TITLE PAGE

Role of the Local Anesthetic Receptor in the State-Dependent Inhibition of Voltage-Gated Sodium Channels by the Insecticide Metaflumizone

Richard T. von Stein and David M. Soderlund

Department of Entomology (DMS); Graduate Field of Environmental Toxicology (RTvS, DMS)

New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456, USA

RUNNING TITLE PAGE

a) Running title: Metaflumizone action at local anesthetic receptor

b) Correspondence:

David M. Soderlund

Department of Entomology

New York State Agricultural Experiment Station

Cornell University

630 W. North St.

Geneva, NY 14456, USA

315-787-2363

315-787-2326 (fax)

E-mail: dms6@cornell.edu

c) Manuscript counts:

Text pages: 24 (Abstract thru references)

Tables: 1

Figures: 8

References: 40

Abstract: 245

Introduction: 749

Discussion: 1,408

d) Abbreviations:

Na_v, voltage-gated sodium; SCI, sodium channel inhibitor; LA, local anesthetic; HEPES, 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide

Abstract

Sodium channel inhibitor (SCI) insecticides selectively target voltage-gated sodium (Na_v) channels in the slow-inactivated state by binding at or near the local anesthetic (LA) receptor within the sodium channel pore. Metaflumizone is a new insecticide for the treatment of fleas on domesticated pets and has recently been reported to block insect sodium channels in the slow-inactivated state, thereby implying that it is also a member of the SCI class. Using the two-electrode voltage clamp technique, we examined metaflumizone inhibition of rat $Na_v1.4$ sodium channels expressed in *Xenopus* oocytes. Metaflumizone selectively inhibited $Na_v1.4$ channels at potentials that promoted slow inactivation and shifted the voltage dependence of slow inactivation in the direction of hyperpolarization. Metaflumizone perfusion at a hyperpolarized holding potential also shifted the conductance-voltage curve for activation in the direction of depolarization and antagonized use-dependent lidocaine inhibition of fast-inactivated sodium channels, actions not previously observed with other SCI insecticides. We expressed mutated $Na_v1.4/F1579A$ and $Na_v1.4/Y1586A$ channels to investigate whether metaflumizone shares the DIV-S6 binding determinants identified for other SCI insecticides. Consistent with previous investigations of SCI insecticides on rat $Na_v1.4$ channels, the F1579A mutation reduced sensitivity to block by metaflumizone, whereas the Y1586A mutation paradoxically increased the sensitivity to metaflumizone. We conclude that metaflumizone selectively inhibits slow-inactivated $Na_v1.4$ channels and shares DIV-S6 binding determinants with other SCI insecticides and therapeutic drugs. However, our results suggest that metaflumizone interacts with resting and fast-inactivated channels in a manner that is distinct from other compounds in this insecticide class.

Introduction

Sodium channel inhibitor (SCI) drugs, an important group of therapeutic agents that include local anesthetics, class I anticonvulsants, and class I antiarrhythmics, have been in clinical use for over a century. Elucidating the molecular details of their action on sodium channels remains relevant to modern drug discovery efforts (Scholz, 2002). Voltage-gated sodium channels are also the molecular targets for SCI insecticides, a structurally diverse group of insect control agents of increasing importance (Wing *et al.*, 2005; Silver and Soderlund, 2005a). Metaflumizone (Fig. 1A), a new agent to control fleas on domesticated animals, is identified as a novel type of SCI insecticide based on its state-dependent inhibition of insect sodium channels (Salgado and Hayashi, 2007; Silver *et al.*, 2009). Indoxacarb (Fig. 1C), the first and only other commercialized SCI insecticide, is a proinsecticide that is converted in insects to its active metabolite, DCJW (Fig. 1C) (Wing *et al.*, 2005). Other SCI insecticides include dihydropyrazoles (e.g. RH-4841; Fig. 1B) and arylalkylbenzhydrolpiperidines (BZPs) that were not commercialized because of their high toxicity to mammals (Silver and Soderlund, 2005a).

SCI insecticides exhibit little or no effect on sodium channels under experimental conditions that promote resting, activated, or fast-inactivated channel conformations, but they cause a slowly-developing inhibition of sodium currents under experimental conditions that promote slow inactivation of channels (Salgado, 1992, Zhao *et al.*, 2003, Silver and Soderlund, 2005b, 2007). Moreover, SCI insecticides typically cause hyperpolarizing shifts in the voltage dependence of slow inactivation and impede recovery from slow inactivation. Thus, SCI insecticides appear to selectively bind to and stabilize sodium channels in a slow-inactivated state. The state-dependent action of SCI insecticides is analogous to that of SCI drugs that

typically exhibit a preferential interaction with activated or fast-inactivated channels (Ragsdale *et al.*, 1996). Unlike therapeutic drugs, the apparent association kinetics of SCI insecticides are very slow, on the order of several minutes. Such slow kinetics preclude the direct inhibition of channels in transient conformations, such as the activated or fast-inactivated states. Therefore, SCI insecticides do not exhibit any frequency- or use-dependent inhibition in response to repetitive stimulation (Silver and Soderlund, 2005b; Salgado and Hayashi, 2007), a hallmark of many SCI drugs.

SCI insecticides share common molecular determinants for binding to mammalian sodium channels with SCI drugs. SCI drugs bind at the "local anesthetic (LA) receptor" in the inner pore, involving residues in the S6 segments of homology domains I, III and IV (for review, see Mike and Lukacs, 2010). Two residues (F1579 and Y1586) in DIV-S6 of rat $Na_v1.4$ channels, identified previously as critical determinants of state-dependent SCI drug block, also are important determinants of SCI insecticide action (Silver and Soderlund, 2007). Alanine substitution at F1579 significantly reduces the extent of channel inhibition by both SCI drugs (Ragsdale *et al.*, 1996; Wang *et al.*, 1998; Wagner *et al.*, 2001) and insecticides (Silver and Soderlund, 2007). Mutations analogous to Y1586A in rat $Na_v1.4$ also significantly reduce the affinity of inactivated rat $Na_v1.2$ (Ragsdale *et al*., 1996) and $Na_v1.3$ channels (Li *et al*., 1999) for some classes of therapeutic SCI drugs. However, the Y1586A mutation paradoxically enhances the extent of $Na_v1.4$ channel inhibition by SCI insecticides (Silver and Soderlund, 2007). Interestingly, the F1817A mutation in the German cockroach $BgNa_v1-1a$ sodium channel (analogous to F1579A in rat $Na_v1.4$) apparently enhances DCJW and metaflumizone inhibition, whereas the Y1824A mutation in $BgNa_v1-1a$ (analogous to Y1586A in $Na_v1.4$) selectively decreases inhibition by metaflumizone (Silver *et al*., 2009). Together, these data suggest that SCI

insecticides have different binding determinants on mammalian and insect channels.

