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## Differential state-dependent modification of inactivation-deficient Na<sub>v</sub>1.6 sodium channels by the pyrethroid insecticides *S*-bioallethrin, tefluthrin and deltamethrin

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### Abstract

Pyrethroid insecticides disrupt nerve function by modifying the gating kinetics of transitions between the conducting and nonconducting states of voltage-gated sodium channels. Pyrethroids modify rat Na<sub>v</sub>1.6 + β1 + β2 channels expressed in *Xenopus* oocytes in both the resting state and in one or more states that require channel activation by repeated depolarization. The state dependence of modification depends on the pyrethroid examined: deltamethrin modification requires repeated channel activation, tefluthrin modification is significantly enhanced by repeated channel activation, and *S*-bioallethrin modification is unaffected by repeated activation. Use-dependent modification by deltamethrin and tefluthrin implies that these compounds bind preferentially to open channels. We constructed the rat Na<sub>v</sub>1.6Q3 cDNA, which contained the IFM/QQQ mutation in the inactivation gate domain that prevents fast inactivation and results in a persistently open channel. We expressed Na<sub>v</sub>1.6Q3 + β1 + β2 sodium channels in *Xenopus* oocytes and assessed the modification of open channels by pyrethroids by determining the effect of depolarizing pulse length on the normalized conductance of the pyrethroid-induced sodium tail current. Deltamethrin caused little modification of Na<sub>v</sub>1.6Q3 following short (10 ms) depolarizations, but prolonged depolarizations (up to 150 ms) caused a progressive increase in channel modification measured as an increase in the conductance of the pyrethroid-induced sodium tail current. Modification by tefluthrin was clearly detectable following short depolarizations and was increased by long depolarizations. By contrast modification by *S*-bioallethrin following short depolarizations was not altered by prolonged depolarization. These studies provide direct evidence for the preferential binding of deltamethrin and tefluthrin (but not *S*-bioallethrin) to Na<sub>v</sub>1.6Q3 channels in the open state and imply that the pyrethroid receptor of resting and open channels occupies different conformations that exhibit distinct structure–activity relationships.

### Keywords

Sodium channel; Na<sub>v</sub>1.6; Use-dependent modification; Deltamethrin; *S*-bioallethrin; Tefluthrin

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## 1. Introduction

Effects on voltage-gated sodium channels underlie both the insecticidal actions of pyrethroids and their neurotoxicity to nontarget organisms (Soderlund, 2012; Soderlund et al., 2002). Pyrethroids modify the gating of voltage-gated sodium channels, which mediate the transient increase in the sodium permeability of the nerve membrane that underlies the rising phase of the nerve action potential (reviewed in: Bloomquist, 1993; Narahashi, 1996; Soderlund, 1995). Voltage- and patch-clamp analyses of isolated sodium currents show that pyrethroids retard the kinetics of sodium channel activation, inactivation, and deactivation to produce persistently open channels. The hallmark of pyrethroid modification in all experimental systems is the induction of a sodium tail current following membrane depolarization–repolarization cycles under voltage clamp whose persistence is correlated with pyrethroid structure.

The majority of studies of pyrethroid action on sodium currents in neurons under voltage-clamp conditions have been performed by equilibrating preparations with insecticide at hyperpolarized membrane potentials and assessing the effects of pyrethroids upon depolarization (Bloomquist, 1993; Narahashi, 1996; Soderlund, 1995). This approach is biased toward the detection of the modification of channels in the closed state by pyrethroids. Consequently, only a few studies have explored possible use-dependent modification by assessing the impact of trains of depolarizing prepulses on the amplitude of the pyrethroid-induced sodium tail current. Use-dependent enhancement of modification is detected in assays of fenvalerate on sodium currents in crayfish giant axons (Salgado and Narahashi, 1993), assays of deltamethrin on the tetrodotoxin (TTX)-sensitive and TTX-resistant components of the sodium current in rat dorsal root ganglion neurons (Tabarean and Narahashi, 2001), and assays of tetramethrin and permethrin on sodium currents in honeybee antennal olfactory neurons (Kadala et al., 2011). By contrast, repetitive depolarization did not alter the time-dependent increase in the sodium tail current elicited by perfusion of squid giant axon preparations with phenothrin (de Weille et al., 1988).

Most of the evidence for use-dependent sodium channel modification comes from studies of pyrethroid action on cloned sodium channels expressed transiently in oocytes of the frog *Xenopus laevis*. Modification by some pyrethroids of both insect and mammalian channels expressed in *X. laevis* oocytes either depends on or is enhanced by trains of high-frequency depolarizing prepulses (reviewed in: Soderlund, 2010). Recent studies of the rat Na<sub>v</sub>1.6 sodium channel isoform in the *Xenopus* oocyte system show that the relative importance of resting and use-dependent channel modification depends on the pyrethroid examined (Tan and Soderlund, 2010) and that use-dependent effects require coexpression of the Na<sub>v</sub>1.6  $\alpha$  subunit isoform with the auxiliary  $\beta$ 1 and  $\beta$ 2 subunits (Tan and Soderlund, 2011a).

The use-dependent effects of pyrethroids on insect sodium channels expressed in *Xenopus* oocytes have been interpreted as evidence for the existence of a high-affinity receptor conformation on channels in the open state (Vais et al., 2000). Despite the inferred importance of pyrethroid binding to open channels there is little direct evidence for open-channel modification by pyrethroids. Assays of *Drosophila melanogaster* Para sodium channels expressed in *Xenopus* oocytes employed *Anemonia sulcata* toxin II (ATX-II) to eliminate fast inactivation and document pyrethroid modification of persistently open channels (Vais et al., 2000). However, the allosteric coupling of the ATX-II and pyrethroid binding sites on mammalian sodium channels (Lombet et al., 1988) suggests that the pyrethroid receptor of ATX-II-modified Para channels may exhibit pharmacological properties that differ from those of native channels.

To study pyrethroid interactions with open channels directly in the absence of pharmacological manipulation we introduced mutations in the inactivation gate region of the rat Na<sub>v</sub>1.6 sodium channel that are known to eliminate fast inactivation (West et al., 1992; Zhou and Goldin, 2004) and employed these inactivation-deficient channels to assess pyrethroid modification in the *Xenopus* oocyte expression system. We employed three pyrethroids that exhibit compound-specific differences in the relative importance of resting and use-dependent modification of native Na<sub>v</sub>1.6 sodium channels in the oocyte expression system. Deltamethrin modification requires repeated channel activation, tefluthrin modification is significantly enhanced by repeated channel activation, and *S*-bioallethrin modification is unaffected by repeated activation (Tan and Soderlund, 2010). Our results provide direct evidence that the differences in resting and use-dependent modification of native Na<sub>v</sub>1.6 channels among these three pyrethroids result from their differential modification of resting and open channels. Our results imply that the pyrethroid receptor of resting and open channels occupies different conformations that exhibit distinct structure–activity relationships.

