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RESEARCH ARTICLE

Actions of the pyrethroid insecticide bifenthrin on sodium channels expressed in rat cerebral cortical neurons

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Abstract

Voltage-gated sodium channels are important sites for the neurotoxic actions of pyrethroid insecticides in mammals. Here, we studied the mode of action of bifenthrin on the native sodium channels in cerebral cortical neurons prepared from newborn rat brain, where the toxic effects are largely generated. Bifenthrin caused a pronounced late current that persisted at the end of a depolarizing pulse, a slowly-decaying tail current following repolarization and significant resting modification (25.3% modification at 10 μM). No significant bifenthrin-induced effect was observed at the peak current. Bifenthrin also caused a concentration-dependent hyperpolarizing shift in steady-state activation and inactivation as well as slowed recovery from channel inactivation. Repetitive depolarization increased the potency of bifenthrin with high frequency. There was approximately 64% inhibition of modification upon repetitive activation by 10-Hz trains of depolarizing pulses. These results suggest that bifenthrin binds to and modifies sodium channels in both the closed and open states and exhibits the behavior between type I and type II.

Keywords

Bifenthrin, patch clamp, pyrethroid, state-dependent modification, voltage-gated sodium channel

History

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Introduction

Pyrethroids are synthetic insecticidal compounds that are frequently and widely used, and they have a high potency and selectivity as nerve poisons against various arthropods with relatively lower toxic effects on mammals (Casida et al., 1983). Historically, pyrethroids are classified into two major groups based on their structure and toxicology, including those lacking the α-cyano substituent (type I) and those with the α-cyano substituent (type II). Type I pyrethroids produce T syndrome (whole body tremor), whereas type II pyrethroids produce CS syndrome (choreoathetosis with salivation). However, several commercially-important pyrethroids produce a combination of type I and type II syndromes, including both tremors and salivation, and they are classified accordingly as type I/II (Verschoyle & Aldridge, 1980). Bifenthrin, the non-cyano-containing pyrethroid that produces type I poisoning syndrome, is one of the most popular pyrethroids effective against a broad spectrum of insect pests of economically important crops (Ray & Fry, 2006).

The basic mechanism of action of pyrethroids involves binding to the nerve membrane sodium channels and modifying their gating kinetics, causing a disruption of nerve function (Narahashi, 1985). Voltage-gated sodium channels (VGSCs) are the primary targets for the central neurotoxic effects of pyrethroids in mammals (Soderlund, 2012). As a class, pyrethroids do not act similarly on VGSCs. The kinetics of the activation, inactivation and deactivation of pyrethroid-modified channels have been employed to evaluate whether a given compound is classified as type I or type II (Breckenridge et al., 2009). Sodium channel activation and inactivation for type I pyrethroids are more rapid than for type II pyrethroids (Choi & Soderlund, 2006). Type I pyrethroid cismethrin modifies the sodium channels in the closed state, whereas type II pyrethroids cypermethrin and deltamethrin modify the open but not inactivated sodium channels (Soderlund, 2010).

The majority of studies during the past decade have employed transient expression in *Xenopus laevis* oocytes to assess the action of pyrethroids on individual sodium channel of defined subunit structure (Choi & Soderlund, 2006; McCavera & Soderlund, 2012; Tan & Soderlund, 2010, 2011;Tan et al., 2011). The results of these studies provide evidence for compound-specific, state-dependent modification of sodium channels by pyrethroids.

Although the *Xenopus* oocyte system offers the opportunity to identify the functional and pharmacological properties of each sodium channel isoform, the properties of channels expressed in the oocyte membrane may not fully reproduce those in the neuronal environment. A study provided evidence that the actions of tefluthrin and deltamethrin on Nav1.6 channels in HEK293 cells differ from the effects of these compounds on Nav1.6 channels in *Xenopus* oocytes (He & Soderlund, 2011).

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There is also evidence that target sites other than sodium channels contribute to pyrethroid-induced neurotoxicity, such as voltage-sensitive calcium channels (Clark & Symington, 2007; Hildebrand et al., 2004) and voltage-sensitive chloride channels (Burr & Ray, 2004; Forshaw et al., 2000). These channels are critical to the neuronal processes in mammalian neurons. Thus, a physiologically relevant model of functional neuronal networks, with intact neurons and containing all of the relevant pyrethroid-sensitive channels, is necessary to understand the pyrethroid-induced modulation of sodium channels.

Cerebral cortical neurons in primary culture offer a model system in which the effects of pyrethroids on neuronal activity can be examined directly on a physiologically relevant timescale. The purpose of this study was to determine the modes of action of a widely used insecticide of the type I pyrethroid class, bifenthrin on VGSC currents. To do this, we studied the state-dependent modification of sodium channels. To the best of our knowledge, this is the first voltage-clamp characterization and quantification of pyrethroid-induced modifications in cerebral cortical neurons.

