M26643 |
| Humhh1a | MIFFMLVIFLGSFYL**V**NLILAVV | M77235 |
| Eel | MVFFIMVIFLGSFYL**I**NLILAVV | M22252 |
| Squid | VIYFIVINFFGSLYL**M**NLMLAVV | D14525 |

**Fig. 2.** Alignment of sodium channel sequences in the IS6 transmembrane segment. The V421M mutation associated with pyrethroid resistance in the Pyr-R strain and comparable positions in other sodium channels are shown in bold. GenBank accession numbers are listed in the right column.

5A). Inactivation of currents in UCR-S and Pyr-R neurons became evident around −73 and −63 mV, respectively. Prepulse potentials required for 50% inactivation were −50.1 ± 0.3 ($n$ = 17) for UCR-S channels and −42.9 ± 0.4 mV ($n$ = 10) for Pyr-R channels. The steady-state inactivation curve for Pyr-R neurons was shifted ~7 mV in the positive direction; no difference was observed in slope factors, which were 5.94 ± 0.1 and 5.86 ± 0.2 for susceptible and resistant strains, respectively.

TABLE 1
Toxicity of permethrin to UCR-S and Pyr-R adult *H. virescens*

| Parameter | Strain | | |
|---|---|---|---|
| | UCR-S | Ace-R[a] | Pyr-R |
| $n$[b] | 300[c] | 49[d] | 114[d] |
| Slope | 2.47 ± 0.24 | 3.01 ± 0.64 | 3.07 ± 0.65 |
| $EC_{50}$ (μg/vial)[e] | 1.1 | 1.0 | 25.1 |
| Confidence intervals (μg/vial) | 0.9–1.3 | 0.7–1.3 | 18.1–34.7 |
| Resistance ratio[f] | | | 23.5 (UCR-S)<br>25.1 (Ace-R) |

[a] Pyrethroid-susceptible strain containing Woodrow83 background (Gilbert et al., 1996).
[b] Number of adult *H. virescens* tested excluding control.
[c] All males tested.
[d] Males and females tested.
[e] $P < .05$ $LC_{50}$.
[f] $EC_{50}$ of Pyr-R divided by either $EC_{50}$ for UCR-S or Ace-R.

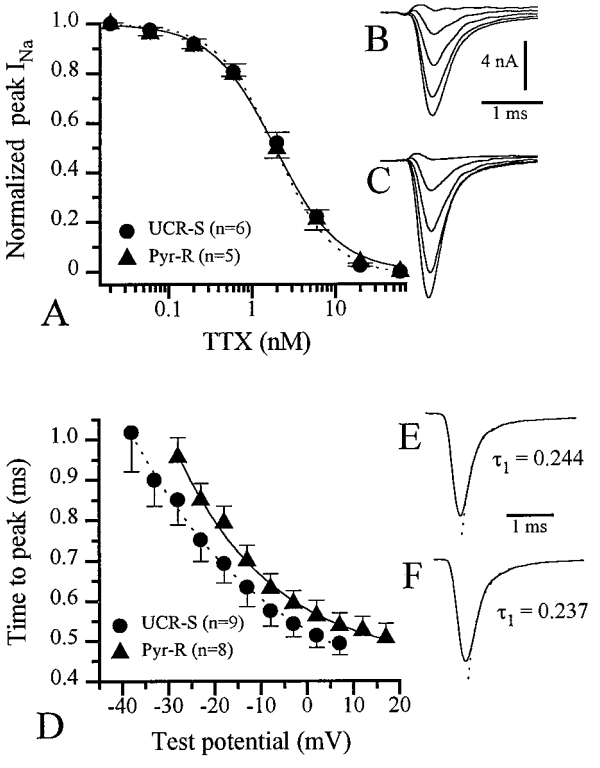


**Fig. 3.** TTX sensitivity and kinetics of *H. virescens* sodium channel gating. A, concentration-response curves for UCR-S and Pyr-R sodium channels show that TTX completely abolishes voltage-activated currents in the concentration range 0.1 to 10 nM, with $K_d$ ~2 nM. B and C, sample current families recorded from UCR-S and Pyr-R neurons, respectively, after application of 0.6, 2, 6, and 60 nM TTX. Largest current in each family is the untreated control. D, kinetics of sodium channel activation in UCR-S and Pyr-R neurons. Time to the peak current from the onset of the depolarization step was measured and plotted as a function of test potential. E and F, fast decay of the sodium current, fitted with double exponentials. $\tau_1$ values associated with typical sodium currents (normalized) are shown.

Recovery from inactivation was examined using a double-pulse protocol in which a conditioning pulse was followed by the second test potential of identical amplitude but varying latency (Fig. 5B, inset). Latencies of between 1 and 2 ms were necessary to achieve full recovery from inactivation, with ~90% recovery observed after about 1 ms. As can be seen in Fig. 5B, no difference in recovery from inactivation was observed for sodium channels in UCR-S ($n$ = 8) and Pyr-R ($n$ = 5) strains.