## 2. Materials and methods

### 2.1. Sodium channel subunit cDNAs

Cloned rat voltage-sensitive sodium channel subunit cDNAs were obtained from the following sources: the Na<sub>v</sub>1.6 $\alpha$  subunit (Dietrich et al., 1998) from L. Sangameswaran (Roche Bioscience, Palo Alto, CA) and the  $\beta$ 1 and  $\beta$ 2 subunits (Isom et al., 1992, 1995) from W.A. Catterall (University of Washington, Seattle, WA). The cDNA for the Na<sub>v</sub>1.6Q3 sodium channel  $\alpha$  subunit, containing the IFM to QQQ mutation at amino acid sequence positions 1477–1479, was prepared by oligonucleotide-mediated site-directed mutagenesis on the parental Na<sub>v</sub>1.6 cDNA using a commercial kit (QuikChange XL, Stratagene, La Jolla, CA) and the structure of the Na<sub>v</sub>1.6Q3 cDNA was confirmed by DNA sequencing. Plasmid cDNAs were digested with restriction enzymes to provide linear templates for cRNA synthesis *in vitro* using a commercial kit (mMessage mMachine, Ambion, Austin, TX). The integrity of synthesized cRNA was determined by electrophoresis in 1% (w/v) agarose–formaldehyde gels.

### 2.2. Expression in oocytes

Freshly dissected *X. laevis* ovaries obtained from a commercial source (Nasco, Ft. Atkinson, WI) were employed as a source of stage V–VI oocytes, which were isolated as described elsewhere (Smith and Soderlund, 2001). Each data set was derived from oocytes isolated from three different ovaries. Oocytes were injected with a 1:1:1 (mass ratio) mixture of  $\alpha$  subunit,  $\beta$ 1 subunit and  $\beta$ 2 subunit cRNAs (0.5–5 ng/oocyte); this mixture provided a ~9-fold molar excess of  $\beta$ 1 and  $\beta$ 2 cRNAs to ensure the preferential expression of desired binary or ternary  $\alpha + \beta$  complexes (Tan and Soderlund, 2009, 2011b). Injected oocytes were incubated in ND-96 medium (in mM: 96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 5 HEPES; adjusted to pH 7.6 at room temperature with NaOH) supplemented with 6% horse serum (Sigma–Aldrich, St. Louis, MO), 0.5% streptomycin/penicillin, and 1% sodium pyruvate (Goldin, 1992) at 16 °C for 3–5 days until electrophysiological analysis of sodium currents.

### 2.3. Electrophysiology

Sodium currents were recorded from oocytes perfused with ND-96 at 21–23 °C in the two-electrode voltage clamp configuration using an Axon Geneclamp 500B amplifier (Molecular Devices, Sunnyvale, CA). Microelectrodes were pulled from borosilicate glass capillary tubes (1.0 mm O.D.; 0.5 mm I.D.; World Precision Instruments Inc., Sarasota, FL) and filled with 3 M KCl. Filled electrodes had resistances of 0.3–1.0 M $\Omega$  when immersed in ND-96 medium. Currents were filtered at 2 kHz with a low-pass 4-pole Bessel filter and digitized at

50 kHz (Digidata 1320A; Molecular Devices). To determine the voltage dependence of activation, oocytes were clamped at a membrane potential of  $-100$  mV and currents were measured during test pulses to potentials to  $40$  mV in  $5$ -mV increments. Maximal peak transient currents were obtained upon depolarization to  $-10$  mV. For determinations of time-dependent modification of  $\text{Na}_v1.6\text{Q3}$  channels, oocytes were clamped at a membrane potential of  $-100$  mV and currents were measured during and after test pulses increasing in duration from  $3$  ms to  $150$  ms in  $3$ ,  $5$  or  $10$  ms increments. All experiments employed  $30$ -s intervals between pulses or pulse trains to permit complete recovery from pyrethroid modification. Capacitive transients and leak currents were subtracted using the P/4 method (Bezaniilla and Armstrong, 1977).

#### 2.4. Assays with pyrethroids

*S*-bioallethrin (92.9% purity) and deltamethrin (99.5%) were obtained from Bayer CropScience (Research Triangle Park, NC) and tefluthrin (98.8%) was obtained from Syngenta (Bracknell, Berks., UK). Pyrethroids were prepared as stock solutions in dimethyl sulfoxide (DMSO) and diluted with ND-96 immediately before use to final concentrations of  $10$   $\mu\text{M}$  (deltamethrin) or  $100$   $\mu\text{M}$  (*S*-bioallethrin and tefluthrin), the highest concentrations achievable in ND-96 perfusion medium for each compound. The final DMSO concentration in the bath did not exceed  $0.1\%$ , a concentration that had no effect on sodium currents. Oocytes were perfused at  $0.45$  ml/min with pyrethroid in ND-96 for  $6$  min and then with ND-96 for  $3$  min prior to recording to stabilize the effective concentration and prevent the continued partitioning of the insecticide from the medium to the oocyte membrane during recording (Choi and Soderlund, 2006). Washout of *S*-bioallethrin, tefluthrin and deltamethrin was negligible under these conditions. All experiments with pyrethroids employed a disposable capillary perfusion system (Tatebayashi and Narahashi, 1994) and custom-fabricated single-use recording chambers (Smith and Soderlund, 2001) to prevent cross-contamination between oocytes. Net sodium currents following pyrethroid exposure were derived by digital subtraction of control current traces prior to pyrethroid exposure from current traces obtained following pyrethroid exposure in the same oocyte.

#### 2.5. Data analysis

Data were acquired and analyzed using pClamp 10.2 (Molecular Devices) and Origin 8.1 (OriginLab Corp., Northampton, MA). The Boltzmann equation [ $y = (A_1 - A_2)/(1 + e^{(x-x_0)/dx}) + A_2$ ] was used to fit conductance–voltage data. Conductances of net pyrethroid sodium tail currents, normalized to the peak current measured prior to insecticide perfusion in the same oocyte using the same pulse protocol, were employed as indices of relative sodium channel modification. Statistical analyses were performed in Prism 5.0 (GraphPad Software, La Jolla, CA). Comparisons of two or more mean values to a common control data set employed one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for statistical significance. Comparisons of two means employed Student's *t*-test.

### 3. Results

#### 3.1. Modified kinetics and gating of $\text{Na}_v1.6\text{Q3}$ sodium channels

We modified the sequence of the inactivation gate region of the  $\text{Na}_v1.6$  sodium channel  $\alpha$  subunit, formed by the intracellular linker between the third and fourth homology domains, by site-directed mutagenesis, replacing the hydrophobic isoleucine–phenylalanine–methionine (IFM) motif at amino acid sequence positions  $1477$ – $1479$  with three positively charged glutamine (QQQ) residues (Fig. 1A). We expressed either the native  $\text{Na}_v1.6\alpha$  subunit or the mutated  $\alpha$  subunit (designated  $\text{Na}_v1.6\text{Q3}$ ) in *Xenopus* oocytes in combination with the conspecific  $\beta 1$  and  $\beta 2$  auxiliary subunits and recorded sodium currents under voltage-clamp conditions in response to step depolarizations from a hyperpolarized

membrane potential.  $\text{Na}_v1.6$  channels (Fig. 1B) activated and inactivated rapidly within the first 10 ms of a depolarizing pulse and gave no detectable persistent current during depolarization or tail current following repolarization. By contrast  $\text{Na}_v1.6\text{Q3}$  channels (Fig. 1C) activated more slowly than  $\text{Na}_v1.6$  channels, giving a steady-state current by 15–20 ms after depolarization and remaining persistently open during prolonged depolarizations. Following repolarization to the holding potential,  $\text{Na}_v1.6\text{Q3}$  channels also exhibited a pronounced tail current that decayed completely within the first two to three ms following repolarization. Fits of these tail currents to a single exponential decay function yielded a first-order time constant ( $\tau_{\text{tail}}$ ) of  $1.4 \pm 0.3$  ms (mean  $\pm$  SE,  $n = 9$ ).