Materials and methods

Animals and cell culture

Cortical neurons were cultured from the cortex of postnatal day 0 (P0) rats as previously described (Johnson et al., 1999) with slight modifications. The newborn rats were decapitated, and the cerebral cortex was dissected in cold phosphate-buffered saline (PBS). Then, the dissected tissues were treated with trypsin for 20 min at 37 °C in a water bath. The cells were plated on a poly-D-lysine-coated substrate in Neurobasal medium plus B27 supplements (Gibco, Life Technologies, Gaithersburg, MD) at a density of 7×10^5 cells/ml. The cultures were maintained at 37 °C in 5% CO_2.

Electrophysiology

Neurons were used after 7–10 days of *in vitro* cell culture. Ionic currents were measured with a Multiclamp 700 A amplifier (Molecular Devices, Foster City, CA). Current traces were filtered at 2.9 kHz with a built-in four-pole Bessel filter and then digitized to the computer at a sampling rate of 20 kHz (DigiData 1322A, Molecular Devices). Series resistance compensation was not used. Patch-clamp pipettes were pulled from borosilicate glass capillaries on a vertical pipette puller (P97, Sutter Instrument Co, Novato, AS). The pipette solution contained (millimolar): CsCl, 50; NaCl, 10; HEPES, 10; CsF, 60 and EGTA, 20. The pH was adjusted to 7.2 with CsOH. The external solution contained the following (millimolar): NaCl, 140; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10 and NaH_2PO_4, 1.25. The pH was adjusted to 7.4 with NaOH. All compounds were purchased from Sigma-Aldrich. The resistance of the microelectrodes filled with the internal solution ranged from 4 to 8 MΩ in standard extracellular solutions.

Assays with pyrethroids

The stock solution of bifenthrin (98.0%; Dr. Ehrensotrfer GmbH, Augsburg, Germany) was dissolved in dimethylsulfoxide (DMSO) to achieve the final concentrations to ensure that with each dose the DMSO added would consistently contribute 0.1%. The doses and 0.1% DMSO as an untreated control were delivered to the intracellular membrane surface by perfusion into the bath within 5 mm of the microelectrode pipette tip.

Data analyses

The data were obtained using a patch-clamp amplifier (Molecular Devices, Multiclamp 700 A) and analyzed by Clampfit 9.0, Origin 8.0 and GraphPad Prism 5. The data were represented as the mean ± SEM. The statistical analysis was performed using either the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post *hoc* test for statistical significance. Comparisons with values of $p < 0.05$ were considered statistically significant.

Results

Kinetic properties of pyrethroid-modified currents

We assessed the modification of channels in the resting state by holding at a hyperpolarized membrane potential during equilibration with bifenthrin and sampling the pyrethroid-modified current during and after the first depolarizing pulse. Figure 1 illustrates the concentration-dependent modification of sodium currents by bifenthrin. Bifenthrin slowed the time course of transient current decay during a depolarizing pulse,

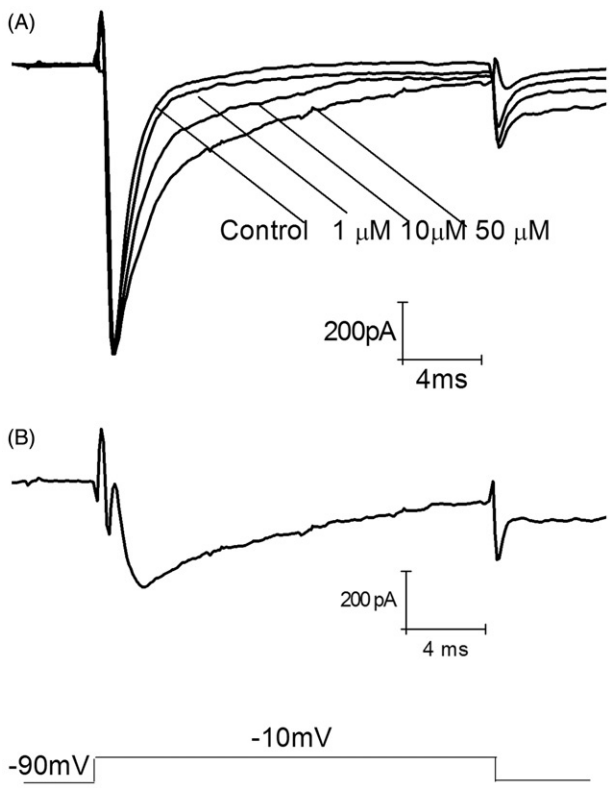

Figure 1. Concentration-dependent modification of sodium currents in cerebral cortical neurons by bifenthrin. (A) The traces were recorded from a single cell prior to bifenthrin exposure (control) and following equilibration with increasing concentrations of bifenthrin. (B) Net sodium current modified by 50 μM bifenthrin was reconstructed by the subtraction of the control current from bifenthrin-modified current measured in the same cell (as in Figure 1A).

significantly increasing τ_{inact} (approximately 12.2 ms for 50 μM bifenthrin) for peak current decay relative to control values (approximately 1.0 ms) and producing a late current that persisted throughout a 50-ms depolarization. A reconstructed current carried only by the bifenthrin-modified channel population is shown in Figure 1(B).