SCI insecticides such as indoxacarb and RH-4841 are conformationally-restricted, nearly planar molecules (Wellinga *et al.*, 1977; Grosscurt *et al.*, 1979; Hasan *et al.*, 1996). Metaflumizone, which lacks the central heterocyclic moiety of other SCI insecticides, is a flexible molecule that may assume binding conformations that are not available to other SCI insecticides. The present study was designed to characterize the action of metaflumizone on $Na_v1.4$ sodium channels expressed in *Xenopus laevis* oocytes and test the hypothesis that metaflumizone shares the DIV-S6 binding determinants identified for other SCI insecticides on $Na_v1.4$ channels. We report here that metaflumizone is an effective state-dependent inhibitor of $Na_v1.4$ channels and show that substituting alanine for F1579 or Y1586 modulates metaflumizone sensitivity in a manner that is consistent with other SCI insecticides (Silver and Soderlund, 2007). However, our data suggest that metaflumizone interacts with mammalian sodium channels in multiple conformational states and may bind to channels without inhibiting them in a manner that is distinct from other compounds in this insecticide class.

Materials and Methods

Molecular Biology.

We used the rat $Na_v1.4$ channel to enable us to place our findings in the context of previous studies (Silver and Soderlund, 2005b, 2006, 2007). We coexpressed the $Na_v1.4$ α subunit with the rat $\beta1$ subunit because these channels exhibit aberrant inactivation gating when expressed alone in *Xenopus laevis* oocytes (Balser *et al.*, 1996). The cloned rat $Na_v1.4$ cDNA was obtained from R.G. Kallen (University of Pennsylvania, Philadelphia, PA) and the rat $\beta1$ subunit cDNA was obtained from W.A. Catterall (University of Washington, Seattle, WA). The F1579A and Y1586A mutations were introduced by site-directed mutagenesis as described previously (Silver and Soderlund, 2007) using a commercial kit (QuikChange XL, Stratagene, La Jolla, CA). Plasmid cDNAs were digested with restriction enzymes to provide linear templates for $Na_v1.4$ parental and mutated cRNA synthesis *in vitro* using a commercial kit (mMessage mMachine, Ambion, Austin, TX). The integrity of the cRNAs was determined by electrophoresis in 1% agarose-formaldehyde gels.

Oocyte Preparation and Injection.

Oocytes from female *Xenopus* frogs (Nasco, Ft. Atkinson, WI) were washed with Ca^{2+}-free OR-2$^+$ (containing in mM: 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, adjusted pH to 7.6 with NaOH) before being digested in 1 mg/mL collagenase (type 1A, Sigma-Aldrich, St. Louis, MO) for approximately 60 min to loosen the surrounding follicle cells. Healthy stage V and VI oocytes were isolated and manually defolliculated with a pair of fine-tipped surgical forceps before being incubated overnight at 16 °C in ND-96 (containing in mM: 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, adjusted to pH 7.6 with NaOH) solution supplemented with 6%

horse serum (Sigma-Aldrich), 0.5% penicillin/streptomycin and 2.5 mM sodium pyruvate

(Goldin, 1992). Oocytes were pressure-injected with 50 nL of a 2:1 (mass ratio) mixture of β1

and parental or mutated rat $Na_v1.4$ channel α subunit cRNA. The α-subunit concentration ranged

from 10 to 50 ng/mL, which yielded sodium current amplitudes between -10 and -30 μA. The

injected oocytes were maintained at 16 °C in supplemented ND-96 media for 2-5 days before

use.

Electrophysiology.

Electrophysiological experiments were conducted in disposable chambers fabricated from

0.25 in. Plexiglass and a glass coverslip sealed together with ethyl cyanoacrylate as described

elsewhere (Smith and Soderlund, 1998). Recording microelectrodes were pulled from

borosilicate glass capillaries (1.0 mm *o.d.*, 0.78 mm *i.d.*; World Precision Instruments Inc.) on a

P-87 Flaming Brown puller (Sutter Instruments, Novato, CA) and filled with 3 M KCl. Filled

electrodes had resistances that ranged between 0.5 and 0.7 MΩ when submerged in ND-96

saline. The two-microelectrode voltage-clamp technique was used to record macroscopic

sodium currents at room temperature (22-24 °C) using a Geneclamp 500B (Molecular Devices,

Foster City, CA) amplifier and pClamp 10.2 software (Molecular Devices, Sunnyvale, CA).

Currents were filtered at 5 kHz with a low-pass 4-pole Bessel filter and digitized at 50 kHz with

a Digidata 1320A (Molecular Devices). Capacitive transient currents were subtracted using the

P/N (N = 4) method (Bezanilla and Armstrong, 1977). Electrophysiological recordings were

initiated at least 5 min after clamping oocytes at a hyperpolarized membrane potential of -120

mV. Oocytes were perfused with ND-96 saline at about 0.45 ml/min using a custom-fabricated

disposable passive capillary perfusion system similar to that described previously (Tatebayashi

and Narahashi, 1994). The entire bath volume could be replaced in ~1 min. All perfusion

capillaries, recording chambers and microelectrodes were used only once to prevent potential

cross-contamination between experiments.

The voltage dependence of activation was determined by clamping oocytes at a

membrane potential of -120 mV and recording sodium currents during 20-ms depolarizing test

pulses ranging from -80 mV to 40 mV in 10-mV increments. To determine the voltage

dependence of steady-state fast inactivation, oocytes were clamped at a holding potential of -120

mV and stimulated with a 200-ms conditioning prepulse to potentials ranging from -100 mV to 0

mV in 10-mV increments followed by a 20-ms test pulse to -10 mV. The voltage dependence of

slow inactivation was determined by clamping oocytes at -120 mV and giving a 100-s

conditioning prepulse to potentials ranging from -90 mV to 0 mV in 10-mV increments followed

by a 50-ms hyperpolarization to -120 mV to completely recover fast-inactivated channels and

then a 20-ms test pulse to -10 mV ($Na_v1.4$ and $Na_v1.4$/F1579A) or 0 mV ($Na_v1.4$/Y1586A). To

avoid potential time-dependent cumulative effects, oocytes were held at -120 mV for >2 min

between conditioning pulses. The effects of metaflumizone on activation and inactivation gating

was assessed after 15 min of metaflumizone perfusion at a holding potential of -120 mV while

sodium currents were measured every minute with a 20-ms test depolarization to -10 mV. The

time course of inhibition by the insecticides was studied by clamping oocytes at a holding

potential of -30 mV ($Na_v1.4$), -35 mV ($Na_v1.4$/F1579A) or -50 mV ($Na_v1.4$/Y1586A) and giving

a 20-ms test depolarization to -10 mV preceded by a 2-s hyperpolarization to -120 mV that

recovered a fraction (~45%) of sodium channels to the resting state, thus enabling a sodium

current of adequate amplitude to be recorded (Salgado, 1992; Silver and Soderlund, 2005b,

2007). For determinations of use dependence, oocytes were clamped at a holding potential of -

120 mV and stimulated at 20 Hz with trains of 0-20 brief (25-ms) conditioning depolarizing

pulses to -50 mV, separated by 25-ms interpulse intervals, followed by a 20-ms test pulse to -10

mV. Pulse trains were separated by a 20-s interval at -120 mV to avoid possible time-dependent

cumulative effects (Ragsdale *et al.*, 1996). Experiments in the presence of lidocaine were

initiated after 10 min of lidocaine perfusion at a holding potential of -120 mV. For experiments

characterizing the effects of SCI insecticides on use-dependent lidocaine inhibition, oocytes were

clamped at -120 mV and perfused with a given insecticide for 15 min prior to changing the bath

perfusate to one containing both the insecticide and lidocaine together for 10 min before pulse

protocols were initiated. Additional details of voltage protocols used are provided in the figure

legends.