**Pyr-R Neurons Show Reduced Excitability.** We have demonstrated that voltage-dependent activation of Pyr-R sodium channels is shifted ~13 mV in the positive direction relative to UCR-S channels. It follows that neurons expressing such channels would be less excitable, requiring more positive potentials to reach threshold for action potential generation. Also noted was relatively sluggish behavior of Pyr-R individuals in laboratory culture compared with the UCR-S strain. Therefore, we tested the hypothesis that Pyr-R neurons may have altered excitability by quantifying action potential thresholds.

Neurons were sampled in the whole cell current-clamp configuration for average action potential threshold values in

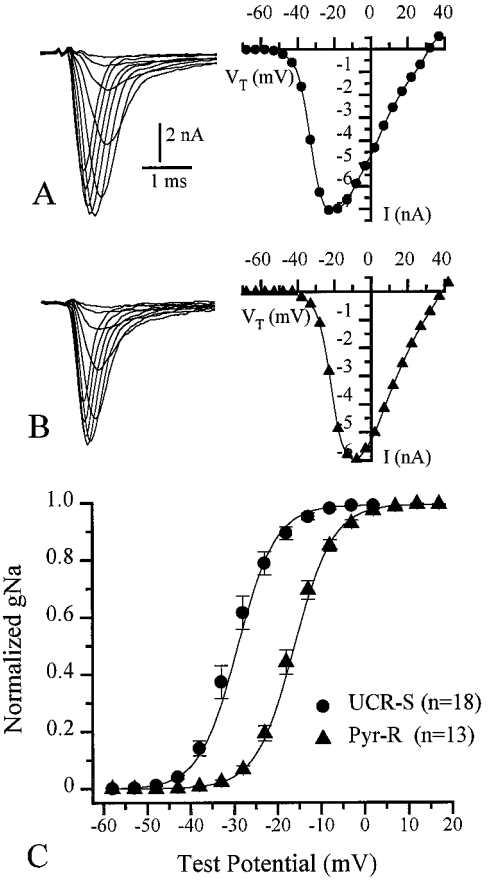


**Fig. 4.** Current-voltage relationships for sodium channels of UCR-S (A) and Pyr-R (B) central neurons. Families of peak currents shown at left were evoked by test potentials every 5 mV from −48 to −3 mV for UCR-S and from −38 to 7 mV for Pyr-R. Typical current/voltage curves for a wider range of voltage steps are shown at right. C, average sodium conductance as a function of test potential for UCR-S and Pyr-R strains. Each value for sodium conductance ($g_{Na}$) was calculated using the equation, $g_{Na} = I_{Na}/(V_T - E_{rev})$ and normalized to maximum sodium conductance. $I_{Na}$ was peak current resulting from applied test potentials ($V_T$) and $E_{rev}$ was the reversal potential of sodium current. Curves were fitted with Boltzmann distribution; error bars = S.E.M.

response to injection of positive current pulses. As shown in Fig. 6, current injection into a UCR-S neuron leads to a linear voltage response between −60 and approximately −36 mV. Upon reaching −36 mV, current injection produces much larger incremental depolarizations leading to the action potential (Fig. 6A). Neurons from a Pyr-R neuron show a linear voltage response until the membrane reaches approximately −25 mV. The average threshold value for 16 UCR-S neurons, defined as the point at which the plot deviates from linearity, was −39.7 mV ± 1.2 mV, whereas the average threshold value from a sample of 15 Pyr-R neurons was −28.4 ± 1.6 mV (Fig. 6B). These data indicate that the action potential threshold is elevated by ~11 mV for neurons of the Pyr-R strain, and verifies the prediction from voltage clamp data and behavioral observations that Pyr-R neurons are significantly less excitable than their UCR-S counterparts.

**Sodium Channels of Pyr-R Strain Are Less Sensitive to Permethrin.** Pyrethroids modify sodium channels through inhibition of inactivation and deactivation, the latter evident as an extreme prolongation of the tail current (Narahashi, 1996). We found that exposure to permethrin prolonged sodium channel tail currents ($I_{Na\text{-}tail}$) of both UCR-S and Pyr-R central neurons (Fig. 7). Notably, compared with UCR-S channels, Pyr-R sodium channels were less sensitive to permethrin.

To quantify this difference in sensitivity, we devised an assay protocol suitable for establishing concentration-response curves while controlling for two additional variables affecting the permethrin response. First, the pronounced lipophilicity of pyrethroids precluded attainment of true equilibrium conditions during the time course of our experiments. Second, the effects of pyrethroids are use dependent, because of the long periods of gating charge immobilization after channel activation (Salgado and Narahashi, 1993). To control for these factors, we applied an identical pulse protocol to neurons of each strain. Test depolarizations ($V_T$) were chosen to cause maximum peak sodium current for each cell sampled. Accordingly, the magnitude of $V_T$ applied was more positive for Pyr-R neurons (−8 mV) than for UCR-S neurons (−18 mV), because of the shift in voltage-dependent gating observed in the resistant strain (see Fig. 4). Upon application of each successive permethrin concentration to the bath, we applied $V_T$ at 10-s intervals continuously for ~3 to 5 min, during which time $I_{Na\text{-}tail}$ increased rapidly to a new steady-state value.

As illustrated in Fig. 7A, a neuron previously exposed to 0.3 μM permethrin has achieved a steady-state tail current.