No significant bifenthrin-induced effect was observed for the peak ($p = 0.2$ in 10 μM, $n = 12$; $p = 0.4$ in 100 μM, $n = 5$, Figure 2D), but there was a statistically significant increase for the late part of the current (measured at the end of the 100-ms depolarizing pulse), revealing a slower I_{Na} inactivation ($p < 0.05$ in 100 μM, $n = 5$, Figure 2B). However, bifenthrin shifted the current–voltage curve of the peak current in the negative conductance region toward hyperpolarization. The membrane potential at which the sodium current attained a maximum was −30 mV in the control and −40 mV with 100 μM bifenthrin.

Effects of bifenthrin on voltage-dependent activation, inactivation and recovery

For activation, the currents at each test potential were converted to conductance (G) using the formula $G = I/(V_m - V_{rev})$, where V_m is the membrane potential and V_{rev} is reversal potential. The peak conductance value for each test potential was normalized to G_{max} (the maximum conductance) and plotted against the test potential to produce a voltage–conductance relationship curve, which was fitted by the Boltzmann equation $G/G_{max} = 1/\{1+\exp[(V_m - V_{0.5})/K]\}$ (Wu et al., 2009), where $V_{0.5}$ is the half activation voltage and K is a slope factor. Bifenthrin caused a concentration-dependent hyperpolarizing shift in the activation–voltage curve (approximately 7 mV) with a slope factor increase (reduction in the numerical slope factor). Although the trend of both of these effects was consistent across concentrations, there was only a significant effect of bifenthrin on the $V_{0.5}$ value differences (Table 1).

The steady-state inactivation was obtained with the double pulse protocols. Neurons were held at −80 mV, and the currents were elicited with a 50-ms test pulse to 0 mV proceeded by a 1000-ms prepulse to potentials between −120 and 0 mV. The peak amplitudes were normalized and plotted versus the prepulse potentials. The curves were well fitted with the Boltzmann equation: $I/I_{max} = 1/\{1+\exp[(V_m - V_{0.5})/K]\}$, where I/I_{max} is the normalized current, $V_{0.5}$ is the half inactivation voltage and K is the slope factor. Bifenthrin also caused a concentration-dependent hyperpolarizing shift in the $V_{0.5}$ for steady-state inactivation. The magnitude of these shifts was bigger than those found for activation and the binary comparisons were more statistically significant ($p < 0.0001$). The other effect of bifenthrin on channel gating was the enhancement of a small but reproducible component of the current that did not inactivate following depolarizing prepulses above 0 mV (Figure 3C). This inactivation-resistant current, which represented approximately 0.07% of the total current in the absence of the insecticide,