Fig. 2 compares the voltage dependence of activation of  $\text{Na}_v1.6$  and  $\text{Na}_v1.6\text{Q3}$  channels. Fits of the plots of sodium conductance as a function of test potential from individual experiments with different oocytes to the Boltzmann equation gave a midpoint potential for activation ( $V_{0.5}$ ) of  $-22.9 \pm 0.2$  mV and a slope factor ( $K$ ) of  $6.55 \pm 0.14$  for  $\text{Na}_v1.6$  channels ( $n = 10$ ). Parallel experiments with  $\text{Na}_v1.6\text{Q3}$  channels gave a  $V_{0.5}$  value of  $-21.0 \pm 1.1$  mV and a slope factor of  $5.66 \pm 0.30$  ( $n = 14$ ). The slope factor for the activation of  $\text{Na}_v1.6\text{Q3}$  channels differed significantly from that for  $\text{Na}_v1.6$  channels (unpaired  $t$ -test;  $P < 0.05$ ).

### 3.2. Effects of pyrethroids on the kinetics and gating of $\text{Na}_v1.6\text{Q3}$ sodium channels

Fig. 3A illustrates the effects of tefluthrin on sodium currents measured in oocytes expressing  $\text{Na}_v1.6\text{Q3}$  channels, which were representative of the effects of all three pyrethroids examined in this study. Specifically, we found no effect of pyrethroids on the rate of onset, magnitude, or persistence of currents carried by  $\text{Na}_v1.6\text{Q3}$  channels during a depolarizing pulse. The sole evidence for modification of  $\text{Na}_v1.6\text{Q3}$  by pyrethroids was the enhancement and prolongation of the sodium tail current measured upon membrane repolarization. Fig. 3B compares representative sodium tail currents recorded from control oocytes and oocytes exposed to *S*-bioallethrin, tefluthrin or deltamethrin. The tail currents observed in each oocyte following insecticide exposure were composites of the endogenous (control) tail current and the prolonged tail current resulting from pyrethroid modification. As illustrated in Fig. 3B, tail currents caused by the three pyrethroids differed in their decay kinetics. Fits of tail currents induced by *S*-bioallethrin and tefluthrin to a first-order decay model gave apparent  $\tau_{\text{tail}}$  values of  $4.0 \pm 0.6$  and  $6.9 \pm 0.9$  ms, respectively (mean  $\pm$  SE,  $n = 9$ ). The  $\tau_{\text{tail}}$  values for *S*-bioallethrin and tefluthrin were significantly greater than the control value (one-way ANOVA with Dunnett's *post hoc* test;  $P < 0.05$ ). By contrast, deltamethrin produced tail currents that were extremely persistent, requiring up to 1 s for complete decay. Fits of deltamethrin-induced tail currents to single or multiple decay models gave highly variable results.

Fig. 4 illustrates the effects of *S*-bioallethrin, tefluthrin, and deltamethrin on the voltage dependence of activation of  $\text{Na}_v1.6\text{Q3}$  channels and Table 1 summarizes the statistical analyses of these data. Deltamethrin and *S*-bioallethrin appeared to shift the threshold for channel activation slightly in the direction of hyperpolarization, but  $V_{0.5}$  and  $K$  values measured in the presence of each of the three pyrethroids did not differ significantly from controls.

### 3.3. Effect of depolarization time on the extent of modification of $\text{Na}_v1.6\text{Q3}$ channels by pyrethroids

We equilibrated oocytes with *S*-bioallethrin, tefluthrin or deltamethrin at a hyperpolarized membrane potential and then assessing the effect of depolarizations of varied length on the amplitudes of the sodium tail current observed following repolarization. Fig. 5 shows superimposed current traces obtained from an oocyte before and after equilibration with

tefluthrin (100  $\mu\text{M}$ ) that were recorded during and after a series of depolarizations ranging from 5 ms to 25 ms. In the absence of insecticide both the maximum steady-state current during depolarization and the tail current following repolarization increased with depolarization time for depolarizations up to 20 ms and then remained constant for up to 150 ms (data for long depolarizations not shown). By contrast, composite tail currents measured following tefluthrin exposure increased in amplitude relative to the peak current during depolarization as a function of depolarization time. The traces shown in Fig. 5 also illustrate the slower decay of tefluthrin-induced tail currents compared to control tail currents at all depolarization times.

Fig. 6 summarizes data from multiple experiments such as that shown in Fig. 5 for depolarizations of 10–150 ms. Conductances of control tail currents, normalized to the maximal steady-state sodium current conductance obtained during the immediately preceding depolarization, did not vary over the range of depolarizations examined. By contrast, normalized conductances of tefluthrin-induced composite tail currents increased progressively with the duration of depolarization up to  $\sim 100$  ms.

We calculated the net conductance of each pyrethroid-induced tail current after subtraction of the corresponding control tail current, measured in the same oocyte prior to pyrethroid exposure, and normalized those values to the maximal sodium conductance measured during the immediately preceding depolarization. Fig. 7A summarizes net pyrethroid-specific tail currents measured in oocytes following exposure to tefluthrin (100  $\mu\text{M}$ ), *S*-bioallethrin (100  $\mu\text{M}$ ) or deltamethrin (10  $\mu\text{M}$ ). Consistent with the results of shown in Fig. 6, net tefluthrin-induced tail currents were clearly detectable following short (10 ms) depolarizations and increased progressively with depolarization time up to 100 ms (Fig. 7A). At 150 ms, the normalized conductance of the tefluthrin-specific component of tail currents were approximately 2.6-fold greater than those measured at 10 ms. Net deltamethrin-induced tail currents were smaller than those measured for tefluthrin following a 10-ms depolarization but also increased progressively with depolarization times up to 100 ms. At 150 ms, the normalized conductance of the deltamethrin-induced tail currents were approximately 3.5-fold greater than those measured at 10 ms. By contrast, depolarizations of up to 150 ms did not increase the amplitude of the net tail currents induced by *S*-bioallethrin (Fig. 7A).