Chemicals.

Metaflumizone and lidocaine were purchased from Sigma-Aldrich. RH-4841 was

provided by G. Carlson (Rohm and Haas Company, Spring House, PA) and DCJW was provided

by K. Wing (DuPont Agricultural Product, Newark, DE). Stock solutions of insecticides (10

mM) and lidocaine (200 mM) were prepared in dimethyl sulfoxide (DMSO) and diluted in ND-

96 just prior to use to a final concentration of 10 µM (insecticides) and 200 µM (lidocaine). The

final DMSO concentration never exceeded 0.2% *v/v*. Initial time-matched vehicle control

experiments verified that 0.2% *v/v* DMSO has no effects on sodium channel gating.

Data Analysis and Statistics.

Data analysis was performed using Origin 8.1 (OriginLab Corp., Northampton, MA).

Conductance-voltage and sodium channel inactivation data was fitted using the Boltzmann

equation $[y = (A_1-A_2)/\{1 + \exp[(V-V_{1/2})/k]\} + A_2]$ where $V_{1/2}$ is the midpoint of the curve, k is the slope factor, and A_1 and A_2 are the maximum and minimum values in the fit, respectively. Time constants for sodium-current inhibition were obtained by fitting the mean data with a single-exponential decay function $[y = A_1*\exp(-x/\tau_1) + y_0]$, where x is time, τ_1 is the time constant, and y_0 is the non-inactivating component. All fitted data are expressed as means and standard errors.

Statistical analyses were performed using Prism 5.0 (GraphPad Software, La Jolla, CA). Statistical significance between two means was assessed using either paired or unpaired Student's t-test, depending on the design of the experiment. Statistical significance of two or more mean values from a single control data set was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* analysis. Differences were considered statistically significant when $P < 0.05$.

Results

Effects of Metaflumizone on Gating Properties of Wildtype Na$_v$1.4 Sodium Channels.

We first characterized the effects of 10 µM metaflumizone, the highest concentration

achievable in ND-96 saline, on the voltage-dependent gating of Na$_v$1.4 sodium channels (Fig. 2).

Statistical analyses of these data are summarized in Table 1. Perfusion of metaflumizone for 15

min at a holding potential of -120 mV caused a 4.7 mV depolarizing shift in the midpoint

potential (V$_{1/2}$) of activation but did not affect the slope factor of the voltage response curve for

activation fitted with the Boltzmann equation (Fig. 2A; Table 1). Consequently, the amplitude of

sodium currents measured at sub-maximal activation potentials were reduced in the presence of

metaflumizone compared to the controls (Fig. 2B). At test potentials of -30 mV and -20 mV, the

reduction in the amplitude of the sodium current by metaflumizone was statistically significant.

Metaflumizone had no effect on the voltage dependence of steady-state fast inactivation (Fig.

2C). By contrast, metaflumizone increased the extent of maximal slow inactivation at

depolarized potentials, shifted the midpoint potential for slow inactivation by 3.5 mV in the

direction of hyperpolarization, and increased the slope of the voltage response compared to the

control (Fig. 2D).

Other SCI insecticides selectively inhibit voltage-gated sodium channels at depolarized

membrane potentials that promote slow inactivation (Salgado, 1990, 1992; Silver and Soderlund,

2005b). Figure 3 illustrates the inhibition of sodium currents through Na$_v$1.4 sodium channels by

10 µM metaflumizone (Fig. 3A), RH-4841 (Fig. 3B) and DCJW (Fig. 3C) after 15 min at a

holding potential of -30 mV. Figure 3D shows the time course of sodium current inhibition by

these compounds. Inhibition by metaflumizone and RH-4841 increased progressively over the 15

min of exposure, whereas inhibition by DCJW reached an apparent steady state after ~6 min of

perfusion. Inhibition by metaflumizone and RH-4841 was slower in onset and less extensive after 15 minutes than that caused by DCJW.

Metaflumizone and Other SCI Insecticides Are State-Dependent Inhibitors

Changes in the transmembrane voltage of the cell determine the conformational state of voltage-gated ion channels, so state-dependent inhibition is often manifested as a voltage-dependent phenomenon. Figure 4 illustrates the voltage- (*i.e.* state) dependent inhibition of sodium currents through $Na_v1.4$ channels after 15 min of insecticide exposure. Insecticides had little or no effect on the peak current amplitude when oocytes were clamped at a holding potential of -120 mV, where channels were almost exclusively in the resting state. However, after 15 min of exposure at a holding potential of -60 mV, peak sodium currents were reduced to variable extents by all insecticides tested. Holding the membrane at -30 mV significantly increased the extent of inhibition by metaflumizone and RH-4841, whereas the extent of inhibition at -30 mV by DCJW was not significantly different from that at -60 mV.

Metaflumizone Interacts with the Local Anesthetic (LA) Receptor on $Na_v1.4$ Channels

Previous studies (O'Reilly *et al.*, 2000; Bai *et al.*, 2003) have demonstrated that mutations in DIV-S6 that alter local anesthetic binding also modulate slow inactivation gating in a residue-specific manner. Therefore, we initially characterized the effects of the F1579A and Y1586A mutations on the voltage dependence of slow inactivation in the absence of insecticides (Fig. 5A). At depolarized potentials slow inactivation was more complete in $Na_v1.4$/Y1586A channels (~95% inactivation at 0 mV) than in $Na_v1.4$/F1579A (~80% inactivation at 0 mV) or $Na_v1.4$ channels (~82% inactivation at 0 mV). Neither mutation significantly affected the

midpoint potential for the voltage dependence of slow inactivation curve, although the slope was

significantly steeper for both $Na_v1.4/F1579A$ ($V_{1/2}$ = -44.1 ± 0.5 mV, k = 5.0 ± 0.4) and

$Na_v1.4/Y1586A$ ($V_{1/2}$ = -45.7 ± 0.4 mV, k = 5.6 ± 0.3) channels compared to $Na_v1.4$ ($V_{1/2}$ = -44.9

± 0.7 mV, k = 9.3 ± 0.6). To avoid possible effects on insecticide potency caused indirectly by

mutation-induced changes in the propensity of channels to occupy high affinity slow-inactivated

states, oocytes were clamped at holding potentials of -35 mV ($Na_v1.4/F1579A$), or -50 mV

($Na_v1.4/Y1586A$) for studies of insecticide action. These holding potentials were empirically

determined to produce fractional slow inactivation (~55%) equivalent to that in $Na_v1.4$ channels

clamped at -30 mV (Fig. 5B).

We used the F1579A and Y1586A mutations to determine if metaflumizone

shares common molecular determinants on mammalian sodium channels with other SCI

insecticides. Figure 6 shows the time course of inhibition of $Na_v1.4/F1579A$ (Fig. 6A) and

$Na_v1.4/Y1586A$ (Fig. 6B) channels by metaflumizone, RH-4841 and DCJW and Fig. 7 compares

the extent of inhibition at 15 minutes for $Na_v1.4$, $Na_v1.4/F1579A$, and $Na_v1.4/Y1586A$ by these

compounds. $Na_v1.4/F1579A$ channels were significantly less sensitive to inhibition than $Na_v1.4$

channels whereas $Na_v1.4/Y1586A$ channels were significantly more sensitive to inhibition than

$Na_v1.4$ channels.