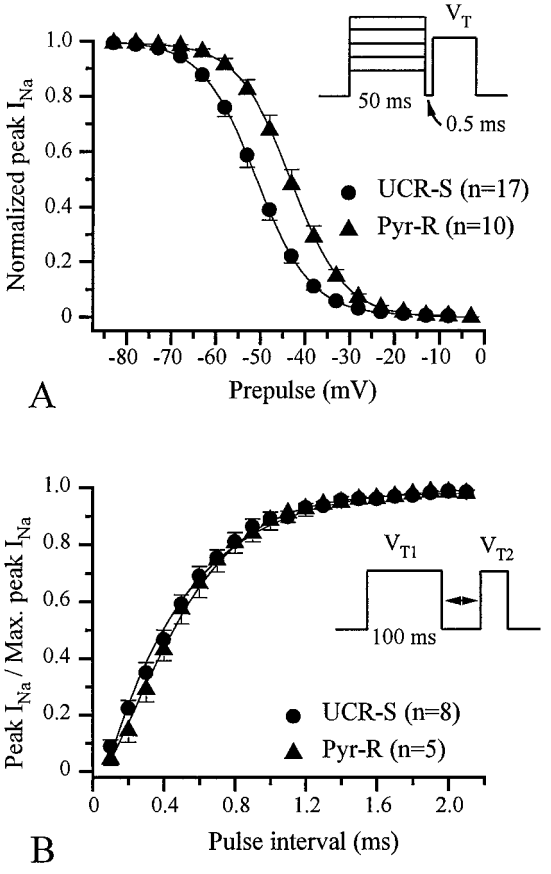


**Fig. 5.** Steady-state inactivation (A) and recovery from inactivation (B) of sodium channels in UCR-S and Pyr-R neurons. A, each peak current was evoked by a test potential ($V_T$) after a 50-ms prepulse as shown in the upper right. The peak current resulting from the test pulse was normalized to the maximum peak current and plotted as a function of the prepulse potential. Steady-state inactivation ($h\infty$) curves were fitted with the Boltzman equation: $h\infty = \{(1 - C)/\{1 + \exp[(V_P - V_{1/2})/k]\}\} + C$, where $V_{1/2}$ is the voltage at half-inactivation; $V_P$ and $k$ are the prepulse potential and slope factor, respectively. C, the noninactivated fraction of current if any remains. B, to measure recovery from inactivation, peak currents were evoked by $V_{T2}$ applied at various time intervals after $V_{T1}$ (inset). Peak currents (Peak $I_{Na}$) were normalized to the maximum peak sodium current (Max. peak $I_{Na}$) and plotted as a function of pulse intervals (bar = S.E.M.).

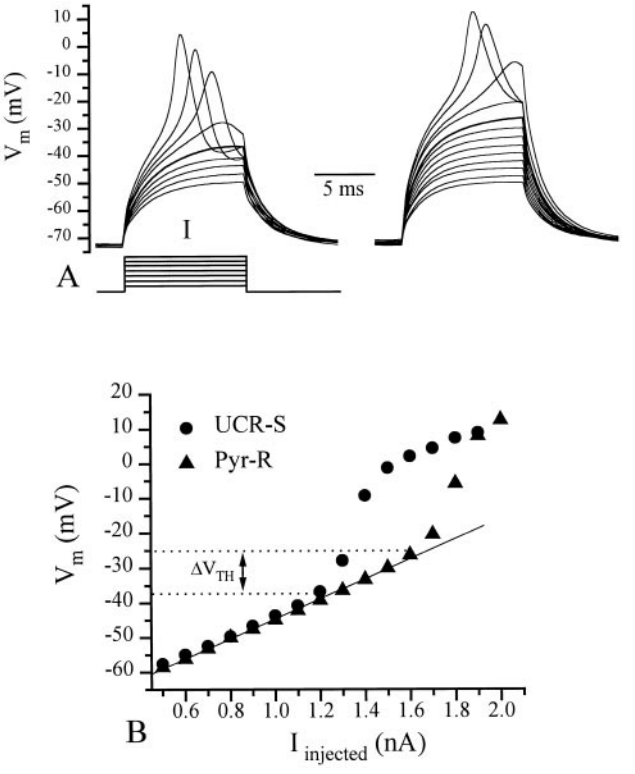


**Fig. 6.** Comparison of action potential threshold ($V_{TH}$) for UCR-S and Pyr-R neurons measured under current clamp conditions. A, current pulses ($I_{injected}$; 10-ms duration) of increasing magnitude elicited progressively more positive voltage responses ($V_m$) until the active membrane response led to an action potential in UCR-S (left) and Pyr-R (right) neurons. The relationship between injected current pulses and membrane potential response was linear until $V_{TH}$ was reached (B). As shown in B, $V_{TH}$ is about 11 mV more positive in the Pyr-R cell compared with UCR-S cell.

Addition of 0.6 $\mu$M permethrin led to a rapid increase in $I_{Na\text{-}tail}$ to a new steady-state value within 3 min. Subsequent addition of 1 $\mu$M permethrin led to further inhibition of deactivation and consequent increase in tail current to a new $I_{Na\text{-}tail}$ value. UCR-S neurons showed pronounced tail-current increases at permethrin concentrations as low as 60 nM (Fig. 7B). On the other hand, much higher concentrations—>1 $\mu$M—were necessary to elicit comparable effects in the Pyr-R strain (Fig. 7C).