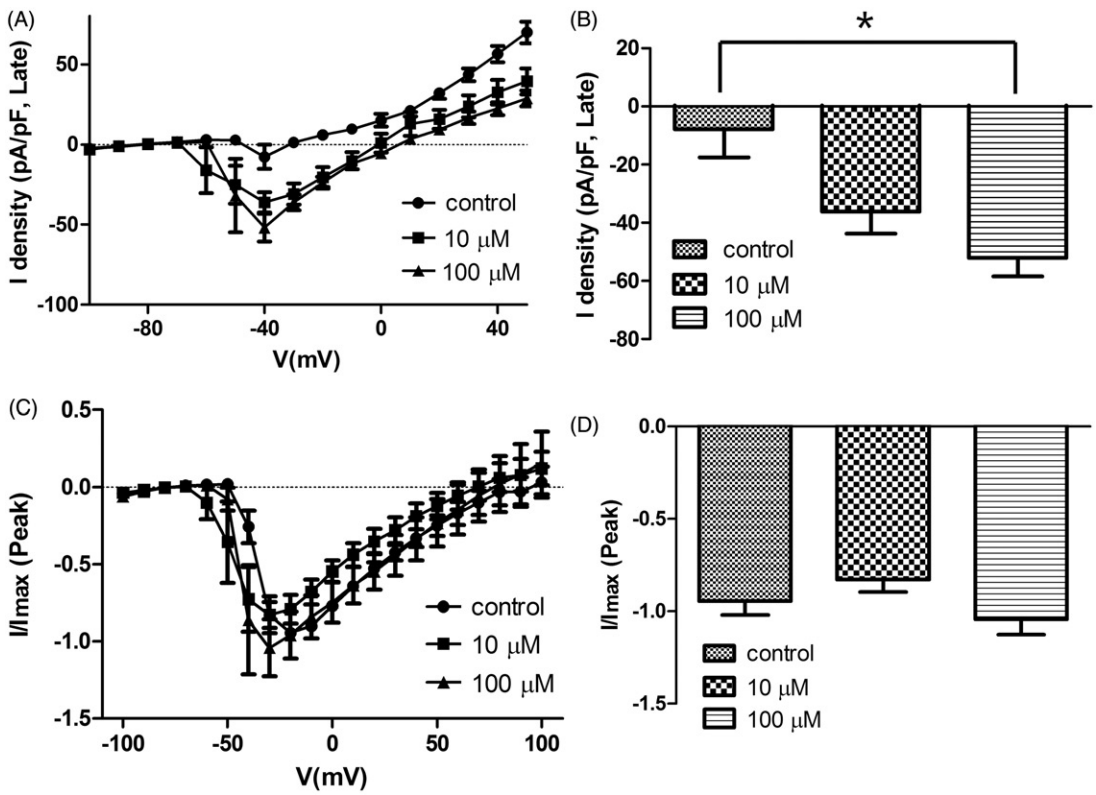

Figure 2. Comparison of the voltage dependence of the late currents ((A) measured at the end of the 100-ms depolarization) and peak currents (C) induced by bifenthrin. The cells were clamped at a membrane potential of −80 mV, and the currents were measured during a 100-ms depolarizing test pulse to potentials from −100 mV to 100 mV in 10-mV increments. The effects of bifenthrin on the maximal current density attained at −40 mV in the late currents (B) and the maximal normalized amplitude of the peak currents (D) are presented. * Indicates that 100 μM bifenthrin treatment is significantly greater than control ($n = 5$ for 100 μM bifenthrin and $n = 12$ for control, unpaired t test, $p < 0.05$).

Table 1. Effects of bifenthrin on the voltage dependence of the activation and steady-state inactivation of sodium channels in cerebral cortical neurons[a].

Condition	Activation			Inactivation		
	$V_{0.5}$	K	n	$V_{0.5}$	K	n
Control	-37.8 ± 2.2[b]	4.00 ± 1.30	18	-45.18 ± 1.2[c]	4.82 ± 0.80	21
+ Bifenthrin						
10 μM	-44.6 ± 1.3[b]	3.051 ± 0.62	12	-56.0 ± 1.1[c]	4.45 ± 0.50	15
100 μM	-44.9 ± 1.8[b]	1.791 ± 0.612	17	-59.3 ± 1.3[c]	4.17 ± 0.68	14

[a]Values calculated from fits of the data from the indicated number of individual experiments to the Boltzmann equation; $V_{0.5}$, midpoint potential (mV) for the voltage-dependent activation or inactivation; K, slope factor.
[b and c]Values significantly different from control (one-way ANOVA, $p < 0.05$ and $p < 0.0001$, respectively).

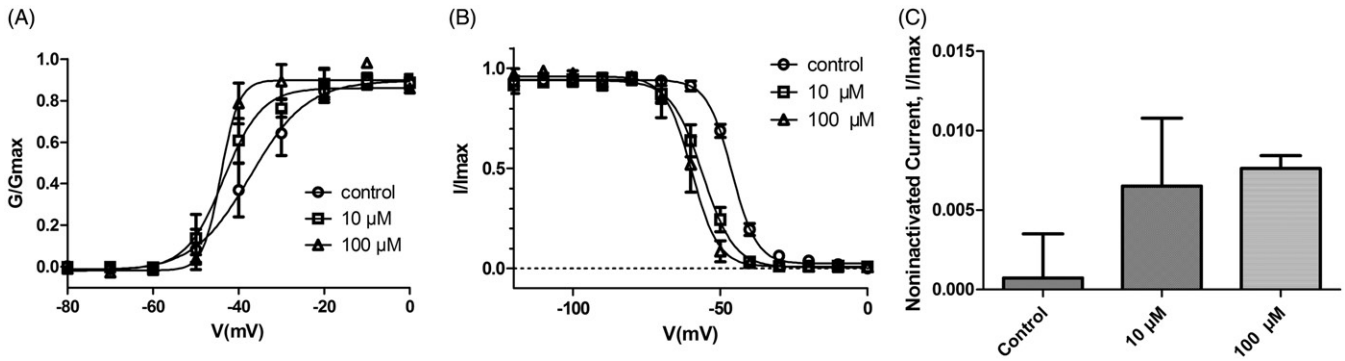

Figure 3. Concentration-dependent modification of the voltage dependence of the activation (A) and steady-state inactivation (B) of sodium channels in cerebral cortical neurons by bifenthrin. (C) Effects of bifenthrin on the amplitude of the inactivation-resistant component of sodium currents. Values are the means ± SEM.

was increased approximately 10-fold in the presence of bifenthrin. The enhancement of the inactivation-resistant current by bifenthrin was correlated with a change in the slope factor (K) of the inactivation curves for these compounds (Table 1), but this effect was not statistically significant.