Metaflumizone Binding in the Absence of Inhibition Selectively Attenuates Lidocaine

Inhibition of Fast-Inactivated $Na_v1.4$ Channels.

SCI drugs have been shown previously to interfere with the ability of SCI insecticides to

inhibit mammalian voltage-gated sodium channels under experimental conditions where no

inhibition by the drugs is observed (Silver and Soderlund, 2005b). However, whether SCI

14

insecticides are reciprocally capable of modulating the extent of inhibition by SCI drugs remains unclear. We therefore assessed the effects of SCI insecticides on $Na_v1.4$ sodium channel inhibition by lidocaine to elucidate whether these insecticides bind to non-slow-inactivated channel states without inhibiting them. Figure 8A shows the effects of high frequency (20 Hz) stimulation on the extent of sodium channel inhibition by 200 μM lidocaine alone and in the presence of 10 μM metaflumizone, RH-4841, or DCJW. Figure 8B summarizes the statistical analyses of steady-state use-dependent inhibition. Repeated depolarization did not affect the stability of the peak transient sodium current in the absence of SCI drugs and insecticides. Lidocaine reduced peak currents by $16.6 \pm 1.1\%$ and $40.0 \pm 1.0\%$ after 0 and 10 depolarizing prepulses, respectively. None of the SCI insecticides exhibited any detectable use-dependent inhibition of sodium channels under these conditions (data not shown). However, metaflumizone significantly antagonized the extent of lidocaine inhibition after 10 depolarizing prepulses. Metaflumizone also slightly reduced the extent of tonic sodium channel inhibition by lidocaine after 0 prepulses, but this effect was not statistically significant. Neither RH-4841 nor DCJW interfered with the tonic or use-dependent inhibition of sodium currents by lidocaine.

Discussion

This is the first report of the action of metaflumizone on mammalian voltage-gated sodium channels. The gating properties of $Na_v1.4+\beta1$ channel complexes that we describe here are in good agreement with the properties of these channels reported in previous studies. Minor differences between our data and previously published data can likely be explained by differences in experimental conditions, such as the choice of expression system or the details of the pulse protocols employed.

Molecules that modify ion channel function often interact preferentially with channels in one conformational state. Negative holding potentials placed $Na_v1.4$ channels predominantly in the resting state, making them virtually insensitive to inhibition by the highest soluble concentration of metaflumizone or any other SCI insecticide tested. Holding the membrane potential at -60 mV caused partial inactivation of channels and facilitated insecticide inhibition. In contrast to DCJW, the extent of inhibition by metaflumizone and RH-4841 was significantly greater at -30 mV, where channel inactivation was more complete than at -60 mV. These findings are consistent with previous studies of metaflumizone and other SCI insecticides (Silver and Soderlund, 2005b; Salgado and Hayashi, 2007) and have been interpreted as evidence that SCI insecticides preferentially interact with slow-inactivated channels.

Fast inactivation in sodium channels terminates sodium conductance within milliseconds after depolarization and involves occlusion of the inner pore of the channel by the highly conserved inactivation gate in the intracellular linker between domains III and IV (Goldin, 2003). However, channel opening is not a prerequisite for fast inactivation because channels can enter the fast-inactivated state from the resting state following brief depolarization to potentials

below the activation threshold (Ragsdale *et al.*, 1996). Under our experimental conditions, metaflumizone had no effect on the voltage dependence of fast inactivation that was measured with standard 200-ms prepulses. This result is consistent with data from recent studies with metaflumizone on cloned cockroach sodium channels expressed in *Xenopus* oocytes (Silver *et al.*, 2009) and the current hypothesis that SCI insecticides inhibition occurs selectively only under experimental conditions that promote slow inactivation.

Slow inactivation of sodium channels occurs following sustained depolarization or prolonged high-frequency stimulation and exhibits development and recovery kinetics on the order of seconds to minutes (for reviews see Vilin and Ruben, 2001; Ulbricht, 2005). The molecular basis of slow inactivation is poorly understood, but it is different from fast inactivation because internal protease perfusion or amino acid substitutions that disable the fast-inactivation gate do not interfere with slow inactivation (Goldin, 2003). Slow inactivation of $Na_v1.4$ channels in our assays developed at potentials negative to channel opening but positive to fast inactivation, as previously reported (O'Reilly *et al.*, 2001; McNulty and Hanck, 2004). Metaflumizone enhanced the extent of slow inactivation and caused a modest hyperpolarizing shift in the voltage dependence of slow inactivation. These effects provide further evidence that SCI insecticides preferentially target and stabilize channels in a slow-inactivated state (Salgado, 1992). However, the 100-s conditioning pulses used to measure slow inactivation were too short to allow the slow inhibition by metaflumizone to develop completely. Therefore, our data are likely to underestimate the magnitude of the effects of metaflumizone on the voltage dependence of slow inactivation. In contrast to our data, Silver *et al.* (2009) reported that metaflumizone caused a modest depolarizing shift in the voltage dependence of slow inactivation of cloned cockroach sodium channels expressed in *Xenopus* oocytes. This discrepancy may reflect

differences in the pulse protocols used to characterize the voltage dependence of slow

inactivation in the two studies.

Substituting alanine at F1579 or Y1586 in the rat $Na_v1.4$ channel produced marked

effects on metaflumizone and RH-4841 sensitivity that are consistent with previous studies in

$Na_v1.4$ with other SCI insecticides (Silver and Soderlund, 2007). The F1579A mutation reduced

the sensitivity to metaflumizone, RH-4841, and DCJW, an effect that is also consistent with the

impact of this mutation on the state-dependent inhibition of SCI drugs (Wang *et al*., 1998). These

data indicate that metaflumizone and RH-4841 share common molecular determinants on

mammalian sodium channels with other SCI insecticides and therapeutic drugs acting at the LA

receptor. The Y1586A mutation significantly increased the sensitivity of $Na_v1.4$ to

metaflumizone and RH-4841, which is consistent with results from previous studies with this

sodium channel isoform and other compounds in this insecticide class. Mutations at this position

appear to strongly influence the state-dependent inhibition of local anesthetics but not of other

therapeutic SCI drugs (for review see Mike and Lukacs, 2010). Studies of the impact of different

amino acid substitutions at Y1717 in rat $Na_v1.3$ channels (analogous to Y1586 in $Na_v1.4$)

expressed in *Xenopus* oocytes suggest that this residue does not directly contribute to the LA

receptor (Li *et al.*, 1999). A molecular model of the LA receptor in $Na_v1.4$ channels indicates

that the convergence of S6 segments constrains space in the inner pore, especially in the region

of Y1586 (Lipkind and Fozzard, 2005). We hypothesize that the much smaller alanine side chain

in the Y1586A mutation indirectly increases SCI insecticide inhibition of rat $Na_v1.4$ channels by

reducing steric hindrance, thereby enhancing binding interaction between the insecticide and

other inner-pore residues as suggested previously (Silver and Soderlund, 2007).