Tail current magnitudes, measured 12 ms after the end of the test depolarization, were taken ~3 to 5 min after each concentration of permethrin applied (Fig. 7, B and C). These values were plotted as a function of permethrin concentration (Fig. 7D). Each concentration-dependent, steady-state $I_{Na\text{-}tail}$ value was expressed as a percentage of the modified channels (%M) using the following equation (Tatebayashi and Narahashi, 1994):

$$\%M = \{[\, I_{Na\text{-}tail}/(V_H - E_{rev})]/[\text{peak } I_{Na}/(V_T - E_{rev})]\} \times 100$$

where peak $I_{Na}$ is a peak sodium current before permethrin exposure, and $V_T$, $V_H$, and $E_{rev}$ are the test potential, holding potential and the reversal potential of sodium current, respectively. Sodium channels of the Pyr-R neurons were clearly less sensitive to permethrin. The effective range of permethrin concentration for modification of UCR-S neurons was 0.1 to 10 $\mu$M; the lowest effective concentrations (0.1–0.2 $\mu$M) modified a few percent of sodium channels, shown previously as sufficient to cause altered excitability in neurons (Tatebayashi and Narahashi, 1994); 1 $\mu$M permethrin modified ~10% of UCR-S sodium channels. In contrast, 1 $\mu$M permethrin had no effect on Pyr-R channels, and >20 $\mu$M was needed to modify ~10% of Pyr-R sodium channels. Concentrations of permethrin exceeding 30 $\mu$M led to unstable currents and consequently were not tested. Overall, in the range of permethrin concentrations causing ~5 to ~20% modification of channels, Pyr-R sodium channels showed a ~21-fold reduced sensitivity.

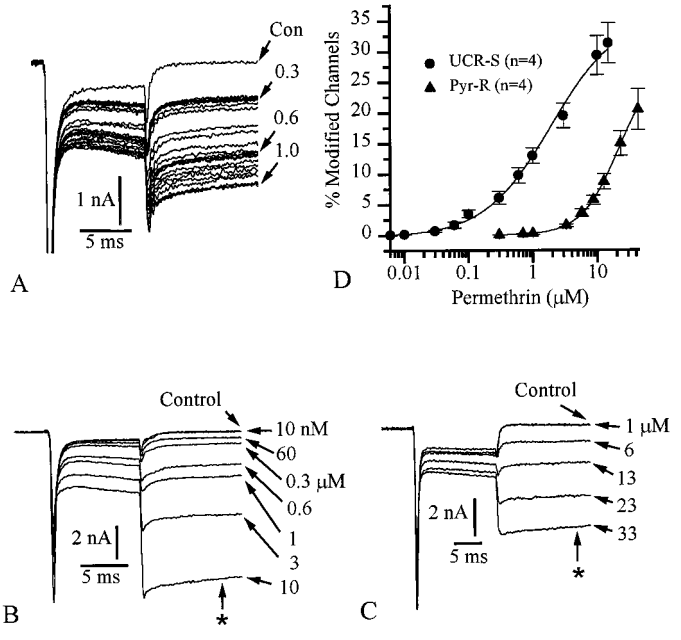


**Fig. 7.** Differential sensitivity of sodium currents in UCR-S and Pyr-R *H. virescens* neurons to permethrin. A, example of prolonged tail currents caused by sequential applications of 0.3, 0.6, and 1 $\mu$M permethrin to a UCR-S neuron. Depolarizing pulses ($V_T$ = −18 mV) to achieve maximal channel activation were applied at ~10-s intervals; the holding potential ($V_H$) was −108 mV. Control sodium current (Con) completely deactivated upon returning to $V_H$, but each application of progressively higher permethrin concentrations produced rapid increases of tail current to new steady-state values within ~3 to 5 min. B, tail currents recorded from a UCR-S neuron following exposure increasing concentrations of permethrin ($V_T$ = −18 mV). C, tail currents recorded from Pyr-R neurons following permethrin exposure ($V_T$ = −8 mV). D, percentage of modified channels plotted as a function of permethrin concentration. Each $I_{Na\text{-}tail}$ measured 12 ms after the end of $V_T$ (*) was used to calculate % modified channels; see text for details (error bars = S.E.M.).