To investigate the kinetics of recovery from the inactivated state, neurons were held at −80 mV, and a 5-ms conditioning depolarizing pulse of −10 mV was fully applied to inactivate the transient sodium channels. Then, a 5-ms test pulse of −10 mV was applied after a series of −80 mV intervals varying from 2 to 60 ms. The peak value of the current evoked by the conditioning pulse was designated as I_{max}, while the peak value of the current evoked by the test pulse was designated as I. The ratio of I to I_{max} represented the recovery of the current from inactivation. The plot of I/I_{max} versus the duration of the −80 mV intervals was well fitted with a monoexponential equation: $I/I_{max} = A + B^*[1-\exp(-t/\text{Tau})]$, where I_{max} is the maximal current amplitude, I is the current after a recovery period of Δt, Tau is the time constant and A is the amplitude coefficient. There was a significant change of the Tau value between the control and multiple dose groups (Figure 4B). The results showed that bifenthrin delayed the recovery of I_{Na} from inactivation.

Properties of the bifenthrin -induced sodium tail currents

There was no tail current developed after the depolarizing pulse under control conditions. In contrast, when neurons were superfused with bifenthrin (10 μM), a pronounced tail current appeared after the membrane repolarization (Figure 5A). Fits of these tail currents to a single exponential decay function yielded a first-order time constant (τ_{tail}) of 44.1 ± 7.9 ms ($n = 5$).

The maximal amplitude of the tail current occurring after a 3-ms pulse to −10 mV was measured for membrane potentials ranging from −80 to +60 mV (Figure 5B). An inward I/V curve is more apparent in the presence of bifenthrin than under the control conditions, and the reversal potential was shifted toward a more positive value.

We employed the normalized conductance of the pyrethroid-induced sodium tail current (Tatebayashi & Narahashi, 1994) to calculate the fraction of sodium channels that were modified following the equilibration with 10 μM bifenthrin (Figure 5C). On average, 25.37 ± 3.44% of the channels were modified in the presence of bifenthrin, which was significantly different from the mean value obtained in the experiment without bifenthrin ($p < 0.01$, $n = 5$).

Use-dependency of the bifenthrin effect on cerebral cortical neurons

The modification of both insect and mammalian sodium channels by some pyrethroids is enhanced by repeated depolarizations (Soderlund, 2010). We, therefore, determined the effect of trains of high-frequency depolarizing prepulses on the modification of peak I_{Na}. Trains of short depolarizations (10 ms to −10 mV) were applied at frequency of 1, 3 or

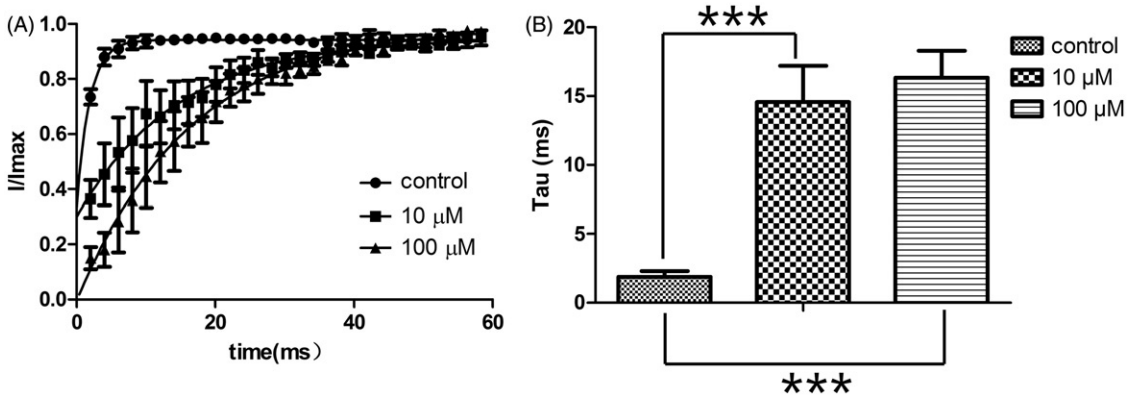

Figure 4. Effects of bifenthrin on the recovery of sodium channels in cerebral cortical neurons. (A) Effects of different concentrations of bifenthrin on the recovery from inactivation curves of I_{Na}. Data were presented as the mean ± SEM. (B) Tau values for experiments with bifenthrin are presented. *** Indicates that bifenthrin treatment is significantly greater than control ($n = 7$ for 10 μM, $n = 5$ for 100 μM and $n = 10$ for control, unpaired t test, $p < 0.0001$).