Perfusion of metaflumizone at a hyperpolarized holding potential reduced the voltage-

dependent probability of sodium channel activation, as indicated by the depolarizing shift in the midpoint potential of the conductance-voltage (*G-V*) curve. Consequently, the amplitude of sodium currents measured during test pulses to sub-maximal activation potentials (*i.e.* -30 mV and -20 mV) were significantly reduced in the presence of metaflumizone compared to the control. These results imply that metaflumizone interacts with $Na_v1.4$ channels in the resting state. Similarly, Salgado and Hayashi (2007) reported a slight decrease in the maximal amplitude of sodium currents through *Drosophila melanogaster* Para/TipE channels expressed in *Xenopus* oocytes after perfusion of 10 μM metaflumizone at a holding potential where channels were predominantly in the resting state. However, effects of other SCI insecticides on activation gating are not observed in assays with rat $Na_v1.4$ channels expressed in oocytes (Silver and Soderlund, 2005b), TTX-S or TTX-R sodium channels in rat DRG neurons (Tsurubuchi *et al.*, 2001; Tsurubuchi and Kono, 2003; Zhao *et al.*, 2003) or sodium channels in cockroach DUM neurons (Lapied *et al.*, 2001). Taken together, these results imply that metaflumizone, unlike other SCI insecticides, interacts with $Na_v1.4$ channels in the resting state. Additional experiments are needed to determine how the F1579A and Y1586A mutations impact the effect of metaflumizone on the voltage dependence of activation. The results of these experiments may help to clarify if the effects of metaflumizone on activation and slow inactivation involve a single binding site.

The effects of metaflumizone on activation gating led us to examine whether metaflumizone or other SCI insecticides interact with resting and fast-inactivated channels despite the lack of evidence for block from these states. We used lidocaine as a model SCI to establish conditions for block from the resting state and the use-dependent enhancement of block of fast-inactivated channels. Although none of the insecticides alone exhibited any use

dependence in response to high-frequency pulse trains that caused significant fast inactivation

from the resting state and enhancement of lidocaine inhibition, metaflumizone was unique in its

ability to interfere with use-dependent inhibition by lidocaine. These results offer further

evidence that binding of SCI insecticides to channels is not always accompanied by channel

inhibition (Silver and Soderlund, 2005b; Silver and Soderlund, 2007) and suggest that

metaflumizone, unlike other rigid, nearly planar SCI insecticides, is able to bind to the LA

receptor in multiple conformational states. Interestingly, metaflumizone did not significantly

affect the level of resting inhibition by lidocaine that was evident upon depolarization following

0 prepulses. Resting and inactivated sodium channels may possess fundamentally distinct drug

binding sites (Hille, 1977; Lenkey *et al.*, 2011). Thus, the ability of metaflumizone to antagonize

lidocaine inhibition of fast-inactivated but not resting sodium channels offers further evidence

for different drug receptor conformations on resting and inactivated sodium channels.

Selective binding to sodium channels in slow-inactivated states may also produce

therapeutically useful effects. The antihypertensive drug mibefradil (McNulty and Hanck, 2004),

the anticonvulsant lacosamide (Errington *et al.*, 2008), and the investigational compound for

neuropathic pain Z123212 (Hildebrand *et al.*, 2011) preferentially target sodium channels in the

slow-inactivated state. However, the properties that distinguish between neurotoxic and

therapeutic SCIs are presently unknown. Therefore, SCI insecticides represent additional novel

and unique chemical probes that may be useful for understanding the molecular details of ligand-

receptor interactions that underlie the toxic or therapeutic effects of agents that target slow-

inactivated channels.

Acknowledgments

Authorship Contributions

Participated in research design: von Stein and Soderlund

Conducted experiments: von Stein

Performed data analysis: von Stein

Wrote or contributed to writing of the manuscript: von Stein and Soderlund

References

Bai C-X, Glaaser IW, Sawanobori T, Sunami A (2003) Involvement of local anesthetic binding sites on IVS6 of sodium channels in fast and slow inactivation. *Neurosci Lett* **337:** 41-45.

Balser JR, Nuss HB, Orias DW, Johns DC, Marban E, and Tomaselli GF (1996) Local anesthetic as effectors of allosteric gating. *J Clin Invest* **98:** 2874-2886.

Bezanilla F and Armstrong CM (1977) Inactivation of the sodium channel. *J Gen Physiol* **70:** 549-566.

Bruhova I, Tikhonov DB, and Zhorov BS (2008) Access and binding of local anesthetics in the closed sodium channel. *Mol Pharmacol* **74:** 1033-1045.

Errington AC, Stöhr T, Heers C, and Lees G (2008) The investigational anticonvulsant lacosamide selectively enhances slow inactivation of voltage-gated sodium channels. *Mol Pharmacol* **73:** 157-169.

Goldin AL (1992) Maintenance of *Xenopus laevis* and oocyte injection. *Methods Enzymol* **207:** 266-297.

Goldin AL (2003) Mechanisms of sodium channel inactivation. *Curr Opin Neurobiol* **13:** 284-290.

Grosscurt AC, van Hes R, and Wellinga, K (1979) 1-Phenylcarbamoyl-2-pyrazolines, a new class of insecticides. 3. Synthesis and insecticidal properties of 3,4-diphenyl-1-phenylcarbamoyl-2-pyrazolines. *J Agric Food Chem* **27:** 406-409.

Hasan R, Nishimura K, Okada M, Akamatsu M, Inoue M, and Ueno T (1996) Stereochemical

22

basis for the insecticidal activity of carbamoylated and acylated pyrazoline. *Pestic Sci* **46:** 105-112.

Hildebrand ME, Smith PL, Bladen C, Eduljee C, Xie JY, Chen L, Fee-Maki M, Doering CJ, Mezeyova J, Zhu Y, Belardetti F, Pajouhesh H, Parker D, Arneric SP, Parmar M, Porreca F, Tringham E, Zamponi GW, and Snutch TP (2011) A novel slow-inactivation-specific ion channel modulator attenuates neuropathic pain. *PAIN*® **152:** 833-843.

Hille B (1977) Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor interaction. *J Gen Physiol* **69:** 497-515.

Lapied B, Grolleau F, and Sattelle DB (2001) Indoxacarb, an oxadiazine insecticide, block insect neuronal sodium channels. *Brit J Pharmacol* **132:** 587-595.

Lenkey N, Karoly R, Epresi N, Vizi ES, Mike A (2011) Binding of sodium channel inhibitors to hyperpolarized and depolarized conformations of the channel. *Neuropharmacology* **60:** 191-200.

Li HL, Galue A, Meadows L, and Ragsdale DS (1999) A molecular basis for the different local anesthetic affinities of resting versus open and inactivated states of the sodium channel. *Mol Pharmacol* **55:** 134-141.

Lipkind GM and Fozzard HA (2005) Molecular modeling of local anesthetic drug binding by voltage-gated sodium channels. *Mol Pharmacol* **68:** 1611-1622.

McNulty MM and Hanck DA (2004) State-dependent mibefradil block of Na$^+$ channels. *Mol Pharmacol* **66:** 1652-1661.

Mike A and Lukacs P (2010) The enigmatic drug binding site for sodium channel inhibitors. *Curr Mol Pharmacol* **3:** 129-144.

O'Reilly JP, Wang S-Y, and Wang GK (2000) A point mutation in domain 4-segment 6 of the skeletal muscle sodium channel produces an atypical inactivation state. *Biophys J* **78:** 773-784.