**Enhanced Sensitivity of Pyr-R Sodium Channels to *α*-Scorpion Toxin.** Sodium channels are modified by a variety of natural and synthetic agents. As many as six nonoverlapping binding sites have been defined, many of which interact allosterically (Lombet et al., 1988). Given the differences in voltage-dependent gating and pyrethroid sensitivity observed here for Pyr-R sodium channels, we examined whether they might exhibit altered sensitivity to other classes of modifiers. In particular, we compared the sensitivities of UCR-S and Pyr-R sodium channels to the $\alpha$-scorpion toxin Lqh$\alpha$IT (Eitan et al., 1990). Lqh$\alpha$IT dramatically slowed inactivation of both UCR-S and Pyr-R sodium channels in a concentration-dependent manner (Fig. 8 insets). To quantify modification of sodium currents by the toxin, elevation of steady-state sodium current ($I_{Na(s\text{-}s)}$) was normalized to maximum amplitude and plotted as a function of Lqh$\alpha$IT concentration (Fig. 8). The median effective dose ($ED_{50}$) for UCR-S and Pyr-R channels was 0.71 ± 0.03 and 1.84 ± 0.06 nM, respectively. Slope factors, 2.12 ± 0.1 for UCR-S and 2.15 ± 0.1 for Pyr-R, were not significantly different. These results show that Pyr-R sodium channels were about 2.6 times more sensitive than their UCR-S counterparts to Lqh$\alpha$IT.

**Sodium Channel Density Is Unchanged in Pyr-R Neurons.** Low levels of pyrethroid resistance in *D. melanogaster* mutants have been associated with a reduction in sodium channel density and lower excitability of neuronal

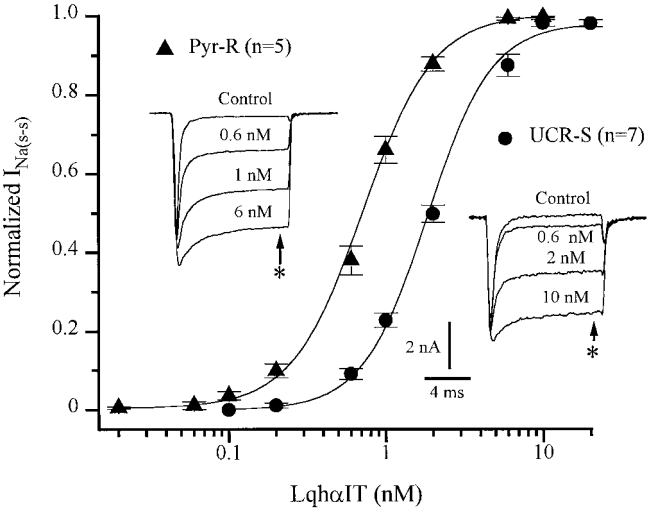


**Fig. 8.** Differential effects of the $\alpha$-scorpion toxin Lqh$\alpha$IT on sodium currents in UCR-S and Pyr-R neurons. Inset (lower right), inhibition of sodium-channel inactivation in a UCR-S neuron caused by exposure of 0.6, 2, and 10 nM Lqh$\alpha$IT. Inset (upper left), effects of 0.6, 1, and 6 nM Lqh$\alpha$IT on a Pyr-R neuron. All steady-state currents modified were normalized to a maximum steady-state sodium current induced by this toxin and plotted as a function of Lqh$\alpha$IT concentration. Error bars indicate S.E.M. Sodium channels in Pyr-R neurons are about 2.6 times more sensitive to the toxin.

membranes (Kasbeker and Hall, 1988). We compared sodium-channel density in UCR-S and Pyr-R neurons by normalizing the maximum peak sodium current to relative cell size, expressed as a function of whole cell capacitance ($C_m$). We found that, after the initial 3-day incubation period, sodium-channel density gradually decreased in culture, but that relative channel densities of UCR-S and Pyr-R neurons were not significantly different (Fig. 9).

## Discussion

Our examination of sodium channels in central neurons of Pyr-R insects has yielded evidence for structural change resulting from the mutation V421M reported previously (Park et al., 1997). These data provide a clear mechanistic basis for pyrethroid resistance—reduced sensitivity at the molecular target site. The involvement of sodium channel regulatory elements, implicated in other instances of pyrethroid resistance (Kasbeker and Hall, 1988), is not indicated, because relative channel density is unchanged. Our study has been restricted to characterization of sodium channels in central neurons, and therefore other genetic factors (e.g., metabolic processes) could also contribute to the overall magnitude of resistance. Nevertheless, our findings together with those of previous reports on the Pyr-R strain strongly support the hypothesis that structural change in the sodium channel is a major factor in the resistance observed. This evidence includes nervous system insensitivity to pyrethroids (Payne, 1987), cross-resistance to pyrethroids and DDT (Brown et al., 1996), genetic linkage between resistance and the *hscp* sodium channel (Taylor et al., 1993), and the mutation V421M in the I-S6 transmembrane segment of the channel (Park et al., 1997).

The V421M substitution fits an emerging pattern of S6 mutations implicated in *kdr*-like pyrethroid resistance. The most common mutation (Leu-to-Phe) was first identified by Williamson et al. in the II-S6 transmembrane segment of *kdr* houseflies and is now documented in numerous species of Pyr-R insects (Williamson et al., 1996; Dong, 1997; Park et al., 1997; Park and Taylor, 1997). This resistance factor occurred in the original housefly strain reported to possess pyrethroid/DDT cross-resistance (Busvine, 1951) and seems to be a particularly stable allele. A mutation at the homologous position, but involving a Leu-to-His mutation instead of a Leu-to-Phe mutation, was detected in a Pyr-R strain of *H. virescens* different from Pyr-R (Park and Taylor, 1997). Finally, a III-S6 mutation is associated with pyrethroid resistance in the temperature-sensitive *para*$^{74}$ *D. melanogaster* mutant (Pittendrigh et al., 1997). Based on the prevalence of S6 mutations in Pyr-R insects, the V421M substitution is well placed as a causal factor in the resistance observed in the Pyr-R strain.