To determine whether depolarization time-dependent modification by *S*-bioallethrin had already reached a steady-state level by 10 ms, the shortest depolarization time used in the experiments shown in Fig. 7A, we assessed the impact of 100  $\mu\text{M}$  *S*-bioallethrin on the extent of channel modification following depolarization at 3-ms increments. As shown in Fig. 7B, the conductances of *S*-bioallethrin-induced tail currents also did not vary with depolarization time following very short depolarizations.

#### 4. Discussion

Fast inactivation of voltage-gated sodium channels terminates the transient sodium current associated with membrane depolarization. Fast inactivation involves a ‘hinged lid’ mechanism, in which the conserved intracellular inactivation gate domain between homology domains III and IV occludes the inner pore of the channel (Goldin, 2003). A hydrophobic isoleucine–phenyl-alanine–methionine (IFM) motif in this domain is essential for the operation of the inactivation gate. Replacement of these hydrophobic residues with glutamine (Q) permits the selective decoupling of sodium channel activation and fast inactivation, producing channels that remain open during prolonged depolarizations (West et al., 1992). We inserted the IFM/QQQ mutation in the rat  $\text{Na}_v1.6$  sequence to create inactivation-deficient rat  $\text{Na}_v1.6\text{Q3}$  sodium channels and employed these channels to

explore directly the interaction of pyrethroid insecticides with sodium channels in the open state.

We expressed rat Na<sub>v</sub>1.6Q3 channels in combination with the rat  $\beta$ 1 and  $\beta$ 2 auxiliary subunits in *Xenopus* oocytes to facilitate the direct comparisons to our previous studies of Na<sub>v</sub>1.6 +  $\beta$ 1 +  $\beta$ 2 channels (Tan and Soderlund, 2010, 2011a,b). The kinetics and voltage-dependent activation gating of rat Na<sub>v</sub>1.6Q3 channels in the present study were consistent with the properties of rat Na<sub>v</sub>1.2Q3, rat Na<sub>v</sub>1.4Q3, and mouse Na<sub>v</sub>1.6Q3 sodium channels described in previous studies (Featherstone et al., 1996; West et al., 1992; Zhou and Goldin, 2004). Depolarizing pulses from hyperpolarized holding potentials activated sodium currents that rapidly reached a steady state, which persisted until the membrane was returned to a hyperpolarized potential. In addition to prolonged sodium currents during depolarization, oocytes expressing Na<sub>v</sub>1.6Q3 channels exhibited a prominent, rapidly decaying sodium tail current upon repolarization of the membrane. This tail current represents open-channel deactivation (*i.e.*, closing of the sodium channel activation gate), a process that is normally obscured with normal channels by the onset of fast inactivation during a depolarizing pulse (Featherstone et al., 1996; Groome et al., 2002).

*S*-bioallethrin, tefluthrin and deltamethrin enhanced and prolonged the sodium tail currents carried by Na<sub>v</sub>1.6Q3 channels but had no effect on the kinetic properties of the persistent currents measured during a depolarizing pulse. The decay kinetics of the composite (control plus pyrethroid-induced) tail currents caused by *S*-bioallethrin and tefluthrin in the present study agreed well with the decay constant of tail currents measured for tefluthrin in assays with Na<sub>v</sub>1.6 channels (Tan and Soderlund, 2010). However, deltamethrin-induced tail currents measured in assays with Na<sub>v</sub>1.6Q3 channels was much more persistent than that measured with Na<sub>v</sub>1.6 channels (Tan and Soderlund, 2010).

We varied the length of depolarizing pulses applied to oocytes expressing Na<sub>v</sub>1.6Q3 channels to investigate the duration of channel opening as a determinant of the extent of pyrethroid modification. The time-dependent increase in the pyrethroid-specific component of sodium tail currents caused by tefluthrin and deltamethrin provided direct evidence that these compounds bind with higher affinity to the open state of the channel than to the resting state. These results confirm that open-channel modification causes the use-dependent effects of these compounds on Na<sub>v</sub>1.6 channels (Tan and Soderlund, 2010). Moreover, values for the factor of tail current increase caused by long depolarizations in assays with tefluthrin and deltamethrin (2.6-fold and 3.5-fold, respectively) corresponded closely to the factors of use-dependent enhancement of modification by these compounds in assays with native Na<sub>v</sub>1.6 channels (2.8-fold and 3.7-fold, respectively) (Tan and Soderlund, 2010). In contrast to the results obtained with tefluthrin and deltamethrin, net *S*-bioallethrin-induced tail currents measured in assays with Na<sub>v</sub>1.6Q3 channels did not vary with depolarization time. This result confirms the lack of enhanced binding of *S*-bioallethrin to open channels that we inferred from the lack of use-dependent modification of Na<sub>v</sub>1.6 channels by this compound (Tan and Soderlund, 2010).

The three pyrethroids in this study also differed in the extent of channel modification obtained at the shortest depolarization times examined. Although the extent of modification of Na<sub>v</sub>1.6Q3 channels by tefluthrin and deltamethrin agree generally with the relative resting modification of Na<sub>v</sub>1.6 channels by the same compounds (Tan and Soderlund, 2010), it is likely that even at short (5–10 ms) depolarizations the modification of Na<sub>v</sub>1.6Q3 channels represents a mixture of binding of tefluthrin and deltamethrin to closed and open channels. By contrast, *S*-bioallethrin produced substantially greater modification of Na<sub>v</sub>1.6Q3 channels following short depolarizations than we found previously for Na<sub>v</sub>1.6 channels in the resting state (Tan and Soderlund, 2010). Our data showing the lack of effect of very

short depolarization times on the extent of Na<sub>v</sub>1.6Q channel modification by *S*-bioallethrin tend to rule out rapid open-channel modification as an explanation for the greater sensitivity of Na<sub>v</sub>1.6Q3 channels to this compound. We therefore hypothesize that the IFM/QQQ mutation somehow alters the access of *S*-bioallethrin to the pyrethroid receptor or its binding at that site when channels are in the resting state.

Taken together, results of the present study and previous studies provide strong evidence that the pyrethroid receptor on voltage-gated sodium channels occupies different conformations on closed and open channels that exhibit unique structure–activity relationships for pyrethroid binding. It would therefore be of interest to expand current efforts to model the pyrethroid receptor (Du et al., 2009; O’Reilly et al., 2006; Usherwood et al., 2007) to include models of the receptor on closed channels. Docking experiments with pyrethroids that interact preferentially with either closed or open channels would be valuable to identify possible structural determinants of state-dependent binding.

The conclusions drawn here regarding the relative importance of closed- and open-channel modification by pyrethroids are derived solely from studies of channels expressed in *Xenopus* oocytes. Recently, we reported that use-dependent modification of rat Na<sub>v</sub>1.6 + β1 + β2 channels expressed in HEK293 cells by tefluthrin and deltamethrin is apparently less significant than in oocytes (He and Soderlund, 2011). In the HEK293 cell environment tefluthrin caused only resting modification whereas deltamethrin caused mixed resting and use-dependent modification. Extension of studies of inactivation-deficient channels such as Na<sub>v</sub>1.6Q3 to include other cellular expression environments, such as HEK293 cells, are needed to identify the role that cellular context may play in modulating the differential effects of pyrethroids on sodium channels in the resting and open state.