O'Reilly JP, Wang S-Y, and Wang GK (2001) Residue-specific effects on slow inactivation at V787 in D2-S6 of Na$_v$1.4 sodium channels. *Biophys J* **81:** 2100-2111.

Ragsdale DS, McPhee JC, Scheuer T, and Catterall, WA (1996) Common molecular determinants of local anesthetic, antiarrhythmic, and anticonvulsant block of voltage-gated Na$^+$ channels. *Proc Natl Acad Sci USA* **93:** 9270-9275.

Salgado VL (1990) Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. *Pestic Sci* **28:** 389-411.

Salgado VL (1992) Slow voltage-dependent block of sodium channels in crayfish nerve by dihydropyrazole insecticides. *Mol Pharmacol* **41:** 120-126.

Salgado VL and Hayashi JH (2007) Metaflumizone is a novel sodium channel blocker insecticide. *Vet Parasitol* **150:** 182-189.

Scholz A (2002) Mechanism of (local) anesthetics on voltage-gated sodium and other ion channels. *Br J Anaesth* **89:** 52-61.

Silver KS and Soderlund DM (2005a) Action of pyrazoline-type insecticides at neuronal target sites. *Pestic Biochem Physiol* **81:** 136-143.

Silver KS and Soderlund DM (2005b) State-dependent block of rat Na$_v$1.4 sodium channels expressed in *Xenopus* oocytes by pyrazoline-type insecticides. *Neurotoxicology* **26:** 397-406.

Silver KS and Soderlund DM (2006) Differential sensitvity of rat voltage-sensitive sodium channel isoforms to pyrazoline-type insecticides. *Toxicol Appl Pharmacol* **214:** 209-217.

Silver KS and Soderlund DM (2007) Point mutations at the local anesthetic receptor site modulate the state-dependent block of rat $Na_v1.4$ sodium channels by pyrazoline-type insecticides. *Neurotoxicology* **28:** 655-663.

Silver KS, Nomura Y., Salgado VL, and Dong K (2009) Role of the sixth transmembrane segment of domain IV of the cockroach sodium channel in the action of sodium channel blocker insecticides. *Neurotoxicology* **30:** 613-621.

Smith TJ and Soderlund DM (1998) Action of the pyrethroid insecticide cypermethrin on rat brain IIa sodium channels expressed in *Xenopus* oocytes. *Neurotoxicology* **19:** 823-832.

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.

Tsurubuchi Y, Zhao X, Nagata K, Kono Y, Nishimura K, Yeh JZ, and Narahashi T (2001) Modulation of tetrodotoxin-resistant sodium channels by dihydropyrazole insecticide RH-3421 in rat dorsal root ganglion neurons. *Neurotoxicology* **22:** 743-753.

Tsurubuchi Y and Kono Y (2003) Modulation of sodium channels by the oxadiazine insecticide indoxacarb and its *N*-decarbomethoxylated metabolite in rat dorsal root ganglion neurons. *Pest Manag Sci* **59:** 999-1006.

Ulbricht W (2005) Sodium channel inactivation: molecular determinants and modulation. *Physiol Rev* **85:** 1271-1301.

Vilin YY and Ruben PC (2001) Slow inactivation in voltage-gated sodium channels, *Cell*

Biochem Biophys **35:** 171-190.

Wagner LE, Gingrich KJ, Kulli JC, and Yang J (2001) Ketamine blockade of voltage-gated sodium channels. *Anesthes* **95:** 1406-1413.

Wang GK, Quan C, and Wang S-Y (1998) A common local anesthetic receptor for benzocaine and etidocaine in voltage-gated µ1 Na$^+$ channels. *Pflugers Arch* **435:** 293-302.

Wellinga K, Grosscurt AC and van Hes R (1977) 1-Phenylcarbamoyl-2-pyrazoline: a new class of insecticides. 1. Synthesis and insecticidal properties of 3-phenyl-1-phenylcarbamoyl-2-pyrazoline. *J Agric Food Chem* **25:** 987-992.

Wing KD, Andaloro JT, McCann SF, and Salgado VL (2005) Indoxacarb and the sodium channel blocker insecticides: chemistry, physiology, and biology in insects, in *Comprehensive Molecular Insect Science* (Gilbert LI, Iatrou K, and Gill SS eds) pp 30–53, Elsevier, New York.

Zhao X, Ikeda T, Yeh JZ, and Narahashi T (2003) Voltage-dependent block of sodium channels in mammalian neurons by the oxadiazine insecticide indoxacarb and its metabolite DCJW. *Neurotoxicology* **24:** 83-96.

FOOTNOTES

Current address for RTvS: Environmental and Occupational Health Science Institute, Rutgers,

The State University of New Jersey, 170 Frelinghuysen Rd., Piscataway, NJ 08854.

Reprint Requests: David M. Soderlund, 630 W. North Street, 620 Barton Laboratory

Geneva, NY 14456, USA. E-mail: dms6@cornell.edu

Legends for Figures

Figure 1: Structures of SCI insecticides. (A) Metaflumizone. (B) RH-4841 and (C) Indoxacarb and DCJW.

Figure 2: Effects of metaflumizone (10 μM) on the voltage dependence of activation, steady-state fast inactivation and slow inactivation. (A) Conductance-voltage plots of activation measured upon depolarization from -120 mV to test potentials ranging from -80 mV to 40 mV. Peak sodium currents were transformed to conductances (G) using the equation $G = I_{Na}/(V_{test} − V_{rev})$, where I_{Na} is the peak sodium current during test depolarization (V), and V_{rev} is the sodium current reversal potential. Data were normalized to maximum peak conductance (G_{Max}) and fitted using the Boltzmann equation; values are means ± SE from 5 oocytes. The asterisks indicate test potentials of sub-maximal activation at which the measured sodium current amplitude in the presence of metaflumizone was significantly (*, $P < 0.05$; **, $P < 0.001$; paired t-test) different from the control. (B) Representative normalized sodium current traces recorded from an oocyte before or after metaflumizone exposure upon depolarization from -120 mV to -20 mV. (C) Voltage dependence of steady-state fast inactivation; conditioning pulses (200 ms) from -120 mV to potentials ranging from -100 mV to 0 mV were followed immediately by 20-ms test pulses to -10 mV. Peak sodium currents were normalized to the maximum current obtained during the inactivation protocol for that oocyte and plotted versus the conditioning potential; curves were fitted using the Boltzmann equation; values are means ± SE from 5 oocytes. (D) Voltage dependence of slow inactivation; amplitudes of peak transient currents measured during a 20-ms depolarization to -10 mV following a 100-s conditioning prepulse from a holding

potential (V_{hold}) of -120 mV to potentials ranging from -90 to 0 mV and a 50-ms

hyperpolarization to -120 mV were normalized to the maximum current obtained during the

inactivation protocol for that oocyte and plotted as a function of the conditioning potential.

Values are means ± SE from 6 (control) or 5 (metaflumizone) separate experiments in different

oocytes; curves are fitted to the Boltzmann equation.