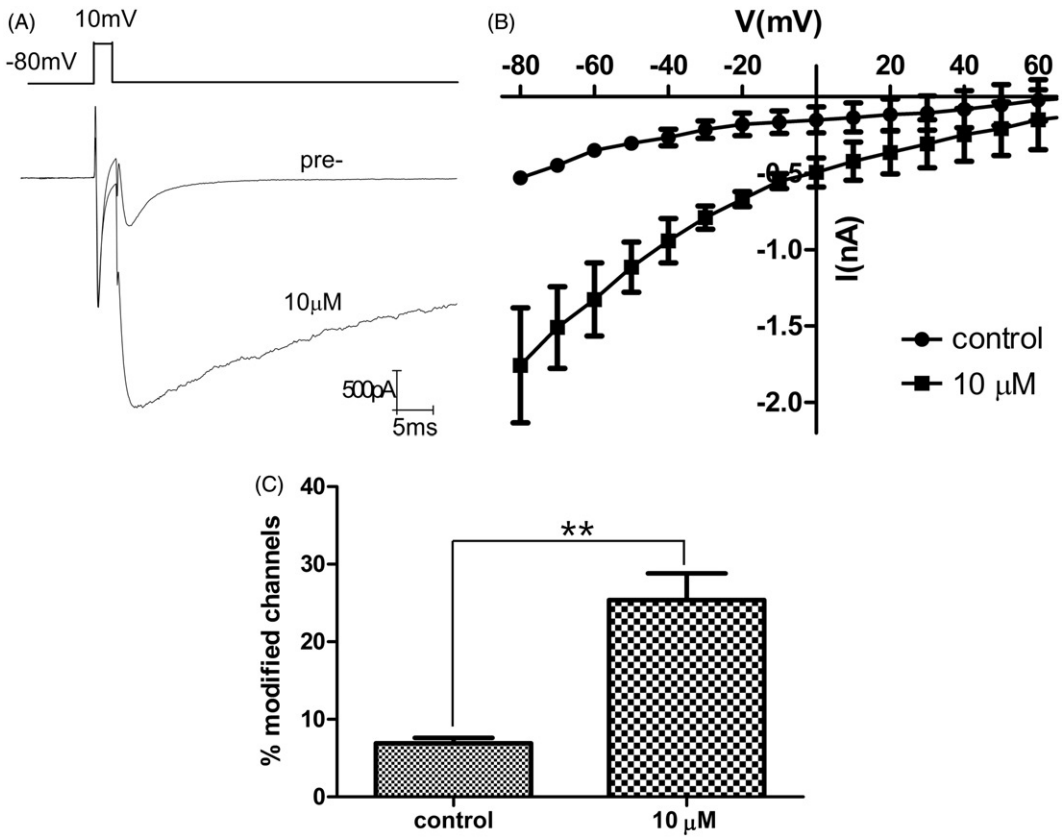

Figure 5. Bifenthrin-induced sodium tail current in cerebral cortical neurons. (A) Under control conditions, no tail current could be detected upon repolarization from a 3-ms depolarizing pulse from −80 to +10 mV as a consequence of the fast deactivation properties of sodium channels. However, after superperfusion with a solution containing 10 μM bifenthrin, a prominent tail current was observed upon repolarization to −80 mV, essentially as a result of deactivationslowing down. (B) Mean current–voltage curve for tail current amplitude in the control (filled circles) and in the presence of bifenthrin (10 μM). (C) The fraction of sodium channels modified by bifenthrin. ** Indicates that bifenthrin treatment is significantly greater than control ($n = 5$ for control and 10 μM, respectively, unpaired t test, $p < 0.01$).

10 Hz. Averaged normalized amplitudes (I/I_{max}, where I_{max} is the amplitude of the current during the first pulse) were obtained from 1 Hz-, 3 Hz- or 10 Hz-train protocols.