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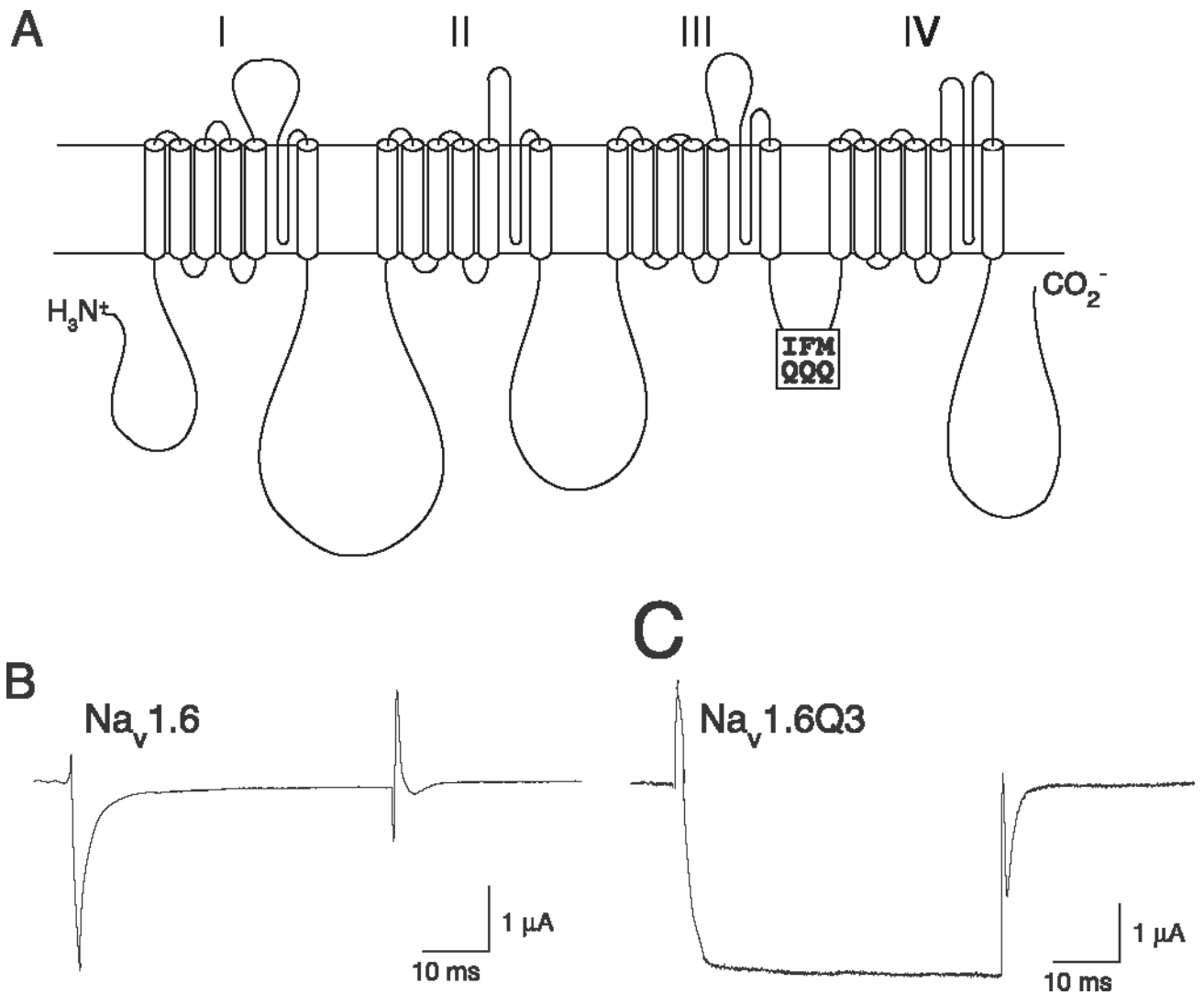
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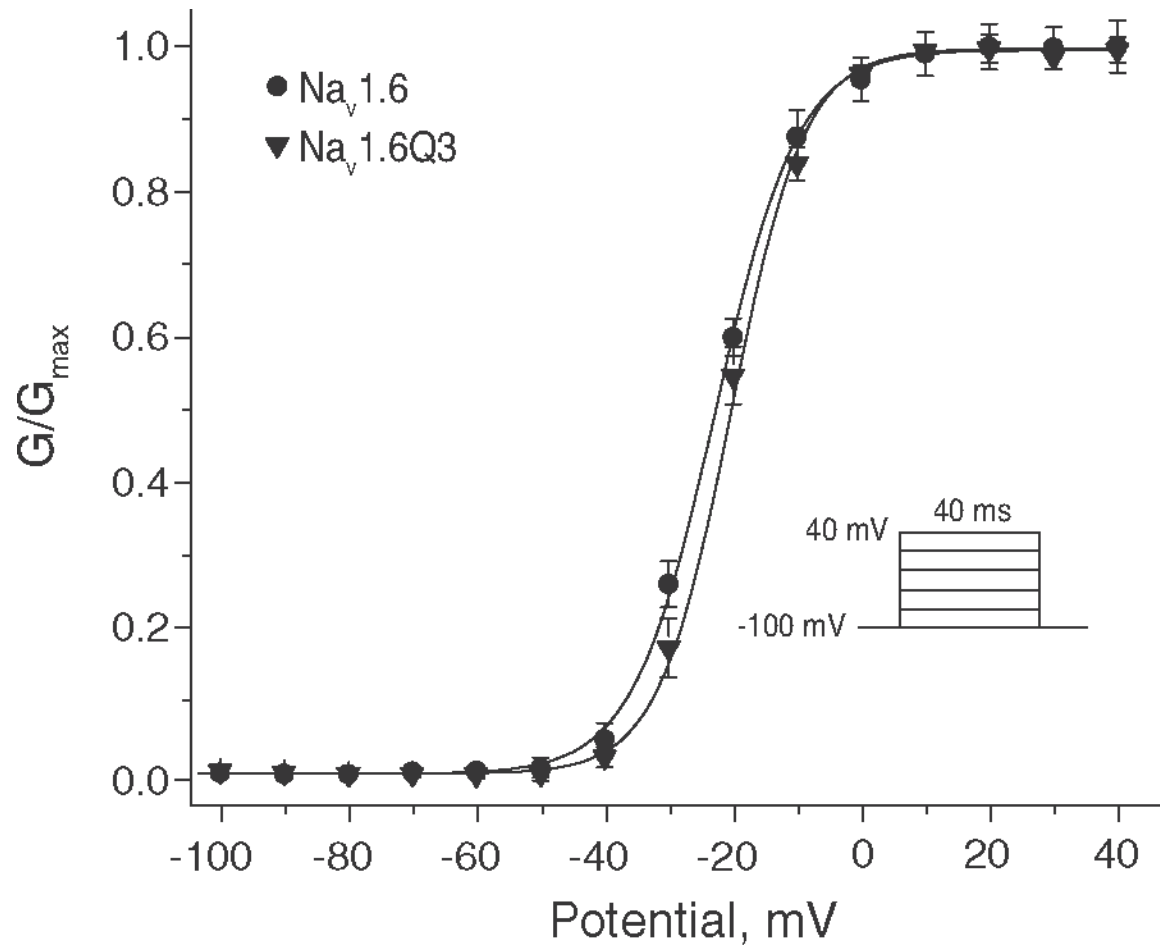