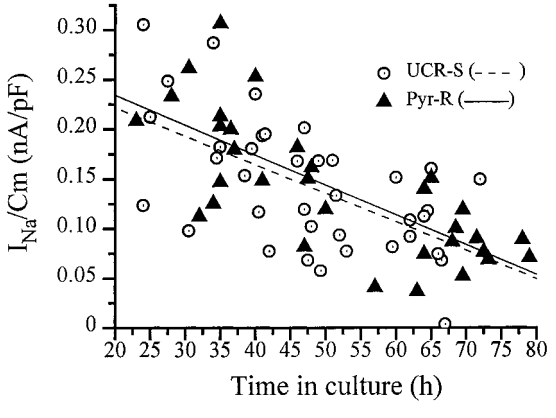


**Fig. 9.** Relative sodium channel densities in UCR-S and Pyr-R neurons calculated as maximum peak sodium current divided by whole cell capacitance ($C_m$). Relative density decreases as a function of time in culture, but no significant difference is evident between UCR-S and Pyr-R strains.

Does the V421M substitution account for the differences in sodium channel properties we have observed in this study? This seems likely, based on extensive sequencing of the *hscp* gene and the nature of the alterations we have observed. V421M is the only sequence polymorphism found in *hscp* sequence comparisons between UCR-S and Pyr-R specimens encompassing 80% of the channel sequence (Park et al., 1997). Regions of the *hscp* channel remaining to be defined lie only at the extreme 5′ and 3′ ends of the molecule (see Fig. 1), areas unlikely to account for the changes reported here. More to the point, the altered gating properties we have observed correspond to those obtained independently by others examining mutations at the same location. A virtually copositional mutation of the rat $\mu$1 sodium channel (N434A; corresponds to 422 in the *hscp* channel; see Fig. 2) shifts voltage- dependent activation ~23 mV positive and fast inactivation ~12 mV positive (Wang and Wang, 1997). These shifts are similar to those we have associated with the V421M substitution. Position 421 also lies in the putative batrachotoxin binding site [site 2; (Trainer et al., 1996)], which is allosterically coupled to pyrethroid binding. Mutations N434K and I433K (the latter copositional with 421 in the *hscp* channel) in the rat $\mu$1 sodium channel abolish sensitivity to batrachotoxin (Wang and Wang, 1998). Based on this corroborative evidence, the V421M substitution seems well situated to account for reduced pyrethroid sensitivity and shifts in voltage-dependent gating properties we have found.

We also have observed altered sensitivity of Pyr-R channels to the $\alpha$-scorpion toxin Lqh$\alpha$IT, a site 3 ligand. Biochemical studies have shown that site 3 is allosterically coupled to pyrethroid binding (Trainer et al., 1997). In addition, a portion of the site 3 receptor lies in the I-S5-S6 extracellular loop, directly adjacent to the I-S6 transmembrane segment (Thomsen and Catterall, 1989). Modification of site 3 affinity by the V421M mutation thus has a reasonable biochemical and structural basis.

What is the relationship between reduced pyrethroid sensitivity and altered voltage-dependent gating in Pyr-R sodium channels? The V421M mutation might alter the pyrethroid binding site directly, or allosterically by modifying voltage-dependent conformational states of the channel. Interestingly, examination of the *kdr* mutation L1014F occurring in the II-S6 transmembrane segment of the housefly sodium channel (Smith et al., 1997) showed reduction of pyrethroid sensitivity without obvious changes in gating properties. This suggests that reduced pyrethroid sensitivity is not necessarily a function of gating-property alteration. However, the same mutation in the rat IIA sodium channel produced a clear shift in the voltage-dependent activation curve (Vais et al., 1997). This discrepancy serves to emphasize that each channel may respond differently to particular point mutations.

As a first step in the analysis of site-insensitive pyrethroid resistance, we have characterized sodium channels of *H. virescens* in their native environment, central neurons. Further mutational analysis will be facilitated by expression of sodium channels in heterologous systems. Heterologous expression should provide additional evidence for a causal relationship between the V421M substitution and altered channel properties and should allow us to distinguish between direct effects of mutations on the pyrethroid binding site and indirect effects occurring through altered voltage-dependent conformational states of the channel. However, clear answers to these questions may require extensive mutagenesis experiments. Recent work of Wang and Wang (1997) on the rat muscle $\mu$1 sodium channel has implications for this issue. They found that the N434A mutation produced a large effect on voltage-dependent gating, whereas N434K did not. Nevertheless, both mutations abolished batrachotoxin sensitivity. It is possible that heterologous expression of the channel may introduce other modulatory influences on the channel arising from differences in postranslational processing, second messenger modulation, and possible contributions of accessory subunits. Such changes will be possible to recognize given the data on neuronal properties reported here.