Figure 3: Inhibition of Na$_v$1.4 sodium currents by SCI insecticides. (A-C) Representative

traces of sodium currents through Na$_v$1.4 channels recorded before and after 15 min of perfusion

with 10 µM metaflumizone (A), RH-4841 (B), or DCJW (C). (D) Time course of sodium current

inhibition caused by metaflumizone, RH-4841, and DCJW. Oocytes were held at a holding

potential of -30 mV and stimulated once every minute with a test pulse (20 ms) to -10 mV that

was preceded by a 2-s hyperpolarization to -120 mV. After 4 min of stable control recordings at -

30 mV, insecticides were perfused into the bath for 15 min; peak sodium currents were

normalized to the mean sodium current amplitude obtained during the 4 min of stable control

currents prior insecticide perfusion. Data were fitted using a first-order exponential decay

function that yielded a single time constant (τ_l); values are means ± SE of 4 or more individual

experiments in separate oocytes.

Figure 4: Voltage-dependent inhibition of Na$_v$1.4 sodium currents by SCI insecticides.
Oocytes were clamped at a membrane potential of -120 mV, -60 mV, or -30 mV and stimulated

every minute with a test pulse (20 ms) to -10 mV during 15 min of insecticide perfusion; a 2-s

hyperpolarization to -120 mV preceded each test pulse in experiments at holding potentials -60

mV and -30 mV. Fractional inhibition was derived from the amplitude of the peak transient

sodium current at the end of the perfusion period normalized to the mean sodium current

amplitude in that oocyte obtained after 4 min of stable control recordings prior to insecticide

application.

Figure 5: Voltage dependence and development of steady-state slow inactivation for Na$_v$1.4,

Na$_v$1.4/F1579A and Na$_v$1.4/Y1586A sodium channels. (A) The voltage dependence of slow

inactivation was examined using the standard protocol described in Fig. 2C and shown in the

inset. Amplitudes of peak transient currents measured during a 20-ms depolarization to -10 mV

(Na$_v$1.4 and Na$_v$1.4/F1579A) or 0 mV (Na$_v$1.4/Y1586A) following a 100-s conditioning prepulse

from a holding potential (V_{hold}) of -120 mV to potentials ranging from -90 to 0 mV and a 50-ms

hyperpolarization to -120 mV were normalized to the maximum current obtained during the

inactivation protocol for that oocyte and plotted as a function of the conditioning potential.

Values are means ± SE from 4-6 experiments in separate oocytes; curves are fitted to the

Boltzmann equation. (B) The development of steady-state slow inactivation was assessed using a

two-pulse protocol (see inset) in which oocytes were clamped at a V_{hold} of -30 mV (Na$_v$1.4), -35

mV (Na$_v$1.4/F1579A), or -50 mV (Na$_v$1.4/Y1586A) and stimulated every minute with a 20-ms

test pulse to -10 mV (Na$_v$1.4 and Na$_v$1.4/F1579A) or 0 mV (Na$_v$1.4/Y1586A) that was preceded

by a 2-s repolarization to -120 mV. Currents were normalized to peak test pulses elicited from a

V_{hold} of -120 mV prior to depolarization; values are means ± SE from 9 experiments in separate

oocytes. Curves were fitted with a single-exponential decay function.

Figure 6: Effects of the F1579A and Y1586A mutations on the inhibition of Na$_v$1.4 sodium

channels by SCI insecticides. (A) Time course of sodium current inhibition in oocytes

expressing $Na_v1.4$/F1579A channels by metaflumizone, RH-4841, and DCJW. Oocytes were

held at a holding potential of -35 mV and stimulated once every minute with a test pulse (20 ms)

to -10 mV that was preceded by a 2-s hyperpolarization to -120 mV. After 4 min of stable

control recordings at -35 mV, insecticides were perfused into the bath for 15 min; peak sodium

currents were normalized to the mean sodium current amplitude obtained during the 4 min of

stable control recordings prior to insecticide perfusion. Data were fitted using a first-order

exponential decay function that yielded a single time constant (τ_l); values are means ± SE of 4-5

individual experiments in separate oocytes. (B) Time course of sodium current inhibition in

oocytes expressing $Na_v1.4$/Y1586A channels by metaflumizone, RH-4841, and DCJW. Oocytes

were held at a holding potential of -50 mV and stimulated once every minute with a 20-ms test

pulse to 0 mV that was preceded by a 2-s hyperpolarization to -120 mV. After 4 min of stable

control recordings at -50 mV, insecticides were perfused into the bath for 15 min; peak sodium

currents were normalized to the mean sodium current amplitude during the 4 min of stable

control recordings prior to insecticide perfusion. Data were fitted using a first-order exponential

decay function that yielded a single time constant (τ_l); values are means ± SE of 3 or more

individual experiments in separate oocytes.

**Figure 7: Relative sensitivity of $Na_v1.4$, $Na_v1.4$/F1579A, and $Na_v1.4$/Y1586A sodium
channels to inhibition by SCI insecticides.** Values are fractional inhibition following 15
minutes of insecticide perfusion and are derived from data in Figs. 3 and 6. Values for
$Na_v1.4$/F1579A and $Na_v1.4$/Y1586A channels marked with asterisks were significantly different
from values for inhibition of $Na_v1.4$ channels by the same compound (one-way ANOVA with
Dunnett' *post hoc* analysis, $P < 0.05$).

31

Figure 8: Use-dependent lidocaine inhibition of sodium channels in the absence and presence of SCI insecticides. (A) Effects of 10 µM metaflumizone, RH-4841 or DCJW on the extent of inhibition by lidocaine (200 µM) following 0-20 depolarizing prepulses (25 ms) from -120 mV to -50 mV at 20 Hz. Values were normalized to the mean peak sodium current amplitude measured from the same oocyte prior to the perfusion of lidocaine. Data points are means ± SE of 5 (lidocaine, metaflumizone+lidocaine, RH-4841+lidocaine) or 4 (DCJW+lidocaine) separate experiments with different oocytes; curves were fitted to mean data points using a single-exponential decay function. (B) Comparison of the extent of resting (after 0 prepulses) and fast-inactivated (after 10 prepulses) sodium channel inhibition by lidocaine in the absence or presence of metaflumizone, RH-4841, or DCJW. Values for use-dependent inhibition marked with an asterisk (*) are significantly ($P < 0.05$) different from use-dependent inhibition by lidocaine alone.

Tables

Table 1: Activation and inactivation gating parameters of Na$_v$1.4 channels in the absence or presence of metaflumizone[a].

Condition	Activation			Fast Inactivation			Slow Inactivation		
	V$_{1/2}$	k	n	V$_{1/2}$	k	n	V$_{1/2}$	k	n
Control	-24.9 ± 0.7	5.9 ± 0.6	5	-53.3 ± 0.2	5.0 ± 0.2	5	-44.9 ± 0.7	9.3 ± 0.6	6
Metaflumizone	-20.2 ± 0.7[*]	6.4 ± 0.6	5	-53.5 ± 0.2	5.2 ± 0.2	5	-48.4 ± 0.9[†]	7.5 ± 0.8[†]	5

[a] Values calculated from fits of data obtained in the absence or presence of 10 µM metaflumizone (Fig. 2) to the Boltzmann

equation; V$_{1/2}$, midpoint potential (mV) for voltage-dependent activation or inactivation; k, slope factor.

[*] Values are significantly different from control (paired t-test, $P < 0.05$).

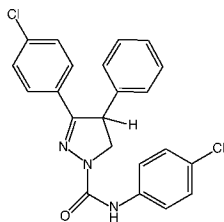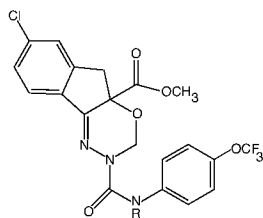[†] Values are significantly different from control (unpaired t-test, $P < 0.05$).