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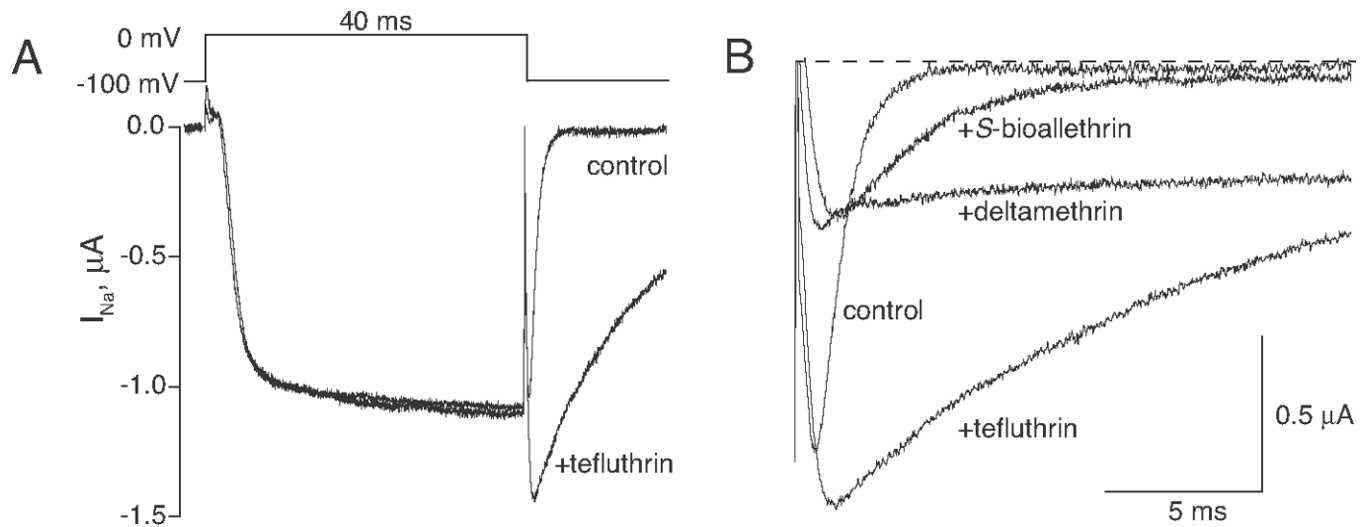


**Fig. 1.** (A) Diagram of the extended transmembrane structure of a voltage-gated sodium channel  $\alpha$  subunit protein showing the location of the IFM/XXX mutation introduced into the  $Na_v1.6Q3$  channel. (B) Representative sodium current trace recorded from an oocytes expressing  $Na_v1.6$  channels during a 40-ms step depolarization from  $-100$  mV to  $-10$  mV. (C) Representative sodium current trace recorded from an oocytes expressing  $Na_v1.6Q3$  channels during a 40-ms step depolarization from  $-100$  mV to  $-10$  mV.



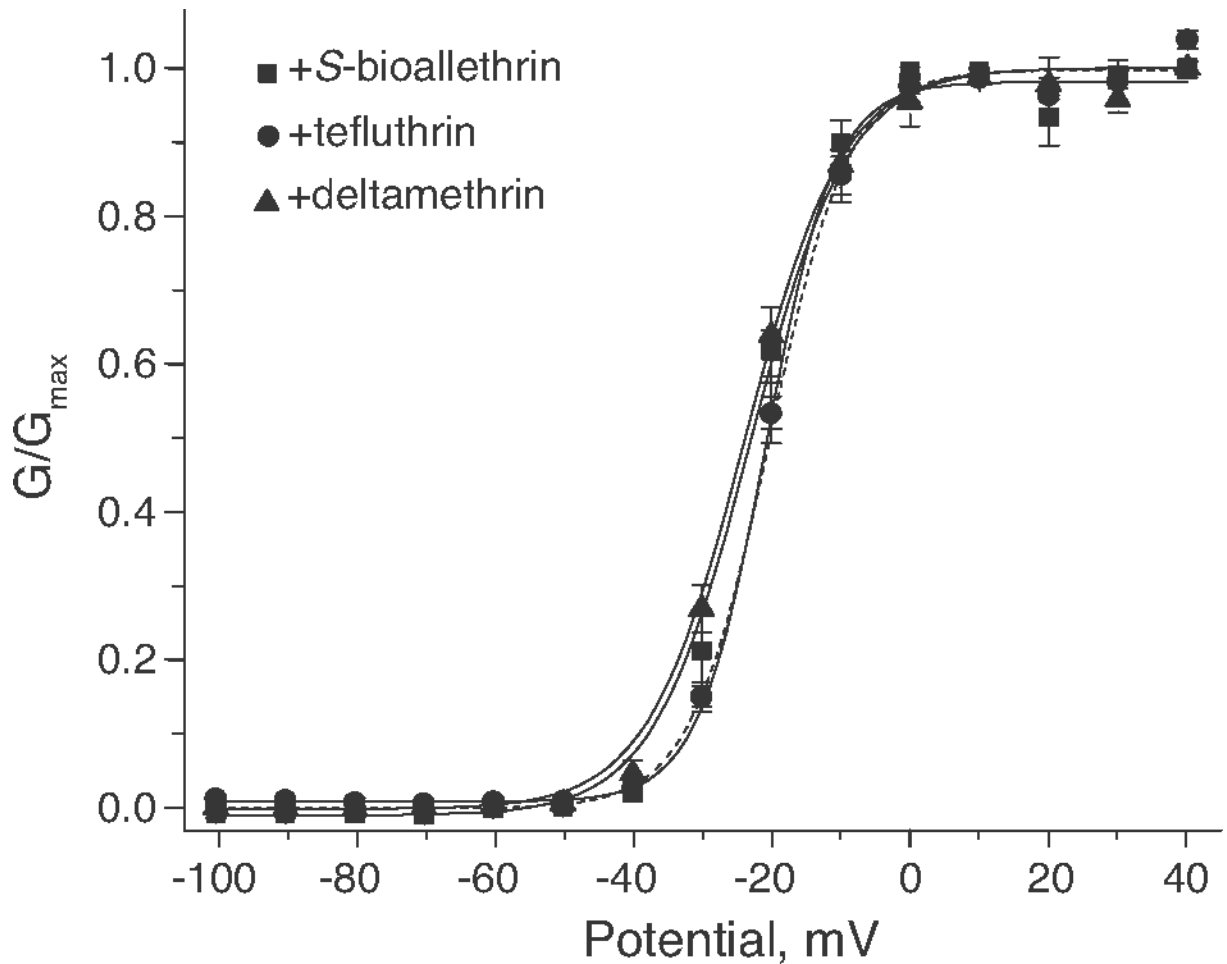
**Fig. 2.**

Voltage dependence of activation of Na<sub>v</sub>1.6 and Na<sub>v</sub>1.6Q3 sodium channels expressed in *Xenopus* oocytes. Normalized conductance ( $G/G_{max}$ ) was derived from the current-voltage relationship obtained using the indicated pulse protocol by dividing peak transient current ( $I_{Na}$ ) by the driving force ( $V - V_{rev}$ ) and normalizing to the maximum conductance observed in each cell. Values for  $G/G_{max}$  were plotted as a function of test potential and curves were drawn by fitting the mean values to the Boltzmann equation. Each data point is the mean of 10 (Na<sub>v</sub>1.6) or 14 (Na<sub>v</sub>1.6Q3) determinations with different cells; bars show SE values larger than the data point symbols.