The stability of insecticide resistance genes in insect populations is likely to be related to their fitness costs (Taylor and Feyereisen, 1996). In this study, we showed that altered voltage-dependent activation of Pyr-R channels is associated with decreased cellular and behavioral excitability. Specifically, Pyr-R neurons exhibit a higher threshold (~11 mV) for action potential generation and relatively sluggish behavior compared with their counterparts in the UCR-S strain. This suggests a possible fitness cost associated with the V421M substitution. Although comparative measures of fitness among the sodium-channel genotypes in isogenic lines are complex and not yet quantified, some reduction in fitness of Pyr-R *H. virescens* (Campanhola et al., 1991) may result from decreased neuronal excitability associated with the V421M mutation. Indeed, population genetic analysis of pyrethroid resistance mutations revealed that the frequency of the V421M allele in field populations of *H. virescens* decreased during the interval 1990 to 1996/1997, whereas the incidence of the L1029H mutation increased during the same period (Park, 1998). As mentioned above, examination of the homologous Leu-to-Phe mutation in housefly sodium channels showed no significant effect on gating characteristics of sodium channels (Smith et al., 1997). This may be a consequence of negligible fitness costs associated with this mutation and thus may help to explain the stability of this mutation in diverse populations of resistant insects.

It has been almost 50 years since the first report of cross-resistance to pyrethroids and DDT, subsequently termed knockdown resistance (Busvine, 1951; Farnham, 1977). Our findings provide some of the first detailed molecular and physiological correlates of this type of resistance, and support the hypothesis that the V421M mutation modifies either the pyrethroid binding site directly or allosterically. Further studies are under way to elucidate the precise molecular mechanisms leading to reduced pyrethroid sensitivity. With the identification of associated sodium channel mutations, hypotheses testing should lead to a greater understanding of the molecular mechanisms underlying resistance, as well as insights into the mechanism by which pyrethroids modify sodium channels.

**Acknowledgments**

We thank Yong Zhao and Karen Bryson for technical assistance, and Profs. Michael Gurevitz and Eliahu Zlotkin for gifts of scorpion toxin.

**References**

Bezanilla F and Armstrong CM (1977) Inactivation of the sodium channel. I. Sodium current experiments. *J Gen Physiol* **70:**549–566.
Brown TM, Bryson PK and Payne GT (1996) Synergism by propynyl aryl ethers in permethrin-resistant tobacco budworm larvae, *Heliothis virescens*. *Pestic Sci* **43:** 323–331.
Busvine JR (1951) Mechanism of resistance to insecticide in houseflies. *Nature (Lond)* **168:**193–195.
Campanhola C, McCutchen BF, Baehricke EH and Plapp FW (1991) Biological constraints associated with resistance to pyrethroids in the tobacco budworm (Lepidoptera, Noctuidae). *J Econ Ent* **84:**1404–1411.
Collins FH and Paskewitz SM (1995) Malaria: Current and future prospects for control. *Annu Rev Entomol* **40:**195–219.
Dong K (1997) A single amino acid change in the para sodium channel protein is associated with knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect Biochem Mol Biol* **27:**93–100.
Dong K and Scott JG (1994) Linkage of kdr-type resistance and the para-homologous sodium channel gene in German cockroaches (*Blattella germanica*). *Insect Biochem Mol Biol* **24:**647–654.
Eitan M, Fowler E, Herrmann R, Duval A, Pelhate M and Zlotkin E (1990) A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: Purification, primary structure, and mode of action. *Biochemistry* **29:**5941–5947.
Farnham AW (1977) Genetics of resistance of house flies (*Musca domestica* L.) to pyrethroids. I Knockdown resistance. *Pestic Sci* **8:**631–636.
ffrench-Constant RH, Steichen JC, Rocheleau TA, Aronstein K, and Roush RT (1993) A single-amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in *Drosophila* populations. *Proc Natl Acad Sci USA* **90:**1957–1961.
Fournier D, Mutero A, Pralavorio M and Bride JM (1993) Drosophila acetylcholinesterase: Mechanisms of resistance to organophosphates. *Chem Biol Interact* **87:** 233–238.
Gilbert RD, Bryson PK and Brown TM (1996) Linkage of acetylcholinesterase insensitivity to methyl parathion resistance in *Heliothis virescens*. *Biochem Genet* **34:**297–312.
Hayashi JH and Levine RB (1992) Calcium and potassium currents in leg motoneurons during postembryonic development in the hawkmoth *Manduca sexta*. *J Exp Biol* **171:**15–42.
Kasbeker DP and Hall LM (1988) A *Drosophila* mutation that reduces sodium channel number confers resistance to pyrethroid insecticides. *Pestic Biochem Physiol* **32:**135–145.
Lee D, Brown TM and Adams ME (1996) Altered biophysical and pharmacological profiles of sodium channels in pyrethroid-resistant insects. *Soc Neurosci Abstr* **22:**60.
Lombet A, Mourre C and Lazdunski M (1988) Interaction of insecticides of the pyrethroid family with specific binding sites on the voltage-dependent sodium channel from mammalian brain. *Brain Res* **459:**44–53.
Loughney K, Kreber R and Ganetzky B (1989) Molecular analysis of the *para* locus, a sodium channel gene in *Drosophila*. *Cell* **58:**1143–1154.
Miller TA, Kennedy JM and Collins C (1979) CNS insensitivity to pyrethroids in the resistant *kdr* strain of house flies. *Pestic Biochem Physiol* **12:**224–230.
Narahashi T (1996) Neuronal ion channels as the target sites of insecticides. *Pharmacol Toxicol* **79:**1–14.
Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima H, Kurasaki M, Takahashi H, Numa S (1986) Existence of distinct sodium channel messenger RNAs in rat brain. *Nature (London)* **320:**188–192.
Park Y (1998) Evolutionary succession of pyrethroid resistance mutations in a sodium channel of *Heliothis virescens* F. Ph.D. dissertation, University of Arizona, Tucson, AZ.
Park Y and Taylor MF (1997) A novel mutation L1029H in sodium channel gene *hscp* associated with pyrethroid resistance for *Heliothis virescens* (Lepidoptera: Noctuidae). *Insect Biochem Mol Biol* **27:**9–13.
Park Y, Taylor MFJ and Feyereisen R (1997) A valine421 to methionine mutation in IS6 of the *hscp* voltage-gated sodium channel associated with pyrethroid resistance in *Heliothis virescens* F. *Biochem Biophys Res Comm* **239:**688–691.
Payne GT (1987) Inheritance and mechanisms of permethrin resistance in the tobacco budworm (Lepidoptera: Noctuidae). Ph.D. dissertation, Clemson University, Clemson, SC.
Payne GT, Blenk RG and Brown TM (1988) Inheritance of permethrin resistance in the tobacco budworm (Lepidoptera: Noctuidae). *J Econ Ent* **81:**65–73.
Pimprale SS, Besco CL, Bryson PK and Brown TM (1997) Increased susceptibility of pyrethroid-resistant *Heliothis virescens* (Lepidoptera: Noctuidae) to chlorfenapyr. *J Econ Ent* **90:**49–54.
Pittendrigh B, Reenan R, ffrench-Constant RH and Ganetzky B (1997) Point mutations in the Drosophila sodium channel gene para associated with resistance to DDT and pyrethroid insecticides. *Mol Gen Genet* **256:**602–610.
Raymond M (1985) Presentation d'un programe d'analyse log-probit a pour micro-ordinnateur. *Cah ORSTOM ser Ent med et Parasitol* **22:**117–121.