33

Figure 1

A
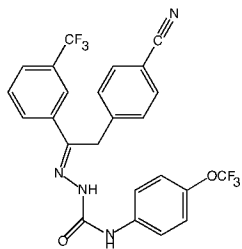
Metaflumizone

B

RH-4841

C

Indoxacarb
  R = CO$_2$CH$_3$
DCJW
  R = H

Figure 2

A

Control
Metaflumizone
(10 µM)

G/G_{max}

20 ms

Voltage (mV)

B

-20 mV

I/I_{max}

5 ms

Metaflumizone
Control

C

I/I_{max}

200 ms   -10 mV

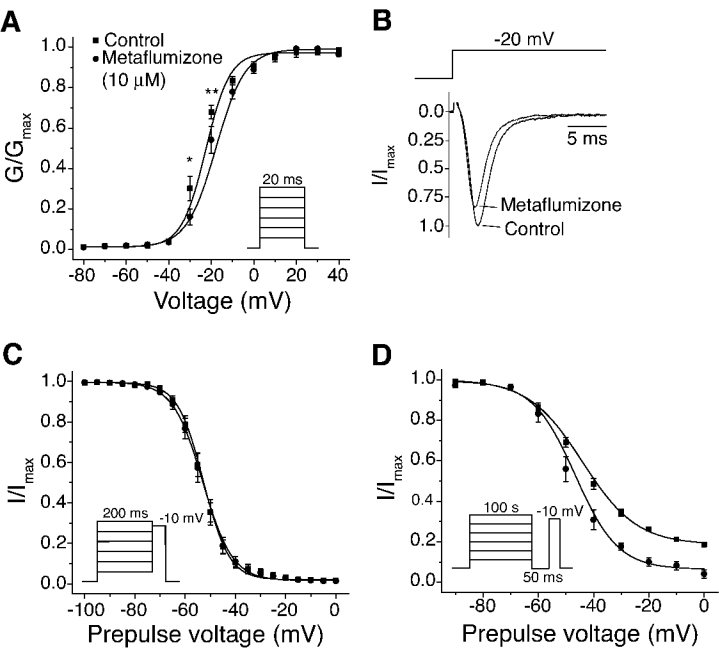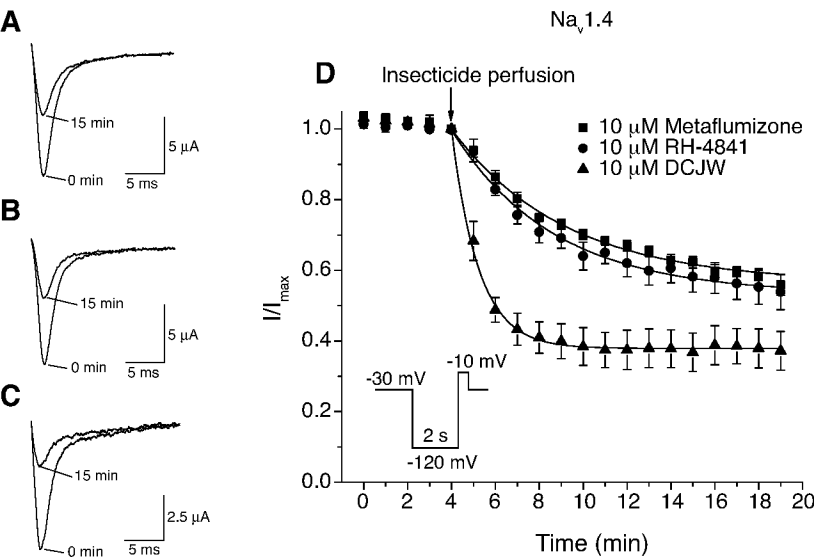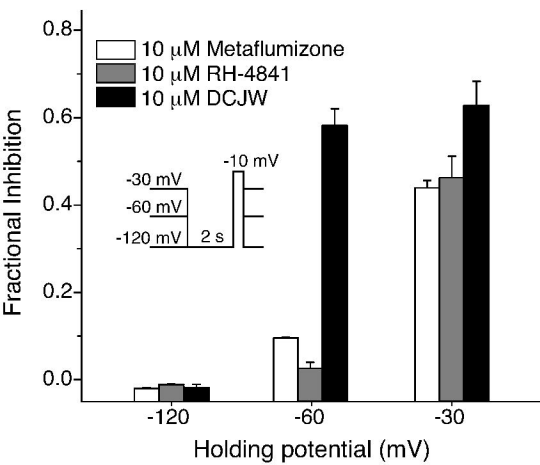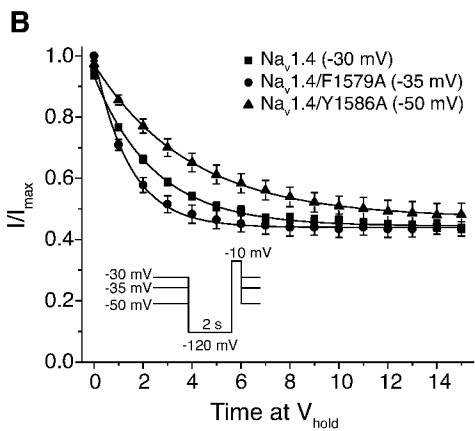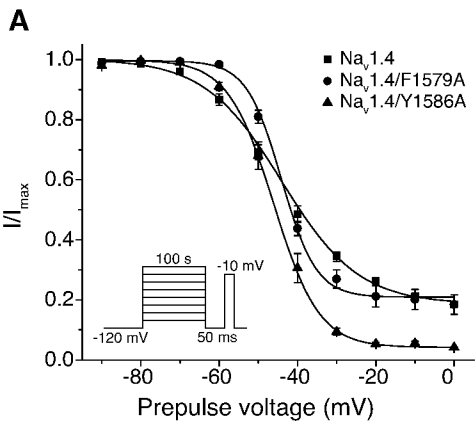Prepulse voltage (mV)

D

I/I_{max}

100 s   -10 mV

50 ms

Prepulse voltage (mV)

Figure 3

Figure 4

Figure 5

Figure 6

A   F1579A

Insecticide perfusion

-35 mV   -10 mV

2 s

-120 mV

■ 10 μM Metaflumizone
● 10 μM RH4841
▲ 10 μM DCJW

B   Y1586A

Insecticide perfusion

-50 mV   -10 mV

2 s

-120 mV

Figure 7

Figure 8

A

I/I_max

(graph with x-axis "Prepulse number" ranging 0 to 20, y-axis "I/I_max" ranging 0.0 to 1.0)

-10 mV
-50 mV
25 ms
n

■ Control
■ 200 μM Lidocaine
○ 10 μM Metaflumizone + lidocaine
△ 10 μM RH-4841+lidocaine
▽ 10 μM DCJW+lidocaine

B

I/I_max

(bar chart with x-axis "Prepulse number" showing values 0 and 10, y-axis "I/I_max" ranging 0.5 to 1.0)

□ Lidocaine
▨ Metaflumizone+lidocaine
▧ RH-4841+lidocaine
▦ DCJW+lidocaine

*