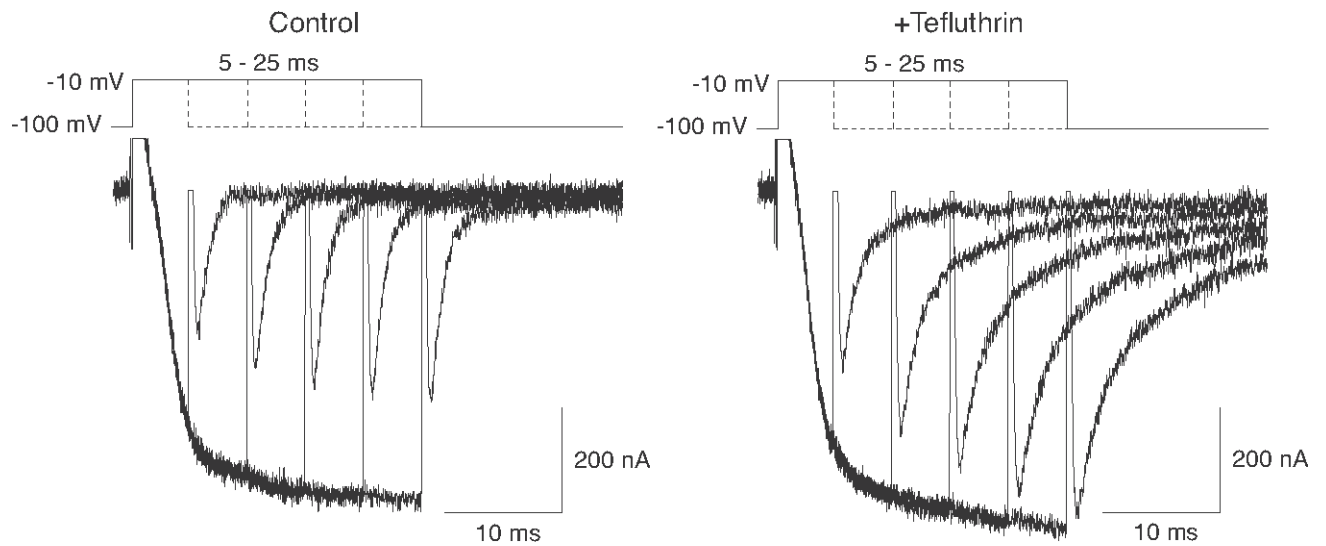
**Fig. 3.**

Pyrethroid-modified currents carried by  $Na_v1.6Q3$  channels expressed in *Xenopus* oocytes. (A) Representative sodium currents recorded from a single oocyte before (control) and after equilibration with 100  $\mu M$  tefluthrin using the indicated pulse protocol. (B) Comparison of representative sodium tail currents recorded from different oocytes either before pyrethroid exposure (control) or after equilibration with *S*-bioallethrin (100  $\mu M$ ), tefluthrin (100  $\mu M$ ) or deltamethrin (10  $\mu M$ ). Currents were recorded for 20 ms immediately following a 40-ms step depolarization from -100 mV to -10 mV. The dashed line indicates zero current.