Salgado VL and Narahashi T (1993) Immobilization of sodium channel gating charge in crayfish giant axons by the insecticide fenvalerate. *Mol Pharmacol* **43:**626–634.
Sawicki R (1978) Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. *Nature* **275:**443–444.
Smith TJ, Lee SH, Ingles PJ, Knipple DC and Soderlund, DM (1997) The L1014F point mutation in the house fly Vssc1 sodium channel confers knockdown resistance to pyrethroids. *Insect Biochem Mol Biol* **27:**807–812.
Tatebayashi H and Narahashi T (1994) Differential mechanism of action of the pyrethroid tetramethrin on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J Pharmacol Exp Ther* **270:**595–603.
Taylor M and Feyereisen R (1996) Molecular biology and evolution of resistance of toxicants. *Mol Biol Evol* **13:**719–734.
Taylor MF, Heckel DG, Brown TM, Kreitman ME and Black B (1993) Linkage of pyrethroid insecticide resistance to a sodium channel locus in the tobacco budworm. *Insect Biochem Mol Biol* **23:**763–775.
Thomsen WJ and Catterall WA (1989) Localization of the receptor site for alpha-scorpion toxins by antibody mapping: implications for sodium channel topology. *Proc Natl Acad Sci USA* **86:**10161–10165.
Trainer VL, Brown GB and Catterall WA (1996) Site of covalent labeling by a photoreactive batrachotoxin derivative near transmembrane segment IS6 of the sodium channel alpha subunit. *J Biol Chem* **271:**11261–11267.
Trainer VL, McPhee JC, Boutelet BH, Baker C, Scheuer T, Babin D, Demoute JP, Guedin D and Catterall WA (1997) High affinity binding of pyrethroids to the alpha subunit of brain sodium channels. *Mol Pharmacol* **51:**651–657.
Vais H, Williamson MS, Hick CA, Eldursi N, Devonshire AL and Usherwood PN (1997) Functional analysis of a rat sodium channel carrying a mutation for insect knock-down resistance (kdr) to pyrethroids. *FEBS Lett* **413:**327–332.
Wang S-Y and Wang GK (1998) Point mutations in segment I-S6 render voltage-gated Na+ channels resistant to batrachotoxin. *Proc Natl Acad Sci USA* **95:**2653–2658.
Wang SY and Wang GK (1997) A mutation in segment I-S6 alters slow inactivation of sodium channels. *Biophys J* **72:**1633–1640.
Williamson MS, Denholm I, Bell CA and Devonshire AL (1993) Knockdown resistance (kdr) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*). *Mol Gen Genet* **240:**17–22.
Williamson MS, Martinez-Torres D, Hick CA and Devonshire AL (1996) Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Mol Gen Genet* **252:**51–60.

**Send reprint requests to:** Dr. Michael E. Adams, Departments of Entomology and Neuroscience, 5419 Boyce Hall, University of California, Riverside, CA 92521. E-mail: adams@ucrac1.ucr.edu