Modification by bifenthrin was strongly enhanced by repetitive depolarizations. The number of prepulses at which the peak current decreased to a steady-state level of use-dependent inhibition depends on the stimulus frequency and drug concentration. Without bifenthrin, the extent of the modification decreased with prepulse number <20 prepulses and then remained stable; but for bifenthrin, it took up to 40 prepulses to reach a stable level. In the absence of the insecticide, repeated stimulation caused a slight decline in the amplitudes of peak transient sodium currents, but the current amplitudes stabilized at above 75% of the initial amplitude (for 10 Hz). In the presence of bifenthrin, repetitive depolarizations produced a 74% decrement in the peak current. A summary of

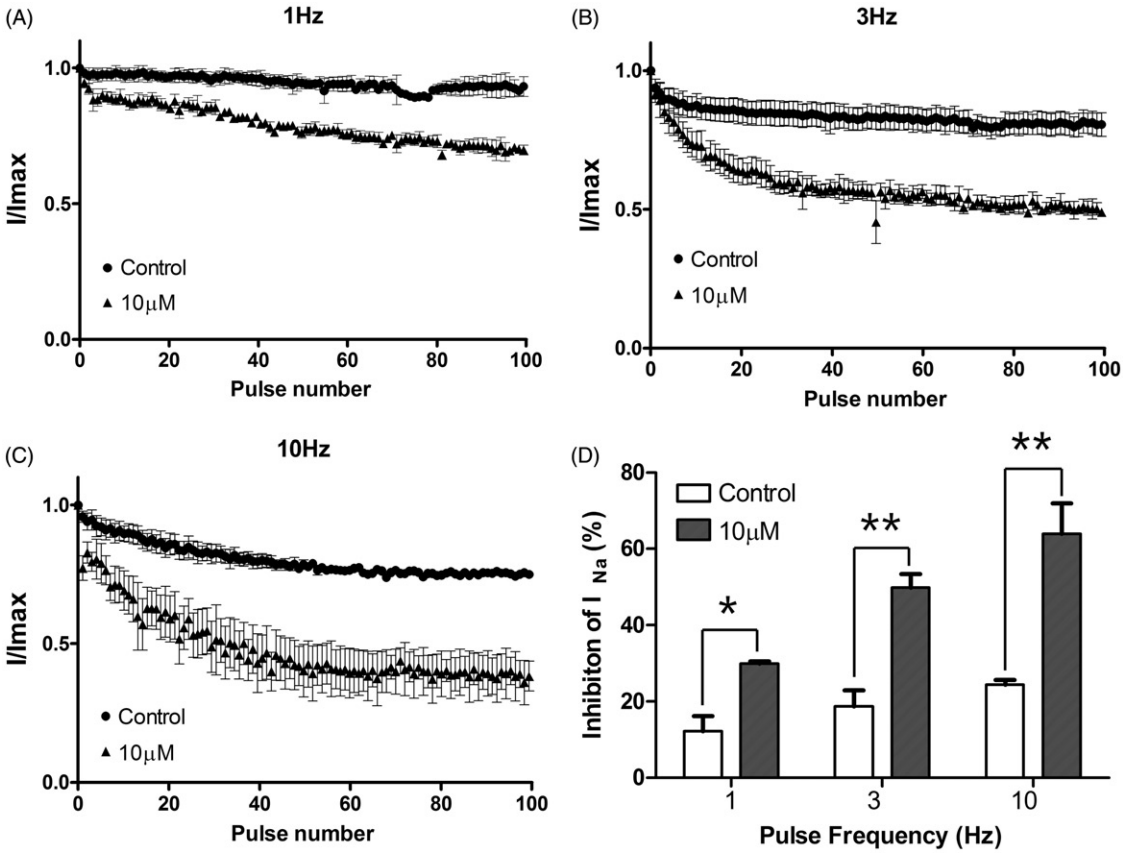

Figure 6. Use-dependent inhibition of sodium tail currents by bifenthrin in cerebral cortical neurons. Na^+ currents evoked by trains of depolarizing pulses of either 1 Hz (A), 3 Hz (B) or 10 Hz (C) were recorded in the absence (control) and the presence of 10 μM bifenthrin. (D) Summary of the effects of stimulation frequency on use-dependent block of the sodium current (*I*) in the presence of 10 μM bifenthrin. * and ** Indicate that 10 μM bifenthrin treatment is significantly greater than control ($n = 5$ for each group, unpaired *t* test, $p < 0.05$ and $p < 0.01$, respectively).

the steady-state use-dependent inhibition by bifenthrin is shown in Figure 6(D).

Discussion

The pyrethroids are a widely used class of insecticides to which there is significant human exposure. Their acute toxicity is dominated by pharmacological actions upon the central nervous system (CNS), predominantly mediated by voltage-gated sodium channels. They are generally regarded as safe to humans, but our examinations of the voltage-dependent sodium current in cerebral cortical neurons suggest a strong sensitivity of mammalian neurons to bifenthrin.