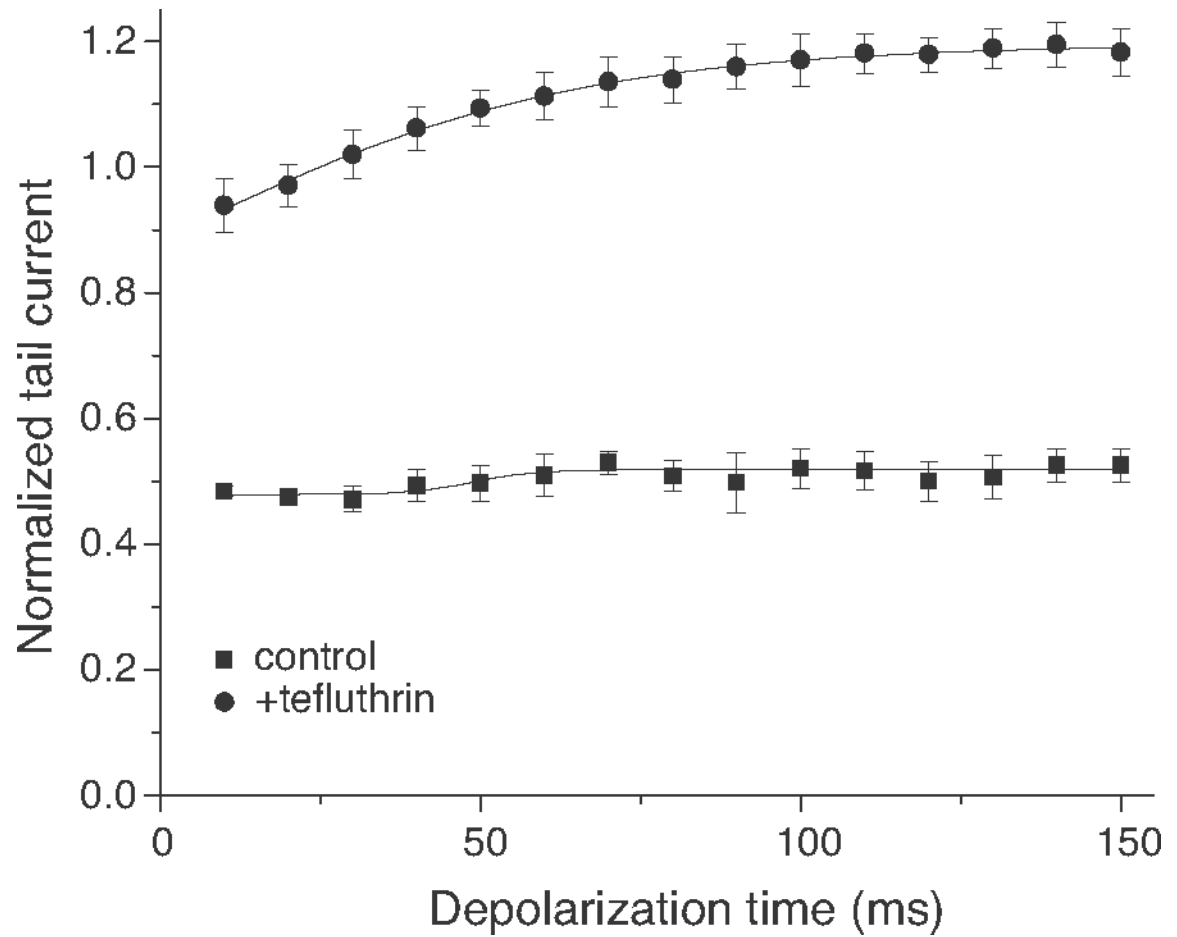


**Fig. 4.**

Effects of *S*-bioallethrin (100  $\mu$ M), tefluthrin (100  $\mu$ M), and deltamethrin (10  $\mu$ M) on the voltage dependence of activation of Na<sub>v</sub>1.6Q3 sodium channels expressed in *Xenopus* oocytes. Normalized conductance ( $G/G_{\max}$ ) values were plotted as a function of test potential and curves were drawn by fitting the mean values to the Boltzmann equation. Each data point is the mean of 5 (*S*-bioallethrin), 9 (tefluthrin) or 6 (deltamethrin) determinations with different cells; bars show SE values larger than the data point symbols. The dashed line shows the curve (from Fig. 2) obtained by fitting mean control values to the Boltzmann equation.

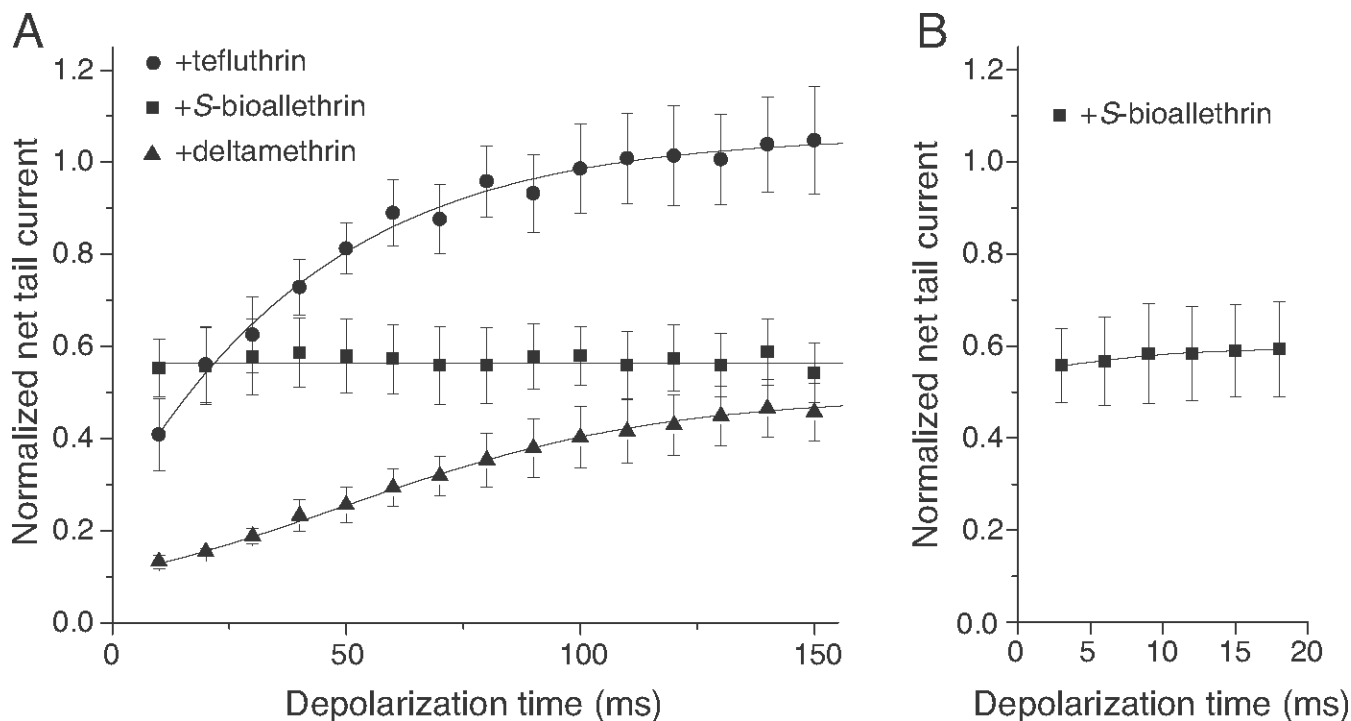


**Fig. 5.** Effect of increasing depolarization time on the amplitude of control (left) and tefluthrin-induced (right) sodium tail currents. Representative traces were recorded from a single oocyte expressing  $\text{Na}_v1.6\text{Q3}$  channels either before or after equilibration with tefluthrin ( $100\ \mu\text{M}$ ) during and after depolarizations of 5–25 ms in 5-ms increments from a holding potential of  $-100\ \text{mV}$  to a test potential of  $-10\ \text{mV}$ .



**Fig. 6.** Effect of increasing depolarization time on sodium tail currents recorded from oocytes expressing  $\text{Na}_v1.6\text{Q3}$  sodium channels before (control) or after equilibration with tefluthrin ( $100 \mu\text{M}$ ). Each data point is the mean of 6 determinations with different cells; bars show SE values larger than the data point symbols.





**Fig. 7.** Effect of increasing depolarization time on the pyrethroid-specific component of sodium tail currents recorded from oocytes expressing  $Na_v1.6Q3$  sodium channels. (A) Normalized net tail currents measured following equilibration with *S*-bioallethrin (100  $\mu$ M), tefluthrin (100  $\mu$ M) or deltamethrin (10  $\mu$ M) and step depolarizations of 10–150 ms in 10-ms increments. (B) Normalized net tail currents measured following equilibration with *S*-bioallethrin (100  $\mu$ M) and step depolarizations of 3–18 ms in 3-ms increments. Values are means of 6 determinations with different cells and are corrected for control tail currents measured prior to pyrethroid exposure in the same oocyte; bars show SE values larger than the data point symbols.

**Table 1**

Effects of *S*-bioallethrin, tefluthrin, and deltamethrin on the voltage dependence of activation of rat a  $\text{Na}_v1.6\text{Q3}$  sodium channels expressed in *Xenopus* oocytes.<sup>a</sup>

Condition	$V_{0.5}$	$K$	$n$
Control	$-21.0 \pm 1.1$	$5.66 \pm 0.30$	14
+ <i>S</i> -bioallethrin (100 $\mu\text{M}$ )	$-23.0 \pm 0.4$	$5.16 \pm 0.27$	5
+Tefluthrin (100 $\mu\text{M}$ )	$-21.4 \pm 1.0$	$5.39 \pm 0.26$	9
+Deltamethrin (10 $\mu\text{M}$ )	$-23.8 \pm 0.4$	$6.54 \pm 1.11$	6

<sup>a</sup>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 voltage-dependent activation;  $K$ , slope factor.