To date, the major findings about the actions of pyrethroids have been obtained with the extensive use of *Xenopus* oocytes that expressed insect and mammalian sodium channels (Choi & Soderlund, 2006; McCavera & Soderlund, 2012; Spencer, 2009; Tan et al., 2011). By contrast, the studies involving sodium channels kept in their native intracellular neuronal environment are limited in number (Kadala et al., 2011). As described here, the sub-lethal effects of type I pyrethroids that have been reported are mainly interpreted as central nervous system perturbations (Ray & Fry, 2006). Cerebral cortical neurons provide a convenient system to study the modes of actions of pyrethroids on sodium channels in their intracellular environment.

We reconstructed the currents carried only by the bifenthrin-modified channels by subtracting the control current measured before bifenthrin exposure from the current measured in the same neuron after equilibration with bifenthrin. In this case, the fast inactivation of I_{Na} was apparently slowed down during the pulse because the current amplitude at the end of the pulse was significantly larger (Figure 1). Bifenthrin caused a slowly-decaying tail current following the repolarization with first-order kinetics. One-fourth of the channels appeared modified by 10 μM bifenthrin after a single, short depolarizing pulse at V_{max} (Figure 5C). The peak amplitude of the sodium current during a single depolarization was not significantly affected (Figure 2D). Taken together, the behavior of bifenthrin was more ambiguous. This compound yielded currents with virtually identical kinetic properties: the moderate degree of retardation in the activation and inactivation kinetics, like the type I compounds, but more slowly-decaying tail currents (Soderlund, 2010). Therefore, bifenthrin is characterized as having properties intermediate between type I and type II.

The appearance of modified currents during the first depolarizing pulse after equilibration, the shift in the voltage-dependence of the activation of the bifenthrin-modified channels, a decaying tail current following repolarization and the development of a slowly-activating, non-inactivating current suggest that bifenthrin modifies sodium channels in the closed state (Soderlund, 2010).

The application of the trains of depolarizing prepulses greatly enhanced the modification of sodium channels by bifenthrin. Bifenthrin produced a significant use-dependent

effect. The extent of the inhibition was increased substantially by the application of the trains of short depolarizing prepulses. This finding suggests a significant fraction of the channels is converted into the slow inactivated state, and, thus becomes susceptible to drug binding/modulation. The interaction of bifenthrin with this channel state that results in delayed recovery is manifested as an enhanced inhibition on repeated channel gating (''use-dependence''). The most likely explanation for this phenomenon is that bifenthrin preferentially interacts with a channel open state. Our findings suggest that bifenthrin binds to and modifies sodium channels in both the closed and open states.

Bifenthrin-induced sodium current modifications during a step depolarization in cerebral cortical neurons were not consistent with earlier works on oocytes expressing Nav1.8 sodium channels (Choi & Soderlund, 2006), where no detectable inactivation during a 40-ms pulse was observed and tail current decay was faster (τ_{tail} ~4 ms), and the resting modification was only 14% at 100 μM. These differences may be contigent on the native intracellular neuronal environment and the various patterns of sodium channels expressed in the central nervous system. There is evidence that the action of pyrethroids at sites other than the sodium channel may contribute to their effects *in vivo*. Pyrethroids have been shown to act on voltage-sensitive calcium channels and voltage-sensitive chloride channels, thereby contributing to pyrethroid-induced toxicity and enhanced excitability in the central nervous system (Burr & Ray, 2004; Hildebrand et al., 2004). These targets may play a role in pyrethroid toxicity. Moreover, mammalian genomes contain nine sodium channels α subunit cDNA sequences (designated Nav1.1–Nav1.9) that encode sodium-selective, voltage-gated ion channels (Goldin et al., 2000). Four of these α subunit isoforms (Nav1.1, Nav1.2, Nav1.3 and Nav1.6) are expressed in the CNS (Goldin, 2001). Previous studies have shown that Nav1.6 sodium channels are more sensitive than Nav1.8 (distributed in the peripheral nervous system) sodium channels and nearly as sensitive as Nav1.3 channels (Tan & Soderlund, 2010). This relationship provides evidence of the likely reason for the mixed behavior of bifenthrin.

Conclusions

In summary, our work describes bifenthrin-induced sodium current modifications in cerebral cortical neurons for the first time. The results of this study suggest that the activation and deactivation kinetics of bifenthrin-modified channels is classified as mixed and this compound interacts with both closed and open sodium channels. Considering that the classification of pyrethroid syndromes is based on the effects on the central nervous system, cerebral cortical neurons will be a reliable model with predictive value for the action of pyrethroids on sodium channels *in vivo*, within this kinetic framework of the type I/type II classification scheme, despite the apparent segregation of each of the four unclassified pyrethroids into one of these two groups of compounds identified on the basis of their kinetic properties in our assays.

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Declaration of interest

The authors report no declarations of interest.

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